Title: Whole-genome sequencing confirms multiple species of Galapagos giant tortoises

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Abstract

Galapagos giant tortoises are endemic to the Galapagos Archipelago, where they are found in isolated populations. While these populations are widely considered distinguishable in morphology, behavior, and genetics, the recent divergence of these taxa has made their status as species controversial. Here, we apply multispecies coalescent methods for species delimitation to whole genome resequencing data from 38 tortoises across all 13 extant taxa to assess support for delimiting these taxa as species. In contrast to previous studies based solely on divergence time, we find strong evidence to reject the hypothesis that all Galapagos giant tortoises belong to a single species. Instead, a conservative interpretation of model-based and divergence-based results indicates that these taxa form a species complex consisting of a minimum of 9 species, with some analyses supporting as many as 13 species. There is mixed support for the species status of taxa living on the same island, with some methods delimiting them as separate species and others suggesting multiple populations of a single species per island. These results make clear that Galapagos giant tortoise taxa represent different stages in the process of speciation, with some taxa further along in that evolutionary process than others. A better understanding of the more complex parts of that process is urgently needed, given the threatened status of Galapagos giant tortoises.

Lay Summary

Species delimitation is a challenging problem in evolutionary biology, but one that is central to the field. Distinguishing species can affect conservation management practices, from conservation status assessments to strategies for breeding programs. More fundamentally, understanding species boundaries affects our ability to assess biodiversity and to study evolutionary processes. The Galapagos Archipelago presents several radiations of closely related taxa that inspired Charles Darwin to develop his theory of evolution by natural selection and later led to foundational case

studies in speciation. The Galapagos giant tortoises were one such inspiration. Nearly two centuries later, there is still an ongoing debate about the taxonomic status of these tortoises, with opinions on their status ranging from barely differentiated populations to separate species. Here, we present the first genomic species delimitation of Galapagos giant tortoises and provide convincing evidence that this group is a complex consisting of between 9 and 13 species. These results provide valuable guidance to conservation stakeholders in the Galapagos, while also adding an important case study to the delimitation of island species.

Introduction

Speciation is a complex biological process driven at least in part by ecological context such as physical barriers to gene flow, adaptation to local environments, and population-specific

92 demographic dynamics. In recently diverged lineages, the relative roles of the evolutionary forces
93 responsible for divergence can be challenging to describe accurately because of shared ancestral
94 polymorphisms in the descendant lineages and introgression due to ongoing gene flow (Shaffer &
95 Thomson 2007). Adding to these difficulties are the small founding populations that characterize
96 the origin of some species, especially in island settings, which can speed up the divergence of
97 lineages through the rapid loss or fixation of alleles (Kimura & Ohta 1969). The iconic radiation
98 of Galapagos giant tortoises (a clade within the genus *Chelonoidis*) is a compelling example of the
99 complexities of species delimitation in a case of recent diversification: their divergence has been
100 molded by a combination of vicariance and colonization events (Caccone *et al*. 1999, 2002;
101 Poulakakis *et al*. 2012, 2020) brought about by both natural and anthropogenic environmental
102 changes and making accurate species delimitation challenging.

103 The Galapagos Islands, the conceptual home of the theory of evolution by natural selection,
104 provide a crucible for the study of speciation and taxonomic complexity. Within the Galapagos
105 Archipelago there are many examples that show clear evidence of recently diverged but genetically
106 distinct species, including finches (Grant & Grant 2003), lava lizards (Benavides *et al.* 2009),
107 iguanas (MacLeod *et al.* 2015), mockingbirds (Arbogast *et al.* 2006), moths (Schmitz *et al.* 2007),
108 and tortoises (Caccone *et al.* 1999). This archipelago therefore provides a model system to
109 understand the complex realities of speciation, allowing us to explore multiple lines of molecular,
110 morphological, and ecological evidence when proposing species delimitations.

111 There are 16 Galapagos giant tortoise taxa, three of which (*niger*, *abingdonii*, and an
112 unnamed taxon from Santa Fé Island) are recently extinct (Figure 1). Morphological, behavioral,
113 and genetic differences have been documented among the taxa (Gaughran *et al.* 2018, Jensen *et*
114 *al.* 2021, Chiari 2021, Hunter *et al.* 2013). The most striking morphological difference is carapace

115 shape, which ranges from a domed shape to a saddleback shape with an elevated anterior carapace
116 opening. These carapace shapes have a genetic underpinning, as evidenced by a clear phylogenetic
117 signal (Jensen *et al.* 2022) and a strong inheritance in juveniles of different taxa raised in a common
118 environment (Pritchard 1996). The taxa also differ in coloration on the head and neck, as well as
119 in limb length (reviewed in Chiari 2021) and aggressive behaviors (Schafer & Krekorian 1983).
120 Mitochondrial, microsatellite, and genomic data all suggest that geographically distinct
121 populations represent genetically differentiated lineages (e.g., Caccone *et al.* 2002, Beheregaray
122 *et al.* 2003, Gaughran *et al.* 2018, Miller *et al.* 2018).

123 Galapagos giant tortoises are descended from individuals that likely floated from
124 continental South America along the Humboldt Current to the newly formed Galapagos Islands
125 around 3 million years ago (Ma; Caccone *et al.* 2002). As more volcanic islands emerged over
126 time, tortoises migrated from existing islands in the east to newer ones in the west (Poulakakis *et*
127 *al*. 2020). Divergence dates from mitochondrial DNA suggest that the progressive colonization
128 from the first colonized island likely occurred after 2 Ma and continued up to the emergence of
129 the youngest island to the west, Fernandina, around 60,000 years ago (Geist *et al*. 2014). The
130 layout of the archipelago has changed dramatically over this time period due to volcanic emergence
131 and subsidence, and sea level changes. During the Pleistocene the central archipelago had a larger
132 landmass that has since fragmented into some of the islands seen today (Geist *et al.* 2014). Thus,
133 the diversification of Galapagos giant tortoises has been triggered by a combination of dispersal
134 to isolated islands and vicariance as landmasses fragmented (Parent *et al.* 2008). In addition, within
135 the last 200 years, humans have moved tortoises between islands (Caccone *et al.* 2002, Poulakakis
136 *et al.* 2008) and land use change has brought previously isolated lineages into contact (Russello *et*
137 *al.* 2005), resulting in low levels of recent gene flow between previously isolated populations.

138 Because of this recent and dynamic evolutionary background, the taxonomy of the
139 Galapagos giant tortoises has been the subject of almost endless debate since the time of Darwin
140 (e.g., Darwin 1839, Günther 1877, Van Denburgh 1907, Pritchard 1996, Zug 1998, Caccone *et al.*
141 1999, Kehlmaier *et al.* 2021). Most taxonomic proposals have recognized these lineages as either
142 a single species with 2-14 subspecies, or as many as 14 separate species (reviewed in Pritchard
143 1996). Following work by Caccone *et al*. (1999), they have been listed as multiple species based
144 on genetic data accumulated over the last several decades (Rhodin *et al.* 2017). More recently,
145 however, two publications have advocated collapsing all taxa into a single species with multiple
146 subspecies (Loire *et al.* 2013, Kehlmaier *et al.* 2021), which was adopted by the IUCN Tortoise
147 and Freshwater Turtle Specialist Group in 2021 (Rhodin *et al.* 2021).
148 To address this question, we turned to whole genome resequencing data and methods that
149 quantitatively assess the degree to which taxa are independently evolving under a multispecies
150 coalescent (MSC) framework (Zhang *et al.* 2011, Jackson *et al.* 2017, Morales *et al.* 2018, Mays
151 *et al*. 2019, Leaché *et al.* 2019, Marshall *et al.* 2021). Here, we apply several of these methods of
152 species delimitation to the Galapagos giant tortoises, and thereby provide an assessment of
153 taxonomic considerations in this endangered clade.
154
155 **Methods**
156 Terminology, study design and data
157 The aim of this paper is to assess genomic evidence for the distinctiveness and taxonomic
158 status of Galapagos giant tortoise taxa using phylogenetic and coalescent frameworks. To remain
159 agnostic about the taxonomic status of the various tortoise populations prior to presenting our
160 results, we avoid designating the taxa as *species* or *subspecies* and refer to them using only the

161 epithets (e.g., *phantasticus* rather than *Chelonoidis phantasticus* or *Chelonoidis niger*
162 *phantasticus*). We mapped existing Illumina short-read data (NCBI Bioproject PRJNA761229;
163 Jensen *et al.* 2021, Jensen *et al.* 2022) to the Pinta Island Galapagos giant tortoise reference
164 genome (NCBI assembly ASM359739v1, Quesada *et al.* 2019). Samples in this short-read data
165 set included two individuals of *phantasticus*, and three individuals each of *guntheri*, *microphyes*,
166 *vandenburghi*, *vicina*, *becki* (PBL population), *becki* (PBR population), *darwini*, *chathamensis*,
167 *donfaustoi*, *porteri*, *duncanensis*, and *hoodensis*, for a total of 38 Galapagos giant tortoise
168 individuals plus one outgroup (Chaco tortoise, *C. chilensis*) individual. We called variant sites for
169 each individual using the BCFtools variant calling pipeline (Li *et al*. 2009) and created a consensus
170 fasta file for each individual from the VCF. We then generated a random set of 1kb loci separated
171 by 100kb. We filtered, masked, and generated phased haplotypes for each locus. Details on these
172 terminology choices, study design, and data are in the Supplemental Methods.
173
174 <u>SNAPP tree generation</u>
175 To assess the phylogenetic relationships among the extant populations of Galapagos giant
176 tortoises, we used SNAPP v1.5.2 (Bryant *et al.* 2012) and MODEL_SELECTION v1.5.3 (Baele
177 *et al.* 2012), as implemented in BEAST2 v2.6.7 (Bouckaert *et al.* 2014), to test 21 different
178 phylogenetic models (Supplemental Table S1) on a dataset comprising 1,000 SNPs from 38
179 Galapagos giant tortoise individuals, plus the *C. chilensis* individual as an outgroup. We then
180 applied the priors from the best model to an extended dataset comprising 5,000 SNPs for
181 phylogenetic reconstruction and downstream species delimitation comparisons. A more detailed
182 description of these methods is presented in Supplemental Methods.
183

184 Species delimitation models

185 We implemented the Bayesian species delimitation model in BPP v4.0 (Flouri *et al.* 2018)
186 to test hypotheses about the number of species in our data set. We ran the species delimitation
187 analyses with 50, 200, 500, or 1000 phased loci, with each locus a randomly selected alignment of
188 1000 bp (see Supplemental Methods). We ran BPP species delimitation with the SNAPP guide
189 tree (i.e., "A10" analysis) and without a guide tree (i.e., "A11" analysis), repeating each run three
190 times with different random seeds to assess convergence among runs. Because the Galapagos
191 Islands are geologically young (<3.5 Ma) and the deepest divergence time among Galapagos giant
192 tortoise lineages is estimated to be between 2 and 3 Ma (Caccone *et al*. 2002), we chose a small
193 but diffuse prior for divergence time ($\tau \sim$ IG(3, 0.001)), which corresponds to a mean of 83,333
194 generations (approximately 2.08 million years). In addition, given the low estimates of effective
195 population size for Galapagos giant tortoises (Garrick *et al*. 2015, Jensen *et al*. 2021) and because
196 the carrying capacity must be low for large-bodied terrestrial vertebrates on these semi-desert
197 islands, we used a prior scaled effective population size of $\theta \sim$ IG(3, 0.001), which is equivalent
198 to N_e=20,833. However, because there is uncertainty about historical population size, we also re-
199 ran the analyses with scaled effective population size priors that were larger ($\theta \sim$ IG(3, 0.005),
200 N_e=104,167) or smaller ($\theta \sim$ IG(3, 0.0001), N_e=2083), keeping the priors for τ the same. In each
201 of run, we used a burn-in of 100,000, a sampling frequency of 2, and collected 300,000 samples.

202 To assess if the chosen loci and included taxa affected our analyses, we ran the above
203 analyses with a different set of 50, 200, 500, and 830 loci and including the Chaco tortoise. The
204 inclusion of this outgroup allowed the model to explore a species delimitation of two (i.e.,
205 Galapagos giant tortoises and Chaco tortoise). Because the divergence time estimate of the Chaco
206 tortoise and Galapagos giant tortoises is around 12 Ma (Caccone *et al.* 1999, Kehlmaier *et al.*

207 2017), we used a divergence time prior of $\tau \sim IG(3, 0.01)$. Finally, we re-ran the analyses with 50
208 unphased loci to ensure that our phasing method was not affecting the results.
209
210 Genealogical divergence index (*gdi*) from BPP
211 The use of species delimitation models in BPP is thought to be a robust method for
212 sympatric species delimitation, but there are concerns that using this MSC-based model selection
213 for species delimitation may delimit populations rather than species, especially if the taxa are
214 allopatric (Jackson *et al.* 2017, Leaché *et al*. 2019). Therefore, we also calculated the *gdi*, a
215 distance-based method with heuristic cutoffs for species delimitation (Jackson *et al.* 2017). The
216 *gdi* is calculated from the divergence time in coalescent units ($2\tau/\theta$) and reflects the probability
217 that two sequences from a purported species coalesce before the divergence time with a sister
218 species (τ) (Leaché *et al.* 2019). As such, it is a coalescent-based measure of genetic divergence,
219 as opposed to a sequence-based measure. Using the effective population size and divergence time,
220 the *gdi* can be calculated in a pairwise manner for sister species *a* and *b*, and can range from 0
221 (panmixia) to 1 (strong divergence). Based on empirical data and simulations, Jackson *et al.* (2017)
222 proposed a general heuristic for delimiting species based on the *gdi*. Namely, $gdi < 0.2$ indicates a
223 single species, and $gdi > 0.7$ indicates different species. Values for *gdi* between these cutoffs
224 indicate ambiguous delimitation.
225 To calculate these indices, we used BPP for parameter estimation of τ and θ, under the
226 guide tree and fixed MSC model ("A00") function in BPP. For these analyses, we used the 830
227 phased loci available in the data set with the Chaco outgroup. Using the phylogeny from our
228 SNAPP analysis as a fixed guide tree, we estimated τ for each node and θ for each branch and tip,
229 by running the MCMC with a burn-in of 100,000, a sample frequency of 2, and a total sample of

200,000. We used the same loci and priors as in the species delimitation modeling. We then used the MCMC output to create posterior distributions of τ (for each node) and θ (for each tip), which we then used to calculate *gdi*. Using posterior distributions allowed us to calculate 95% credibility intervals. Following the suggestion of Leaché *et al*. 2019, we also successively collapsed each node of the phylogeny, re-ran the A00 parameter estimation model, and calculated *gdi* for each collapsed taxon.

PHRAPL

We then assessed the presence of gene flow between all pairs of lineages within each of the two main clades of the tree (domed and saddleback) and tested for a collapse event using PHRAPL (Jackson *et al.* 2017) implemented in R version 4.2 (R Core Team, 2022). PHRAPL works by estimating the probability that a set of gene trees are observed under a given model by calculating the frequency at which they are observed in a distribution of expected tree topologies, weighting the probability by Akaike information criterion (AIC). In this way, PHRAPL can determine the most likely demographic history. Three demographic models for each pairwise comparison were constructed to test varying divergence and gene flow scenarios between populations of Galapagos giant tortoises (Supplemental Figure S1). Model 1 was a two species isolation only model, which tested for divergence between two populations with no ongoing migration. Model 2 was also a two-species model and tested for constant symmetrical migration between the two populations. The final model (Model 3) was the same as Model 2 but we removed the divergence between the two populations using the *setCollapseZero* function in PHRAPL. This single species model therefore allowed for constant symmetrical gene flow between the populations with no divergence events. Input gene trees were generated for each of the 830 phased

253 loci in RaxML (v 8.2.12, Stamatakis 2014) with 20 replicate searches, rapid hill-climbing and the
254 GTRGAMMA model. The Chaco tortoise was included as an outgroup to root the tree. PHRAPL
255 is a demographic model and requires individuals to be assigned to a population prior to model
256 construction. Here, individuals were assigned to a population based on geographic location, as
257 detailed in Figure 1. In this way, the tortoises on each island were represented by a single
258 population except that six geographically separate populations were recognized on Isabela Island
259 and two on Santa Cruz Island. each island lineage was assigned to a distinct population, with the
260 exception of Isabela Island having six populations, based on the six geographically separated
261 lineages, and Santa Cruz island having two populations. Gene trees were subsampled at random
262 with replacement 100 times, sampling 2 individuals per lineage in each replicate. The outgroup
263 was not included in the subsampling and so was not included in the PHRAPL runs. Simulation of
264 100,000 gene trees was conducted using a grid of parameter values for divergence time (*t*) and
265 migration (*m*; Supplemental Table S2). The initial divergence within the Galapagos tortoise
266 radiation is estimated to be within the last 2 Ma, but the species pairs we compare here are more
267 recent (Poulakakis *et al.* 2020). We set the parameters of our grid search to capture this by limiting
268 the maximum divergence (*t*) to 1 Ma. Migration rates were equal to 4*Nm*, where *Nm* is the number
269 of migrants per generation, with the lowest migration being equivalent to one migrant every 800
270 years and the highest equivalent to one migrant every 25 years. This range of values was designed
271 to capture the putatively complex historical gene flow among populations. Akaike weights (wAIC)
272 were used to compare models and calculate model probabilities ranging from 0 (low support) to 1
273 (high support). To present the best supported hypothesis overall for each pairwise comparison, the
274 summed wAIC of the two-species models (Models 1 and 2) was compared with the wAIC of the
275 single species model (Model 3). We interpreted a summed wAIC of > 0.9 across the two-species

276 models as strong support for a two species hypothesis, a wAIC > 0.9 of the one species model as
277 strong support for a single species hypothesis, and all other scenarios as ambiguous. In addition to
278 identifying the top model chosen by PHRAPL, we also calculated the *gdi* value between taxon
279 pairs following the approach taken in Jackson *et al*. 2017 and using the *CalculateGdi* function in
280 PHRAPL. The *gdi* value from PHRAPL differs from that from BPP in that it is calculated using
281 the model averaged divergence rate (*t*), migration rate into population 1 (*M1*), and migration into
282 population 2 (*M2*). Because our models did not estimate the direction of migration, *M1=M2* in our
283 analyses. We interpreted the *gdi* values using the same thresholds described above.
284
285 **Results**
286 SNAPP
287 Of the 21 SNAPP models tested, Model 6 ($\alpha = 5$, $\beta = 150$, $\lambda = 39$) presented the greatest
288 marginal likelihood estimate (–14,448.23), with a Bayes factor equivalent to –299.07 relative to
289 the default, reference model (*i.e.*, Model 7; Table S1). Using the framework of Kass & Raftery
290 (1995), the Bayes factor in support for Model 6 is "decisive" compared to the reference model.
291 The final tree derived from the parameters used for Model 6 (combined posterior effective sample
292 size = 1,085; cladogram shown in Figure 2, phylogram with scaled branch lengths in Supplemental
293 Figure S2) has two primary sub-clades, which reflect the two carapace morphologies: the larger
294 clade contains the nine taxa with mostly domed carapaces, whilst the smaller clade contains the
295 four taxa with mostly saddleback carapaces. Most nodes (82%) are highly supported, with
296 posterior probabilities ≥ 0.95 (Figure 2).
297
298 Species delimitation models in BPP

When we ran an "unguided" species delimitation (A11 analysis in BPP) with our most realistic prior of $\theta \sim IG(3, 0.001)$, the analysis supported 13 species with P>0.98 when using 50, 200, 500, or 1000 phased loci across all runs (Supplemental Table S3). When a smaller θ prior was used, 13 species were almost always supported with P>0.99 (Supplemental Table S3). When using a slightly larger θ prior, the analysis generally supported 12 or 13 species when 200, 500, and 1000 loci were used, but as few as 9 species when only 50 loci were used (Supplemental Table S3). In cases where there was support for fewer than 13 species, the analysis typically supported the collapse of two or more taxa on Isabela Island into one species. In four out of 45 runs, the A11 analysis supported a single species of Galapagos giant tortoise (Supplemental Tables S3 and S5), but these runs likely represent poor mixing (see Discussion). When the Chaco tortoise outgroup was included, most runs supported more than 10 species (Supplemental Table S4). Importantly, a two-species model (with Galapagos giant tortoises as one species and the Chaco tortoise as another species) was never supported. However, runs with the Chaco tortoise did not always converge on the same distributions of posterior support when started with different random seeds (Supplemental Table S4). Results were nearly identical when we used the unphased loci (Supplemental Tables S5-S6) and running the "A10" analysis with the guide tree (Supplemental Tables S7-S8). Using our most realistic priors, therefore, the BPP analyses supported delimitation of 13 species (Figure 2).

gdi calculation from BPP parameter estimates

Overall, the median *gdi* estimated in BPP ranged from 0.023 (*phantasticus*) to 0.534 (*hoodensis*; Figure 2, Table S11). None of the taxa exceeded a *gdi* of 0.7, which has been proposed as a heuristic for strongly delimited species. Five taxa (*becki (PBR)*, *donfaustoi*, *hoodensis*,

322 *duncanensis*, and *chathamensis*) had *gdi* in the ambiguous delimitation range of 0.2 < *gdi* < 0.7.
323 The remaining eight taxa had *gdi* below 0.2. Estimates of *gdi* were effectively identical across
324 priors (Supplemental Tables S12–S13). In many cases, the *gdi* differed dramatically between sister
325 taxa, emphasizing the asymmetry commonly observed in this statistic.

326 When taxa do not meet the heuristic *gdi* threshold for species delimitation, Leaché *et al*.
327 (2019) recommended progressively collapsing taxon pairs, rerunning the MCMC and calculating
328 the *gdi* of the new groups. If the collapsed taxon represents a better-supported species, the
329 expectation is that the *gdi* would increase. This occurred when the taxa on central and southern
330 Isabela Island were collapsed (*gdi*=0.226, Figure 3A and Supplemental Table S16) and when those
331 on Santa Cruz Island were collapsed (*gdi*=0.291, Figure 3C and Supplemental Table S14). In all
332 other cases, however, the collapsed taxa had *gdi* lower than the separated taxa (Figure 3B and 3D,
333 Supplemental Tables S14-S18). Overall, the *gdi* estimates provide ambiguous support for
334 delimiting most taxa on different islands as separate species but favor treating some populations
335 on the same island as conspecific (Figure 2).

336

337 PHRAPL

338 For the PHRAPL analysis we focus on results of pairwise comparisons for populations within the
339 two primary clades (Figure 2). For the saddleback clade, there are four lineages spread across four
340 separate islands and so all pairwise comparisons were included here. Within the domed clade,
341 Isabela and Santa Cruz islands host five and two lineages respectively, while Santiago has a single
342 living lineage. For this clade we have presented the results in Figure 2 as within Santa Cruz Island
343 for its two lineages, within central and southern Isabela Island for its four lineages that form a
344 clade and within the clade of Santiago and its sister taxa, *becki (PBR)* and *becki (PBL)* on northern

Isabela Island. Results for model wAIC and *gdi* values for all possible pairwise comparisons within the saddleback and domed morphology clades are presented in Supplemental Table S20. Within the saddleback clade, all pairwise comparisons supported a two-species model when wAIC values were summed, supporting four species across this clade. The *gdi* values in this clade were generally in the ambiguous range of $0.2<gdi<0.7$, except for the *gdi* of *hoodensis* and *phantasticus* (*gdi*=0.179; Figure 2 and Table S20). For the Santa Cruz Island tortoises (*porteri* and *donfaustoi*), PHRAPL supported a two-species model with a wAIC > 0.9, but the *gdi* was 0.181 (Figure 2). Similarly, within the central and southern Isabela Island clade there was full support for two-species models in pairwise comparisons, yet mixed support from the *gdi* values across pairwise comparisons of the four lineages. The pairwise comparisons between taxa on Santa Cruz and Isabela Islands also all favored a two-species model, and all *gdi* estimates fell between 0.2 and 0.7 (Figure 2). Similarly, all comparisons between the Santiago lineage (*darwini*) and the taxa on Isabela and Santa Cruz Islands strongly supported a two-species model, with five out of eight *gdi* comparisons above 0.2. In total, the species delimitation models in PHRAPL support the delimitation of 13 species, while the *gdi* estimates from PHRAPL suggest between 7 and 9 species, depending on the interpretation of heuristic thresholds.

Discussion

Despite considerable progress in achieving a more unified concept of species (Mayden 1997; de Queiroz 1998, 2007; Hey 2006), the empirical application of such a concept presents challenges, particularly in cases of recent and/or incomplete divergence (e.g., de Queiroz 2005a, Carstens *et al.* 2013, Jackson *et al.* 2017). The Galapagos giant tortoises represent such a case.

367 Here, we build on decades of research on the molecular evolution of these tortoises and provide
368 genomic evidence that the Galapagos giant tortoise complex consists of multiple distinct species.
369 Our phylogenetic analysis using SNAPP supported the topology previously inferred from
370 genome-wide sequence data (Jensen *et al.* 2022), except for the relationships among the most
371 recently diverged taxa (*guntheri*, *vandenburghi* and *microphyes*) and the placement of the Santa
372 Cruz Island taxa. Given this strong support, we used this nuclear phylogeny as a guide tree in
373 subsequent analyses. Notably, the topology of these trees inferred from nuclear loci deviates in
374 some ways from mitochondrial trees for these taxa (Poulakakis *et al.* 2012, 2020), an incongruence
375 that highlights the complex and rapid evolutionary history of Galapagos giant tortoises.
376 Our first line of evidence for the distinctiveness of Galapagos giant tortoise species is the
377 model-based species delimitation analysis in BPP. This analysis supported 13 species of Galapagos
378 giant tortoises across different numbers of loci when our most realistic priors were applied. Across
379 different priors we observe stronger support for 13 species as more loci are used, which may
380 indicate that the greater information content from more loci provides better biological resolution.
381 Theory and simulations suggest that BPP will increasingly favor a two-species model when
382 hundreds or thousands of loci are used, even under high rates of migration (Leaché *et al*. 2019).
383 Under our most realistic prior set, however, even 50 loci strongly support the delimitation of 13
384 species both with and without a guide tree.
385 In cases where fewer than 13 species were supported, the model favored collapsing two or
386 more of the taxa in a clade of four taxa on Isabela Island (i.e., *guntheri*, *microphyes*, *vandenburghi*,
387 and *vicina*). These taxa are geographically adjacent to each other on the central and southern
388 volcanoes of Isabela Island, which suggests that recent divergence or gene flow is a factor. The
389 delimitation models sometimes collapsed taxa that were not sister taxa on our phylogeny,

390 emphasizing the finding from this and other studies that the phylogeny of taxa on Isabela Island is
391 complex (Poulakakis *et al.* 2012, Jensen *et al*. 2022) and likely needs genomic data from a larger
392 number of individuals to be resolved.

393 In four runs of our A11 analysis including only Galapagos giant tortoise individuals the
394 analysis supported a single species of Galapagos giant tortoise. These runs are likely the result of
395 poor mixing, which is a known issue in the reversible-jump Markov Chain Monte Carlo (rjMCMC)
396 algorithm of BPP (Yang and Rannala 2010, Flouri *et al.* 2018). Mixing can be especially
397 problematic when sampling the fully resolved or fully collapsed tree, where the algorithm can get
398 "stuck" (Giarla *et al.* 2014). That we never find support for a single Galapagos giant tortoise
399 species when the Chaco tortoise individual is added to the analysis nor when we provide a guide
400 tree further supports the conclusion that the single-species runs of the A11 analysis are the result
401 of poor mixing.

402 We also modeled species delimitation in PHRAPL, comparing support for models of each
403 taxonomic pair as one species, two species in isolation, and two species with gene flow. We found
404 two-species models favored in all cases (Figure 2, Supplemental Table S20). The migration rates
405 chosen accounted for migration occurring up to 800 generations ago and there was mixed support
406 across populations for the two-species model that allowed migration vs. no migration. Jackson *et*
407 *al*. (2017) described the process of model selection in PHRAPL to be less accurate for isolation
408 only models (here, Model 1) with recent divergence times (t<2). Given that we tailored the
409 divergence values to be <1 to suit the evolutionary time scale of the Galapagos giant tortoise
410 lineages, there may be some bias in results where an isolation-with-migration model is selected
411 over the isolation-only model. This is a limitation of PHRAPL in that it has difficulty
412 distinguishing incomplete lineage sorting from gene flow, and so will favor the isolation-with-

413 migration model over the model of recent isolation. Furthermore, PHRAPL is more accurate with
414 a larger dataset (Jackson *et al.* 2017). The sample size for each taxon in our data set is small (n=2–
415 3 individuals per taxon). Because PHRAPL uses subsampling of individuals to generate expected
416 tree topologies, having a larger sample size that better represents the whole population could help
417 disentangle the history of the unresolved populations. Current work is underway to increase the
418 number of reference sequences for extant populations.

419 The species delimitation model in BPP has been criticized as oversplitting species and
420 recovering population structure rather than species (Jackson *et al.* 2017), as has species
421 delimitation modeling in PHRAPL (Leaché *et al*. 2019). Some have argued that the *gdi* better
422 reflects species differences by explicitly incorporating information about divergence time and
423 either population size (when calculated from BPP parameter estimates) or migration rates (when
424 calculated in PHRAPL) (Jackson *et al.* 2017, Leaché *et al*. 2019). Using the *gdi* thresholds of
425 $gdi<0.2$ for single species and $gdi>0.7$ for two species, we found ambiguous support ($0.2<gdi<0.7$)
426 in the BPP *gdi* estimates for *becki (PBR)*, *donfaustoi*, *hoodensis*, *chathamensis*, and *duncanensis*.
427 All other taxa had a $gdi<0.2$, creating multiple scenarios in which one sister taxon had $gdi>0.2$ and
428 the other taxon had $gdi<0.2$. In PHRAPL, the *gdi* estimates for most taxa were also in the
429 ambiguous range of $0.2<gdi<0.7$, with some taxon pairs falling below 0.2 (i.e., *phantasticus* and
430 *hoodensis*, *porteri* and *donfaustoi*, and some of the domed taxa on Isabela and Santiago Islands).

431 Although these thresholds were proposed in the literature from a small sample of species,
432 work in other natural populations has shown that well-accepted species can fall into the ambiguous
433 range of 0.2–0.7, including horned lizards (Leaché *et al.* 2021), penguins (Mays *et al.* 2019), and
434 flying lizards (Reilly *et al.* 2022). Furthermore, there appears to be significant variation in *gdi*
435 within clades and between them. For example, in a survey of bird species the median *gdi* was 0.346

436 (1st quartile: 0.012; 3rd quartile: 0.742), while the surveyed mammalian species had a median *gdi*
437 value of 0.799 (1st quartile: 0.716; 3rd quartile: 0.955) (Jackson *et al*. 2017). This suggests that
438 appropriate heuristic cutoffs for *gdi* may differ among taxa, given that divergence times,
439 population sizes and life histories differ substantially across these clades. Likewise, island
440 radiations may also have a different underlying distribution of *gdi* given their shallow divergence
441 times and propensity for rapid adaptation. Before a *gdi* heuristic can be confidently applied to
442 Galapagos giant tortoise species, more thorough surveys of *gdi* among testudine species and across
443 island radiations are needed.

444 When sister taxa are not species, Leaché *et al*. (2019) found that collapsing the taxa and
445 recalculating *gdi* results in a larger *gdi*, signaling better support for the resulting species. This
446 behavior of *gdi* can therefore be used to interpret the *gdi* without relying as heavily on heuristic
447 thresholds. When we replicate this iterative process in BPP with our taxon set, we find that this
448 pattern occurs when we collapse all central and southern Isabela Island taxa (i.e., *guntheri*,
449 *microphyes*, *vandenburghi*, *vicina*) and when we collapse the Santa Cruz taxa (*porteri* and
450 *donfaustoi*). All other collapses, however, result in estimates of *gdi* that are smaller rather than
451 larger than the *gdi* of one or both sister taxa (Figure 3).

452 Applying population genetic principles to the *gdi* equation reveals why this occurs.
453 Because *gdi* is calculated from the ratio of τ to θ, it will remain constant when these values change
454 proportionally (e.g., the *gdi* will be 0.33 when τ is 0.001 and θ is 0.005, and when τ is 0.002 and θ
455 is 0.01). When sister taxa are collapsed, both τ and θ will increase. By definition, in recent
456 radiations τ will increase only marginally at each successive node. On the other hand, θ will be
457 inflated when calculated from a highly structured sample (e.g., differentiated species) because the
458 number of segregating sites is expected to be higher in a combined sample. Thus, the lower *gdi*

459 values that we found for successive collapsing of taxa from different islands further supports the
460 idea that these taxa do not belong to a single species.

461 The analyses presented in this study represent the most thorough attempt to date to tackle
462 the question of species delimitation in Galapagos giant tortoises using genetic data. Several prior
463 attempts to delimit Galapagos giant tortoise species focused on population genetic clustering,
464 mitochondrial monophyly, and amount of sequence divergence (e.g., Caccone *et al.* 1999,
465 Poulakakis *et al.* 2015, Loire *et al*. 2013, Kehlmaier *et al*. 2021). While these types of information
466 can provide some evidence concerning species boundaries, they lack a strong foundation in
467 modern population genetic theory as applied to species delimitation.

468 One such study claimed that genomic differentiation did not exist among *becki*,
469 *vandenburghi*, and *porteri* (Loire *et al.* 2013), but a re-analysis of the data with appropriate filtering
470 showed clear genomic differentiation of the three taxa (see Supplemental Figure S6 in Gaughran
471 *et al*. 2018). Another study comparing only mitochondrial DNA data between Galapagos giant
472 tortoises and extinct Caribbean giant tortoises argued that the recent divergence times of Galapagos
473 giant tortoise taxa relative to other tortoise species disqualified them from species status
474 (Kehlmaier *et al.* 2021). This view of species delimitation, however, ignores the complex reality
475 of speciation (see Donoghue 1985, Mallet 1995, Hey 2006). In addition, it ignores the fact that
476 some clades, such as those that colonized islands or other new environments, may be subject to
477 different demographic and selective forces that are associated with adaptive radiations. Outside of
478 tortoises, such a view would require a drastic re-delimitation of recently radiated species, with a
479 disproportionate effect on small, endangered island populations.

480 To date, all published genetic evidence, including the data from Loire *et al*. (2013),
481 supports the idea that Galapagos tortoise taxa are genetically distinct populations. Given the

482 statistical power of genome-wide SNPs to accurately detect differentiation (Gaughran *et al.* 2018),
483 this pattern of genetic distinctiveness appears unlikely to be overturned. Moving forward, genomic
484 discussions of Galapagos giant tortoise taxonomy should recognize that the preponderance of
485 evidence supports several genetically distinct taxa. However, whether these genetically distinct
486 populations deserve to be considered species is a more nuanced question.

487 The unified species concept (de Queiroz 2005b, 2007) provides one way to understand this
488 question. It highlights the idea of an independently evolving metapopulation lineage as the
489 keystone property of every species concept, with other properties (e.g., morphological
490 distinctiveness, genetic divergence, reproductive isolation) representing lines of evidence that a
491 taxon is an independently evolving lineage. The unified species concept thereby provides a
492 conceptual framework for evaluating candidate species that are morphologically similar, recently
493 diverged, or continuing to hybridize. The evidence we present here, combined with decades of
494 work documenting the distinctiveness of these taxa across multiple axes, shows how the Galapagos
495 giant tortoise taxa meet many criteria discussed as important aspects of modern species concepts.

496 As described above, the diverse methods we apply here are not magic solutions to species
497 delimitation, and each is the subject of ongoing debate. Still, interpreting our results holistically
498 provides some clarification on species delimitation in Galapagos giant tortoises and highlights
499 some areas of the taxonomy that remain difficult to resolve with our current data. Importantly, our
500 results largely refute the single-species model of Galapagos giant tortoise taxonomy that was
501 recently adopted by the IUCN Turtle Taxonomy Working Group (Rhodin *et al.* 2021). Instead, our
502 modeling in BPP delimits at least 12 species and our modeling in PHRAPL delimits 13 species.
503 On the other hand, the *gdi* results from BPP suggest 9 species, while those from PHRAPL suggest
504 between 7 and 9 species (Figure 2). We find significant support for the species status of some taxa,

505 especially the cases in which there is a single taxon per island. On the other hand, we find mixed
506 support for taxa inhabiting the same island as different species (i.e., Isabela Island and Santa Cruz
507 Island). Although model selection in BPP and PHRAPL supports the delimitation of species within
508 islands, the pattern of *gdi* results suggests that Santa Cruz Island may be home to a single species
509 and that the taxa of central and southern Isabela Island may also be a single species. Future work,
510 incorporating more samples for each taxon, will likely resolve the ambiguous delimitations by
511 making clear if the populations on Santa Cruz Island and central/southern Isabela Island have been
512 most affected by constant migration, secondary contact after divergence, or other demographic
513 processes.

514 Fundamentally, the taxonomic designation of Galapagos giant tortoises is both a scientific
515 and a philosophical question, and one that is deserving of genuine debate in the literature. Still,
516 this specific debate must be informed by decades of broader debates on species definitions and
517 methods of species delimitation. The rich literature around this topic highlights the fact that
518 speciation is a complex process: it can proceed at different rates and under different circumstances,
519 and our own temporally-biased observations mean that we necessarily study taxa at different stages
520 of this process. Galapagos giant tortoise taxa are evidently at different stages of lineage separation
521 and divergence and therefore offer an exciting system in which to study both species boundaries
522 and the process of speciation.

523

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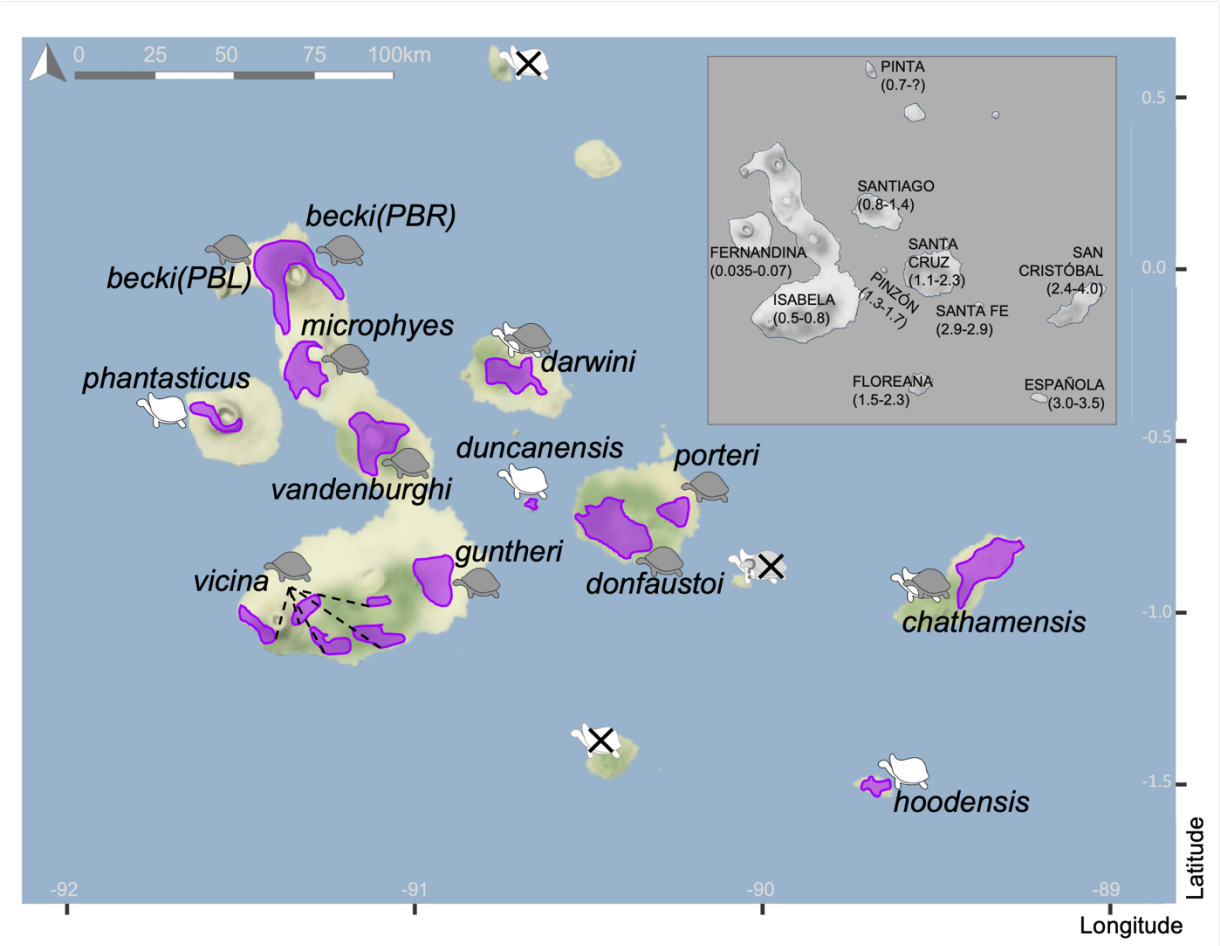

Figure 1. Galapagos giant tortoises are found on seven islands and form at least 13 genetically distinct taxa. Purple shapes indicate current ranges of populations. Carapace morphology (domed = gray, saddleback = white) is shown for each population, with "intermediate" shell shape indicated with overlapping icons. Icons with an "X" indicate extinct tortoise populations. Isabela Island and Santa Cruz Island are both home to multiple genetically distinct, allopatric populations. Inset map shows the names of islands with recently extant Galapagos giant tortoise taxa, with geological ages of each island in million years below.

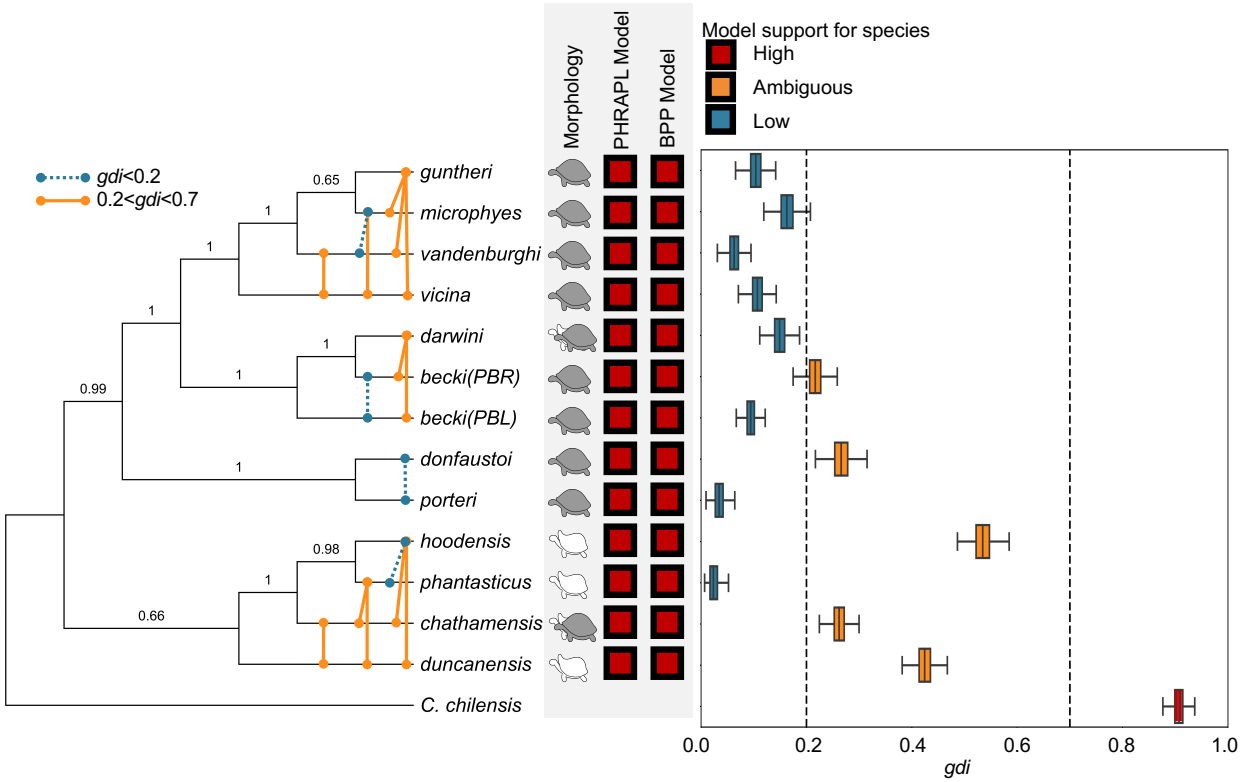

Figure 2. Multiple lines of evidence confirm the Galapagos giant tortoise species complex consists of multiple genetically divergent, independently evolving species. On the left, the cladogram generated from genome-wide SNPs in SNAPP, with branch posterior support values. Within-clade PHRAPL pairwise *gdi* values are indicated on the tree by colored lines connecting lineages. In the center, support for species delimitation models in PHRAPL and BPP. On the right, a boxplot of the *gdi* values estimated from the posterior distributions of τ and θ generated by BPP for each Galapagos giant tortoise taxon (red: *gdi* > 0.7, meeting the heuristic threshold for strongly delimited species; blue: *gdi* < 0.2, below the heuristic threshold for a single species; orange: 0.2 < *gdi* < 0.7, which is considered ambiguous species delimitation). Model-based species delimitation in BPP and PHRAPL model selection supported 13 species (not shown).

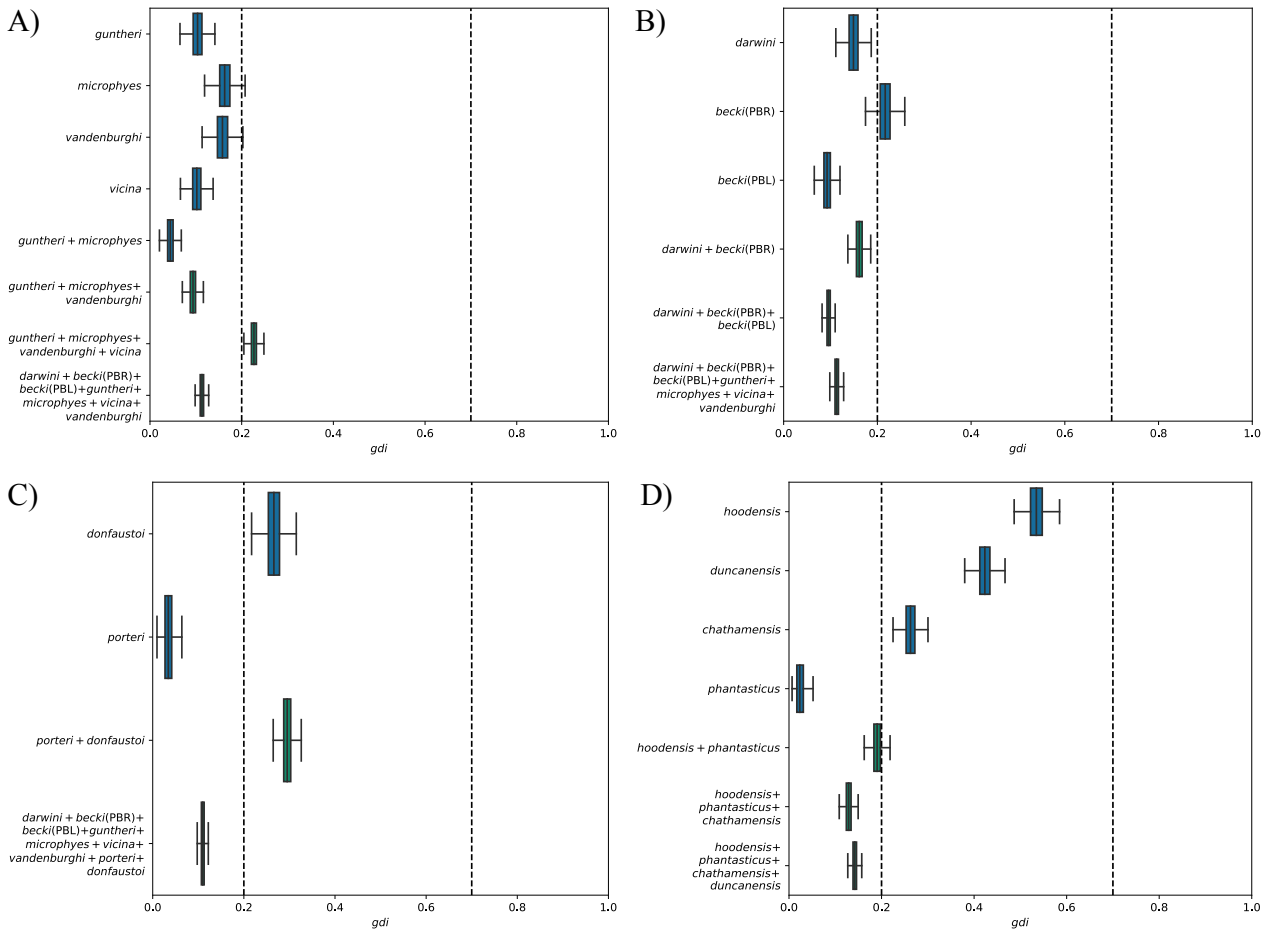

Figure 3. **Estimate *gdi* for progressively collapsed taxa shows support for some delimited species but not others.** *gdi* calculated for each taxon, including when sister taxa are collapsed following the method of Leaché *et al.* (2019). Blue boxes are single named taxa; green boxes are two or more taxa collapsed into a single "species." A) Isabela Island clade with progressive taxonomic collapse, showing a higher *gdi* above 0.2 when the four taxa from central and southern Isabela Island are collapsed. B) Santiago Island and northern Isabela Island clade with progressive taxonomic collapse, showing lower *gdi* when taxa are collapsed. C) Santa Cruz Island clade with progressive taxonomic collapse, showing a higher *gdi* above 0.2 when the two taxa are collapsed. D) Saddleback clade with progressive taxonomic collapse, showing lower *gdi* when taxa are collapsed.

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716 **Author Contributions**

717 SJG, RG, AC, and ELJ designed the study. SJG, RG, AO, MJ, AC, and ELJ carried out the
718 methodology. SJG, RG, MJ, NF, AO, JMM, NP, KdQ, and ELJ contributed to the investigation.
719 SJG, RG, AO, MJ, NF, and ELJ visualized the results. SJG, AC, and ELJ acquired funding for the
720 work. Project administration and supervision was provided by SJG, AC, and ELJ. SJG, RG, AO,
721 MJ, AC, and ELJ wrote the original draft, and all authors contributed to reviewing and editing
722 subsequent drafts.

723

724 **Data Accessibility**

725 The whole genome resequencing data from Jensen *et al*. (2022) is available under NCBI BioProject
726 PRJNA761229. Code for running BPP and PHRAPL, as well as alignments for loci used in these
727 analyses, are available at: https://github.com/sjgaughran/tortoise-species-delimitation. Output files
728 from SNAPP and BPP are available at: https://doi.org/10.5061/dryad.63xsj3v84

729 **Conflict of Interest**

730 The authors declare no conflicts of interest.

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740 **Supplemental Methods**

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742 Terminology, study design and data

743 We use several terms to refer to the demographic and evolutionary units of tortoises living
744 in the Galapagos. We use the term *population* in the evolutionary genetics sense of a group of
745 interbreeding individuals existing together in time and space (e.g., Hedrick 2009). We use the term
746 *lineage* in a slightly broader sense, to expand the time component of *population*, thereby including
747 ancestor-descendant relationships. We use the systematic term *taxon* to refer broadly to an
748 evolutionary unit (e.g., lineage) without specifying whether that unit is a species or a subspecies.
749 Because our goal is to evaluate the status of Galapagos giant tortoise taxa as species, we avoid
750 using the terms *species* and *subspecies* as *a priori* descriptors. To maintain this agnosticism, we
751 use only the epithet to refer to each taxon (e.g., *phantasticus* rather than *Chelonoidis phantasticus*
752 or *Chelonoidis niger phantasticus*).

753 We designed a whole-genome study of population distinctiveness and species delimitation,
754 taking advantage of existing illumina short-read whole genome resequencing data (Jensen *et al.*
755 2021, Jensen *et al*. 2022), which are available under NCBI Bioproject PRJNA761229. The samples
756 in our data set represent all extant taxa, which have previously been classified as populations,
757 species, or subspecies. The samples were originally selected for sequencing as representatives of
758 the genetic and geographic populations (Figure 1) that have previously been shown to exist using
759 microsatellite (Ciofi *et al.* 2002, Ciofi *et al.* 2006) and reduced representation SNP (Miller *et al.*
760 2018) data. Previous genetic research suggests that the *becki* taxon consists of two geographically
761 and genetically distinct populations, referred to in the literature as "PBL" (Piedras Blancas) and
762 "PBR" (Puerto Bravo) (Garrick *et al.* 2014). A prior phylogeny constructed from these genome

763 data in Jensen *et al*. (2022) showed the named taxa to be monophyletic, with the exception of
764 *becki*. Within *becki*, PBR individuals formed a clade sister to *darwini* and PBL individuals did not
765 form a clade, with one sample closest to PBR and *darwini* and the other two closest to other taxa
766 from Isabela. We have therefore chosen to test *becki (PBL)* and *becki (PBR)* as potentially distinct
767 taxa, and have retained the potentially admixed PBL individual to avoid biasing our analyses.

768 We mapped these reads to a reference genome for *abingdonii* (Quesada et al. 2019) using
769 bwa-mem (Li 2013). Jensen *et al.* (2021) previously investigated the potential for reference bias
770 in using the *abingdonii* assembly and found no evidence for such a bias. We called SNPs using the
771 BCFtools variant calling pipeline (Li et al. 2009). We then filtered the VCF for bi-allelic SNPs
772 that had a minimum GQ of 25, a minimum map quality score of 25, and at least 2 reads for each
773 allele in the genotype. We then created a consensus fasta file for each individual using BCFtools
774 and masked each consensus sequence for all missing genotypes from the VCF to ensure that
775 missing data were not erroneously assigned to the reference allele. We also masked every fasta
776 with a mask of repetitive regions downloaded from the UCSC Genome Browser and with a
777 mappable regions mask generated by Jensen *et al*. (2021). From these filtered and masked fastas,
778 we generated a random set of 1kb loci separated by 100kb. We retained loci that had less than 10%
779 missing data, GC content between 30% and 70%, and at least one variable site. Because BPP
780 requires phased data when using more than ~100 loci, we phased each locus using PHASE v. 2.1.1
781 (Stephens *et al*. 2001, Stephens and Scheet 2005). We then used several methods to delimit species
782 under different phylogenetic and coalescent models.

783

784 <u>SNAPP Tree Generation</u>

785 We used SNAPP v1.5.2 (Bryant *et al.* 2012) and MODEL_SELECTION v1.5.3 (Baele *et*
786 *al.* 2012), as implemented in BEAST2 v2.6.7 (Bouckaert *et al.* 2014), to generate a guide tree
787 reflecting the phylogenetic relationships among the extant populations of Galapagos giant tortoises
788 for downstream species delimitation comparisons. We first tested 21 different phylogenetic
789 models on a dataset comprising 1,000 random, unlinked SNPs—and corresponding genotypes—
790 from 39 samples. We obtained this dataset from a previous study conducted by Jensen et al. (2022)
791 and it included one Chaco tortoise (*C. chilensis*) sample to serve as an outgroup to the Galapagos
792 giant tortoises. We ensured the Galapagos giant tortoises presented genetic variation across loci
793 and we allowed no missing data.

794 We grouped the samples into populations, assumed forward and backward mutation rates
795 equivalent to the unit ($u = v = 1$), and used combinations of shape ($\alpha = 5, 12, 30$), scale ($\beta = 50,$
796 $60, 70, 80, 110, 150$), and speciation rate ($\lambda = 0.01, 10, 39$) parameters as priors to constitute the
797 models to be tested (see Table S1). Path sampling runs for each model consisted of 24 steps,
798 100,000 MCMC generations sampled every 100 generations, and a 50% burn-in. We compared
799 the resulting marginal likelihood estimates from each run and selected the best model via Bayes
800 factor delimitation (Kass & Raftery 1995) after using the default model ($\alpha = 12, \beta = 110, \lambda = 0.01$)
801 as a reference.

802 For the best model, we then used an additional set of 4,000 random, unlinked biallelic SNPs
803 (5,000 SNPs total) for final phylogenetic reconstruction. For this dataset, we performed four
804 independent runs consisting of 2,000,000 MCMC generations sampled every 1,000 generations
805 and a 10% burn-in. We used Tracer v1.7.2 (Rambaut *et al.* 2018) to assess statistical convergence
806 across runs and LogCombiner v2.6.7 (Drummond & Rambaut 2007) to summarize the posterior
807 trees.

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809 Divergence time from BPP

810 We can calculate the absolute divergence time in generations from τ, the rate-scaled

811 divergence time in BPP, as:

812

813 $T = \tau/\mu$

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815 Where μ is the per-generation mutation rate. Although the per-generation mutation rate in

816 Galapagos giant tortoises is unknown, we use $6x10^{-9}$ reflecting the *de novo* mutation rate recently

817 measured in the painted turtle (Bergeron *et al*. 2023).

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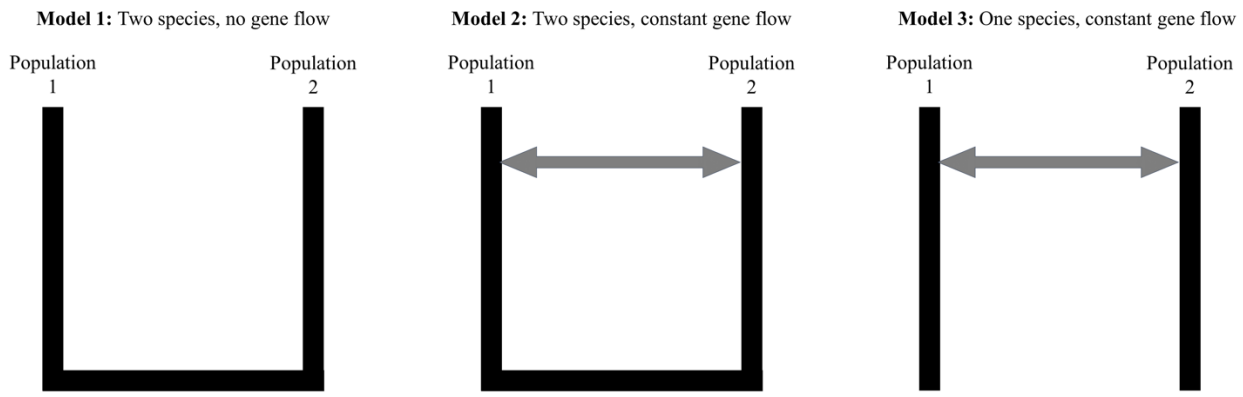
831 **Supplemental Figures and Tables**

832



833

834 **Figure S1.** The three models used to test one and two species models under differing gene flow

835 scenarios in PHRAPL. The grey arrow indicates bi-directional gene flow between the two

836 populations.

837

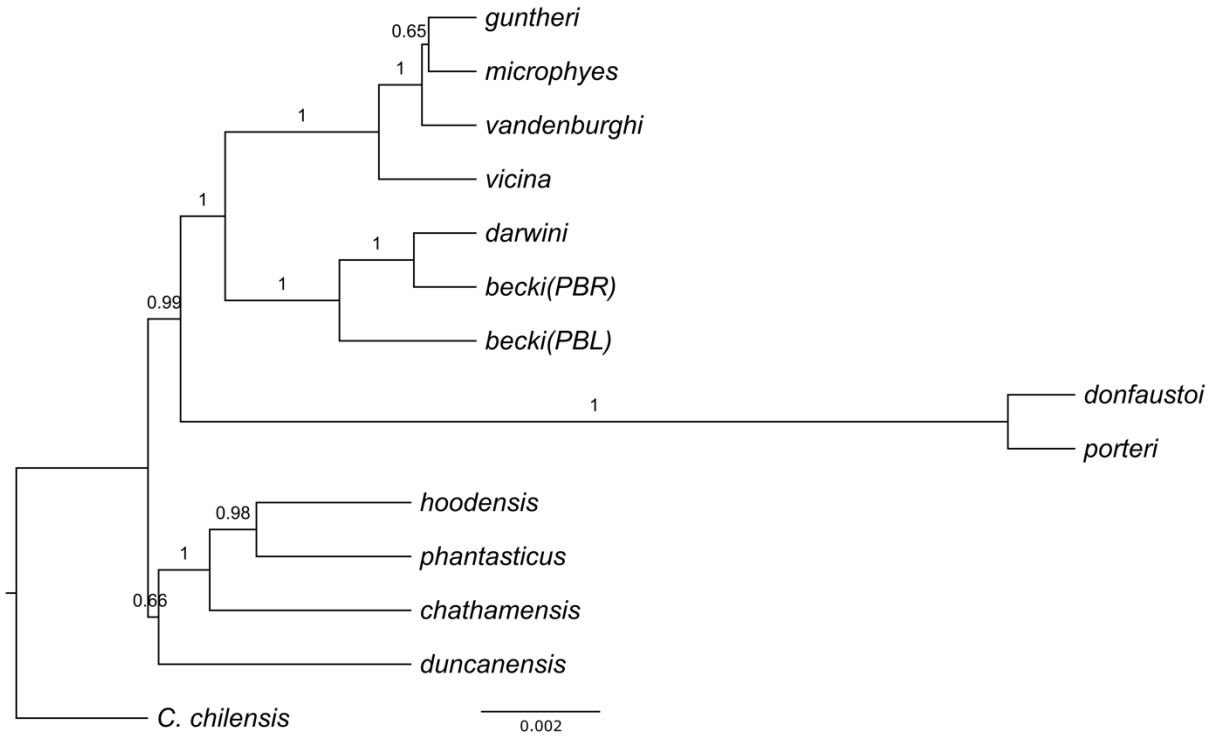


Figure S2. Phylogeny with scaled branch lengths generated from 5,000 genome-wide SNPs in SNAPP from the best supported model ($\alpha = 5$, $\beta = 150$, $\lambda = 39$) with branch posterior support values indicated. The scale bar indicates the number of nucleotide substitutions per site.

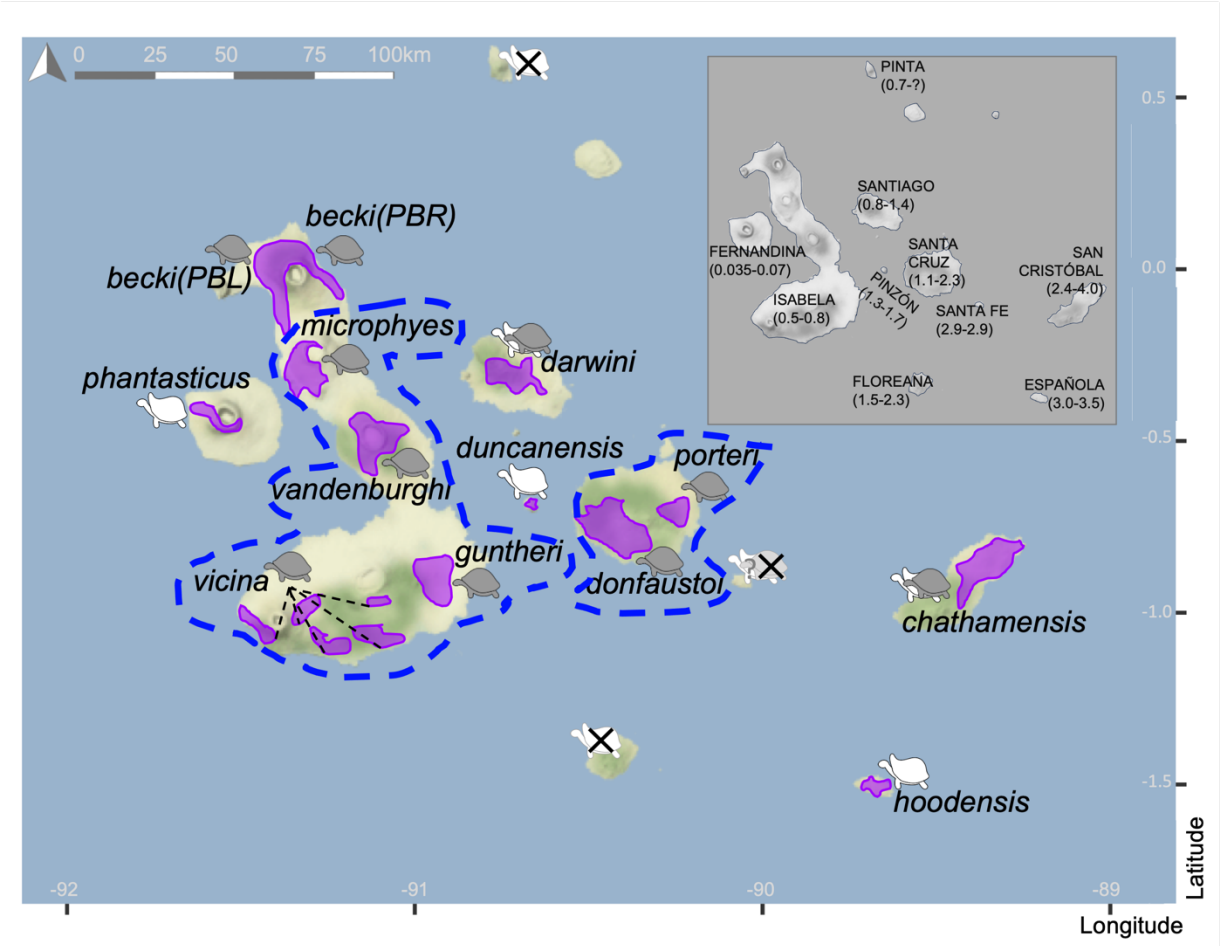


Figure S3. Galapagos giant tortoise species ranges when the Santa Cruz Island taxa (*porteri* and *donfaustoi*) are collapsed and the central and southern Isabela Island (*microphyes*, *vandenburghi*, *guntheri*, and *vicina*) are collapsed. Purple shapes indicate current ranges of populations. Blue dotted lines surround taxa that are collapsed into a single species according to some of our analyses. Carapace morphology (domed = gray, saddleback = white) is shown for each population, with "intermediate" shell shape indicated with overlapping icons. Icons with an "X" indicate extinct tortoise populations. Inset map shows the names of islands with recently extant Galapagos giant tortoise taxa, with geological ages of each island in million years below.

861 **Table S1.** Summary statistics from 21 different phylogenetic models tested on 1,000 random,
862 unlinked biallelic SNPs—and corresponding genotypes—from 39 tortoises arranged into
863 populations. Each run consisted of 24 steps, 100,000 MCMC generations sampled every 100
864 generations, and a 50% burn-in. The default, reference model for Bayes factor delimitation is
865 shown with an asterisk (*).

866

Model	α	β	λ	Effective Sample Size	Marginal Likelihood	Rank	Bayes Factor
1	5	50	0.01	1,330.47	–14,556.42	8	–82.70
2	5	50	10	1,994.26	–14,471.91	4	–251.73
3	5	50	39	1,204.93	–14,456.56	2	–282.42
4	5	150	0.01	1,816.32	–14,552.45	7	–90.64
5	5	150	10	1,808.11	–14,463.95	3	–267.65
6	5	150	39	1,574.37	–14,448.23	1	–299.07
7*	12	110	0.01	1,749.29	–14,597.77	9	–
8	12	110	10	2,007.71	–14,509.09	6	–177.35
9	12	110	39	1,430.58	–14,496.23	5	–203.09
10	30	60	0.01	2,476.20	–15,036.56	21	+877.57
11	30	60	10	2,939.10	–14,929.72	18	+663.89
12	30	60	39	2,235.82	–14,917.57	17	+639.60
13	30	70	0.01	2,597.08	–14,985.58	20	+775.62
14	30	70	10	2,258.89	–14,882.03	16	+568.52
15	30	70	39	2,420.92	–14,872.60	15	+549.67
16	30	80	0.01	2,347.20	–14,941.95	19	+688.35
17	30	80	10	2,904.94	–14,841.68	14	+487.81
18	30	80	39	2,179.97	–14,831.56	13	+467.57
19	30	150	0.01	2,060.26	–14,764.49	12	+333.44
20	30	150	10	1,920.77	–14,675.53	11	+155.51
21	30	150	39	2,119.72	–14,663.75	10	+131.95

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Table S2. Parameter values for migration (*m*) and divergence (*t*) used for the grid search within PHRAPL. The maximum divergence time is equivalent to 1Ma.

m	0.005	0.02	0.08	0.12	0.25	0.4	0.8
t	0.01	0.05	0.1	0.25	0.5	0.75	1

892 **Table S3.** Posterior output from the A11 analysis species delimitation model in BPP using phased

893 loci from the 38 Galapagos giant tortoise individuals. Support for collapsed taxa as species is

894 included in the final column. The "Medium θ" prior is the most realistic prior.

895

***θ* prior**	**No. loci**	**Seed**	**Posterior for number of species**	**Posteriors for collapsed taxonomic groups or notes**
Small θ	50 loci	Seed 1	P[13]=0.997	
		Seed 2	P[13]=0.998	
		Seed 3	P[13]=0.999	
	200 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[13]=1.000	
	500 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[1]=1.000	single_species=1.000
	1000 loci	Seed 1	P[7]=0.162 P[6]=0.079 P[1]=0.752	single_species=0.752 *darwini*+*beck*(PBR)=0.248 *porteri*+*donfaustoi*=0.243 *phantasticus*+*hoodensis*=0.243 *guntheri*+*vandenburghi*+ *microphyes*+*vicina*=0.162 *guntheri*+*vandenburghi*+ *microphyes*+*vicina*+ *becki (PBL)*=0.162
		Seed 2	P[13]=1.000	
		Seed 3	P[13]=1.000	
Medium θ	50 loci	Seed 1	P[13]=0.988 P[12]=0.012	*guntheri*+*vandenburghi*=0.010
		Seed 2	P[13]=0.991	
		Seed 3	P[13]=0.992	
	200 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[13]=1.000	
	500 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[13]=1.000	
	1000 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[13]=1.000	
Large θ	50 loci	Seed 1	P[13]=0.282	*guntheri*+*vandenburghi*=0.259

			P[12]=0.413 P[11]=0.261	*becki (PBR)+darwini*=0.175 *guntheri+microphyes*=0.161 *vandenburghi+vicina*=0.079 *guntheri+vandenburghi+ microphyes*=0.077 *porteri+donfaustoi*=0.068 *guntheri+vandenburghi+ vicina*=0.068 *guntheri+vicina*=0.023
		Seed 2	P[13]=0.192 P[12]=0.304 P[11]=0.297 P[10]=0.155 P[9]=0.048	*guntheri+vandenburghi*=0.197 *becki (PBR)+darwini*=0.176 *guntheri+vandenburghi+ microphyes*=0.161 *guntheri+vandenburghi+ microphyes+vicina*=0.148 *guntheri+microphyes*=0.112 *porteri+donfaustoi*=0.094 *vicina+vandenburghi*=0.058 *vicina+vandenburghi+ guntheri*=0.052 *vicina+guntheri*=0.036
		Seed 3	P[13]=0.060 P[12]=0.118 P[11]=0.146 P[10]=0.541 P[9]=0.127	*guntheri+vandenburghi+ microphyes+vicina*=0.632 *becki (PBR)+darwini*=0.160 *guntheri+microphyes*=0.079 *guntheri+vandenburghi+ microphyes*=0.077 *porteri+donfaustoi*=0.073 *guntheri+vandenburghi*=0.064 *vicina+vandenburghi*=0.038 *vicina+vandenburghi+ guntheri*=0.032 *vicina+guntheri*=0.010
	200 loci	Seed 1	P[13]=0.924 P[12]=0.076	*guntheri+vandenburghi*=0.073
		Seed 2	P[13]=0.897 P[12]=0.103	*guntheri+vandenburghi*=0.103
		Seed 3	P[13]=0.957 P[12]]=0.043	*guntheri+vandenburghi*=0.043
	500 loci	Seed 1	P[13]=0.258 P[12]=0.484 P[11]=0.053 P[10]=0.204	*guntheri+microphyes*=0.524 *guntheri+microphyes+ vandenburghi+vicina*=0.204 *vandenburghi+vicina*=0.040 *guntheri+microphyes+ vandenburghi*=0.012
		Seed 2	P[13]=1.000	

		Seed 3	P[1]=1.000	single_species=1.000
	1000 loci	Seed 1	P[13]=1.000	
		Seed 2	P[13]=1.000	
		Seed 3	P[11]=0.710 P[10]=0.290	*guntheri*+*microphyes*+ *vandenburghi* =0.710 *guntheri*+*microphyes*+ *vandenburghi*+*vicina*=0.290

915 **Table S4.** Posterior output from the A11 analysis species delimitation model in BPP using phased
916 loci from the 38 Galapagos giant tortoise individuals and the Chaco tortoise individual. Support
917 for collapsed taxa as species is included in the final column. The "Medium θ" prior is the most
918 realistic prior.
919

θ prior	No. loci	Seed	Posterior for number of species	Posteriors for collapsed taxonomic groups or notes
Small θ	50 loci	Seed 1	P[12]=0.993	*guntheri+microphyes+ vandenburghi*=0.993
		Seed 2	P[13]=0.273 P[12]=0.716	*guntheri+microphyes+ vandenburghi*=0.716 *guntheri + vandenburghi*=0.270
		Seed 3	P[12]=0.949 P[11]=0.051	*guntheri+microphyes+ vandenburghi*=0.995 *phantasticus+becki (PBL)*=0.045
	200 loci	Seed 1	P[14]=0.809 P[12]=0.187	*guntheri+microphyes+ vandenburghi*=0.187
		Seed 2	P[14]=1.000	
		Seed 3	P[12]=0.790 P[11]=0.210	*guntheri+microphyes+ vandenburghi*=0.790 *guntheri+microphyes+ vandenburghi+vicina*=0.210
	500 loci	Seed 1	P[14]=1.000	
		Seed 2	P[14]=0.723 P[12]=0.121 P[11]=0.025 P[10]=0.025 P[2]=0.095	all_single_species=0.095 *guntheri+vandenburghi+ microphyes*=0.121 *guntheri+vandenburghi+ microphyes+vicina*=0.051 *porteri+donfaustoi*=0.026
		Seed 3	P[14]=1.000	
	830 loci	Seed 1	P[14]=1.000	
		Seed 2	P[14]=1.000	
		Seed 3	P[14]=1.000	
Medium θ	50 loci	Seed 1	P[14]=0.018 P[13]=0.335 P[12]=0.646	*guntheri+microphyes+ vandenburghi*=0.351 *guntheri+vandenburghi*=0.316 *guntheri+vandenburghi+ vicina*=0.289 *vandenburghi+vicina*=0.018
		Seed 2	P[14]=0.012	*guntheri+microphyes+*

			P[13]=0.159 P[12]=0.785 P[11]=0.045	*vandenburghi*=0.537 *guntheri+vandenburghi+ vicina*=0.226 *vandenburghi+vicina*=0.079 *guntheri+vandenburghi*=0.063 *guntheri+microphyes+ vandenburghi+vicina*=0.045 *guntheri+microphyes*=0.041 *microphyes+vandenburghi*=0.012
		Seed 3	P[14]=0.016 P[13]=0.216 P[12]=0.767	*guntheri+microphyes+ vandenburghi*=0.717 *guntheri+vandenburghi*=0.177 *guntheri+vandenburghi+ vicina*=0.051 *guntheri+vandenburghi*=0.040
	200 loci	Seed 1	P[14]=0.992	
		Seed 2	P[14]=0.961 P[13]=0.039	*guntheri+vandenburghi*=0.039
		Seed 3	P[14]=0.524 P[13]=0.076 P[12]=0.352 P[10]=0.042	*guntheri+microphyes+ vandenburghi*=0.352 *guntheri+vandenburghi*=0.072 *guntheri+microphyes+ vandenburghi+vicina*=0.048 *porteri+donfaustoi*=0.042
	500 loci	Seed 1	P[14]=0.810 P[13]=0.017 P[12]=0.053 P[11]=0.056 P[9]=0.014 P[8]=0.048	*guntheri+microphyes+ vandenburghi+vicina+ becki (PBL)*=0.064 *porteri+donfaustoi*=0.062 *guntheri+microphyes+ vandenburghi+vicina*=0.056 *guntheri+microphyes+ vandenburghi*=0.053 *darwini+becki (PBR)*=0.048 *guntheri+microphyes*=0.017
		Seed 2	P[14]=0.892 P[13]=0.025 P[12]=0.050 P[11]=0.033	*guntheri+microphyes+ vandenburghi*=0.050 *guntheri+microphyes+ vandenburghi+vicina*=0.033 *guntheri+microphyes*=0.025
		Seed 3	P[14]=1.000	
	830 loci	Seed 1	P[14]=1.000	
		Seed 2	P[14]=1.000	
		Seed 3	P[14]=1.000	
Large $\boldsymbol{\theta}$	50 loci	Seed 1	P[12]=0.044 P[11]=0.388 P[10]=0.549	*guntheri+microphyes+ vandenburghi+vicina*=0.896 *porteri+donfaustoi*=0.607

			P[9]=0.022	*guntheri+microphyes+ vandenburghi*=0.098 *becki (PBL)+becki (PBR)*=0.031
		Seed 2	P[12]=0.081 P[11]=0.442 P[10]=0.464 P[9]=0.011	*guntheri+microphyes+ vandenburghi+vicina*=0.671 *porteri+donfaustoi*=0.690 *guntheri+microphyes+ vandenburghi*=0.285 *becki (PBL)+becki (PBR)*=0.042 *guntheri+vicina+ vandenburghi*=0.023 *guntheri+microphyes*=0.020 *vandenburghi*+vicina=0.017
		Seed 3	P[12]=0.013 P[11]=0.342 P[10]=0.613 P[9]=0.032	*guntheri+microphyes+ vandenburghi+vicina*=0.872 *porteri+donfaustoi*=0.737 *guntheri+microphyes+ vandenburghi*=0.111 *becki (PBL)+becki (PBR)*=0.048 *microphyes+guntheri*=0.010
	200 loci	Seed 1	P[11]=0.683 P[10]=0.248 P[9]=0.070	*guntheri+microphyes+ vandenburghi+vicina*=1.000 *porteri+donfaustoi*=0.317 *darwini+becki (PBR)*=0.070
		Seed 2	P[12]=0.690 P[11]=0.253 P[10]=0.057	*guntheri+microphyes+ vandenburghi+vicina*=0.310 *guntheri+microphyes+ vandenburghi* =0.690 *porteri+donfaustoi*=0.057
		Seed 3	P[11]=1.000	*guntheri+microphyes+ vandenburghi+vicina*=1.000
	500 loci	Seed 1	P[14]=1.000	
		Seed 2	P[11]=1.000	*guntheri+microphyes+ vandenburghi+vicina*=1.000
		Seed 3	P[11]=1.000	*guntheri+microphyes+ vandenburghi+vicina*=1.000
	830 loci	Seed 1	P[11]=1.000	*guntheri+microphyes+ vandenburghi+vicina*=1.000
		Seed 2	P[14]=1.000	
		Seed 3	P[11]=1.000	*guntheri+microphyes+ vandenburghi+vicina*=1.000

922 **Table S5.** Posterior output from the A11 analysis species delimitation model in BPP using
923 unphased loci from the 38 Galapagos giant tortoise individuals. Support for collapsed taxa as
924 species is included in the final column. The "Medium θ" prior is the most realistic prior.

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***θ* prior**	**No. loci**	**Seed**	**Posterior for number of species**	**Posteriors for collapsed taxonomic groups or notes**
Small ***θ***	50 loci	Seed 1	P[13]=0.986 P[12]=0.014	*porteri+donfaustoi*=0.013
		Seed 2	P[13]=0.997	
		Seed 3	P[13]=0.995	
Medium ***θ***	50 loci	Seed 1	P[13]=0.992	
		Seed 2	P[13]=0.993	
		Seed 3	P[1]=1.000	single_species=1.000
Large ***θ***	50 loci	Seed 1	P[13]=0.577 P[12]=0.357 P[11]=0.063	*guntheri+vandenburghi*=0.228 *darwini+becki (PBR)*=0.056 *microphyes+guntheri*=0.037 *vandenburghi+vicina*=0.036 *guntheri+vicina*=0.036 *porteri+donfaustoi*=0.026 *guntheri+vandenburghi+ vicina*=0.025
		Seed 2	P[13]=0.517 P[12]=0.334 P[11]=0.066 P[10]=0.071 P[9]=0.012	*guntheri+vandenburghi*=0.232 *darwini+becki (PBR)*=0.066 *guntheri+vandenburghi+ microphyes+vicina*=0.075 *microphyes+guntheri*=0.050 *porteri+donfaustoi*=0.031 *guntheri+vicina*=0.030 *guntheri+vandenburghi+ vicina*=0.029 *vandenburghi+vicina*=0.027
		Seed 3	P[13]=0.493 P[12]=0.391 P[11]=0.106 P[10]=0.010	*guntheri+vandenburghi*=0.248 *darwini+becki (PBR)*=0.092 *microphyes+guntheri*=0.064 *guntheri+vicina*=0.039 *guntheri+vandenburghi+ vicina*=0.039 *vandenburghi+vicina*=0.036 *porteri+donfaustoi*=0.024 *guntheri+vandenburghi+ microphyes* =0.022

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928 **Table S6.** Posterior output from the A11 analysis species delimitation model in BPP using
929 unphased loci from the 38 Galapagos giant tortoise individuals and the Chaco tortoise individual.
930 Support for collapsed taxa as species is included in the final column. The "Medium θ" prior is the
931 most realistic prior.

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***θ* prior**	**No. loci**	**Seed**	**Posterior for number of species**	**Posteriors for collapsed taxonomic groups or notes**
Small ***θ***	50 loci	Seed 1	P[13]=0.036 P[12]=0.964	*guntheri*+*vandenburghi*+ *vicina*=0.964 *guntheri*+*vandenburghi*=0.036
		Seed 2	P[13]=0.173 P[12]=0.826	*guntheri*+*vandenburghi*+ *vicina*=0.449 *guntheri*+*vandenburghi*+ *microphyes*=0.378 *guntheri*+*vandenburghi*=0.173
		Seed 3	P[12]=0.987	*guntheri*+*vandenburghi*+ *microphyes*=0.628 *guntheri*+*vandenburghi*+ *vicina*=0.359
Medium ***θ***	50 loci	Seed 1	P[13]=0.401 P[12]=0.589	*guntheri*+*vandenburghi*+ *microphyes*=0.118 *guntheri*+*vandenburghi*+ *vicina*=0.472 *guntheri*+*vandenburghi*=0.400
		Seed 2	P[14]=0.010 P[13]=0.157 P[12]=0.832	*guntheri*+*vandenburghi*+ *microphyes*=0.735 *guntheri*+*vandenburghi*=0.146 *guntheri*+*vandenburghi*+ *vicina*=0.097 *guntheri*+*microphyes*=0.011
		Seed 3	P[14]=0.010 P[13]=0.110 P[12]=0.879	*guntheri*+*vandenburghi*+ *vicina*=0.573 *guntheri*+*vandenburghi*+ *microphyes*=0.301 *guntheri*+*vandenburghi*=0.080 *guntheri*+*microphyes*=0.024 *vandenburghi*+*microphyes*=0.011
Large ***θ***	50 loci	Seed 1	P[11]=0.241 P[10]=0.634	*guntheri*+*vandenburghi*+ *microphyes*+*vicina*=0.980

			P[9]=0.123	*porteri+donfaustoi*=0.623 *becki (PBL)+becki (PBR)*=0.232 *darwini+becki (PBR)*=0.042
		Seed 2	P[12]=0.022 P[11]=0.385 P[10]=0.430 P[9]=0.163	*guntheri+vandenburghi+ microphyes+vicina*=0.841 *porteri+donfaustoi*=0.536 *becki (PBL)+becki (PBR)*=0.344 *guntheri+vandenburghi+ microphyes* =0.159 *darwini+becki (PBR)*=0.011
		Seed 3	P[11]=0.235 P[10]=0.497 P[9]=0.263	*guntheri+vandenburghi+ microphyes+vicina*=0.997 *porteri+donfaustoi*=0.624 *becki (PBL)+becki (PBR)*=0.349 *darwini+becki (PBL)+becki (PBR)*=0.019 *darwini+becki (PBR)*=0.019

949 **Table S7.** Posterior output from the A10 analysis species delimitation model in BPP using phased
950 loci from the 38 Galapagos giant tortoise individuals. Nodes in the guide tree with a posterior
951 probability <0.95 were counted as a single species. The "Medium θ" prior is the most realistic
952 prior. A maximum of 13 species (i.e., each Galapagos giant tortoise taxon) was possible.

***θ* prior**	**No. loci**	**Seed**	**Number of species with >0.95 posterior support**
Small ***θ***	50 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	200 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	500 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	1000 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
Medium ***θ***	50 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	200 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	500 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
	1000 loci	Seed 1	13
		Seed 2	13
		Seed 3	13
Large ***θ***	50 loci	Seed 1	8
		Seed 2	9
		Seed 3	9
	200 loci	Seed 1	11
		Seed 2	10
		Seed 3	11
	500 loci	Seed 1	12
		Seed 2	12
		Seed 3	12
	1000 loci	Seed 1	12

		Seed 2	13
		Seed 3	13

Table S8. Posterior output from the A10 analysis species delimitation model in BPP using phased loci from the 38 Galapagos giant tortoise individuals and the Chaco tortoise individual. Nodes in the guide tree with a posterior probability <0.95 were counted as a single species. The "Medium θ" prior is the most realistic prior. A maximum of 14 species (i.e., each Galapagos giant tortoise taxon and the Chaco tortoise) was possible.

θ prior	**No. loci**	**Seed**	**Number of species with >0.95 posterior support**
Small θ	50 loci	Seed 1	10
		Seed 2	11
		Seed 3	12
	200 loci	Seed 1	14
		Seed 2	13
		Seed 3	11
	500 loci	Seed 1	10
		Seed 2	12
		Seed 3	10
	830 loci	Seed 1	14
		Seed 2	10
		Seed 3	14
Medium θ	50 loci	Seed 1	12
		Seed 2	12
		Seed 3	12
	200 loci	Seed 1	14
		Seed 2	14
		Seed 3	12
	500 loci	Seed 1	14
		Seed 2	14
		Seed 3	14
	830 loci	Seed 1	14
		Seed 2	14
		Seed 3	14
Large θ	50 loci	Seed 1	9
		Seed 2	10
		Seed 3	10
	200 loci	Seed 1	12

		Seed 2	12
		Seed 3	11
	500 loci	Seed 1	11
		Seed 2	13
		Seed 3	12
	830 loci	Seed 1	13
		Seed 2	11
		Seed 3	12

Table S9. Divergence time estimates in generations from BPP, with prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001). Median and 95% credible interval calculated from the posterior distribution of τ. Divergence time calculated using a per-generation mutation rate of 6×10^{-9}.

Taxon	**Median divergence time in generations (95% credible interval)**
darwini	2667 (2000–3333)
becki (PBR)	2667 (2000–3333)
guntheri	1333 (1167–1667)
microphyes	1333 (1167–1667)
vandenburghi	1500 (1333–1667)
vicina	2333 (2000–2667)
becki (PBL)	3833 (3167–4667)
donfaustoi	3167 (2500–4000)
porteri	3167 (2500–4000)
hoodensis	4500 (3500–5500)
phantasticus	4500 (3500–5500)
duncanensis	5667 (4833–6833)
chathamensis	6333 (5500–7833)
C. chilensis	368,500 (352,667–392,167)

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987 **Table S10**. Effective population size estimates from BPP, with prior $\theta \sim$ IG(3, 0.001) and prior τ

988 $\sim$ IG(3, 0.001) and an assumed mutation rate of 6.0 x 10^{-9}. Median and 95% credible interval

989 calculated from the posterior distribution of θ. Effective population size calculated using a per-

990 generation mutation rate of 6×10^{-9}.

Taxon	**Median effective population size (95% credible interval)**
darwini	8208 (6000–10,667)
becki (PBR)	5458 (4083–6958)
guntheri	6416 (4833–8833)
microphyes	3917 (3125–5083)
vandenburghi	12,125 (8583–19,042)
vicina	27,333 (25,542–29,208)
becki (PBL)	19,750 (15,208–25,875)
donfaustoi	5083 (3958–6625)
porteri	45,125 (26,083–94,333)
hoodensis	2917 (2333–3542)
phantasticus	94,042 (45,208–223,375)
duncanensis	5750 (4875–7125)
chathamensis	9333 (7833–11,375)
C. chilensis	77,917 (71,292–85,292)

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996 **Table S11**. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ

997 in BPP, with prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini	0.149 (0.140–0.159)
becki (PBR)	0.218 (0.187–0.247)
guntheri	0.104 (0.077–0.131)
microphyes	0.162 (0.132–0.194)
vandenburghi	0.063 (0.040–0.087)
vicina	0.107 (0.082–0.134)
becki (PBL)	0.094 (0.075–0.115)
donfaustoi	0.266 (0.231–0.278)
porteri	0.034 (0.016–0.058)
hoodensis	0.534 (0.497–0.572)
phantasticus	0.023 (0.010–0.049)
duncanensis	0.423 (0.393–0.455)
chathamensis	0.262 (0.236–0.290)

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1006 **Table S12**. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ

1007 in BPP, with prior $\theta \sim$ IG(3, 0.005) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini	0.137 (0.111–0.166)
becki (PBR)	0.197 (0.168–0.227)
guntheri	0.083 (0.060–0.108)
microphyes	0.134 (0.106–0.160)
vandenburghi	0.048 (0.029–0.069)
vicina	0.092 (0.070–0.116)
becki (PBL)	0.091 (0.072–0.112)
donfaustoi	0.235 (0.223–0.271)
porteri	0.028 (0.008–0.049)
hoodensis	0.515 (0.477–0.551)
phantasticus	0.028 (0.010–0.052)
duncanensis	0.429 (0.398–0.460)
chathamensis	0.268 (0.241–0.296)

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1016 **Table S13**. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ

1017 in BPP, with prior $\theta \sim$ IG(3, 0.0001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini	0.152 (0.124–0.180)
becki (PBR)	0.223 (0.193–0.255)
guntheri	0.109 (0.077–0.143)
microphyes	0.171 (0.133–0.210)
vandenburghi	0.064 (0.041–0.090)
vicina	0.107 (0.081–0.135)
becki (PBL)	0.094 (0.074–0.115)
donfaustoi	0.271 (0.233–0.309)
porteri	0.032 (0.012–0.058)
hoodensis	0.537 (0.502–0.573)
phantasticus	0.025 (0.013–0.046)
duncanensis	0.413 (0.381–0.446)
chathamensis	0.256 (0.228–0.283)

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1025 **Table S14**. Estimates of *gdi* for each taxon after the first round of sister taxa were collapsed. The

1026 *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP, with

1027 prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)	0.161 (0.144–0.180)
guntheri+microphyes	0.044 (0.029–0.062)
vandenburghi	0.158 (0.127–0.190)
vicina	0.105 (0.080–0.131)
becki (PBL)	0.093 (0.074–0.113)
donfaustoi+porteri	0.291 (0.268–0.314)
hoodensis+phantasticus	0.190 (0.170–0.211)
duncanensis	0.427 (0.396–0.460)
chathamensis	0.263 (0.235–0.291)

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1039 **Table S15**. Estimates of *gdi* for each taxon after the second round of sister taxa were collapsed.

1040 The *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP,

1041 with prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)+becki (PBL)	0.096 (0.086–0.106)
guntheri+microphyes+vandenburghi	0.094 (0.078–0.111)
vicina	0.102 (0.077–0.129)
donfaustoi+porteri	0.295 (0.273–0.318)
hoodensis+ phantasticus+chathamensis	0.129 (0.115–0.145)
duncanensis	0.423 (0.392–0.456)

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1044 **Table S16**. Estimates of *gdi* for each taxon after the third round of sister taxa were collapsed. The

1045 *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP, with

1046 prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)+becki (PBL)	0.099 (0.089–0.110)

guntheri+microphyes+vandenburghi+vicina	0.226 (0.211–0.243)
donfaustoi+porteri	0.298 (0.275–0.322)
hoodensis+ phantasticus+duncanensis+ chathamensis	0.144 (0.134–0.156)

Table S17. Estimates of *gdi* for each taxon after the fourth round of sister taxa were collapsed. The *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP, with prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)+becki (PBL)+ guntheri+microphyes+vandenburghi+vicina	0.113 (0.103–0.124)
donfaustoi+porteri	0.287 (0.264–0.310)
hoodensis+phantasticus+duncanensis+ chathamensis	0.144 (0.132–0.155)

Table S18. Estimates of *gdi* for each taxon after the fifth round of sister taxa were collapsed. The *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP, with prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)+becki (PBL)+ guntheri+microphyes+vandenburghi+vicina+ donfaustoi+porteri	0.110 (0.102–0.118)
hoodensis+ phantasticus+duncanensis+ chathamensis	0.142 (0.132–0.153)

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1061 **Table S19**. Estimates of *gdi* for each taxon after the sixth round of sister taxa were collapsed. The

1062 *gdi* was calculated from the posterior distributions of θ and τ of the collapsed taxa in BPP, with

1063 prior $\theta \sim$ IG(3, 0.001) and prior $\tau \sim$ IG(3, 0.001).

Taxon	**Median *gdi* (95% credible interval)**
darwini+becki (PBR)+becki (PBL)+ guntheri+microphyes+vandenburghi+vicina+ donfaustoi+porteri+ hoodensis+ phantasticus+duncanensis+chathamensis	0.988 (0.984–0.991)
C. chilensis	0.92 (0.901–0.939)

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1066 **Table S20:** Pairwise comparisons analyzed in PHRAPL across the two main clades of the phylogenetic tree, along with the wAIC for
1067 each of the three PHRAPL models. Models with a high Akaike weight (wAIC) > 0.9 for a model set are highlighted in gray. The
1068 cumulative sum of wAIC for all two species models is given for each comparison, with wAIC > 0.9 also highlighted in gray. Mean *gdi*
1069 < 0.2 are shown in bold.

Taxon 1	**Taxon 2**	**wAIC Model 1** **2 species, no gene flow**	**wAIC Model 2** **2 species, constant gene flow**	**wAIC Model 3** **1 species**	**Sum wAIC of 2 Species Models**	**Mean *gdi***
Within Saddleback Clade						
duncanensis	*hoodensis*	0.984	0.016	0.000	1.000	0.387
chathamensis	*hoodensis*	0.465	0.535	0.000	1.000	0.384
chathamensis	*duncanensis*	0.722	0.278	0.000	1.000	0.217
hoodensis	*phantasticus*	0.539	0.461	0.000	1.000	**0.179**
duncanensis	*phantasticus*	0.022	0.978	0.000	1.000	0.319

chathamensis	*phantasticus*	0.000	1.000	0.000	1.000	0.319
Within Isabela Island (Domed Clade)						
becki(PBL)	*becki*(PBR)	0.916	0.084	0.000	1.000	**0.182**
vicina	*guntheri*	0.227	0.773	0.000	1.000	0.447
vicina	*vandenburghi*	0.000	1.000	0.000	1.000	0.304
vicina	*microphyes*	0.005	0.995	0.000	1.000	0.345
vicina	*becki*(PBL)	0.127	0.873	0.000	1.000	**0.179**
vicina	*becki*(PBR)	0.000	1.000	0.000	1.000	0.307
guntheri	*vandenburghi*	0.001	0.999	0.000	1.000	0.455
guntheri	*microphyes*	0.000	1.000	0.000	1.000	0.460
guntheri	*becki*(PBL)	0.702	0.298	0.000	1.000	0.404

guntheri	*becki*(PBR)	0.000	1.000	0.000	1.000	0.536
vandenburghi	*microphyes*	0.790	0.210	0.000	1.000	**0.180**
vandenburghi	*becki*(PBL)	0.000	1.000	0.000	1.000	0.280
vandenburghi	*becki*(PBR)	0.000	1.000	0.000	1.000	0.316
microphyes	*becki*(PBL)	0.000	1.000	0.000	1.000	0.314
microphyes	*becki*(PBR)	0.006	0.994	0.000	1.000	0.344
Within Santa Cruz Island (Domed Clade)						
donfaustoi	*porteri*	0.489	0.511	0.000	1.000	**0.181**
Santa Cruz Island with Isabela Island (Domed Clade)						
donfaustoi	*becki*(PBL)	0.745	0.255	0.000	1.000	0.386

donfaustoi	*becki*(PBR)	0.004	0.996	0.000	1.000	0.458
donfaustoi	*vandenburghi*	0.665	0.335	0.000	1.000	0.386
donfaustoi	*microphyes*	0.000	1.000	0.000	1.000	0.484
donfaustoi	*guntheri*	0.000	1.000	0.000	1.000	0.276
donfaustoi	*vicina*	0.488	0.512	0.000	1.000	0.400
porteri	*becki*(PBL)	0.000	1.000	0.000	1.000	0.324
porteri	*becki*(PBR)	0.653	0.347	0.000	1.000	0.384
porteri	*vandenburghi*	0.665	0.335	0.000	1.000	0.386
porteri	*microphyes*	0.831	0.169	0.000	1.000	0.386
porteri	*guntheri*	0.000	1.000	0.000	1.000	0.281

porteri	*vicina*	0.000	1.000	0.000	1.000	0.344
Isabela and Santa Cruz Islands compared with Santiago Island (Domed Clade)						
darwini	*becki*(PBL)	0.004	0.996	0.000	1.000	0.278
darwini	*becki*(PBR)	0.000	1.000	0.000	1.000	0.295
darwini	*donfaustoi*	0.597	0.403	0.000	1.000	**0.181**
darwini	*porteri*	0.203	0.797	0.000	1.00	**0.181**
darwini	*guntheri*	0.188	0.812	0.000	1.000	**0.178**
darwini	*microphyes*	0.000	1.000	0.000	1.000	0.375
darwini	*vandenburghi*	0.000	1.000	0.000	1.000	0.372
darwini	*vicina*	0.000	1.000	0.000	1.000	0.318

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