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 modern methods of species delimitation to whole genome resequencing data from 38 tortoises across all 13 extant taxa to assess support for delimiting these taxa as species. 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 5 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 there is 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.

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

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

There are 16 Galapagos giant tortoise taxa, three of which (*niger*, *abingdonii*, and an unnamed taxon from Santa Fé Island) are recently extinct (Figure 1). Morphological, behavioral, and genetic differences have been documented among the taxa (Gaughran *et al.* 2018, Jensen *et al.* 2021, Chiari 2021, Hunter *et al.* 2013). The most striking morphological difference is carapace shape, which ranges across lineages from a domed shape to a saddleback shape with an elevated anterior carapace opening. These carapace shapes have a genetic underpinning, as evidenced by a clear phylogenetic signal (Jensen *et al.* 2022) and a strong inheritance in juveniles of different taxa raised in a common environment (Pritchard 1996). The taxa also differ in coloration on the head and neck, as well as in limb length (reviewed in Chiari 2021). Domed and saddleback taxa also display different aggressive behaviors (Schafer & Krekorian 1983). Mitochondrial, microsatellite, and genomic data all suggest that geographically distinct populations represent genetically differentiated lineages (e.g., Caccone *et al.* 2002, Beheregaray *et al.* 2003, Gaughran *et al.* 2018, Miller *et al.* 2018).

Galapagos giant tortoises are descended from individuals that likely floated from continental South America along the Humboldt Current to the newly formed Galapagos Islands some time around 4 million years ago (Caccone *et al.* 2002). As more volcanic islands emerged over time, tortoises migrated from existing islands in the east to newer ones in the west (Poulakakis *et al*. 2020). Divergence dates from mitochondrial DNA suggest that the progressive colonization from the first colonized island likely occurred after 2 million years ago, and continued up to the emergence of the youngest island to the west, Fernandina, around 60,000 years ago (Geist *et al.* 2014). The layout of the archipelago has changed dramatically over this time period due to volcanic emergence and subsidence, and sea level changes. During the Pleistocene the central archipelago had a larger landmass that has since fragmented into some of the islands seen today (Geist *et al.*

2014). Thus, the diversification of Galapagos giant tortoises has been triggered by a combination of dispersal to isolated islands and vicariance as landmasses fragmented (Parent *et al.* 2008). In addition, within the last 200 years, humans have moved tortoises between islands (Caccone *et al.* 2002, Poulakakis *et al.* 2008) and land use change has brought previously isolated lineages into contact (Russello *et al.* 2005), resulting in low levels of recent gene flow between previously isolated populations.

Because of this recent and dynamic evolutionary background, the taxonomy of the Galapagos giant tortoises has been the subject of almost endless debate since the time of Darwin (e.g., Darwin 1839, Günther 1877, Van Denburgh 1907, Pritchard 1996, Zug 1998, Caccone *et al.* 1999, Kehlmaier *et al.* 2021). Most taxonomic arguments have classified these lineages as either a single species with 2-14 subspecies, or as many as 14 separate species (reviewed in Pritchard 1996). Following work by Caccone *et al*. (1999), they have been listed as multiple species based on genetic data accumulated over the last several decades (Rhodin *et al.* 2017). More recently, however, two publications have advocated for collapsing all taxa into a single species with multiple subspecies (Loire *et al.* 2013, Kehlmaier *et al.* 2021), which was adopted by the IUCN Tortoise and Freshwater Turtle Specialist Group in 2021 (Rhodin *et al.* 2021).

To address this question, we turned to whole genome resequencing data and methods that quantitatively assess the degree to which taxa are independently evolving under a multispecies coalescent (MSC) framework (Zhang *et al.* 2011, Jackson *et al.* 2017, Morales *et al.* 2018, Mays *et al*. 2019, Leaché *et al.* 2019, Marshall *et al.* 2021). Here, we apply several of these methods of species delimitation to the Galapagos giant tortoises, and thereby provide an assessment of taxonomic considerations in this endangered clade.

Methods

Terminology, study design and data

The aim of this paper is to assess genomic evidence for the distinctiveness and taxonomic status of Galapagos giant tortoise taxa using phylogenetic and coalescent frameworks. In an attempt to remain agnostic about the taxonomic status of the various tortoise populations prior to presenting our results, we avoid using the words *species* and *subspecies* in reference to the named taxa, instead using only the epithets (e.g., *phantasticus* rather than *Chelonoidis phantasticus* or *Chelonoidis niger phantasticus*). Details on these terminology choices, study design, and data are in the supplementary methods.

SNAPP tree generation

To first confirm the genetic relationships among the extant populations of Galapagos giant tortoises, we used the SNAPP v1.5.2 (Bryant *et al.* 2012) model within the BEAST2 v2.6.7 software (Bouckaert *et al.* 2014) on a downsampled data set of 3000 SNPs. The full methodology describing eight models tested is in the supplementary methods.

Species delimitation models

We implemented the Bayesian species delimitation model in BPP v4.0 (Flouri *et al.* 2018) to test hypotheses about the number of species in our data set. Because using hundreds or thousands of loci can lead to over-splitting of taxa along population lines (Leaché *et al.* 2019), we followed the study design of Jackson *et al*. (2017) and Leaché *et al*. (2019) by limiting our species delimitation analysis to a randomly selected set of 50 alignments of 1000 bp each (see Supplementary Methods). We ran BPP species delimitation with a guide tree ("A10") and without

a guide tree ("A11"). Because the Galapagos Islands are geologically young (<3.5 million years [Ma]) and the deepest divergence time among Galapagos giant tortoise lineages is estimated to be between 2 and3 Ma (Caccone *et al*. 2002), we chose a small but diffuse prior for divergence time ($\tau \sim$ IG(3, 0.001)), which corresponds to a mean of 50,000 generations (approximately 1.25 million years). In addition, given the low estimates of effective population size for Galapagos giant tortoises (Garrick *et al*. 2015, Jensen *et al*. 2021) and because the carrying capacity must be low for terrestrial vertebrates on these semi-desert islands, we used a prior scaled effective population size of $\theta \sim$ IG(3, 0.001), which is equivalent to N_e=12,500. However, because there is uncertainty about historical population size, we also re-ran the analyses with scaled effective population size priors that were larger ($\theta \sim$ IG(3, 0.005), N_e=62,500) or smaller ($\theta \sim$ IG(3, 0.0001), N_e=1250), keeping the priors for τ the same. In each of these runs, we used a burnin of 100,000, a sampling frequency of 2, and collected 300,000 samples.

Genealogical divergence index (*gdi*)

The use of species delimitation models in BPP is thought to be a robust method for sympatric species delimitation, but there are concerns that using this MSC-based model selection for species delimitation may delimit populations rather than species, especially if the taxa are allopatric (Jackson *et al.* 2017, Leaché *et al*. 2019). Therefore, we also calculated the *gdi*, a distance-based method with heuristic cutoffs for species delimitation (Jackson *et al.* 2017). The *gdi* is calculated from the divergence time in coalescent units ($2\tau/\theta$) and reflects the probability that two sequences from a purported species coalesce before the divergence time with a sister species (τ) (Leaché *et al*. 2019). As such, it is a coalescent-based measure of genetic divergence, as opposed to an sequence-based measure (e.g., d_{XY}). Using the effective population size and

divergence time, the *gdi* can be calculated in a pairwise manner for sister species *a* and *b*, and can range from 0 (panmixia) to 1 (strong divergence). Based on empirical data and simulations, Jackson *et al.* (2017) proposed a general heuristic for delimiting species based on the *gdi*. Namely, *gdi* < 0.2 indicates a single species, and *gdi* > 0.7 indicates different species. Values for *gdi* between these cutoffs indicate ambiguous delimitation. On the other hand, Rannala & Yang (2020) proposed a more nuanced set of heuristics for designating sister taxa as two species: *gdi* > 0.25 for both taxa, $M = Nm < 0.1$, and divergence time greater than 10^4 generations. For our data set, which lacks migration rate estimates, we suggest the following criteria: If *gdi* > 0.7 for one taxon and *gdi* > 0.2 for the sister taxon and the divergence time is greater than 10^4 generations, two species are supported. If *gdi* < 0.2 for one taxon and *gdi* < 0.7 for the sister taxon, one species is supported. If *gdi* > 0.2 but < 0.7 for both taxa, the evidence is ambiguous. The reasoning behind our criteria is described in more detail in the supplemental methods.

To calculate these indices, we used BPP for parameter estimation of τ and θ, under the guide tree and fixed MSC model ("A00") function in BPP. Using the phylogeny from our SNAPP analysis as a fixed guide tree, we estimated τ for each node and θ for each branch and tip, by running the MCMC with a burnin of 100,000, a sample frequency of 2, and a total sample of 300,000. 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. Furthermore, we calculated divergence time in generations from τ as additional support for putative species (see supplemental methods).

<u>PHRAPL</u>

We then assessed the presence of gene flow between all pairs of lineages in each clade 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. Four demographic models for each pairwise comparison were constructed to test varying divergence and gene flow scenarios between populations of Galapagos giant tortoises. Model 1 was a two species isolation only model, which tested for divergence between two populations with no subsequent gene flow. Model 2 was a single species model, where there was no divergence event. Model 3 was a two species model where gene flow occurred after divergence but stopped at some point and does not occur at present, representative of ancestral gene flow between the two populations. The final model (Model 4) tested for a two species hypothesis with recent gene flow. This was modeled as no gene flow occurring immediately after the divergence event and beginning more recently and continuing into the present day. Gene trees were generated from the same 50 1000bp nuclear genomic blocks used in the BPP analyses. PHRAPL is a demographic model and requires individuals to be assigned to a population prior to model construction. Here, individuals were assigned to a population based on geographic location, as detailed in Figure 1. In this way, each island lineage was assigned to a distinct population, with the exception of Isabela Island having six populations, based on the six geographically separated lineages, and Santa Cruz island having two populations. Gene trees were subsampled at random with replacement 20 times, sampling 2 individuals per lineage in each replicate. Simulation of 100,000 gene trees was conducted using a grid of parameter values for divergence time (t) and

migration (*m*). The initial divergence within the Galapagos tortoise radiation is estimated to be within the last 1.5 Ma, but the species pairs we compare here are more recent (Poulakakis *et al.* 2020). We set the parameters of our grid search to capture this by limiting the maximum divergence (*t*) to 1 Ma. Akaike weights (wAIC) were used to compare models and calculate model probabilities ranging from 0 (low support) to 1 (high support). To present the best supported hypothesis overall for each pairwise comparison, the sum wAIC of all two species models (models 1, 3 and 4) was compared with the wAIC of the single species model (model 2). We interpreted a summed wAIC of > 0.9 across the two species models as strong support for a two species hypothesis, a wAIC > 0.9 of the one species model as strong support for a single species hypothesis, and all other scenarios as ambiguous.

Results

<u>SNAPP</u>

Of the eight SNAPP models we ran, models 5 ($\alpha = 5$, $\beta = 150$), 6 ($\alpha = 30$, $\beta = 60$), and 8 ($\alpha = 30$, $\beta = 80$) showed clear trace plot convergence for the initial sub-run chain lengths, thus were run with longer chains up to 100,000, with model 8 producing the best-supported tree (Figure 2). The tree has two primary sub-clades, which reflect the two carapace morphologies: the larger clade contains the nine taxa with mostly domed carapaces, whilst the smaller clade contains the four taxa with mostly saddleback carapaces. All nodes are highly supported, with a minimum posterior probability of 0.74 (Figure 2).

<u>Species delimitation models in BPP</u>

When we ran an "unguided" species delimitation (A11 in BPP) with a prior of θ ~ IG(3, 0.001), the model gave nearly equal posterior probability to 12 (P=0.537) or 13 (P=0.462) distinct species. The 12 species model collapsed *microphyes* and *vandenburghi* as a single species, with all other named taxa delimited as separate species. When using a slightly larger θ prior, the model assigned 0.99 posterior support to 13 species, but the much smaller θ prior resulted in 1.0 posterior support for 9 species, with all Isabela taxa except *becki*-PBR (i.e., *guntheri*, *microphyes*, *vandenburghi*, *vicina*, and *becki*-PBL) collapsed into one species. When a guide tree was used (A10 in BPP), the model with a midrange θ prior assigned 1.00 posterior support to 10 species (collapsing the Isabela taxa *vicina*, *guntheri*, *vandenburghi* and *microphyes*). The model with a much smaller θ prior also assigned 1.0 posterior support to 10 species, collapsing *microphyes*, *vandenburghi*, and *guntheri* into a single species, as well as *becki*-PBR and *darwini* into a single species. The model with a larger θ prior assigned 0.97 posterior support to 13 species. Across different models and priors, therefore, the BPP analyses supported delimitation of between 9 and 13 species (Table 1).

<u>*gdi* calculation from parameter estimates</u>

Overall, the median *gdi* ranged from 0.152 (*vandenburghi*) to 0.851 (*hoodensis*; Figure 2, Table S3). The four saddleback taxa (*phantasticus*, *hoodensis*, *duncanensis*, *chathamensis*) met our criteria for species delimitation, with *gdi* of 0.304, 0.851, 0.704, and 0.499, respectively. On the other hand, the *gdi* for *darwini* (0.187) and *becki*-PBR (0.282) indicate these taxa belong to a single species, as did the *gdi* for *guntheri* (0.427) and *vandenburghi* (0.152). Delimitation of the remaining taxa (*microphyes*, *vicina*, *becki*-PBL, *donfaustoi*, and *porteri*) was ambiguous, with $0.2 < gdi < 0.7$. The calculations using a larger θ prior produced smaller *gdi* estimates, with ambiguous

delimitation for the saddleback taxa and support for a single species in the saddleback clade (Supplementary Table S4). Using a smaller θ prior produced larger *gdi* estimates, with the four saddleback taxa meeting our criteria for species and the delimitation for all other taxa remaining ambiguous (Supplementary Table S5).

To further explore this, we followed the suggestion of Leaché *et al*. (2019) to progressively collapse species pairs in our phylogeny and rerun the MCMC and *gdi* calculation, with the expectation that *gdi* would increase if collapsed taxa represented better-supported species. Contrary to those expectations, *gdi* generally decreased in our sample set as each node was collapsed into a broader "species" (Supplementary Tables S7-S11 and Supplementary Figures S2-5). In the case of *darwini* and *becki*-PBR, the *gdi* of the collapsed pair was the same as the *gdi* for *becki*-PBR and slightly greater than the *gdi* for *darwini*. For the tortoises on Santa Cruz Island, the *gdi* of the collapsed pair was greater than that calculated for *porteri* but less than that calculated for *donfaustoi*. Finally, the *gdi* calculated from collapsing *hoodensis* and *phantasticus* was much less than the *gdi* calculated for *hoodensis* but about the same as that calculated for *phantasticus*. Overall, the *gdi* estimates do not support Galapagos giant tortoises as a single species, but also do not support delimiting all 13 extant taxa as species (Figure 2, Table 1).

PHRAPL

For the PHRAPL analysis we focus on results of pairwise comparisons for the two primary clades (Figure 2). For the saddleback clade, there are four lineages spread across four separate islands and so all pairwise comparisons were included here. Within the domed clade, Isabela and Santa Cruz islands host five and two lineages respectively, while Santiago has a single living lineage. For this clade we have presented the results as within Santa Cruz Island for its two lineages, within

Isabela Island for its six lineages and pairwise comparison for Santiago to its nearest sister taxon (*becki*-PBR, Isabela Island). Results for all possible pairwise comparisons within the saddleback and domed morphology clades are presented in Supplementary Table 12. Within the saddleback clade, all pairwise comparisons supported a two species hypothesis across the taxa when wAIC values were summed (Figure 2), supporting four distinct species across this clade. For the Santa Cruz Island tortoises (*porteri* and *donfaustoi*), PHRAPL did not support either a one or a two species hypothesis with a wAIC > 0.9, so we consider the result to be ambiguous (Table 1). Similarly, within Isabela Island there was mixed support for both one and two species models across pairwise comparisons of the five lineages, thus we also deemed the result for within these lineages to be ambiguous. The pairwise comparisons between taxa on Santa Cruz and Isabela Islands all favored a two-species model, although only seven out of ten with strong support. Similarly, six out of eight comparisons between the Santiago lineage (*darwini*) and the taxa on Isabela and Santa Cruz Islands strongly supported a two-species model, one weakly favored two species, and the last weakly favored a one-species model. In total, given that the four saddleback lineages were supported as distinct species, PHRAPL supports at least five species of Galapagos giant tortoises across the tree. In addition, the PHRAPL results largely support three additional species for the populations inhabiting Isabela, Santa Cruz, and Santiago Islands.

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.

Here, we build on decades of research on the molecular evolution of these tortoises and provide genomic evidence that the Galapagos giant tortoise complex consists of multiple distinct species.

First, we used a genome-wide SNP dataset to assess support for a phylogeny of 38 samples representing 13 named lineages. Our SNAPP analysis here supported the topology previously inferred from genome-wide sequence data (Jensen *et al.* 2022), with the exception of small differences in the sister relationships among the most recently diverged taxa (*guntheri*, *vandenburghi* and *microphyes*). Given this strong support, we used this nuclear phylogeny as a guide tree in subsequent analyses. Notably, the topology of these trees inferred from nuclear loci deviates in some ways from mitochondrial trees for these taxa (Poulakakis *et al.* 2012, Poulakakis *et al.* 2020), an incongruence that highlights the complex and rapid speciation history of Galapagos giant tortoises.

Our first line of evidence for the distinctiveness of Galapagos giant tortoise species is the model-based species delimitation analysis in BPP. Both "guide tree" (A10) and "unguided" (A11) models supported between 9 and 13 species of Galapagos giant tortoises. The exact number of species supported was affected by the prior distribution of scaled effective population size, and to some degree by the use of a guide tree. Because our guide topology may not reflect the true evolutionary history of these taxa and our best estimates of historical effective population size fall closest to the midrange θ prior, we believe the "unguided" model with the prior $\theta \sim$ IG (3, 0.001) is the most appropriate model. This model was evenly split between inferring 12 or 13 species, supporting the collapse of *microphyes* and *vandenburghi* into a single species about half the time. These two taxa are geographically adjacent to each other on the central volcanoes of Isabela Island, which suggests that recent divergence or gene flow is a factor. Furthermore, these taxa are not

sister taxa in our phylogeny, emphasizing the finding from this and other studies that the phylogeny of taxa on Isabela Island is difficult to resolve (Poulakakis *et al.* 2012, Jensen *et al*. 2022).

Theory and simulations suggest that BPP will increasingly favor a two species model when hundreds or thousands of loci are used, even under high rates of migration (Leaché *et al*. 2019). That we infer strongly delimited species with a modest number of loci (n=50) suggests that overfitting is not a likely explanation for our results. Still, the species delimitation model in BPP has been criticized as oversplitting species and recovering population structure rather than species (Jackson *et al.* 2017). Some have argued that the *gdi* better reflects species differences by explicitly incorporating information about population size and divergence time (Jackson *et al*. 2017). When we calculate *gdi* for Galapagos giant tortoise taxa, we find strong support for the species status of the saddleback taxa (*phantasticus*, *hoodensis*, *duncanensis*, and *chathamensis*), no support for the species status of *darwini*/*becki*-PBR or *guntheri*/*vandenburghi*, and ambiguous support for all others.

When sister taxa are not species, Leaché *et al*. (2019) found that collapsing the taxa and recalculating *gdi* results in a larger *gdi*, signaling better support for the resulting species. However, when we replicate this iterative process with our taxon set, we find that *gdi* generally becomes smaller rather than larger with each collapse of sister taxa (supplemental figures S1-S4). Applying population genetic principles to the *gdi* equation reveals why this occurs. Because *gdi* is calculated from the ratio of τ to θ, it will remain constant when these values change 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 is 0.01). When sister taxa are collapsed, both τ and θ will increase. By definition, in recent radiations τ will increase only marginally at each successive node. On the other hand, θ will be inflated when calculated from a highly structured sample (e.g., differentiated species) because the number of segregating

sites is expected to be higher in a combined sample. Thus, the lower *gdi* values that we found for successive collapsing of taxa further supports the idea that the taxa in our sample do not belong to a single species.

Additionally, work in natural populations has shown that well-accepted species can fall into the ambiguous range of 0.2–0.7, including horned lizards (Leaché *et al.* 2021), penguins (Mays *et al.* 2019), and flying lizards (Reilly *et al.* 2022). Furthermore, there appears to be significant variation in *gdi* within clades and between them. For example, in a survey of bird species the median *gdi* was 0.346 (1st quartile: 0.012; 3rd quartile: 0.742), while the surveyed mammalian species had a median *gdi* value of 0.799 (1st quartile: 0.716; 3rd quartile: 0.955) (Jackson *et al*. 2017). This suggests that appropriate heuristic cutoffs for *gdi* may differ among taxa, given that divergence times, population sizes and life histories differ substantially across these clades. Likewise, island radiations may also have a different underlying distribution of *gdi* given their shallow divergence times and propensity for rapid adaptation. Here, we use a heuristic that is more conservative than that of Rannala & Yang (2020), but less conservative than the thresholds proposed by Jackson *et al*. (2017), reflecting our belief that greater nuance is required in interpreting *gdi*. Still, before a *gdi* heuristic can be confidently applied to Galapagos giant tortoise species, a more thorough survey of *gdi* among testudine species and across island radiations is needed.

When we applied model-based species delimitation in PHRAPL to the Galapagos giant tortoise complex, we found a mixed signal of two species models favored in most but not all cases (Figure 2, Supplemental Table 12). Specifically, the four saddleback taxa are each strongly supported as distinct species, but support for the various domed taxa as species is more ambiguous. The taxa on Santa Cruz Island (*porteri* and *donfaustoi*) have ambiguous support for one or two

species models, as do various pairs of the domed taxa on Isabela Island (*guntheri, vandenburghi, microphyes, vicina, becki-PBL* and *becki-PBR*). In several cases, pairs of taxa are strongly supported as distinct species (e.g., *guntheri* and *microphyes*) but the same taxa are ambiguously supported as distinct from more distantly related taxa (e.g., *becki*-PBL). The cases of Isabela Island and Santa Cruz Island are particularly complex due to especially recent divergence and the potential for gene flow among taxa within the islands. Furthermore, PHRAPL is sensitive to parameter choice in the grid search (Smith & Carstens 2020) and is more accurate with a larger dataset (Jackson *et al.* 2017). The sample size for each taxon in our sample set is small (n=2–3 individuals per taxon). Because PHRAPL uses subsampling of individuals to generate expected tree topologies, having a larger sample size that better represents the whole population could help disentangle the history of the unresolved populations. Current work is underway to increase the number of reference sequences for extant populations.

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

One such study claimed that genomic differentiation did not exist among *becki*, *vandenburghi*, and *porteri* (Loire *et al.* 2013), but a re-analysis of the data with appropriate filtering showed clear genomic differentiation of the three taxa (see Supplemental Figure S6 in Gaughran *et al*. 2018). Another study argued that the recent divergence times of Galapagos giant tortoise

taxa relative to other tortoise species disqualified them from species status (Kehlmaier *et al.* 2021). This view of species delimitation, however, ignores the complex reality of speciation (see Donoghue 1985, Mallet 1995, Hey 2006). In addition, it ignores the fact that some clades, such as those that colonized islands or other new environments, may be subject to different demographic and selective forces that are associated with adaptive radiations. Outside of tortoises, such a view would require a drastic re-delimitation of recently radiated species, with a disproportionate effect on small, endangered island populations.

To date, all published genetic evidence, including the data from Loire *et al*. (2013), supports the idea that Galapagos tortoise taxa are genetically distinct populations. Given the statistical power of genome-wide SNPs to accurately detect differentiation (Gaughran *et al.* 2018), this pattern of genetic distinctiveness appears unlikely to be overturned. Moving forward, genomic discussions of Galapagos giant tortoise taxonomy should recognize that the preponderance of evidence supports several genetically distinct taxa. However, whether these genetically distinct populations deserve to be considered species is a more nuanced question.

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

Our diverse set of analyses here clearly rejects a single-species model of Galapagos giant tortoises, as was recently adopted by the IUCN Turtle Taxonomy Working Group (Rhodin *et al.* 2021). Instead, our modeling in BPP delimits at least 12 species; a conservative interpretation of the PHRAPL results supports at least five species; and the *gdi* results suggest at least five species (Table 1, Figure 2). We find significant support for species designations of some taxa, especially the cases in which there is a single taxon per island. On the other hand, we find mixed support for delimiting taxa inhabiting the same island as different species (i.e., Isabela Island and Santa Cruz Island). Whether these taxa represent populations of a single species, very recently diverged species, or species with gene flow (e.g., after secondary contact) remains an open question. Future work, incorporating more samples for each taxon, will likely resolve the ambiguous delimitations by making clear if these populations have been most affected by constant migration, secondary contact after divergence, or other demographic processes.

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

Table 1. Summary table of named Galapagos giant tortoise taxa and support for their species status. Nuclear monophyly: "yes" if all samples form a monophyletic group in Jensen *et al.* (2022), "no" otherwise. BPP (unguided model): "yes" if delimited as a species with >0.9 support in the A11 model of BPP using realistic priors, "no" if collapsed with another taxon with >0.9 support, "ambiguous" otherwise. *gdi*: "yes" if *gdi* >0.7 for one taxon and >0.2 for the sister taxon, "no" if *gdi* <0.2 for one taxon and <0.7 for sister taxon, "ambiguous" otherwise. PHRAPL: "yes" if summed wAIC of two species models returned >0.9 support for sister-taxa pairwise comparisons or across island pairwise comparisons in the cases of Isabela and Santa Cruz islands, "no" if wAIC >0.9 of the one species model and "ambiguous" if wAIC <0.9 for both one and two species models.

Scientific Name	**Native Island (Nearest Volcano on Island)**	**Nuclear monophyly (Jensen et al. 2022)**	**BPP (unguided model)**	***gdi***	**PHRAPL**
duncanensis	Pinzón	Yes	Yes	Yes	Yes
chathamensis	San Cristóbal	Yes	Yes	Yes	Yes
hoodensis	Española	Yes	Yes	Yes	Yes
phantasticus	Fernandina	Yes	Yes	Yes	Yes
darwini	Santiago	Yes	Yes	No	Yes
becki-PBR	Isabela (Volcano Wolf)	Yes	Yes	No	Ambiguous
becki-PBL	Isabela (Volcano Wolf)	No	Yes	Ambiguous	Ambiguous
vicina	Isabela (Volcano Cerro Azul & Sierra Negra)	Yes	Yes	Ambiguous	Ambiguous

microphyes	Isabela (Volcano Darwin)	Yes	Ambiguous	Ambiguous	Ambiguous
guntheri	Isabela (Volcano Cerro Azul)	Yes	Yes	No	Ambiguous
vandenburghi	Isabela (Volcano Alcedo)	Yes	Ambiguous	No	Ambiguous
donfaustoi	Santa Cruz	Yes	Yes	Ambiguous	Ambiguous
porteri	Santa Cruz	Yes	Yes	Ambiguous	Ambiguous

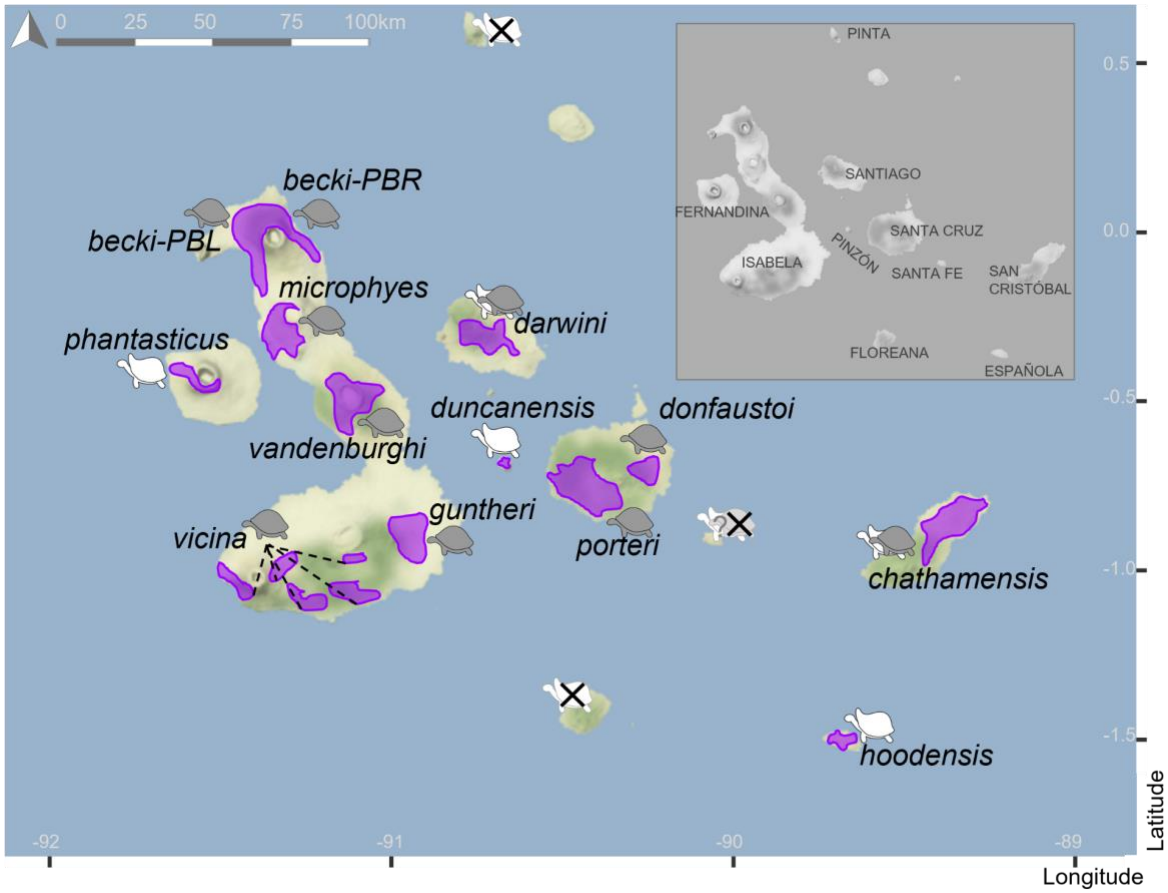

Figure 1. Galapagos giant tortoises are found on seven islands and form at least 13 genetically distinct taxa. Purple shapes indicate current ranges of populations. Carapace morphology (domed = gray, saddleback = white) is shown for each population, with "intermediate" shell shape indicated with overlapping icons. 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.

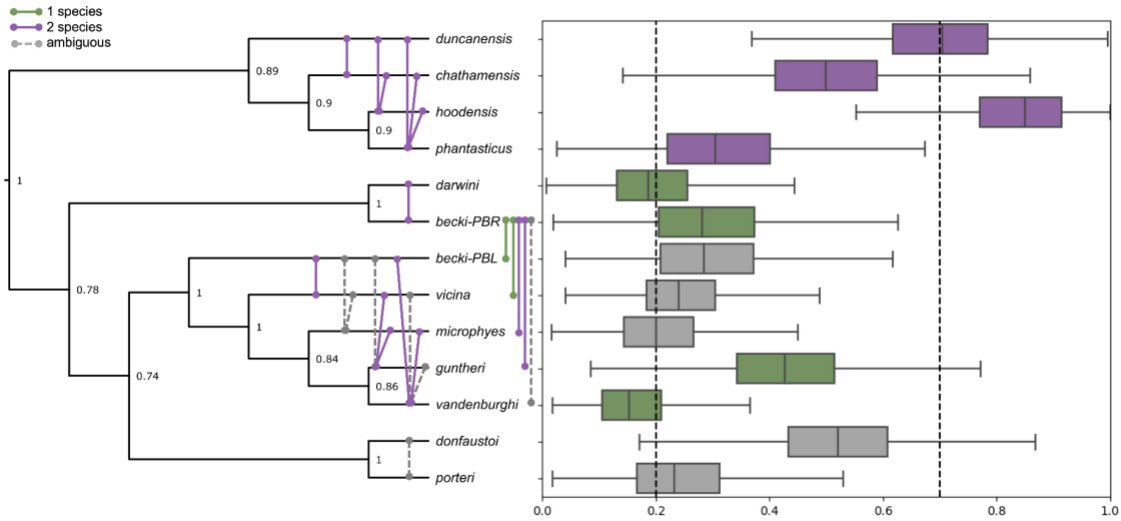

Figure 2. Multiple lines of evidence confirm the Galapagos giant tortoise species complex consists of multiple genetically divergent, independently evolving species. On the left, the phylogeny generated from genome-wide SNPs in SNAPP, with node posterior support values. Best supported wAIC results from PHRAPL models are indicated on the tree by colored lines connecting lineages (purple: 2 species model >0.9; grey: neither model supported above 0.9; green: 1 species model >0.9). 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 (purple: two species, with *gdi* > 0.7 for one taxon and *gdi* > 0.2 for the sister taxon; green: one species, with *gdi* < 0.2 for one taxon and *gdi* < 0.7 for the sister taxon; grey: ambiguous, with *gdi* > 0.2 but < 0.7 for both taxa). Model-based species delimitation in BPP supported 12 or 13 species with approximately equal probability (not shown).

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Author Contributions

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

Data Accessibility

The whole genome resequencing data from Jensen *et al*. (2022) is available under NCBI BioProject PRJNA761229. Code for running BPP and PHRAPL, as well as alignments for loci used in these analyses, are available at: https://github.com/sjgaughran/tortoise-species-delimitation

Conflict of Interest

The authors declare no conflicts of interest.

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Supplemental Methods

Terminology, study design and data

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

We designed a whole-genome study of population distinctiveness and species delimitation, taking advantage of existing illumina short-read whole genome resequencing data (Jensen *et al.* 2021, Jensen *et al*. 2022), which are available under NCBI Bioproject PRJNA761229. Samples in this data set included two individuals of *phantasticus*, and three individuals each of *guntheri*, *microphyes*, *vandenburghi*, *vicina*, *becki* (PBL population), *becki* (PBR population), *darwini*, *chathamensis*, *donfaustoi*, *porteri*, *duncanensis*, and *hoodensis*, for a total of 38 Galapagos giant tortoise individuals These named taxa have previously been classified as populations, species, or subspecies. The samples were originally selected for sequencing as representatives of the genetic and geographic populations (Figure 1) that have previously been shown to exist using microsatellite (Ciofi *et al.* 2002, Ciofi *et al.* 2006) and reduced representation SNP (Miller *et al.* 2018) data. Previous genetic research suggests that the *becki* taxon consists of two geographically

and genetically distinct populations, referred to in the literature as “PBL” (Piedras Blancas) and “PBR” (Puerto Bravo) (Garrick *et al.* 2014). A prior phylogeny constructed from these genome data in Jensen *et al*. (2022) showed the named taxa to be monophyletic, with the exception of *becki*. Within *becki*, PBR individuals formed a clade sister to *darwini* and PBL individuals did not form a clade, with one sample closest to PBR and *darwini* and the other two closest to other taxa from Isabela. We have therefore chosen to test *becki*-PBL and *becki*-PBR as potentially distinct taxa, and have retained the potentially admixed PBL individual to avoid biasing our analyses.

We mapped these reads to a reference genome for *abingdonii* (Quesada et al. 2019) using bwa-mem (Li 2013). Jensen *et al.* (2021) previously investigated the potential for reference bias in using the *abingdonii* assembly and found no evidence for such a bias. We then followed the sequence processing methods described in Jensen *et al*. (2022) to generate a random set of 1kb loci separated by 100kb to be used in the BPP and PHRAPL analyses. As in Jensen *et al*. (2022), we retained loci that had more than 5 parsimony informative sites, less than 10% missing data, and GC content between 30% and 70%. We then used several methods, described below, to delimit species under different phylogenetic and coalescent models. For the SNAPP analysis, the input data were a set of 3,000 SNP loci, downsampled from variants detected and filtered in Jensen *et al*. (2022).

<u>SNAPP Tree Generation</u>

Eight models were constructed (see Supplementary Table S1) to cover a range of potential gamma prior distributions. Models 1 and 4 used mutation rate parameters of u = 3.101 and v = 0.596 from Austin *et al*. (2010) and Iannucci *et al.* (2021), whilst the other six runs used the default

values of u = v = 1. Only model 1 used the default prior parameters for α and β (α = 12, β = 110) with the others using alternative parameter values (α = 5, 30; β = 50, 60, 70, 80, 150).

For each model, computational loads were minimized by running 10 shorter Markov chain Monte Carlo (MCMC) chains and combining them into one using the LogCombiner plug-in. The standard sub-run chain length ran was 10,000, and longer chains were run depending upon inspection of trace plots. By viewing these trace plots in Tracer v1.7.2 (Rambaut *et al.* 2018), we could both visually and statistically identify those with satisfactory convergence, indicated by having an effective sample size (ESS) in excess of 200, minimal standard deviation, and minimal standard error of the mean values. Models that produced trace plots with acceptable summary statistics were subsequently run with extended chain lengths. The burn-in was set to 20% and altering this did not yield noticeable changes to model outputs. For models which showed satisfactory convergence on trace plots, combined trees were constructed and visualized using the TreeAnnotator and FigTree v1.4.4 plug-ins in BEAST2.

<u>Divergence time from BPP</u>

Rannala and Yang (2020) pointed out drawbacks for relying solely on the heuristic for *gdi* in species delimitation, and also recommend including a minimum divergence time of 10,000 generations for two species. Because τ is the rate-scaled divergence time in BPP, we can calculate the absolute divergence time in generations as:

$$T = \tau/\mu$$

Where μ is the per-generation mutation rate (see Jensen et al. 2021 for discussion of mutation rate in Galapagos giant tortoises).

Interpretation and exploration of *gdi* estimates

As the name suggests, the genealogical divergence index (*gdi*) provides a normalized value of the divergence (in terms of gene tree monophyly) between a target taxon and its sister taxon, taking into account divergence time between the two taxa (τ) and the target taxon's scaled effective population size (θ). The resulting *gdi* is specific to the target taxon, meaning sister taxa can have a different *gdi* when their effective population sizes differ. As Leaché *et al*. (2019) and Rannala and Yang (2020) point out, this can lead to a scenario in which the *gdi* of one taxon in a pair reaches the threshold for species delimitation but the *gdi* for the sister taxon does not. This scenario seems particularly likely in island radiations, in which divergence times are often shallow and population size changes are common due to founder effects and limited geographic ranges.

To address this issue, we adopted an approach in which the inferences for sister taxa cannot be contradictory (i.e., either both or neither are inferred to be species, or the inference for both is considered ambiguous.Thus, we adopted the following criteria for species delimitation using the *gdi*: if *gdi* > 0.7 for one taxon and *gdi* > 0.2 for its sister taxon and the divergence time is greater than 10^4 generations, both are considered supported as separate species. If *gdi* < 0.2 for one taxon and *gdi* < 0.7 for the sister taxon, a single species is considered supported. If *gdi* > 0.2 but < 0.7 for both taxa, the delimitation is considered ambiguous. Finally, if *gdi* < 0.2 for one taxon and *gdi* > 0.7 for the sister taxon, the delimitation is considered ambiguous (no cases in our data set).

Leaché *et al.* 2019 suggested an additional method of interpreting the *gdi*, which involves progressively collapsing sister taxa and re-calculating the *gdi* from the collapsed taxa. Those

authors suggest that such an approach will lead to an increased *gdi* as populations with low *gdi* are progressively collapsed into taxa that resemble unambiguous species. We proceeded through six rounds of collapsing sister taxa and re-calculating *gdi* to investigate if collapsing any of our named taxa resulted in higher *gdi* (i.e., stronger support as species when taxa were collapsed).

Supplemental Figures and Tables

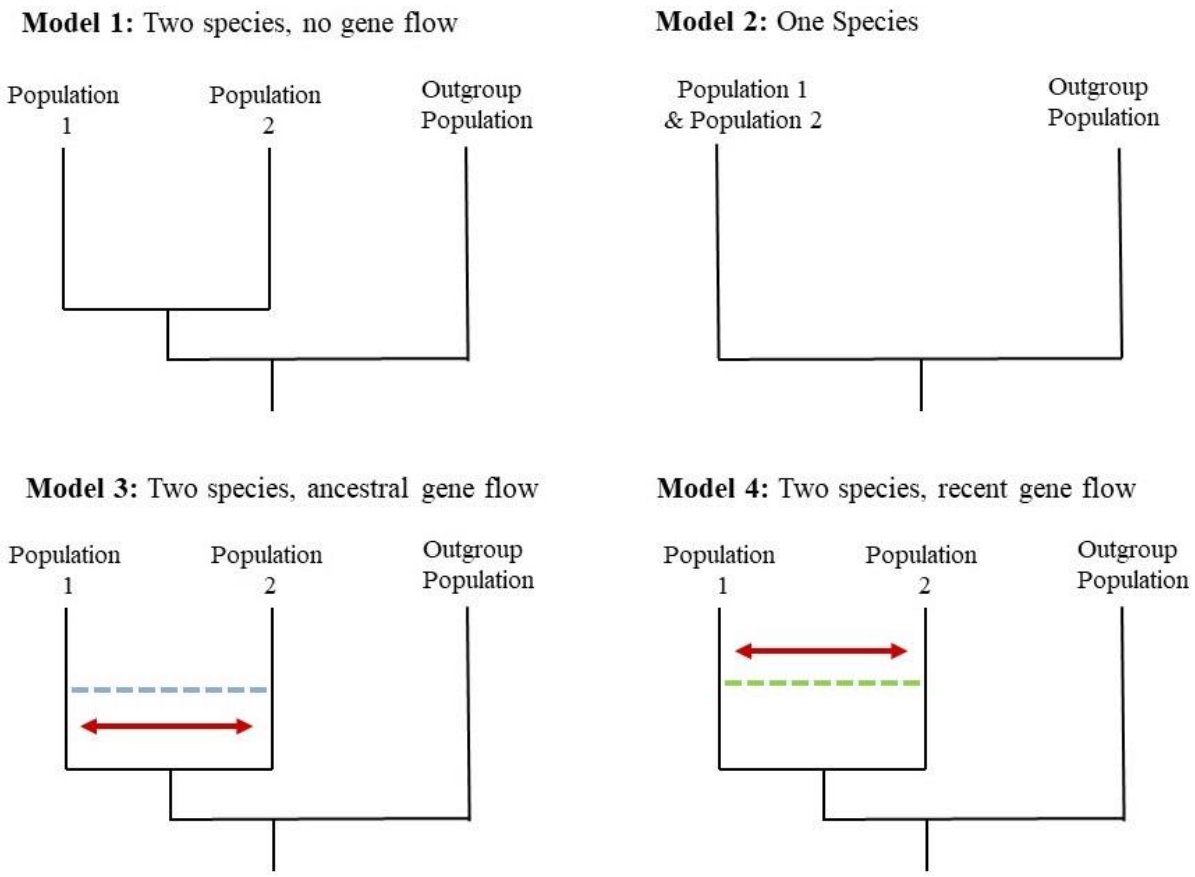

Figure S1. The four models used to test one and two species models under differing gene flow scenarios in PHRAPL. The red arrow indicates bi-directional gene flow between the two populations. In Model 3, the dotted blue line indicates the end of gene flow between the populations and in Model 4 the green dotted line indicates the beginning of gene flow.

Table S1. details of the 8 models for which SNAPP analyses were run, detailing choice of prior distribution and parameters, MCMC chain length, and mutation rate parameters. The standard MCMC chain length of 10,000 was run for all models initially, with longer chains being run if trace log convergence was sufficient. Apart from models 1 and 4, all models utilized the default mutation rate parameters in SNAPP of u = v = 1.

Model number	Prior distribution	Prior parameters	Sub-run chain lengths	Mutation rate parameters
1	Gamma	α = 12, β = 110	10,000	u = 3.101, v = 0.596
2	Gamma	α = 30, β = 150	10,000	u = 1, v = 1
3	Gamma	α = 5, β = 50	10,000	u = 1, v = 1
4	Gamma	α = 30, β = 150	10,000	u = 3.101, v = 0.596
5	Gamma	α = 5, β = 150	10,000	u = 1, v = 1
			50,000	
6	Gamma	α = 30, β = 60	10,000	u = 1, v = 1
			20,000	
			30,000	
			50,000	
			100,000	
7	Gamma	α = 30, β = 70	10,000	u = 1, v = 1
8	Gamma	α = 30, β = 80	10,000	u = 1, v = 1
			50,000	
			100,000	

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

Table S3. 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.

Taxon	**Median divergence time in generations (95% credible interval)**
darwini	35,700 (16,500–57,400)
becki-PBR	35,700 (16,500–57,400)
guntheri	26,800 (13,900–40,900)
microphyes	26,800 (13,900–40,900)
vandenburghi	30,700 (17,800–45,100)
vicina	38,600 (24,100–54,700)
becki-PBL	52,900 (39,200–68,900)
donfaustoi	42,900 (24,200–62,100)
porteri	42,900 (24,200–62,100)
hoodensis	35,600 (19,800–53,600)
phantasticus	35,600 (19,800–53,600)
duncanensis	46,700 (31,300–63,900)
chathamensis	55,100 (40,300–72,100)

Table S3. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ 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	0.187 (0.056–0.422)
becki-PBR	0.282 (0.100–0.575)
guntheri	0.427 (0.200–0.682)
microphyes	0.200 (0.070–0.415)
vandenburghi	0.152 (0.048–0.342)
vicina	0.240 (0.107–0.450)
becki-PBL	0.284 (0.099–0.557)
donfaustoi	0.520 (0.278–0.770)
porteri	0.233 (0.075–0.490)
hoodensis	0.851 (0.770–0.981)
phantasticus	0.304 (0.098–0.612)
duncanensis	0.704 (0.448–0.904)
chathamensis	0.499 (0.260–0.756)

Table S4. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ 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	0.064 (0.014–0.179)
becki-PBR	0.092 (0.021–0.137)
guntheri	0.137 (0.032–0.302)
microphyes	0.076 (0.017–0.192)
vandenburghi	0.066 (0.014–0.172)
vicina	0.130 (0.044–0.266)
becki-PBL	0.207 (0.065–0.424)
donfaustoi	0.247 (0.090–0.452)
porteri	0.105 (0.020–0.294)
hoodensis	0.527 (0.287–0.734)
phantasticus	0.165 (0.044–0.372)
duncanensis	0.498 (0.314–0.705)
chathamensis	0.383 (0.206–0.584)

Table S5. Estimates of *gdi* for each taxon, calculated from the posterior distributions of θ and τ 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	0.274 (0.094–0.538)
becki-PBR	0.377 (0.140–0.693)
guntheri	0.526 (0.276–0.794)
microphyes	0.265 (0.089–0.517)
vandenburghi	0.202 (0.075–0.417)
vicina	0.261 (0.107–0.499)
becki-PBL	0.322 (0.131–0.605)
donfaustoi	0.594 (0.344–0.841)
porteri	0.305 (0.095–0.581)
hoodensis	0.945 (0.663–0.999)
phantasticus	0.437 (0.171–0.789)
duncanensis	0.749 (0.466–0.940)
chathamensis	0.494 (0.237–0.772)

Table S6. Estimates of *gdi* for each taxon after the first 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	0.293 (0.154–0.486)
guntheri+*microphyes*	0.072 (0.014–0.183)
vandenburghi	0.145 (0.043–0.319)
vicina	0.244 (0.100–0.451)
becki-PBL	0.295 (0.102–0.570)
donfaustoi+*porteri*	0.291 (0.161–0.463)
hoodensis+*phantasticus*	0.317 (0.113–0.607)
duncanensis	0.723 (0.479–0.911)
chathamensis	0.490 (0.240–0.757)

Table S7. Estimates of *gdi* for each taxon after the second 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	0.298 (0.155–-0.495)
guntheri+*microphyes*+*vandenburghi*	0.051 (0.014–0.131)
vicina	0.250 (0.109–0.445)
becki-PBL	0.284 (0.096–0.372)
donfaustoi+*porteri*	0.300 (0.164–0.474)
hoodensis+ *phantasticus*+*duncanensis*	0.099 (0.035–0.227)
+*chathamensis*	0.564 (0.343–0.794)

Table S8. Estimates of *gdi* for each taxon after the third 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	0.268 (0.132–0.452)
guntheri+*microphyes*+*vandenburghi*+*vicina*	0.062 (0.018–0.133)
becki-PBL	0.337 (0.144–0.589)
donfaustoi+*porteri*	0.266 (0.139–0.431)
hoodensis+ *phantasticus*+*duncanensis*+ *chathamensis*	0.073 (0.033–0.142)

Table S9. 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	0.319 (0.174–0.515)
guntheri+*microphyes*+*vandenburghi*+*vicina*+ *becki*-PBL	0.037 (0.009–0.081)
donfaustoi+*porteri*	0.392 (0.259–0.554)
hoodensis+*phantasticus*+*duncanensis*+ *chathamensis*	0.073 (0.014–0.145)

Table S10. 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	0.404 (0.266–0.575)
guntheri+*microphyes*+*vandenburghi*+*vicina*+ *becki*-PBL+*donfaustoi*+*porteri*	0.015 (0.007–0.031)
hoodensis+ *phantasticus*+*duncanensis*+ *chathamensis*	0.092 (0.039–0.185)

Table S11. Estimates of *gdi* for each taxon after the sixth 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)**
guntheri+*microphyes*+*vandenburghi*+*vicina*+ *becki*-PBL+*donfaustoi*+*porteri*+*darwini*+ *becki*-PBR	0.012 (0.005–0.022)
hoodensis+ *phantasticus*+*duncanensis*+ *chathamensis*	0.255 (0.176–0.358)

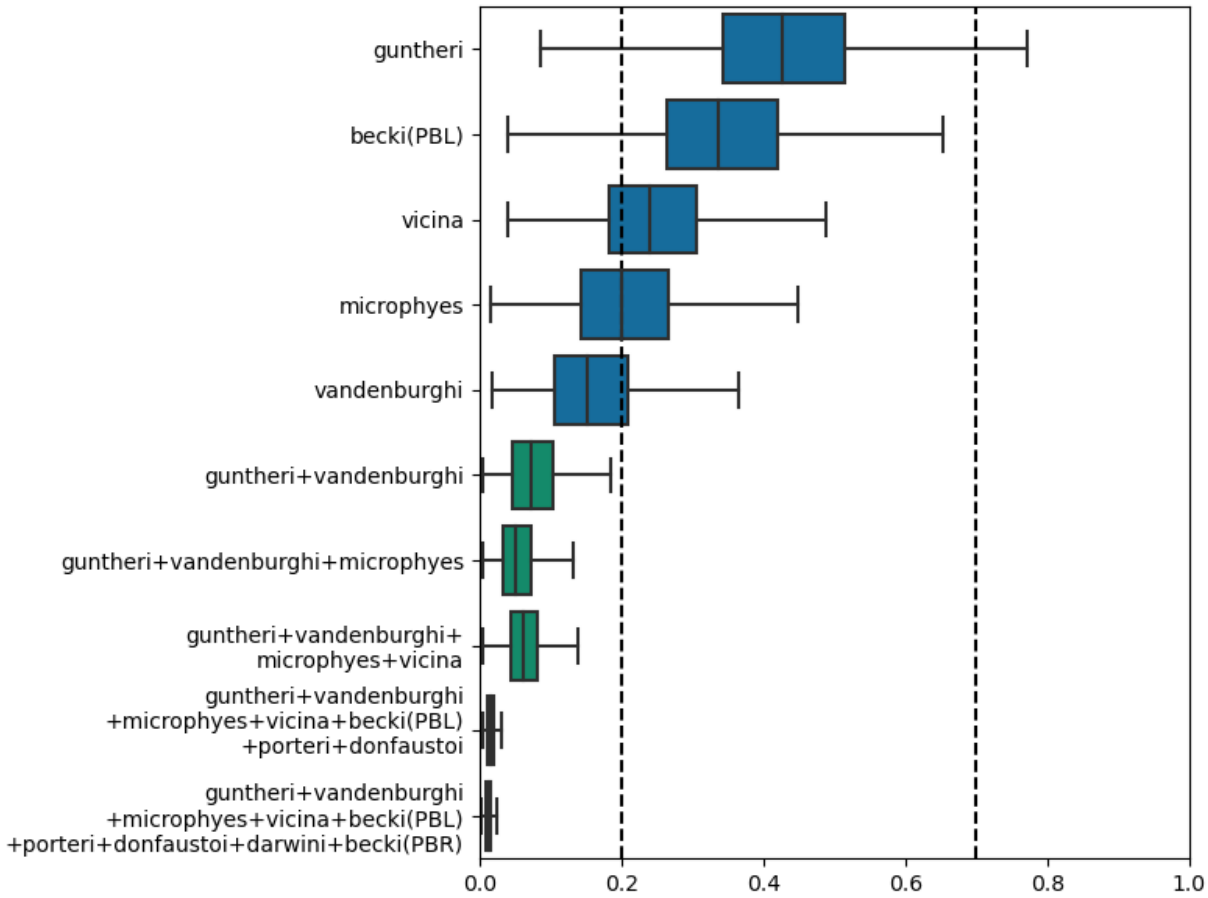

Figure S2. *gdi* calculated for Isabela Island clade with progressive collapse. Blue boxes are single named taxa; green boxes are two or more taxa collapsed into a single "species."

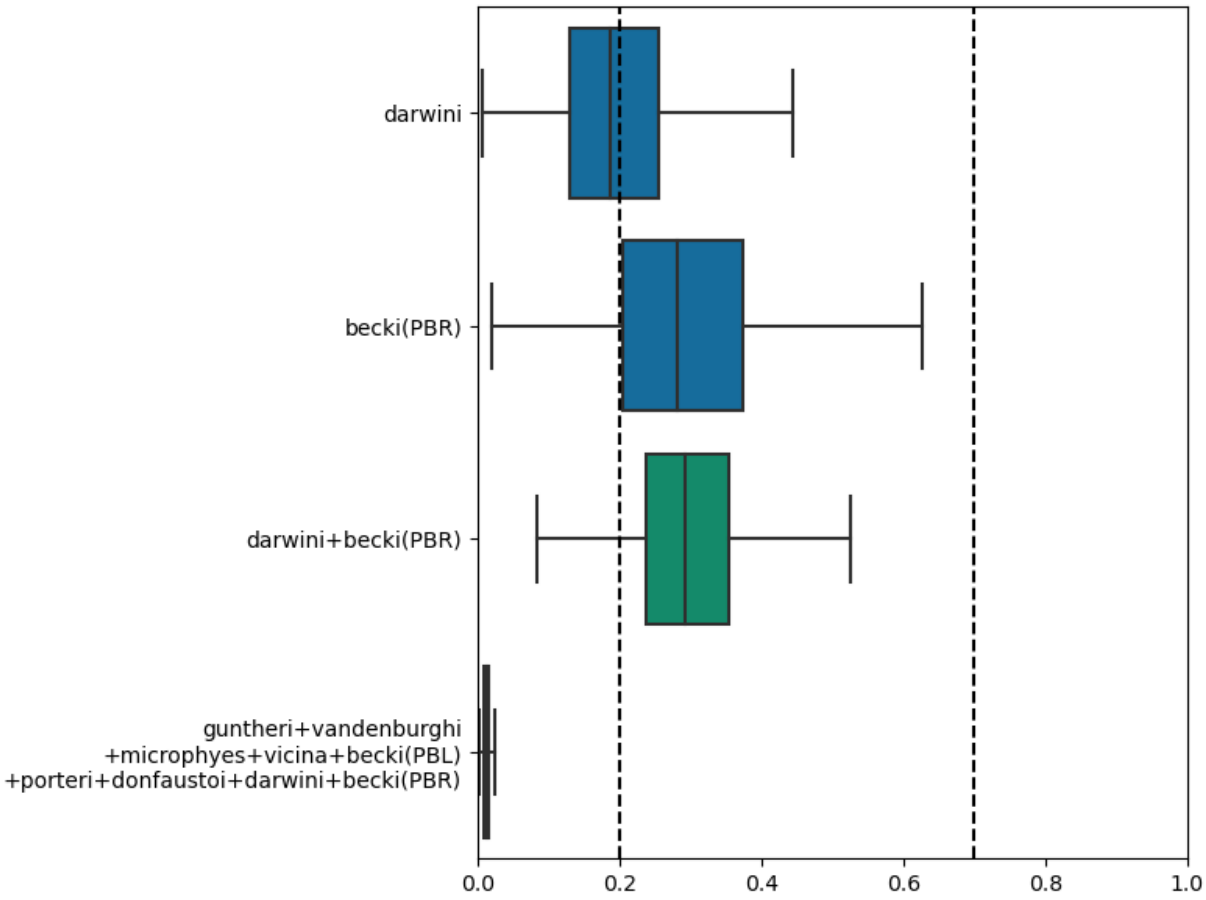

Figure S3. *gdi* calculated for *darwini+becki*-PBR clade with progressive collapse. Blue boxes are single named taxa; green boxes are two or more taxa collapsed into a single "species."

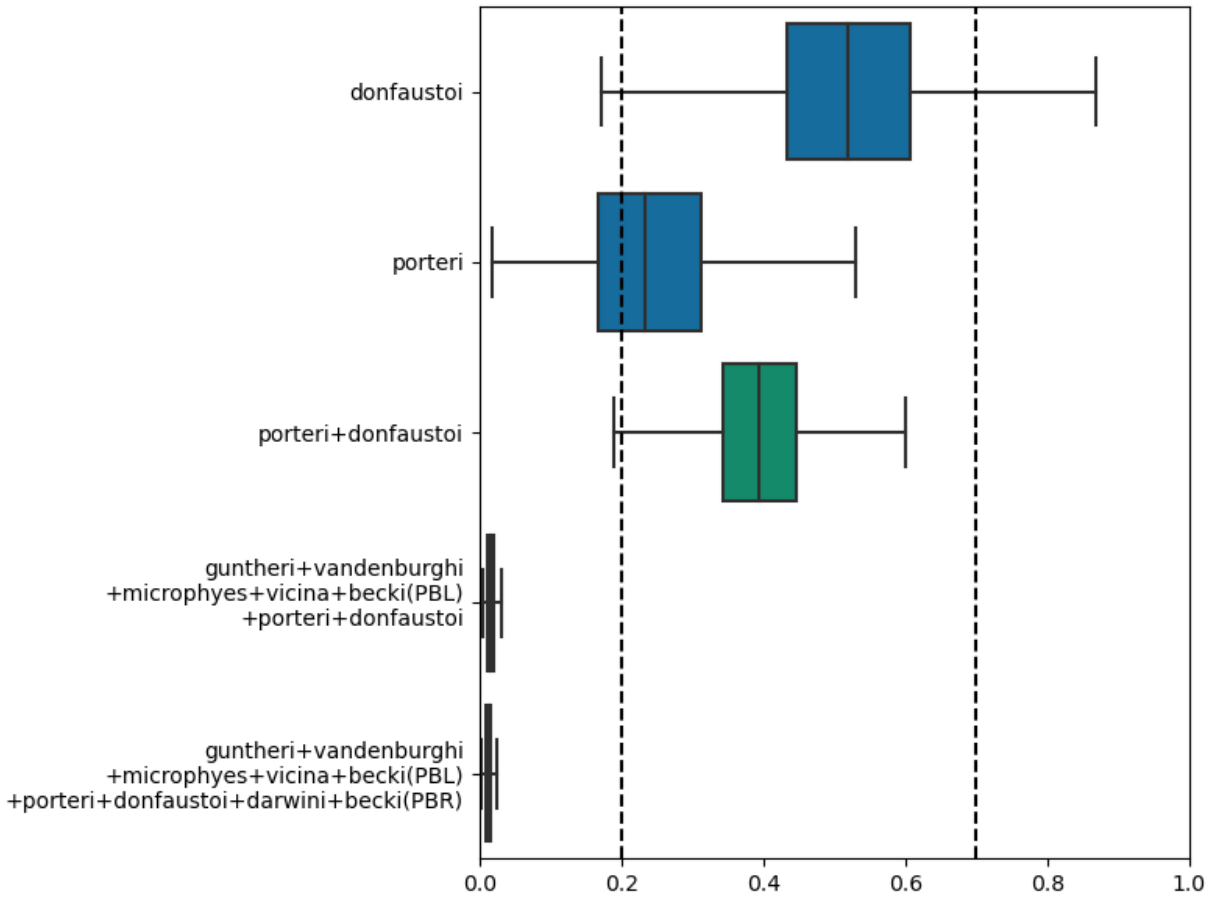

Figure S4. *gdi* calculated for Santa Cruz clade with progressive collapse. Blue boxes are single named taxa; green boxes are two or more taxa collapsed into a single "species."

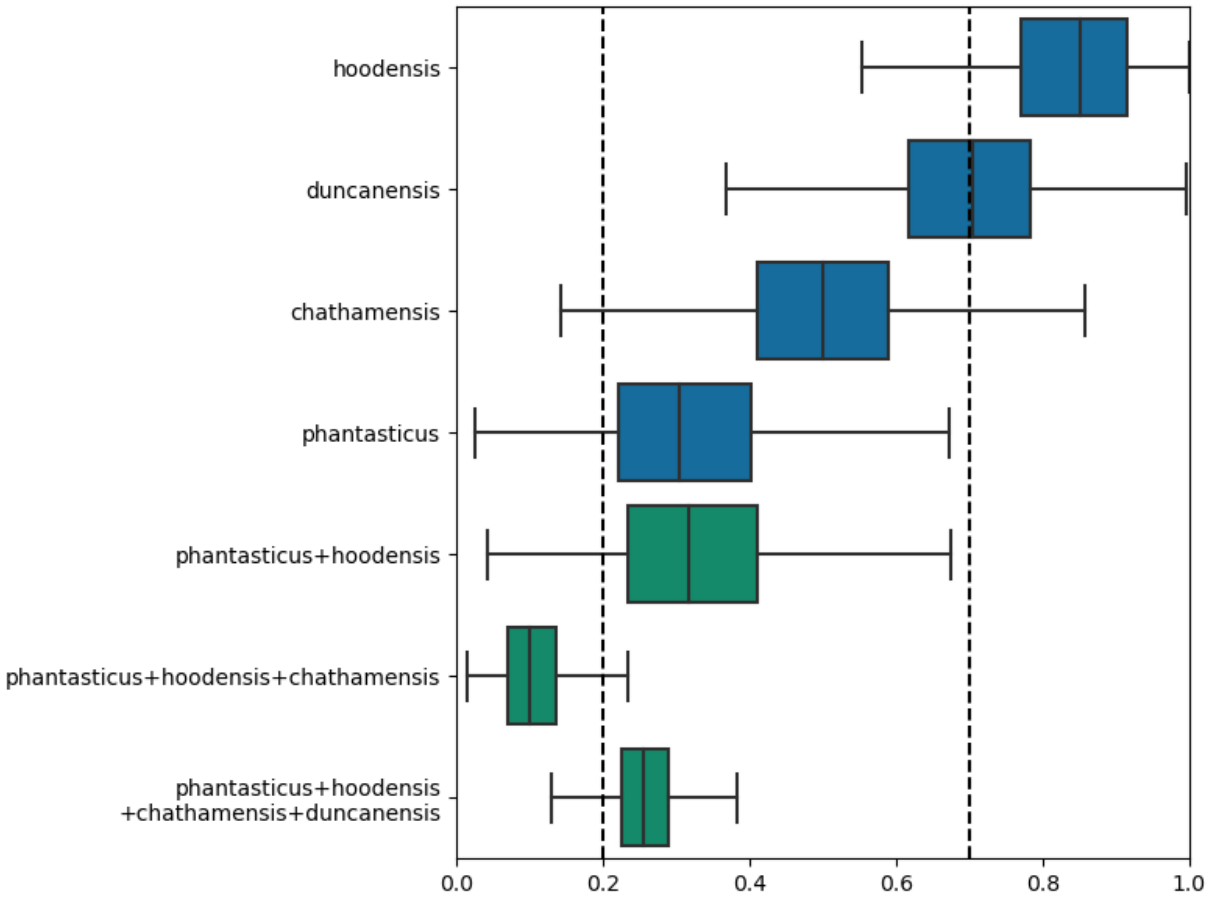

Figure S5. *gdi* calculated for saddleback clade with progressive collapse. Blue boxes are single named taxa; green boxes are two or more taxa collapsed into a single "species."

Table S12: Pairwise comparisons analyzed in PHRAPL across the two main clades of the phylogenetic tree, along with the wAIC for each of the four PHRAPL models. Models with a high weighted AIC > 0.9 for a model set are highlighted in gray. The cumulative sum of wAIC for all two species models is given for each comparison, with values above 0.9 highlighted in blue.

Taxon 1	**Taxon 2**	**wAIC Model 1** **2 species, no gene flow**	**wAIC Model 2** **1 species**	**wAIC Model 3** **2 species, ancestral gene flow**	**wAIC Model 4** **2 species, recent gene flow**	**Sum wAIC of 2 Species Models**
Within Saddleback Clade						
duncanensis	*hoodensis*	0.056	2.2e-39	0.030	**0.914**	**1**
chathamensis	*hoodensis*	0.002	9.8e-18	**0.945**	0.053	**1**
chathamensis	*duncanensis*	0.137	0.008	0.850	0.004	**0.991**
hoodensis	*phantasticus*	0.791	3.6e-28	0.182	0.028	**1**
duncanensis	*phantasticus*	4.0e-11	2.7e-30	0.081	**0.919**	**1**
chathamensis	*phantasticus*	0.452	0.009	0.128	0.411	**0.991**

Within Isabela Island (Domed Clade)						
becki-PBL	*becki*-PBR	4.9e-5	**0.994**	0.005	0.0002	0.0052
vicina	*guntheri*	0.264	8.3e-7	0.026	0.709	**0.999**
vicina	*vandenburghi*	0.259	0.372	0.153	0.215	0.627
vicina	*microphyes*	0.103	0.381	0.452	0.063	0.618
vicina	*becki*-PBL	2.3e-5	0.005	**0.971**	0.023	**0.994**
vicina	*becki*-PBR	0.0001	**0.995**	6.0e-5	0.004	0.0041
guntheri	*vandenburghi*	0.216	0.633	0.125	0.024	0.365
guntheri	*microphyes*	0.0005	1.0e-6	**0.994**	0.005	**0.99**
guntheri	*becki*-PBL	0.175	0.291	0.0006	0.533	0.71
guntheri	*becki*-PBR	**0.994**	0.002	0.001	0.002	**0.997**

vandenburghi	*microphyes*	0.660	0.053	0.253	0.033	**0.946**
vandenburghi	*becki*-PBL	0.188	1.6e-11	0.467	0.345	**1**
vandenburghi	*becki*-PBR	0.067	0.220	0.384	0.329	0.78
microphyes	*becki*-PBL	0.743	0.225	0.024	0.008	0.775
microphyes	*becki*-PBR	0.004	0.001	0.054	**0.940**	**1**
Within Santa Cruz Island (Domed Clade)						
donfaustoi	*porteri*	8.5e-11	0.733	0.232	0.035	0.267
Santa Cruz Island with Isabela Island (Domed Clade)						
donfaustoi	*becki*-PBL	0.381	0.496	0.099	0.022	0.502
donfaustoi	*becki*-PBR	**0.999**	3.3e-7	3.5e-7	7.1e-8	**1**

donfaustoi	*vandenburghi*	0.167	3.0e-31	0.799	0.034	**1**
donfaustoi	*microphyes*	0.709	0.130	0.014	0.147	0.87
donfaustoi	*guntheri*	0.117	0.478	0.033	0.372	0.522
donfaustoi	*vicina*	0.333	7.1e-34	0.658	0.009	**1**
porteri	*becki*-PBL	**0.970**	0.019	0.007	0.004	**0.981**
porteri	*becki*-PBR	0.018	0.067	0.762	0.153	**0.933**
porteri	*vandenburghi*	0.401	2.4e-31	0.593	0.005	**0.999**
porteri	*microphyes*	0.017	0.024	0.880	0.078	**0.975**
porteri	*guntheri*	0.009	0.186	0.803	0.002	0.814
porteri	*vicina*	0.186	0.119	0.693	0.001	0.88

Isabela and Santa Cruz Islands compared with Santiago Island (Domed Clade)						
darwini	*becki*-PBL	0.029	4.1e-10	**0.932**	0.038	**0.999**
darwini	*becki*-PBR	0.707	1.3e-31	0.121	0.173	**0.999**
darwini	*donfaustoi*	**0.969**	1.2e-28	0.0289	0.002	**0.999**
darwini	*porteri*	0.007	0.291	0.003	0.698	0.708
darwini	*guntheri*	0.706	1.3e-31	0.120	0.172	**0.996**
darwini	*microphyes*	0.848	0.019	0.015	0.118	**0.981**
darwini	*vandenburghi*	0.010	0.679	0.064	0.246	0.32
darwini	*vicina*	0.148	2.0e-18	0.802	0.049	**0.999**

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