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# Demographic history and patterns of molecular evolution from whole genome sequencing in the radiation of Galapagos giant tortoises

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## Abstract

Whole genome sequencing provides deep insights into the evolutionary history of a species, including patterns of diversity, signals of selection, and historical demography. When applied to closely related taxa with a wealth of background knowledge, population genomics provides a comparative context for interpreting population genetic summary statistics and comparing empirical results with the expectations of population genetic theory. The Galapagos giant tortoises (*Chelonoidis* spp.), an iconic rapid and recent radiation, offer such an opportunity. Here, we sequenced whole genomes from three individuals of the 12 extant lineages of Galapagos giant tortoise and estimate diversity measures and reconstruct changes in coalescent rate over time. We also compare the number of derived alleles in each lineage to infer how synonymous and nonsynonymous mutation accumulation rates correlate with population size and life history traits. Remarkably, we find that patterns of molecular evolution are similar within individuals of the same lineage, but can differ significantly among lineages, reinforcing the evolutionary distinctiveness of the Galapagos giant tortoise species. Notably, differences in mutation accumulation among lineages do not align with simple population genetic predictions, suggesting that the drivers of purifying selection are more complex than is currently appreciated. By integrating results from earlier population genetic and phylogeographic studies with new findings from the analysis of whole genomes, we provide the most in-depth insights to date on the evolution of Galapagos giant tortoises, and identify discrepancies between expectation from population genetic theory and empirical data that warrant further scrutiny.

KEYWORDS
*Chelonoidis*, genetic diversity, multiple sequentially Markovian coalescent, population genetics

## 1 | INTRODUCTION

The ability to sequence whole genomes at relatively low cost is allowing greater insight into molecular evolutionary patterns and population history of non-model organisms (Ekblom & Galindo, 2011; Ellegren, 2014). Sequencing members of a group of closely related taxa, rather than a single species, can provide additional comparative context for interpreting population genetic summary statistics, outcomes of population structure analyses, and coalescent models (Brawand et al., 2014; Svardal et al., 2021; Teng et al., 2017). Such context is important because for many analyses different processes can produce similar patterns, and teasing apart which type of evolutionary

  

force (e.g., selection, drift, mutation and/or gene flow) is the main contributor to an observed pattern can be very difficult in the absence of additional independent information (Beichman et al., 2018; Mazet et al., 2016). For example, Tajima's *D* is expected to have a value of zero when a population is at equilibrium with no selection, but deviate from zero due to selection or demographic changes (Tajima, 1989). Likewise, longer runs of homozygosity can be due to recent inbreeding, historically small population size, or selective sweeps (Ceballos et al., 2018). In recent years, sequentially Markovian coalescent (SMC) models have gained popularity to estimate historical demography, but they have also been extensively critiqued for their sensitivity to the confounding effects of historical population structure and admixture (Mazet et al., 2016; Orozco-terWengel, 2016). Without additional context, the relative roles of these evolutionary forces in shaping the results of these analyses can be difficult to interpret.

The radiation of Galapagos giant tortoises (*Chelonoidis* spp., Figure 1) offers an ideal comparative context to examine evolutionary forces, as it is a system of closely related, recently diverged species that share a genomic architecture. There is a wealth of existing information about the geological history of the Galapagos archipelago that has provided important context for reconstructing colonization and divergence processes in this group (e.g., Geist et al., 2014; Grehan, 2001; Poulakakis et al., 2012, 2020), as has information on the recent human-mediated impacts that have shaped contemporary tortoise populations (Pritchard, 1996). Population genetic and phylogenetic studies of this system have progressed over the last two decades (reviewed in Caccone, 2021), benefiting from increasingly comprehensive geographic and genetic sampling, and advances in the number and type of available genetic markers, from sequencing a handful of genes and genotyping microsatellites (Caccone et al., 1999, 2002; Ciofi et al., 2002, 2006; Edwards et al., 2013, 2014; Garrick et al., 2012; Russello et al., 2007, 2010), to reduced-representation sequencing (Gaughran et al., 2018; Jensen, Edwards, et al., 2018; Miller et al., 2018), full mitochondrial genome sequencing (Jensen, Miller, et al., 2018; Poulakakis et al., 2020) and publication of a reference genome (Quesada et al., 2019). Thus, our understanding of the differentiation among, and levels of diversity within the species has been built up over time, with each generation of genetic technology providing additional data and insights (see "Study System" in Materials and methods for a brief summary).

Our background knowledge on the history of the Galapagos giant tortoises can also be leveraged to add to a growing understanding of

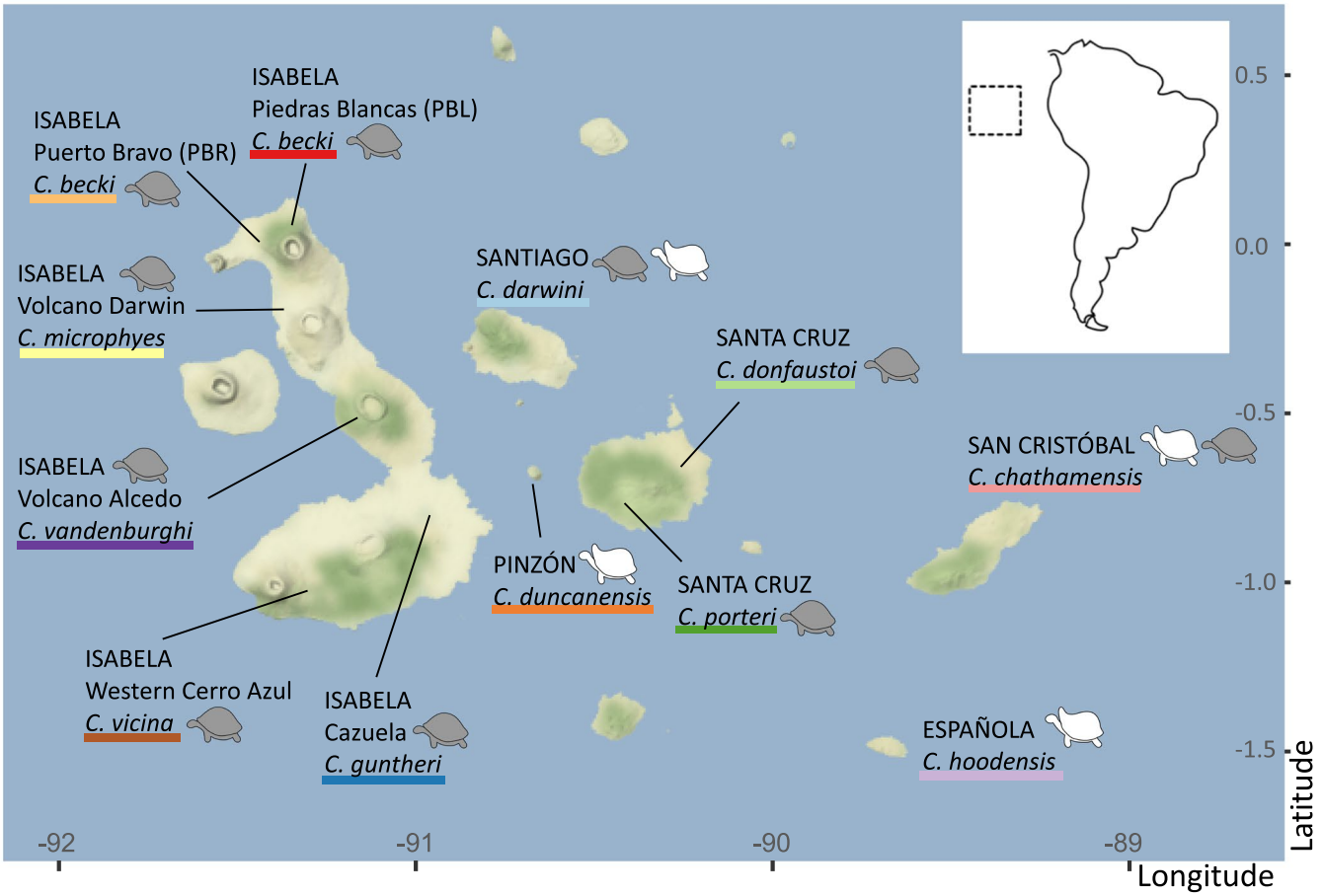


**FIGURE 1** F Map of the Galapagos archipelago indicating the locations of each lineage of *Chelonoidis* spp. sampled. Island names are in all capitals, and taxon names in italics. Tortoise icons depict the general morphology of each lineage, either domed (grey) or saddle-backed (white), with both icons together indicating semi-saddle-backed lineages [Colour figure can be viewed at wileyonlinelibrary.com]

the correlates of patterns of molecular evolution in natural populations. There has recently been renewed interest in studying differences in the genome-wide substitution rates and nonsynonymous mutation accumulation rates across lineages, which may reflect differences in the efficiency of purifying selection in different populations (Do et al., 2015; Simons & Sella, 2016). Nearly neutral theory predicts that amino acid substitutions—presumed to be slightly deleterious—will occur at a higher rate in small populations due to increased drift (Ohta, 1995). In studies of natural populations, however, these patterns are not always straightforward, with higher rates of mutation accumulation showing up in some small populations (Robinson et al., 2018; Xue et al., 2015), but not in others (Do et al., 2015; Gaughran, 2020; van der Valk et al., 2021). Specific demographic scenarios, as well as the distribution of fitness effects of alleles and the proportion of additive versus recessive alleles, all seem to affect the pattern of mutation accumulation in natural populations (Do et al., 2015; van der Valk et al., 2021). Given the apparent complexity of this phenomenon and the difficulty in appropriately parameterizing the relevant population genetic models, more empirical studies of the patterns of molecular evolution in natural populations are needed to help interpret these statistics, and the Galapagos giant tortoises provide a useful case.

In this study, we analyse whole nuclear genome sequences from three individuals of each of the extant species of Galapagos giant tortoises, and use these new data to build upon our understanding of within population diversity and demographic history. We also compare the number of derived alleles in each lineage to infer how synonymous and nonsynonymous mutation accumulation rates correlate with population history. Ultimately, this work highlights the complexity of evolutionary forces that lead to molecular change. Our results signal that we still have a poor understanding of these molecular processes in natural populations, but provides a starting point to compare patterns of molecular evolution across species radiations. Ultimately, more studies are required in empirical systems, as well as further development of theory, to better understand molecular evolutionary processes and eventually apply this to conservation issues.

## 2 | MATERIALS AND METHODS

### 2.1 | Study system

Tortoises rafted to the Galapagos archipelago in a single successful colonization event from the South American mainland (Caccone et al., 1999), probably first establishing on San Cristóbal or Española Island (or a joint-paleoisland). Following initial establishment, giant tortoises colonized the rest of the archipelago, with divergence among lineages in the radiation beginning ~1.5 million years ago (Ma), roughly following the island progression rule, moving east to west in accordance with the pattern of island emergence (Poulakakis et al., 2020). A combination of vicariance and dispersal have contributed to divergence among the 14 named species (Poulakakis et al., 2012). Today, most of the islands occupied by tortoises have only a single species, with two exceptions. Santa Cruz Island has two allopatric species, *C. porteri* and *C. donfaustoi*, which arose through separate colonization events (Caccone et al., 2002; Poulakakis et al., 2015; Russello et al., 2005), and Isabela Island, which consists of five volcanos and has a different species associated with each. The species on the southern (*C. guntheri*, *C. vicina*) and central volcanoes (*C. microphyes*, *C. vandenburghi*) probably diverged from a common ancestor that colonized Isabela Island from Santa Cruz within the last 400,000 years (Poulakakis et al., 2020). The northernmost volcano on Isabela Island, Volcano Wolf, has a single species, *C. becki*, that consists of two genetically distinct lineages (referred to as Piedras Blancas [PBL] and Puerto Bravo [PBR]), which arose from two colonization events from Santiago Island (Garrick et al., 2014). *Chelonoidis becki* is the only species in the radiation that is known to have more than one genetic population. Thus, for the other Galapagos giant tortoise species, there is the rare opportunity to estimate population genetic parameters (e.g., the effective population size) for the species as a whole.

Galapagos giant tortoises were heavily exploited by humans and suffered negative impacts from invasive species throughout the 18th, 19th and 20th centuries, which drove three species to extinction (*C. abingdonii* on Pinta, *C. niger* on Floreana, and an unnamed species from Santa Fé Island), and several others to the brink (Pritchard, 1996). Conservation programmes, including captive breeding and head-starting, have been instrumental in restoring self-sustaining tortoise populations on some islands, while intensive eradication of invasive species has eliminated or reduced threatening processes (e.g., Cayot, 2008; Cayot et al., 1994; Gibbs et al., 2021). Population bottlenecks were experienced by all the Galapagos giant tortoise species, but with varying degrees of severity. For example, *C. hoodensis* from Española was reduced to 15 individuals, which all share a single mitochondrial DNA control region haplotype, and have the lowest microsatellite allelic diversity and heterozygosity among the extant species (Garrick et al., 2015). This species has been brought back through captive breeding, and now numbers >2,000 individuals in the wild, but genetic diversity cannot recover as quickly as population size (Milinkovitch et al., 2013). Other species of Galapagos giant tortoise maintained populations in the hundreds or thousands throughout the period of decline, and while some have relatively high levels of diversity, others do not, potentially due to their more recent founder events (Garrick et al., 2015).

Galapagos giant tortoises are long-lived organisms with distinct carapace morphologies. They reach sexual maturity around 18–25 years of age, living up to 200 years. Although their age of first reproduction is around 15–20 years, they can continue to reproduce up to 100 years (Gibbs & Goldspiel, 2021). This life history creates difficulties for assigning a generation time in population genetic analyses, as the long overlapping generations cannot be accurately represented by a single point value that some analyses require (Lehtonen & Lanfear, 2014).

There are distinctive morphological types in Galapagos giant tortoises that are correlated with the environment they occupy (Chiari,

2021). Some lineages (for instance, *C. porteri* and *C. vandenburghi*) are characterized by having a "domed" carapace. They usually live in high elevation, upland areas with lush vegetation. Other lineages (for instance, *C. hoodensis* and *C. duncanensis*) have carapaces with an upward arched front opening, resulting in a "saddle-backed" shape that is associated with more arid habitat. There is large variation in morphology within some lineages that fall between the two types (for instance, *C. chathamensis* and *C. darwini*), which are classified as being "semi-saddle backed".

## 2.2 | Data collection

We selected from an archive of samples collected for previous studies (Caccone et al., 1999, 2002; Ciofi et al., 2002) three individuals of each of the 12 extant lineages of Galapagos giant tortoise (i.e., the 11 named species, including both the PBL and PBR lineages of *C. becki*), plus one Chaco tortoise (*C. chilensis*) as an outgroup, which has been previously identified as the closet living ancestor (Caccone et al., 1999). DNA was extracted from blood using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Shotgun sequencing libraries were prepared by the Yale Center for Genome Analysis and sequenced on an Illumina NovaSeq.

## 2.3 | Sequence processing and alignment

Sequences were processed and aligned to the *C. abingdonii* reference genome (assembly ASM359739v1, Quesada et al., 2019). *Chelonoidis abingdonii* is an extinct species of Galapagos giant tortoise that is most closely related to *C. hoodensis* (Caccone et al., 1999). Although using an in-group reference genome can bias downstream analyses, the very recent divergence among all the species studied minimizes this, and there is no evidence that one species has better alignment than the others (see Results). We excluded mitochondrial contigs from the reference genome, and used the BAM pipeline in PALEOMIX (version 1.2.14, Schubert et al., 2014) to trim, align and filter the data. PALEOMIX employs standard bioinformatic tools and native scripts in a streamlined workflow, including sequence trimming using ADAPTERREMOVAL (version 2.3.1, Lindgreen, 2012), alignment using BWA mem (version 0.7.17, Li & Durbin, 2009), PCR duplicate removal using PICARD MarkDuplicates (version 2.6.0, http://broadinstitute.github.io/picard/) and paleomix rmdup_collapsed, and indel realignment using GATK IndelRealigner (McKenna et al., 2010). The resulting BAM files were filtered for map quality (MQ ≥ 30), retaining only primary alignments, and with an insert size between paired end reads of less than 800 bp using BAMTOOLS (version 2.5.1, Barnett et al., 2011). The BAM files were also filtered to remove repetitive regions of the genome using a reference genome "mapability mask file" generated through the program SEQBILITY (https://github.com/lh3/misc), and regions identified by REPEATMASKER (Smit et al., 1996). Finally, we also removed contigs less than 100 kb in length, as these tended to have lower mean mapping quality scores (contigs retained $n$ = 2,598, total length 2,226,678,034 bp, equal to 96.8% of the genome). The mean coverage and mean mapping quality of the BAM files were calculated using QUALIMAP2 (Okonechnikov et al., 2015).

We used the filtered BAM files as input to detect variants and call genotypes using BCFTOOLS mpileup/call (Danecek et al., 2021), and filtered the resulting vcf file with VCFTOOLS (Danecek et al., 2011) to only call genotype supported by a minimum depth of six with a minimum genotype quality score of 18, and sites were filtered to drop loci with any missing data that had more than two alleles, or that had coverage greater than 1 standard deviation above the mean. We used the --indep-pairwise function in PLINK v1.9 (Chang et al., 2015) to prune out loci in linkage disequilibrium (LD) using a sliding window size of 50 kb, step size of five loci, and $r^2$ threshold of 0.5.

## 2.4 | Population structure

The distinctiveness of the 12 previously recognized Galapagos giant tortoise lineages was re-assessed using principal components analysis (PCA), implemented in PLINK v2.0 using the --pca var-wts option, and plotted in *R*. We measured population differentiation using $F_{ST}$ (Weir & Cockerham, 1984), calculated in PIXY (Korunes & Samuk, 2021) using a vcf file that included invariant sites. $F_{ST}$ was estimated in 10,000 bp windows along the genome, which were then averaged and visually represented as a two-dimensional neighbour-net phylogenetic network (Bryant & Moulton, 2004) using SplitsTree (Huson & Bryant, 2006). Another measure of lineage differentiation, between group nucleotide divergence ($D_{XY}$), was also estimated in PIXY.

## 2.5 | Within population diversity

Genome-wide heterozygosity and nucleotide diversity ($\pi$) are common measures of genetic diversity that are thought to positively correlate with long-term effective population size, and to be relatively slow to respond to population bottlenecks (Allendorf, 1986; Charlesworth, 2009). Genome-wide heterozygosity for each individual was calculated using the --het command in VCFTOOLS to identify the number of heterozygous sites, which was divided by the total number of sites (including invariant), and then averaged within each lineage. We used PIXY to calculate $\pi$, again, using a vcf file that included invariant sites, and calculated an average within each lineage.

## 2.6 | Demographic history

To reconstruct changes in coalescent rate through time for each of the 12 lineages, which can be correlated to changes in effective population size ($N_e$), we used the multiple sequentially Markovian

coalescent (MSMC) model implemented in MSMC2 (Schiffels & Durbin, 2014; Schiffels & Wang, 2020). In this method, the SMC model is run within each individual tortoise's unphased diploid genome and a composite likelihood approach is used to combine the models across individuals with a given lineage. We followed the general protocol outlined in Schiffels and Wang (2020). Briefly, we used the variants detected by BCFTOOLS mpileup/call, filtering out sites that had base quality below 30 (-q 30) and map quality below 30 (-Q 30), and removing indels. The MSMC2 recommended pipeline uses a mask file in addition to the ones we applied to the BAM files, which for each individual masks regions that are more than twice or less than half the average depth of coverage for the sample. In this case, sites with too high coverage may represent repetitive elements in the genome, while sites with unexpectedly lower coverage may have allelic dropout or low confidence genotype calls. Contigs that were entirely invariant in any sample, and therefore could not be included in the model, were removed from analyses for all samples. The *y*-axes of MSMC plots are sometimes scaled by mutation rate to obtain estimates of $N_e$ from the inverse coalescent rates. Because our results show clear signs that the MSMC coalescent rate does not always correspond to $N_e$ (see Results and Discussion), we chose to leave the *y*-axis as inverse coalescent rate. We chose to scale the *X*-axis by a per generation mutation rate of $1 \times 10^{-8}$, which falls between the estimated mutation rates of $3.3 \times 10^{-9}$ in Shaffer et al. (2013) and $2.5 \times 10^{-8}$ in Loire et al. (2013), and a generation time of 25 years, by which age individuals have reached sexual maturity (Gibbs & Goldspiel, 2021; Throp, 1975).

### 2.7 | Mutation accumulation

To compare mutation accumulation across lineages, we annotated variants and calculated the *R*-statistics developed by Do et al. (2015) using the pipeline described in Gaughran (2020) and available at https://github.com/sjgaughran/mutation-accumulation. This method relies on two assumptions: that synonymous derived alleles are neutral and that nonsynonymous derived alleles are nearly neutral or slightly deleterious. Under the nearly neutral model of molecular evolution, slightly deleterious alleles are more likely to rise to high frequencies in populations with smaller $N_e$ compared to those with larger $N_e$, given the increased role of drift in smaller populations. Accordingly, the rate of derived nonsynonymous mutation accumulation—as measured by the *R* statistic—is expected to be higher in smaller populations (Do et al., 2015).

To calculate the *R* statistic, variants in protein-coding genes were first annotated with SNPeff database built from the *C. abingdonii* reference genome (Quesada et al., 2019). In the SNPeff annotation pipeline, a database of all coding sequence (CDS) regions was created based on a GFF genome annotation file. Each variant in the vcf file was then compared to the database to determine if the alternate allele caused an amino acid change (nonsynonymous) or was silent (synonymous). Variants were polarized by considering the Chaco tortoise allele as ancestral (with sites that were heterozygous in the Chaco tortoise removed from the calculations). In addition to identifying synonymous and nonsynonymous alleles, we also annotated the degree of change in amino acids of nonsynonymous variants. We followed the classification system devised by Li et al. (1984) that calculates the difference between amino acids using the Grantham distance (*D*; Grantham, 1974) and categorizes changes as conservative ($D < 50$), moderately conservative ($50 < D < 100$), moderately radical ($100 < D < 150$), or radical ($D \geq 150$).

*R*-statistics are based on a pairwise comparison that calculates a ratio of the sum of derived alleles found only in lineage X (and not in Y) to those found in lineage Y (and not in X). When the ratio is greater than 1, we can infer that lineage X has accumulated more derived mutations, whereas when it is <1 we can infer that lineage X has accumulated fewer derived mutations. Statistical significance was assessed through bootstrapping. Briefly, the vcf file was split into 1,000 subfiles containing equal numbers of contiguous SNPs. A random set of 1,000 subfiles were then sampled with replacement and the *R*-statistics were calculated. This bootstrapping was repeated 1,000 times to create a 95% confidence interval. When the interval did not include 1, the result was considered significant. In addition, by calculating $R_{X/Y}$ for different classes of variants (e.g., synonymous, nonsynonymous), we can calculate the $R'_{X/Y}$ class statistic that, similar to *dN/dS*, normalizes for differences in mutation rate, generation time, and genotyping error. This can also be extended to other classes, such as the radical and conservative amino acid changes described above. In this study, we employ this normalizing statistic in three ways:

$$R\prime_{X/Y}^{nonsyn} = R_{X/Y}^{nonsynonymous} / R_{X/Y}^{synonymous}$$

$$R\prime_{X/Y}^{rad/s} = R_{X/Y}^{radical} / R_{X/Y}^{synonymous},$$

and

$$R\prime_{X/Y}^{rad/con} = R_{X/Y}^{radical} / R_{X/Y}^{conservative}.$$

## 3 | RESULTS

### 3.1 | Population genomic sequencing and diversity

We obtained an average of 138 million reads per individual (range 10–208 million), that resulted in an average coverage in the filtered BAM files of 13.8× (range 9.5×–22.1×) across all 36 Galapagos giant tortoise individuals. There was no evidence of bias in alignment to the *C. abingdonii* reference genome, with an average of 99.2% of reads aligning to the reference genome across all 36 individuals (range: 95.6% [in individual CRU_38] to 99.7% [in AGO_32]), with an average mapping quality of 59.94 (Table S1). There were only five individuals with less than 99% of reads mapped to the *C. abingdonii* reference genome, and they were distributed across the lineages (one *C. guntheri*, one *C. porteri*, two *C. vicina*, and one *C. vandenburghi*). Alignment to the *G. evgoodei* reference genome ranged from 83.1% to 95.7% of reads (mean 93.3%), with an average mapping quality of 58.6 after filtering the BAM (Table S1).

Genome-wide heterozygosity differed by 1.86× between the lineage with the lowest value (*C. hoodensis*: 0.00022) and highest value (*C. becki*-PBL: 0.00041, Table 1). π followed similar patterns, as expected due to its mathematical similarity with heterozygosity (Table 1).

## 3.2 | Population structure and demographic reconstructions

The PCA, based on 1,283,467 SNP loci that passed filtering, showed individuals to cluster within lineages, and had clustering among lineages similar to that found in previous studies (Miller et al., 2018), with PC1 and PC2 explaining 2.9% and 2.7% of variance in the data set, respectively (Figure 2). PC3 and PC4 account for nearly the same amount of variance (2.4% and 2.3%, respectively, Figure 2). The southern (*C. guntheri*, *C. vicina*) and central Isabela Island lineages (*C. microphyes*, *C. vandenburghi*) cluster closely together, while the others are spread out along PC1, 2, 3 and 4. However, the southern and central Isabela lineages clearly separate when analysed without the other lineages (Figure 2).

$F_{ST}$ values ranged from 0.402 between *C. hoodensis* and *C. microphyes*, to 0.074 between *C. vandenburghi* and *C. guntheri*, with an average of 0.240 (Table S2). The neighbour-net phylogenetic network (Figure 3) depicted these patterns by grouping the southern (*C. guntheri*, *C. vicina*) and central Isabela Island lineages (*C. microphyes*, *C. vandenburghi*) on one end with comparatively short edges between them. *Chelonoidis duncanensis*, *C. hoodensis* and *C. chathamensis* are each out on a long edge, while *C. porteri* and *C. donfaustoi* share a portion of edge that connects them to the other species.

**TABLE 1** Diversity measures averaged for each lineage of Galapagos giant tortoise (*Chelonoidis* spp.), estimated from whole genome sequence of three individuals each

| Island | Lineage | Mean observed heterozygosity | π |
|---|---|---|---|
| Santiago | *darwini* | 0.000396 | 0.000350 |
| Santa Cruz | *donfaustoi* | 0.000275 | 0.000249 |
| Santa Cruz | *porteri* | 0.000382 | 0.000366 |
| Española | *hoodensis* | 0.000224 | 0.000211 |
| Pinzón | *duncanensis* | 0.000317 | 0.000290 |
| San Cristóbal | *chathamensis* | 0.000390 | 0.000387 |
| Isabela | *becki* –PBL lineage | 0.000417 | 0.000371 |
| Isabela | *becki* –PBR lineage | 0.000314 | 0.000294 |
| Isabela | *guntheri* | 0.000289 | 0.000274 |
| Isabela | *vicina* | 0.000266 | 0.000273 |
| Isabela | *vandenburghi* | 0.000294 | 0.000285 |
| Isabela | *microphyes* | 0.000285 | 0.000253 |
| Mean across lineages | | 0.000321 | 0.000300 |

*Note*: π, nucleotide diversity.

$D_{XY}$ ranged from 0.00050 between *C. porteri* and *C. chathamensis*, and 0.00030 between *C. guntheri* and *C. microphyes*, with an average of 0.00042 (Table S2).

On the MSMC plot (Figure 4), the *x*-axis is depicted as years ago, with the result in generations ago being scaled by the mutation rate. However, this conversion does not necessarily accurately represent true dates (due to the reliance on estimates of mutation rate and generation time; see Discussion), so we present the results as "MSMC years ago". The inverse coalescent rates recovered by MSMC often correspond to changes in $N_e$ and can be scaled to reflect $N_e$ using the per generation mutation rate, however in this study we feel this conversion may not be appropriate (see Discussion). Here, we found that the population curves of all Galapagos giant tortoise lineages follow the same trajectory prior to around 400,000 MSMC years ago when some lineages begin to diverge (Figure 4). *Chelonoidis porteri* and *C. donfaustoi*, the two species that co-occur on Santa Cruz Island, have similar, fluctuating trajectories until ~20,000 MSMC years ago. The southern (*C. guntheri*, *C. vicina*) and central Isabela Island lineages (*C. microphyes*, *C. vandenburghi*) appear to share a demographic trajectory through 10,000 MSMC years ago (Figure 4). Despite PBL and PBR being two lineages within a single species (*C. becki*), they have very different trajectories across the MSMC analysis (Figure 4). PBL, and to a lesser extent *C. chathamensis*, both show dramatic increases in inverse coalescent rate not seen in the other lineages (Figure 4).

## 3.3 | Mutation accumulation in Galapagos giant tortoises

Most of our comparisons of synonymous mutation accumulation are not significantly different across the 12 Galápagos giant tortoise lineages. However, *C. hoodensis* and *C. duncanensis* do show significantly higher rates of synonymous mutation accumulation compared to the other lineages (Table 2a). Conversely, *C. vicina* has a significantly lower rate of synonymous mutations compared to most other lineages (Table 2a). Because of this, we use the $R'_{X/Y}$ statistic, which controls for differences in neutral mutation or substitution rate (see Methods).

The pairwise $R'_{X/Y}$ statistic shows numerous significant differences in the rates of non-synonymous mutation accumulation across lineages (Table 2b). For example, *C. porteri* has significantly lower rates of nonsynonymous mutation accumulation compared to many other lineages (*C. chathamensis*, *C. guntheri*, *C. vicina*, *C. vandenburghi*, and *C. microphyes*). *Chelonoidis duncanensis* also has lower rates compared to *C. chathamensis*, *C. vandenburghi*, and *C. microphyes*. Although most lineages on Isabela Island do not have significantly different rates of nonsynonymous mutation accumulation, *C. vandenburghi* and *C. microphyes* have higher rates than *C. donfaustoi*, *C. porteri*, *C. duncanensis*, and *C. becki's* PBL lineage.

Finally, we looked at two measures of mutation accumulation that attempt to capture the magnitude of effects from the derived allele: $R'^{rad/s}_{X/Y}$ and $R'^{rad/con}_{X/Y}$. Both statistics show similar patterns,

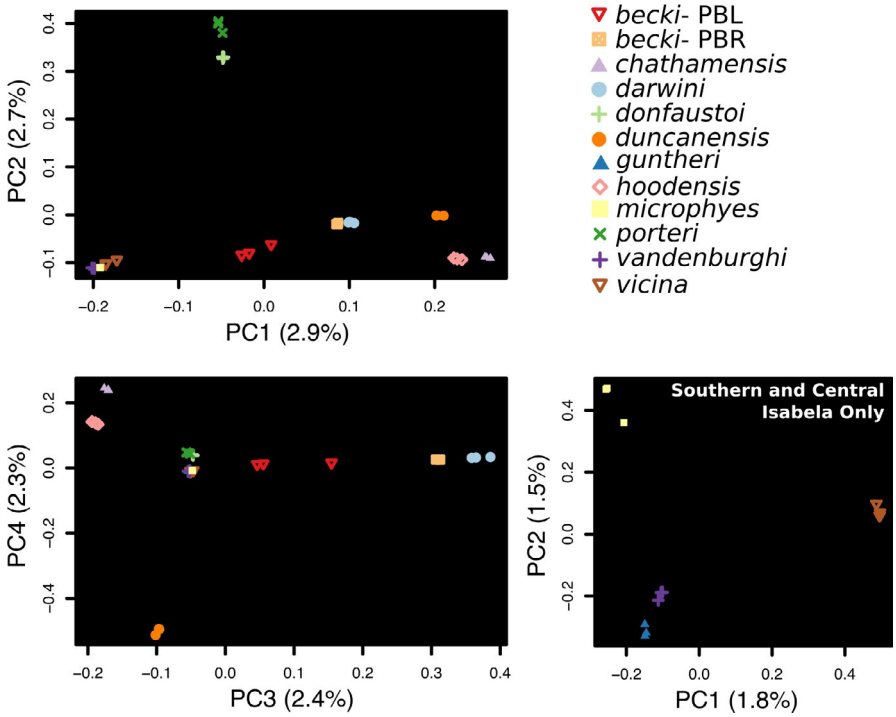


**FIGURE 2** Principle components analysis (PCA) plots from PLINK for 36 individuals representing the 12 *Chelonoidis* spp. lineages, and a subanalysis including only the lineages from southern (*C. guntheri*, *C. vicina*) and central Isabela Island (*C. microphyes*, *C. vandenburghi*). Each individual is represented as a point. Percentages on the axes correspond to the amount of variation explained by that axis [Colour figure can be viewed at wileyonlinelibrary.com]

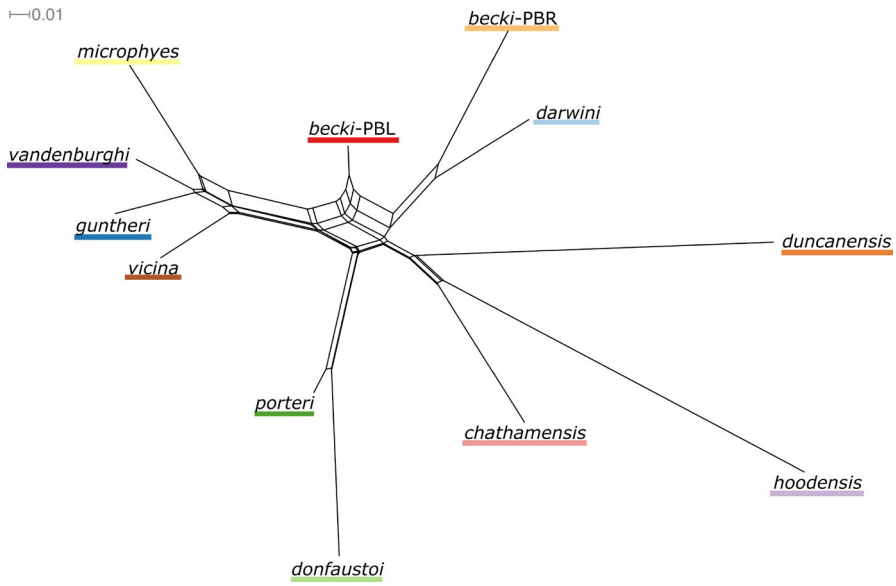


**FIGURE 3** Neighbor-net depicting the relationships among lineages, based on $F_{ST}$ values. The lengths of the edges on the network depict the degree of genetic differentiation [Colour figure can be viewed at wileyonlinelibrary.com]

in which *C. darwini* has a higher rate of radical non-synonymous mutation accumulation compared to *C. chathamensis* and *C. guntheri*, while *C. chathamensis* has a lower rate compared to *C. darwini*, *C. donfaustoi*, *C. porteri*, *C. hoodensis*, *C. duncanensis*, and *C. becki's* PBR lineages (Table 2c).

## 4 | DISCUSSION

Sequencing the whole genomes of Galapagos giant tortoises has presented us with new, deeper insights into the levels of diversity within lineages, and their demographic histories. The comparative context that an evolutionary radiation provides further allowed us to disentangle the evolutionary forces contributing to some of the complex patterns we observe and identified some contrasting patterns between the observed data and the expected ones based on theoretical predictions.

### 4.1 | Population structure and divergence

Our results on the distinctiveness of the lineages highlight the hierarchical patterns of differentiation among lineages. The large range in $F_{ST}$ values, from 0.402 to 0.074, is reflected in the elongated shape of the neighbor-net phylogenetic network (Figure 3), with some lineages clustered on short edges, and others out on very long edges.

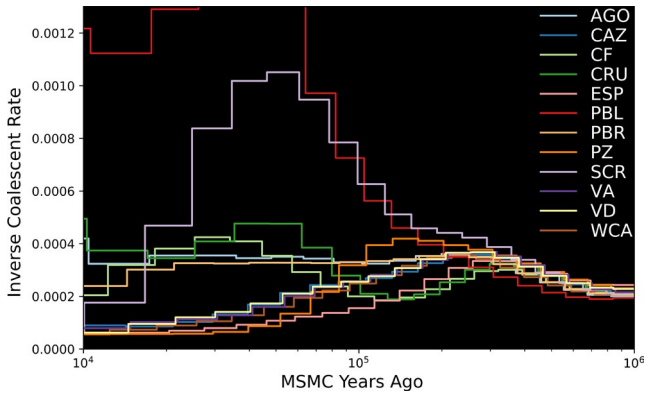


**FIGURE 4** MSMC plots of the 12 *Chelonoidis* spp. lineages, representing all extant species. Results have been scaled by a generation time of 25 years and a per generation genomic mutation rate of $1 \times 10^{-8}$ [Colour figure can be viewed at wileyonlinelibrary.com]

The lineage with the strongest differentiation from the other lineages, *C. hoodensis*, is found on the oldest island, Española (3–3.5 million years old, Geist et al., 2014), while the weakest differentiation was among the lineages from southern and central Isabela, an island that only formed between 500–800 thousand years ago (KYA) (Geist et al., 2014). The four lineages from southern and central Isabela originated from a single colonization event, with differentiation occurring in situ around 130 KYA, based on reconstructions from the mitochondrial genome (Poulakakis et al., 2020).

The patterns of divergence can also be inferred from MSMC plots. At times before divergence, lineages will share a common trajectory, but distinct trajectories can appear once lineages split and follow different evolutionary paths. In our study, we see that patterns of divergent trajectories in the MSMC analysis are broadly congruent with what has been found in previous studies that generated dated phylogenies using mitochondrial genes or whole mitochondrial genomes (Poulakakis et al., 2012, 2020). However, the MSMC analysis places the earliest divergence among lineages at around 300–400 KYA, which is substantially more recent than 1.5 Ma estimated by Poulakakis et al. (2020). This difference is probably due to a combination of factors that are both methodological and biological. For the mitochondrial genome phylogeny, nodes were dated using calibration points for lineage divergence among outgroups based on studies of fossils (Pereira et al., 2017). The MSMC analysis, on the other hand, was given a constant mutation rate based on genome-wide estimates of substitution rate from other testudines lineages, and a generation time of 25 years, to transform our time parameter from generations ago to years ago. A slower mutation rate or a longer generation time would linearly shift our time estimates in the MSMC to older time points. Given that Galapagos giant tortoises can breed for over 100 years and have overlapping generations, the parameters given to the MSMC are a necessary simplification that may not fully represent the biology of the system. Thus, estimates from our MSMC analysis are best viewed in relative, rather than absolute, terms.

## 4.2 | Reconstructions of population histories

The levels of genetic diversity within each Galapagos giant tortoise species, and the demographic reconstructions from the MSMC, reflect the long-term demographic history of the lineages. On Española Island, *C. hoodensis* has probably always had a small population size. This species has the lowest genome-wide heterozygosity, is fixed for a single mitochondrial haplotype (Caccone et al., 2002), and has had a steady decline in inverse coalescent rate since the time it diverged from the other lineages in the MSMC analysis. *Chelonoidis darwini* on Santiago Island, on the other hand, historically had a large, steady population size in the MSMC and maintained high levels of diversity.

The lineage with one of the largest population sizes today, *C. porteri*, likewise has high levels of heterozygosity. Found on the same island as *C. porteri*, *C. donfaustoi* is the most endangered lineage, with a census population size estimated to be just 400 individuals (IUCN 2020), with correspondingly low levels of diversity. These lineages have contrasting current statuses, yet show similar trajectories in the MSMC analysis (Figure 4), which could be due to them experiencing similar environmental conditions historically and/or be indicative of a history of shared gene flow. Although *C. porteri* and *C. donfaustoi* cluster relatively close together in PCA and DAPC analyses using nuclear SNPs in Miller et al. (2018) and in the PCA and neighbor-net in this study (Figures 2 and 3), it was previously assumed that this affinity was due to contemporary gene flow, not shared ancestry. These species have highly distinct mitochondrial haplotypes that place them in different clades in the Galapagos giant tortoise phylogeny, separated by the deepest divergences within the radiation, and are as differentiated as other species in the radiation based on microsatellite genotypic data (Caccone et al., 2002; Poulakakis et al., 2012, 2020; Russello et al., 2005). Evidence that these two lineages arose through separate colonizations of Santa Cruz Island, from different sources, led to them being described as different species (Poulakakis et al., 2015). However, the potential that they have a more shared history than previously understood presents an exciting challenge for future work to fully explore the history of these lineages.

Previous studies have found the *C. duncanensis* lineage to have a puzzling history. Pinzón Island is only 18 $km^2$ in area, and thus is unlikely to have supported a very large population of tortoises. Yet, previous demographic reconstructions have found *C. duncanensis* to have very high historical $N_e$ (Garrick et al., 2015; Jensen, Miller, et al., 2018). However, results from the whole mitochondrial genome in Poulakakis et al. (2020) interpreted in the light of geological reconstructions of connectivity among islands during glacial maxima (Ali & Aitchison, 2014), seemed to address this apparent paradox. Briefly, ancestral area reconstructions suggested that when the *C. duncanensis* lineage was historically large, it may have occupied a united landmass of modern-day Pinzón, Santa Cruz and Floreana Islands, before retreating and becoming isolated on Pinzón. The MSMC results here are congruent with this possible history, showing *C. duncanensis* to have a large population size ~150,000 MSMC years ago, and then gradually declining. The diversity measures in *C. duncanensis* (low heterozygosity and $\pi$) may reflect this demographic

**TABLE 2A** Pairwise $R_{X/Y}$ of synonymous sites

| Island | Lineage | *darwini* | *donfaustoi* | *porteri* | *hoodensis* | *duncanensis* | *chathamensis* | *becki*-PBL | *becki*-PBR | *guntheri* | *vicina* | *vandenburghi* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Santiago | *darwini* | – | | | | | | | | | | |
| Santa Cruz | *donfaustoi* | ns | – | | | | | | | | | |
| Santa Cruz | *porteri* | ns | ns | – | | | | | | | | |
| Española | *hoodensis* | 0.919 | 0.916 | 0.882 | – | | | | | | | |
| Pinzón | *duncanensis* | 0.852 | 0.848 | 0.817 | ns | – | | | | | | |
| San Cristóbal | *chathamensis* | ns | ns | 0.925 | ns | 1.127 | – | | | | | |
| Isabela | *becki*– PBL | ns | ns | ns | 1.110 | 1.195 | ns | – | | | | |
| Isabela | *becki*– PBR | ns | ns | ns | ns | 1.157 | ns | ns | – | | | |
| Isabela | *guntheri* | ns | ns | ns | ns | 1.167 | ns | ns | ns | – | | |
| Isabela | *vicina* | 1.104 | 1.102 | ns | 1.200 | 1.292 | 1.140 | 1.090 | 1.123 | 1.139 | – | |
| Isabela | *vandenburghi* | ns | ns | ns | 1.091 | 1.175 | ns | ns | ns | ns | 0.887 | – |
| Isabela | *microphyes* | ns | ns | 0.917 | ns | 1.125 | ns | 0.936 | ns | ns | 0.839 | ns |

*Note*: The column is lineage *X* and the row is lineage *Y*. $R_{X/Y}$ values >1.000 show elevated rates of derived synonymous mutation accumulation in lineage *X* (column lineage), and $R_{X/Y}$ values <1.000 show lower rates of derived synonymous mutation accumulation in lineage *X*.

history as well as its known recent history, as it experienced a severe bottleneck down to just 150–200 individuals in the 20th century (MacFarland et al., 1974).

*Chelonoidis chathamensis* from San Cristóbal Island shows a massive fluctuation in its MSMC trajectory. The inverse coalescent rates recovered by MSMC are often scaled using the per-generation mutation rate and presented as $N_e$. However, increases in inverse coalescent rate may not always correspond to increases in $N_e$, and can be caused instead by historical population subdivision or admixture events (Brandvain et al., 2014; Nadachowska-Brzyska et al., 2016). The recent finding of a now-extinct second mitochondrial lineage on San Cristóbal Island (E. L. Jensen et al., Unpublished data) suggests gene flow and/or population subdivision as possible drivers of the increase in inverse coalescent rate seen for *C. chathamensis* instead of a dramatic increase in $N_e$. *Chelonoidis chathamensis* also shows an interesting pattern of significantly lower rates of radical amino acid changes ($R'_{X/Y}$) compared to many other lineages (Table 2c), although the significance of this is unclear.

The other lineage with a fluctuation in its MSMC is *C. becki*-PBL. Found on the northernmost volcano of Isabela Island, the two lineages within *C. becki* have a complex and dynamic history. The lineages arose through two colonization events, one ~200 kya eventually becoming PBL and the other ~50 kya which became PBR, originating from the same source on neighbouring Santiago Island. The lineages have coexisted since the second colonization event, and over time, are expected to fuse due to introgressive hybridization (Garrick et al., 2014). Some introgression between PBL and PBR may be seen in the PCA (Figure 2). PBL shows the greatest spread among individuals in a lineage, with one PBL individual closer to PBR and *C. darwini* than the others along PC1 and 3. However, this more recent gene flow is unlikely to be the cause of the observed fluctuation in inverse coalescent rate in PBL. Yet, an increase in $N_e$ to such an elevated level, substantially higher than any of the other lineages at any point in time, also seems unlikely. A situation that could lead to this pattern is if the modern PBL population was the descendant of a hybrid lineage that formed after tortoises colonizing northern Isabela from Santiago interbred with tortoises already present there, which subsequently underwent a complete range shift, or went extinct. *Chelonoidis vandenburghi* mitochondrial haplotypes have been found in the PBL lineage (Garrick et al., 2014), but whether that is due to ancient gene flow from a scenario similar to that described above or recent admixture is unclear.

The history of the *C. vandenburghi* lineage is particularly interesting. Previous studies have shown this species to have fewer and more closely related mitochondrial haplotypes than the other southern and central Isabela lineages. Beheregaray et al. (2003) suggested that this was due to a volcanic eruption ~100 kya that caused complete or near extinction of the population there, which was replaced by migrants from Sierra Negra (where the present-day *C. guntheri* lineage lives) that carried a single mitochondrial haplotype. Evidence from microsatellites further suggested a bottleneck in the *C. vandenburghi* lineage, but the MSMC reconstruction here (Figure 4) does not show a decline. Notably, though, SMC analyses are insensitive to

**TABLE 2B** Pairwise $R'_{X/Y}$ (derived nonsynonymous over synonymous sites)

| Island | Lineage | *darwini* | *donfaustoi* | *porteri* | *hoodensis* | *duncanensis* | *chathamensis* | *becki-PBL* | *becki-PBR* | *guntheri* | *vicina* | *vandenburghi* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Santiago | *darwini* | – | | | | | | | | | | |
| Santa Cruz | *donfaustoi* | ns | – | | | | | | | | | |
| Santa Cruz | *porteri* | ns | ns | – | | | | | | | | |
| Española | *hoodensis* | ns | ns | ns | – | | | | | | | |
| Pinzón | *duncanensis* | ns | ns | ns | ns | – | | | | | | |
| San Cristóbal | *chathamensis* | ns | ns | 0.891 | ns | 0.906 | – | | | | | |
| Isabela | *becki-* PBL | ns | ns | ns | ns | ns | ns | – | | | | |
| Isabela | *becki-* PBR | ns | ns | ns | ns | ns | ns | ns | – | | | |
| Isabela | *guntheri* | ns | ns | 0.905 | ns | ns | ns | ns | ns | – | | |
| Isabela | *vicina* | ns | ns | 0.897 | ns | ns | ns | ns | ns | ns | – | |
| Isabela | *vandenburghi* | ns | 0.897 | 0.861 | ns | 0.883 | ns | 0.895 | ns | ns | ns | – |
| Isabela | *microphyes* | ns | 0.903 | 0.865 | ns | 0.890 | ns | 0.900 | ns | ns | ns | ns |

*Note*: The column is species *X* and the row is species *Y*. $R'_{X/Y}$ values >1.000 show elevated rates of amino acid mutation accumulation in species *X* (column species), and $R'_{X/Y}$ values <1.000 show lower rates of amino acid mutation accumulation in species *X*.

short-term historical bottlenecks, even when they are intense (Henn et al., 2016).

### 4.3 | Patterns of molecular evolution

Remarkably, here we found that patterns of molecular evolution differ among the closely related lineages of Galapagos giant tortoises. These differences occur in the rates of both neutral synonymous mutation accumulation and non-neutral mutation accumulation, suggesting that multiple complex factors may have led to rapid changes in patterns of molecular evolution in Galapagos giant tortoises.

When examining only neutral synonymous sites, we observed that the *C. duncanensis* and *C. hoodensis* lineages, both of which have saddle-backed morphology, have elevated rates of synonymous mutation accumulation. This rate is expected to reflect the neutral mutation rate (Do et al., 2015; Simons & Sella, 2016), which suggests that these two species have higher average per year synonymous mutation rates compared to many other lineages, which are domed. Elevated observed mutation rates can occur either due to increased molecular mutation rate or decreased (shorter) generation time (Martin & Palumbi, 1993). A shorter generation time in these saddle-backed lineages seems like a more plausible explanation, although we do not yet understand the mechanism.

Since we found differences in neutral (synonymous) mutation accumulation across some lineages, we used the $R'_{X/Y}$ set of statistics for comparisons of nonsynonymous mutation accumulation, which control for differences in mutation rates across lineages (Do et al., 2015). $R'_{X/Y}$ statistics can reflect differences in the strength or pattern of selection on nonsynonymous sites. We found numerous pairs of Galapagos giant tortoise lineages with significant differences in rates of overall nonsynonymous mutation accumulation, as well as differences when the type of amino acid change (i.e., radical change or similar amino acid) was considered.

Once the elevated per-year mutation rate is taken into account, the *C. duncanensis* lineage's rate of nonsynonymous mutations is actually lower than other lineages. One interpretation is that purifying selection is more efficient in populations with large $N_e$, which *C. duncanensis* had between 100,000–200,000 MSMC years ago. However, *C. duncanensis* had among the lowest $N_e$ from 50,000 MSMC years to the end of the MSMC analysis. The *C. porteri* lineage also has very low rates of nonsynonymous mutation accumulation but had the lowest $N_e$ of all the lineages from 150,000–300,000 MSMC years ago followed by a larger $N_e$ in the last 100,000 MSMC years (Figure S1). Another possible explanation is that deleterious alleles have been purged from smaller populations. However, we found that most lineages have approximately the same rate of radical (i.e., presumably large-effect) mutation accumulation as measured by $R'^{rad/s}_{X/Y}$, which suggests that the differences in overall $R'_{X/Y}$ between species are driven by small-effect alleles.

Smaller populations are predicted to have higher rates of nearly-neutral mutation accumulation due to less efficient

**TABLE 2C** Pairwise $R'_{X/Y}{}^{rad/con}$ (derived radical over conservative change sites)

| Island | Lineage | *darwini* | *donfaustoi* | *porteri* | *hoodensis* | *duncanensis* | *chathamensis* | *becki-PBL* | *becki-PBR* | *guntheri* | *vicina* | *vandenburghi* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Santiago | *darwini* | – | | | | | | | | | | |
| Santa Cruz | *donfaustoi* | ns | – | | | | | | | | | |
| Santa Cruz | *porteri* | ns | ns | – | | | | | | | | |
| Española | *hoodensis* | ns | ns | ns | – | | | | | | | |
| Pinzón | *duncanensis* | ns | ns | ns | ns | – | | | | | | |
| San Cristóbal | *chathamensis* | 1.645 | 1.513 | 1.511 | 1.774 | 1.544 | – | | | | | |
| Isabela | *becki-* PBL | ns | ns | ns | ns | ns | ns | – | | | | |
| Isabela | *becki-* PBR | ns | ns | ns | ns | ns | 0.662 | ns | – | | | |
| Isabela | *guntheri* | 1.475 | ns | ns | 1.521 | ns | ns | ns | ns | – | | |
| Isabela | *vicina* | ns | ns | ns | ns | ns | ns | ns | ns | ns | – | |
| Isabela | *vandenburghi* | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | – |
| Isabela | *microphyes* | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |

*Note*: The column is lineage *X* and the row is lineage *Y*. $R'_{X/Y}{}^{rad/con}$ values >1.000 show elevated ratios of radical amino acid changes to conservative amino acid changes in lineage *X* (column lineage), and $R'_{X/Y}{}^{rad/con}$ values <1.000 show lower ratios of radical amino acid changes to conservative amino acid changes in lineage *X*.

purifying selection (Ohta, 1973), but this pattern does not always emerge in natural populations. For example, Do et al. (2015) found that while both Denisovans and Neanderthals had much smaller $N_e$ compared to modern humans, only Denisovans had significantly elevated nonsynonymous mutation. Similarly, van der Valk et al. (2021) found that mammal populations with historically small effective population sizes carried significantly lower genetic load than larger populations, possibly due to long-term purging of deleterious alleles. In addition, population genetic theory predicts that slightly deleterious mutations will accumulate or be purged at different rates depending on if they contribute additively or recessively to genetic load (Do et al., 2015; Simons & Sella, 2016), a phenomenon that is still not understood in natural populations. The fact that some smaller populations in our study have lower rates of mutation accumulation, along with these previous empirical and theoretical findings, suggests that the process of nearly neutral mutation accumulation is complex in natural populations. The pattern of mutation accumulation may therefore be affected by recent inbreeding (e.g., Xue et al., 2015), historically small populations that allowed for the purging of strongly deleterious alleles (e.g., Robinson et al., 2018), or shifts in the distribution of fitness effects (Castellano et al., 2019). None of these scenarios alone, however, provide an obvious explanation for our observed patterns. Our empirical results therefore raise questions about both the plausibility of the assumptions involved in models of purifying selection and about our ability to accurately capture these evolutionary phenomena using genome-wide averages. Due to the confounding variables discussed above, we cannot draw conclusions about whether purging or long-term demography has driven lower mutation accumulation in our small-$N_e$ populations. Additional empirical studies from other species radiations are needed to better understand how these evolutionary processes play out in natural populations with complex histories.

## 4.4 | Conservation implications

Our results from the analysis of whole genome sequence data from multiple representatives of the 11 living Galapagos giant tortoise species furthers our ability to conserve these organisms by providing a richer understanding of genome-wide levels of diversity. Additionally, the more accurate understanding we are developing on the history of each lineage is also critical for conservation. For example, estimates of the carrying capacity of Española Island have suggested that it could support quite a large Galapagos giant tortoise population (Gibbs et al., 2014), yet our reconstructions here indicate that *C. hoodensis* maintained a relatively constant and small population size throughout time, a result also found in previous studies (Garrick et al., 2015). Understanding why the population would be naturally small when the island was estimated to be able to support larger numbers is an important avenue for future study and has implications for determining what census population size is an appropriate goal for conservation programs.

## 4.5 | Conclusions

The accumulated wealth of information about the history of the Galapagos archipelago and tortoise lineages have enabled us to interpret the results from whole genomes within a richer context than would be available for less well-studied organisms. For example, the massive increases in inverse coalescent rate seen in the MSMC analysis for *C. chathamensis* and *C. becki*-PBL do not probably represent exceptionally large historical $N_e$, and are more likely to have been caused by historical gene flow or population substructure. Our finding of differences in the rates of molecular evolution in this group reinforces the distinctness of each lineage, and is particularly interesting because it suggests these changes can occur rapidly during species radiations. Still, the fact that lower levels of nearly neutral mutation accumulation do not obviously track with larger estimated population size makes it clear that more theoretical and empirical studies are needed to understand the impacts of purifying selection on molecular evolution. Future advances in understanding the distribution of fitness effects and the functional implications of derived mutations will be key to a fuller understanding of molecular evolution in natural populations, including those of conservation concern.

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## AUTHOR CONTRIBUTIONS

Evelyn L. Jensen - conceptualization, methodology, investigation, formal analysis, data curation, writing the original draft, writing-review and editing, visualization. Steven J. Gaughran - conceptualization, methodology, formal analysis, writing-original draft, writing-review and editing, and visualization. Ryan C. Garrick - conceptualization, writing-review and editing. Michael A. Russello - conceptualization, writing-review and editing. Adalgisa Caccone - conceptualization, resources, writing-original draft, writing-review and editing, supervision, project administration, funding acquisition.

## DATA AVAILABILITY STATEMENT

Fastq files for all individuals are available on the NCBI Short Read Archive as bioproject PRJNA761229.

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