



Original Article

# Temporal Mitogenomics of the Galapagos Giant Tortoise from Pinzón Reveals Potential Biases in Population Genetic Inference

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## Abstract

Empirical population genetic studies generally rely on sampling subsets of the population(s) of interest and of the nuclear or organellar genome targeted, assuming each is representative of the whole. Violations of these assumptions may impact population-level parameter estimation and lead to spurious inferences. Here, we used targeted capture to sequence the full mitochondrial genome from 123 individuals of the Galapagos giant tortoise endemic to Pinzón Island (*Chelonoidis duncanensis*) sampled at 2 time points pre- and postbottleneck (circa 1906 and 2014) to explicitly assess differences in diversity estimates and demographic reconstructions based on subsets of the mitochondrial genome versus the full sequences and to evaluate potential biases associated with diversity estimates and demographic reconstructions from postbottlenecked samples alone. Haplotypic diversities were equal between the temporal samples based on the full mitochondrial genome, but single gene estimates suggested either decreases or increases in diversity depending upon the region. Demographic reconstructions based on the full sequence were more similar between the temporal samples than those based on the control region alone, or a subset of 3 regions, where the trends in population size changes shifted in magnitude and direction between the temporal samples. In all cases, the estimated coalescent point was more distant for the historical than contemporary sample. In summary, our results empirically demonstrate the influence of sampling bias when interpreting population genetic patterns and punctuate the need for careful consideration of potentially conflicting evolutionary signal across the mitochondrial genome.

**Subject areas:** Conservation genetics and biodiversity

**Keywords:** Bayesian skyline plot, demographic history, historical DNA, mitogenome, museum specimens

Empirical population genetic studies that subsample the genome generally rely on 2 key assumptions, namely, that individuals sampled for the study are representative of the population(s) of interest and that the genetic markers employed provide signal that is representative of the genome as a whole (Avise 1994). Violation of either assumption may lead to spurious results, but short of sequencing the full genome of all individuals in a population or species, decisions must be made about which portion of the nuclear and/or organelle genomes to assay and in which individuals.

Studies of natural populations typically sample subsets of individuals to estimate genetic parameters across the *spatial* extent of the population distribution. Although this can vary greatly across systems and studies, the vast majority of this work relies on sampling populations at a *single time point* or *across a very narrow temporal scale* (but see Bouzat et al. 1998; Wisely et al. 2002; Miller and Waits 2003; Eldridge et al. 2004; Nyström et al. 2006; Ugelvig et al. 2011). However, having only one temporal snapshot may provide a biased view of population history, particularly if the sampled population has experienced a bottleneck in the recent past.

Regarding genetic marker choice, animal mitochondrial DNA has long been used as a source of historical information for many population-level studies (Avise et al. 1987). This choice can be attributed to a host of attractive properties, including the high copy number of mitochondrial genomes per cell providing ease of amplification from conventional DNA sources, and opportunity for recovery from noninvasively collected tissues (e.g., hair, feathers, feces) and archival materials (e.g., museum specimens, subfossils; reviewed in Wandeler et al. 2007 and Andrews et al. Forthcoming). Lack of recombination due to clonal inheritance, nearly neutral evolution, and a clock-like evolutionary rate (but see Galtier et al. 2009 and references therein) in animals have led to the almost universal treatment of the mitochondrial genome (mitogenome) as a single locus. Thus, as long as there is sufficient variation in the specific region(s) sequenced to address the objectives of a particular study, there may not be strong justification for investing resources in sequencing a longer portion of mitochondrial DNA. For example, the mitochondrial control region (CR) has been commonly targeted for population-level studies in animals, given its high mutation rate relative to other areas in the mitogenome. However, the assumption that the insights into population history provided by the CR, or a subset of protein-coding mitochondrial genes, reflect those provided by sequencing the whole mitogenome has not been, to our knowledge, directly tested.

The iconic Galapagos giant tortoises (*Chelonoidis* sp.) are a species complex that has been extensively studied using mitochondrial DNA (Caccone et al. 1999, 2002; Beheregaray et al. 2003; Russello et al. 2005, 2007, 2010; Poulakakis et al. 2008, 2012; Garrick et al. 2012, 2015; Jensen et al. 2016). Most studies have focused on the CR only, although multiple mitochondrial DNA regions have been sequenced from exemplar individuals from the 11 extant (Caccone et al. 2002, 2004) and 4 extinct species (Poulakakis et al. 2012) to reconstruct the evolutionary history of the species complex. The species endemic to Pinzón Island (*Chelonoidis duncanensis*, formerly referred to as *Chelonoidis ephippium*), in particular, has exceptionally high mitochondrial DNA CR haplotype diversity compared with the other species (Garrick et al. 2015), despite having experienced a bottleneck down to ~150–200 individuals in the 20th century due to human exploitation (MacFarland et al. 1974). The persistence of high levels of genetic diversity despite a significant demographic contraction has been attributed to a large historical effective population size, despite the small area of Pinzón Island [currently 18 km<sup>2</sup> but

may have been larger historically, see Ali and Aitchison (2014) and Geist et al. (2014)]. Previous diversity estimates and demographic reconstructions for the Pinzón giant tortoise have relied heavily on mitochondrial CR sequences collected from a postbottlenecked sample. However, the availability of a population-level sampling ( $n = 78$ ) of Pinzón giant tortoises collected before the bottleneck, in tandem with the advent of high-throughput DNA sequencing approaches has afforded a unique opportunity to further explore the seemingly incongruous diversity patterns in this species, and more broadly, to explicitly evaluate the potential effects of sampling biases in estimating population genetic parameters.

In this study, we used hybrid capture to sequence the mitogenome from a large sample of Pinzón Galapagos giant tortoises prebottleneck (collected c. 1906,  $n = 78$ ) and roughly one generation postbottleneck (collected 2014,  $n = 45$ ). Using these data, we evaluated 1) differences in diversity estimates and demographic reconstructions based on subsets of the mitochondrial genome versus the full sequences and 2) potential biases associated with diversity estimates and demographic reconstructions from postbottlenecked samples alone.

## Methods

### Study System

*Chelonoidis duncanensis* is endemic to Pinzón, a small island located in the center of the Galapagos archipelago. All analyses to date indicate that the species is comprised of a single unstructured population (Beheregaray et al. 2003; Jensen et al. 2015). Thus, in this case, the terms species and population refer to the same unit of biological organization. Due to hunting by humans for food, *C. duncanensis* declined over the 18th–19th centuries, ultimately resulting in a bottleneck in which the census population size was estimated to have been as low as 150–200 individuals in the 1960s (MacFarland et al. 1974). For this study, we used samples collected from Pinzón Island at 2 different time points: 1) in 1906 by the California Academy of Sciences (Van Denburgh 1914), representing the prebottlenecked population and 2) in 2014 (this study). Despite the collection of these samples being separated by more than 100 years, only roughly one generation has elapsed between them due to the population's history. It is thought that there was no successful recruitment from ~1890 to 1965 due to invasive black rats depredating all the hatching tortoises (Beck 1903; Pritchard 1996). Starting in 1965, a head start program collected eggs and hatchlings from natural nests in the wild and reared the young Pinzón giant tortoises in captivity, repatriating them to the wild when they were sufficiently large to no longer be at risk of predation from black rats, helping the population to recover to its current size of ~500 individuals (Cayot 2008). Given this population history, the individuals sampled in 2014 are most likely first-generation offspring of the bottleneck survivors, with the exception of 2 wild-born hatchlings which may be second-generation (Aguilera et al. 2015).

### Historical Sample Collection and DNA Extraction

Using a Dremel rotary tool with a cutting blade, wedge cuts of bone were made from femurs of 78 whole specimens accessioned in the California Academy of Sciences collection (Supplementary Table 1). DNA was extracted in a dedicated ancient DNA lab at the University of British Columbia Okanagan, Kelowna, BC. Samples were ground while submerged in liquid nitrogen using a Spex 6770 freezer mill, and then, a modified version of extraction protocol Y described by Gamba

et al. (2016) was followed to demineralize the bone, concentrate the lysate, and recover the DNA using a MinElute (Qiagen) column.

### Contemporary Sample Collection and DNA Extraction

Blood samples from contemporary individuals (Supplementary Table 2) were collected in December 2014 during a survey of the population on Pinzón Island. A small blood sample (0.1–1.0 mL) was collected from the brachial artery for 43 adults (mean carapace length 75 cm) and 2 hatchlings. Blood was stored in tubes containing a lysis buffer (100 mM Tris–HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS; Longmire et al. 1997) and stored at ambient temperature in the field and at 4 °C upon arrival in the lab. Genomic DNA was extracted using a NucleoSpin QuickBlood kit (Machery–Nagel) following the manufacturer’s protocols, with the addition of RNase A (Qiagen).

### Design of Baits for Targeted Capture of the Mitochondrial Genome

An existing 15 648 bp sequence of a draft mitochondrial genome for *C. duncanensis* (M. Miller and A. Caccone, unpublished data) was used to design capture baits. The sequence was provided to MYcroarray (Ann Arbor, MI) for bait design, which included evaluating bait specificity. Baits were 80 bp long with 20 bp between overlapping baits (60 bp overlap, 4× bait coverage per locus). Baits passed filtering if they were expected to have at most 10 hits between 62.5 °C and 65 °C and 4 hits above 65 °C, and fewer than 2 passing baits on each flank.

### Library Preparations and Captures

The historical and contemporary DNA samples were sent to MYcroarray to construct the libraries and perform the captures. Contemporary DNA samples were sheared via sonication before library preparation. Each sample was uniquely barcoded using dual indexes as part of a blunt-end library preparation. Individual libraries were pooled in equimolar amounts before capture. The captures for the historical samples were performed on pools of 4 individuals using 0.5× ng of baits, while the contemporary samples were pooled into groups of 8 before captures using 0.65× ng of baits. Captured libraries were then amplified and quantified before being pooled for sequencing, using a partial lane of Illumina HiSeq 2500 paired-end 150 bp.

### Sequence Processing and Assembly

We used captured sequences from 3 contemporary individuals (tortoise ID #s: A092Cen, C031Cen, G100Cen, Supplementary Table 2) in separate de novo assemblies in GENEIOUS 8.1.6 (Kearse et al. 2012) and used the consensus sequence for the 3 individuals to produce a reference. This de novo reference was required as initial assemblies of the captured sequences to the original reference used for bait design had poor mapping scores, likely due to several insertions/deletions of multiple base pairs identified when comparing the de novo reference to the original one. To further assess this discrepancy, we evaluated 2 of these multiple base-pair insertions/deletions using Sanger sequencing. The 2 regions were sequenced in 7 individuals each representing a different species of Galapagos giant tortoise (including *C. duncanensis*) and compared with both the original and de novo references (for details see Supplementary Methods). In all cases, the Sanger sequences matched the de novo reference (Supplementary Figure 1). As an additional line of evidence

to support the validity of the de novo reference, we calculated the A + T percentage of the sequence and found it to be the same as previously detected in studies sequencing a portion of the mitochondrial genome in Galapagos giant tortoises (59.5% in this study, 59.4% in Caccone et al. 2002). The de novo mitogenome reference fasta file was indexed using BWA *index* (Li and Durbin 2009) and SAMTOOLS *faidx* (Li et al. 2009).

Sequences were processed using the BAM pipeline in PALEOMIX (version 1.2.6, Schubert et al. 2014). Briefly, sequences were trimmed using ADAPTERREMOVAL (version 2.1.7, Lindgreen 2012) and overlapping paired-end reads were merged for historical individuals. Unmerged reads were excluded for historical individuals because we expected that the insert size of endogenous historical DNA would be short enough for merging (<289 bp, given the 150 bp read length and requiring an 11 bp overlap between the paired-end reads). For the contemporary sequences, all reads that passed filtering were retained. Reads were mapped to the consensus de novo reference sequence using BWA *aln* (Li and Durbin 2009) with seeding disabled. PCR duplicates were filtered using the function *MarkDuplicates.jar* in PICARD (version 2.6.0, <http://broadinstitute.github.io/picard/>) and *paleomix rmdup\_collapsed*, a function in PALEOMIX (version 1.2.6, Schubert et al. 2014). For the historical sequences, MAPDAMAGE2.0 (Jónsson et al. 2013) was used to rescale the quality scores of bases that were potentially the result of postmortem DNA damage. The mapping of BAM files was further refined using GATK *IndelRealigner* (McKenna et al. 2010). Genotype calling was performed using SAMTOOLS *mpileup* (with the settings -gd -q 15 -t DP) with consensus genotypes determined using BCFTOOLS *call* (Li et al. 2009) and exported in vcf format. Filtering of the vcf files was done using VCFTOOLS (Danecek et al. 2011), requiring a minimum read depth of 6×.

### Population Genetic Analyses

The mitogenome sequence was annotated using MITOS v1 (Bernt et al. 2013) to determine locations of genes (names and abbreviations of genes are in Supplementary Table 3). Using the annotation, we made subsets of the alignment with the CR only, and the CR + cytochrome b (Cytb) + 16S rRNA (16S). We used these subsets in addition to the full length of the recovered sequence (full MT genome) in the analyses described below. These datasets were chosen because the CR is commonly analyzed on its own in intraspecific studies, even for Bayesian skyline analysis (e.g., Moodley et al. 2017), and the CR, Cytb, and 16S rRNA were used in the most recent and comprehensive phylogeographic study of Galapagos giant tortoises (Poulakakis et al. 2012).

The sequences for the 3 datasets were sorted into haplotypes and used to construct haplotype networks using statistical parsimony (95% confidence criterion, gaps treated as a fifth state), as implemented in TCS V1.21 (Clement et al. 2000). The haplotype networks were imported into TCSBU for reformatting (Múrias dos Santos et al. 2016). Molecular diversity indices including haplotype and nucleotide diversities, population differentiation between the temporal samples measured by  $\Phi_{ST}$ , and descriptors of population size changes (Tajima’s (1989)  $D$ , Fu’s (1997)  $F_s$ , and mismatch distributions (Rogers and Harpending 1992)) were calculated for the 3 different datasets (CR, CR + Cytb + 16S, full MT genome) in the historical and contemporary samples in ARLEQUIN 3.5.2.2 (Excoffier et al. 2007).

In our original study design, we included fewer contemporary than historical samples because we anticipated that a large proportion of the historical samples would not yield enough sequence data to be retained in the analyses. Contrary to this expectation, only one

historical sample failed to produce enough sequence (see Results). This resulted in unequal sample sizes for the historical and contemporary groups. These circumstances afforded us the opportunity to assess whether the difference in inferences between historical and contemporary samples was due to the disparity in sample size between the groups by evaluating a subset of the historical sample equal in size to the contemporary one. Thus, we randomly selected 45 historical samples by assigning each a number using a random number generator and selecting the 45 with the smallest assigned number. For this subset of the historical samples, we calculated Tajima's  $D$  and Fu's  $F_s$  as above, as those measures may be impacted by sampling size (Subramanian 2016).

To evaluate changes in effective population size over time, we employed Bayesian skyline analysis (Drummond et al. 2005) in BEAST2 (Bouckaert et al. 2014). We ran the analysis on the historical and contemporary sequences separately, using the 3 different datasets: 1) the full MT genome split into 5 partitions (CR, each of 3 codon positions for the coding genes, and RNAs, including transfer RNAs and 12S and 16S); 2) CR + Cytb + 16S as 3 partitions (not separating by codon); and 3) CR only. For each partition in each dataset, the best fitting substitution model was identified from among 88 possibilities, using the Akaike information criterion (AIC) as implemented in JMODELTEST (Posada 2008) (see Supplementary Table 4 for selected models). For the 2 datasets with multiple partitions, we ran 3 initial chains for  $4.0 \times 10^7$  Markov Chain Monte Carlo (MCMC) generations, using a previously calculated substitution rate for the CR partition of  $8.5 \times 10^{-7}$  substitutions per lineage per generation, assuming a generation time of 25 years (Beheregaray et al. 2004). These preliminary runs were used to estimate the clock rates for the other partitions, setting an upper bound to 1.0. The mean rate estimated for each partition (Supplementary Table 4) was then fixed in subsequent runs. The substitution models and clock rates were unlinked among partitions, while the trees were linked. Searches used the coalescent Bayesian Skyline prior, a random starting tree and the number of population groups as 10, with other priors set to default. Final searches were  $8.0 \times 10^7$  MCMC generations long, sampling parameters every 5000 steps, and discarding the first 10% as burn-in. Convergence of 3 independent chains was assessed via effective sample size values, and Bayesian skyline analysis was performed in TRACER v1.6 (Rambaut et al. 2014). To allow direct comparisons between the Bayesian skyline analyses for the historical and contemporary samples, we applied the clock rates estimated for one sample to the other. We also used our randomly selected subset of 45 historical samples and reran the Bayesian skyline analyses for the 3 datasets to determine the impact of sample size, using the previously determined clock rates for the full set of historical samples and the contemporary samples. A total of 15 different Bayesian skyline plots (BSPs) were constructed, using the 3 sample sets (contemporary,  $n = 45$ ; historical,  $n = 77$ ; historical subset,  $n = 45$ ), the 3 datasets (CR only, CR + Cytb + 16S, and full MT genome), and with 2 clock rates for the CR + Cytb + 16S and full MT genome datasets, determined from both contemporary and historical data.

### Revisiting the Garrick et al. Dataset

Close evaluation of the sequence data used in Garrick et al. (2015) showed errors in the alignment of the last 3 base pairs of the CR. To verify what impact this error had on the findings of that study, we recalculated the number of CR haplotypes and haplotypic diversity ( $H_d$ ) on an alignment of the 27 original sequences with the last 3 base pairs trimmed (original sequence data are available from Dryad doi:10.5061/dryad.7h8q2). We also regenerated the extended

Bayesian skyline plot (EBSP), using the trimmed alignment for the CR and original sequences for the PAX nuclear intron, following the specifications given in Garrick et al. (2015).

## Results

The de novo assemblies of the mitogenome using 3 contemporary individuals each produced a single-long contig that, when aligned with one another, had no discrepancies, resulting in a 16043-bp-long sequence. As it did not circularize, the sequence is not quite complete; however, we still refer to analysis of this 16043-bp-long sequence as the "full MT genome." This nearly complete mitochondrial genome was recovered for all 45 contemporary and for 77 of the 78 historical individuals. Mean sequencing depth was 67× for the contemporary and 188× for the historical samples, and missing data were low (2.7% for contemporary, 1.8% for historical).

To compare results obtained from the 16043-bp fragment with the ones obtained from shorter mitochondrial DNA fragments, we divided the sequence data into 3 datasets: CR (666 bp), CR + Cytb + 16S (3408 bp), and the full MT genome (16043 bp). There were a total of 12 CR haplotypes, 21 CR + Cytb + 16S haplotypes, and 48 full MT genome haplotypes across the historical and contemporary samples (Table 1). Individuals that differed only at sites with missing data were conservatively grouped into the same haplotype. For the CR, all 5 haplotypes found in the contemporary sample were also found in the historical sample (Figure 1). The 7 CR haplotypes found only in the historical sample were no more than 5 mutational differences apart from haplotypes identified in the contemporary sample (Figure 1).

For the CR + Cytb + 16S data, a high frequency of shared haplotypes between the temporal samples was observed, though unshared haplotypes were also found in both the historical and contemporary samples (10 and 4, respectively; Figure 1). A similar pattern was seen for the full MT genome network based on 48 haplotypes (Figure 1). This network has some reticulation and shows 32 hypothetical (or unsampled) haplotypes connecting the extant haplotypes sampled in this study.

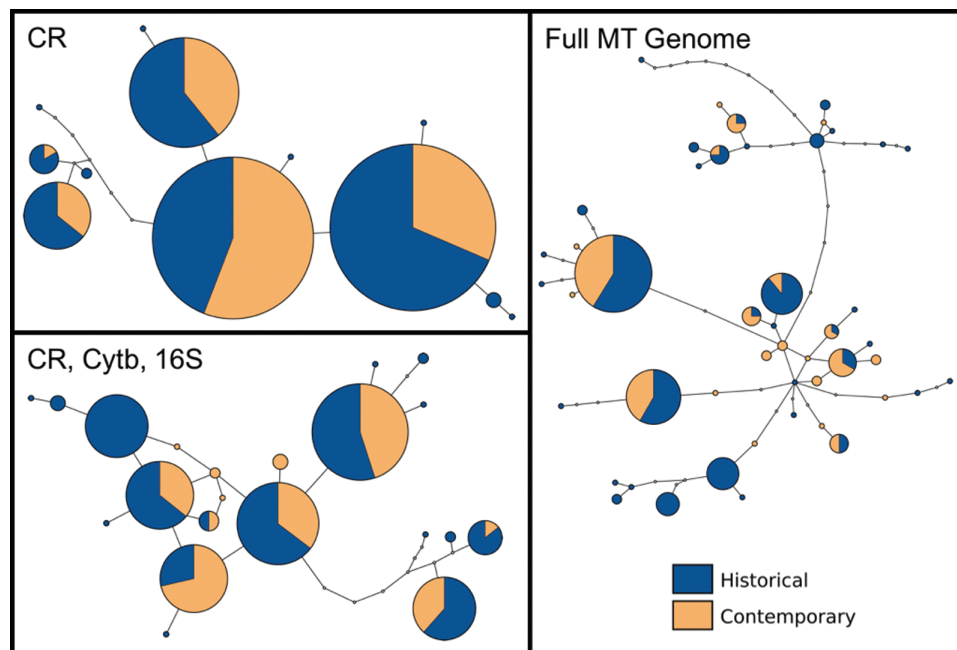
The number of segregating sites in each gene within the historical sample ranged from 0 to 16 and from 0 to 8 in the contemporary sample, with the highest number in both samples in the CR (Supplementary Table 3). The nucleotide diversity was greater in the historical sample across the MT genome as a whole, and in each gene, except for ND5, 12S, and trnA (Figure 2). Although  $H_d$  for the full MT genome was equal in the 2 temporal samples ( $H_d = 0.953$ , Figure 2), there were large differences in  $H_d$  when considering each gene separately (Figure 2). For most genes, the historical sample had greater  $H_d$  than the contemporary sample, except in the cases of ND5, ND6, and 12S. Genetic differentiation estimates ( $\Phi_{ST}$ ) between historical and contemporary samples were low and not significant for each of the datasets (CR only [0.004], CR + Cytb + 16S [0.014], the full MT genome [0.010]).

The metrics of population size change generally suggested a stable population size (Table 1), except when evaluating the full MT genome, which showed significantly negative Fu's  $F_s$  ( $-8.096$ ,  $P = 0.03$ ) for the full historical sample. Tajima's  $D$  and Fu's  $F_s$  were not significantly different from zero for either the subset historical or contemporary sample for any of the datasets. The mismatch distributions are qualitatively multimodal for each dataset in the full historical and contemporary samples (Figure 3), but only the raggedness values for the full MT genome were significant (Table 1).

**Table 1.** Number of haplotypes and segregating sites and descriptors of population size changes for the full historical ( $n = 77$ ), subset of historical ( $n = 45$ ), and contemporary ( $n = 45$ ) samples of Pinzón giant tortoise when evaluating the CR only, the CR, Cytb, and 16S rRNA genes, and the full mitochondrial (MT) genome

		Full historical	Subset historical	Contemporary
CR	$N_{hap}$	12	10	5
	$S$	16	13	8
	Tajima's D	-0.467 (NS)	-0.511 (NS)	0.103 (NS)
	Fu's $F_s$	-1.168 (NS)	-0.555 (NS)	1.738 (NS)
	$R$	0.086 (NS)		0.129 (NS)
CR, Cytb, 16S	$N_{hap}$	17	13	11
	$S$	22	18	14
	Tajima's D	-0.508 (NS)	-0.313 (NS)	-0.236 (NS)
	Fu's $F_s$	-2.797 (NS)	-1.670 (NS)	-1.274 (NS)
	$R$	0.022 (NS)		0.061 (NS)
Full MT Genome	$N_{hap}$	35	24	22
	$S$	70	58	44
	Tajima's D	-1.051 (NS)	-1.118 (NS)	-0.799 (NS)
	Fu's $F_s$	-8.096 ( $P = 0.030$ )	-4.543 (NS)	-4.056 (NS)
	$R$	0.017 ( $P = 0.049$ )		0.037 ( $P = 0.044$ )

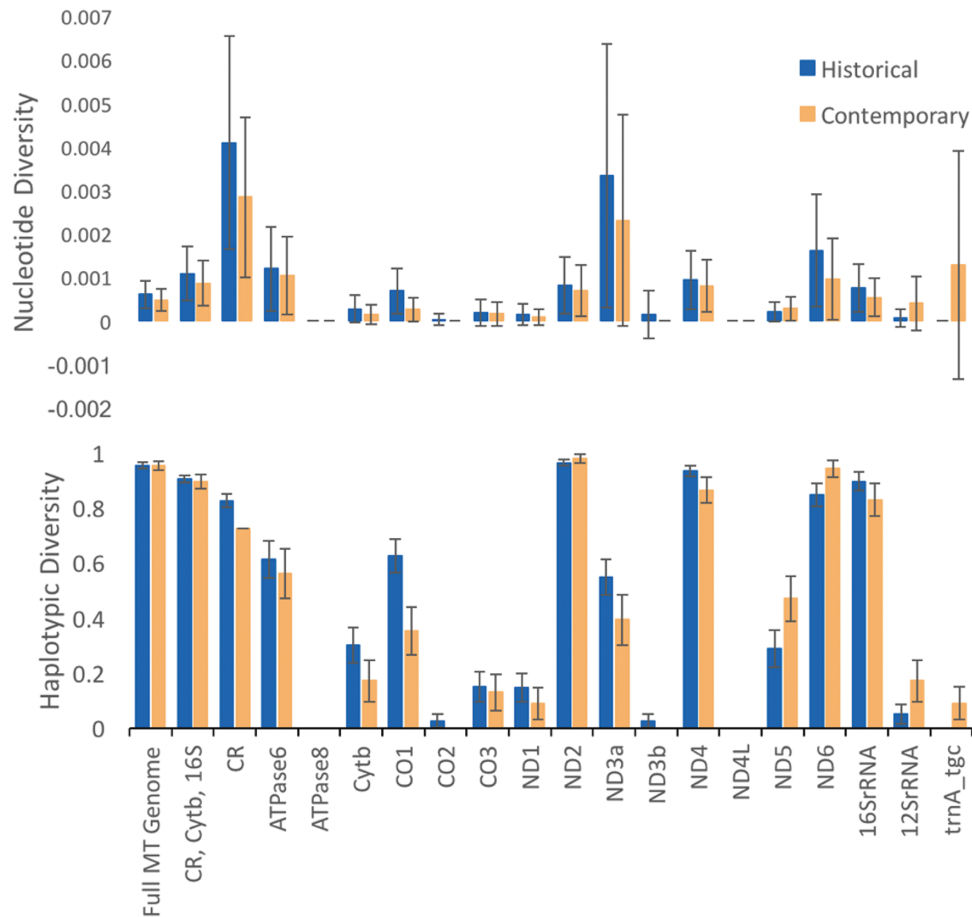
Significant  $P$  values are given in parentheses. Raggedness was not calculated for the subset of the historical sample.  $N_{hap}$ , number of haplotypes detected;  $S$ , number of segregating sites;  $r$ , raggedness index; NS, not significant at  $P < 0.05$ .



**Figure 1.** Haplotype networks generated using statistical parsimony, for the CR only, CR, Cytb, and 16S rRNA genes and the full mitochondrial (MT) genome for historical and contemporary samples of Pinzón giant tortoises. Colored circles represent unique haplotypes sampled in this study, with the overall frequency of a given haplotype indicated by circle size, which is to scale across the networks. Each of these circles is drawn as a pie chart, where different colors indicate the haplotype's frequency in the historical or contemporary samples. Small open circles represent hypothetical (i.e., unsampled or extinct) haplotypes, and each thin black line between a pair of haplotypes represents one mutational change. Reticulations (i.e., closed loops) represent uncertainty in relationships and/or homoplasy.

Trends in the BSPs varied widely between historical and contemporary samples and among datasets (Figure 4). In all cases, confidence limits are broad (Supplementary Figures 2–4), and a constant population size cannot be ruled out. The BSPs based on the CR only data show opposite trends when constructed from the historical samples (historically stable population size, recent increase) versus the contemporary samples (historically stable population size, recent decrease) (Figure 4A). The coalescent point is much more distant for the historical (~2200 generations) than the contemporary sample

(~1200 generations, Figure 4A). For BSPs based on the CR + Cytb + 16S data and the full MT genome, different clock rates produced the same relative differences between historical and contemporary samples, but the absolute values for effective population size and coalescent point were shifted (Figure 4B). The CR + Cytb + 16S BSP for the historical samples showed a slight increase in population size historically, with a dramatic increase in recent generations, before decreasing slightly (Figure 4B). Each of the BSPs based on the full MT genome indicated a steady increase in population size



**Figure 2.** Nucleotide diversity and haplotypic diversity across the full mitochondrial (MT) genome, a concatenated sequence of the CR, Cytb, and 16S genes, and each mitochondrial gene separately for the historical and contemporary samples of Pinzón giant tortoise. Error bars indicate the standard deviation. The gene abbreviations follow [Supplementary Table 3](#). The majority of transfer RNA genes did not have any variable sites and are excluded from the plots.

historically, with a rapid increase and then decrease in population size more recently ([Figure 4C](#)).

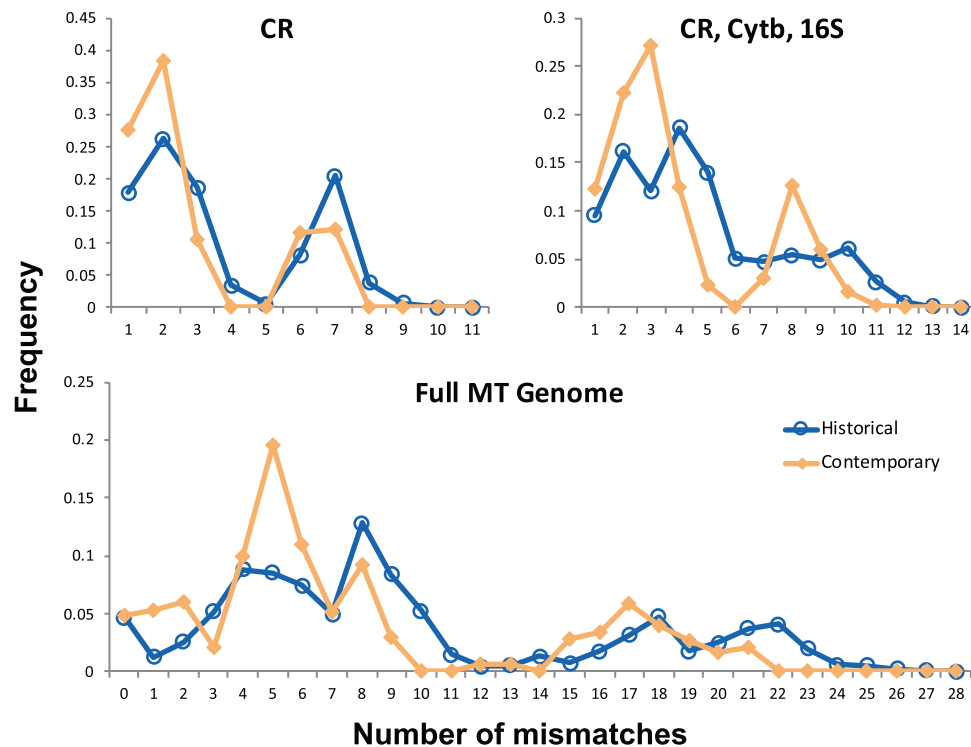
The subset of 45 historical individuals randomly selected had a total of 24 haplotypes across the full MT genome ([Table 1](#)). For the CR region BSP, the analysis of a subset of historical sequences still shows a recent increase in population size. However, the extent of this increase is much lower than the analysis of the full set of historical individuals ([Figure 4A](#)). The analysis conducted on the CR + Cytb + 16S dataset using the same subset of historical samples showed a recent decrease in population size, more similar to the BSP constructed from the contemporary samples than when using all the historical samples ([Figure 4B](#)). For the full MT genome analysis, the magnitude of population size changes differs between the analysis of the full and subset of historical samples, with the subset having a higher peak effective population size, before decreasing to an even lower effective population size at time zero than the full set of historical individuals ([Figure 4C](#)).

Reanalysis of the Pinzón giant tortoise CR sequences from [Garrick et al. \(2015\)](#) found 10 unique haplotypes, fewer than the 13 originally reported, yet haplotype diversity was similar (0.88 [original] vs. 0.85 [this study]). The 5 runs of the EBSF based on the mitochondrial CR and nuclear PAX intron, each suggested that a stable population size cannot be ruled out (the 95% highest posterior density intervals for the number of population size change events included zero), counter to initial findings ([Garrick et al.](#)

[2015](#)). However, the shape of the EBSF ([Supplementary Figure 5](#)) is very similar to that presented in the original article and suggests a high recent effective population size ( $N_e$  at time zero is ~13 500).

## Discussion

There is a broad literature on the topic of mito-nuclear discordance (e.g., [Funk and Omland 2003](#); [Chan and Levin 2005](#); [Petit and Excoffier 2009](#); [Toews and Brelsford 2012](#)), but conflict in population-level signal among mitochondrial genes has not received much consideration. In this study, each of the 3 datasets (CR only, CR + Cytb + 16S, and the full MT genome) reconstructed different population histories in the Bayesian skyline analysis and did not agree on whether the contemporary sample has less diversity than the historical sample, as might have been expected due to the bottleneck. For some of the mitochondrial genes, there was actually higher  $H_d$  in the contemporary sample than the historical sample (e.g., ND5, 12S rRNA), although for the MT genome as a whole, the  $H_d$  values were equal ([Figure 2](#)). If we had only sequenced the CR, the data would suggest that the bottleneck has caused a decrease in haplotypic diversity, while analysis of the ND5 gene would lead to the opposite conclusion that there is greater  $H_d$  in the contemporary population ([Figure 2](#)). Over the MT genome sequence, there was no difference in haplotypic diversity. This suggests that either the bottleneck was not severe enough for much mitochondrial DNA sequence



**Figure 3.** Mismatch distributions based on evaluating the CR only, the CR, Cytb, and 16S genes, and the full mitochondrial (MT) genome sequences from historical or contemporary samples of Pinzón giant tortoises.

variation to be lost or that the bottleneck was so recent that a genetic signature has not had time to develop. Based on these results, it is plausible that studies that rely on the CR only, and/or 1 or 2 other mitochondrial genes, may be overestimating or underestimating the differences in diversity between temporal samples or among geographically separated populations.

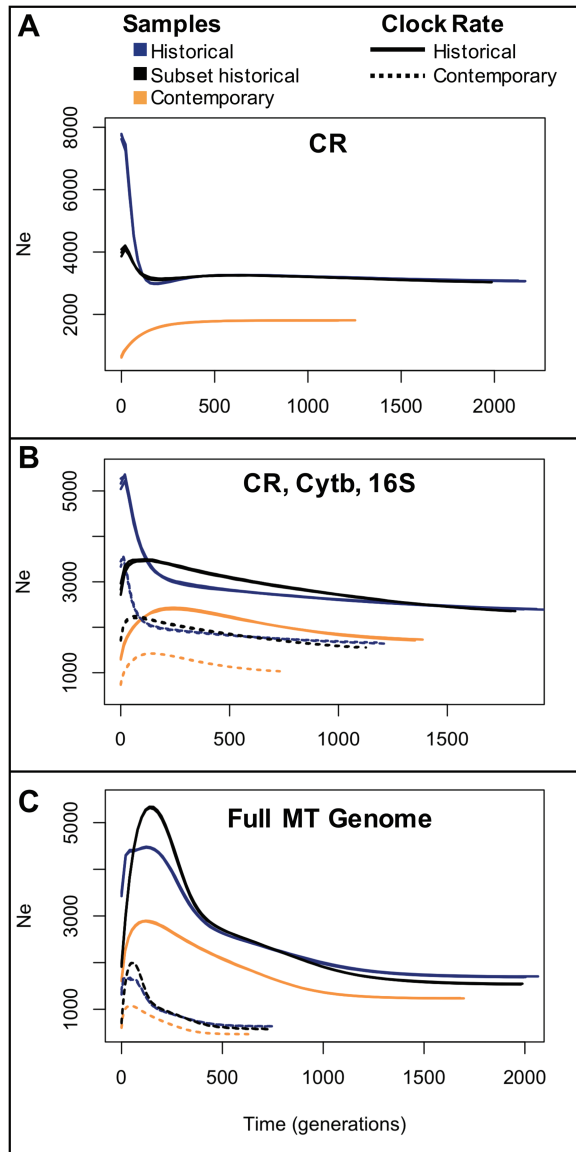
Due to limitations in the samples available for population genetic and phylogenetic studies, reconstructions are often based on samples from a single point in time. Here, our reconstructions of population history from Bayesian skyline analysis based on the historical and contemporary groups of samples are quite different (Figure 4). Although the broad confidence intervals for the BSP (Supplementary Figures 2–4) make the literal interpretation of any changes in effective population size speculative, the differences between the reconstructions from the temporal samples illustrate the difficulties of drawing conclusions from a single time point alone, particularly a postbottlenecked sample. BSP from the postbottleneck contemporary sample reconstructs lower effective population sizes throughout time, including at time zero (Figure 4).

To evaluate whether the differences between the historical and contemporary samples in the measures of population size change and BSPs were simply due to sample size, we selected a random subset of 45 historical individuals for comparison. The historical subset produced a BSP more similar to the contemporary one than the full historical sample for the CR + Cytb + 16S dataset, indicating that sample size could be driving some of the differences in reconstruction between the temporal samples. However, the historical subset BSPs for the CR only and the full MT genome are more similar to the reconstruction from the full historical sample than the contemporary one. Taken together, these findings indicate that the disparity in sample size alone is not sufficient to explain the differences in reconstructions of population history. However, it is important to

note that it is possible that there is not enough power in this BSP analyses to reconstruct historical trends in the effective population size, as simulations have shown that samples of <100 sequences may fail to capture the full extent of population expansions (Grant 2015). However, our dataset of >16 000 bp of sequence from a sample of 77 individuals is larger than what is commonly used for BSP analyses in the literature (Grant 2015).

Of the other metrics of population size change, only the analysis of the full MT genome showed a significant signature of population expansion based on Fu's  $F_s$  (full historical sample only) and the raggedness index of the mismatch distribution (for both the full historical and contemporary samples, Table 1). Although the significant Fu's  $F_s$  value for the full historical sample was no longer significant when analyzing the historical subset for the MT genome, the role of sample size on this metric and Tajima's  $D$  was largely unpredictable in this system across datasets (Table 1). The CR only and CR + Cytb + 16S datasets did not show a signature of population expansion for any of the groups. However, the mismatch distributions for the full historical and contemporary samples are qualitatively multimodal. We did not calculate Tajima's  $D$ , Fu's  $F_s$ , or mismatch distributions for each of the genes separately as the low number of segregating sites in each (Supplementary Table 3) did not yield enough power to detect changes in population size (Ramos-Onsins and Rozas 2002).

Despite the lack of consensus among results in this study regarding the precise demographic history of Pinzón tortoises, it is clear that they have not experienced prior cycles of bottlenecks and expansions and have likely only recently become rare, as opposed to having existed as a small population historically. Garrick et al. (2015) classified Pinzón giant tortoises as being “newly rare,” while many of the other species of Galapagos giant tortoise were found to be “naturally rare.” The EBSP in Garrick et al. (2015), our reanalysis of that dataset here, and the BSP based on the mitogenome all concur in



**Figure 4.** BSPs based on mitochondrial sequences for Pinzón giant tortoises including the full set of historical samples ( $n = 77$ ), the subset of historical samples ( $n = 45$ ), and the contemporary samples ( $n = 45$ ), showing the point estimates (median) of the effective population size ( $N_e$ ) over time, where time before present ( $x$  axis) is in generations. Panels **A–C** show the results of this analysis for the CR, CR + Cytb + 16S, and the full mitochondrial (MT) genome (analyzed as 5 partitions; the CR, the 3 codon positions for the coding genes, and RNA genes, including transfer RNAs and 12S and 16S). Three independent runs of each set of parameters are overlaid. Solid lines are using the clock rates estimated from the historical dataset; dashed lines are from clock rates estimated from the contemporary one, except for the CR only, where the clock rate was fixed a priori. Color figure is available in the online version.

suggesting a very high recent effective population size for the Pinzón tortoises. This seemingly contradictory result, given the documented demographic history, could be due to their biogeographic history. The population of Galapagos giant tortoises on Pinzón Island was likely founded through vicariance rather than dispersal from neighboring islands, as changes in sea levels separated Pinzón Island from a larger landmass (Poulakakis et al. 2012; Ali and Aitchison 2014; Geist et al. 2014). Paleogeographic reconstructions in Poulakakis

et al. (2012) suggest that Pinzón giant tortoises diverged from the population on Santa Cruz Island around 1.26 million years ago. However, this timeframe is well before the earliest coalescent point for any of the BSP, which was  $\sim 2000$  generations ago for the full MT genome BSP for the historical samples (Figure 4C). Assuming a generation time of 25 years in a naturally reproducing population, coalescence would have occurred  $\sim 50,000$  years ago. This implies that the population expansion detected in the BSP for the full MT genome (Figure 4C) occurred well after the Pinzón species' divergence due to vicariance. Moreover, there is no evidence of current or historical population subdivision on the island that could inflate  $N_e$  by way of secondary contact. Thus, the biological explanation for the high effective population size remains unclear.

In summary, our results empirically demonstrate the influence of sampling bias when interpreting population genetic patterns and punctuate the need for careful consideration of the potential for conflicting evolutionary signal across the mitochondrial genome. Our results suggest that some of the inferences regarding the population history of the Pinzón giant tortoise are influenced by the portion of the mitochondrial genome evaluated and that the population-level information content of the mitochondrial genome in these tortoises does not saturate after sequencing the CR or a specific subset of genes. This suggests that additional insights can be gained from investigating a larger portion of the mitochondrial genome. Furthermore, the insights into population history provided by sampling the contemporary population are not the same as when sampling the population just one generation prior. Genomic approaches employing targeted capture make sequencing a large portion of the mitochondrial genome much more feasible than ever before, particularly from historical samples, affording ample opportunities for refining reconstructions of population history and further exploring influences of sampling biases in population genetic studies.

## Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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## Data Availability

Haplotype sequences are available on GenBank (accession numbers MG912791–MG912838).

## References

- Aguilera WT, Málaga J, Gibbs JP. 2015. Conservation: giant tortoises hatch on Galapagos island. *Nature*. 517:271.
- Ali JR, Aitchison JC. 2014. Exploring the combined role of eustasy and oceanic island thermal subsidence in shaping biodiversity on the Galápagos. *J Biogeogr*. 41:1227–1241.
- Andrews KR, De Barba M, Russello MA, Waits L. Forthcoming. Advances in high-throughput DNA sequencing and genotyping for non-invasive, archival, and environmental samples. In: Hohenlohe PA, Rajora O, editors. *Population genomics: wildlife*. New York (NY): Springer Publishing.
- Avise JC. 1994. *Molecular markers, natural history and evolution*. New York (NY): Chapman & Hall.
- Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu Rev Ecol Syst*. 18:489–522.
- Beck R. 1903. *In the home of the giant tortoise*. New York (NY): New York Zoological Society.
- Beheregaray LB, Ciofi C, Caccone A, Gibbs JP, Powell JR. 2003. Genetic divergence, phylogeography and conservation units of giant tortoises from Santa Cruz and Pinzón, Galápagos Islands. *Conserv Genet*. 4:31–46.
- Beheregaray LB, Gibbs JP, Havill N, Fritts TH, Powell JR, Caccone A. 2004. Giant tortoises are not so slow: rapid diversification and biogeographic consensus in the Galápagos. *Proc Natl Acad Sci USA*. 101:6514–6519.
- Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritsch G, Pütz J, Middendorf M, Stadler PF. 2013. MITOS: improved de novo metazoan mitochondrial genome annotation. *Mol Phylogenet Evol*. 69:313–319.
- Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput Biol*. 10:e1003537.
- Bouzat JL, Lewin HA, Paige KN. 1998. The ghost of genetic diversity past: historical DNA analysis of the greater prairie chicken. *Am Nat*. 152:1–6.
- Caccone A, Gentile G, Burns CE, Sezzi E, Bergman W, Ruelle M, Saltonstall K, Powell JR. 2004. Extreme difference in rate of mitochondrial and nuclear DNA evolution in a large ectotherm, Galápagos tortoises. *Mol Phylogenet Evol*. 31:794–798.
- Caccone A, Gentile G, Gibbs JP, Fritts TH, Snell HL, Betts J, Powell JR. 2002. Phylogeography and history of giant Galápagos tortoises. *Evolution*. 56:2052–2066.
- Caccone A, Gibbs JP, Ketmaier V, Suatoni E, Powell JR. 1999. Origin and evolutionary relationships of giant Galápagos tortoises. *Proc Natl Acad Sci USA*. 96:13223–13228.
- Cayot L. 2008. The restoration of giant tortoise and land iguana populations in Galápagos. *Galápagos Res*. 65:39–43.
- Chan KM, Levin SA. 2005. Leaky prezygotic isolation and porous genomes: rapid introgression of maternally inherited DNA. *Evolution*. 59:720–729.
- Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Mol Ecol*. 9:1657–1659.
- Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, et al.; 1000 Genomes Project Analysis Group. 2011. The variant call format and VCFtools. *Bioinformatics*. 27:2156–2158.
- Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol Biol Evol*. 22:1185–1192.
- Eldridge MDB, Rummery C, Bray C, Zenger KR, Browning TL, Close RL. 2004. Genetic analysis of a population crash in brush-tailed rock-wallabies (*Petrogale penicillata*) from Jenolan Caves, south-eastern Australia. *Wildl Res*. 31:229–240.
- Excoffier L, Laval G, Schneider S. 2007. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online*. 1:47–50.
- Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*. 147:915–925.
- Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annu Rev Ecol Evol Syst*. 34:397–423.
- Galtier N, Nabholz B, Glémin S, Hurst GD. 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Mol Ecol*. 18:4541–4550.
- Gamba C, Hanghøj K, Gauntz C, Alfarhan AH, Alquraishi SA, Al-Rasheid KA, Bradley DG, Orlando L. 2016. Comparing the performance of three ancient DNA extraction methods for high-throughput sequencing. *Mol Ecol Resour*. 16:459–469.
- Garrick RC, Benavides E, Russello MA, Gibbs JP, Poulakakis N, Dion KB, Hyseni C, Kajdacs B, Márquez L, Bahan S, et al. 2012. Genetic rediscovery of an ‘extinct’ Galápagos giant tortoise species. *Curr Biol*. 22:R10–R11.
- Garrick RC, Kajdacs B, Russello MA, Benavides E, Hyseni C, Gibbs JP, Tapia W, Caccone A. 2015. Naturally rare versus newly rare: demographic inferences on two timescales inform conservation of Galápagos giant tortoises. *Ecol Evol*. 5:676–694.
- Geist DJ, Snell H, Snell H, Goddard C, Kurz MD. 2014. A paleogeographic model of the Galápagos islands and biogeographical and evolutionary implications. In: Harpp KS, Mittelstaedt E, d’Ozouville N, Graham DW, editors. *The Galápagos: a natural laboratory for the earth science*. New Jersey (NJ): John Wiley & Sons, Inc. p. 145–166.
- Grant WS. 2015. Problems and cautions with sequence mismatch analysis and Bayesian skyline plots to infer historical demography. *J Hered*. 106:333–346.
- Jensen EL, Mooers AØ, Caccone A, Russello MA. 2016. I-HEDGE: determining the optimum complementary sets of taxa for conservation using evolutionary isolation. *PeerJ*. 4:e2350.
- Jensen EL, Tapia W, Caccone A, Russello MA. 2015. Genetics of a head-start program to guide conservation of an endangered Galápagos tortoise (*Chelonoidis ephippium*). *Conserv Genet*. 16:823–832.
- Jónsson H, Ginolhac A, Schubert M, Johnson PL, Orlando L. 2013. mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. *Bioinformatics*. 29:1682–1684.
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 28:1647–1649.
- Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 25:1754–1760.
- Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics*. 25:2078–2079.
- Lindgreen S. 2012. AdapterRemoval: easy cleaning of next-generation sequencing reads. *BMC Res Notes*. 5:337.
- Longmire J, Maltbie M, Baker R. 1997. Use of ‘lysis buffer’ in DNA isolation and its implication for museum collections. *Occas Pap Mus Texas Tech Univ*. 163:1–3.
- Macfarland CG, Villa J, Toro B. 1974. The Galápagos giant tortoises (*Geochelone elephantopus*) Part I: status of the surviving populations. *Biol Conserv*. 6:118–133.
- McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernysky A, Garimella K, Altshuler D, Gabriel S, Daly M, et al. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 20:1297–1303.
- Miller CR, Waits LP. 2003. The history of effective population size and genetic diversity in the Yellowstone grizzly (*Ursus arctos*): implications for conservation. *Proc Natl Acad Sci USA*. 100:4334–4339.
- Moodley Y, Russo IRM, Dalton DL, Kotze A, Muya S, Haubensak P, Balint B, Munimanda GK, Deimel C, Setzer A, et al. 2017. Extinctions, genetic erosion and conservation options for the black rhinoceros (*Diceros bicornis*). *Sci Rep*. 7:16.
- Múrias dos Santos A, Cabezas MP, Tavares AI, Xavier R, Branco M. 2016. tcsBU: a tool to extend TCS network layout and visualization. *Bioinformatics*. 32:627–628.
- Nyström V, Angerbjörn A, Dalén L. 2006. Genetic consequences of a demographic bottleneck in the Scandinavian arctic fox. *Oikos*. 114:84–94.

- Petit RJ, Excoffier L. 2009. Gene flow and species delimitation. *Trends Ecol Evol.* 24:386–393.
- Posada D. 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol.* 25:1253–1256.
- Poulakakis N, Glaberman S, Russello M, Beheregaray LB, Ciofi C, Powell JR, Caccone A. 2008. Historical DNA analysis reveals living descendants of an extinct species of Galápagos tortoise. *Proc Natl Acad Sci USA.* 105:15464–15469.
- Poulakakis N, Russello M, Geist D, Caccone A. 2012. Unravelling the peculiarities of island life: vicariance, dispersal and the diversification of the extinct and extant giant Galápagos tortoises. *Mol Ecol.* 21:160–173.
- Pritchard PCH. 1996. The Galápagos tortoises—nomenclatural and survival status. *Chelonian Res Monogr.* 1:1–85.
- Rambaut A, Suchard M, Xie D, Drummond A. 2014. Tracer v1.6. Available from: <http://beast.bio.ed.ac.uk/Tracer> (accessed February 25, 2017).
- Ramos-Onsins SE, Rozas J. 2002. Statistical properties of new neutrality tests against population growth. *Mol Biol Evol.* 19:2092–2100.
- Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Mol Biol Evol.* 9:552–569.
- Russello MA, Beheregaray LB, Gibbs JP, Fritts T, Havill N, Powell JR, Caccone A. 2007. Lonesome George is not alone among Galápagos tortoises. *Curr Biol.* 17:R317–R318.
- Russello MA, Glaberman S, Gibbs JP, Marquez C, Powell JR, Caccone A. 2005. A cryptic taxon of Galápagos tortoise in conservation peril. *Biol Lett.* 1:287–290.
- Russello MA, Poulakakis N, Gibbs JP, Tapia W, Benavides E, Powell JR, Caccone A. 2010. DNA from the past informs ex situ conservation for the future: an “extinct” species of Galápagos tortoise identified in captivity. *PLoS One.* 5:e8683.
- Schubert M, Ermini L, Der Sarkissian C, Jónsson H, Ginolhac A, Schaefer R, Martin MD, Fernández R, Kircher M, McCue M, *et al.* 2014. Characterization of ancient and modern genomes by SNP detection and phylogenomic and metagenomic analysis using PALEOMIX. *Nat Protoc.* 9:1056–1082.
- Subramanian S. 2016. The effects of sample size on population genomic analyses—implications for the tests of neutrality. *BMC Genomics.* 17:123.
- Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics.* 123:585–595.
- Toews DP, Brelsford A. 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Mol Ecol.* 21:3907–3930.
- Ugelvig LV, Nielsen PS, Boomsma JJ, Nash DR. 2011. Reconstructing eight decades of genetic variation in an isolated Danish population of the large blue butterfly *Maculinea arion*. *BMC Evol Biol.* 11:201.
- Van Denburgh J. 1914. Expedition of the California Academy of Sciences to the Galápagos Islands 1906–1906. *Proceeding of the California Academy of Sciences.* 2:203–374.
- Wandeler P, Hoeck PE, Keller LF. 2007. Back to the future: museum specimens in population genetics. *Trends Ecol Evol.* 22:634–642.
- Wisely SM, Buskirk SW, Fleming MA, McDonald DB, Ostrander EA. 2002. Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. *J Hered.* 93:231–237.