# Learning from the past, examining the present and planning for the future: Genetic approaches to the conservation of giant Galápagos tortoises

by

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**Abstract**

Population genetics allows us to interpret the historical information contained in DNA, telling the story of population dynamics, demography and divergences, both recent and ancient, providing insights difficult or otherwise impossible to obtain. My PhD thesis research addresses fundamental questions in conservation genetics and demonstrates the utility of incorporating genetic information into conservation planning. Prioritizing taxa to receive conservation efforts is a difficult and contentious issue. Numerous methods have been proposed to rank taxa based on the importance of the phylogenetic diversity they contribute. However, all of these metrics share a flaw, in that complementarity among taxa is not taken into account when determining rankings. Here I propose a new method, I-HEDGE, which is an improvement on existing metrics as it integrates evolutionary isolation, probability of extinction and complementarity. Another area I address is that all too often the genetic impacts of conservation activities, including captive breeding and head-start programs, go unmonitored, which can result in losses of genetic diversity. The giant Galápagos tortoises endemic to Pinzón Island narrowly escaped extinction in the 20$^{th}$ century thanks to an intensive head-start program, now operating for 50 years. I evaluated two cohorts of the head-start program in detail using microsatellite markers to determine how representative they are of the extent and distribution of genetic variation in the wild population, which is one of the goals of the program. The cohorts were not representative of the sample of wild adults used for comparison, but the Pinzón tortoises appear to have retained a remarkable amount of genetic variation despite their near extinction. The genomic consequences of a rapid population decline and recovery, such as that experienced by the Pinzón tortoise, have rarely been empirically evaluated. This study system has the advantage

of a large number of historical specimens collected in 1906, allowing a direct evaluation of genomic patterns pre- and post-decline. By estimating effective population sizes and patterns of diversity in the historical and contemporary populations, it became clear that despite their near extinction, the Pinzón tortoises have retained high levels of diversity thanks to their demographic history and quick recovery.

## Preface

Several individuals have contributed to Chapters 2, 3 and 4 of this thesis. Manuscript versions of these chapters are or will be co-authored when published.

A version of Chapter 2 has been published:
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The co-authors and I shared in the identification and design of this research. I conducted all of the analyses and drafted the manuscript. The co-authors reviewed drafts of the manuscript.

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A version of Chapter 4 will be co-authored when published. The author line is expected to be:
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# Introduction

## 1.1 Conservation genetics

Written in the DNA of every individual are not just the instructions for building the organism, but also the history of its ancestors. When populations of individuals are analyzed together, their DNA can tell an even larger story of population dynamics, demography and divergences, both recent and ancient. Genetic approaches allow us to interpret the historical information contained in DNA, which can provide insights difficult or impossible to obtain in other ways. For example, determining whether two allopatric populations exchange migrants could be accomplished by a long-term capture-mark-recapture study, or through the one-time genetic sampling and analysis of the populations. Other questions relevant to conservation, such as "has this population recently declined, or has it always been small?" can only be answered through genetic approaches, in the absence of long-term records.

The DNA of populations can also influence the future, as it is the genetic variation within populations and the resulting differential fitness that is the raw material on which natural selection acts. There is a clear relationship between the amount of genetic diversity in a population and population persistence (Frankham 1997, 2005). In a changing world, it is the ability to adapt that will ultimately determine which species persist into the future. The ability to survive novel disease threats is also positively correlated with the level of genetic diversity in a population (Smith et al. 2009). Intraspecific genetic diversity has an intrinsic value that makes it worthy of conservation (Frankel 1974), and is one of the recognized levels of biodiversity (Vié et al. 2009). Thus, maintaining existing levels of intraspecific genetic diversity is a common goal across conservation programs.

Modern conservation biology combines pure and applied sciences to the goal of preserving biodiversity at ecosystem, species and genetic levels (Meine et al. 2006). With the threat to biodiversity worldwide outstripping the resources available for conservation, conservation actions need to be deliberate and defensible. As a sub-discipline of both conservation biology and population genetics, conservation genetics seeks to inform conservation actions through the use of genetic information. Genetic evaluations of species and populations can clarify priorities, allowing resources to be allocated appropriately. By default, conservation is a crisis-driven discipline where immediate action is often required. In many situations, actions that are practical can take precedent over those that are more theoretically sound. Thus, it is critical that whenever possible, current programs are evaluated to ensure that actions are generating the desired outcomes. Genetic approaches can reveal invisible threats to the long-term success of species or populations, such as inbreeding, bottlenecks, introgression and loss of adaptive variation.

### 1.2 Historical perspectives

Population genetics allows inferences regarding the processes underlying the patterns observed in DNA. More direct elucidation of the relationship between current genetic diversity and past demographic events is possible with a temporal sampling approach that surveys natural populations pre- and post- bottleneck. The ability to implement just such an approach has been limited until recently with the arrival of the genomics era.

Natural history collections throughout the world are filled with biological samples that were collected over the past two centuries, representing a treasure trove for evolutionary studies.

Unfortunately, the degraded nature of historical DNA has limited its utility in studies using "traditional" genetic markers (e.g. microsatellites, Sanger-sequenced mitochondrial DNA). Early studies making use of historical DNA were fraught with practical issues, such as contamination with modern DNA, resulting in spurious findings that caused controversies (discussed in Austin et al. 1997, Leonard 2008). Over time, recommendations have been developed that overcome some of the issues of earlier studies (Cooper and Wayne 1998, Wandeler et al. 2007). Despite the many difficulties, insights have been gained through genetic analysis of historical DNA that are important for both conservation and evolutionary understanding (e.g. Cooper et al. 1996, Ozawa et al. 1997). Emerging genomics technologies are pushing this research area much further than has been possible to date. As most next-generation sequencing platforms, by design, yield short sequences (~50-300 base pairs), the short fragments of historical DNA are not a constraint. The first population genomic study using museum specimens was published in October, 2013, and was loftily but aptly titled "Unlocking the vault" (Bi et al. 2013). Empirical evolutionary studies incorporating genomic data from temporal samples of even the most long-lived species are now possible, including the giant Galápagos tortoises.

### 1.3 Galápagos tortoises

The Galápagos archipelago is one of the world's biodiversity hotspots, and home to high profile, highly endangered taxa, such as the endemic species of giant tortoises (Bensted-Smith 2002). Many of the tortoise populations experienced precipitous declines during the 18$^{th}$ and 19$^{th}$ centuries (MacFarland et al. 1974), and are only now recovering thanks to extensive and multi-faceted conservation and restoration programs (Cayot et al. 1994). The

Galápagos tortoises make an excellent study system for conservation genetics research, being of high conservation importance and cultural interest.

The Galápagos archipelago lies ~1000 km to the west of mainland South America at the equator. The geological history of the islands is fairly well-known (Geist et al. 2014), with the islands emerging as a result of the Nazca Plate moving over a volcanic hotspot as the plate slides eastward. The very first Galápagos Islands emerged at least 9 million years (MY) ago, but have now subsided into the ocean, slowly sinking as they moved eastward away from the hotspot. The oldest current islands, Española, Santa Fé and San Cristóbal, date to a minimum estimated age of 3.0 - 2.4 MY. The youngest islands in the west are still volcanically active, including Fernandina, which emerged between 60,000 and 30,000 years ago.

Since Charles Darwin visited the Galápagos Islands (Darwin 1882), there has been interest in the biogeography of the island's biota. The Galápagos Islands are home to numerous endemic species, none so charismatic as the giant tortoises. Early studies of Galápagos tortoises determined that the two morphologies, saddle-backed and dome-shaped carapaces, were more indicative of the ecology of each species rather than evolutionary relationships among them (Fritts 1984). The evolutionary relationships among the surviving species of Galápagos tortoise and their closest relatives in mainland South America were first described using genetic sequence data by Caccone et al. (1999). Over time, higher resolution phylogenies among the extinct and extant species of Galápagos tortoise have been developed

(Ciofi et al. 2002, Beheregaray et al. 2004), culminating in a comprehensive portrayal of the phylogenetic and biogeographic history of this group (Poulakakis et al. 2012).

Tomás De Berlanga, the bishop of Panama, discovered the Galápagos Islands in 1535 when his ship was blown off course (Slevin 1959). Exploitation of the islands' resources began in the late 1600's, with tortoise harvesting for meat and oil over the ensuing centuries decimating the populations. The records from ship's logbooks suggest that whalers took more than 100,000 tortoises between 1831 and 1867 alone (Townsend 1926). Non-native species, including goats, pigs, dogs and rats, were introduced that competed for food or preyed directly upon the native species, including tortoises, and destroyed habitat (Schofield 1989). The final blows to many of the tortoise populations came not from hungry sailors, but from zoologists, eager to collect specimens for their museum or institute (Pritchard 1996).

The first protection laws were passed by the Ecuadorian government in 1934 to preserve the Galápagos fauna and set aside some of the islands as nature reserves. The whole archipelago became a national park in 1959; and at that time surveys were initiated to determine the status of the remaining tortoise populations. MacFarland et al. (1974) reported the findings of those surveys and concluded that if the impacts of the invasive species could be eliminated, all but two of the tortoise species could remain at stable population sizes or even begin increasing. The two other species, from Española and Pinta Islands, had too few individuals remaining for natural recovery, and the recommendation was made to bring the surviving individuals into captivity for breeding.

Unfortunately, only a single individual on Pinta could be located. This adult male tortoise lived out the rest of his days in captivity, and became a conservation icon *Lonesome George*. However, during a population genetic evaluation of the Volcano Wolf population on Isabela Island, an individual with Pinta ancestry was discovered among purebred native individuals (Russello et al. 2007a), which sparked further study of the Volcano Wolf population. Eventually more individuals were discovered with mixed ancestry, not just from Pinta, but also from the extinct Floreana species (Poulakakis et al. 2008, Garrick et al. 2012, Edwards et al. 2013). It is only through genetic assignment tests that these individuals were detected, and plans are currently underway to use genetics to guide breeding these admixed individuals together to develop populations that are enriched for Pinta and Floreana ancestry.

The Española tortoise breeding program was founded by just 12 females and three males, and went on to become an unqualified success (Milinkovitch et al. 2013). The species now numbers almost 2000 in the wild, thanks to over 50 years of captive breeding and repatriation (Gibbs et al. 2014). However, with such a small number of founders, it is important to ensure that each contributed equally to the captive breeding program to maximize the genetic diversity in the offspring. Genetic parentage analysis of captive bred individuals revealed that breeding success was highly skewed among founders (Milinkovitch et al. 2004), but fortunately, steps were taken to encourage a more equal contribution in subsequent years of the breeding program (Milinkovitch et al. 2013).

Conservation genetics has played an important role in the recovery of the Española species, and will be central in the future efforts to rebreed the Pinta and Floreana species. One species

of Galápagos tortoise that would also benefit from conservation genetic analysis is the species from Pinzón, which is the study system for the latter part of this thesis.

### 1.4 Thesis overview

This thesis addresses fundamental issues in conservation genetics. In Chapter 2, I take a novel, integrative approach to the difficult topic of prioritizing units for conservation. One proposed basis for determining priorities is ensuring that extinctions do not result in inordinate losses of evolutionary history. Numerous methods have been proposed to rank taxa based on the importance of the phylogenetic diversity they contribute. However, all of these metrics share the shortcoming that complementarity among taxa is not taken into account when determining rankings. My thesis work proposes a new method, I-HEDGE, which is a development on existing metrics as it integrates evolutionary isolation, probability of extinction and complementarity.

In Chapter 3, I conduct an empirical evaluation to see whether head-starting of tortoises is an effective strategy for maintaining the extent and distribution of genetic variation in the natural population. Head-starting is an interactive *in situ* / *ex situ* conservation strategy that may have unintended negative genetic impacts if head-start cohorts are not representative of the population. A head-start program has been operating for the Pinzón Island giant Galápagos tortoise for decades without an explicit assessment of the genetic impacts. I used genotypic data to evaluate two cohorts to determine the genetic variation with each cohort, and how representative they are of the wild population. I find that the head-start program has been only partially successful in its goal of maintaining genetic diversity, because although

the cohorts have high genetic variation, they are not fully representative of the wild population.

Finally, in Chapter 4, I directly evaluate the genomic consequences of rapid population decline and recovery in the Pinzón tortoise using historical samples and recently developed genomic methods. By reconstructing demographic history and determining the extent and distribution of genetic variation in the temporal samples, I find that the large historical effective population size and rapid recovery of the population have maintained high levels of diversity post-bottleneck.

This body of work contributes to the field of conservation genetics by further demonstrating the utility of incorporating genetic information into conservation prioritization and the implementation of conservation interventions such as head-starting, and by providing a unique empirical evaluation of rapid population decline and recovery. This study also provides data that will be used to inform the conservation and management of Galápagos tortoises.

# Chapter 2: I-HEDGE: Determining optimum complementary sets of taxa for conservation using evolutionary isolation

## 2.1 Background

The Noah's Ark problem embodies the difficulties of deciding what to conserve in the face of limited resources (Weitzman 1998). It is generally recognized that the extinction of some species represents a greater loss of biodiversity than others (an example is the extinction of one among many species of rat versus extinction of the panda, see Vane-Wright et al. 1991). In the midst of the current biodiversity crisis, if prioritization is required, conservation efforts should perhaps be directed towards ensuring that extinctions do not result in inordinate losses of evolutionary history (Vane-Wright et al. 1991). Methods first pioneered by Faith (1992) have been further developed and refined to evaluate the relative importance of species based on their contribution to total genetic diversity (Weitzman 1992, Witting and Loeschcke 1993, Redding 2003, Steel et al. 2007, Faith 2008, Haake et al. 2008, Minh et al. 2009, Hartmann 2013). These methods were initially created for the analyses of phylogenetic trees, but have recently been extended for use with phylogenetic networks that better represent genetic diversity among populations and recently diverged species (Volkmann et al. 2014).

Current metrics consider the expected contribution of each taxon to future subsets of taxa (i.e. scenarios where some taxa are lost). One is the "fair proportion" or "evolutionary distinctness" metric (Redding 2003, Isaac et al. 2007, Jetz et al. 2014) extended to networks, where all future subset sizes and identities are considered equally likely (referred to here as the Shapley index, SH, following Haake et al. 2008). Another, heightened evolutionary

distinctiveness (HED), explicitly weighs future subsets by their probability using estimates of the current extinction probabilities of all other taxa (Steel et al. 2007).

Rankings based on these metrics alone do not necessarily constitute rational prioritizations for conservation. One issue is that a secure species on a long branch may have a high HED score, because its own low probability of extinction [p(ext)] does not contribute to its own score. Also, as laid out clearly by Faith (2008), the above metrics are not designed to identify the best ordering or *subset* of taxa to protect, since complementarity is not taken into account. For example, two closely related species may both be at high risk of extinction, meaning each would contribute to future diversity if its relative were to go extinct. However, if one of the two were successfully protected, its sister should drop in value because the shared component of diversity is now retained.

The first issue above has been addressed by the development of metrics such as HEDGE ("heightened evolutionary distinctiveness and globally endangered", Steel et al. 2007), which is the product of a taxon's HED score and p(ext). HEDGE scores represent the increase in expected phylogenetic diversity if the taxon's p(ext) is changed from its current value to a p(ext) of zero (i.e. it is "saved" from extinction; see also Faith (2008)). Here, we present an extension of HEDGE that addresses the issue of complementarity. If the species that has the highest HEDGE score is indeed saved from extinction, then the HED score of neighbouring taxa should decrease to reflect this new p(ext) of the shared part of the network. We propose a modified, iteratively calculated, version of HEDGE (I-HEDGE), which is calculated by "saving" the top ranked taxon after calculating HEDGE in each round by setting its

extinction probability to near zero, and then recalculating HEDGE until all species have been "saved". This procedure produces the optimal ranked list for conservation prioritization, taking into account complementarity and based on both phylogenetic diversity and extinction probability.

To demonstrate this procedure, we use the example of the giant Galápagos tortoises (genus *Chelonoidis*), a recent island radiation with complex phylogeography and hierarchical levels of divergence (Figure 2.1). Recently diverged groups, such as island radiations, are the type of system where a network-based ranking approach will be most relevant. Tortoises initially colonized Galápagos approximately 3 million years ago from mainland South America, and subsequently radiated across all major islands and volcanoes as they formed (Caccone et al. 2002, Poulakakis et al. 2012). Historically, 15 species were formally described and were abundantly distributed across the Galápagos archipelago (MacFarland et al. 1974), exhibiting divergence times spanning a wide temporal range (<0.28 mya – 1.7 mya; Caccone et al. 2002, Poulakakis et al. 2012). Populations were decimated throughout the $18^{th}$-$20^{th}$ centuries through human exploitation and the negative impacts of invasive species. Four species have gone extinct, and several others have become highly endangered (MacFarland et al. 1974). Over the past 50 years, conservation efforts have been extensive, targeted primarily at the most imperilled species. Although effective at preventing the extinction of two additional species and increasing population sizes of others, these conservation strategies have been designed and implemented without reference to genetic divergence and distinctiveness of individual populations, raising concerns that this approach may not maximize genetic diversity in the future.

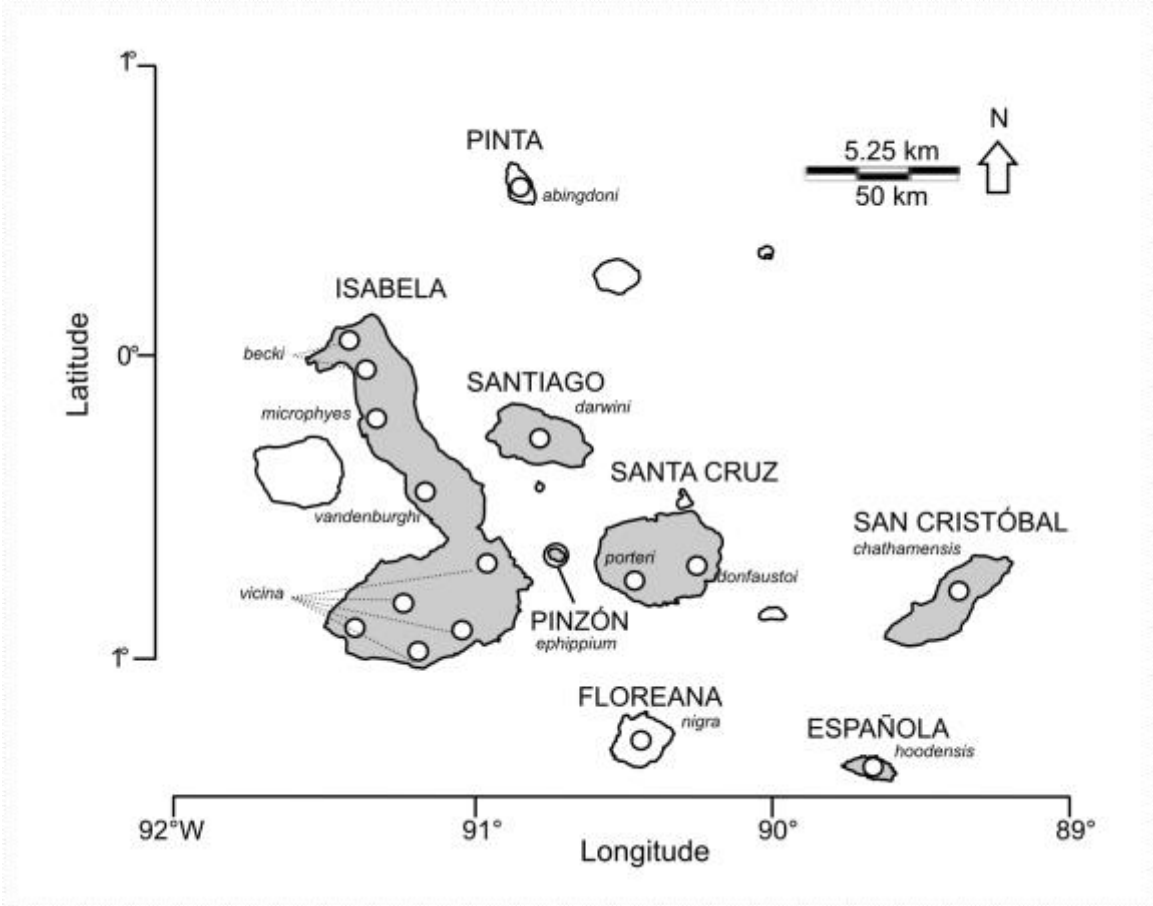


Figure 2.1 Map of the Galápagos archipelago showing locations of *Chelonoidis* tortoise populations.
Names of islands are in capital letters; species epithets are indicated in italics. Circles indicate locations for giant tortoise populations. Islands shaded in grey have extant populations of giant tortoises.

Here, we present I-HEDGE, a procedure to determine the optimum complementarity set for conservation prioritization, and explore its utility in the network-based context of ranking the giant Galápagos tortoise species. We compare the I-HEDGE approach to the Shapley index, a simpler, non-complementarity method on networks. The Shapley index is directly equal (Volkmann et al. 2014) to the Fair Proportion metric used by the Zoological Society of London in their Edge of Existence program (Isaac et al. 2007). The prioritizations that result

are discussed in light of past and current conservation strategies directed towards giant Galápagos tortoises.

## 2.2 Materials and methods

### 2.2.1 Data set

Previous studies of giant Galápagos tortoises have resulted in the development of a database of mitochondrial control region (CR) sequence data from population-level samples of all extant and several extinct species (Caccone et al. 2002, Russello et al. 2005, Russello et al. 2007a, Poulakakis et al. 2008, Garrick et al. 2012, Poulakakis et al. 2012, Edwards et al. 2013). Here, we made use of that database (DRYAD entry doi:10.5061/dryad.7h8q2), consisting of 334 individuals from extant species sampled across 15 sites, in addition to 33 individuals from two extinct species (Russello et al. 2007a, Poulakakis et al. 2008, Russello et al. 2010, Garrick et al. 2012, Edwards et al. 2013) (see Table 2.1). We have included the extinct species in our study because they may not be extinct for much longer, as the Galápagos National Park has initiated a program to rebreed them from living individuals with admixed ancestry (see Discussion). Here, we have performed the analyses using the currently accepted taxonomy, including recognizing the recently described species, *C. donfaustoi* (Poulakakis et al. 2015), as distinct from *C. porteri* on Santa Cruz Island.

Table 2.1 Sample information and SH and I-HEDGE rankings from the network-based analyses.

| Island | Species | N | SH | I-HEDGE |
|---|---|---|---|---|
| Pinta | *abingdoni* | 12 | 4 | 1 |
| Floreana | *nigra* | 20 | 6 | 2 |
| Santa Cruz | *donfaustoi* | 20 | 1 | 3 |
| San Cristóbal | *chathamensis* | 19 | 3 | 4 |
| Española | *hoodensis* | 15 | 2 | 5 |
| Pinzón | *ephippium* | 27 | 5 | 6 |
| Santa Cruz | *porteri* | 23 | 7 | 7 |
| Isabela | *microphyes* | 21 | 8 | 8 |
| Isabela | *vandenburghi* | 28 | 9 | 9 |
| Isabela | *becki* | 45 | 10 | 10 |
| Santiago | *darwini* | 21 | 11 | 11 |
| Isabela | *vicina* | 116 | 12 | 12 |

*N* sample size, *SH* Shapley index, *I-HEDGE* iterative heightened evolutionary distinctness globally endangered index

### 2.2.2 Network construction

Pairwise differentiation among species was calculated with the fixation index $\Phi_{ST}$ (Excoffier et al. 1992) using the Kimura 2-parameter (K2P) genetic distance and a gamma value of 0.5 (empirically-determined for CR sequences; Beheregaray et al. 2004), as implemented in ARLEQUIN v3.5.1.2 (Excoffier et al. 2005). The pair-wise differentiation matrix was then represented as a two-dimensional NeighbourNet network (Bryant and Moulton 2004) using SPLITSTREE (Huson and Bryant 2006) and default settings. This network representation produces sets of distances among subsets of taxa (termed "splits") that can be used to calculate expected genetic contribution of individual tips (Volkmann et al. 2014).

### 2.2.3 Prioritization metrics

As outlined by Volkmann et al. (2014), the expected future contribution of a taxon to total genetic diversity can be calculated by evaluating the split distance of a taxon to possible

future subsets of taxa on a genetic network. The two metrics of future expected genetic diversity adapted to networks by Volkmann et al. (2014) are SH (Haake et al. 2008) and HED (Steel et al. 2007). SH is based on game theory (Shapley 1953), and calculates the predicted amount of diversity a taxon contributes to all possible subsets of taxa. HED is similar to SH, but weights each future subset of taxa based on the probability of that subset (Steel et al. 2007). These probabilities are calculated by considering the probability of extinction (e.g. over the next 100 years) of each taxon in the network.

We used the scripts developed and published by Volkmann et al. (2014) to calculate SH, and modified the HED script to calculate I-HEDGE in the R statistical package (http://www.R-project.org/). HED values are used to calculate HEDGE, which is the product of HED and the p(ext) for the taxon. For the calculation of HED and HEDGE, it is important to use the best available information for the p(ext) of each taxon. Calculating informed probabilities of extinction is a nontrivial matter, and there is substantial literature on the topic of population viability analyses (Beissinger and Westphal 1998, Menges 2000, Reed et al. 2002). When species or population specific information is not available, it is possible to use proxies, such as those outlined in Mooers et al. (2008) that convert IUCN Red List (IUCN 2014) statuses to p(ext) (e.g. Vulnerable = 0.1, Endangered = 0.667, Critically Endangered = 0.999), or other measures (O'Grady et al. 2004). The giant Galápagos tortoises are a special case where, despite being highly endangered, realistically they have a low actual p(ext) due to the intensive management they receive. In this case, the IUCN Red List statuses do not correlate to census population size, nor do they convert to a realistic probability of extinction for each species. We used a flat p(ext) for each of the extant taxa (arbitrarily set to 0.5) to reflect these

circumstances. For the extinct species, p(ext) was set to 1 to reflect that, in fact, these species are extinct. I-HEDGE was calculated as follows. HEDGE was calculated initially for the entire set of taxa using p(ext) described above. The top-ranked taxon (eg., species X) was placed at the top of the I-HEDGE list. Next, assuming that species X will be "saved", its extinction probability was then set to 0.001 and the HEDGE calculation was re-run. The top-ranked taxon from the second run that was not already prioritized was then given the overall second ranked position on the I-HEDGE list, its extinction probability was set to 0.001, and the procedure was repeated until all but one taxon was prioritized. R scripts that automate the calculation of I-HEDGE from networks and trees are available on GitHub (https://github.com/Eljensen/I-HEDGE).

The relationship between the species rankings from SH and I-HEDGE were then compared via simple Spearman's rank correlation.

### 2.3 Results

Pairwise values of $\Phi_{ST}$ ranged from 0.11 (*becki – darwini*) to 1 (*hoodensis – chathamensis*) among the species (Appendix A.1). The network (Figure 2.2) is non-treelike, and many of the terminals are roughly equally distant from the center of the network.

The ranking positions for the species estimated on this network were similar for both the SH and I-HEDGE metrics (Spearman's rank correlation $\rho$ =0.8601, $p$<0.0001). The ranking of the bottom seven species was identical between the metrics, while the top six varied by as many as four positions in the rankings (Table 2.1).

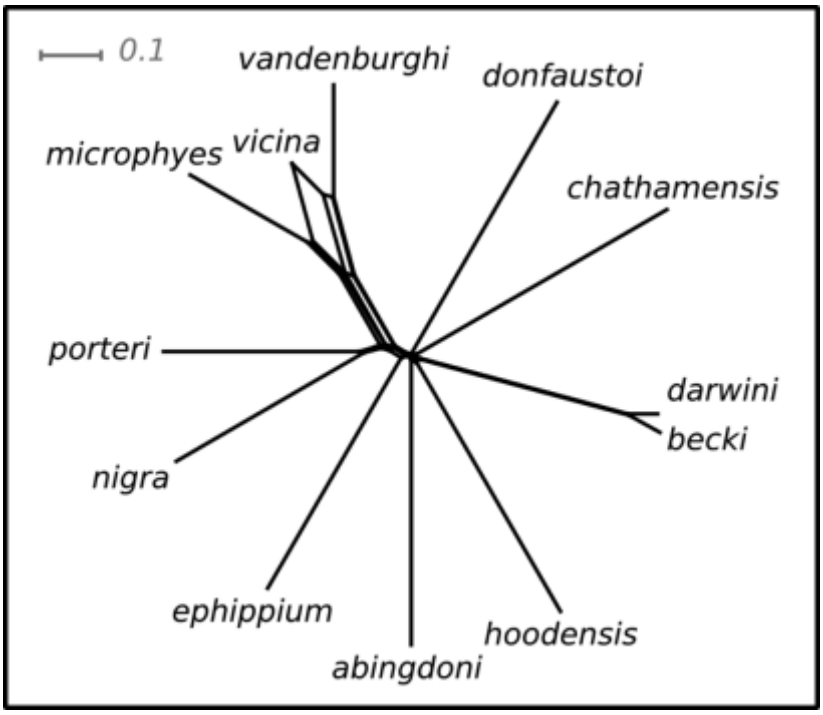


Figure 2.2 NeighbourNet depicting the relationships among species.
The lengths of the edges on the network depict the degree of genetic differentiation.

## 2.4 Discussion

Often conservation decision-makers are time and resource limited. The flexibility of input data for the I-HEDGE method is one of its strengths. The best available information regarding the p(ext) should be used, but in the absence of specific information, proxies can be used. Similarly, the network can be constructed from any type of differentiation matrix, including those generated from genotypic or phenotypic data. Furthermore, the network-based approach presented here can be applied below the species level to prioritize among populations or conservation units.

For the giant Galápagos tortoises, the SH and I-HEDGE ranking schemes produced similar results. Such robustness would be welcome, but the results depend on both network shape (here, quite starlike) and the patterns of imperilment across its tips. Here, we used a constant

p(ext) for the extant species, but the results were also consistent using p(ext) values based on IUCN red lists statuses (data not shown) which vary from Vulnerable to Critically Endangered (van Dijk et al. 2014). More simulation work and more case studies are needed to explore the sensitivity of these indices to variation in p(ext) and network/tree shape. Certainly, the iterative calculation of I-HEDGE should provide useful fine-tuning of the ranked list. The straight calculation of SH or HED values describes a property of the terminal unit, the average distance linking that unit to possible future networks. Such values should not be interpreted as an ordered list of priorities for conservation, since complementarity is not taken into account (Faith 2008). In contrast, I-HEDGE produces a ranked list that can be used to identify the order of species that if conserved, would preserve the most future expected genetic diversity under a given set of extinction probabilities for tips. In the unlikely event of a tie, other factors could be taken into account (population size, logistics, available funding, etc.) to raise one taxon over the other. Indeed, we recognize that such other factors may take precedence over the priorities suggested by I-HEDGE. Nevertheless, by taking into account evolutionary isolation, probability of extinction and complementarity, I-HEDGE is an integrative index and provides a rational basis for conservation prioritization.

The greatest increase in phylogenetic diversity for the giant Galápagos tortoises would be achieved by restoring the two extinct species, *C. abingdoni* and *C. nigra*. This result is due to the fact that they currently contribute no phylogenetic diversity and, if re-established, would each contribute an edge of substantial length on the network. Evaluating the contributions of these species to overall diversity is timely, as individuals with admixed ancestry have been discovered that share as much as half their genomes with these recently extinct species from

Floreana Island (Poulakakis et al. 2008, Russello et al. 2010, Garrick et al. 2012) or Pinta Island (Russello et al. 2007a, Edwards et al. 2013). The Galápagos National Park has begun an initiative to retrieve these admixed individuals and use them for both selective breeding and repatriation to their respective islands. Our finding that the greatest increase in phylogenetic diversity can be achieved by rebreeding lineages of the two extinct species provides additional support to the initiative.

Over the last 50 years, the species that have received the most intensive management are *C. hoodensis*, which was rescued from a population low of 15 individuals to its current size numbering nearly 2000 through captive breeding (Milinkovitch et al. 2013, Gibbs et al. 2014) and *C. ephippium*, which was the focus of a head-start program (Cayot 2008). Our finding that these species rank fifth and sixth, respectively (or first and fourth when the extinct species are excluded from the analysis, data not shown), for I-HEDGE further substantiates the extreme efforts that were put into recovering them from the brink of extinction.

As the shape of the network directly impacts the ranking of the terminal units, it is important to use genetic markers that are appropriate to the scale of divergence among taxa and reflect genome wide genetic diversity. Here, we made use of an existing, expansive mitochondrial control region dataset that has proven informative across multiple studies at both the within- and among-population/species levels in giant Galápagos tortoises (Caccone et al. 2002, Russello et al. 2005, Russello et al. 2007a, Poulakakis et al. 2008, Garrick et al. 2012, Poulakakis et al. 2012, Edwards et al. 2013). We evaluated a previously published

microsatellite dataset for giant Galápagos tortoises (Garrick et al. 2015) for use in this study, but the network generated depicted relationships that were highly incongruent with all previous studies of this group based on nuclear and mitochondrial DNA character data (see Appendix A.2) (Caccone et al. 2004, Poulakakis et al. 2012). Homoplasy of microsatellite fragment lengths has never been investigated in giant Galápagos tortoises, but studies of other taxa have found this to be quite common in comparisons among recently diverged groups (Garza and Freimer 1996, Angers et al. 2000, van Oppen et al. 2000, Anmarkrud et al. 2008). Given the wide range of divergence times between giant Galápagos tortoises (<0.28 mya – 1.7 mya; Poulakakis et al. 2012), it is quite likely that this source of homoplasy may have contributed to the reconstruction of spurious relationships that would influence downstream rankings. We therefore decided that the microsatellite data were not appropriate to use in this context, and suggest that marker choice should be given careful consideration on a system-by-system basis prior to implementing this network-based approach. For example, Volkmann et al. (2014) used two case studies to initially illustrate the calculation of SH and HED from networks, one using mitochondrial control region data for a broadly distributed species with subspecific variation, and another finer-scale example using microsatellite genotypic data for an endemic species with a highly restricted distribution. We recognize that basing conservation priorities on the information in a single locus is not ideal, and moving forward, genome-wide single nucleotide polymorphism data may be best suited to this approach, providing broad-scale coverage that enables more precise estimation of population-level parameters, including structure within and among populations and species.

### 2.4.1 Summary

The giant Galápagos tortoises are among the most charismatic emblems of evolutionary biology, and flagship species for conservation. Our results support both past and ongoing recovery efforts, and reinforce the emphasis that has been placed on rescuing *C. ephippium* and *C. hoodensis* from the brink of extinction over the past 50 years. The possible rebreeding of lineages of two recently extinct species *C. abingdonii* and *C. nigra*, if successful, may contribute substantially to the total genetic diversity of the giant Galápagos tortoises. As the Anthropocene progresses, it is important that conservation decisions are deliberate and based on the best available information. Metrics that explicitly measure a taxon's expected genetic contributions to future biodiversity, especially those that incorporate complementarity (such as I-HEDGE, introduced here) may be useful tools for managers interested in stewarding the breadth of genetic diversity under the Noah's Ark paradigm. As a general prioritization program moves forward, it will be important to identify both the axes of worth (ecological, evolutionary, current utility), and, for each, identify appropriate metrics (e.g., reliable measures of genetic diversity).

# Chapter 3: Genetics of head-start program cohorts to guide conservation of an endangered Galápagos tortoise (*Chelonoidis ephippium*)

## 3.1 Background

In the face of on-going biodiversity loss, an ever increasing number of species are in need of conservation interventions to prevent their extirpation or extinction (Vié et al. 2009). In many cases, the most effective conservation measures will be those that take place within the species' natural environment (*in situ*), such as habitat protection or hunting limitations, since such measures address the direct causes of species' decline. However, when *in situ* species conservation measures are not possible or sufficient to tip the scale from population decline to recovery, *ex situ* strategies, often in the form of captive management, are required. The potential of managing populations of endangered species outside their natural distribution met with initial optimism (Foose 1993), followed by acrimonious debate (Caughley 1994, Snyder et al. 1996) as to its conservation value. However, in extreme situations, such as when species are extinct in the wild (Iyengar et al. 2007) or *in situ* natural recruitment has ceased (Saltzgiver et al. 2012), *ex situ* captive breeding and/or head-starting may be the only way to prevent extinction. In order for *ex situ* conservation measures to effectively contribute to the long-term persistence of species, however, programs must be scientifically managed to allow for the release of a genetically and demographically healthy group back into the wild (Russello and Amato 2007).

*Chelonoidis ephippium* [also known as *Geochelone ephippium* (Ernst and Barbour 1989), *Geochelone nigra duncanensis* (Pritchard 1996) and *Chelonoidis nigra duncanensis* (Bonin

et al. 2006)] is a giant tortoise restricted to Pinzón Island in the Galápagos archipelago (Figure 3.1). Despite historical population sizes numbering in the thousands, the species was thought to have gone nearly extinct in the early 20th century (Townsend 1931) due to exploitation by humans with recovery limited by high hatchling mortality caused by introduced rats (*Rattus rattus*, MacFarland et al. 1974). Over the next three decades, a small number of tortoises were eventually located on the island and have been the focus of a captive "head-start" program. This conservation strategy entails collecting eggs or recently hatched individuals in natural nests on-island, transporting them to the Galápagos National Park breeding facility on Santa Cruz Island, rearing hatchlings *ex situ* to age 4-5 years old, and repatriating them back to Pinzón Island. Given the rugged and remote nature of Pinzón Island, the collection of head-start individuals is largely opportunistic, with the number of individuals and manner of collection varying widely among years. During the last population survey in 2004, it was estimated that ~500 repatriated individuals were residing alongside an aging and dwindling native population consisting of fewer than 65 individuals (C. Márquez, unpublished data). Although individuals repatriated in the 1970's have demonstrated normal breeding behavior and preliminary nest building activity (C. MacFarland, unpublished data), little to no recruitment has occurred due to continued depredation by black rats (Metzger and Marlow 1986). The elimination of black rats from Pinzón Island is viewed as the last step in ensuring a self-sustaining tortoise population. Accordingly, a black rat eradication campaign commenced in 2012, which involved a helicopter blanketing the island with two rounds of specially formulated rat poison. At present, giant tortoises of Pinzón Island are listed under CITES Appendix I and considered "extinct in the wild" by the IUCN Red List of Threatened

Species, although this ranking is not accurate and is currently under revision (L. Cayot, personal communication).

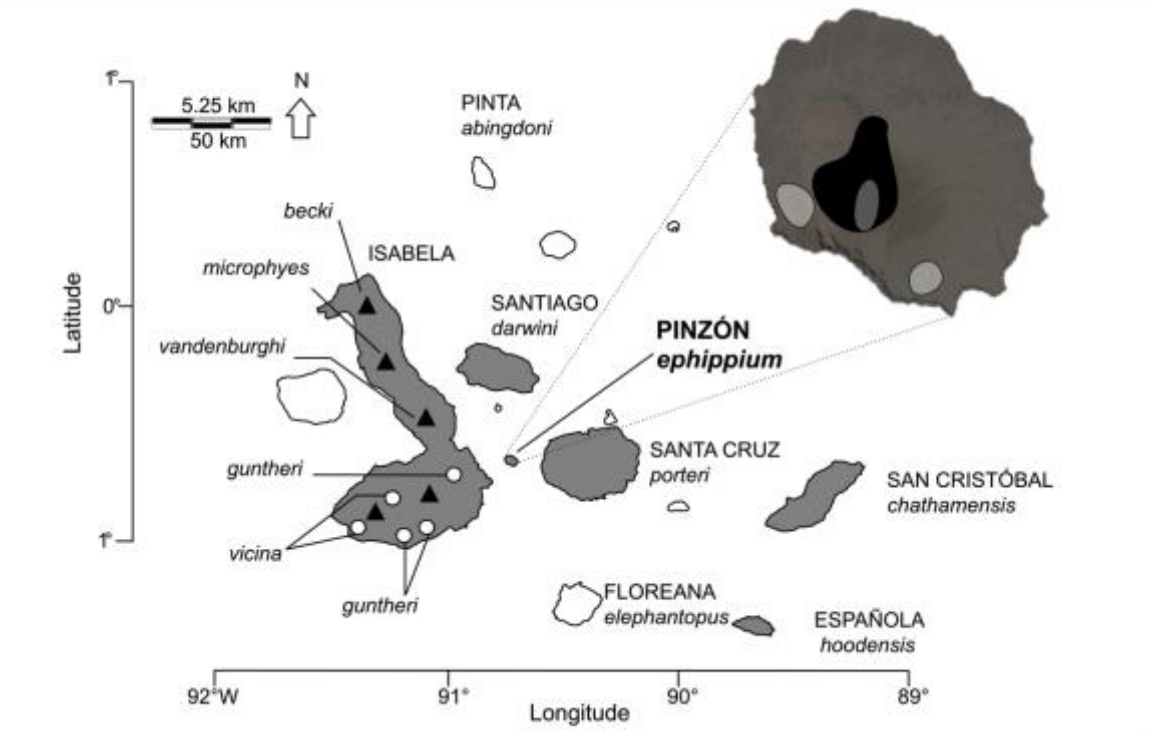


Figure 3.1 Map of Pinzón Island within the Galápagos archipelago.
Islands shaded in grey have extant populations of giant tortoises. Italicized names represent current taxonomic designations of the tortoises, names of the islands are in captials. Triangles indicate volcanos on Isabela Island. On Pinzón Island, the black and light grey shading indicate the collection areas for wild adults and head-start individuals, respectively.

Following the black rat eradication, the potential for establishment of a self-sustaining breeding population of giant tortoises on Pinzón Island brings into focus the need for an explicit evaluation of the demographic and genetic parameters associated with this endemic species. Previous studies have shown how skewed breeding success over the course of a 40-year old *ex situ* conservation program significantly reduced the effective population size of an already critically endangered species of Galápagos tortoise on Española Island (*C. hoodensis;* Milinkovitch et al. 2004, Milinkovitch et al. 2007, Milinkovitch et al. 2013). The

Pinzón tortoise case provides a unique opportunity to evaluate, at a turning point in the program, the distribution of genetic variation in the resident population and investigate the degree to which "head-start" individuals represent the gene pool of the on-island breeders. Such information will guide repatriation efforts to maximize effective population size and minimize genetic consequences of small population size.

Here, we collected microsatellite genotypic data from 156 hatchlings from two head-start cohorts and from 15 adults recently brought into captivity as a hedge to any deleterious consequences of the rat eradication campaign. These data were combined with those from previous population samplings of wild adults (Beheregaray et al. 2003a) to: 1) reconstruct patterns of genetic diversity and demographic history of the adult population on Pinzón Island; 2) quantify the extent and distribution of genetic variation in head-start cohorts from two years; 3) conduct comparative population genetic analyses to examine the degree to which head-start cohorts and captive adult founders genetically represent the contemporary native population, and 4) perform sibship analysis to estimate the number of breeders that gave rise to the head-start cohorts and infer family structure. Research results are discussed within the general context of genetic management of interactive *in situ*/*ex situ* conservation strategies.

## 3.2 Materials and methods

### 3.2.1 Sample collection

In December 2010 we collected blood samples from 171 captive individuals that originated on Pinzón Island, including hatchings representing cohorts of head-start individuals collected

on-island in 2007 (n=39) and 2009 (n=117) and adults that were brought into captivity in 2010 to act as founders of a breeding program if necessary (n=15). All samples were collected and transported in accordance with The University of British Columbia Animal Care Certificate # A10-0243, CITES import permit # 10CA02233/CWHQ-1, and CITES export permit # 0212280.

These data were combined with the current database that included 57 adults sampled on Pinzón Island (Beheregaray et al. 2003a) that, at the time of sampling, had a curved carapace length >55 cm, an indication of sexual maturity (MacFarland et al. 1974). The combined sampling was divided into four groups for downstream analyses: 1) 2007 cohort of head-start individuals ("2007", n= 37); 2) 2009 cohort of head-start individuals ("2009", n=117); 3) all adults ("adults", n=72), including those previously sampled on-island (n=57) (Beheregaray et al. 2003a) and wild individuals recently brought into captivity (n=15); and 4) adults recently brought into captivity only, which are a subset of the adult sample ("captive", n=15). It is possible that our sample of adults includes both wild-born native individuals and those that were part of early head-start cohorts repatriated since 1970 that have now reached maturity. Although the wild individuals were sampled in 1997, given the long life span of tortoises (>100 years) these individuals are likely still active breeders on the island.

### 3.2.2 Data collection and quality

All newly sampled individuals were genotyped at a panel of 9 microsatellite loci (GAL45, GAL50, GAL75, GAL94, GAL100, GAL127, GAL136, GAL159, GAL263; Ciofi et al. 2002) that has been demonstrated to be highly informative at the intra- and inter-specific levels across a range of studies (Ciofi et al. 2002, Beheregaray et al. 2003a, Beheregaray et

al. 2003b, Russello et al. 2005, Ciofi et al. 2006, Russello et al. 2007a, Russello et al. 2007b, Russello et al. 2010, Benavides et al. 2012, Garrick et al. 2012, Edwards et al. 2013). Moreover, use of these loci allowed direct integration with data collected from an earlier study on Pinzón tortoises (Beheregaray et al. 2003a). Allele calls between data sets were calibrated by regenotyping a subset (n=8) of the individuals from Beheregaray et al. (2003a). The genotypic data were examined for the presence of null alleles using MICROCHECKER (Van Oosterhout et al. 2004). Deviation from Hardy-Weinberg equilibrium (HWE) was assessed using exact tests, as implemented in GENEPOP 3.3 (Raymond and Rousset 1995, Rousset 2008). Linkage disequilibrium (LD) was investigated for all pairs of loci using GENEPOP 3.3 (Raymond and Rousset 1995, Rousset 2008). Significance levels were adjusted for multiple comparisons using the false discovery rate (Benjamini and Hochberg 1995), as advocated by Narum (2006) for use in conservation genetic studies.

### 3.2.3 Within population genetic variation and relatedness

Gene diversity ($N_g$) and rarefied allelic richness ($N_a$) were calculated for each group in FSTAT (Goudet 2001); observed ($H_o$) and unbiased expected heterozygosity ($UH_e$) were calculated for each group in GENALEX 6.5 (Peakall and Smouse 2006, Peakall and Smouse 2012). Pairwise relatedness was calculated according to the method of Queller and Goodnight (1989) in GENALEX 6.5 (Peakall and Smouse 2006, Peakall and Smouse 2012); from this, population mean relatedness was calculated using 999 permutations and 999 bootstrap replicates. In addition, the observed distribution of pairwise relatedness among adults was plotted with those of 1000 simulated dyads for each of four relatedness categories (unrelated, half sibs, full sibs, and parent–offspring) using iREL (Gonçalves da Silva and Russello 2011). The inbreeding coefficient, $F_{is}$, was calculated for each group as

implemented in Genetix (Belkhir et al. 2004), with significance assessed using 1000 permutations.

### 3.2.4 Among population genetic variation and demographic history

Genetic differentiation among groups was estimated by pairwise comparisons of $\theta$ (Weir and Cockerham 1984), as calculated in GENETIX (Belkhir et al. 2004), and evaluated using 1000 permutations. Significance levels were adjusted for multiple comparisons using the false discovery rate (Benjamini and Hochberg 1995). The significance of genotypic differentiation among groups was also tested using a log-likelihood G-test that does not assume HWE within samples using FSTAT (Goudet 2001) and based on 1000 permutations. A list of private alleles was tabulated in GENALEX (Peakall and Smouse 2006, Peakall and Smouse 2012) and private allele frequency corrected for sample size was calculated in HPRARE (Kalinowski 2005).

Genetic signatures of demographic contraction were assessed for the adults using the heterozygote excess and the mode-shift tests, both implemented in BOTTLENECK 1.2.02 (Piry et al. 1999) and the M-ratio test using M_P_VAL.exe and critical_M.exe (Garza and Williamson 2001). For the heterozygote excess test, 1000 iterations were used with the Wilcoxon test under the two phase model with 70% stepwise mutations. For the M-ratio we calculated $\theta$ ($\theta = 4N_e\mu$) using mutation rates ($\mu$) that are considered both slow ($1.5 \times 10^{-4}$) and fast ($1.5 \times 10^{-3}$) for herpetofauna (Zhang and Hewitt 2003), and various pre-bottleneck $N_e$ (50, 500, 1000 and 1,500), resulting in $\theta$ ranging from 0.03 to 9. Multiple values of $\theta$ were used to assess how robust conclusions were to permutation of that parameter. We used 3.5

base steps for multi-step mutations, and the amount of mutations greater than single step, $P_g$, was 0.2 as suggested by Garza and Williamson (2001).

To determine whether population substructure exists, the Bayesian method of Pritchard et al. (2000) was used as implemented in STRUCTURE 2.3.4. Runs were 1,000,000 *Markov Chain Monte Carlo* replicates in length after a burn-in period of 500,000, using correlated allele frequencies under a straight admixture model. We varied the number of clusters ($K$) from 1 to 5 with 40 iterations per value of $K$. The most likely number of clusters was determined by plotting the log probability of the data ($\ln \Pr(X|K)$) across the range of $K$ values tested and selecting the $K$ where the value of $\ln \Pr(X|K)$ plateaued, as suggested in the Structure manual. We also calculated $\Delta K$ (Evanno et al. 2005), as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2011). Additionally, the model-free Discriminant Analysis of Principle Components (DAPC; Jombart et al. 2010) was carried out using the *dapc* function of the *Adegenet* package (Jombart 2008) in the R statistical package (http://www.R-project.org/). The number of clusters within the data set ($K$) was selected using the *find.clusters* function and Bayesian Information Criterion (BIC). The chosen number of $K$ was based on the minimum number of clusters after which the BIC decreased by a negligible amount.

### 3.2.5 Sibship analyses

Sibship analyses were performed combining the 2007 and 2009 head-start individuals as the offspring group and no known potential fathers or mothers, as implemented in COLONY version 2.0 (Jones and Wang 2010). To evaluate which mating strategy was most appropriate and best fit the data (both sexes monogamous, one sex polygamous the other monogamous,

both sexes polygamous), three different runs were performed and the likelihood of the observed genotypic data was compared across runs. We assumed inbreeding and used the sibship complexity prior; run lengths were carried out using the "short with high precision" option. Using the most likely mating strategy, we constructed a frequency distribution of inferred full-sibling family sizes.

## 3.3 Results

### 3.3.1 *In situ* population genetic variation and demographic history

Deviation from Hardy-Weinberg equilibrium in the form of heterozygote deficit was detected in five of the nine loci (GAL 45, 50, 75, 100 and 136), a subset of which (GAL 45, 50 and 136) were also flagged as potentially having null alleles due to homozygote excess. Significant linkage disequilibrium was found in eight of 36 pairwise comparisons of loci. These patterns have been identified in previous studies of Pinzón tortoises (Beheregaray et al. 2003a). As the microsatellite loci were originally developed from a Pinzón individual (Ciofi et al. 2002), null alleles or other artefactual causes of heterozygote deficit are unlikely. For this reason, we retained all nine loci and, where possible, used analyses that did not assume HWE.

Genetic variation within the adult group (Table 3.1) is on the high end of what has been reported for other Galápagos tortoise species (Ciofi et al. 2002). The strong signal of heterozygote deficit contributed to several findings in the adult group, namely, significant inbreeding ($F_{is}$= 0.11, $P$<0.05), and no genetic signature of population decline in the heterozygote excess test. However, the M-ratio (0.626), which does not depend upon

heterozygosity, is smaller than the critical M (0.773-0.656) for all values of $\theta$, robustly indicating a population bottleneck over a range of mutation rates (slow or fast) and effective population sizes (50 to 1,500). The mode-shift test revealed a normal distribution, suggesting a stable population size.

The observed distribution of relatedness values among the adults most closely approximated the distribution expected for randomized, unrelated individuals (Figure 3.2). However, observed pairwise relatedness values exceeded both the lower and higher thresholds of the randomized distribution, indicating overrepresentation of unrelated and more highly related individuals in the adult population (Figure 3.2).

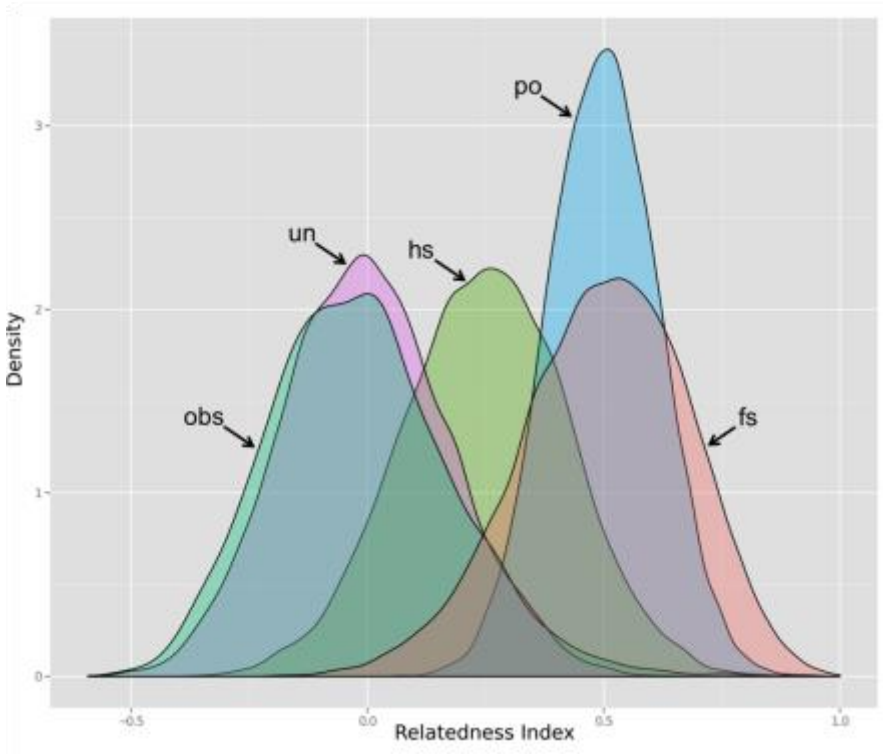


Figure 3.2 Plot of the frequency distribution of observed (obs) and expected Queller and Goodnight (1989) pairwise relatedness values among the adults.
Expected distributions were generated using simulations of unrelated individuals (un), full-siblings (fs), half-siblings (hs) and parent-offspring pairs (po) in iREL (Gonçalves da Silva and Russello 2011) **.**

There was no indication of population substructure, with no substantial decrease in BIC from 1-8 clusters in the *find.clusters* analysis. The Bayesian clustering analysis also determined one cluster to be the most likely, as the ln probability of $K$ increased linearly from $K$=1. The $\Delta K$ method suggested $K$=2 as most likely, although due to the manner in which it is calculated, the $\Delta K$ method of Evanno et al. (2005) is incapable of inferring a $K$=1.

### 3.3.2 Genotypic variation, family structure and representation of head-start cohorts

Comparable levels of genetic variation were found in the head-start cohorts regardless of hatchling year, with similar levels also found in the adults (Table 3.1). There was no evidence of inbreeding within the head-start cohorts (Table 3.1), and neither group had relatedness values significantly different from 0 (data not shown).

Table 3.1 Genetic variation within groups of Pinzón tortoises.

| | $N$ | $N_g$ | $N_a$ | $H_o$ | $UH_e$ | $R_{Q\&G}$ | $F_{is}$ | $P_A$ |
|---|---|---|---|---|---|---|---|---|
| **2007** | 39 | 0.69 | 7.83 | 0.735 | 0.689 | 0.02 | -0.07* | 1 (0.43) |
| **2009** | 117 | 0.70 | 8.25 | 0.706 | 0.679 | 0.00 | -0.01 | 8 (0.77) |
| **Adults** | 72 | 0.69 | 9.60 | 0.613 | 0.691 | 0.01 | 0.11* | 24 (2.26) |

Sample size ($N$); gene diversity ($N_g$); rarefied allelic richness ($N_a$); observed heterozygosity ($H_o$); unbiased expected heterozygosity ($H_e$); mean relatedness ($R_{Q\&G}$); inbreeding coefficient ($F_{is}$); private alleles with rarefied private allele frequency in parentheses ($P_A$). * $p$<0.05

Pairwise $\theta$ values were low among the head-start cohorts and the adults, ranging from 0.006 to 0.01 (Table 3.2), although each value was significant, except between the 2007 and 2009 cohorts under the log-likelihood G-test. While low values, these significant differences in the distributions of genetic variation suggest that the head-start hatchlings are not entirely representative of the variation of the wild population (Table 3.2). This result is also

supported by the high incidence of private alleles. When the head-start groups were combined and analyzed relative to the adult group, 13 alleles were private to the head-start individuals and 24 were private to the adults; 39 out of 72 adults had at least one of these private alleles.

Table 3.2 Genetic divergence ($\theta$) among groups below the diagonal, significance of $\theta$, determined through 1000 permutations, indicated above the diagonal.

| | **2007** | **2009** | **Adult** |
|---|---|---|---|
| **2007** | | + | + |
| **2009** | 0.006 | | + |
| **Adult** | 0.008 | 0.010 | |

(+) Significant after controlling for false discovery rate

Of the three mating systems evaluated, the model with both sexes polygamous had the highest log likelihood. Under this model, 52 full-sibling families were reconstructed, ranging from one to ten family members. The single individual families (i.e. individuals with no first order relatives in the sample) were the most common (Figure 3.3). A small number of full-sibling families (n=7) contained individuals from both the 2007 and 2009 cohorts. A total of 63 breeders were inferred as parents in the sibship analysis (32 males, 31 females).

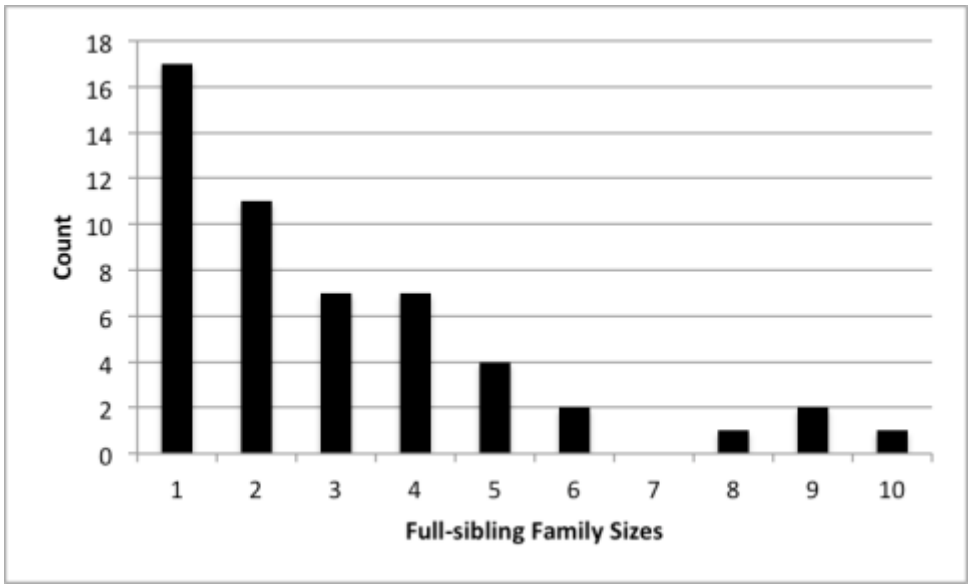


Figure 3.3 Frequency distribution of full-sibling family sizes in the 2007 and 2009 head-start cohorts combined inferred using COLONY v.2.0 (Jones and Wang 2010).
The x-axis indicates the number of members in a full-sibling family; the y-axis indicates the number of families.

### 3.3.3 Relatedness and *in situ* representation of captive adult founders

The 15 captive adults, recently removed from the wild as a hedge against possible deleterious effects of the rodenticide campaign, had higher gene diversity (0.72), but lower allelic richness (6.9), and moderate values for heterozygosity and inbreeding ($H_o$ = 0.700, $UH_e$ = 0.715, $F_{is}$ = 0.02) relative to the rest of the adults. The mean relatedness among the captive adults was negative (-0.06), but not significantly different from 0 (data not shown). The captive adults were not significantly differentiated from the remaining adults ($\theta$ = -0.004).

## 3.4 Discussion

### 3.4.1 *In situ* diversity

Given what is known about the severe population decline experienced by the Pinzón tortoises in the 19$^{th}$ and 20$^{th}$ centuries (MacFarland et al. 1974), it is remarkable that so much genetic

diversity has been retained. However, the finding of significant heterozygote deficit across multiple loci suggests a complex demographic history.

A population that has recently undergone a bottleneck is expected to have heterozygote excess, and conversely, an expanding population can display heterozygote deficit (Maruyama and Fuerst 1985, Cornuet and Luikart 1996). Microsatellite data may be informative for demographic events within the past 10-50 generations (Peery et al. 2012), which, assuming a generation length of 25 years for Galápagos tortoises, covers the time period from ~760 to 1760. We found evidence for a population bottleneck using the M-ratio test, which appears to be in conflict with the observed heterozygote deficit. Yet, simulation studies have shown that the M-ratio test may be more robust than heterozygosity-based approaches for detecting bottlenecks under a number of scenarios including when a population has made a demographic recovery, mutation rates are high, or pre-bottleneck sizes were large (Williamson-Natesan 2005). The latter case may be relevant for *C. ephippium*, as recent reconstructions based on slower-evolving mitochondrial DNA and a nuclear intron (PAX-P1) revealed that Pinzón tortoises had a very large historical effective population size that was increasing over the last 8000 generations (Garrick et al. 2015). This work built upon an earlier study using mitochondrial DNA (Beheregaray et al. 2003a) that likewise detected signatures of demographic expansion in Pinzón tortoises based on mismatch distributions. Taken together, there are temporal signals indicating historical population expansion over thousands of generations and recent population decline for Pinzón tortoises, consistent with our findings of both heterozygote deficit and significant M-ratio test.

A Wahlund effect (Wahlund 1928) due to unrecognized population substructure within the sample can also cause an observed heterozygote deficit. Here, no population substructure was found based on results from both Bayesian clustering analyses implemented in STRUCTURE (Pritchard et al. 2000) that assume markers are in HWE, as well as the model-free Discriminant Analysis of Principle Components (Jombart et al. 2010). The possibility remains, however, that our sample includes closely related individuals from several different families contributing to a "family" Wahlund effect (Castric et al. 2002). To examine this possibility, we evaluated the observed distribution of pairwise relatedness values relative to simulated expected distributions of unrelated, half-sibling, and first-order–related (full sibling, parent-offspring) individuals. Ideally, an independent sample would be used to generate the allele frequency distribution upon which the simulations are based. Although this design was not possible in the current study, this approach still offers some qualitative insights. Here, the observed distribution is slightly skewed towards lower relatedness than that expected for randomized, unrelated individuals, and has a longer tail at the higher end of relatedness (Figure 3.2). This pattern may indicate the existence of "families" of closely related individuals within our adult sample that have lower among-family relatedness than expected in a random sample of unrelated individuals, mimicking a Wahlund effect on a finer-scale (Castric et al. 2002). It is not known if Galápagos tortoises exhibit nest site fidelity, but if so, this behavior could generate weak genetic structure among nest sites, contributing to a "family" Wahlund effect. Moreover, in the progeny, this "family" Wahlund effect can lead to the pattern of heterozygote excess (Pudovkin et al. 1996), which we observed in both the 2007 and 2009 head-start cohorts (Table 3.1). Overall, the "family" Wahlund effect is a plausible and interesting explanation for the pattern of heterozygote

deficit observed here as well as in the previous study (Beheregaray et al. 2003a) that did not have the benefit of sampling putative progeny.

Another cause of heterozygote deficit is the mating of close relatives, which increases homozygosity. Here, we found the adult sample to have a significant $F_{is}$ value, which is consistent with a previous study that found the Pinzón population has inbreeding coefficients higher than many of the other populations of Galápagos tortoise (Garrick et al. 2015). Given the recent decrease in population size and its currently small size, inbreeding is also a plausible explanation for the observed heterozygote deficit. Additional studies using more loci and genome-level scans as well as a larger and geographically representative sample of the *in situ* population are required to further tease apart the complex demographic history and intriguing patterns of genetic diversity in Pinzón tortoises.

### 3.4.2 Genetic diversity in head-start cohorts

Two key targets when managing an interactive *in situ*/*ex situ* conservation program are to maintain a genetically healthy population and to ensure that the *ex situ* population captures the genetic diversity present in the wild population (Lacy 1994). The head-start program implemented by the Galápagos National Park has increased the population size while maintaining moderate levels of genetic variation within the *in situ* and *ex situ* populations of the Pinzón tortoise (Table 3.1). The 2007 and 2009 cohorts of head-start individuals, although somewhat differentiated from each other and the adult group, have allelic richness and heterozygosity comparable to or higher than levels detected in the adult group. However, it is concerning that the head-start cohorts do not represent the extent and distribution of *in situ* variation (Table 3.2). There are a large number of private alleles detected in the 2007 and

2009 head-start cohorts, which suggests that our sample of adults is not representative of the on-island breeders, despite constituting a large proportion of the known wild population. Likewise, the large number of alleles private to the adult group indicates that many of those individuals have not contributed offspring to the sampled head-start cohorts.

Although precise geographic information of nest locations from which the head-start individuals were collected is not available, collection sites tended to be on the southwestern, coastal area of Pinzón Island, extending inland towards the central crater (Figure 3.1). The adults in Beheregaray et al. (2003a) were sampled exclusively in the central crater. The disparity of geographic sampling locations between our *in situ* and *ex situ* samples may partially explain the observed differentiation. Future egg collection trips should endeavor to sample from all known nesting areas in order to capture the extent and distribution of extant variation.

The sibship analysis determined both sexes to be polygamous as the most likely mating system for Pinzón tortoises, which is consistent with what is known about their behavior (Milinkovitch et al. 2004). Of the 52 likely full-sibling families, seven have individuals from both 2007 and 2009, which potentially indicates sperm storage by the female or remating with the same male. Sperm storage is common in other Chelonians (Pearse and Avise 2001), but has not been demonstrated in Galápagos tortoises. Although this pattern requires further investigation, if accurate, the detection of sperm storage may have important implications for better understanding colonization history of giant tortoises in the Galápagos archipelago, as

well as how genetic diversity may be retained during severe population bottlenecks (Murray 1964, Karl 2008).

The most common full-sibling family sizes are at the lower end of the spectrum, meaning that most individuals have no or few full siblings within the sample (Figure 3.3). This finding reflects positively on the ability of the head-start program to capture genetic diversity and not result in inflated relatedness. However, there are several full-sibling families that are overrepresented in the head-start cohorts, with eight to ten members. The large number of inferred breeders (32 males, 31 females) is consistent with the high levels of genetic diversity in the head-start cohorts.

Cohorts of hatchlings have been head-started nearly every year since 1965, ranging in number from just a few individuals, to the largest cohort in 2009 at 117 individuals. As we have been able to include just two cohorts in our study, it should be noted that the patterns found in those groups might not hold true over the entire history of the program. However, as the on-island collection locations for the head-start individuals have remained the same over the years and the collection strategy has been consistently opportunistic, we likely captured a reasonable snapshot of the head-start program.

### 3.4.3 Genetic representation of captive founders

The 15 captive adults were brought into the breeding facility to act as an insurance population prior to the outset of the Pinzón Island rat eradication program. In ideal situations, the founders of captive breeding programs are individually chosen based on their specific genetic traits (Witzenberger and Hochkirch 2011). Although these founders were chosen without prior genetic information, they constitute a reasonable set of founders for conserving

a broad representation of existing on-island diversity. These eight females and seven males constitute the group with the highest gene diversity in this study, and have high observed heterozygosity, a low inbreeding coefficient and low mean relatedness. During their time in captivity, these individuals have already bred and produced a small number of offspring (Tapia, pers. obs.). The plans for these captive adults depend upon the outcome of the rat eradication program. Results from follow-up monitoring trips will inform whether the captive adults are returned to Pinzón Island or retained *ex situ* to sustain the breeding program.

### 3.4.4 Conservation implications

The head-start program for giant tortoises on Pinzón Island was successful in its demographic goal of increasing population size, however it has been only partially effective at maintaining genetic diversity. Inbreeding and relatedness have been minimized in the head-start cohorts, but they do not represent the extent and distribution of species-level genetic variation. As it is likely that only trivial levels of recruitment outside of the head-start program has occurred in the past century (Metzger and Marlow 1986), it is critical that the source locations of eggs and hatchlings for the head-start program are broadened to encompass genetic contributions from adults who, up to this point, may not have been represented. Despite being a reasonably well-studied group, there are major gaps in our knowledge regarding the reproductive biology of Galápagos tortoises, and the questions of nest site fidelity and sperm storage are just two examples. A better understanding of reproductive biology would help in the effective design of conservation strategies.

Given the uncertain status of the Pinzón Island tortoise, it will be important to maintain the head-start program both to address the underrepresentation of genetic diversity as well as to act as insurance until the eradication of black rats. The interactive *in situ*/*ex situ* conservation program, centered around head-starting, paired with the rat eradication program holds great promise for establishing and maintaining a demographically and genetically healthy population of giant tortoises on Pinzón Island.

# Chapter 4: Looking through the bottleneck: genomic analysis of historical and contemporary patterns of genetic variation in the Pinzón Island Galápagos tortoise

## 4.1 Background

Populations and species are in decline globally in what has been termed the "biodiversity crisis" (Wilson 1985). Genetic diversity within species is one of the fundamental levels of biodiversity recognized by the Convention on Biological Diversity (www.cbd.int), and is lost when population sizes are reduced. Understanding the factors associated with the genetic consequences of population decline is important, as levels of genetic diversity are associated with the probability of long-term population persistence (Frankham 1997, 2005), ability to survive a novel disease threat (Smith et al. 2009), and adaptation to changing environmental conditions (Barrett and Schluter 2008, Jump et al. 2009, Pauls et al. 2013).

Declining populations often experience a genetic bottleneck where, as the effective population size shrinks, rare alleles tend to be lost rapidly (Nei et al. 1975). Immediately after a bottleneck, heterozygosity can increase relative to expectations based on the number of alleles present (Nei et al. 1975, Leberg 1992, Cornuet and Luikart 1996). If a population persists at a small size, however, inbreeding and drift result in an overall decrease in heterozygosity over time (Nei et al. 1975). These impacts can culminate in genetic differentiation between pre- and post-bottleneck populations (i.e., along a temporal axis), with a similar genetic signature to differentiation between subdivided, geographically structured populations (i.e., along a spatial axis; Bryant et al. 2016, Hoffmann et al. 2016). The specific genetic consequences of a bottleneck depend upon its severity and duration, as well as species' life history traits and the demographic history of the population in question,

including whether it has experienced bottlenecks in the past (Nei et al. 1975, Maruyama and Fuerst 1985, Tajima 1989a, Leberg 1992). The rate of post-bottleneck recovery also influences the genetic patterns in populations (Maruyama and Fuerst 1984). Many empirical and experimental studies have focused on the decline phase of bottlenecks, and have demonstrated the importance of the length and severity of a population contraction on post-bottleneck genetic diversity (e.g. Spencer et al. 2000, England et al. 2003). Far fewer studies have focused on the recovery phase to empirically validate the impacts of rate of recovery or immigration, or to evaluate the impacts of human-mediated population enhancement.

Active management of populations, including the use of approaches such as captive breeding, head-starting and assisted translocations, are becoming increasingly common to facilitate recovery. Such actions can be particularly effective when used together with other conservation measures that address the original factors leading to population decline (Tenhumberg et al. 2004, Conde et al. 2011, Seddon et al. 2014). However, active management interventions can have unintended genetic consequences. The negative genetic impacts that can occur in captive breeding programs are well known (e.g. inbreeding, genetic drift, adaptation to captivity; Snyder et al. 1996), and can be proactively avoided through careful planning and managing of captive populations (Frankham 2008, Williams and Hoffman 2009, Witzenberger and Hochkirch 2011). Head-starting, the collection of eggs or young from the wild for captive rearing before repatriation to the wild, can bolster population sizes by increasing juvenile survival. However, since the parents of head-started individuals are often unknown, the population can become unintentionally skewed towards over-representing certain families if head-started individuals are collected in a biased manner.

Investigations into the genetic consequences of population decline and the potential efficacy of recovery efforts typically rely on estimates of levels of genetic diversity, effective population size and structure inferred from the post-bottlenecked population. However, when assessing the population only following its decline, such estimates may provide a biased view of population history and represent a "shifted baseline" upon which to base conservation decisions (Pacioni et al. 2015). A more direct way to assess the impacts of decline and recovery would be to use temporal sampling of the pre- and post-bottlenecked populations. Although few in number, such studies have provided important insights and demonstrated the value of a temporal approach, yet they have largely relied on a limited number of genetic markers to characterize patterns (e.g. fragment of the mitochondrial control region and/or 5-24 microsatellite loci; Bouzat et al. 1998, Wisely et al. 2002, Eldridge et al. 2004, Nyström et al. 2006, Ugelvig et al. 2011).(Miller and Waits 2003)

Recently-developed methods for historical DNA analysis, paired with targeted capture approaches and next-generation sequencing, are now increasing opportunities for collecting genome-wide data from temporally spaced population samples. Next-generation sequencing has allowed for the economical collection of vast quantities of data, and most platforms, by design, yield short sequences (~50-300 base pairs). Thus, the short fragments of somewhat degraded historical DNA are not a constraint, allowing older or less well preserved specimens to be included in population genetic studies. However, DNA extracted from historical or ancient specimens often contains a large proportion of exogenous DNA. The development of capture approaches is now allowing DNA from the target species to be enriched while simultaneously generating a reduced representation genomic library, resulting

in more efficient use of sequencing effort (Bi et al. 2013, Carpenter et al. 2013, Gasc et al. 2016). These technical advances are enabling new uses of the vast wealth of natural history collections around the world to study evolutionary processes, taxonomy, systematics, ecology and conservation issues (Wandeler et al. 2007, Habel et al. 2014, Holmes et al. 2016, Raupach et al. 2016, Yeates et al. 2016).

With the tools now available to collect high quality genome-wide data from pre- and post-bottleneck populations, comprehensive assessments of the consequences of population bottlenecks are possible in cases where samples exist. The Pinzón giant tortoises (*Chelonoidis ephippium*) present just such an opportunity. Endemic to to Pinzón Island in the Galápagos (Figure 1), Pinzón tortoises declined to near extinction in the 20$^{th}$ century, but recovered through an intensive head-start program. Historically, Pinzón tortoises numbered in the thousands, but exploitation by humans in the early to mid 1800's dramatically reduced the population size. Records indicate that between the years 1846-1863, whalers collected at least 356 individuals from Pinzón Island, to be used as a source of fresh meat at sea (Townsend 1931). In 1891, black rats (*Rattus rattus*) were first documented on the island (reported in Patton et al. 1975), and by 1903 the absence of young tortoises was attributed to predation by the introduced rats (Beck 1903). By 1928, *C. ephippium* was thought to be nearly extinct (Townsend 1931). Surveys conducted in the 1960's located 100 individuals, with the census population size estimated to be 150-200 (MacFarland et al. 1974). In 1965, no individuals with a curved carapace length smaller than 50 cm were observed (MacFarland et al. 1974), and it was speculated that no successful recruitment had occurred since the introduction of rats in the 1890's (Pritchard 1996). At that point, the Pinzón giant tortoise

was essentially a species of "living dead", that would face extinction when the last of the remaining, aging adults died.

Faced with the potential loss of a keystone species, the Charles Darwin Research Station initiated a head-start program in 1965, later managed in collaboration with the Galápagos National Park Directorate, where eggs or hatched but not yet emerged individuals were collected from Pinzón Island and reared in captivity (Cayot 2008). At age 4 or 5, individuals were repatriated to Pinzón Island once they were large enough to minimize the risk of predation by rats. This program successfully raised and repatriated over 800 juvenile tortoises over the past 50 years. Recognizing that the head-start program would have to operate in perpetuity unless something was done about the cause of hatchling mortality in the wild (Cayot 2008), a rat eradication campaign was carried out in December 2012, after an initial attempt in the late 1980's was unsuccessful (Cayot et al. 1993). By 2014 the eradication project was declared a success, and the first instance of wild recruitment was observed (Tapia Aguilera et al. 2015). Thus, this work has rescued the species from certain extinction. This history of decline and recovery in *C. ephippium* provides a rare opportunity to perform a direct evaluation of the relationship between current genetic diversity and past demographic events, including the impact of the head-start program, due the existence of a large number of historical specimens (n = 86 complete adult specimens) collected from Pinzón during the 1905-1906 California Academy of Sciences expedition to Galápagos (Van Denburgh 1914).

In this study, we collected genome-wide single nucleotide polymorphism (SNP) data from 78 historical (c. 1906) samples that, given the long generation times in Galápagos tortoises (estimates of 25-40 years; MacFarland et al. 1974), are likely representative of the pre-bottleneck population, as well as from 150 contemporary (c. 2014) samples of *C. ephippium*. Full mitochondrial genome sequences were also collected for the 78 historical and a subset of 45 contemporary individuals. The major goals were to empirically test population genetic theory associated with rapid decline and recovery with respect to predictions from population genetic theory. The predictions are that 1) there will be a loss of allelic diversity post-bottleneck; 2) despite the loss in allelic diversity, levels of heterozygosity will be maintained post-bottleneck; 3) the effective population size will be reduced post-bottleneck; and 4) temporal population structure will develop between the pre- and post-bottleneck populations. In addition to empirically testing bottleneck theory, we use patterns in the historical population to evaluate the degree to which the extent and distribution of genetic variation in the contemporary population has been impacted by the head-start program. Furthermore, for comparison to documented history and to evaluate potential biases associated with using point estimates from post-bottlenecked populations alone, we reconstructed the demographic history of the population using these broad historical and contemporary samplings of nuclear and mitochondrial genomes. Lastly, as this study is among the first to collect temporal population genomic data using a combination of methods, we provide technical insights into the study design that may prove helpful to others.

## 4.2 Materials and methods

### 4.2.1 Sample collection

#### 4.2.1.1 Contemporary tortoise population

In December 2014, we spent six days collecting blood samples from tortoises on Pinzón Island. All parts of the island suspected to have tortoises were surveyed. Each tortoise we encountered was measured along the curved length of its carapace and its location recorded using GPS. A small blood sample (0.1–1 mL) was collected from the brachial artery. 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. All samples were collected in partnership with the Galápagos National Park, and in accordance with the University of British Columbia animal care protocol #A14-0239. Samples were transported under CITES export permit #15EC000001/VS and import permit #14CA03454/CWHQ-1C.

#### 4.2.1.2 Historical tortoise population

Whole specimens of *C. ephippium* were collected from the wild in December 1905 through August 1906; details of the collections and the expedition are given in Van Denburgh (1914). The field notes suggest that specimens were collected from various parts of the island. In 2004, femurs attached to carapaces were sampled from 78 specimens accessioned in the California Academy of Sciences. All individuals were adults (carapace length ranging from 87 to 53 cm), and 57 were noted as being female, while the remaining 21 were noted as being male (Van Denburgh 1914). A Dremel rotary tool with a cutting blade was used to initially scrape off the surface of the bone. A wedge cut was performed targeting 100–200 mg of material (mean 164 mg). Samples were stored in dry tubes at ambient temperatures until DNA was extracted in 2016.

### 4.2.2 Molecular and bioinformatic methods

#### 4.2.2.1 Restriction site associated DNA sequencing

We used restriction site associated DNA sequencing (RAD-Seq) to simultaneously identify and genotype SNPs in the Pinzón tortoises. We generated RAD libraries for 150 individuals sampled in the year 2014 using a modified version of the Etter et al. (2011) protocol. Genomic DNA was extracted from these contemporary samples using a NucleoSpin QuickBlood kit (Machery-Nagel) following the manufacturer's protocols, with the addition of RNaseA (Qiagen). RAD libraries were constructed using 500 ng of input DNA of each sample digested with the *Sbf1* restriction enzyme (New England Biolabs Inc.), and pooled into three libraries of 48 or 52 individually barcoded samples. The barcodes used were six base pairs (bp) long, and each differed by at least two bases (Hohenlohe et al. 2010, Miller et al. 2012). A sonicator (Bioruptor® NGS; Diagenode) was used to shear the DNA to a mean length of ~500 bp, and automated size selection was performed using a Pippin Prep™ (Sage Science) to isolate DNA fragments between 350 and 600 bp. Each library was initially sequenced using one full lane of paired end 150 bp Illumina HiSeq 2500 in rapid run mode; additional sequencing of two libraries was required to achieve the target number of high quality reads. Two samples (tortoise IDs # A025 and G154) were run in duplicate in separate libraries to assess error rates downstream.

#### 4.2.2.2 Assembly and SNP discovery

The STACKS V1.3 suite of scripts (Catchen et al. 2011, Catchen et al. 2013) was used for sequence assembly and SNP discovery. The *process_radtags* module was used to separate reads by barcode, remove low-quality reads and those that lacked the *Sbf1* recognition

sequence, and trim reads to 140 bp. Only paired-end reads for which both mate pairs met quality checks were retained. The *clone_filter* module was used to identify and remove polymerase chain reaction (PCR) duplicates based on paired-end reads having identical sequence that, due to the random shearing during library preparation, are unlikely to have originated from separate genomic DNA molecules. After this step, only forward reads were used. The *denovo_map.pl* module was used to execute the STACKS components *ustacks*, *cstacks* and *sstacks*. A subset of 30 individuals (10 from each library) was used to test six parameter sets. For all tests, a minimum of three identical reads were required to create a stack (-m 3), but the number of mismatches allowed between loci was varied (-M 2 or 3) as was the number of mismatches allowed between sample tags when generating the catalog (-n 1, 2 or 3). The catalogs produced by the six parameter sets had varying numbers of RAD tags, but similar numbers of variable sites, inbreeding coefficients and nucleotide diversity values (data not shown), indicating that outcomes were insensitive to choice of parameter set. We therefore used one of the middling parameter sets (-m3 -n 2, -M 2) and applied it to the full dataset. Following *denovo_map.pl*, the *rxstacks* module was run using a lnl cut off of -15, with the conf_filter and prune_haplos options turned on. The *populations* module was then run using a minimum stack depth of five, with loci required to be present in 70% of individuals, a minimum minor allele frequency of 0.05 and a lnl cut off of -30.

#### 4.2.2.3 Design of baits for targeted capture of RAD loci in historical samples

The dataset of RAD tags that were identified in the *populations* module as having variable sites meeting the above criteria were used to develop baits to capture these targeted loci in the historical samples. The 140 bp RAD tag sequences were 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, 4x bait coverage per locus). Baits were BLASTed (Altschul et al. 1990) against the *Chrysemys picta* genome (Shaffer et al. 2013) to estimate hybridization melting temperature and bait specificity. The *Chrysemys picta* genome was the closest complete genome available at the time of bait generation, but is in a different family than *C. ephippium*; thus we used relatively relaxed stringency when assessing hybridization specificity. Baits passed filtering if they were expected to have at most 10 hits between 62.5–65 °C and four hits above 65 °C, and fewer than two passing baits on each flank.

#### 4.2.2.4 Design of baits for targeted capture of the mitochondrial genome

An existing 15,648 bp sequence of a draft mitochondrial genome for *C. ephippium* (Caccone, unpublished data) was used to design capture baits using the same procedure as above.

#### 4.2.2.5 Historical sample extraction, library preparation and targeted capture

DNA was extracted from the wedge cuts of bone in a dedicated ancient DNA lab at The University of British Columbia Okanagan, Kelowna BC, using a modified version of extraction protocol Y described by Gamba et al. (2015). Samples were ground while submerged in liquid nitrogen using a Spex 6770 freezer mill (5 minute precooling, 1 minute of grinding at 10x per second). Samples were demineralized in a solution of 3 mL 0.5M EDTA pH 8.0, 150 µL 10% SDS and 100 µL of 20mg/ml Proteinase K and incubated overnight at 56 °C. The lysate was concentrated to 250 µL using Amicon Ultra-4 30kDa tubes by centrifugation. The resulting 250 µL of lysate was mixed with 5x volume of buffer PB and added in three steps to a MinElute (Qiagen) column and centrifuged, removing the flow through after each step. The column was washed twice with 750 µL of PE and

centrifuged, allowing desalting for 5 minutes during the first wash. The elution was performed using 50 μL of ultra-pure water preheated to 56 °C.

The historical DNA samples were sent to MYcroarray (Ann Arbor, MI) to construct the libraries and perform the captures. Each sample was uniquely barcoded using dual indexes as part of a blunt-end library preparation. Individual libraries were pooled in equimolar amounts prior to capture. Whole mitochondrial genome and reduced representation nuclear locus captures were done separately. Captures were performed on pools of four individuals using 0.5x ng of baits, which were then amplified and quantified before being pooled for sequencing.

#### 4.2.2.6 Contemporary sample library preparation and targeted capture

A subset of the contemporary samples, including 43 adults (mean curved carapace length 75 cm), and two wild-born hatchlings, were chosen for mitochondrial genome capture. MYcroarray performed the library preparation and captures. Contemporary DNA samples were sheared via sonication and prepared into dual-indexed sequencing libraries using standard protocols. Individual libraries were pooled in equimolar amounts into groups of eight prior to capture using 0.65x ng of baits. The captures of nuclear loci from the historical individuals were pooled with the mitochondrial captures for both the historical and contemporary and sequenced in a single, partial lane on an Illumina HiSeq 2500 platform.

### 4.2.3 Captured sequence data processing and assembly

#### 4.2.3.1 Reference sequences

The reference "genome" used for the nuclear captures consisted of the 140 bp long target sequences as well as 100 bp of flanking sequence on either end pulled from a draft genome of *C. abingdonii* (Caccone, unpublished data). These 340 bp long sequences in fasta format were indexed using BWA (version 0.7.11) *index* (Li and Durbin 2009) and SAMTOOLS (version 1.1) *faidx* (Li et al. 2009). For the mitochondrial genome, we used captured sequences from three contemporary individuals (tortoise ID #'s: A093, C031, G100) in separate *de novo* assemblies in GENEIOUS 8.1.6 (Kearse et al. 2012) to produce a reference. This *de novo* reference was required as initial assemblies with the original reference had poor mapping scores due to multiple insertions/deletions. The *de novo* mitochondrial reference fasta file was also indexed using BWA *index* (Li and Durbin 2009) and SAMTOOLS *faidx* (Li et al. 2009).

#### 4.2.3.2 Sequence processing

Sequences were processed using the BAM pipeline in PALEOMIX (version 1.2.6, Schubert et al. 2014), which employs other, standard bioinformatics tools alongside native scripts to support the pipeline. Briefly, the demultiplexed fastq files from the sequencing provider were trimmed of adapter sequences and low quality/ambiguous bases using ADAPTERREMOVAL (version 2.1.7, Lindgreen 2012). As part of the trimming process, overlapping paired-end reads were merged. In the mapping stage, unmerged reads were excluded for historical individuals, since the insert size for endogenous historical DNA is expected to be short enough for merging (<289 bp), as recommended for historical samples in the PALEOMIX documentation. For the contemporary sequences, all reads that passed filtering were retained. Processed reads were mapped to the reference sequences 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 that is part of the PALEOMIX pipeline. For the historical sequences, MAPDAMAGE2.0 (Jonsson et al. 2013) was used to rescale the quality scores of bases that were potentially the result of post-mortem DNA damage. The alignments of BAM files were further improved using GATK *IndelRealigner* (McKenna et al. 2010).

To allow the nuclear sequences to be compared between the historical captures and the contemporary RAD data, the fastq files retained following the *clone_filter* step in the STACKS workflow were also run through PALEOMIX, using the same procedure as above excluding the DNA damage correction, starting at the mapping stage.

Genotype calling for the nuclear and mitochondrial assemblies was performed on the combined BAM files generated from the historical and contemporary populations, and the contemporary nuclear BAM files alone using SAMTOOLS *mpileup* (with the settings -gd -q 15 -t DP) and BCFTOOLS *call* (using -mv for the nuclear assemblies and -c for the mitochondrial assemblies; Li et al. 2009) and exported in vcf format. Filtering of the vcf files was done using VCFTOOLS (Danecek et al. 2011). For the mitochondrial sequences, filtering consisted of requiring a minimum read depth of 6x. For the nuclear SNPs, some historical individuals had very little data (fewer than 100,000 reads mapped), and were removed from subsequent analyses. The nuclear loci were filtered as follows: a minimum read depth of 6x was required, the locus had to be present in at least 50% of the retained historical individuals and have a minor allele frequency of at least 0.05 across the total

sample. All loci with a mean depth of coverage greater than two standard deviations above the mean depth were removed. Loci were assessed for departure from Hardy-Weinberg equilibrium (HWE), with significance assessed using the correction for false discovery rate described by Benjamini and Yekutieli (2001). HWE tests were performed on the historical and contemporary samples separately, and loci that showed significant deviation in either sample were removed. Finally, we thinned the loci, only retaining the first SNP in each 140 bp RAD tag.

A separate round of SNP variant detection was done using just the contemporary samples starting from the SAMTOOLS *mpileup* step. This procedure produced a larger SNP dataset than was possible from the combined data and could be used to assess whether the subset of SNP loci genotyped in both samples provide the same signal as a larger pool of loci. Filtering was the same as above (minimum depth of 6, mean depth within two standard deviations of the mean, minor allele frequency of at least 0.05, meeting HWE expectations, selecting the first SNP per RAD tag), except that loci had to be present in 50% of contemporary individuals.

We assessed genotyping error of the nuclear SNP loci by calculating the number of genotype mismatches between two pairs of replicate contemporary individuals that had been processed separately from DNA extraction onwards.

To assess differences in allelic variation in the temporal samples, we evaluated the number of variable sites unique either to the contemporary or historical populations, by running a

separate round of filtering, starting with a vcf file containing all the variable sites genotyped in at least 50% of the historical and at least 50% of the sample overall, and not in the flanking region. Then, assessing the historical and contemporary samples separately, we filtered for maximum mean depth, minor allele frequency of at least 0.025, retaining only RAD tags with four or fewer variable sites, and no significant departure from HWE. The resulting vcf file produced the total number of variable sites that met filtering criteria in the focal sample. We then assessed those loci for variation in the other sample.

### 4.2.4 Population genetic analyses: Mitochondrial DNA genomes

Mitochondrial sequences were sorted into haplotypes, aligned, and a haplotype network was constructed using statistical parsimony (95% confidence criterion, gaps treated as a fifth state), implemented in TCS V1.21 (Clement et al. 2000). The haplotype network was imported into TCSBU for reformatting (Murias dos Santos et al. 2016). Molecular diversity indices (number of polymorphic sites, nucleotide diversity), population differentiation between the temporal samples measured by $F_{ST}$, and descriptors of population size changes (Tajima's (1989b) $D$, Fu's (1997) $F_S$) and mismatch distributions (Rogers and Harpending 1992), including the estimation of the raggedness index, $r$, were calculated in ARLEQUIN V3.5 (Excoffier et al. 2005). To provide a point of comparison with previous Galápagos giant tortoise studies that have used a 795 bp region of the mitochondrial d-loop, haplotypes were trimmed to match this segment, and diversity indices were re-calculated using the same approaches as above.

To evaluate changes in effective population size over time, we employed Bayesian skyline analysis (Drummond et al. 2005) as implemented in BEAST2 (Bouckaert et al. 2014). We

ran the analysis on the historical and contemporary sequences separately. The mitochondrial genome sequence was first annotated using MITOS v1 (Bernt et al. 2013) to determine protein coding and non-coding (i.e. rRNA and tRNA) partitions. A separate partition for the d-loop was identified by aligning sequences from previous studies to the full mitochondrial genomes obtained here, in SEQUENCHER 5.0.1 (Gene Codes Corporation). For each of the three partitions, the best fitting substitution model (HKY+I for coding, HKY+G for non-coding, TN93 for d-loop) was identified from among 88 possibilities using the Akaike information criterion (AIC) as implemented in JMODELTEST (Posada 2008). We used a previously calculated substitution rate for the d-loop partition of $8.5 \times 10^{-7}$ substitutions per lineage per generation, assuming a generation time of 25 years (Beheregaray et al. 2004). Preliminary runs were used to estimate the clock rates for the other partitions, setting an upper bound to 1.0. The mean rates estimated for each dataset were then fixed in subsequent runs and applied to both datasets to allow direct comparisons of the Bayesian skyline plots (historical dataset estimates of clock rates were $2.15 \times 10^{-7}$ for coding regions, $6.91 \times 10^{-8}$ for non-coding regions; contemporary dataset estimates were $3.79 \times 10^{-7}$ for the coding, $3.59 \times 10^{-7}$ for the non-coding). The substitution models and clock rates were unlinked among partitions, while the trees were linked. Searches used the coalescent Bayesian Skyline prior and a random starting tree, with other priors set to default. Final searches were $4.0 \times 10^{7}$ Markov Chain Monte Carlo (MCMC) generations long, sampling parameters every 5000 steps and discarding the first 10% as burn-in. Convergence of three independent chains was assessed via effective sample size values and Bayesian skyline analyses as implemented in TRACER v1.6 (Rambaut et al. 2014).

### 4.2.5 Population genetic analyses: Nuclear SNPs

#### 4.2.5.1 Within sample diversity

We used the genotypic data to calculate standard measures of within-population genetic diversity, including heterozygosity using GENODIVE V2.0b27 (Meirmans and Van Tienderen 2004). Individual inbreeding coefficients were calculated using VCFTOOLS (Danecek et al. 2011). Pairwise relatedness (Queller and Goodnight 1989) was calculated within the historical and contemporary samples separately using the *Related* package (Pew et al. 2015) in R. Effective population sizes for the historical and contemporary samples were calculated using the bias-corrected version of the linkage-disequilibrium method (Hill 1981, Waples 2006, Waples and Do 2010), as implemented in NeESTIMATOR V2 (Do et al. 2013) using an allele frequency cut-off of 0.05. Heterozygosity, the inbreeding coefficient, pairwise relatedness and effective population size were also calculated using the larger SNP dataset for the contemporary population to allow us to assess the impact of the number of loci on the results.

#### 4.2.5.2 Analyses between temporal samples

The presence of substructure within the combined sample (historical and contemporary) was assessed using Bayesian clustering analysis, as implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). Run length was set to 300,000 MCMC replicates after a burn-in period of 100,000 using correlated allele frequencies under a straight admixture model. We varied the number of clusters ($K$) assumed from one to five, with five iterations of each. The most likely number of clusters was determined by plotting the log probability of the data (ln $\Pr(X|K)$) across the range of $K$ values tested and selecting the $K$ where the value of ln

Pr($X|K$) plateaued, as suggested in the STRUCTURE manual, and using the delta $K$ statistic (Evanno et al. 2005), as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2011). We also used the model-free discriminant analysis of principle components (DAPC, Jombart et al. 2010), as implemented in *Adegenet* (Jombart 2008) in the R statistical package (http://www.R-project.org/). The number of clusters within the data set ($K$) was selected using the *find.clusters* function and Bayesian information criterion (BIC). The chosen value of $K$ was based on the minimum number of clusters after which the BIC decreased by a negligible amount.

To assess whether there are differences in the genetic diversity captured in the head-start program over time, we used size as a proxy for age and pulled out two groups of individuals from the contemporary dataset and repeated a subset of the above analyses. The groups were "adult" individuals with a curved carapace length >65cm (n=83) and "young" individuals that are <35cm and >15 cm (n=29). Individuals <15cm were excluded because they are wild-born hatchlings that were not part of the head-start program. These groups are somewhat arbitrary, but were chosen to represent non-overlapping groups of individuals that were head-started in the early years of the program ("adults"), or very recently ("young"), to allow comparisons of levels of diversity within each time point, and patterns of pairwise relatedness within and among the groups.

The $Phi_{ST}$ metric of population differentiation was calculated between samples in GENODIVE with significance assessed using 999 permutations. Exact tests for differences in allele frequencies between the historical and contemporary samples, and between the

historical sample and subsamples of the contemporary population were performed in GENEPOP v4.5 (Raymond and Rousset 1995), with significance assessed using an adjusted p-value based on the correction for false discovery rate described by Benjamini and Yekutieli (2001).

To evaluate the possibility of spatial patterns within the contemporary sample, we compared the straight line geographic distance between individuals at the time of sampling, calculated using the GEOGRAPHIC DISTANCE MATRIX GENERATOR (Ersts 2012), and their pairwise relatedness. Only individuals with a curved carapace length >50 cm (i.e. mature adults, n=99) were used in this analysis to exclude recently repatriated cohorts that have not yet had time to disperse away from the release site. No spatial data are available for the historical samples, precluding a similar analysis with that population.

## 4.3 Results

### 4.3.1 Sequencing, bait design and capture

Sequencing of the initial RAD libraries generated from the contemporary population produced a total of 706 million 150 bp, paired-end reads for the 150 unique individuals plus two replicate samples. The mean number of retained forward reads per individual after the *clone_filter* step was 1.7 million. The final catalog consisted of 194,683 RAD tags, of which 9890 were variable and met filtering criteria. These 9890 RAD tag sequences were used for initial capture bait design, with 8,918 passing filtering and becoming part of the final set of capture targets.

The *de novo* assemblies of the mitochondrial genome using three contemporary individuals each produced a single long contig that aligned together with few discrepancies, resulting in a 16,042 bp long mitochondrial genome. This sequence did not circularize, indicating that it is not quite complete.

The mean read length for the historical libraries was 99 bp. For the historical samples, a mean of 128,000 reads mapped to the mitochondrial references after removing PCR duplicates, while for the contemporary samples the mean was 7,300. For the nuclear captures, there was substantial variation in the number of reads for the historical individuals, resulting in 39 of 78 individuals being retained after removing PCR duplicates, averaging 205,000 mapped reads.

### 4.3.2 Mitochondrial genome analyses

The nearly complete mitochondrial genome (16,042 bp) was recovered for all 45 contemporary and 77 of the 78 historical individuals. Mean depth was 67 for the contemporary and 188 for the historical samples (Appendix B) and missing data were low (2.7% for contemporary, 1.8% for historical). There were 76 variable sites and no gaps, resulting in 48 haplotypes, 35 in the historical and 22 in the contemporary population, with only nine shared between the temporal samples (Figure 4.1). Individuals that differed only at sites with missing data were conservatively grouped into the same haplotype. Seven contemporary and ten historical individuals shared the most common haplotype. The haplotype network has some reticulation, and shows 32 inferred haplotypes connecting the sampled haplotypes. Haplotype diversity ($H_d$) was nearly equal in the two samples (Table 4.1). The dloop region had 12 haplotypes, all of which were found in the historical sample,

while a subset of five haplotypes was found in the contemporary sample (Table 4.1). Pairwise $F_{ST}$ between the historical and contemporary population was 0.008 (non-significant). For both populations, Tajima's D and Fu's FS were negative, but only the Fu's FS for the historical was marginally significant (Table 4.1). Mismatch distributions were multimodal for both populations (Figure 4.2), with a raggedness index of 0.02 and 0.04 for the historical and contemporary samples, respectively (Table 4.1).

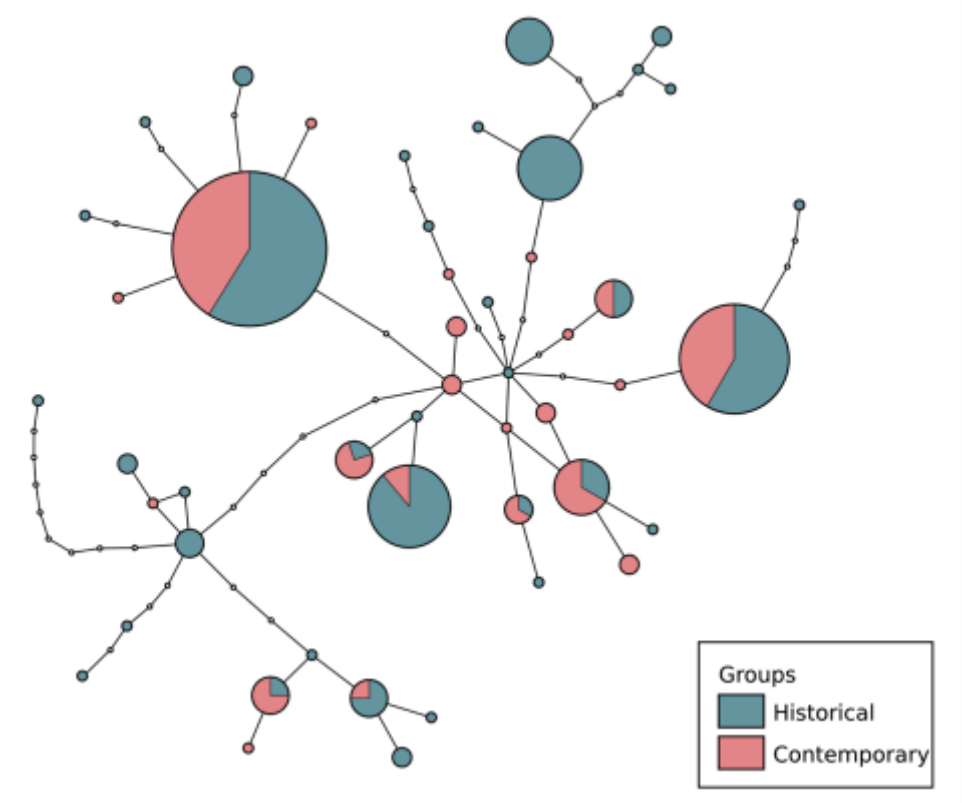


Figure 4.1 Mitochondrial genome haplotype network generated using statistical parsimony. Circles indicate haplotypes in the sample, with the overall frequency of the haplotype indicated by the circles' size and the frequency in each sample indicated as the proportion of the colors. Inferred but unsampled haplotypes are indicated as small open circles in the network.

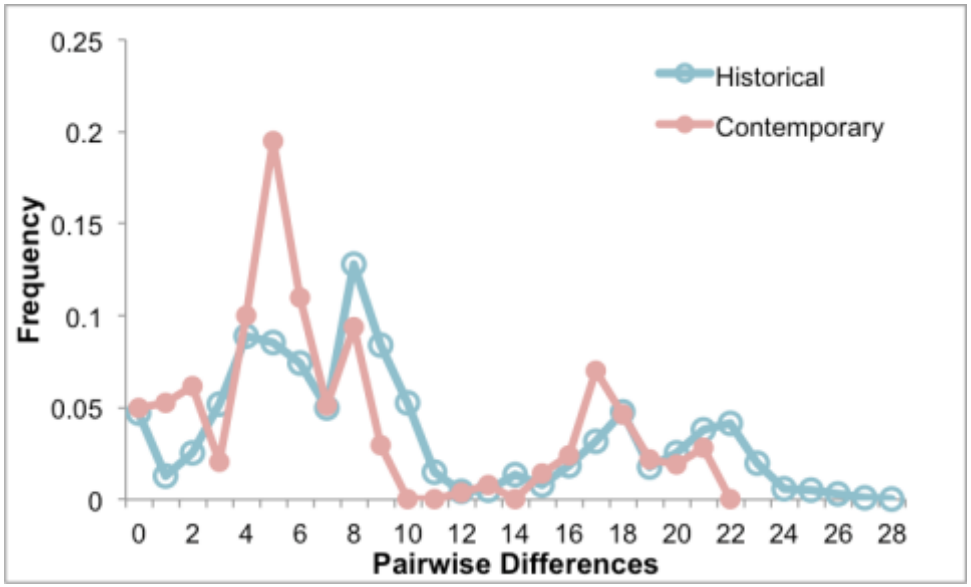


Figure 4.2 Mismatch distributions between mitochondrial genome haplotypes in each population.

Table 4.1 Diversity indices for the full mitochondrial genome and dloop region, and demographic tests calculated from the mitochondrial genome sequences. P-values are indicated in parentheses.

| | **Dloop** | | | | **Full Mitochondrial Genome** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **$N_{hap}$** | **PS** | **$H_d$** | **$\pi$** | **$N_{hap}$** | **PS** | **$H_d$** | **$\pi$** | **Tajima's D** | **Fu's FS** | ***r*** |
| **Historical** | 12 | 16 | 0.82 | 0.0036 | 35 | 70 | 0.953 | 0.0006 | -1.051 (0.158) | -8.096 (0.042) | 0.017 (0.048) |
| **Contemporary** | 5 | 8 | 0.72 | 0.0024 | 22 | 44 | 0.951 | 0.0005 | -0.774 (0.269) | -3.986 (0.099) | 0.040 (0.080) |

*$N_{hap}$*, Number of haplotypes detected; *PS*, number of polymorphic sites; *$H_d$* haplotype diversity; $\pi$, nucleotide diversity; *r*, raggedness index.

The Bayesian skyline plots revealed similar trends in demographic history for both the historical and contemporary datasets, with a static population historically that increased in effective population size more recently before sharply decreasing (Figure 4.3). In both cases, the confidence intervals for the more recent time points are large (Appendix B). Overall, the historical dataset reconstructs higher effective population sizes at all time periods, and has a more distant coalescent point. The different clock rates produced the same relative differences between the historical and contemporary populations, but the absolute values for effective population size and coalescent point were shifted.

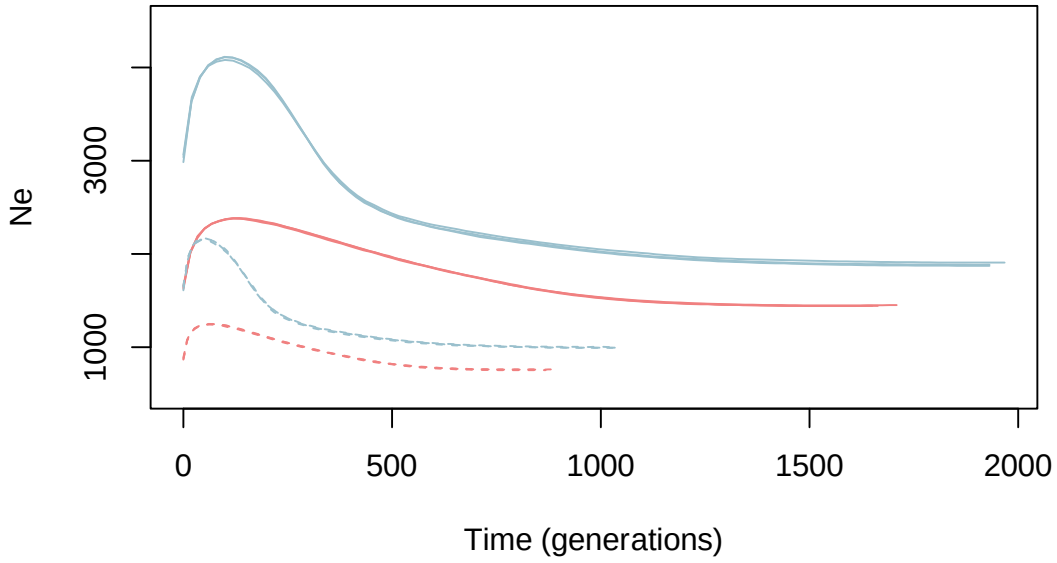


Figure 4.3 Bayesian skyline plots from mitochondrial genome sequences showing the median reconstructions of effective population size (*Ne*) over time, where time is in generations starting from the present (time 0).
Three independent runs of each set of parameters are overlaid. Solid lines are using the clock rates estimated from the historical dataset, dashed lines are from clock rates estimated from the contemporary dataset. Blue is the historical data, pink is the contemporary.

### 4.3.3 Nuclear SNP analyses

The final filtered dataset from the combined variant detection consisted of 2,496 SNP loci (Table 4.2) genotyped in 39 historical and 150 contemporary individuals. Our variant

detection and filtering steps allowed for loci to be fixed in one of the samples; 75 loci were non-polymorphic in the contemporary sample and 67 were non-polymorphic in the historical sample. The mean depth was 14.1 and 15.4, and mean missing data were 10% and 34% for the contemporary and historical individuals, respectively. Genotype completeness was 66% for the historical population and 81% for the contemporary population. Genotyping error rates were 6.3% and 7.2%, as assessed using two pairs of duplicate contemporary individuals. The allele balance (mean proportion of reads supporting each allele call) was equal in both the historical (0.51, SD 0.1) and contemporary data (0.51, SD 0.05). The final filtered dataset for just the contemporary sample consisted of 7,785 SNP loci (Table 4.3) with a mean depth of 13.5, 11% mean missing data and 6.1% and 6.5% genotype error rates between the duplicate individuals.

Table 4.2 Filtering down of SNP loci from combined variant detection.

| **Filtering Criteria** | **# SNPs Retained** |
|---|---|
| Site present in ≥50% of historical individuals | 8826 |
| Filter out loci with mean depth >2x standard deviations above the mean (mean depth 18.5, depth cut off 62.7) | 8498 |
| Minor allele frequency 0.05 | 4846 |
| Meeting HWE expectations in both populations (adjusted p-value 0.0055) | 3448 |
| Require to be genotyped in ≥50% of individuals (to ensure locus is not in flanking region of RAD tag) | 2787 |
| Thin loci to 1 SNP per 140 bp RAD tag | 2496 |

Table 4.3 Filtering down of SNP loci from the contemporary only variant detection.

| **Filtering Criteria** | **# SNPs Retained** |
|---|---|
| Site present in ≥50% of individuals | 11757 |
| Filter out loci with a mean depth of 2x standard deviations above the mean (mean depth 16.3, depth cut off 45.6) | 11413 |
| Minor allele frequency 0.05 | 9488 |
| Meeting HWE expectations (adjusted p-value 0.005) | 8720 |
| Thin loci to 1 SNP per 140 bp RAD tag | 7785 |

The assessment of the total variable sites within each sample after filtering found the historical sample to have more polymorphic sites (2899) than the contemporary sample (2728). There were 368 (12.7%) polymorphic sites in the historical sample that were fixed in the contemporary, and 105 (3.8%) polymorphic sites in the contemporary that were fixed in the historical. The frequency of the allele present in one sample, but absent in the other, was generally low, averaging 0.13 and 0.12 in the historical and contemporary, respectively (Appendix B). In a small proportion of cases (3% in the historical, and 7% in the contemporary), it was the minor allele that was fixed in the other sample (Appendix B)

#### 4.3.3.1 Within temporal samples

Levels of heterozygosity and inbreeding were very similar between the temporal samples, as was the mean relatedness (Table 4.4). The distributions of pairwise relatedness values within each sample were closely matched (Figure 4.4A), with similar distributions also found in comparing the relatedness among and between the adult and young groups in the contemporary population (Figure 4.4B). The estimated effective population size for the historical population was an order of magnitude larger than the contemporary population (867.6 and 59.6 respectively, Table 4.4). Diversity statistics were very similar for the contemporary population when calculated using 2,496 or 7,785 loci (Table 4.4).

Table 4.4 Within-population diversity metrics for the historical, contemporary, and groups within contemporary populations.

| | Sample | N | Hobs | F | Mean $R_{Q\&G}$ | $N_e$ (95% CI) |
|---|---|---|---|---|---|---|
| **2,496 loci** | Historical | 39 | 0.300 | -0.029 | -0.025 | 867.6 (678.5, 1201.1) |
| | Contemporary | 150 | 0.312 | -0.059 | -0.009 | 59.6 (59.3, 59.8) |
| | Adult | 83 | 0.304 | -0.033 | 0.004 | -- |
| | Young | 29 | 0.327 | -0.111 | 0.003 | -- |
| **7,785 loci** | Contemporary | 150 | 0.322 | -0.057 | -0.009 | 60.7 (60.6, 60.8) |

*N*, sample size; Hobs, observed heterozygosity; *F*, inbreeding coefficient; $R_{Q\&G}$ Queller and Goodnight relatedness; $N_e$, effective population size.

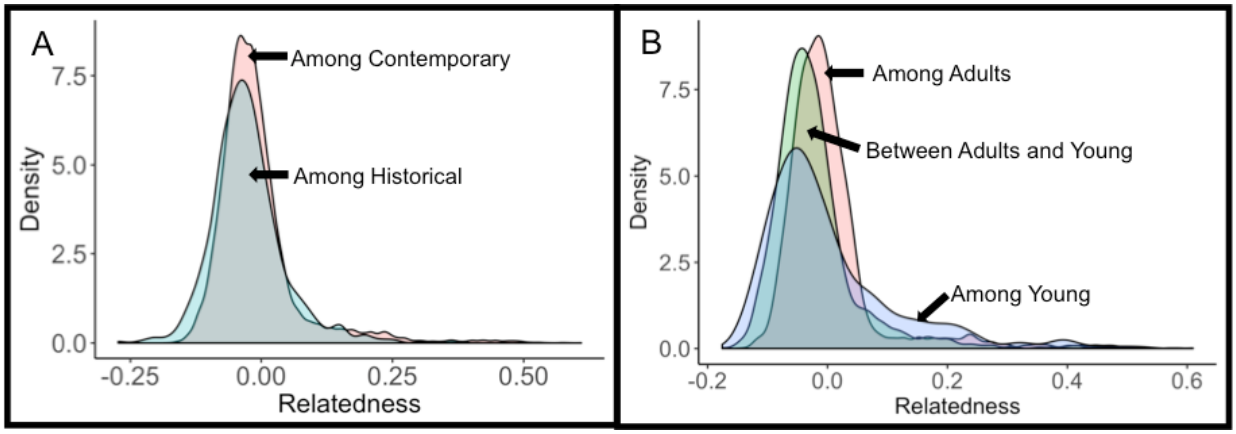


Figure 4.4 Frequency distribution of pairwise relatedness (Queller and Goodnight 1989) between individuals within A. the historical and contemporary samples; and B. between and among the adult and young groups of contemporary individuals.

#### 4.3.3.2 Between temporal samples

Both STRUCTURE and DAPC separated the historical and contemporary individuals into two populations (Figure 4.5, Appendix B), with no substructure within either detected when further evaluating the historical and contemporary populations alone (data not shown). Metrics of population differentiation were low, with slightly higher values between the historical and young contemporary individuals than between the historical and adult contemporary (Table 4.5). There were significant differences in allele frequencies between the samples, again with a pattern of more differences between the historical and young contemporary than the historical and adult contemporary (Table 4.5).

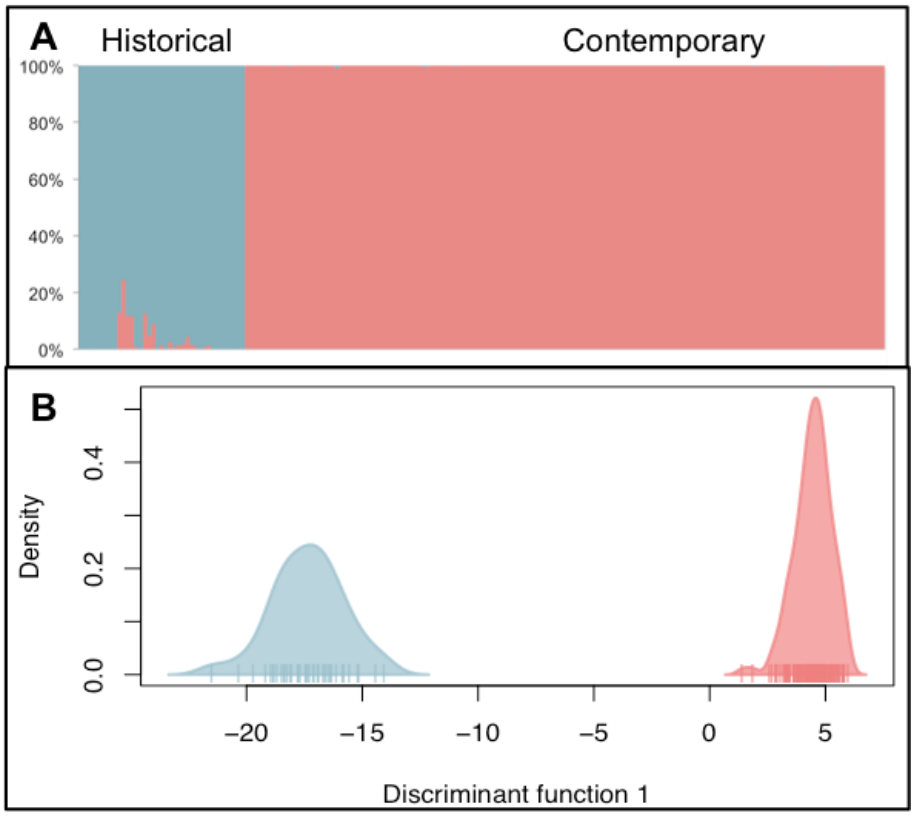


Figure 4.5 Population substructure of the historical and contemporary samples.
A. STRUCTURE bar plot, averaged over 5 iterations, depicting K=2. Each color represents an inferred genetic cluster; each bar on the x-axis represents and individual with the y-axis displaying the proportion of membership in each genetic cluster. B. DAPC plot showing separation between the two groups from the *find.clusters* analysis that corresponds to the historical and contemporary populations. Blue is historical, pink is contemporary.

Table 4.5 Measures of population differentiation between the historical and contemporary population and groups within the contemporary population.

| | **DAF** | ***Phi*$_{ST}$** |
|---|---|---|
| **Historical and Contemporary** | 0.062 | 0.053* |
| **Historical and Adult** | 0.059 | 0.053* |
| **Historical and Young** | 0.068 | 0.063* |
| **Adult and Young** | 0.022 | 0.010* |

*DAF*, the proportion of loci with significantly different allele frequencies (adjusted p-value 0.0059), * denoting significance at p<0.0001 for the *Phi*$_{ST}$.

Analysis of relatedness by distance among the contemporary individuals greater than 50 cm indicates that there are no spatial patterns of related individuals being clustered together on the island (Figure 4.6).

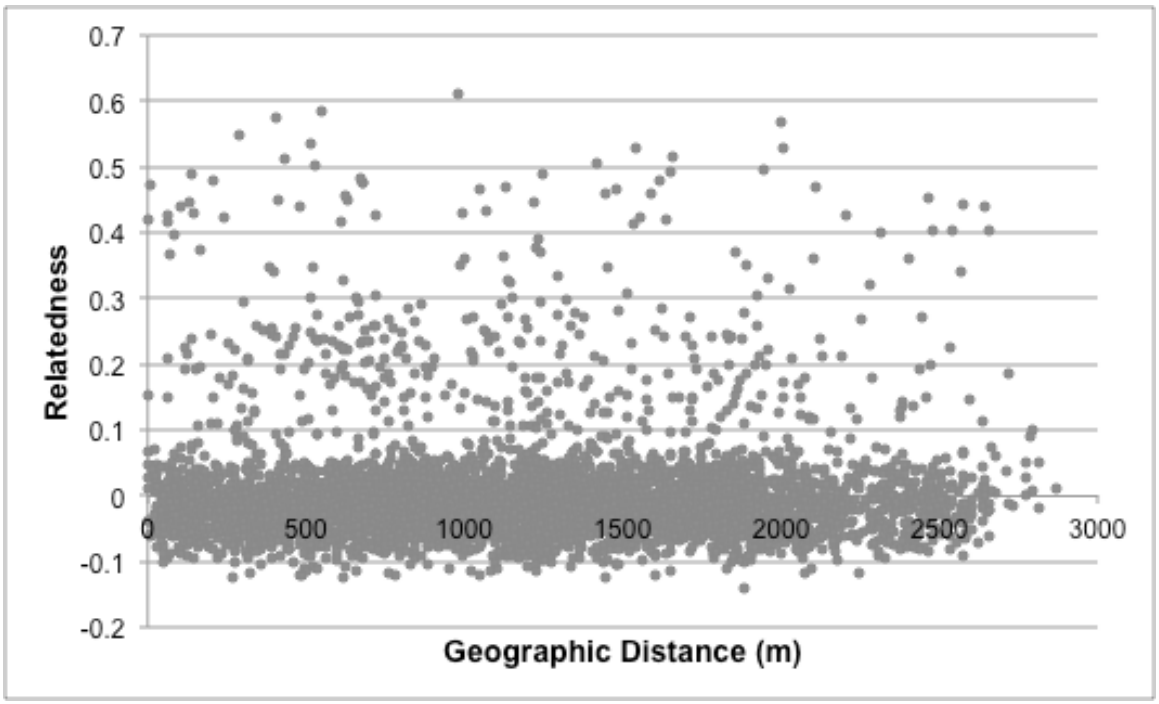


Figure 4.6 Pairwise relatedness by distance among contemporary individuals with a curved carapace length >50cm.
Relatedness (Queller and Goodnight 1989) was calculated from the 2,496 SNP loci.

## 4.4 Discussion

Much attention has been paid towards documenting the genetic consequences of population decline (Frankham 2005, Bouzat 2010), yet rarely have there been opportunities to test theoretical population genetic predictions using an equivalent sampling of the pre-bottlenecked populations. In the case of Pinzón tortoises, the harvesting of so many individuals in the early 20$^{th}$ century for museum collections, despite potential impacts to population status at the time, has provided an unprecedented sample for directly assessing

genetic patterns associated with population decline and recovery. Here, the extent and distribution of genetic variation recovered in the historical and contemporary populations closely follow the expectations for a recent bottleneck with respect to the loss of allelic variation, maintenance of heterozygosity, decline in effective population size, and increase in structure over time. Moreover, the novel ability to reconstruct demographic history using a pre-bottleneck population sampling suggests that some estimates that rely (necessarily) on post-bottlenecked samplings may be downwardly biased. Lastly, this study also constitutes the first use of historical specimens to assess the genetic impact of head-starting as an active management strategy, providing important insights into the effectiveness of this strategy as a conservation intervention.

### 4.4.1 Pre- and post-bottleneck variation

The changes in genetic diversity metrics observed between the 1906 and 2014 populations closely match the four theoretical expectations for a bottleneck we set out to test. Between the temporal samples, allelic variation was lost, particularly at sites with a low minor allele frequency (Appendix B), as indicated by the large number of variable sites identified in the historical population that are fixed in the contemporary population. Likewise, the number of haplotypes and polymorphic sites across the mtDNA genome was significantly lower in the contemporary population (Table 4.1). Heterozygosity was constant across the samples, and even slightly elevated in the contemporary young tortoises (Table 4.4), which is consistent with predictions of a transient, slight increase in heterozygosity in generations immediately following a bottleneck (Nei et al. 1975, Leberg 1992, Cornuet and Luikart 1996). The estimated effective population size from the SNP data is reduced by an order of magnitude lower in the contemporary population compared to the historical population (Table 4.4).

Temporal genetic structure has also developed as a likely result of the significant changes in allele frequencies, as indicated by the small, but significant, $Phi_{ST}$ values and the STRUCTURE and DAPC analyses that separated the historical and contemporary samples into two populations (Figure 4.5). Importantly, the absence of substructure within either sample suggests panmixia on the island during each time point.

### 4.4.2 Demographic history

Understanding long-term demographic history can provide important insights into contemporary bottlenecked populations. Here, we used full mitochondrial genome sequences to gain insights into demographic history using several approaches: mismatch distributions, neutrality metrics, and Bayesian skyline plot analysis. Mismatch distributions and the neutrality metrics provide insights into whether a population is stable or increasing in size. For the historical sample, most of the analyses indicate a stable population size (multimodal mismatch distribution, significant raggedness, insignificant Tajima's D), but the negative and significant Fu's FS suggests expansion. For the contemporary population, all indications are of a stable population size, except for the mismatch distribution, which, although qualitatively multimodal, does not have significant raggedness. Thus, the impression overall is of a stable population size, but expansion cannot be ruled out.

The Bayesian skyline plots from the historical and contemporary datasets reconstruct the same overall demographic history: a historically stable population size that expanded and recently declined rapidly. Based on the median reconstructions, the timing of the expansion is relatively more recent in the Bayesian skyline plot from the historical data, which also has a higher effective population size than the contemporary when applying the same clock rates

(Figure 4.3). Each of the Bayesian skyline plot trajectories begin in the past with the estimated most recent common ancestor. This coalescence point appears to be more recent for the contemporary dataset than the historical, which can be attributed to the difference in the maximum number of pairwise differences between sequences in each sample (22 for contemporary, 28 for historical). The higher number of pairwise differences between samples in the historical population could be due to the greater sample size, as there is equal haplotype diversity in the contemporary population for the mitochondrial genome.

Despite these findings, the broad confidence intervals (Appendix B) associated with the Bayesian skyline plots make interpretation of any recent changes in effective population size tentative. Moreover, caution must be exercised given that not all clock rates were independently derived. Here we did have the advantage of an independently estimated mutation rate for the dloop (Beheregaray et al. 2004) that we fixed for that partition while estimating the clock rate for the other two (coding and non-coding). Interestingly, the clock rates estimated for each partition are different for the historical and contemporary datasets, with the historical being slower than the contemporary, and the non-coding partition being slower than the coding (although only slightly for the contemporary). Importantly, applying the clock rates estimated from one dataset to the other did not impact the relative reconstructions of demographic history, but the slower rates did increase the estimated effective population sizes in absolute terms. This latter result suggests that relying on estimates from post-bottlenecked populations may downwardly bias estimates of historical effective population size (also found in Pacioni et al. 2015), although additional studies employing simulation would be required to further test this hypothesis.

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 only recently become rare, as opposed to having existed as a small population historically. Garrick et al. (2015) classified Pinzón tortoises as being "newly rare", while many of the other species of Galápagos tortoise were found to be "naturally rare". The extended Bayesian skyline plot in Garrick et al. (2015) showed the Pinzón tortoises to have a recent effective population size of over 15,000. As we have used mitochondrial genome sequence for our Bayesian skyline plot analyses, which has ¼ the effective population size of the nuclear genome, the estimate from the historical dataset using the rates estimated from the historical data is comparable to that previous estimate (maximum point on the historical data, historical rates plot is ~4,110, Figure 4).

The estimates of effective population size calculated from the SNP data are not directly comparable to the values from the Bayesian skyline plot analyses due to the different quantities that are being measured. The linkage disequilibrium method of effective population size estimation used with the SNP data takes advantage of the fact that genetic drift generates associations between alleles at different loci at a rate inversely proportional to the effective population size (Hill 1981). Thus, calculating the linkage disequilibrium between pairs of alleles and loci and assuming a recombination rate of 0.5, the effective population size of the parental generation can be estimated. Skyline plot analyses are a completely different approach, where the genealogy among haplotype sequences is estimated, and effective population size at each coalescence event is estimated according to a relationship with the time interval between coalescences and the number of lineages at the

beginning of each interval (Strimmer and Pybus 2001). Bayesian skyline plots (Drummond et al. 2005) extend this method to generate confidence intervals for the estimates of effective population size that represent both phylogenetic and coalescent uncertainty. Thus, while it is not possible to directly compare the effective population sizes from the Bayesian skyline plot at time zero to the point estimates of effective population size from the SNP data, there are complementary indications of a high effective population size historically followed by recent decline.

### 4.4.3 Genetic legacy of the head-start program

The demographic recovery of the Pinzón tortoise was achieved through active management in the form of a head-start program, which carried the possibility of further skewing genetic contributions to subsequent generations due to unequal representation of the surviving individuals. Even if offspring of each of the survivors are represented in the head-start program, genetic diversity could have become skewed due to the overrepresentation of certain families in the head-start generations. Both of these concerns were relevant in this case due to differences across the decades regarding how thoroughly each nesting zone was searched when collecting eggs and hatchlings. To test this, we calculated pairwise relatedness in the historical and contemporary populations and compared the distributions of relatedness between the samples. We found that the distribution of pairwise relatedness over the whole contemporary population matches the distribution for the natural population in 1906 (Figure 4.4A), indicating that the head-start program collected eggs in a way that was not biased towards certain families. This finding applied to the contemporary population as a whole. To test whether there was consistency between the early years of the head-start program and more recent years, we used the “adult” and “young” subsets of the

contemporary population, and compared patterns of relatedness within and among those subsets. The similar distributions of relatedness within and among the young and adult groups (Figure 4.4B) indicate that over the 50-years of the head-start program, genetic diversity seems to have been captured consistently.

The finding of low mean relatedness in the contemporary population is consistent with the low incidence of large full-sibling families reconstructed in the 2007 and 2009 cohorts in Chapter 3. The number of nests located and number of individuals in each cohort varied widely over the years, with more than 50 nests located some years, and fewer than 10 in others (Pritchard 1996), so there was real concern that some age classes would be less diverse. There is no indication of inbreeding in the contemporary population as a whole, or in the adult and young groups, and the rapid recovery in population size achieved through the head-start program suggests that inbreeding is unlikely to be a concern in the future. Overall, the head-start program was successful in preserving as much genetic diversity as possible, given the initial bottleneck from over-exploitation in previous centuries.

### 4.4.4 Insights from temporal and genome-wide sampling

In this study, we had the benefit of the historical sample to provide context to the patterns of diversity observed in the contemporary population. Pre-bottleneck, population-level samplings are not available for the vast majority of populations, and so indirect estimates of the severity and genetic impacts of bottlenecks must be relied upon. In some cases, the bottlenecked population may be compared to a stable population of the same species (Whitehouse and Harley 2001), or even to a related species (Akst et al. 2002, Waldick et al. 2002) to indirectly assess the genetic impacts of population decline. Previous archipelago-

wide studies of Galápagos tortoise have taken this approach and compared levels of variation in each species to gain insights into population history (Ciofi et al. 2002, Beheregaray et al. 2003a, Garrick et al. 2015). However, the baseline provided by comparing Pinzón tortoises to the other Galápagos tortoise likely has underestimated the degree to which variation was lost in this species, given that *C. ephippium* maintains higher levels of genetic variation than most of the other extant species despite having gone through a substantial bottleneck. It has only been through direct comparisons with the pre-bottlenecked population sample that it has become apparent that even greater levels of diversity once existed. The historical sample also provided an important point of reference from which to evaluate the impacts of the head-start program on genetic diversity, particularly the degree to which patterns of relatedness were altered (or maintained) by non-targeted head-starting.

The discrepancy in relative haplotype diversities between the dloop and full mitochondrial genome has not been detected before. Although the sequencing and reporting of full mitochondrial genomes is becoming more commonplace (Smith 2016), the collection of population level datasets is still rare, let alone from temporal populations, and other comparable cases are lacking. The dloop haplotype diversity for the contemporary population in this study is similar to what was previously reported from a random sample of individuals (0.76, Beheregaray et al. 2003a). These patterns in the dloop are consistent with expectations, given that haplotypes detected in 2014 are a subset of the haplotypes from 1906. Conversely, when evaluating the mitochondrial genome as a whole, there are 13 haplotypes found only in the 2014.. The latter pattern is likely due to undersampling of diversity in the historical population, an outcome that is not apparent when just considering the dloop. Simulations

have shown that undersampling can result in failure to detect, or underestimation of the magnitude of population expansions in Bayesian skyline plot analysis (Grant 2015). The findings from mismatch distributions are typically less impacted by undersampling, since small sample sizes are still likely to represent deep divergences (Felsenstein 2006). We have taken a restrained approach when interpreting the findings of the Bayesian skyline plots in this study, recognizing that mitochondrial diversity is likely underestimated in our sample.

### 4.4.5 Technical aspects

This study provides several useful insights into the technological considerations of a temporal population genetic study. We chose to use RAD sequencing of the contemporary population to discover variable regions of the genome that could be targeted using hybridization baits in the historical samples. This procedure may have introduced some ascertainment bias, in that only RAD tags known to have variable sites were targeted. Our filtering of variable sites was designed to minimize this potential bias by selecting the first occurring SNP in the RAD tag, which was not necessarily the SNP identified in the initial analysis, perhaps located further along the initial 140 bp read. Since there are 75 loci retained in the final dataset that are not polymorphic in the contemporary population, this approach seems to have been successful. Additionally, in the dataset used for temporal analyses, we applied the minor allele frequency filter to the combined sample, to ensure that the loci retained were informative in both populations.

When designing this study, no closely related genome was available to assist with capture bait design or act as a reference during assembly. The original plan had been to use the 140 bp long target sequences as the reference to map reads to. This strategy was suboptimal,

however, since reads from the historical individuals that only partially overlapped with the target sequence failed to map, resulting in an uneven distribution of coverage that was higher towards the middle of the target and almost zero at the ends. A draft genome of the congener *Chelonoidis abingdonii* became available during the course of our analyses, which allowed us to pull out 100 bp of flanking sequence on either side of the 140 bp targets. Without the flanking regions provided by this genomic resource, we would only have had sufficient read depth to genotype variable sites located in the center of the target region, which would have substantially reduced size of our dataset to ~600 SNP loci (data not shown).

During the filtering process for the SNP dataset, a trade-off had to be made between the number of historical individuals retained and the number of SNP loci that met filtering criteria. The final dataset consisting of 39 historical individuals genotyped at 2,496 SNP loci was still a remarkable outcome that demonstrates the possibilities afforded by targeted capture. The successful sequencing of the full mitochondrial genome in all but one of the historical individuals can be attributed to the higher copy number of organellar DNA, and indicates that the DNA extraction and library preparation was successful for the 77 individuals. If time and funding had permitted, a second round of capture with the nuclear baits to further enrich the libraries and additional sequencing would likely have increased both the number of SNP loci and historical individuals retained in the nuclear dataset. However, the larger number of loci genotyped in the contemporary individuals provided almost exactly the same diversity estimates as the subset of 2,496 loci, lending confidence that our findings would remain unchanged with additional loci. The mean depth for the

historical and contemporary SNP datasets was about equal, with genotyping error rates similar to what would be expected based on the coverage (Fountain et al. 2016).

We were able to directly assess genotyping error rates using two pairs of contemporary samples that had been run in duplicate. Duplicate historical individuals were not included in the study design, so a similar calculation specific to that dataset is not possible. Errors in genotypes can be introduced during data collection steps, including PCR errors during amplification, sequencing errors, or in the case of historical samples, post-mortem DNA degradation. Likewise, genotyping errors can arise during data processing, including alignment errors and filtering steps (e.g. read depths contributing to genotype calling). We expect the levels of genotyping error due to sequencing errors and data processing to be similar between the historical and contemporary datasets, as the sequencing was run using the same chemistry and model of sequencer, and the same data quality filters were used. Post-mortem DNA degradation was accounted for during the data processing by using MAPDAMAGE (Jonsson et al. 2013) to rescale nucleotide quality scores that were suspected to be impacted by DNA degradation. Thus, we expect the genotyping error rates calculated from the contemporary samples to be reasonably representative of error rates in the historical samples. Directly assessing genotyping error rates though duplicate samples would be preferable, and should be factored into the design of future studies.

The historical individuals had lower genotype completion (66%) than the contemporary individuals (81%), but these values are similar to the completeness reported in other studies using historical samples (Shultz et al. 2016). Furthermore, a recent sensitivity analysis found

that missing data levels of even up to 50% did not impact summary statistics (Shafer et al. 2016). Preliminary tests of our dataset retaining only individuals with more complete data also did not change the results (data not shown).

### 4.4.6 Summary

The rapid decline of the Pinzón tortoises culminated in a bottleneck of 150-200 aging adults before the head-start program was initiated and population recovery began. By comparing the 1906 and 2014 populations, we have directly assessed the impacts of the bottleneck, and found patterns consistent with theoretical expectations. Given the quick restoration to a large population size achieved through the head-start program, it is unlikely that inbreeding and genetic drift will impact the population by causing a further reduction in genetic diversity as the population continues to grow through natural recruitment following successful rat eradication. The results from the 1906 sample provide a unique context for understanding patterns in the contemporary population, and for evaluating the success of the head-start program in maintaining natural distributions of genetic variation.

# Chapter 5: Conclusion

## 5.1 Research findings and significance

Resources for conservation are limited, and tough decisions about priorities need to be made. Determining which conservation unit(s) funds and efforts should be directed to, and which should be left to their fate is a contentious issue that has no clear solution. As discussed in Chapter 2, one proposed basis for determining priorities is ensuring that extinctions do not result in inordinate losses of evolutionary history (Vane-Wright et al. 1991). Evolutionary history (e.g. phylogenetic diversity) may perhaps be conserved for its intrinsic value, but also because it is assumed to represent evolutionary potential and trait diversity. Thus, numerous methods have been proposed to rank taxa based on their phylogenetic diversity (e.g. Weitzman 1992, Redding 2003, Haake et al. 2008). Currently, the application of such prioritization metrics is limited to a single example, the Zoological Society of London's EDGE of Existence programme (http://www.edgeofexistence.org). The "EDGE" metric used in that program (Isaac et al. 2007) shares a flaw with all other existing metrics, in that complementarity among taxa is not taken into account when determining rankings. The new method proposed in Chapter 2, I-HEDGE, is an improvement on existing metrics as it integrates evolutionary isolation, probability of extinction and complementarity. The EDGE of Existence programme is currently reviewing its methodology, and is considering I-HEDGE as the possible basis for its new prioritization scheme (Arne Mooers, pers. com.). Tools such as I-HEDGE can be used by nations to demonstrate that they are meeting international conservation objectives, for example the Convention on Biological Diversity (www.cbd.int), that specify the importance of maintaining genetic diversity as well as species diversity.

Very few head-start programs have ever been genetically evaluated, and the research presented here provides a rare retrospective analysis of the genetic impacts of this type of conservation intervention. In Chapter 3, I evaluated two cohorts of the head-start program in detail, reconstructing sibling relationships and assessing how representative the cohorts are of the wild population. Our discovery that our sample of the wild population, despite constituting such a high proportion of the species, did not capture the extent of diversity in the population motivated us to obtain the more comprehensive sample used in Chapter 4. The expedition to Pinzón Island in December 2014 allowed us to collect 262 blood samples from individuals representing all age-classes and thus capturing the full history of the head-start program. Study of 150 of those samples in Chapter 4 indicated that the head-start program, when evaluated as a whole, has been highly successful in perpetuating the full complement of genetic diversity that survived through the bottleneck, and maintaining natural patterns of population structure. The restoration of Pinzón tortoises was a major triumph for the Galápagos National Park and serves as an important success story in conservation. Our detailed analysis of the historical and contemporary population of Pinzón tortoises shows that the species has maintained as much diversity as was possible given the bottleneck, thanks to the quick rebound in population size enabled by the head-start program.

Genomic approaches have opened the door to new areas of inquiry, such as the identification of loci under natural selection, but has also, importantly, allowed for more accurate and precise estimates of population structure and demographic parameters (Allendorf et al. 2010, Ouborg et al. 2010). Genome-wide data can be particularly valuable when collected from historical and ancient DNA samples. The degraded nature of historical DNA has limited its

utility in studies using traditional genetic markers, but as most next-generation sequencing platforms, by design, yield short sequences (~50-300 base pairs), the short fragments of historical DNA are not a constraint. When combined with the treasure-trove of biological samples held in natural history collections, genomics has opened new avenues for evolutionary studies that may provide important insights into the history of populations (Holmes et al. 2016, Yeates et al. 2016). In this research we have used novel approaches to collect genome-wide data from a population of 110-year old museum specimens of Pinzón Island giant Galápagos tortoises. The genetic data collected from the museum samples in Chapter 4 (2,496 SNPs genotyped in 39 individuals and the mitochondrial genome in 77 individuals) is one of the largest historical population genetic datasets to be collected. These data have allowed a detailed picture to emerge of the demographic history of Pinzón tortoises and addressed fundamental questions related to the genomic consequences of rapid population decline and recovery. The results from the direct assessment of pre- and post-bottleneck diversity closely matched theoretical expectations, and provide a valuable empirical example of a bottleneck characterized using temporal samples in a natural population.

## 5.2 Remarks on conservation genomics

When designing a population or conservation genetic study, careful consideration is typically given to the trade-off between the number of individuals to be sampled and the number of genetic markers to be used. With the advent of genomics, it is easier than ever to increase the number of genetic markers, but there are still significant costs associated with data collection that increase with each additional individual genotyped. The study designs in Chapter 3 and 4 represent different points on the spectrum of sample size and number of genetic markers.

Chapter 3 evaluates two cohorts, constituting small snapshots into the head-start program, and a sample from the wild population that did not turn out to be very representative of the population as a whole, whereas Chapter 4 includes samples from across the entire history of the head-start program, sampled in the wild. The results from the cohorts indicated that few full siblings were being head-started, resulting in low relatedness among individuals, a result that is consistent when evaluating the full breadth of the head-start program. However, some of the results were not consistent between the marker sets. For example, the microsatellite study found the wild population of adults to have significant levels of inbreeding and a pattern of heterozygote deficit, while the genome wide SNP dataset and more comprehensive sampling of the wild population did not find either of these patterns. These differences can largely be attributed to the higher resolution provided by thousands of SNPs versus 9 microsatellite loci (Schlotterer 2004). However, increasing the number of SNP loci above a certain point does not seem to provide more information, as the results for the contemporary population based on >7,000 loci were similar to the ~2,500 loci.

The field of population genomics of non-model organisms has not yet matured to the point of having clear conventions for the reporting of bioinformatics methods, and with the ever increasing number of software packages available, determining appropriate analysis methods can be difficult. Recently, Shafer et al. (2016) rigorously tested the impacts of various assembly and variant detection methods on downstream population genetic results, and highlighted the importance of using multiple pipelines to ensure robust inferences, and reference assemblies whenever possible. More such studies are needed to develop best practices and conventions in the literature. We have made efforts to be as explicit as possible when describing the pipeline of bioinformatic methods ultimately used in Chapter 4, but have

not elaborated on all of the various parallel approaches that we evaluated. For example, the variant detection method used in the end was SAMTOOLS *mpileup* + BCFTOOLS *call* (Li et al. 2009), but we had also evaluated FREEBAYES (Garrison and Marth 2012) and GAKT *Haplotype Caller* (McKenna et al. 2010, DePristo et al. 2011). The three software packages produced similar results, and so we chose to go with what seemed to be the more common approach in the literature.

### 5.3 Pinzón tortoises

Head-starting has often been critiqued as a conservation measure that does not address the root cause of population decline, which for many species is high adult mortality (Heppell et al. 1996). However, for the Pinzón tortoise, high hatchling mortality due to non-native rats was the limiting factor in population recovery, and head-starting was an effective conservation strategy until the rats could be eradicated. With natural recruitment now occurring in the wild, the head-start program will no longer be necessary to support the population. If genetic diversity had become skewed over the course of the head-start program, a targeted head-start or breeding program could have been used to increase the representation of certain lineages. However, there are currently no indications that such an intervention is warranted. Estimates of the population size from the capture-mark-recapture study conducted by other researchers during the expedition in 2014 have not yet been published, but the 426 unique individuals encountered and 24 wild born hatchlings observed are a promising indication for the future. Monitoring of Pinzón Island should continue to ensure that rats do not recolonize and to track ecological restoration.

## 5.4 Final remarks

In this dissertation I have demonstrated that population genetics can provide information to improve conservation practices, through thoughtful prioritization of taxa, assessment of past actions, and understanding of the impacts of population decline and recovery. This project has been enabled through collaborations between universities in Canada and the USA, and with conservation practitioners and National Park staff in the Galápagos. With a seemingly ever-increasing number of species in decline, such collaborations will become more important in the future to share knowledge and resources. The case of the Pinzón tortoise demonstrates that remarkable recoveries are possible with dedicated efforts, and serves as an important success story during this era of almost universal biodiversity decline.

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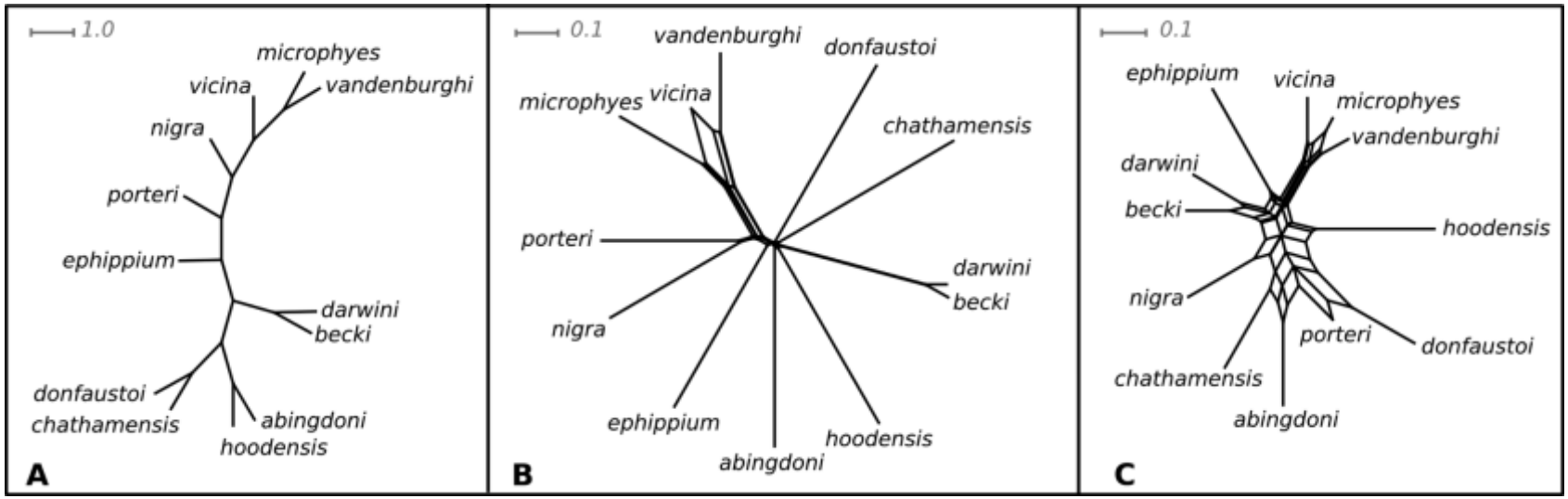
## Appendices

## Appendix A Supplementary materials for Chapter 2

Pairwise values of $\Phi_{ST}$ among species, calculated using the Kimura 2-parameter model

Each value is significant at $p$ <0.001, except between *darwini* and *becki*, which is significant at $p$ <0.05.

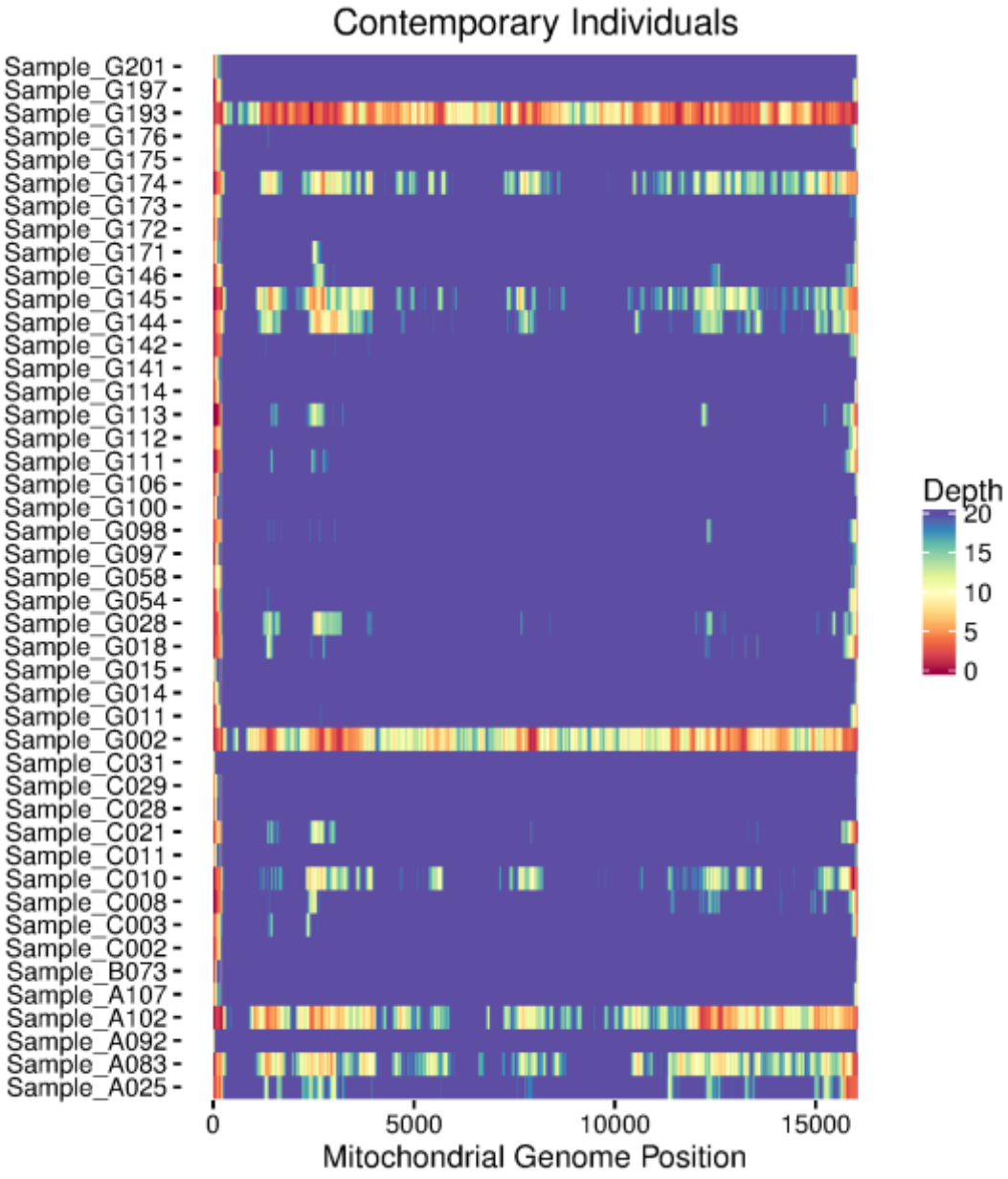
| | *darwini* | *donfaustoi* | *hoodensis* | *porteri* | *becki* | *abingdoni* | *ephippium* | *chathamensis* | *vandenburghi* | *microphyes* | *nigra* | *vicina* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *darwini* | 0.00 | | | | | | | | | | | |
| *donfaustoi* | 0.92 | 0.00 | | | | | | | | | | |
| *hoodensis* | 0.91 | 1.00 | 0.00 | | | | | | | | | |
| *porteri* | 0.84 | 0.91 | 0.93 | 0.00 | | | | | | | | |
| *becki* | 0.11 | 0.92 | 0.92 | 0.87 | 0.00 | | | | | | | |
| *abingdoni* | 0.92 | 0.98 | 0.98 | 0.91 | 0.93 | 0.00 | | | | | | |
| *ephippium* | 0.90 | 0.96 | 0.96 | 0.84 | 0.91 | 0.95 | 0.00 | | | | | |
| *chathamensis* | 0.91 | 0.99 | 1.00 | 0.92 | 0.92 | 0.98 | 0.96 | 0.00 | | | | |
| *vandenburghi* | 0.93 | 0.99 | 0.99 | 0.85 | 0.93 | 0.98 | 0.95 | 0.99 | 0.00 | | | |
| *microphyes* | 0.92 | 0.99 | 0.99 | 0.81 | 0.92 | 0.98 | 0.94 | 0.99 | 0.67 | 0.00 | | |
| *nigra* | 0.86 | 0.94 | 0.96 | 0.70 | 0.88 | 0.94 | 0.89 | 0.95 | 0.90 | 0.88 | 0.00 | |
| *vicina* | 0.85 | 0.88 | 0.89 | 0.74 | 0.87 | 0.89 | 0.85 | 0.89 | 0.27 | 0.36 | 0.71 | 0.00 |

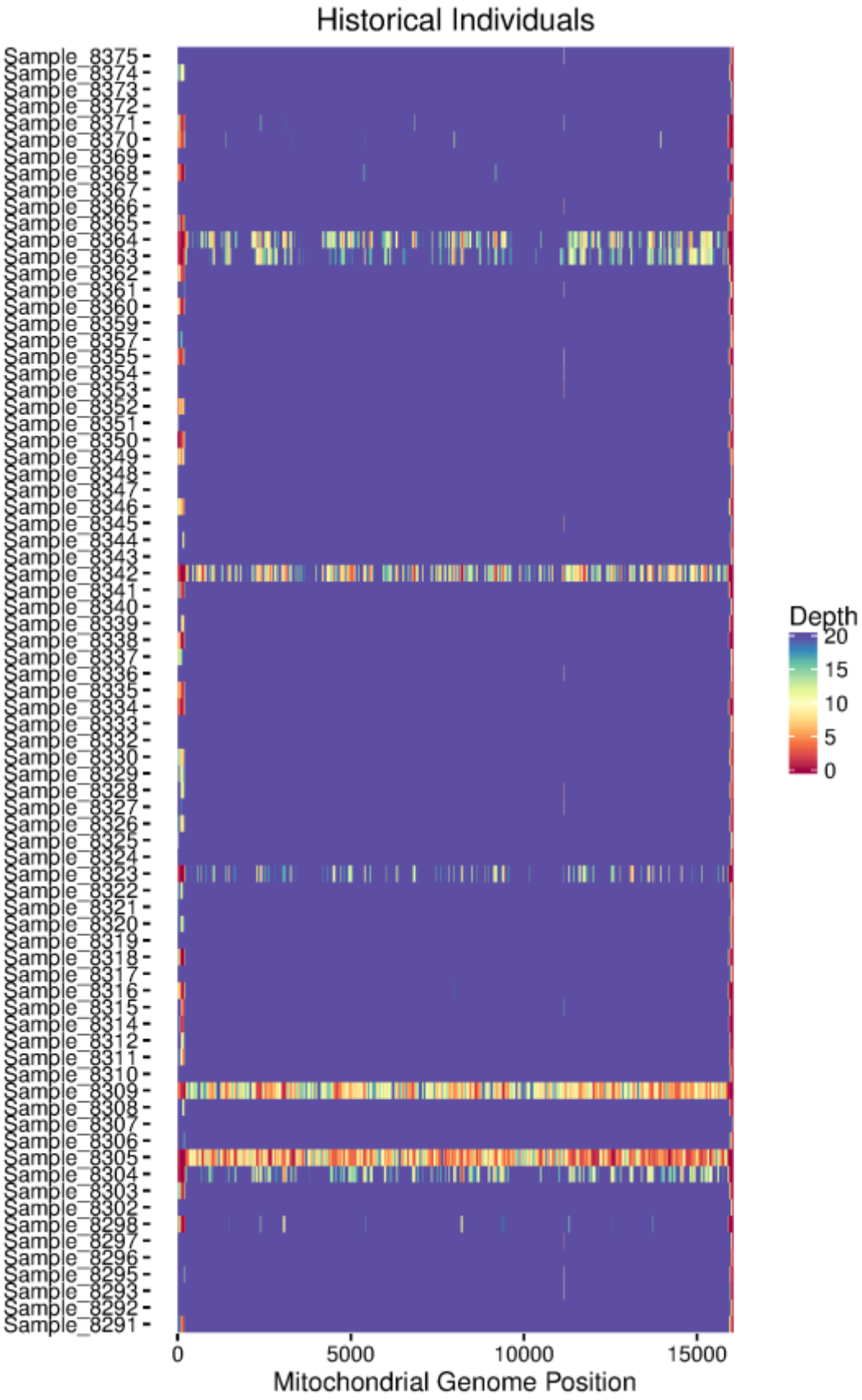



Depictions of (A) the relationships among Galápagos tortoise species resolved by previous studies (Beheregaray et al. 2004, Caccone et al. 2004, Russello et al. 2005, Poulakakis et al. 2008, Poulakakis et al. 2012) which are here presented as an unrooted equal-length tree, (B) the splits-network generated from pairwise $\Phi_{ST}$ values calculated from mitochondrial control region sequences and (C) the splits-network generated from pairwise $D_{est}$ (Jost 2008) values calculated from genotypes at 12 microsatellite loci. While A and B represent similar patterns, C depicts divergent relationships, particularly the placement of *hoodensis* away from *abingdoni*, *chathamensis* away from *donfaustoi*, and *nigra* away from *porteri*.

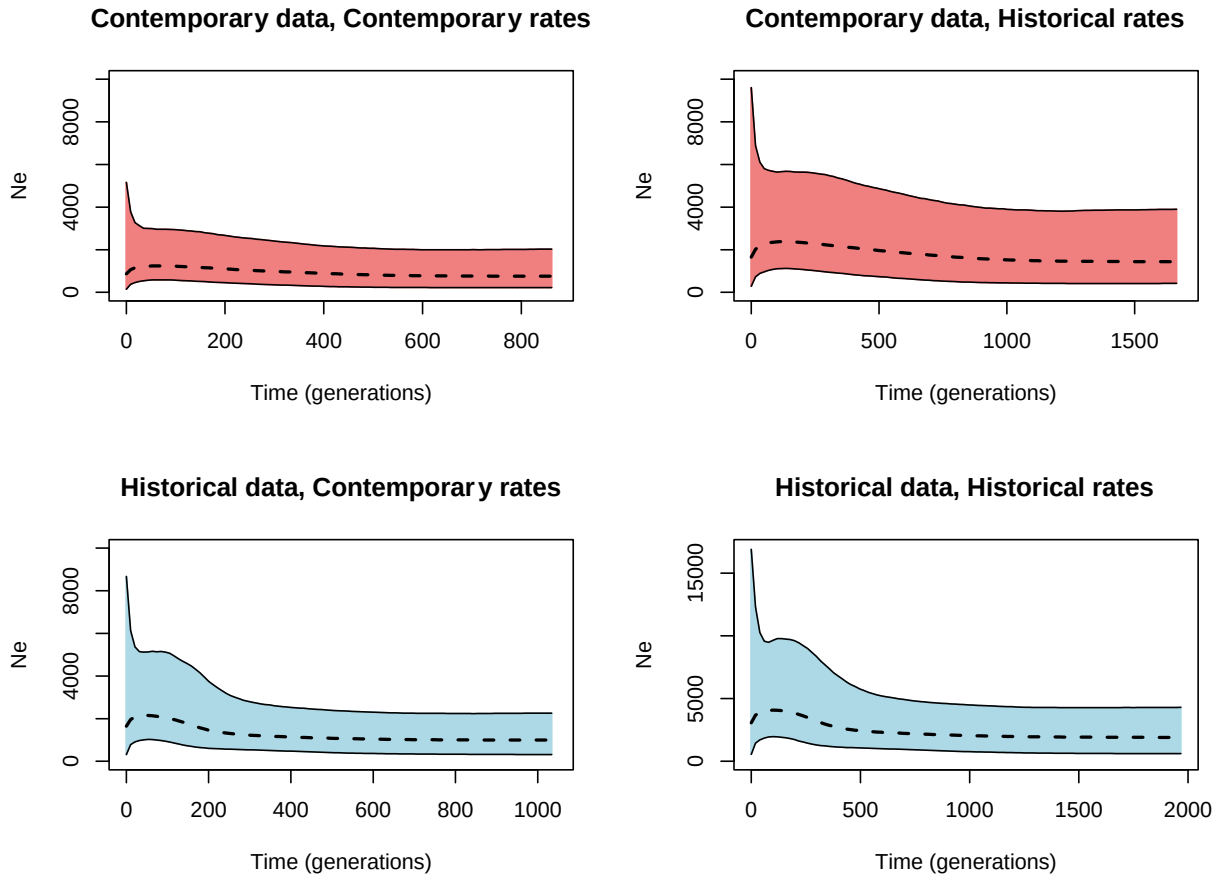
**Appendix B  Supplementary materials for Chapter 4**

Read depth at each position along the mitochondrial genome. Values greater than 20 are shown as equal to 20.

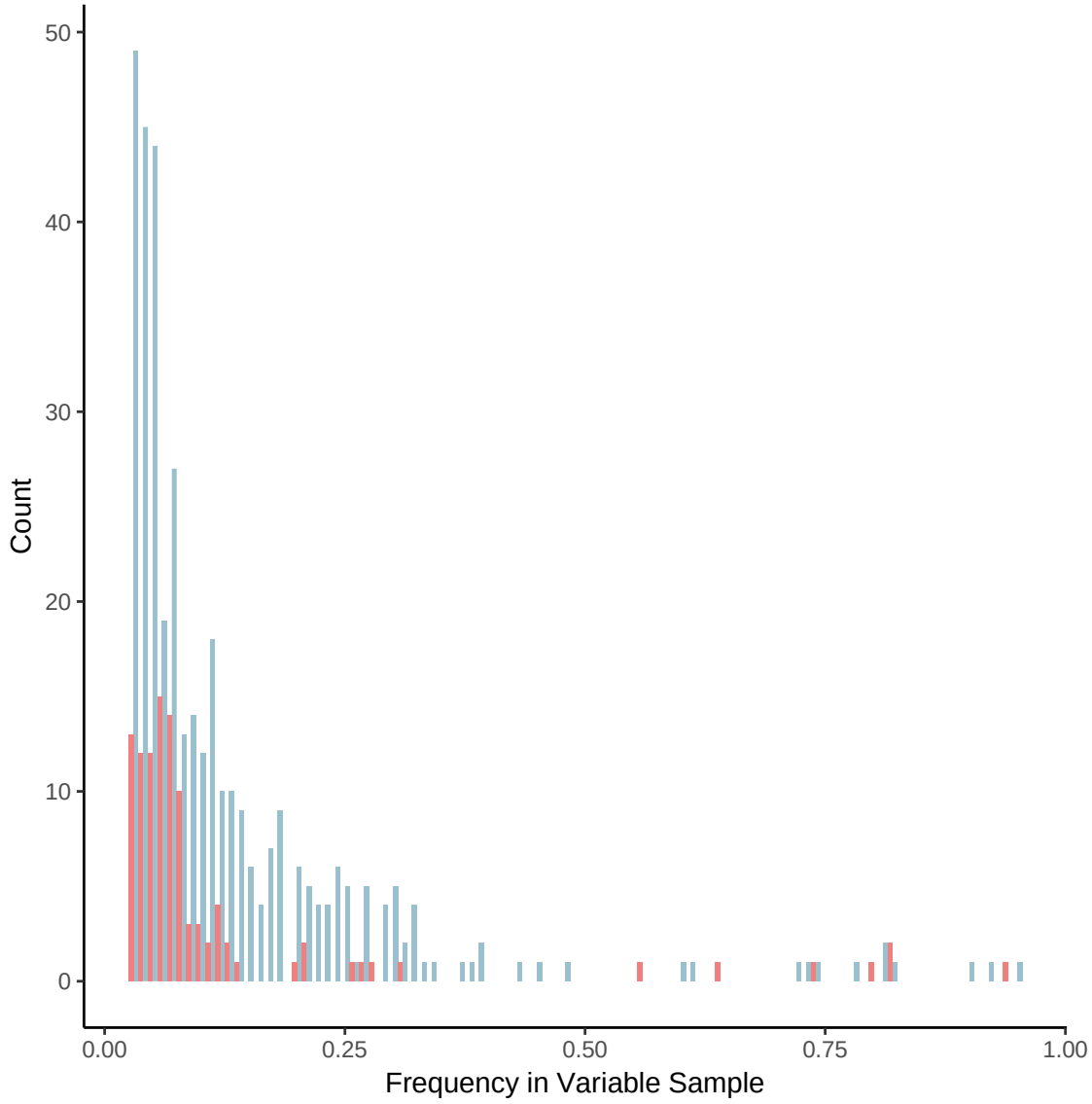



Historical Individuals
Sample_8375
Sample_8374
Sample_8373
Sample_8372
Sample_8371
Sample_8370
Sample_8369
Sample_8368
Sample_8367
Sample_8366
Sample_8365
Sample_8364
Sample_8363
Sample_8362
Sample_8361
Sample_8360
Sample_8359
Sample_8357
Sample_8355
Sample_8354
Sample_8353
Sample_8352
Sample_8351
Sample_8350
Sample_8349
Sample_8348
Sample_8347
Sample_8346
Sample_8345
Sample_8344
Sample_8343
Sample_8342
Sample_8341
Sample_8340
Sample_8339
Sample_8338
Sample_8337
Sample_8336
Sample_8335
Sample_8334
Sample_8333
Sample_8332
Sample_8330
Sample_8329
Sample_8328
Sample_8327
Sample_8326
Sample_8325
Sample_8324
Sample_8323
Sample_8322
Sample_8321
Sample_8320
Sample_8319
Sample_8318
Sample_8317
Sample_8316
Sample_8315
Sample_8314
Sample_8312
Sample_8311
Sample_8310
Sample_8309
Sample_8308
Sample_8307
Sample_8306
Sample_8305
Sample_8304
Sample_8303
Sample_8302
Sample_8298
Sample_8297
Sample_8296
Sample_8295
Sample_8293
Sample_8292
Sample_8291
0
5000
10000
15000
Mitochondrial Genome Position
Depth
20
15
10
5
0

Bayesian skyline plots from the mitochondrial genome sequences showing the median reconstructions of effective population size (Ne) over time (dashed lines) with 95% highest posterior density intervals, where time is in generations starting from the present.
The four plots are from the historical and contemporary samples, using the clock rates estimated from each dataset, as indicated in the title for each plot. Note that the x-axes are different for each plot, and that the y-axis is different in the historical data historical rates plot.



Histogram of the frequency of the allele in the variable sample that is absent in the other sample. Pink are variable in the contemporary sample that are fixed in the historical (105 loci); blue are variable in the historical sample that are fixed in the contemporary (368 loci).



Evaluations of different values of K.

A. The plot of ln P(K) for each value of K evaluated in the STRUCTURE analysis.

B. The plot of deltaK for detecting the number of K groups that best fit the data.

C. Plot of Bayesian Information Criterion from the DAPC analysis depicting the support for each value of K.