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Genetic divergence, phylogeography and conservation units of giant tortoises from Santa Cruz and Pinzón, Galápagos Islands

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Abstract

Island radiations can offer challenging systems for the implementation of conservation policies because descendent populations may exhibit different levels of adaptive divergence, reproductive isolation, and phylogenetic distinctiveness. This seems particularly true for the endangered Galápagos giant tortoises (*Geochelone nigra*), which comprise a lineage that radiated rapidly and concomitantly with the evolution of the archipelago. We used mitochondrial DNA sequences and microsatellite markers to investigate the genetic structure, and to reconstruct genealogical relationships and the history of population colonization of giant tortoises from the Islands of Santa Cruz and Pinzón, including samples of a basal taxon from the Island of San Cristóbal. Populations displayed marked genetic divergence, contrasting demographic histories, and deep phylogeographic structure. The pattern of diversification among populations was consistent with geological and biogeographic history, and to some extent, with adaptive and morphological divergence. Results strongly indicate the presence of a minimum of four conservation units with long-standing evolutionary separation: two in Santa Cruz, one in Pinzón, and one in San Cristóbal. We propose that these findings be effectively integrated with other existing data by the appropriate environmental agencies to evaluate current conservation efforts and implement new strategies aimed at protecting the integrity and diversity of giant tortoise populations.

Introduction

Evolutionary radiations provide favourite models for studying the origins of biological diversity and their association with divergent natural selection. They can also offer challenging systems for implementation of conservation policies because of the difficulties in identifying the biological units that truly represent the outcome of a radiation. An obvious problem is that evolutionary divergence is not expected to be equal among all descendent taxa. Occurrences of deep or shallow evolutionary timeframes depend largely on the geological setting of the area being occupied and on the rise of different ecological roles and adaptations (Givnish and Sytsma 1997). As a consequence, studies intending to identify the biological units for conservation in a radiation must deal with the difficulties of incorporating information from populations that have reached different stages of reproductive isolation and phylogenetic distinctiveness. This can be even more challenging if diversification occurred rapidly and relatively recently. Under these circumstances it becomes appropriate to study radiations using molecular markers that can reveal genetic distance and genealogical information over short periods of time, such as microsatellites and mitochondrial DNA (mtDNA) sequences (e.g. Kornfield and Parker 1997; Petren et al. 1999; Sato et al. 1999; Beheregaray et al. 2002).

The giant Galápagos tortoises are a distinctive example of a vertebrate radiation. They are the largest terrestrial chelonians in the world, are endemic to a remote oceanic archipelago, and represent the only surviving group of giant tortoises where evolutionary divergence is evident among populations (Pritchard 1996). The limited number of comparative studies of this radiation provides no agreement whether distinct tortoise populations should be treated as different species (e.g. Fritts 1984; Zug 1997). Nonetheless, the standard taxonomy usually recognizes 15 subspecies in the *Geochelone nigra* lineage (occasionally referred as *G. elephantopus*) endemic to different islands, or in the case of Isabela Island, restricted to different volcanoes in the same island (Pritchard 1996). Phylogenetic analyses of mtDNA sequences indicate that the group is monophyletic and support the hypothesis that the archipelago was colonized by a mainland South American ancestor that diversified rapidly and in a chronological fashion as a result of the formation and evolution of the Galápagos Islands (approximately between 2 and 0.18 million years [My] ago) (Caccone et al. 1999; Caccone et al. in press).

Despite recent human colonisation of the Galápagos, the tortoise populations have been greatly reduced by different causes. Historically, large numbers of individuals, perhaps as many as 200,000, were killed by whalers and buccaneers to be used as food (Towsend 1925). Additionally, at least 650 animals were removed to other continents by scientific expeditions in more recent times (MacFarland et al. 1974). Major contemporary threats are introduced pests, such as goats, black rats, donkeys, pigs, cats and dogs, which offer strong competition for food or predate intensively eggs and hatchlings (MacFarland et al. 1974; Pritchard 1996). As a result, populations were extirpated on some islands and others were dramatically reduced in number and distribution. Based on previous census estimates, all populations are categorized as endangered and only three of the eleven remaining subspecies appear to have the potential for natural self-replacement (MacFarland et al. 1974; Herrero 1997). The critical status of most populations led to the creation of a number of conservation projects by the Charles Darwin Research Station (CDRS) and the Galápagos National Park Service, including repatriation programs and campaigns for eradication of introduced pests. The giant tortoises are the icon of Galápagos' fabled fauna and a fundamental component of conservation efforts in the islands. The implementation of effective programs for protecting the diversity of this tortoise radiation relies on the identification of populations with long-term evolutionary separation and adaptive significance. These are the biological units that potentially have unique demographic histories and ecological and genetic attributes that are likely to be significant for population persistence and differentiation (Ryder 1986; Moritz 1994; Avise 2000; Crandall et al. 2000).

In this paper we use sequences of the mtDNA control region and data from ten microsatellite loci to investigate the population genetic structure and reconstruct the phylogeographic history of giant tortoises from the islands of Santa Cruz and Pinzón. Santa Cruz, the second largest inland of the archipelago, contains geographically separated populations with 'dome' carapaces classified as *G. nigra porteri*. Evidence based on mtDNA sequences from a small number of individuals (Caccone et al. in press), and reports of an isolated northwestern group of animals with 'saddleback' carapaces (Snow 1964; Pritchard 1996) suggest that Santa Cruz might contain three distinct tortoise populations distributed in the localities of La Caseta, Cerro Fatal and Cerro Montura (Figure 1). Although the population in La Caseta is the second largest of the archipelago (estimated around 2,500 individuals), it is highly threatened due to predation of eggs and young by introduced pests and is probably in decline (MacFarland et al. 1974). The populations in Cerro Fatal and Cerro Montura are thought to be extremely small (around 50 and 10 individuals, respectively) and are also threatened by pests (Snow 1964; MacFarland et al. 1974; Pritchard 1996; Fritts personal communication). Pinzón, on the other hand, is a very small island adjacent to Santa Cruz that contains the sole population of *ephippium*, a distinct taxon with saddleback morphology (Fritts 1984; Metzger and Marlow 1986; Pritchard 1996). Despite intensive repatriation of captive-raised tortoises conducted by the CDRS since 1971, predation by black rats on Pinzón is still a major threat of extinction for the *ephippium* lineage. Virtually no recruitment was observed in this population during the last century and only 150–200 tortoises are thought to exist in the island (Macfarland et al. 1974; Metzger and Marlow 1986). These two islands are in a central position in the archipelago (Figure 1; Snell et al. 1996), and their populations are therefore strategic to understanding the history of colonization and divergence of the radiation. We also analyse individuals from San Cristóbal (*G. nigra chatamensis*), which is one of the two oldest

Table 1. Genetic variability in four populations of Galápagos giant tortoises based on 697 bp of mtDNA control region and ten microsatellite loci. *n* is sample size. The sample from Cerro Montura ($n = 1$) is not included

Island	Population	*n*	Mitochondrial DNA			Microsatellites		
			No. of haplotypes	Haplotypic diversity, *h*	Genetic diversity, Θ	Mean no. of alleles per locus	H_E	H_O
Santa Cruz	La Caseta	66	12	0.80 (0.027)	0.0100 (0.0069–0.0132)	15.6 (2.6)	0.79 (0.05)	0.70 (0.06)
Santa Cruz	Cerro Fatal	16	1	0	0	3.9 (0.5)	0.54 (0.06)	0.54 (0.07)
Pinzón		53	8	0.76 (0.038)	0.0031 (0.0015–0.0048)	10 (1.6)	0.69 (0.07)	0.57 (0.06)
San Cristóbal		27	1	0	0	5.4 (0.8)	0.70 (0.05)	0.61 (0.06)

H_E and H_O are mean expected and observed heterozygosity, respectively.
Values between brackets are the standard errors for *h* and the 95% confidence intervals for Θ.

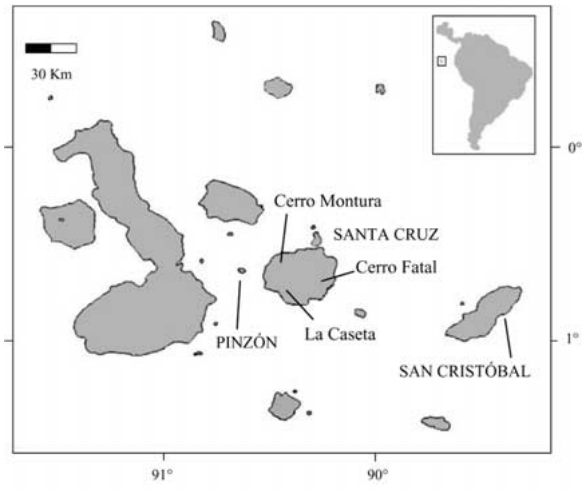

Figure 1. Map of the Galápagos archipelago showing the three islands included in this study and the approximate position of localities sampled in the Island of Santa Cruz (La Caseta, Cerro Fatal and Cerro Montura). The rectangle in the insert shows the position of the archipelago in relation to South America.

Galápagos islands (White et al. 1993) and contains the taxon basal to *porteri* and *ephippium* (Caccone et al. in press). Our specific objectives in this study are: (i) to test for genetic divergence among populations, including within island comparisons in Santa Cruz; (ii) to reconstruct genealogical relationships and the history of population colonization in Santa Cruz and Pinzón. The resulting information is integrated with other studies to identify biological units with long-term evolutionary separation and to guide conservation efforts for giant tortoise populations.

Material and methods

Samples

Blood samples of 162 wild giant tortoises were obtained from two localities in Santa Cruz (La Caseta and Cerro Fatal), one in Pinzón (Central), and one in San Cristóbal (Media Luna). We also included a single individual from the arid region of Cerro Montura (northwestern Santa Cruz), kept at the CDRS in Santa Cruz. Site locations and number of tortoises collected at each site are given in Figure 1 and Table 1, respectively. Blood was taken from the tortoises' forelegs (vena brachialis) using a syringe. Samples were preserved in Tris-EDTA-SDS buffer and stored at 4 °C in the laboratory. The majority of sampled individuals were adults. The sex of 81 tortoises was visually determined by examining the shape of the ventral carapace, indicating a slight excess of males in our sample (57%). Because of the complicated taxonomic history of tortoise populations and current disagreement about their specific status, we decided to use the word 'taxon' instead of 'subspecies' throughout this paper.

Genetic methods

Total DNA was extracted from blood using the Easy DNA extraction kit (Invitrogen). Polymerase chain reaction (PCR) amplification of a ∼ 700 bp of the hypervariable section of the mtDNA control region was performed with primers CytoR4 (5′-GCTTAACT AAAGCACCGGTCTTG-3′) and DL3Rev (5′-AATA TTTGAGTTGTCGTGGG-3′). All samples were

screened for sequence polymorphism in this fragment using the single-stranded conformation polymorphism technique (SSCP) as described in Sunnucks et al. (2000). SSCP offers an inexpensive, simple and precise method for detecting whether or not DNA fragments are identical in sequence (Orita et al. 1989; see Sunnucks et al. 2000 for a review and examples). We used 10 μL radiolabelled PCR reactions for the SSCP containing: 50–100 ng of template DNA, 10 pmol of each primer, 0.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus), each dNTP at 2.5 mM, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100 and 0.05 μL [α-^{33}P] dATP at 1000 Ci/mmol overlaid with mineral oil. PCR cycling conditions were: 94 °C/2 min, followed by 34 cycles at 94 °C/1 min, 52 °C/1 min and 72 °C/1 min, and a final extension of 72 °C for 2 min. Fresh PCR products destined for sequencing were prepared for individuals representing different SSCP gel phenotypes. Product bands were cut from agarose gels, purified with GENECLEAN III (BIO 101) and strands sequenced in both directions in an ABI 377 DNA sequencer following manufacturer's protocols. The number of individuals sequenced per SSCP phenotype is given in Appendix 1. Sequences were edited using SEQUENCER 3.1.1 (Gene Codes Corporation, Ann Arbor, MI) and aligned by eye.

Samples were also screened for nuclear DNA variation at ten microsatellite loci developed for *Geochelone nigra* (primer sequences described in Ciofi et al. in press). PCR primers were labelled with FAM, HEX, and TET fluorescent dyes. Microsatellites were amplified in 10 μL PCR reactions containing 1 $\times$ buffer, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.28 μM of each PCR primer, and 0.2 units of *Taq* polymerase (Applied Biosystems, Roche). Reactions were carried out using an initial step at 94 °C/5 min, followed by 30 cycles of 94 °C/30 s, 54 °C to 62 °C for 30 s (depending on the primer), and 72 °C/45 s, and a final extension of 72 °C for 5 min. PCR products were separated by electrophoresis on a 6% acrylamide slab gel, using an ABI 373 automated sequencer. Results were then analyzed with the GENESCAN and GENOTYPER softwares (Applied Biosystems), which allow sizing and quantification of DNA fragments.

Mitochondrial DNA data analysis

Genetic diversity at the mtDNA level was estimated by $\Theta = 4\ N_e\mu$, the product of effective population size and neutral mutation rate per site. The method, implemented in the software Recombine, is based on a maximum likelihood estimation using Metropolis-Hastings Markov Chain Monte Carlo genealogy sampling and allows for fluctuating population sizes (Kuhner et al. 1995, 1998). Haplotypic diversity (h) was calculated as in Nei (1987). Levels of genetic divergence between samples were calculated with the fixation index ϕ_{ST} (Excoffier et al. 1992), an estimator that includes information on haplotype frequency and molecular distance. For ϕ_{ST} we used the Kimura 2-parameter (K2P) genetic distance (Kimura 1980) with a gamma value of 0.5 (empirically determined by maximum likelihood). This approach is indicated for analyses of the mtDNA control region and for data sets with different rates of transitions and transversions (Kumar et al. 1993). The significance of ϕ_{ST} for population comparisons was assessed by 2,000 permutations. The values of h, ϕ_{ST} and the permutations were computed in ARLEQUIN (Schneider et al. 2000).

We also used ARLEQUIN to investigate the demographic history of tortoise populations by performing mismatch analyses of mtDNA sequences. This method is based on the assumption that events of demographic population growth or decline leave distinctive signatures in the array of DNA sequences (Rogers and Harpending 1992). Mismatch distributions are obtained by estimating the number of nucleotide differences between every pair of individuals and displaying graphically the relative frequencies of the results of these pairwise comparisons. The curves are expected to be multimodal in population samples at demographic equilibrium and unimodal in samples under population expansion (Rogers and Harpending 1992). These distributions are then compared to the one expected under a model of population expansion (Rogers 1995) by calculating the estimator of time to the expansion (τ) and the mutation parameter (θ) according to Schneider and Excoffier (1999). We used the formula $t = \tau/(2\mu)$ to estimate the timing of possible population expansions (t is given in generations; μ is the mutation rate for the control region fragment). Approximate confidence intervals for the parameters of the distributions were obtained by a parametric bootstrap approach, and a comparison of the sum of squared deviations (SSD) between observed and expected distributions provided a test statistic for the estimated expansion model (Schneider and Excoffier 1999). We also calculated the raggedness index (r) of the observed distribution – an index that takes higher values for multimodal distributions found in

stationary populations (Harpending 1994), and tested its significance using a similar parametric approach incorporated in ARLEQUIN.

Evolutionary relationships within and among populations were examined by constructing a haplotype network with the mtDNA sequences based on the statistical parsimony method of Templeton et al. (1992). This method links first haplotypes with the smaller number of differences as defined by a 95% confidence criterion. The analysis was performed in TCS (Clement et al. 2000), a program that can also identify the most ancient haplotypes in the sample using predictions from coalescent theory.

Microsatellite data analysis

We used GENEPOP 3.2a (Raymond and Rousset 1995) to calculate mean number of alleles per locus, allele frequencies, expected (H_E) and observed (H_O) heterozygosities, and test for Hardy-Weinberg equilibrium (HWE) using the Markov chain method.

Levels of nuclear DNA differentiation among populations were estimated using two approaches: (i) computing pairwise comparisons of the fixation index using the estimator θ (Weir and Cockerham 1984) with the program GENETIX (Belkhir et al. 2000); and (ii) conducting assignment tests of individual tortoises based on a method implemented in GENECLASS (Cornuet et al. 1999). The assignment test is a likelihood-based technique which calculates population allele frequencies, computes the likelihood of an individual multi-locus genotype belonging to a candidate set of populations, and assigns that individual to the population where the likelihood of its genotype is the highest. Tortoises with a likelihood $<$ 5% of belonging to their sampled population where not assigned to that locality. We used the 'leave one out' option, a procedure that reduces the bias of adding the current individual in its population when calculating allelic frequencies. Assignments were conducted with a Bayesian method using a simulation procedure with 10,000 randomly generated genotypes. We chose the Bayesian method because it has performed better in computer simulations than other assignment tests (Cornuet et al. 1999), it takes into account the sampling error associated with estimating allele frequencies and consider differences in genetic diversity between populations (Rannala and Mountain 1997).

For the analyses mentioned above involving multiple simultaneous comparisons we corrected the statistical significance levels using the sequential Bonferroni procedure (Rice 1989) with a $\alpha = 0.05$. The sample from Cerro Montura ($n = 1$) was used only for the assignment test and phylogenetic analysis.

Results

Contrasting levels of genetic diversity and departures from HWE

Data on SSCP and sequencing obtained for all samples resulted in an aligned 697 bp fragment of the mtDNA control region. Multiple sequences from SSCP phenotypes (Appendix 1) and comparisons with previous data sets (Caccone et al. 1999; Caccone et al. in press) confirmed the reliability of the SSCP technique: same phenotypes (gel bands) always had identical sequence and different phenotypes had distinct sequences.

Twenty-two haplotypes were detected in our sample of 163 individuals. Levels of mtDNA variation differed considerably among populations (Table 1). All tortoises from Cerro Fatal (Santa Cruz) possessed a single haplotype; the same was observed for the 27 San Cristóbal individuals, represented by only one maternal lineage. In contrast, tortoises from La Caseta (Santa Cruz) and Pinzón had a much larger number of haplotypes (12 and 8, respectively) and high haplotypic diversity ($h = 0.80$ in La Caseta and 0.76 in Pinzón). Our microsatellite data also revealed differences in nuclear DNA variation: the mean number of alleles in Cerro Fatal and San Cristóbal (Table 1) was lower than in the other two populations (Wilcoxon signed-rank tests, $P < 0.05$). However, it should be noted that differences in sample size may be responsible for this trend in variation as the largest samples were accompanied by the largest levels of variation.

Fisher's probability tests indicated that the San Cristóbal and Cerro Fatal populations are probably in HWE (only two out of the ten loci in San Cristóbal and none in Cerro Fatal were out of equilibrium after Bonferroni correction; $P < 0.05$). In contrast, the majority of loci in the other two populations showed departures from HWE, especially in Pinzón. This appeared as a consequence of the large excess of homozygotes observed at most loci (Table 1; Appendix 2), which generated significantly positive values for the inbreeding coefficient F_{is} (data not shown). These deviations are probably not due to null alleles (alleles that do not amplify during PCR because of mutations in the primer region) given that the microsatellite library was developed from a Pinzón individual; nor is it

Table 2. Genetic divergence among four populations of Galápagos giant tortoises as measured by fixation indices*. Results are based on mtDNA control region haplotypes (ϕ_{ST}, below diagonal) and ten microsatellite loci (Θ, above diagonal)

Island	Population	La Caseta	Cerro Fatal	Pinzón	San Cristóbal
Santa Cruz	La Caseta		0.146	0.183	0.146
Santa Cruz	Cerro Fatal	0.862		0.319	0.305
Pinzón		0.828	0.961		0.245
San Cristóbal		0.891	1.0	0.965	

*All pairwise comparisons were statistically significant ($P < 0.001$).

Table 3. Assignment tests in five samples of Galápagos giant tortoises based on ten microsatellite loci. Values are the proportion of individuals 'assigned' to each population, or 'not assigned' to any of the four populations. 'Non assigned' tortoises had multi-locus genotypes with a probability of belonging to the locality where they were collected lower than 5%

		La Caseta	Cerro Fatal	Pinzón	San Cristóbal
Sampled in					
La Caseta					
	Assigned to	0.79	0.00	0.00	0.00
	Not assigned	0.21			
Cerro Fatal					
	Assigned to	0.18	0.76	0	0.00
	Not assigned		0.06		
Pinzón					
	Assigned to	0.00	0.00	0.78	0.00
	Not assigned			0.22	
San Cristóbal					
	Assigned to	0.00	0.00	0.00	0.82
	Not assigned				0.18
Cerro Montura ($n = 1$)					
	Assigned to	0	0	*	0

*2% probability of belonging to Pinzón, 0% to any other population.

due to technical/scoring errors because some samples were amplified twice in different PCRs and produced the same multi-locus genotype. It is possible that the non-equilibrium scenario observed in Pinzón might be a result of the repatriation program conducted in that population; while in La Caseta the Wahlund effect (the inclusion of two or more genetically distinct units into a single sample, Wahlund 1928) could perhaps account for the discrepancies observed (see discussion).

Genetic differentiation among populations

Strong genetic divergence was detected among populations from different islands representing the three described taxa. High genetic differentiation was also revealed between La Caseta and Cerro Fatal populations, from the Island of Santa Cruz. The pronounced differences in haplotypic and allelic frequency distribution among all populations (Appendices 1 and 2) translated in a highly significant fixation index for all possible pairwise comparisons ($P < 0.001$), a result observed for mtDNA and microsatellite data sets (Table 2).

In all populations, a high proportion of tortoises (75 to 82%; Table 3) had multilocus genotypes that were allocated to the populations where they were sampled based on results from assignment tests. All remaining individuals (except for three tortoises from Cerro Fatal) were not assigned to any of the four localities. The existence of a considerable number of non-assigned individuals could be due to an incom-

plete representation of the allelic diversity in each population, or alternatively, due to individual dispersal among localities (an unlikely hypothesis for isolated populations such as San Cristóbal). Evidence of migration between localities was detected only in three tortoises from Cerro Fatal that were genetically assigned to the La Caseta population. The tortoise from Cerro Montura (northwestern Santa Cruz) had a genotype with a 2% probability of belonging to Pinzón and 0% to any other population.

Demographic history

Results from the mismatch analysis provided important insight into the demographic history of tortoises from La Caseta and Pinzón (the other populations were fixed for a single control region lineage). These two populations showed substantially different mismatch distributions (Figure 2) and genetic diversities (Table 1). La Caseta displayed a multimodal distribution with steep waves and a high frequency of diverged haplotypes. The curve obtained differs from the one estimated from a model of population expansion (Figure 2). On the other hand, the distribution in Pinzón had a smoother curve that fits better with the expected for an expanding population, suggesting historical demographic growth in Pinzón. These contrasting demographic scenarios were statistically supported by both SSD and raggedness index (r) tests. The mismatch of La Caseta was significantly different than the expectations for an expansion model (P based on SSD < 0.0001), and its r index was also much larger ($r = 0.18$) than the one for a unimodal distribution ($P < 0.0001$). Conversely, the mismatch in Pinzón fitted well the predicted distribution under a model of population expansion ($P = 0.46$ and 0.64 based on SSD and r tests, respectively). The timing of past population expansion in Pinzón, calculated using a generation period for giant tortoises of 25 years (based on results from repatriation programs) and rates of mtDNA evolution for Testudines of 0.5 and 0.9% per My (Avise et al. 1992; Caccone et al. in press) was approximately 620,000–400,000 years ago.

Genealogical relationships

The mtDNA network revealed three groups of haplotypes with large differences in sequence divergence (Figure 3). The first haplogroup includes all samples from Pinzón and is unique by begin formed solely by closely related haplotypes (0.14 to 1.15% of sequence divergence). Interestingly, the haplotype identified by

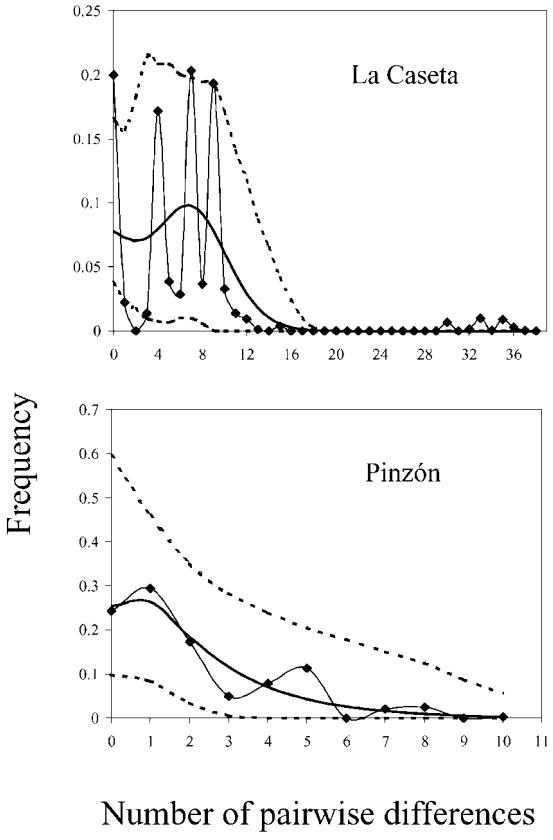

Figure 2. Mismatch distributions of mtDNA haplotypes in giant tortoises from La Caseta and Pinzón. Diamonds represent the observed relative frequencies of nucleotide differences between pairs of individuals (based on 2,145 pairwise comparisons in La Caseta and 1,378 in Pinzón). Thick curve shows the distribution fitted to the data under a model of population expansion, and dashed lines are the 2.5 and 97.5 percentile values of 1,000 simulations.

coalescence criteria as the ancestral sequence of this group (haplotype 18), was also detected in the tortoise sampled at Cerro Montura, in Santa Cruz. The second haplogroup contains only samples from La Caseta and is composed of two moderately diverged branches that have apparently diversified from an abundant ancestor. The third haplogroup is comprised by the divergent haplotypes of Cerro Fatal and San Cristóbal, and also includes a third lineage represented by a single individual sampled in La Caseta (haplotype 12). Haplotype 12 is parsimoniously connected to Cerro Fatal (they differ by 6 substitutions), but is between 29 and 36 steps from any La Caseta haplotypes. The deep sequence divergence among the three haplogroups strongly suggests long-term evolutionary isolation among most populations, especially for the Cerro Fatal and San Cristóbal haplogroup,

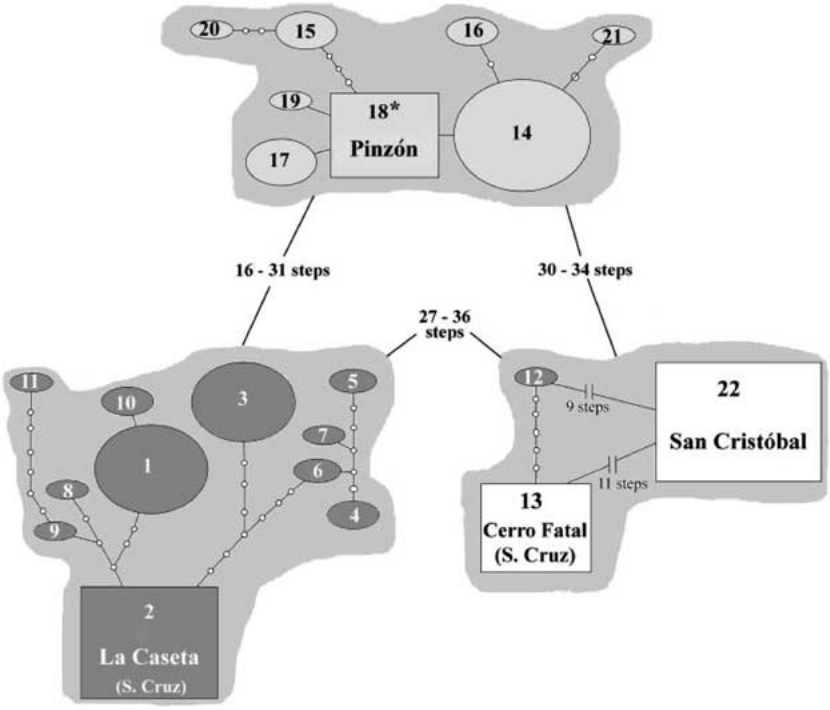

Figure 3. Network showing genealogical relationships among four Galápagos giant tortoise populations based on mtDNA haplotypes. Relationships were estimated with the parsimony method of Templeton et al. (1992). The numbers within ovals correspond to haplotypes (see Appendix 1), and the size of ovals to the number of tortoises with that particular haplotype. Haplotypes separated by single lines are one mutation, or step, apart, and small circles along single lines represent missing haplotypes. Rectangles are haplotypes considered as ancestral based on coalescence criteria. Dark grey ovals are the haplotypes found in La Caseta and grey ovals the ones from Pinzón. Haplotype 18 (marked with an asterisk) was also found in the individual from Cerro Montura.

which differed from other tortoise haplotypes by very long branches (between 3.9 and 5.2% of divergence).

Discussion

We analysed the genetic structure and genealogical relationships of giant tortoise populations from Santa Cruz and Pinzón, Galápagos Islands. An important outcome of this study was the detection of a pattern suggestive of long-standing evolutionary separation among populations. The implications of this finding for reconstructing the biogeographic history of this tortoise radiation and for guiding conservation strategies are explored in an integrated manner in this section.

Genetic divergence between and within islands

As might be expected based on taxonomy, both mtDNA sequences and microsatellite markers showed high levels of genetic differentiation among populations from different islands currently assigned as *porteri* (Santa Cruz), *ephippium* (Pinzón) and *chatamensis* (San Cristóbal). We also detected strong genetic divergence between La Caseta and Cerro Fatal, in the Island of Santa Cruz, from which only a single taxon (*porteri*) has been described. The pattern of population structure disclosed by the fixation index analysis was consistent with assignments tests based on multilocus genotypes, which statistically allocated the majority of individual tortoises to the locality where they were sampled.

Although the differentiation detected by microsatellites was highly significant for all population comparisons, our mtDNA data set revealed much higher structure (i.e. mtDNA values approached or reached the theoretical maximum of unity for the fixation index). A possible cause for this inequality could be the smaller effective population size (*Ne*) of mtDNA relative to nuclear genome and associated higher levels of divergence. Theoretically, nuclear DNA is predicted to display a fourfold suppression in rates of

allele sorting compared to mtDNA (Birky et al. 1989; Avise 2000). A point that should be emphasized is that this expectation depends on the operational sex ratio being equal. In Testudines, sex determination is controlled by temperature during egg incubation: cooler temperatures usually produce more males, warmer temperatures more females (Ciofi and Swingland 1997 and references therein). Sorting of matrilines could proceed faster in an isolated population after a relatively cooler period of incubation resulted in an excess of breeding males. Under this scenario, only a small proportion of females would produce the next generation, depleting even more the *Ne* of the mtDNA in relation to the nuclear genome. A similar reasoning could apply if islands were founded by a small number of females and populations experienced severe bottlenecks during colonization. Another aspect of the biology of tortoises would also exacerbate the inequality in effective size of the two genomes: females are known to store viable sperm for months (Devine 1984; Pearse and Avise 2001). Thus, if a single multiply inseminated female founded a new population, ratio of effective size of nuclear versus mitochondrial genes could be much greater than four. These suggestions are purely speculative given that the historical demography of the two sexes is obviously unknown. We prefer to assume that genetic drift, probably the major factor driving neutral genetic differentiation in island populations, might have accounted for the faster sorting and higher structure of the mtDNA. This seems more appropriate than assuming strong male-biased dispersal between islands, a situation applicable for Galápagos marine iguanas (Rassmann et al. 1997), but unlikely for tortoises.

Phylogeography, demographic history and island colonization

All populations surveyed displayed remarkably deep phylogeographic structure. We propose that the major splits in the matrilineal network correspond to authentic long-term evolutionary disjunctions among populations. This assumption is supported by evidence consistent with several 'aspects of genealogical concordance' (*sensu* Avise 1996), such as the existence of multiple sequence characters diagnosing each haplogroup (between 36 and 16 mutations) and the strong genetic divergence among populations detected by microsatellite markers.

Another aspect of genealogical concordance especially relevant to validate a phylogeographic outcome is agreement with biogeographic history (Avise 1996, 2000). The Galápagos islands were formed as a result of an eastward displacement of the Nazca plate over a 'hot spot' (White et al. 1993). Therefore, a logical biogeographic pattern is that founding events might have occurred from geologically older islands (those on the eastern end of the archipelago and nearer South America) to younger, more westerly located islands. This pattern has been partially corroborated by molecular evolutionary studies on marine iguanas (Rassmann et al. 1997). For giant tortoises, molecular phylogenetic analyses of all extant taxa suggest a complex scenario of island colonization generally consistent with biogeography (Caccone et al. 1999; Caccone et al. in press). The following conclusions made by Caccone and collaborators are particularly relevant here: (i) the two oldest islands (San Cristóbal and Española) contain the basal taxa of giant tortoises, which in separate occasions founded populations on younger islands; (ii) La Caseta and Cerro Fatal were founded by two very divergent lineages. Our fine-scale phylogeographic reconstruction, coupled with results from the mismatch analysis, substantiate these findings and disclose novel information on the colonization and evolution of tortoises in Santa Cruz and Pinzón.

For instance, La Caseta can be distinguished from other populations by having diverged matrilines and high genetic diversity (Θ = 1.0%). Mismatch analysis detected no signal for demographic expansions in La Caseta. On the contrary, its multipeaked mismatch distribution is indicative of a relatively old population with stable demography (Rogers and Harpending 1992; Avise 2000). In contrast, there is statistical support for past demographic expansion in Pinzón, a population formed by closely related lineages with relatively recent coalescence and low genetic diversity (Θ = 0.31%). A scenario of historical growth apparently conflicts with the marked population reduction that occurred around 100 years ago in Pinzón after the arrival of feral black rats (MacFarland et al. 1974; Pritchard 1996). It is possible that this reduction happened so recently that there has not been enough time for a distinct signature to accumulate in the array of DNA sequences of the population (see Rogers 1997 for mismatch models of population decrease following expansion). In addition, theoretical and empirical evidence suggest that the signal of the main expansion detected by mismatch analysis (if one occurred) will be quite robust to further fluctuations in population size (a detailed example is provided by Lavery et al.

1996). We propose that the genetic expansion detected in Pinzón is a relict of a demographic growth that would have followed the onset of the founding population. The discrepancies in genealogical and demographic history between the two populations suggest that Pinzón was founded more recently than La Caseta. This agrees with the size and geographic location of the islands (see Figure 1), and with geology: Santa Cruz emerged not less than 2.2 My ago, while the maximum age for Pinzón is around 1.5 My (White et al. 1993). Moreover, the timing for population expansion in Pinzón was calculated at around 500,000 years ago. This estimate has a large confidence interval, but is notwithstanding consistent with geological history.

The mtDNA network also sheds light into the history of the enigmatic saddleback population from Cerro Montura. Our single individual from this locality had the same matriline identified as the ancestral lineage in Pinzón. To some extent, assignment tests substantiated this result by showing that this tortoise is more related to the ones in Pinzón (2% probability of belonging) than to other populations (0% of probability). One likely explanation is human introduction from the nearby Pinzón. Nevertheless, field notes and photographic material from historic expeditions reveal that saddleback tortoises have occupied Cerro Montura for at least 100 years and that reproduction is probably occurring (Snow 1964; Pritchard 1996). Thus, it is conceivable that these animals are remnant of a larger and natural population, which raises the exciting possibility for coexistence of two morphologically distinct taxa in Santa Cruz. For Galápagos giant tortoises, morphological divergence appears to be a better indicator of current ecological conditions than of evolutionary relationships: mostly due to physiological constraints, saddleback tortoises tend to occupy lower and xeric habitats, whereas the larger domed tortoises are found on islands with higher elevations and more ecological complexity (Fritts 1983, 1984). According to Fritts' hypothesis, recently formed islands, which have arid conditions and sparse vegetation, would be colonized first by saddleback tortoises. As large islands age, ecosystems develop and moisture-dependent habitats are formed at higher elevations, favouring the establishment of domed individuals. Saddlebacks might have been the first colonizers of Santa Cruz. They are currently restricted to the dry zone around Cerro Montura because subsequent colonization by domed tortoises may have out-competed saddlebacks in moister localities. If Fritts' speculation is correct, the *ephippium* lineage on Pinzón may have been founded by migrants from Santa Cruz, a scenario consistent with geology and not rejected by our phylogeographic reconstruction. While we have proposed a sequence of colonization for saddleback tortoises, our current knowledge precludes analogous inferences for the domed population in La Caseta. This taxon seems to have originated either from San Cristóbal or Española, a dichotomy that can be potentially resolved only with the inclusion of extinct taxa into the analysis (Caccone et al. in press).

Finally, the deepest separation in the matrilineal network (the one between Cerro Fatal and other populations in Santa Cruz and Pinzón) can perhaps also be attributed to independent events of island colonization. The extant population most closely related to Cerro Fatal is the San Cristóbal taxon *chatamensis* (Caccone et al. in press), a result confirmed by our analysis based on larger sample sizes. This seems intriguing because Cerro Fatal tortoises are morphologically domed, whereas *chatamensis* is saddleback. Nonetheless, Caccone et al. (in press) suggested that individuals from an extinct domed taxon from San Cristóbal may have founded Cerro Fatal. This was based on evidence that San Cristóbal was once inhabited by a domed population (van Denburgh 1914; Pritchard 1996; Fritts personal communication), which was heavily collected by whalers and became extinct in the 1930s (Banning 1933). This population inhabited the higher and moister habitats of southwestern and central areas of the island, whereas the saddleback tortoises are distributed in arid regions of northeastern San Cristóbal (Pritchard 1996). Accordingly, habitat segregation may have accounted for the divergence of tortoise populations in San Cristóbal and for the subsequent colonization event of Cerro Fatal.

Due to the proximity of some populations, it was interesting to detect remarkably strong phylogeographic structure and uniquely sorted matrilines, a pattern also observed for other populations of Galápagos tortoises (Caccone et al. in press; L. Beheregaray unpublished). Marked phylogeographic structure has also been reported for several terrestrial and freshwater turtles in North America (e.g. Avise et al. 1992; Walker and Avise 1998; Weisrock and Janzen 2000). The deeper divergences among these populations can be attributed to ancient vicariant events (Pliocene or older), while variability in recent scenarios can be related to retention of polymorphisms generated within ancestral refugia (Walker and Avise 1998; Weisrock and Janzen 2000). These explanations

are inconsistent with the relatively recent formation of the Galápagos and with results from our molecular studies. These studies suggest a single colonization event for the archipelago (Caccone et al. 1999), strong founder effects in older islands and no retention of ancestral polymorphism in younger populations (this study; Caccone et al. in press). Overall, there is considerable evidence that most genetic variability of Galápagos tortoises has been generated *in situ* after population colonization, and there is not much support for inter-island dispersal among established populations. Elucidation of the latter phenomenon possibly relies on identification of behavioural and ecological constraints of individual populations. Evidence is so far limited to comparisons between few taxa with divergent carapace shapes, but suggest strong antagonistic behaviour (Schafer and Krekorian 1983) and morphological specializations for particular ecological resources (Fritts 1983).

Gene flow among populations

In spite of the apparent limited dispersal between islands, we were able to identify signals of historic migration between domed populations in Santa Cruz. Three tortoises sampled in Cerro Fatal were assigned to La Caseta based on microsatellite data. This could be indicative of previous migrations by La Caseta males because the three tortoises displayed a typical Cerro Fatal matriline. Another unidirectional event of introgression, but this time involving the female sex, was detected in one tortoise sampled in La Caseta. This animal is from a matriline related to Cerro Fatal (haplotype 12), but is very distant to any lineage found in La Caseta. Given that its microsatellite genotype was assigned to La Caseta, this could actually be a descendant of a female from another population that migrated into La Caseta. These events of dispersal might reflect historical differences in spatial patterns of population structure in Santa Cruz. Today, the vast majority of domed Santa Cruz individuals are confined to the tortoise reserve in the southwest (which includes La Caseta), and are separated from the small eastern population of Cerro Fatal by settled agricultural zones (Pritchard 1996). It is reasonable to assume that domed tortoises occupied other areas along the southern part of the island before human arrival. If animals were concentrated around zones of higher altitudes (the pattern expected for domed tortoises), we may well envision a historical scenario promoting fine-scale genetic structuring and moderate dispersal between neighbour populations. In fact, this is consistent with several pieces of evidence from this study. First, the moderately diverged matriline represented by haplotype 12 could be a remnant of an extinct haplogroup now confined to the tortoise reserve. Second, La Caseta contains two diverged matrilineal groups that diversified historically from a common ancestor (Figure 3), and has high genetic and haplotypic diversity. Third, the homozygous excess in La Caseta suggests a Wahlund effect in that population. These facts indicate that La Caseta could be presently composed of an admixed sample from historically sundered populations.

Implications for conservation of Galápagos giant tortoises

We identified four relevant units for conservation: the taxa *ephippium* (from Pinzón), *chatamensis* (San Cristóbal), and *porteri* (La Caseta) and a fourth unit from Cerro Fatal (eastern Santa Cruz). These four populations display the genetic attributes normally ascribed to 'evolutionarily significant units' (ESUs) (*sensu* Moritz 1994). An ESU is a population unit with a distinct, long-term evolutionary history that should be managed separately and has high conservation priority (Ryder 1986). The application of this concept in conservation biology has received criticism because ESUs can sometimes be identified based solely on evidence of genetic isolation instead of also incorporating data on functional and ecological diversity (Crandall et al. 2000; Pearman 2001). Our work shows that the differentiation among these four units is largely consistent with patterns of genetic divergence, genealogical structure, demographic distinctiveness and biogeography, and to some extent, is also concordant with adaptive and morphological divergence (see above). Nevertheless, because no life-history data is available for the Cerro Fatal population we prefer to consider the three described taxa as distinct ESUs and Cerro Fatal as a different 'management unit' (Moritz 1994). In this context, Cerro Fatal could be elevated to an ESU if further studies reveal morphological and ecological divergence between tortoises from La Caseta and Cerro Fatal. Finally, the isolated and extremely small Cerro Montura population deserves further investigation given that we had only a single individual available for this study. Additional sampling of this population may shed light on the origin of saddleback tortoises in Santa Cruz and clarify the issue of whether Cerro Montura is a valuable

natural population, or represents a recent, perhaps human-induced, introduction from Pinzón.

Another result with implications for conservation was the detection of contrasting levels of genetic diversity among populations. We detected only one control region lineage in San Cristóbal and one in Cerro Fatal, a finding that reinforces the prioritisation of conservation efforts to prevent reductions of the number of breeders and further loss of variability in these populations. In contrast, the fairly abundant population from La Caseta had high mitochondrial and nuclear DNA variability. This population appears to have a history of demographic stability, potential for natural persistence and diversification, and therefore, should be rigorously protected. An unexpected outcome of this study was the discovery of relatively high genetic variability in Pinzón, a population that experienced reductions in size so severe to the point that it was thought to be extinct in the 1920s (MacFarland et al. 1974; Pritchard 1996). Fortunately, considerable genetic variation was maintained in the survivors (i.e. we found eight different maternal lineages in 53 individuals), a result that encourages the continuation of the repatriation of captive-raised tortoises and the eradication of black rats from Pinzón. To conclude, we propose that the information generated here be effectively integrated with other existing data by the appropriate environmental agencies to evaluate current conservation efforts and implement new strategies aimed at protecting the integrity and diversity of Galápagos giant tortoise populations.

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Appendix 1. Frequency of the 22 mtDNA control region haplotypes found in five samples of Galápagos giant tortoises. Last column shows the number of individuals sequenced for each SSCP gel phenotype

Haplotype	La Caseta	Cerro Fatal	Cerro Montura	Pinzón	San Cristóbal	No. sequenced per SSCP phenotype
1	0.242	–	–	–	–	4
2	0.318	–	–	–	–	8
3	0.212	–	–	–	–	5
4	0.045	–	–	–	–	2
5	0.030	–	–	–	–	1
6	0.030	–	–	–	–	1
7	0.015	–	–	–	–	1
8	0.015	–	–	–	–	1
9	0.015	–	–	–	–	1
10	0.045	–	–	–	–	2
11	0.015	–	–	–	–	1
12	0.015	–	–	–	–	1
13	–	1.0	–	–	–	9
14	–	–	–	0.396	–	7
15	–	–	–	0.094	–	2
16	–	–	–	0.057	–	1
17	–	–	–	0.132	–	4
18	–	–	1.0	0.264	–	4
19	–	–	–	0.019	–	1
20	–	–	–	0.019	–	1
21	–	–	–	0.019	–	1
22	–	–	–	–	1.0	10

GenBank entries for haplotypes are: AY097997, AY097999 – AY098001, AY097977, AY098053, AY098057, AY098058, AY098060, AY098064, AY098072.

Appendix 2. Allele frequencies of 10 microsatellite loci in four samples of Galápagos giant tortoises. Alleles (in italics) are designated by the length of PCR products in base pairs

Locus and Sampling site	Alleles																				
GAL45	*87*	*95*	*103*	*105*	*107*	*109*	*111*														
La Caseta	0.667	0	0	0	0.288	0.030	0.015														
Cerro Fatal	0.844	0	0	0	0.156	0	0														
Pinzón	0.1968	0	0	0.066	0.717	0.019	0														
San Cristóbal	0.175	0.250	0.100	0	0.400	0	0.075														
GAL50	*102*	*104*	*108*	*110*	*112*	*114*	*118*	*122*	*124*	*126*	*128*	*130*	*132*	*134*	*136*	*138*	*140*	*142*	*144*	*146*	*148*
La Caseta	0	0.030	0	0	0.068	0.061	0.038	0.030	0	0.045	0.030	0.015	0.045	0.045	0.061	0.015	0.015	0.068	0.008	0.045	0.045
Cerro Fatal	0	0	0	0.063	0	0.031	0	0	0	0	0	0	0	0	0	0	0	0	0	0.063	0
Pinzón	0	0	0.019	0.264	0	0	0	0.330	0.038	0.009	0.009	0	0	0	0	0.094	0.038	0	0	0.047	0
San Cristóbal	0.050	0	0.175	0.050	0	0	0	0	0	0	0	0.025	0	0	0	0	0	0.025	0.175	0.025	0
	150	*152*	*154*	*156*	*158*	*160*	*162*	*164*	*166*	*168*	*170*	*174*									
La Caseta	0.023	0.023	0.038	0.030	0.068	0.030	0.038	0.015	0.023	0.030	0.015	0									
Cerro Fatal	0.375	0	0	0.031	0	0	0	0	0	0	0	0.438									
Pinzón	0	0.028	0	0.028	0.009	0.028	0.019	0	0	0.009	0.028	0									
San Cristóbal	0	0	0	0	0.075	0	0	0	0.275	0	0	0.125									
GAL73	*84*	*88*	*90*	*92*	*94*	*96*	*98*	*100*	*102*	*104*	*106*	*108*	*110*	*112*	*114*	*116*	*118*	*126*	*130*		
La Caseta	0.016	0.047	0.047	0.016	0.117	0.164	0.148	0.133	0.133	0.039	0.039	0.008	0.008	0	0.031	0.023	0.008	0.008	0.016		
Cerro Fatal	0	0	0	0	0.063	0	0.719	0	0	0.156	0.063	0	0	0	0	0	0	0	0		
Pinzón	0	0	0	0	0	0.087	0	0.010	0.087	0.048	0.269	0.279	0.202	0.010	0.010	0	0	0	0		
San Cristóbal	0.605	0	0	0	0	0.395	0	0	0	0	0	0	0	0	0	0	0	0	0		
GAL75	*73*	*75*	*77*	*81*	*83*	*85*	*87*	*89*	*91*	*93*	*95*	*97*	*99*	*103*	*105*	*107*	*109*	*111*	*113*	*119*	*121*
La Caseta	0	0.038	0.123	0.069	0.123	0.108	0.146	0.054	0.100	0.123	0.038	0.046	0.015	0	0.015	0	0	0	0	0	0
Cerro Fata	0	0.313	0.125	0	0.156	0.156	0.063	0.156	0	0.031	0	0	0	0	0	0	0	0	0	0	0
Pinzón	0.009	0	0	0	0	0	0.245	0	0.047	0	0.019	0.217	0	0.009	0	0.236	0.123	0.019	0.009	0.047	0.019
San Cristóbal	0	0	0	0	0.200	0	0	0.575	0	0.200	0.025	0	0	0	0	0	0	0	0	0	0
GAL94	*87*	*89*	*91*	*93*	*95*	*97*	*99*	*101*	*103*	*105*	*107*	*109*	*113*								
La Caseta	0	0	0	0.086	0.266	0.102	0.031	0.305	0.063	0.031	0.086	0.023	0.008								
Cerro Fatal	0	0	0	0	0	0.406	0	0.063	0.156	0	0	0.375	0								
Pinzón	0.160	0.462	0.009	0	0	0.028	0	0.274	0.047	0.019	0	0	0								
San Cristóbal	0	0.184	0	0	0	0	0.263	0.132	0.421	0	0	0	0								

Appendix 2. Continued

Locus and Sampling site	Alleles																		
GAL100	*100*	*104*	*110*	*112*	*116*	*118*	*120*	*122*	*124*	*126*	*128*	*134*	*136*	*150*	*156*	*162*	*164*		
La Caseta	0.017	0	0.042	0.025	0.373	0.042	0.212	0.136	0.008	0.076	0	0.042	0	0.008	0	0.008	0.008		
Cerro Fatal	0	0	0	0	0.033	0	0	0.633	0	0.333	0	0	0	0	0	0	0		
Pinzón	0	0.098	0.333	0.069	0.020	0	0.186	0.118	0.059	0.069	0.049	0	0	0	0	0	0		
San Cristóbal	0.050	0	0.025	0.050	0.400	0	0.200	0.050	0	0.100	0	0.075	0.025	0	0.025	0	0		
GAL127	*95*	*101*	*103*	*105*	*107*	*109*	*111*	*113*	*115*	*117*	*119*	*121*	*123*	*125*	*127*	*129*	*131*		
La Caseta	0.008	0.008	0	0.038	0.045	0.076	0.053	0.008	0.053	0.015	0.030	0.008	0.053	0.038	0.045	0.061	0.023		
Cerro Fatal	0	0	0	0	0.625	0	0	0	0.219	0	0.156	0	0	0	0	0	0		
Pinzón	0	0	0.113	0.019	0.075	0.283	0.142	0.019	0	0.085	0.038	0.009	0.009	0	0.057	0	0.019		
San Cristóbal	0	0	0	0.050	0	0	0	0	0	0	0.075	0	0	0	0	0	0		
	133	*135*	*137*	*139*	*141*	*143*	*145*	*147*	*149*	*151*	*155*	*161*	*163*	*165*	*169*	*171*			
La Caseta	0.098	0.008	0.098	0.030	0.015	0.053	0.038	0.023	0.015	0.015	0.008	0.023	0.008	0	0.008	0			
Cerro Fatal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Pinzón	0.038	0.009	0.019	0.019	0	0	0	0	0.019	0	0	0.009	0	0.009	0	0.009			
San Cristóbal	0	0	0.425	0.150	0.200	0	0	0.100	0	0	0	0	0	0	0	0			
GAL136	*75*	*77*	*81*	*83*	*85*	*87*	*89*	*91*	*93*	*99*	*101*	*103*	*105*	*107*					
La Caseta	0.040	0.008	0	0.661	0.185	0.048	0.032	0	0.016	0	0	0	0.008	0					
Cerro Fatal	0	0	0	0.867	0.133	0	0	0	0	0	0	0	0	0					
Pinzón	0.077	0	0	0.125	0.077	0.048	0.058	0.010	0	0.558	0.010	0.019	0.010	0.010					
San Cristóbal	0.053	0	0.237	0.237	0	0.316	0.079	0	0	0.079	0	0	0	0					
GAL159	*89*	*93*	*95*	*97*	*99*	*101*	*103*	*105*	*107*	*109*	*111*	*113*	*115*	*117*	*119*	*121*	*123*	*125*	*139*
La Caseta	0.023	0.038	0.015	0.045	0.008	0.152	0.129	0.152	0.136	0.098	0.068	0.023	0.023	0.008	0	0.023	0.023	0.023	0.015
Cerro Fatal	0	0	0	0	0	0.063	0	0.719	0	0	0.125	0.094	0	0	0	0	0	0	0
Pinzón	0	0	0	0	0	0	0.085	0	0.009	0.057	0.085	0.179	0.274	0.245	0.066	0	0	0	0
San Cristóbal	0.575	0	0	0	0.025	0	0.400	0	0	0	0	0	0	0	0	0	0	0	0
GAL263	*80*	*82*	*86*	*88*	*90*	*92*	*94*	*96*	*106*	*110*	*112*	*118*	*122*	*130*	*134*				
La Caseta	0.500	0.031	0.008	0.047	0.031	0.102	0	0.063	0.094	0.039	0.031	0.008	0.016	0.008	0.023				
Cerro Fatal	0.438	0.063	0	0.469	0.031	0	0	0	0	0	0	0	0	0	0				
Pinzón	0.057	0	0	0.009	0	0	0.896	0.038	0	0	0	0	0	0	0				
San Cristóbal	0.250	0.325	0	0.275	0	0.150	0	0	0	0	0	0	0	0	0				

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