

Historical DNA analysis reveals living descendants of an extinct species of Galápagos tortoise

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Giant tortoises, a prominent symbol of the Galápagos archipelago, illustrate the influence of geological history and natural selection on the diversification of organisms. Because of heavy human exploitation, 4 of the 15 known species (*Geochelone* spp.) have disappeared. Charles Darwin himself detailed the intense harvesting of one species, *G. elephantopus*, which once was endemic to the island of Floreana. This species was believed to have been exterminated within 15 years of Darwin's historic visit to the Galápagos in 1835. The application of modern DNA techniques to museum specimens combined with long-term study of a system creates new opportunities for identifying the living remnants of extinct taxa in the wild. Here, we use mitochondrial DNA and microsatellite data obtained from museum specimens to show that the population on Floreana was evolutionarily distinct from all other Galápagos tortoise populations. It was demonstrated that some living individuals on the nearby island of Isabela are genetically distinct from the rest of the island's inhabitants. Surprisingly, we found that these "non-native" tortoises from Isabela are of recent Floreana ancestry and closely match the genetic data provided by the museum specimens. Thus, we show that the genetic line of *G. elephantopus* has not been completely extinguished and still exists in an intermixed population on Isabela. With enough individuals to commence a serious captive breeding program, this finding may help reestablish a species that was thought to have gone extinct more than a century ago and illustrates the power of long-term genetic analysis and the critical role of museum specimens in conservation biology.

conservation genetics | evolution | phylogenetics | hybridization | cryptic diversity

The giant tortoises of the Galápagos archipelago (*Geochelone* spp.) are renowned both for their uniqueness and for their contribution to the development of Darwin's theory of natural selection (1). Fifteen formally described taxa of giant Galápagos tortoises are generally recognized, four of which are extinct from the islands of Floreana (*G. elephantopus*), Santa Fe (*Geochelone* sp. undescribed), Fernandina (*G. phantastica*), and Rabida (*Geochelone* sp. undescribed) (Fig. 1) (2). When Charles Darwin visited the island of Floreana in 1835, he learned that giant tortoises were the staple food source of the recent settlers to the island and that, as a consequence, their numbers had become "greatly reduced" (3). Robert Fitz-Roy, captain of the HMS *Beagle*, remarked that the large number of empty tortoise shells lying about the settlement showed "what havoc has been made among these helpless animals" (4). Even before settlers came to Floreana in 1832, there was a long history of tortoise capture and transportation from the island by whalers and buccaneers, who stored the animals in the hulls of their ships for food (2). Darwin reported that single vessels had taken up to 700 individuals at a time. Thus, it is not surprising that the species on Floreana (5) is thought to have disappeared by no later than the mid-1800s (2, 6).

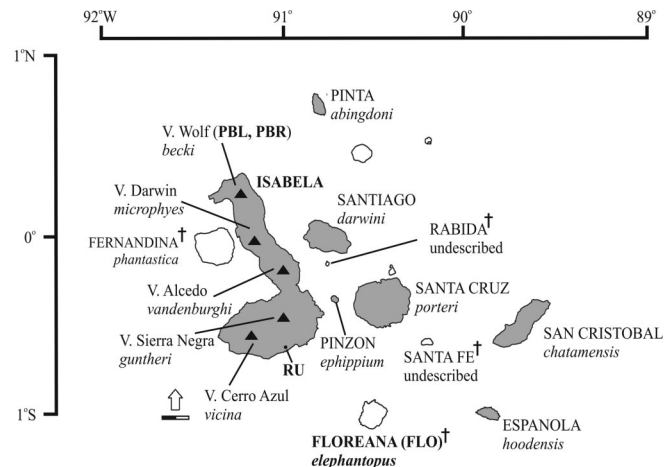


Fig. 1. Distribution of giant tortoises in the Galápagos archipelago. Shaded and non-shaded islands indicate presence of extant and extinct tortoise populations, respectively. Italicized names indicate current taxonomic designations (5, 8). Pinta is represented by a single male kept in captivity. †: extinct species. ▲: volcanoes on Isabela.

The taxonomic ranking of populations on different islands and volcanoes, often morphologically distinct, has been contentious, especially as to whether such populations should be considered different species or subspecies (7). Here, we adopt the taxonomic classifications of Van Denburgh (8) who treats all described taxa of Galápagos tortoises as separate species. This classification scheme is recognized by Ernst and Barbour (5) and is the most consistent with the overwhelming morphological and molecular evidence now available (9–13). Among their most remarkable features is the tremendous variation in carapace morphology among populations from different islands. Some tortoises have a very rounded or "domed" shape, whereas others exhibit a "saddlebacked" shape with a sharply raised anterior opening of the carapace,

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See Commentary on page 15227.

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Table 1. Floreana specimens used in this study

A/A	Reference	Museum	Locality	Sample	Collection Information	Number of Extractions	MtDNA*	Microsatellites (# Successfully Genotyped Loci)
1	R-46606	MCZ	Floreana	Damaged carapace with attached bones	CH Townsend, 1928	2	Yes	No
2	R-4477	MCZ	Floreana	Small carapace with attached bones	Hassler, 1872	2	Yes	Yes (9)
3	R-4478	MCZ	Floreana	Small carapace with attached bones	Hassler, 1872	2	Yes	Yes (8)
4	R-4480	MCZ	Floreana	Ethanol	Hassler, 1872	2	Yes	No
5	R-12049	MCZ	Floreana	Ethanol	Hassler, 1872	2	Yes	No
6	R-11069	MCZ	Floreana	Tail	Hassler, 1872	2	Yes	Yes (6)
7	R-4476	MCZ	Floreana	Ethanol	Hassler, 1872	2	Yes	Yes (4)
8	R-11064	MCZ	Floreana	Carapace	n/a	4	Yes	Yes (7)
9	R-11070	MCZ	Floreana	Arm bone from mounted specimen	Captain Downes, 1834	2	Yes	Yes (10)
10	R-29998	MCZ	Floreana	Damaged carapace with attached bones	CH Townsend, 1928	2	Yes	Yes (7)
11	R-45756	MCZ	Floreana	Damaged carapace with attached bones	CH Townsend, 1928	2	Yes	No
12	R-32590	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	Yes (9)
13	R-46401	AMNH	Floreana	Piece of plastron	CH Townsend, 1928	2	Yes	No
14	R-46402	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	No
15	R-46403	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	Yes (4)
16	R-46404	AMNH	Floreana	Piece of pelvic girdle	CH Townsend, 1928	2	Yes	Yes (7)
17	R-46405	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	Yes (9)
18	R-46406	AMNH	Floreana	Piece of pelvic girdle	CH Townsend, 1928	2	Yes	Yes (7)
19	R-46407	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	Yes (8)
20	R-46408	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	4	Yes	Yes (7)
21	R-46412	AMNH	Floreana	Piece of plastron	CH Townsend, 1928	2	Yes	Yes (8)
22	R-46413	AMNH	Floreana	Piece of plastron	CH Townsend, 1928	2	Yes	Yes (9)
23	R-46419	AMNH	Floreana	Piece of plastron	CH Townsend, 1928	2	Yes	No
24	R-46424	AMNH	Floreana	Vertebra and leg bone	CH Townsend, 1928	2	Yes	Yes (8)
25	R-45290	AMNH	Floreana	Piece of vertebra	CH Townsend, 1928	2	Yes	No

*We failed to amplify mtDNA or nDNA data for specimens R-1905, R12302, and R-32098 from MCZ and for specimens R-32591, R-45291, R-46410, R-46415, R-46417, R-46420, R-46421, and R-46422 from AMNH. For four specimens [R-4479 and R-4668 (from MCZ), R-46418 (from AMNH), and v872 (from the Charles Darwin Research Station)], we amplified the CR sequence once, but we failed to reproduce the result.

a feature that is thought to be an adaptation for feeding on elevated vegetation in dry habitats (14). Based on historical observations and a handful of museum specimens, it appears that the tortoises of Floreana were of the latter type, along with four other formally described species from the islands of Espanola (*G. hoodensis*), Pinta (*G. abingdoni*), Pinzon (*G. ephippium*), and San Critsobal (*G. chathamensis*) (14).

The variation among island forms provided one of the clues that helped Darwin grasp how natural selection can lead to the observed morphological differences between populations or closely related species. This pattern was initially made clear to him by the vice-governor of the Galápagos who claimed that he could recognize which island an individual tortoise came from by its appearance (3). Underlying this observation is the idea that each island houses independently evolving lineages, shaped by their own unique histories of interaction with the environment. Indeed, previous genetic studies have shown that most tortoise populations in the Galápagos were formed from a single colonization event, with subsequently little or no genetic exchange with other islands (9–12, 15). An exception is the largest island of Isabela, where three of the five named taxa were found to be genetically distinct, whereas the two southern-most taxa could not be distinguished. Although the majority of tortoises from the northernmost volcano Volcano Wolf on Isabela represent a genetically distinct taxon (*Geochelone becki*), several individuals show mixed ancestry and a very divergent evolutionary history (10); the precise source of this mixed ancestry has not been fully resolved.

Although extinct since the nineteenth century, specimens from Floreana are well represented in natural history collections, which are to biodiversity research what genome databases and cell lines are to genomics (16). DNA techniques now allow the inclusion of extinct organisms in evolutionary analyses, facilitating investigation of unresolved phylogenetic ques-

tions and providing a broader understanding of changes in the distribution of genetic variation over time. To investigate the evolutionary history of the extinct population on Floreana, we generated genetic data from museum specimens collected on the island and compared them with an expanded molecular database including all other extant taxa of Galápagos tortoises.

Results and Discussion

Mitochondrial control region sequences were generated from 25 museum specimens collected during three separate expeditions to the island from the late 1800s to early 1900s [supporting information (SI) Fig. S1 and Table 1] and then were compared with an expanded database of genetic information from all extant taxa. Twenty mtDNA haplotypes were found among the 25 museum specimens. The resulting phylogeny (Fig. 2) firmly placed 21 of 25 specimens in a distinct “clade” that includes the one previously published sequence from Floreana (12). This “Floreana clade” is related most closely to a lineage consisting of several taxa from the nearby island of Isabela (Fig. 1). The remaining four museum samples cluster within lineages found on the islands of Santa Cruz, Pinzon, and southern Isabela. Their presence on Floreana probably is the result of human-mediated transport from these other islands [see *SI Materials and Methods* for the morphology of these specimens]. There are detailed accounts of whalers and buccaneers dropping stores of tortoises on various islands to lighten the burden of their ships (17). Additionally, as the tortoise population on Floreana declined, expeditions were sent to bring back animals from other islands (6, 18).

It seems, however, that the legacy of such tortoise harvesting is not completely negative. Grouped within the Floreana clade are two haplotypes (PBR12 and PBL16) that previously were described from 12 individuals in the Puerto Bravo (PBR) and

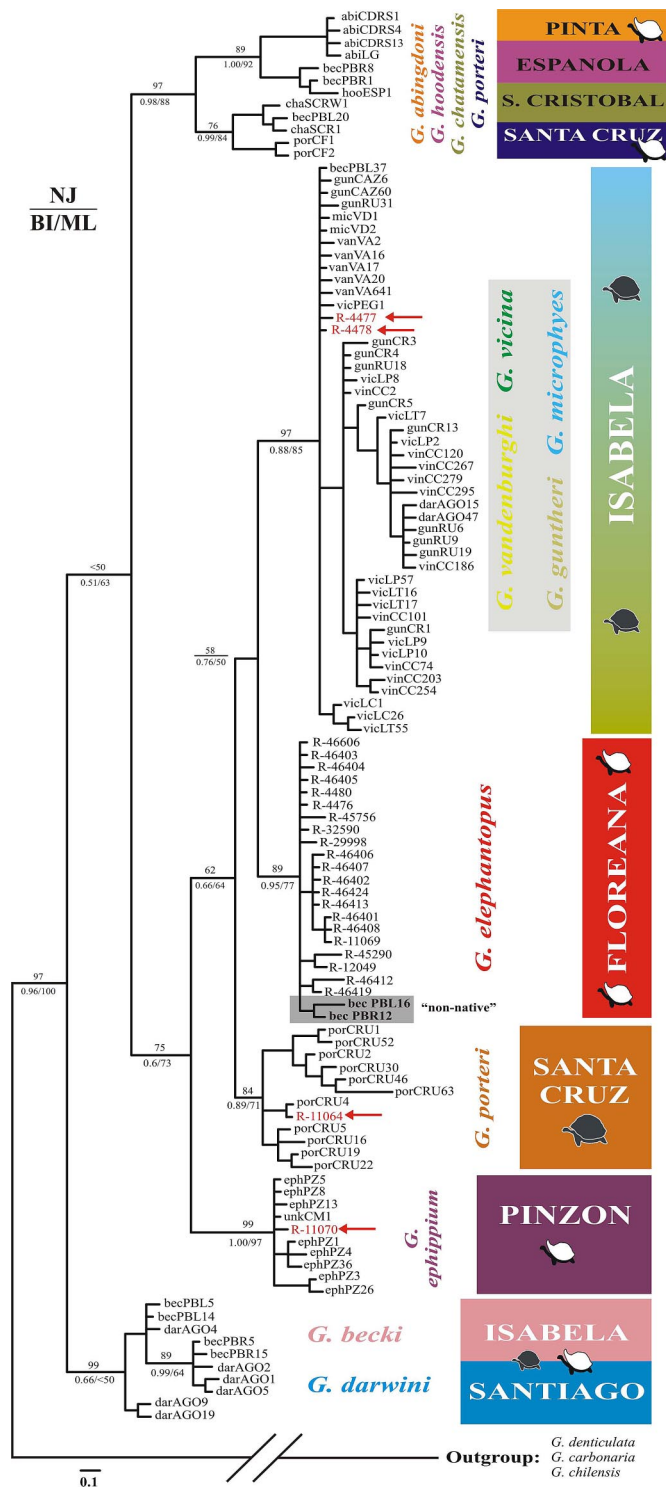


Fig. 2. Bayesian inference (BI) tree of giant Galápagos tortoises based on the mtDNA control region sequences. The analysis was run with four chains for 10^7 generations under the Tamura and Nei +I+G model. Numbers on branches indicate bootstrap values based on 1000 repetitions in neighbor-joining (NJ) (Above) and 100 repetitions in maximum likelihood (ML) (Below Right) and posterior probabilities in Bayesian inference (BI) (Below Left). Only the values of the major lineages are presented. Shaded and unshaded tortoise caricatures signify domed and saddle-backed morphologies, respectively. Red arrows indicate specimens collected on Floreana that cluster with mtDNA lineages other than that of the “Floreana clade.” The gray shading in the “Floreana clade” shows the haplotypes from Volcano Wolf (PBR and PBL) and Roca Union (RU) populations.

Piedras Blancas (PBL) populations on Volcano Wolf in northern Isabela, as well as a single individual from the Roca Union (RU) population on southern Isabela (Fig. 1). These individuals are referred to as “non-native” because they are genetically distinct from the rest of the inhabitants of the same volcano (Table S1) (10). They differ by 17 to 32 nucleotide substitutions from other individuals on Isabela but by only 2 to 9 substitutions from haplotypes within the Floreana clade. These results were reinforced by the Shimodaira-Hasegawa test that rejects the hypotheses that *i*) all individuals of Floreana consist of a monophyletic group ($P < 0.001$), and *ii*) the two “non-native” haplotypes (PBR12, and PBL16) form a clade with the rest of native PBR and PBL haplotypes ($P < 0.001$). Unlike other populations that are exclusively domed or saddlebacked in carapace morphology, the PBR and PBL populations display high morphological diversity (14), whereas the rest of the Isabela tortoises are strictly domed. Until now, these individuals were thought to be the result of human transport from another population (10), but without the Floreana data presented here, the origin of this introduction could not be inferred. Thus, in these living individuals on Isabela, we have identified the genetic signature of a species that went extinct >150 years ago.

The evolutionary history of Floreana and the “non-native” individuals was assessed further by successfully analyzing variation at 10 nuclear microsatellite loci for 17 museum specimens (Table 1 and Table S2). With all 10 loci considered, the combined probability of identity of siblings (P_{ID-sib}) was on the order of 10^{-5} , demonstrating the power of this set of loci to discriminate between siblings (Fig. S2). These data were compared with a genotypic database containing 336 individuals from all extant Galápagos tortoise populations (19, 20). Bayesian clustering analysis revealed $K = 14$ as the “true” number of clusters, recovering a distinct group containing the Floreana and Floreana-like individuals from the populations on Volcano Wolf. Eleven of the 13 “non-native” tortoises on Isabela exhibited a strong signature of Floreana ancestry and a high probability of assignment to the Floreana cluster (q -values = 0.706–0.967) (Fig. 3A). The other two individuals (RU47 and PBL16) did not assign to the Floreana cluster, indicating mixed ancestry with a substantial contribution from the populations in which they were collected. The assignment tests in GeneClass2 exhibited a high degree of overlap with the results of STRUCTURE, indicating similar patterns of mixed ancestry of the “non-native” individuals from PBR, PBL, and RU populations (Table 2).

The triangle plot in Fig. 3B (see also Fig. S3) depicts a fine-scale examination of the history of the “non-native” individuals from Volcano Wolf. This plot was obtained through q -value distributions of 500 simulated genotypes each of parental populations, F1 hybrids, F2 hybrids, and backcrosses for all pairwise comparisons between samples from Floreana (FLO), Volcano Wolf (PBR and PBL), and Volcano Darwin (VD; Isabela) or Santa Cruz. The estimated probability of the “non-native” individuals having a hybrid origin are given in Table 3, indicating that all except PBR11 and PBR16 have high probabilities of having an ancestor in the Floreana population in the past two generations. Combined with the mtDNA results, these data suggest a hybrid origin of the “non-native” individuals from Isabela, revealing that they are recent descendants of a hybridization event between Floreana and native Isabela tortoises. More specifically, the specimens PBR11 and PBR16 are most likely to be second-generation backcrosses of a PBR-FLO mating to the resident PBR population, whereas the other 11 individuals are either F2 or backcross hybrids (Fig. S4).

The detection of Floreana descendants on Isabela provides evidence for the rediscovery of this unique, extinct lineage.

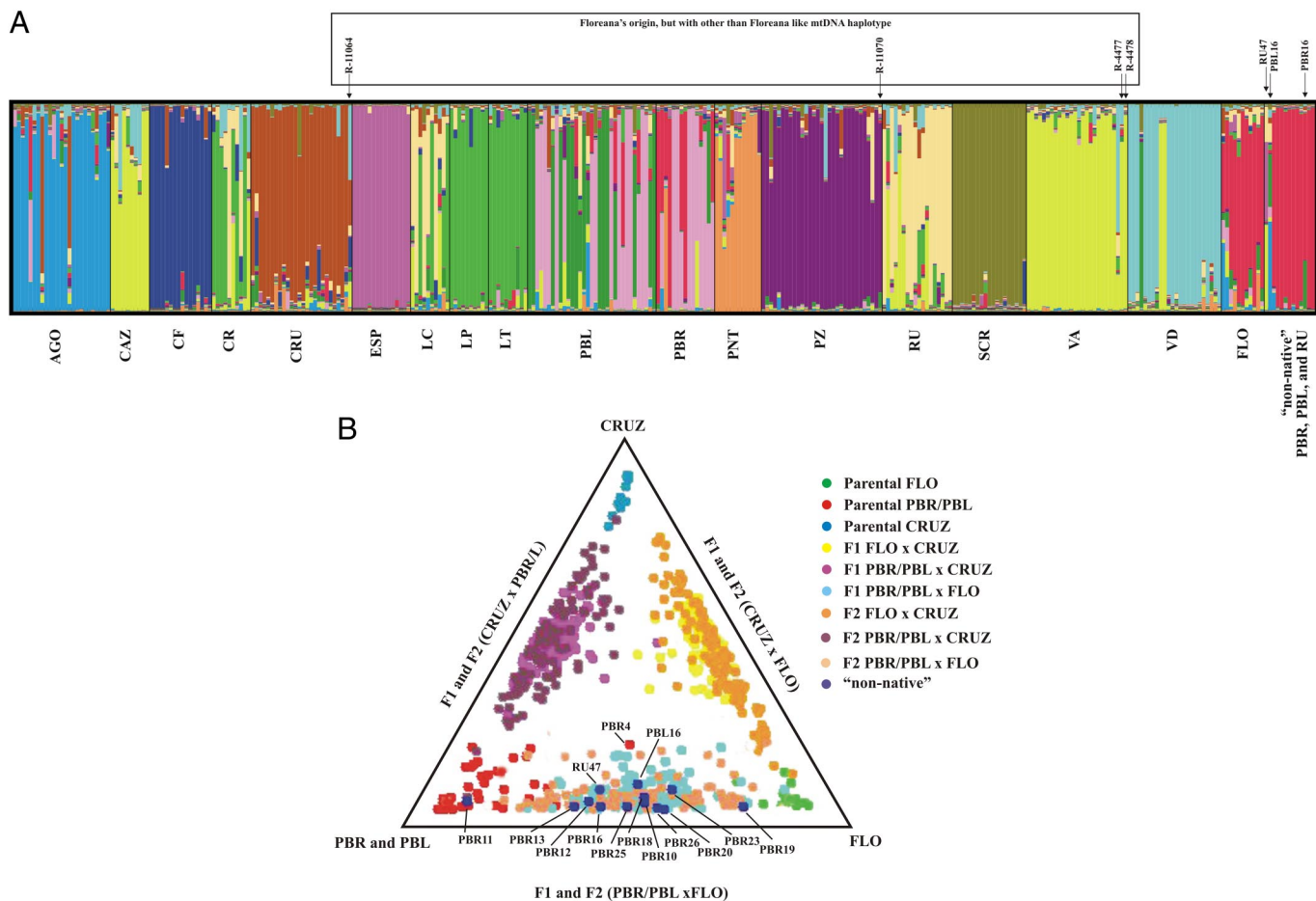


Fig. 3. STRUCTURE assignment plots. (A) A STRUCTURE bar plot indicating the genetic composition of all populations in the current study, highlighting the assignment of individuals sampled on Floreana and “non-native” individuals with Floreana-like mtDNA haplotypes sampled on Volcano Wolf, Isabela. Colors indicate the relative contribution of each of 14 genetic partitions recovered from the data for each individual (column) in each sampled population. Population acronyms: AGO = Santiago, CAZ = La Cazuela, CF = Cerro Fatal, CR = Cabo Rosa, CRU = Santa Cruz, ESP = Española, FLO = Floreana; LC = Los Crateres, LP = Las Pampas, LT = Las Tablas, PBL = Piedras Blancas, PBR = Puerto Bravo, PNT = Pinta, PZ = Pinzón, RU = Roca Union, SCR = San Cristóbal, VA = Volcano Alcedo, VD = Volcano Darwin. (B) A STRUCTURE triangle plot revealing patterns of clustering of empirical and simulated parental, F1, and F2 genotypes for all possible pairwise comparisons involving the Puerto Bravo (PBR) and Piedras Blancas (PBL) Volcano Wolf populations and the Floreana (FLO), and Santa Cruz (CRUZ) populations (see *SI Materials and Methods* for more details). For purposes of display, only the parental, F1, and F2 simulated populations are shown. The 13 “non-native” individuals from PBR, PBL, and RU are indicated.

Moreover, many of these hybrid individuals still maintain the saddlebacked morphology and potentially other characteristics that distinguish the tortoises of Floreana from native populations on Isabela. Perhaps most encouraging is that 40% of the individuals sampled on Volcano Wolf show mixed ancestry. The actual size of this population probably is in the thousands (20), presenting a potentially bountiful stock of individuals with which to initiate a captive breeding program in which targeted mate selection could help restore the genetic integrity of the Floreana population. Several studies from an array of organisms have shown that selective mating of hybrid individuals based on genotypic information can be used to restore the genetic constitution of the original, endangered population (21–23). In addition, the ability to breed Galápagos tortoises in captivity has been demonstrated successfully in other populations (21). Therefore, an attempt to find and remove these non-native individuals from Isabela should be the initial step in the recovery effort. Such a removal is vital to the establishment of a captive breeding program and also will prevent further introgression of these introduced lines into the native gene pool of Isabela tortoises.

Our findings rely heavily on the genetic and morphological information provided by museum specimens, illustrating the great benefits furnished by collections that are well organized and easily accessible. Of equal importance is the availability of a large genetic database on which potential hybrid specimens can be compared. Such a database can come only from long-term study of a system. As species continue to come under threat from human activities, even within World Heritage Sites such as the Galápagos, an increased depth of understanding will be instrumental in refining conservation strategies to protect what remains and potentially to resurrect what has been lost.

Methods

Detailed descriptions of the methods used to extract, amplify, and sequence DNA from the bones of the giant Galápagos tortoises, as well as the phylogenetic and population genetic methods used in analysis, are provided in the *SI Materials and Methods*.

Genetic Data. Bone samples of giant tortoises collected from the island of Floreana in nineteenth and twentieth centuries were obtained from the Museum of Comparative Zoology ($n = 16$) and American Museum Natural

Table 2. Lineage identification of the 13 individuals from PBR, PBL, and RU populations with Floreana-like mtDNA haplotype and the four individuals sampled on Floreana that exhibited non-native mtDNA haplotypes

Individual	Haplotype Name*	Closely Related Haplotype or Population		Mitochondrial DNA				Microsatellite Multilocus Genotypes				Island	STRUCTURE [§]	q
		Haplotype or Population	Distance†	GeneClass2 [‡]		L ₁		L ₂		Population	Island			
				Population	Island	L ₁	L ₂	Population	Island					
R-4477	New1	VD, VA, CAZ	1-2	VA	Isabela	13,444	VD	Isabela	13,597	VA, CAZ	Isabela		0.796	
R-4478	VD2 (28)	VD, VA, CAZ	0-2	VA	Isabela	10,461	VD	Isabela	15,528	VA, CAZ	Isabela		0.917	
R-11070	New1	PZ	1	PZ	Pinzon	16,774	—	—	—	PZ	Pinzon		0.805	
R-11064	New1	CRUZ	1	CRUZ	Santa Cruz	11,539	RU	Isabela	12,725	CRUZ, VA, CAZ, CF	Isabela, Santa Cruz		Mixed	
PBR10	PBR12 (80)	FLO	2-9	FLO	Floreana	19,088	PBR	Isabela	23,995	FLO	Floreana		0.825	
PBR11	PBR12 (80)	FLO	2-9	FLO	Floreana	16,643	PBL	Isabela	16,693	FLO	Floreana		0.889	
PBR12	PBR12 (80)	FLO	2-9	PBR	Isabela	18,889	FLO	Floreana	20,309	FLO	Floreana		0.955	
PBR13	PBR12 (80)	FLO	2-9	PBR	Isabela	20,179	FLO	Floreana	20,467	FLO	Floreana		0.965	
PBR16	PBR12 (80)	FLO	2-9	PBR	Isabela	23,229	PBL	Isabela	25,218	FLO	Floreana		0.689	
PBR18	PBR12 (80)	FLO	2-9	FLO	Isabela	15,875	FLO	Floreana	16,435	FLO	Floreana		0.883	
PBR19	PBR12 (80)	FLO	2-9	FLO	Floreana	18,150	PBR	Isabela	18,785	FLO	Floreana		0.958	
PBR20	PBR12 (80)	FLO	2-9	FLO	Floreana	17,132	PBR	Isabela	18,913	FLO	Floreana		0.937	
PBR23	PBR12 (80)	FLO	2-9	FLO	Floreana	19,590	PBR	Isabela	20,621	FLO	Floreana		0.959	
PBR25	PBR12 (80)	FLO	2-9	PBR	Isabela	19,366	FLO	Floreana	25,004	FLO	Floreana		0.948	
PBR26	PBR12 (80)	FLO	2-9	PBR	Isabela	22,293	FLO	Floreana	24,330	FLO	Floreana		0.967	
PBL16	PBL16 (77)	FLO	5-11	PBL	Isabela	17,216	FLO	Floreana	19,307	PBL, AGO	Isabela, Santiago		Mixed	
RU47	PBR12 (80)	FLO	2-9	RU	Isabela	17,407	PBL	Isabela	21,624	RU, CAZ, ESP	Isabela, Espanola		Mixed	

Abbreviations: AGO, Santiago; CAZ, La Cazuella; CRUZ, Santa Cruz; ESP, Española; FLO, Floreana; PBL, Piedras Blancas; PBR, Puerto Bravo; PZ, Pinzón; RU, Roca Union; VA, Volcano Alcedo; VD, Volcano Darwin.

*The haplotype names as in the study of Beheregaray et al.

†Haplotypes recovered among these individuals.

‡Distance indicates the number of nucleotide substitutions from the most closely related haplotype.

§Population and island assignment are according to the microsatellite genotypic data and algorithms implemented in GeneClass2 and STRUCTURE are indicated by their corresponding likelihood values (L₁ and L₂) and membership coefficients (q), respectively.

New haplotypes were recovered from these individuals.

"Non-native" individuals, Floreana-like haplotype	Population*			
	PBR/PBL Population*	FLO Population*		
	P ₁ †	P ₂ ‡	P ₃ §	P ₄ ¶
PBR10	0.282	0.000	0.002	0.716
PBR11	0.997	0.000	0.000	0.003
PBR12	0.618	0.000	0.002	0.380
PBR13	0.779	0.000	0.000	0.221
PBR16	0.962	0.000	0.000	0.038
PBR18	0.732	0.000	0.003	0.264
PBR19	0.012	0.092	0.176	0.719
PBR20	0.163	0.000	0.181	0.655
PBR23	0.698	0.000	0.000	0.302
PBR25	0.391	0.000	0.002	0.607
PBR26	0.074	0.000	0.010	0.916
PBL16	0.548	0.000	0.020	0.432
RU47	0.383	0.000	0.023	0.594

*PBR/PBL and FLO: empirical and simulated individuals as parental populations as estimated in STRUCTURE.

†P₁: probability of being from assumed population (PBR/PBL).

‡P₂: probability of being from the other population (FLO).

§P₃: probability of having a hybrid origin in the first past generation. Values are from one generation.

¶P₄: probability of having a hybrid origin in the second past generation. Values are from two generations.

History (n = 24) (Table 1). DNA was extracted from ≈ 0.1–0.2 g of bone (see *SI Materials and Methods*). All DNA extractions were carried out by independent researchers in two physically separated laboratories dedicated to the study of ancient DNA at Yale University, after all necessary precautions to prevent contamination by extant specimens.

Approximately 700 bp of DNA sequence from the mitochondrial control region were amplified in four overlapping fragments varying in size from 175 to 258 bp (including the primers). At least two sterile negative controls were used for each reaction to detect contamination throughout the amplification reaction. PCR products then were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced using BigDye Terminator cycle sequencing chemistry (v. 3.1) on an ABI 3730 automated sequencer (Applied Biosystems). Sequences were obtained from at least two amplifications of individual samples.

The variation of the Floreana specimens at 10 nuclear microsatellite loci relative to a genotypic database including 336 individuals from all extant populations of Galápagos tortoises was also analyzed (19, 20). To ensure consistency, the genotypes of the museum specimens were confirmed by amplifying and genotyping a locus five times for each specimen.

Phylogenetic and Population Genetic Analyses. The sequences from the museum specimens were combined with previously published sequences of the extant giant Galápagos tortoises and three outgroup taxa (*G. chilensis*, *G. denticulata*, and *G. carbonaria*). Mitochondrial DNA control region sequences for 116 extinct and extant tortoises were aligned in ClustalX, and Bayesian, maximum likelihood, and neighbor-joining phylogenetic analyses were conducted in MrBAYES (24), RAxML (25), and PAUP* (26), using the Tamura and Nei (27) +I+G model of evolution based on the results of the Akaike Information Criterion (28) as implemented in the program MODELTEST (29). Tests of alternative hypotheses were conducted in which maximum likelihood topologies resulting from the constrained and unconstrained searches were compared using the Shimodaira-Hasegawa log-likelihood test (30) as implemented in PAUP*. The alternative topologies tested include i) all Floreana individuals as a monophyletic group and ii) the two "non-native" haplotypes (PBR12, PBL16) in a monophyletic group with the rest of native PBR and PBL haplotypes.

The probability of two individuals sharing the same multilocus genotype $P_{(D)}$, was estimated using the software GIMLET (31) to explore the discrimination power of the microsatellite locus combination. The distinctiveness of the Floreana population was investigated using the Bayesian clustering approach implemented in STRUCTURE (32). For comparison, the assignment method of Rannala and Mountain (33) also was used, as implemented in GENECLASS2 (v. 2.0) (34). To determine the ancestry of the "non-native" individuals, a model-based clustering method was performed in STRUCTURE that estimates the proportion of an individual's genotype originating from sets of potentially hybridizing populations or taxa. The probability of a hybrid origin in the past two generations was

also estimated. To test the validity of population assignments and to identify the possible range of q -values for potential purebreds and different hybrid classes, a simulation was conducted for parental and hybrid genotypes using HYBRIDLAB (35) following the approach in Russello *et al.* (20).

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- Caccone A, Gibbs JP, Ketmaier V, Suatoni E, Powell JR (1999) Origin and evolutionary relationships of giant Galápagos tortoises. *Proc Natl Acad Sci USA* 96(23):13223–13228.
- Pritchard PCH (1996) The Galápagos tortoises: Nomenclatural and survival status. (Chelonian Research Foundation: Lunenburg, MA), pp 1–85.
- Darwin C (1845) *Journal of Researches into the Natural History and Geology of the Countries Visited During the Voyage of H.M.S. Beagle Round the World* (John Murray: London) p 519.
- FitzRoy R (1839) *Narrative of the Surveying Voyages of His Majesty's Ships Adventure and Beagle Between the Years 1826 and 1836, Describing Their Examination of the Southern Shores of South America, and the Beagle's Circumnavigation of the Globe. Proceedings of the Second Expedition, 1831–36, under the Command of Captain Robert Fitz-Roy, R.N.* (Henry Colburn, London).
- Ernst CH, Barbour RW (1997) *Turtles of the World* (Smithsonian Institution Press, Washington, DC).
- Broom R (1929) On the extinct Galápagos tortoise that inhabited Charles island. *Zoologica* 9:313–320.
- Zug GR (1997) Galápagos tortoise nomenclature: Still unresolved. *Chelonian Conserv Biol* 2:618–619.
- Van Denburgh J (1914) The gigantic land tortoises of the Galápagos archipelago. *Proceedings Calif Acad Sci* 2:203–374.
- Beheregaray LB, *et al.* (2004) Giant tortoises are not so slow: Rapid diversification and biogeographic consensus in the Galápagos *Proc Natl Acad Sci USA* 101(17):6514–6519.
- Caccone A, *et al.* (2002) Phylogeography and history of giant Galápagos tortoises. *Evolution* 56(10):2052–2066.
- Ciofi C, *et al.* (2006) Phylogeographic history and gene flow among giant Galápagos tortoises on southern Isabela Island. *Genetics* 172(3):1727–1744.
- Russello MA, *et al.* (2005) A cryptic taxon of Galápagos tortoise in conservation peril. *Biol Lett* 1(3):287–290.
- Russello MA, *et al.* (2007) Lineage identification of Galápagos tortoises in captivity worldwide. *Anim Conserv* 10(3):304–311.
- Fritts TH (1984) Evolutionary divergence of giant tortoises in Galápagos. *Biol J Linn Soc Lond* 21(1–2):165–176.
- Beheregaray LB, *et al.* (2003) Genes record a prehistoric volcano eruption in the Galápagos. *Science* 302(5642):75.
- Butler D, Gee H, Macilwain C (1998) Museum research comes off list of endangered species. *Nature* 394(6689):115–117.
- Baur G (1889) The gigantic land tortoises of the Galápagos islands. *Am Nat* 23:1039–1057.
- Townsend CH (1925) The Galápagos tortoises in relation to the whaling industry: A study of old logbooks. *Zoologica* 4:55–135.
- Ciofi C, Milinkovitch MC, Gibbs JP, Caccone A, Powell JR (2002) Microsatellite analysis of genetic divergence among populations of giant Galápagos tortoises. *Mol Ecol* 11(11):2265–2283.
- Russello MA, *et al.* (2007) Lonesome George is not alone among Galápagos tortoises. *Curr Biol* 17(9):R317–R318.
- Milinkovitch MC, *et al.* (2004) Genetic analysis of a successful repatriation programme: Giant Galápagos tortoises. *Proc R Soc Lond B Biol Sci* 271(1537):341–345.
- Milinkovitch MC, *et al.* (2007) Giant Galápagos tortoises; molecular genetic analyses identify a trans-island hybrid in a repatriation program of an endangered taxon. *BMC Ecol* 7(1):2.
- Marker-Kraus L, Grisham J (1993) Captive breeding of cheetahs in North-American zoos 1987–1991. *Zoo Biol* 12(1):5–18.
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17(8):754–755.
- Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22(21):2688–2690.
- Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4 (Sinauer Associates, Sunderland, MA).
- Tamura K, Nei M (1993) Estimation of the Number of nucleotide substitutions in the control region of mitochondrial-DNA in humans and chimpanzees. *Mol Biol Evol* 10(3):512–526.
- Akaike H (1974) New look at statistical-model identification. *IEEE Trans Automat Contr* 19(6):716–723.
- Posada D, Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14(9):817–818.
- Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol* 16(8):1114–1116.
- Valiere N (2002) GIMLET: A computer program for analysing genetic individual identification data. *Mol Ecol Notes* 2(3):377–379.
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155(2):945–959.
- Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proc Natl Acad Sci USA* 94(17):9197–9201.
- Piry S, *et al.* (2004) GENECLASS2: A software for genetic assignment and first-generation migrant detection. *J Hered* 95(6):536–539.
- Nielsen EEG, Bach LA, Kotlicki P (2006) HYBRIDLAB (version 1.0): A program for generating simulated hybrids from population samples. *Mol Ecol Notes* 6(4):971–973.