

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).

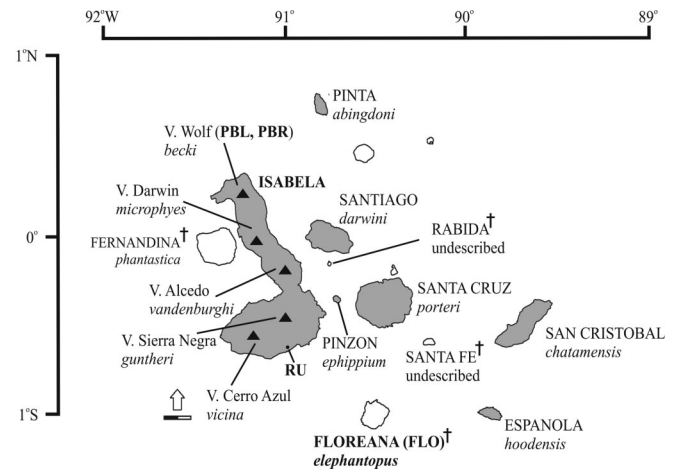


Fig. 1. Distribution of giant tortoises in the Galápagos archipelago. Shaded and non-shaded islands indicate presence of extant and extinct tortoise populations, respectively. Italicized names indicate current taxonomic designations (5, 8). Pinta is represented by a single male kept in captivity. †: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

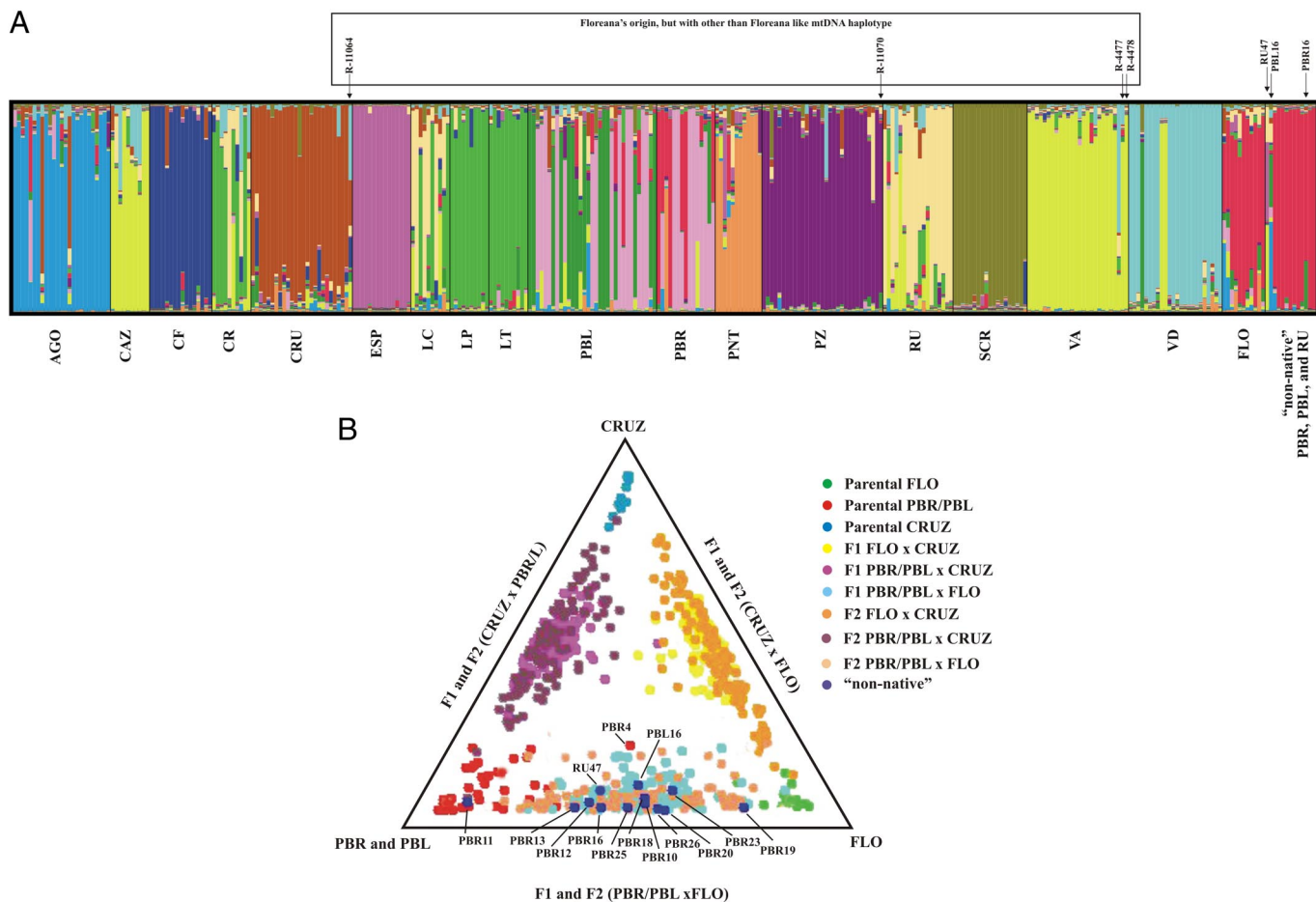


Fig. 3. STRUCTURE assignment plots. (A) A STRUCTURE bar plot indicating the genetic composition of all populations in the current study, highlighting the assignment of individuals sampled on Floreana and “non-native” individuals with Floreana-like mtDNA haplotypes sampled on Volcano Wolf, Isabela. Colors 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	Mitochondrial DNA				GeneClass2 [§]				STRUCTURE [§]					
	Haplotype Name ^a	Closely Related Haplotype or Population	Island	Distance ^b	Population	Island	L ₁	L ₂	Population	Island	L ₂	Population	Island	q
R-4477	New1	VD, VA, CAZ	Isabela	1-2	VA	Isabela	13.444	13.597	VA, CAZ	Isabela	13.597	VA, CAZ	Isabela	0.796
R-4478	VDz (28)	VD, VA, CAZ	Isabela	0-2	VA	Isabela	10.461	15.528	VA, CAZ	Isabela	15.528	VA, CAZ	Isabela	0.917
R-11070	New1	PZ	Pinzon	1	PZ	Pinzon	16.774	—	—	—	—	—	Pinzon	0.805
R-11064	New1	CRUZ	Santa Cruz	1	RU	Isabela	11.539	12.725	CRUZ, VA, CAZ, CF	Isabela, Santa Cruz	12.725	CRUZ, VA, CAZ, CF	Isabela, Santa Cruz	Mixed
PBR10	PBR12 (80)	FLO	Floreana	2-9	FLO	Floreana	19.088	23.995	PBR	FLO	23.995	FLO	Floreana	0.825
PBR11	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	16.643	16.693	PBR	FLO	16.693	FLO	Floreana	0.889
PBR12	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	18.889	20.309	FLO	FLO	20.309	FLO	Floreana	0.955
PBR13	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	20.179	20.467	FLO	FLO	20.467	FLO	Floreana	0.965
PBR16	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	23.229	25.218	PBR	FLO	25.218	FLO	Floreana	0.689
PBR18	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	15.875	16.435	FLO	FLO	16.435	FLO	Floreana	0.883
PBR19	PBR12 (80)	FLO	Floreana	2-9	FLO	Floreana	18.150	18.785	PBR	PBR	18.785	FLO	Floreana	0.958
PBR20	PBR12 (80)	FLO	Floreana	2-9	FLO	Floreana	17.132	18.913	PBR	Isabela	18.913	FLO	Floreana	0.937
PBR23	PBR12 (80)	FLO	Floreana	2-9	FLO	Floreana	19.590	20.621	PBR	Isabela	20.621	FLO	Floreana	0.959
PBR25	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	19.366	25.004	FLO	FLO	25.004	FLO	Floreana	0.948
PBR26	PBR12 (80)	FLO	Floreana	2-9	PBR	Isabela	22.293	24.330	FLO	FLO	24.330	FLO	Floreana	0.967
PBL16	PBL16 (77)	FLO	Floreana	5-11	PBL	Isabela	17.216	19.307	FLO	FLO	19.307	PBL, AGO	Isabela, Santiago	Mixed
RU47	PBR12 (80)	FLO	Floreana	2-9	RU	Isabela	17.407	21.624	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.

^aThe haplotype names as in the study of Beheregaray et al.

^bHaplotypes recovered among these individuals.

^cDistance indicates the number of nucleotide substitutions from the most closely related haplotype.

^dPopulation 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.

Table 3. The probabilities of Isabela "non-native" individuals having a hybrid origin in the past two generations

"Non-native" individuals, Floreana-like haplotype	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 $\approx 0.1-0.2$ 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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