Correspondence

Lonesome George is not alone among Galápagos tortoises

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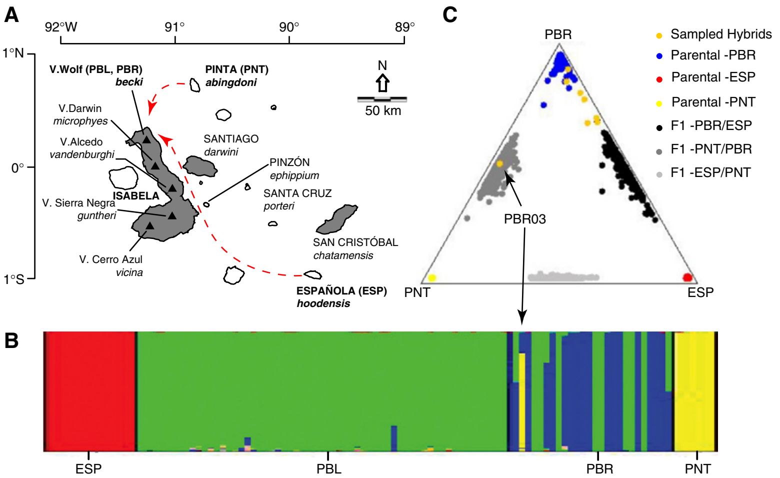

Current Biology

Figure 1. Giant tortoises of the Galápagos.

(A) Distribution of giant tortoises in the Galápagos archipelago. Shaded islands indicate presence of extant tortoise populations and italicized names indicate current taxonomic designations [4,5]. Island names are capitalized with triangles representing volcanoes on Isabela Island. Bold names designate the primary populations of focus in the current study. Red arrows highlight direction of transport/colonization consistent with the observed pattern of hybridization revealed for individual PBR03. (B) A STRUCTURE bar plot indicating the genetic composition of the principal populations in the current study, highlighting the mixed ancestry recovered in the PBR population. The analysis was run according to parameters specified in the supplemental data for all extant populations in Galápagos, but, for the purposes of display, only the clustering of the principal populations are shown above. Colors represent the relative contribution of each of four genetic partitions recovered from the data for each individual (column) in each sampled population. Population acronyms are as in (A). (C) A STRUCTURE triangle plot revealing patterns of clustering of simulated parental and F1 genotypes for all possible pairwise comparisons involving the Volcano Wolf Puerto Bravo (PBR), Pinta (PNT) and Española (ESP) populations. Colors for the parental populations are as in (B), with simulated F1s according to the legend. Clustering of the eight observed PBR individuals with the *G. hoodensis* (ESP)-like mtDNA haplotype (orange) are overlaid on top of the simulated parental and F1 distributions. The likely PNT/PBR F1 hybrid (PBR03) is indicated by arrows in (B) and (C).

The loss of biological diversity continues at an astounding rate, recently punctuated by the reported extinction of the Baiji or Yangtze River Dolphin (*Lipotes vexillifer*) [1]. The conservation landscape is dotted with similarly charismatic species that have become flagships for protection because of their increasing rarity. Although a miniscule fraction of biological diversity is at risk, flagship species effectively demonstrate the critical status of an ever-increasing number of taxa worldwide, potentially justifying the disproportionate amount of conservation resources directed towards their protection. Lonesome George, “the rarest living creature” according to the Guinness World Records, is perhaps the most renowned. The apparent sole survivor of the *Geochelone abingdoni* species of giant Galápagos tortoises from Pinta Island, Lonesome George is a potent conservation icon with much publicity surrounding the search for a mate [2,3]. Here, we report finding an individual of Pinta ancestry in a population on Volcano Wolf on neighboring Isabela Island.

Volcano Wolf harbors two populations, Puerto Bravo (PBR) and Piedras Blancas (PBL), of *G. becki*, one of the eleven extant species of Galápagos tortoises (Figure 1A) [4,5] (see Supplemental data available on-line with this issue for more details of the taxonomic history). Unlike other species that exhibit distinctive domed versus saddle-backed carapace morphology, the PBR population displays relatively high morphological diversity [6]. This diversity is consistent with molecular studies that revealed a complex history for the PBL and PBR populations on Volcano Wolf, including individuals with extremely divergent mitochondrial (mt)DNA haplotypes that are more similar to haplotypes found in other species than to those from the populations in which they were sampled [7]. The PBR population exhibits the highest proportion of individuals with divergent haplotypes: eight of 27 individuals have mtDNA haplotypes closely related to the sole haplotype from *G. hoodensis* on Española Island (Figure 1A) [7]. This non-native mtDNA haplotype differs by 27–30 substitutions from the endemic *G. becki* haplotypes in PBR. Likely shaped by both natural and human-mediated dispersal [7], the degree of nuclear introgression represented within these PBR individuals with divergent mtDNA haplotypes and their relationships to the former inhabitants of Pinta have never been assessed. To date, the ability to detect Pinta ancestry has been precluded by the lack of genotypic information regarding historical population allele frequencies from this locality.

We explored the complex evolutionary history of the *G. becki* PBR population on Isabela Island by analyzing variation at ten nuclear microsatellite loci relative to a genotypic database including 354 individuals from all extant populations of Galápagos tortoises [8]. The nearly extinct *G. abingdoni* on Pinta was included in this reference population database for the first time by way of genotypic data collection from six museum specimens. Bayesian clustering revealed a widespread pattern of mixed ancestry in the PBR population (Figure 1B and Supplemental data). Of particular note, one of eight PBR individuals with a *G. hoodensis* (Española)-like haplotype (PBR03) exhibited a strong signature of *G. abingdoni* ancestry and an assignment to the Pinta population (q-value = 0.743; Figure 1B). The other seven assigned to the PBR population from which they were collected (q-values ≥ 0.994).

The triangle plot in Figure 1C depicts a fine-scale examination of the history of mixed ancestry in the PBR population, obtained through q-value distributions of 500 simulated genotypes each of parental, F1 hybrids, F2 hybrids, and B2 and B3 backcrosses for all pairwise comparisons between *G. becki* (PBR, Isabela), *G. hoodensis* (Española), and *G. abingdoni* (Pinta). PBR03 falls in the center of the PBR–Pinta F1 q-value distribution (Figure 1C). Combined with the results from previous mtDNA analyses [7], these data suggest a hybrid origin of PBR03 resulting from a mating between a *G. becki* female from PBR with a *G. hoodensis* (Española)-like haplotype and a male from Pinta. Unfortunately PBR03 is a male. Our results also indicate that the seven additional PBR individuals with the divergent mtDNA haplotype are most likely at least second-generation backcrosses of PBR-Española F1s to the resident PBR population on Isabela Island.

The detection of Pinta ancestry on Volcano Wolf on Isabela Island provides evidence that Lonesome George is not the only living descendent of *G. abingdoni*. The identification of eight individuals of mixed ancestry among only 27 individuals sampled (estimated Volcano Wolf population size 1,000–2,000) [9] suggests the need to mount an immediate and comprehensive survey of the PBR population to search for additional individuals of Pinta ancestry. Given the failure of Lonesome George to reproduce despite considerable efforts over the past 30 years, it is clear that recovery of the genetically unique *G. abingdoni* will require identification of Pinta-native genotypes elsewhere; our data indicate that this goal may now be attainable. In the event that additional individuals of Pinta ancestry are discovered on Volcano Wolf or among the large number of individuals of unknown origin in captivity [10], an interactive *in situ* and *ex situ* conservation and repatriation program may be enacted for species recovery. This conservation strategy has had demonstrated success for a similarly imperiled sister species (*G. hoodensis*) [11], providing a model program for guiding future management of *G. abingdoni*. More generally, our results highlight the importance of historical DNA analysis, simulation approaches and reference population databases for reconstructing evolutionary patterns, revealing cryptic diversity, and informing conservation management.

Acknowledgments

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Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/17/9/R317/DC1

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SUPPLEMENTAL DATA

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Supplemental experimental procedures and results

Taxonomy

Fifteen formally described taxa of Galápagos tortoises are generally recognized, 11 of which are extant and threatened by human activities [S1]. The taxonomic rank 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 [S2]. Here we adopt the taxonomic classifications of S3 that treat all described taxa of Galápagos tortoises as separate species. This classification scheme is recognized by S1 and is the most consistent with the overwhelming morphological and molecular evidence now available [S4, S5].

Sampling and Data Collection

Blood samples collected from two sites on Volcano Wolf (*Geochelone becki*; PBL n = 62; PBR n = 27) were analyzed in the current study, a sub-set of which was used in previous studies [S5, S6]. Museum specimens were also sampled for six *G. abingdoni* collected on Pinta in an effort to reconstruct an initial estimate of historical population allele frequencies (California Academy of Sciences CAS8110, CAS8113; Charles Darwin Research Station CDRS_P04, CDRS_P13, CDRS_P15, CDRS_V870). Methods for DNA extraction, PCR amplification and sequencing of 697 bp fragment of the mitochondrial DNA (mtDNA) control region (CR), and PCR amplification and genotyping at ten microsatellite loci for all extant samples are reported elsewhere [S6, S7]. Similar methods relating to data collection from historical specimens are reported in S8.

Data Analysis

Degree of sequence similarity of the obtained mtDNA CR haplotypes was assessed relative to a database of 88 haplotypes recovered from approximately 800 individuals sampled from all extant populations throughout the Galápagos [S5, S6] using stand-alone Basic Local Alignment Search Tool (BLAST; ftp://ftp.ncbi.nlm.nih.gov/blast/). Eight PBR individuals were confirmed to have the divergent, Española *G. hoodensis*-like haplotype [S5], which differed from the two Volcano Wolf endemic haplotypes shared by the other nineteen individuals by 27-30 substitutions. Two of the six historical Pinta specimens share the same haplotype as Lonesome George, while three novel mtDNA CR haplotypes were found in the four remaining historical samples. These haplotypes were only one to two nucleotide substitutions different from the haplotype exhibited by Lonesome George. Across ten microsatellite loci, 18 new alleles were identified for the Pinta population, three being private to *G. abingdoni* (up from one private allele exhibited by Lonesome George alone). Genealogical relationships among all sampled haplotypes were reconstructed as a haplotype network using the statistical parsimony method of S9 as implemented in TCS, version 1.06 [S10]. Statistical parsimony reconstructed five disconnected networks with 90% confidence to account for the archipelago-wide mtDNA CR haplotype variation, similar to previous findings [S5]. One of the disconnected networks consisted of a grouping of the single Española *G. hoodensis* haplotype with a haplotype recovered from PBR just three steps away (data not shown). This group was connected to the four haplotypes recovered from Pinta by an additional 13 substitutions. The two haplotypes endemic to PBR were found in a separate, disconnected network, clustering with haplotypes from the neighboring PBL population as well as with those sampled on the nearby island of Santiago (data not shown).

Levels of nuclear DNA differentiation among populations were estimated by pairwise population comparisons of θ [S11] and Rho calculated in GENETIX [S12] and RSTCALC [S13], respectively. The historical Pinta population was significantly genetically differentiated from all other populations, on the basis of both θ and Rho (Table S1). The distinctiveness of the Pinta *G. abingdoni* population was further investigated using the Bayesian clustering approach implemented in STRUCTURE 2.1 [S14]. This model-based method was used to estimate the most appropriate number of populations (K) needed for interpreting the observed multilocus genotypic data. These data were based on 354 individuals sampled from all populations of extant taxa [S7, this study] as well as for the museum specimens of the nearly extinct *G. abingdoni* on Pinta [this study]. The number of populations with the highest posterior probabilities and accompanying lowest variance was identified and proportional membership of each tortoise to the assigned clusters was used as an estimate of distinctiveness of sampling locations. Membership coefficients (q) were estimated following a Markov chain Monte Carlo simulation (MCMC) of 1.0×10^6 repetitions following an initial “burnin” of 5.0×10^5 repetitions, assuming an admixed model with default parameters and correlated allele frequencies. In this case, the posterior probability was maximized (and variance minimized) at $K = 14$, recovering a distinct *G. abingdoni* population in addition to the 13 previously obtained clusters in S7. Once the number of distinct clusters was determined, STRUCTURE 2.1 [S14] was re-run using the identified units as prior information in order to infer the ancestry of all PBR individuals (results reported in main text).

The program HYBRIDLAB [S15] was then used to simulate parental and hybrid genotypes. This simulation was conducted to test the validity of population assignments, determine the ranges of q-values expected for parental and various hybrid types, and establish at what generation backcrosses become indistinguishable from purebred parental individuals [S16]. In this case, multi-locus genotypic data collected from population samplings on Pinta, Española, and PBR on Volcano Wolf (only individuals not previously identified as of mixed ancestry from initial analyses) were used as the parental populations for genotype simulations. Five hundred individuals were simulated for each parental population, as well as for all pairwise combinations of F1 hybrids, F2 hybrids, and B2 and B3 backcrosses. These simulated datasets were analyzed in STRUCTURE 2.1 [S14] using the previously described parameters and plotted in Figure 1c (parental and F1 only). Recent simulation [S17] and empirical hybridization studies [S16] have demonstrated a q-value threshold of 0.10 as an efficient cut-off for parental populations exhibiting F_{ST} values of 0.20. Observed pairwise F_{ST} values between the Pinta, Española, and PBR populations ranged from 0.22-0.41, indicating that the conventional q-value threshold of 0.10 is appropriate in the current study. In order to examine our ability to infer the degree of backcrossing reflective of the recent history of the other seven PBR individuals of mixed ancestry exhibiting the Española *G. hoodensis* -like genotype, the q-value distributions for the parental, F1, F2, B2 and B3 backcrosses between PBR and Española were plotted in Figure S1 and the amount of overlap assessed. The results are consistent with the previous STRUCTURE analyses indicating a probable F1 Pinta-V. Wolf PBR origin for PBR03, and an Española-V. Wolf PBR mixed ancestry for the remaining seven PBR individuals with the Española *G. hoodensis*-like mtDNA haplotype (Figure S1).

Table S1. Pairwise comparisons of θ (below) and Rho (below) values among all extant populations of Galápagos tortoises.

Island/	Pinta	Isabela											Santa Cruz		Pinzón	Santiago	San Cristóbal	Española
Volcano		V. Wolf N.	V. Wolf S.	V. Darwin	V. Alcedo	V. Sierra Negra			V. Cerro Azul									
	PNT	PBL	PBR	VD	VA	CAZ	RU	CR	LC	PEG	LP	LT	CRU	CF	PZN	AGO	SCR	ESP
PNT	-	**0.2119**	**0.2211**	**0.2407**	**0.3173**	**0.3284**	**0.2322**	**0.2214**	**0.1933**	**0.2417**	**0.2855**	**0.2868**	**0.2226**	**0.3092**	**0.2473**	**0.2217**	**0.3785**	**0.4187**
PBL	**0.2303**	-	0.0132	0.1144	0.1257	0.1883	0.0824	0.1018	0.0726	0.0903	0.1096	0.1021	0.1060	0.1606	0.1712	0.0644	0.1580	0.2407
PBR	**0.2164**	0.0186	-	0.1432	0.1389	0.2127	0.1061	0.1109	0.0908	0.1040	0.1260	0.1193	0.1284	0.2017	0.1590	0.0867	0.1719	0.2599
VD	**0.2454**	0.1358	0.1482	-	0.0981	0.1138	0.0846	0.0926	0.1014	0.1024	0.1395	0.1274	0.1490	0.2393	0.2201	0.1677	0.2115	0.3045
VA	**0.2586**	0.1163	0.1474	0.1392	-	0.1122	0.0784	0.1202	0.1148	0.1188	0.1507	0.1641	0.1595	0.2236	0.2192	0.1693	0.2004	0.3177
CAZ	**0.3742**	0.2006	0.2244	0.1723	0.0259	-	0.0554	0.0969	0.1261	0.1822	0.2101	0.3223	0.3160	0.4403	0.3863	0.2321	0.3734	0.5005
RU	**0.2228**	0.0945	0.1098	0.1011	0.0316	0.1150	-	0.0653	0.0384	0.0388	0.0741	0.1088	0.1137	0.2093	0.1885	0.1423	0.1858	0.2965
CR	**0.2828**	0.0769	0.1037	0.1232	0.0785	0.1394	0.0035	-	0.0025	0.0055	0.0591	0.0924	0.2003	0.2178	0.3038	0.1455	0.1917	0.2547
LC	**0.1375**	0.0457	0.0814	0.0490	0.0691	0.1395	0.0263	0.0173	-	-0.0068	0.0061	0.0384	0.0913	0.1612	0.2149	0.1143	0.1466	0.2880
PEG	**0.2528**	0.0578	0.0851	0.1686	0.0958	0.1663	0.0122	0.0190	-0.0054	-	0.0294	0.0585	0.1362	0.2147	0.3081	0.1425	0.1224	0.3377
LP	**0.1218**	0.0826	0.0753	0.1350	0.1673	0.1922	0.0745	0.0550	0.0276	0.0494	-	0.0056	0.1605	0.2322	0.2551	0.1709	0.1736	0.3573
LT	**0.2304**	0.0682	0.0619	0.1833	0.2622	0.1701	0.1260	0.0695	0.0492	0.0725	0.0169	-	0.1324	0.2123	0.2807	0.1588	0.1755	0.3587
CRU	**0.2433**	0.1992	0.2127	0.1998	0.2552	0.1923	0.2024	0.1562	0.1395	0.1932	0.2187	0.2468	-	0.0931	0.2652	0.1076	0.1716	0.3802
CF	**0.2782**	0.3259	0.3407	0.3207	0.3944	0.2883	0.3239	0.3302	0.2317	0.3364	0.3018	0.3788	0.0909	-	0.4138	0.1985	0.3022	0.4631
PZN	**0.2837**	0.1494	0.1687	0.2401	0.3270	0.2649	0.2742	0.2208	0.1827	0.2182	0.2348	0.2275	0.1923	0.2585	-	0.1870	0.4549	0.3444
AGO	**0.4316**	0.1187	0.1394	0.2531	0.3047	0.3402	0.2231	0.1750	0.1758	0.1744	0.1758	0.1412	0.2988	0.3932	0.3693	-	0.1667	0.2246
SCR	**0.2638**	0.1601	0.1723	0.3155	0.2741	0.2601	0.1563	0.2159	0.1734	0.2231	0.2323	0.2534	0.1801	0.2489	0.2371	0.1661	-	0.3437
ESP	**0.3564**	0.1259	0.1009	0.3864	0.3772	0.3983	0.2304	0.3428	0.2070	0.1879	0.1913	0.1964	0.2782	0.3429	0.3713	0.2954	0.2639	-

Bold cells highlight pairwise θ and Rho values for Pinta for all possible comparisons, of which all are highly significant at the $P < 0.01$ level. Acronyms indicating sampling sites are as follows: Pinta (PNT); Piedras Blancas (PBL); Puerto Bravo (PBR); Volcano Darwin (VD); Volcano Alcedo (VA); La Cazuela (CAZ); Roca Union (RU); Cabo Rosa (CR); Los Crateres (LC); Pegas (PEG); Las Pampas (LP); Las Tablas (LT); La Caseta (CRU); Cerro Fatal (CF); Pinzón (PZN); Santago (AGO); San Cristóbal (SCR); Española (ESP).

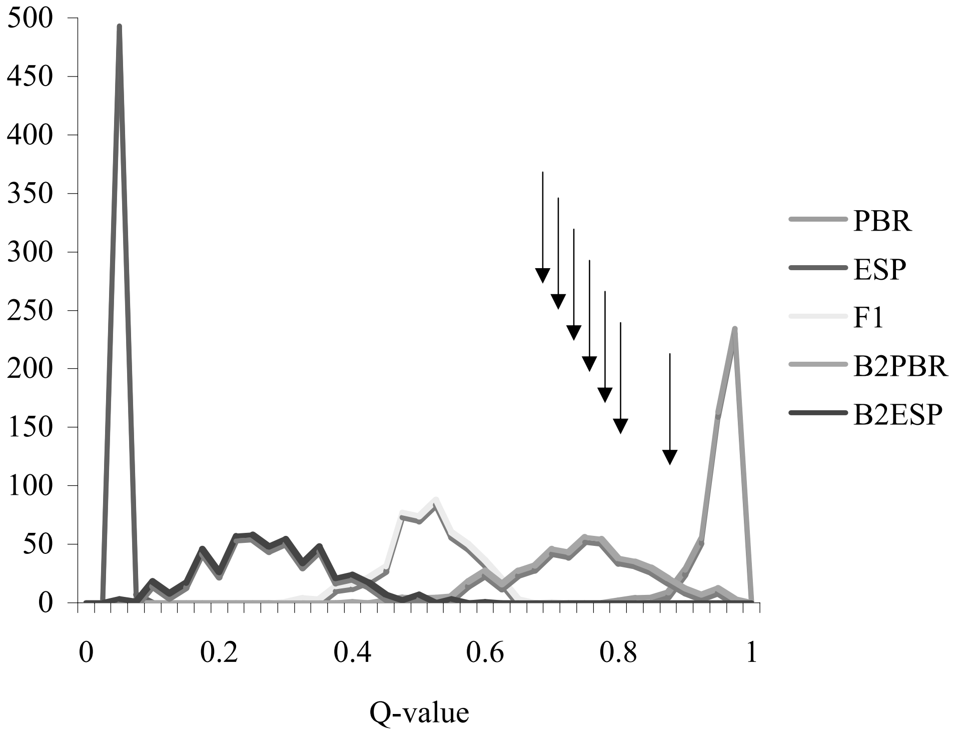

Figure S1. Frequency distributions of Q for all hybrid types. Parental populations were simulated from empirical allele frequencies sampled in Puerto Bravo (PBR) on Volcano Wolf, Isabela Island, and Española Island (ESP). F1 simulated hybrids were generated by crosses between the PBR and ESP parental populations. B2 backcrosses resulted from simulating crosses from PBR/ESP F1's back to either the parental PBR (B2PBR) or ESP (B2ESP). Q-values of simulated F1 hybrids ranged from 0.28 to 0.69 (mean = 0.50), clearly distinct from either parental population. Simulated F2 hybrids also exhibited little (e.g. PBR 1.2%) to no overlap (e.g. Española 0.0%) with the parental populations with q-values ranging from 0.16 to 0.94 (mean = 0.51). The ranges of q-values for B2 and B3 backcrosses (not shown) of the hybrids to the parental populations were as follows: B2-PBR 0.40-0.96 (mean = 0.73); B3-PBR

0.39-0.97 (mean = 0.85); B2-Española 0.03-0.58 (mean = 0.26); B3-Española 0.03-0.41 (mean = 0.15). Simulated B2-PBR and B3-PBR backcrosses exhibited 27.6% and 75.4% overlap with simulated PBR parental individuals, respectively. Hybrid backcrosses to Española showed a much lower degree of overlap with q-values from the parental population (B2-Española 0.8%; B3-Española 13.8%). In general, 80.2% of the simulated genotypes of hybrid origin over three generations are distinguishable from the parental populations on PBR and Española. Of more immediate relevance, 82.6% of simulated genotypes of hybrid origin (F1, B2 and B3) were discernible from the PBR parental population based on this panel of loci and a q-value cutoff threshold < 0.10. The arrows in Figure S1 represent the q-values of the seven PBR individuals of mixed PBR and Española ancestry relative to the simulated distributions. The observed values range from 0.68 to 0.91, with the mean of 0.77 falling in between the means of the B2-PBR and B3-PBR q-value distributions. These data suggest that the seven additional PBR individuals of mixed ancestry are most likely at least second-generation backcrosses of PBR-Española F1s to the resident population on Volcano Wolf.

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