

Lineage identification of Galápagos tortoises in captivity worldwide

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Keywords

Geochelone nigra (*elephantopus*); microsatellite; mitochondrial DNA control region; population assignment; captive breeding; historical DNA.

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Received 15 December 2006; accepted 15 March 2007

doi:10.1111/j.1469-1795.2007.00113.x

Abstract

Ex situ conservation strategies may be substantially informed by genetic data, and yet only recently have such approaches been used to facilitate captive population management of endangered species. The Galápagos tortoise *Geochelone nigra* is an endangered species that has benefited greatly from the application of molecular and population genetic data, but remains vulnerable throughout its range. The geographic and evolutionary origins of 98 tortoises in private collections and zoos on three continents were identified using mitochondrial DNA (mtDNA) control region sequences and multi-locus microsatellite genotype data relative to a large database of representative samplings from all extant populations, including historical population allele frequency data for the *Geochelone nigra abingdoni* taxon on Pinta by way of museum specimens. All but six individuals had mtDNA haplotypes previously sampled, with the novel haplotypes identified as most closely related to robust populations on the islands of Santa Cruz and Isabela. Multi-locus genotypic assignments corroborated the results obtained from the mtDNA analyses, with 83.7% of individuals consistently assigned to the same locality by both datasets. Overall, the majority of captive unknowns sampled were assigned to the La Caseta *Geochelone nigra porteri* population, with no fewer than six individuals of hybrid origin detected. Although a purported Pinta individual was revealed to be of Pinzón ancestry, the two females currently housed with Lonesome George exhibited haplotypic and genotypic signatures that indicate that they are among the most appropriate matches for captive breeding. More generally, molecular approaches continue to represent important tools for assessing conservation value, minimizing hybridization and guiding management programs for preserving the distinctiveness of *G. nigra* taxa in captivity.

Introduction

Endangered species management has increasingly benefited from the insights gained through the application of molecular and population genetic data. From elucidating conservation units and resolving taxonomic uncertainty to reconstructing patterns of population differentiation and their formative processes, the tools of molecular and population genetics are now routinely directed towards addressing issues of conservation relevance (reviewed in DeSalle & Amato, 2004). In spite of the large body of research applying molecular approaches towards the investigation of conservation-related questions *in situ*, comparatively few studies

have applied these tools for guiding *ex situ* population management programs. *Ex situ* conservation strategies directed toward minimizing inbreeding, maximizing mean kinship, establishing parentage, avoiding hybridization and identifying appropriate individuals for reintroduction may all be substantially informed by genetic data, and yet only recently have such approaches been used to facilitate captive population management of endangered species (Doyle *et al.*, 2001; Jones *et al.*, 2002; Norton & Ashley, 2004; Russello & Amato, 2004; Rodriguez-Clark & Sanchez-Mercado, 2006).

The Galápagos tortoise *Geochelone nigra* is an endangered species that has benefited greatly from the application of molecular and population genetic data. Numerous

studies have been directed towards assessing the distinctiveness of the 11 extant taxa and their relative evolutionary relationships, recovering reciprocally monophyletic groups on all major islands, except for the largest island of Isabela, where four of the five named taxa were found to be genetically distinct (Caccone *et al.*, 2002; Ciofi *et al.*, 2002; Beheregaray *et al.*, 2003; Russello *et al.*, 2005). In addition, molecular and population genetic approaches have been instrumental in facilitating and evaluating the success of captive breeding and reintroduction programs within the group (Burns *et al.*, 2003; Milinkovitch *et al.*, 2004, 2007).

Despite the tremendous accomplishments of on-going *in situ* and *ex situ* conservation programs, the Galápagos tortoise remains vulnerable throughout its range (IUCN, 2006), limited to populations on six islands within this remote, oceanic archipelago (MacFarland, Villa & Basilio, 1974). The most extreme example is the *Geochelone nigra abingdoni* taxon on the island of Pinta, which is represented by only a single known male, Lonesome George, currently housed at the Charles Darwin Research Station (CDRS). Additional taxa restricted to the islands of Pinzon (*Geochelone nigra ephippium*), Española (*Geochelone nigra hoodensis*) and eastern Santa Cruz (Cerro Fatal *Geochelone nigra porteri*) are also critically endangered, characterized by small population sizes, impending human-related threats and, in some cases, reduced levels of genetic variation (Milinkovitch *et al.*, 2004; Russello *et al.*, 2005). In general, extant taxa continue to be impacted by issues associated with human population growth in the region, including habitat modification and a long legacy of introduction of non-native organisms such as burros, goats and black rats throughout the islands (MacFarland *et al.*, 1974; Powell & Gibbs, 1995; Pritchard, 1996; Kaiser, 2001).

Within the uncertain conservation climate surrounding the Galápagos tortoise, captive populations represent a critical hedge against extinction and an important source of individuals for population reinforcement. A previous study by our group identified the geographic and evolutionary origin of 59 tortoises of unknown ancestry housed in three enclosures at the CDRS on Santa Cruz utilizing mitochondrial DNA (mtDNA) control region sequences and multi-locus microsatellite genotype data relative to a large database of haplotypic and genotypic data collected for population samplings of all extant populations throughout Galápagos (Burns *et al.*, 2003). This work was of immediate relevance for guiding the captive breeding programs at CDRS. However, the number of *G. nigra* held in collections outside of Galápagos is an order magnitude greater. These tortoises are spread throughout the world's zoos and private collections, many of which are of unknown ancestry and uncertain conservation value.

The current study identifies the geographic and evolutionary origin of tortoises in private collections and zoos on three continents, utilizing mtDNA control region sequences and multi-locus microsatellite genotype data relative to an expanded database of haplotypic and genotypic data collected for population samplings of all extant populations, including population allelic data for the *G. n. abingdoni*

taxon on Pinta by way of museum specimens. Historical information obtained in this study is intended for immediate integration into ongoing *in situ* and *ex situ* conservation programs for the Galápagos tortoise.

Materials and methods

Study area

The Galapagos archipelago includes 123 islands and minor islets between 1°25.00'S–1°40.00'N and 268°00.00'E–270°45.00'E (Snell, Stone & Snell, 1996). Historically, as many as 11 islands have been reported to harbor *G. nigra*, and yet only six retain populations today. Fifteen formally described taxa of *G. nigra* are generally recognized, 11 of which are extant and threatened by human activities and introductions of non-native species (Pritchard, 1996). Expeditions conducted over a 7-year period collectively obtained blood samples from 847 tortoises representing all extant populations in support of a host of population genetic and phylogeographic studies (Caccone *et al.*, 1999; Ciofi *et al.*, 2002; Beheregaraya *et al.*, 2003; Russello *et al.*, 2005).

Sampling

For the current study, blood samples were obtained from 98 tortoises of unknown origin from the following captive collections: Caloosahatchee Aviary and Botanical Garden, FL, USA (CABG; *n* = 25); CDRS, Santa Cruz, Galápagos (CDRS; *n* = 2); mainland Ecuador hotels, universities, zoological and private collections (ECU; *n* = 29); former Witmer Collection on Floreana, Galápagos (FLO; *n* = 29); Prague Zoo, Czech Republic (PRZ; *n* = 2); San Diego Zoo, USA (SDZ; *n* = 7); and Zurich Zoo, Switzerland (ZUZ; *n* = 4). Individuals sampled from CABG were acquired as adults of unknown origin. Seven CABG individuals (CABG05-07, CABG09, CABG12-14) were labeled as originating from colonies established at the New York Zoological Society by Charles H. Townsend in 1928 (Townsend, 1931). The two females currently cohabitating with Lonesome George at the CDRS were originally brought into captivity directly from Volcán Wolf on northern Isabela. Given the complex history and diverse morphology associated with these populations (Caccone *et al.*, 2002), these two females, CDRS106 and CDRS107, were included in the current analysis. Individuals sampled across mainland Ecuador originated from the following institutions, none of which were accompanied by detailed information regarding their history of acquisition: Colegio Americano de Quito (ECU01, ECU02), a private collection in Pumebo Valley (ECU03), Escuela Superior Politécnica del Litoral de Guayaquil (ESPOL; ECU04-ECU06, ECU15-ECU16), Zoo de Baños (ECU07-ECU10), Hotel Rumipamba de las Rosas (ECU11-ECU12), Animal Rescue Center Jambeli (ECU13-ECU14) and the Parque Zoológico Metropolitana de Guayabamba (ECU17-ECU29). Captive individuals residing on Floreana were repossessed by the Galápagos National Park Service

(GNPS) from Margaret Witmer's estate and have since been managed by the GNPS. The origin and history of these animals remain ambiguous to this day. The two individuals at the Prague Zoo originated from zoo Koln am Rhein where they were acquired as juveniles of unknown origin in 1972. Zurich Zoo individuals have been in residence since 1946 (ZUZ01) and 1962 (ZUZ10, ZUZ20, ZUZ30), respectively, all of which were of unknown origin pre-dating their arrival. Where available, studbook information allowed us to target individuals with purportedly pure bloodlines and no history of hybridization. All samples were collected in accordance with local, national and international regulations (IACUC protocol # 2004-10825; CITES permit # 06US784934/9).

mtDNA analysis

DNA was extracted from all blood samples using the Qiagen DNeasy Tissue Kit and the manufacturer's protocols (Qiagen Inc., Valencia, CA, USA). An ~695 base pair fragment of the mtDNA control region was amplified by way of polymerase chain reaction (PCR) following the conditions in Caccone *et al.* (1999). Double-stranded PCR products were sequenced using Big Dye 3.1 terminators on an Applied Biosystems 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The degree of sequence similarity of the obtained fragments relative to a database of 88 haplotypes recovered from the 847 individuals sampled from all extant populations throughout Galápagos (Caccone *et al.*, 2002; Ciofi *et al.*, 2002; Beheregaray *et al.*, 2003; Russello *et al.*, 2005) was assessed using the stand-alone Basic Local Alignment Search Tool (BLAST; <ftp://ftp.ncbi.nlm.nih.gov/blast/>). This approach was also used to estimate the number of pairwise substitutions between each individual and the 88 known haplotypes. A haplotype network was reconstructed under the principle of statistical parsimony as implemented in TCS (Clement, Posada & Crandall, 2000) to determine the relationships of newly identified haplotypes relative to those previously recovered from the wild-sampled individuals.

Microsatellite analysis

Genotypic data were obtained for all individuals at ten microsatellite loci (GAL45, GAL50, GAL73, GAL75, GAL94, GAL100, GAL127, GAL136, GAL159, GAL263; Ciofi *et al.*, 2002) using an Applied Biosystems 3730 DNA sequencer. These data were subsequently analyzed relative to a large database of multi-locus genotypes at overlapping loci for 354 individuals sampled from all populations of extant taxa as well as for six museum specimens of the nearly extinct *G. n. abingdoni* on Pinta (Russello *et al.*, in press).

Individuals of unknown ancestry were assigned to island populations based on their multi-locus genotypes using two separate approaches. First, the exclusion-simulation test of the partial Bayesian assignment method of Rannala & Mountain (1997) was used to assign individuals to the two closest natural populations where the likelihoods of its

genotype occurring were the highest (L_1 and L_2) as implemented in GENECLASS (Cornuet *et al.*, 1999). The exclusion threshold was set to 0.01, relative to a distribution estimated from 10 000 randomly generated genotypes. In addition, the Bayesian model-based clustering method of Pritchard, Stephens & Donnelly (2000) for inferring population structure and assigning individuals to populations was used as implemented in Structure 2.1. Membership coefficients (q) of the captive unknowns in one or more of the reference populations was estimated following a Markov chain Monte Carlo simulation (MCMC) of 1.0×10^6 repetitions following an initial 'burnin' of 5.0×10^4 repetitions. The q of an individual for a particular population represents the fraction of its genome that has ancestry in that population. Given the large body of research directed towards reconstructing population structure and genetic distinctiveness of the extant named taxa of *G. nigra* (Caccone *et al.*, 2002; Ciofi *et al.*, 2002; Beheregaray *et al.*, 2003; Russello *et al.*, 2005), analyses were run using a model that utilized prior population information, as recommended by Pritchard *et al.* (2000).

Results

Population assignment: mtDNA analysis

Overall, 20 mtDNA control region haplotypes were recovered among the 98 sampled individuals of unknown ancestry. All but six of the haplotypes were identical to one of the 88 haplotypes previously identified from extensive samplings of the wild populations throughout Galápagos (Table 1). The six new haplotypes found in the current study differed from existing haplotypes by one or two substitutions (Table 1). Based on pairwise nucleotide differences as well as recovered relationships via a haplotype network reconstructed under statistical parsimony (95% confidence; data not shown), the novel haplotypes were identified as most closely related to La Caseta *G. n. porteri* on Santa Cruz (haplotype 18, ZZ01; haplotype 21, ECU28; haplotype 87, CABG24/CABG29; haplotype 88, PRZ02), and Sierra Negra *Geochelone nigra guntheri* on the island of Isabela (haplotype 54, CABG12; haplotype 55, ECU26).

Overall, approximately half of the individuals (49.0%) of unknown ancestry sampled on three different continents were assigned to the La Caseta *G. n. porteri* population on Santa Cruz based on their mtDNA haplotypes. The vast majority of the remaining individuals exhibited haplotypes sampled on northern Isabela (PBL, $n = 11$; PBR, $n = 4$; VA, $n = 12$; VD, $n = 4$) (Table 1; Fig. 1).

Population assignment: microsatellite analysis

Multi-locus genotypic data were used to assign all individuals of unknown ancestry to a population of origin relative to a large database of 354 field-collected samples representing all extant populations, including museum specimen exemplars from the island of Pinta. The assignment tests of

Table 1 Lineage identification of Galápagos tortoises *Geochelone nigra* of unknown ancestry based on mtDNA and microsatellite data

Mitochondrial DNA control region					Microsatellite multi-locus genotypes									
#	Haplo-type	Popula-tion	Island	Dis-tance	Genbank	Rannala & Mountain (1997)					Pritchard <i>et al.</i> (2000)			
						Popula-tion	Island	L_1	Popula-tion	Island	L_2	Popula-tion	Island	q
CABG01	54	CAZ	Isabela	0	AF548257	CRU	Santa Cruz	20.47	VA	Isabela	23.67	CRU	Santa Cruz	0.635
CABG04	52	PBL	Isabela	0	AF548255	PBL	Isabela	14.62	PBR	Isabela	18.54	PBL	Isabela	0.824
CABG05	88	CRU	Santa Cruz	0	AY268588	CF	Santa Cruz	24.76	CRU	Santa Cruz	26.32	CRU	Santa Cruz	0.389
CABG06	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	21.24	LC	Isabela	22.50	CAZ	Isabela	0.752
CABG07	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	19.74	VA	Isabela	24.48	CAZ	Isabela	0.850
CABG09	55	CAZ	Isabela	0	AF548258	RU	Isabela	18.65	CAZ	Isabela	18.84	CAZ	Isabela	0.791
CABG10	61	VA	Isabela	0	AF548264	PEG	Isabela	19.16	LP	Isabela	19.63	LP	Isabela	0.537
CABG11	54	CAZ	Isabela	0	AF548257	VD	Isabela	18.44	CRU	Santa Cruz	20.19	VD	Isabela	0.476
CABG12	54	CAZ	Isabela	1	AF548257	VA	Isabela	17.65	CAZ	Isabela	18.63	CAZ	Isabela	0.933
CABG13	55	CAZ	Isabela	0	AF548258	VD	Isabela	22.78	VA	Isabela	24.17	CAZ	Isabela	0.468
CABG14	61	VA	Isabela	0	AF548264	VA	Isabela	12.47	PEG	Isabela	19.52	VA	Isabela	0.890
CABG15	54	CAZ	Isabela	0	AF548257	CAZ	Isabela	13.74	VA	Isabela	17.24	CAZ	Isabela	0.855
CABG18	54	CAZ	Isabela	0	AF548257	CAZ	Isabela	21.27	CR	Isabela	22.23	CAZ	Isabela	0.625
CABG19	54	CAZ	Isabela	0	AF548257	LC	Isabela	19.95	RU	Isabela	22.62	LC	Isabela	0.606
CABG21	54	CAZ	Isabela	0	AF548257	PBL	Isabela	25.58	PBR	Isabela	26.37	CAZ	Isabela	0.421
CABG23	88	CRU	Santa Cruz	0	AY268588	VD	Isabela	23.23	CAZ	Isabela	23.48	CAZ	Isabela	0.943
CABG24	87	CRU	Santa Cruz	1	AY268587	CAZ	Isabela	24.77	RU	Isabela	25.83	CAZ	Isabela	0.409
CABG25	88	CRU	Santa Cruz	0	AY268588	LC	Isabela	24.35	CRU	Santa Cruz	25.18	CAZ	Isabela	0.936
CABG27	61	VA	Isabela	0	AF548264	VA	Isabela	19.40	VD	Isabela	19.65	VA	Isabela	0.554
CABG28	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	25.23	LC	Isabela	26.32	CAZ	Isabela	0.940
CABG29	87	CRU	Santa Cruz	1	AY268587	RU	Isabela	15.91	VA	Isabela	18.18	RU	Isabela	0.254
CABG30	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	20.83	CAZ	Isabela	21.07	CAZ	Isabela	0.942
CABG31	54	CAZ	Isabela	0	AF548257	CR	Isabela	14.81	VA	Isabela	16.10	CAZ	Isabela	0.439
CABG296	52	PBL	Isabela	0	AF548255	PBL	Isabela	9.35	LP	Isabela	10.57	CRU	Santa Cruz	0.853
CABG297	52	PBL	Isabela	0	AF548255	PBL	Isabela	8.68	LC	Isabela	12.63	CRU	Santa Cruz	0.568
CDRS106	78	PBR	Isabela	0	AF548281	PBR	Isabela	20.46	PBL	Isabela	27.92	PBR	Isabela	0.936
CDRS107	78	PBR	Isabela	0	AF548281	PBR	Isabela	23.75	PBL	Isabela	29.92	PBR	Isabela	0.931
ECU01	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	17.03	CF	Santa Cruz	21.89	CRU	Santa Cruz	0.633
ECU02	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	16.15	CF	Santa Cruz	19.93	CRU	Santa Cruz	0.525
ECU03	55	CAZ	Isabela	0	AF548258	PEG	Isabela	18.20	LT	Isabela	18.85	CAZ	Isabela	0.416
ECU04	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	15.54	CF	Santa Cruz	18.88	CRU	Santa Cruz	0.853
ECU05	88	CRU	Santa Cruz	0	AY268588	PEG	Isabela	19.17	LC	Isabela	19.54	LC	Isabela	0.424
ECU06	14	CRU	Santa Cruz	0	AF548217	CRU	Santa Cruz	17.46	LC	Isabela	21.69	LC	Isabela	0.730
ECU07	52	PBL	Isabela	0	AF548255	PBL	Isabela	13.99	PBR	Isabela	24.14	PBL	Isabela	0.888
ECU08	61	VA	Isabela	0	AF548264	VA	Isabela	13.89	CAZ	Isabela	15.10	CAZ	Isabela	0.563
ECU09	55	CAZ	Isabela	0	AF548258	CAZ	Isabela	21.83	RU	Isabela	25.54	CAZ	Isabela	0.874
ECU10	28	VD	Isabela	0	AF548231	VD	Isabela	10.88	VA	Isabela	20.56	VD	Isabela	0.929
ECU11	14	CRU	Santa Cruz	0	AF548217	CRU	Santa Cruz	11.49	CF	Santa Cruz	17.76	CRU	Santa Cruz	0.938
ECU12	25	PBR	Isabela	0	AF548228	PBR	Isabela	10.32	PBL	Isabela	14.80	PBL	Isabela	0.675
ECU13	61	VA	Isabela	0	AF548264	LT	Isabela	15.50	LP	Isabela	16.00	LP	Isabela	0.612
ECU14	54	CAZ	Isabela	0	AF548257	VA	Isabela	13.79	VD	Isabela	19.60	VA	Isabela	0.785
ECU15	78	PBR	Isabela	0	AF548281	PBR	Isabela	24.21	AGO	Isabela	27.96	PBR	Isabela	0.759
ECU16	15	CRU	Santa Cruz	0	AF548218	VD	Isabela	21.82	CR	Isabela	23.90	VD	Isabela	0.472
ECU17	86	CRU	Santa Cruz	0	AY268586	CF	Santa Cruz	14.69	CRU	Isabela	14.80	CRU	Santa Cruz	0.732
ECU18	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	15.58	RU	Isabela	17.23	CRU	Santa Cruz	0.428
ECU19	27	VD	Isabela	0	AF548231	VD	Isabela	8.99	CAZ	Isabela	16.90	VD	Isabela	0.940
ECU20	14	CRU	Santa Cruz	0	AF548217	CRU	Santa Cruz	14.87	CF	Santa Cruz	18.35	CRU	Santa Cruz	0.867
ECU21	61	VA	Isabela	0	AF548264	VD	Isabela	21.84	PEG	Isabela	22.95	PEG	Isabela	0.559
ECU22	61	VA	Isabela	0	AF548264	VA	Isabela	19.80	CR	Isabela	20.50	VA	Isabela	0.248
ECU23	52	PBL	Isabela	0	AF548255	PBL	Isabela	16.57	PEG	Isabela	23.51	PBL	Isabela	0.847
ECU24	61	VA	Isabela	0	AF548264	RU	Isabela	16.77	CAZ	Isabela	17.45	CAZ	Isabela	0.591
ECU25	61	VA	Isabela	0	AF548264	PBL	Isabela	17.14	LT	Isabela	17.42	PBL	Isabela	0.239
ECU26	55	CAZ	Isabela	1	AF548258	CAZ	Isabela	17.48	LT	Isabela	19.09	CAZ	Isabela	0.822
ECU27	61	VA	Isabela	0	AF548264	PBL	Isabela	16.10	CRU	Santa Cruz	21.71	PBL	Isabela	0.606
ECU28	21	CRU	Santa Cruz	2	AF548224	CRU	Santa Cruz	15.71	CF	Santa Cruz	19.03	CRU	Santa Cruz	0.880

Table 1. Continued.

Mitochondrial DNA control region						Microsatellite multi-locus genotypes								
#	Haplo-type	Popula-tion	Island	Dis-tance	Genbank	Rannala & Mountain (1997)				Pritchard <i>et al.</i> (2000)				
						Popula-tion	Island	L_1	Popula-tion	Island	L_2	Popula-tion	Island	q
ECU29	52	PBL	Isabela	0	AF548255	PBL	Isabela	15.61	LC	Isabela	20.06	PBL	Isabela	0.742
FLO01	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	23.49	CRU	Santa Cruz	24.07	CRU	Santa Cruz	0.964
FLO02	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	22.85	LP	Isabela	25.38	CRU	Santa Cruz	0.537
FLO03	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	21.96	PBL	Isabela	22.71	CRU	Santa Cruz	0.962
FLO04	88	CRU	Santa Cruz	0	AY268588	CRU	Santa Cruz	23.87	PBL	Isabela	25.01	CRU	Santa Cruz	0.964
FLO05	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	22.06	CRU	Santa Cruz	22.19	CRU	Santa Cruz	0.962
FLO06	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	18.94	CRU	Santa Cruz	22.68	CRU	Santa Cruz	0.944
FLO07	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	22.07	CRU	Santa Cruz	22.85	CRU	Santa Cruz	0.961
FLO08	88	CRU	Santa Cruz	0	AY268588	PBL	Isabela	15.45	AGO	Santiago	17.05	CRU	Santa Cruz	0.914
FLO09	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	23.22	CRU	Santa Cruz	25.51	CRU	Santa Cruz	0.960
FLO10	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	27.09	CRU	Santa Cruz	31.68	CRU	Santa Cruz	0.669
FLO11	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	17.99	CRU	Santa Cruz	21.81	CRU	Santa Cruz	0.937
FLO12	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	25.00	PBR	Isabela	28.81	CRU	Santa Cruz	0.561
FLO13	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	21.09	CRU	Santa Cruz	22.72	CRU	Santa Cruz	0.956
FLO14	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	19.36	CRU	Santa Cruz	25.78	CRU	Santa Cruz	0.937
FLO15	86	CRU	Santa Cruz	0	AY268586	CRU	Santa Cruz	12.21	CF	Santa Cruz	20.21	CRU	Santa Cruz	0.952
FLO16	28	VD	Isabela	0	AF548231	VD	Isabela	10.53	VA	Isabela	15.86	VD	Isabela	0.942
FLO17	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	17.87	CRU	Santa Cruz	20.36	CRU	Santa Cruz	0.947
FLO18	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	17.13	PBR	Isabela	27.04	CRU	Santa Cruz	0.949
FLO19	52	PBL	Isabela	0	AF548255	PBL	Isabela	17.97	CRU	Santa Cruz	22.29	CRU	Santa Cruz	0.949
FLO20	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	19.71	CRU	Santa Cruz	25.78	CRU	Santa Cruz	0.957
FLO21	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	14.40	CRU	Santa Cruz	17.54	CRU	Santa Cruz	0.924
FLO22	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	22.08	CRU	Santa Cruz	25.44	CRU	Santa Cruz	0.961
FLO23	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	19.59	CRU	Santa Cruz	24.49	CRU	Santa Cruz	0.959
FLO24	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	17.03	CRU	Santa Cruz	19.60	CRU	Santa Cruz	0.944
FLO25	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	21.15	CRU	Santa Cruz	24.70	CRU	Santa Cruz	0.956
FLO26	52	PBL	Isabela	0	AF548255	CRU	Santa Cruz	22.43	PBL	Isabela	24.04	CRU	Santa Cruz	0.961
FLO27	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	18.89	CRU	Santa Cruz	25.81	CRU	Santa Cruz	0.934
FLO28	86	CRU	Santa Cruz	0	AY268586	LC	Isabela	21.25	PBL	Isabela	21.44	CRU	Santa Cruz	0.942
FLO29	86	CRU	Santa Cruz	0	AY268586	PBL	Isabela	12.05	AGO	Santiago	20.00	PBL	Isabela	0.896
PRZ01	8	PZN	Pinzón	0	AF548211	PZN	Pinzón	14.99	PBL	Isabela	31.14	PZN	Pinzón	0.949
PRZ02	88	CRU	Santa Cruz	1	AY268588	CRU	Santa Cruz	23.05	AGO	Santiago	29.98	CRU	Santa Cruz	0.746
SDZ01	52	PBL	Isabela	0	AF548255	PBL	Isabela	16.90	AGO	Santiago	25.24	PBL	Isabela	0.610
SDZ02	24	LP	Isabela	0	AF548237	RU	Isabela	18.23	VA	Isabela	19.55	CAZ	Isabela	0.880
SDZ03	55	CAZ	Isabela	0	AF548258	LT	Isabela	18.90	LP	Isabela	19.69	LP	Isabela	0.848
SDZ04	27	VD	Isabela	0	AF548230	VD	Isabela	11.95	CR	Isabela	24.63	VD	Isabela	0.952
SDZ05	55	CAZ	Isabela	0	AF548258	VA	Isabela	22.18	RU	Isabela	24.00	CAZ	Isabela	0.549
SDZ06	54	CAZ	Isabela	0	AF548257	PEG	Isabela	22.05	VD	Isabela	24.29	CAZ	Isabela	0.674
SDZ07	61	VA	Isabela	0	AF548264	VD	Isabela	23.47	VA	Isabela	23.78	CAZ	Isabela	0.453
ZUZ01	18	CRU	Santa Cruz	1	AF548221	CRU	Santa Cruz	17.74	CF	Santa Cruz	19.57	CR	Isabela	0.910
ZUZ10	52	PBL	Isabela	0	AF548255	PBL	Isabela	15.89	PEG	Isabela	22.03	PBL	Isabela	0.852
ZUZ20	52	PBL	Isabela	0	AF548255	PBL	Isabela	14.58	AGO	Santiago	16.88	PBL	Isabela	0.503
ZUZ30	61	VA	Isabela	0	AF548264	VD	Isabela	16.67	CR	Isabela	20.90	CR	Isabela	0.950

Individuals are listed according to one of the following *ex situ* collections in which they currently reside: Caloosahatchee Aviary and Botanical Garden (CABG); Charles Darwin Research Station (CDRS); mainland Ecuador hotels, universities, zoological and private collections (ECU); former Witmer Collection on Floreana, Galápagos (FLO); Prague Zoo (PRZ); San Diego Zoo (SDZ); Zurich Zoo (ZUZ). Unknown tortoises are assigned to a population of origin based on the location of a shared or closely related mtDNA haplotype previously sampled in the wild. Population and island location of reference haplotypes are specified by acronyms as in Fig. 1. Novel haplotypes recovered in this study are indicated by the number of substitutions ('distance') from the most closely related haplotype previously recovered, with associated GenBank accession number. Population and island assignment according to the microsatellite genotypic data and the tests of Rannala & Mountain (1997) and Pritchard *et al.* (2000) are indicated by their corresponding likelihood values (' L_1 ' and ' L_2 ') and membership coefficients (' q '), respectively.

mtDNA, mitochondrial DNA.

PBL, Piedras Blancas on Vólcan Wolf; PBR, Puerto Bravo on Vólcan Wolf; VD, Vólcan Darwin; VA, Vólcan Alcedo; CAZ, La Cazuella; LC, PEG, East Cerro Azul; LP, LT, West Cerro Azul; CR, Cabo Rosa; RU, Roca Union; PNT, Pinta; AGO, Santiago; PZN, Pinzon; CF, Cerro Fatal *porterii*; SCR, San Cristobal; ESP, Espanola; CRU, Santa Cruz.

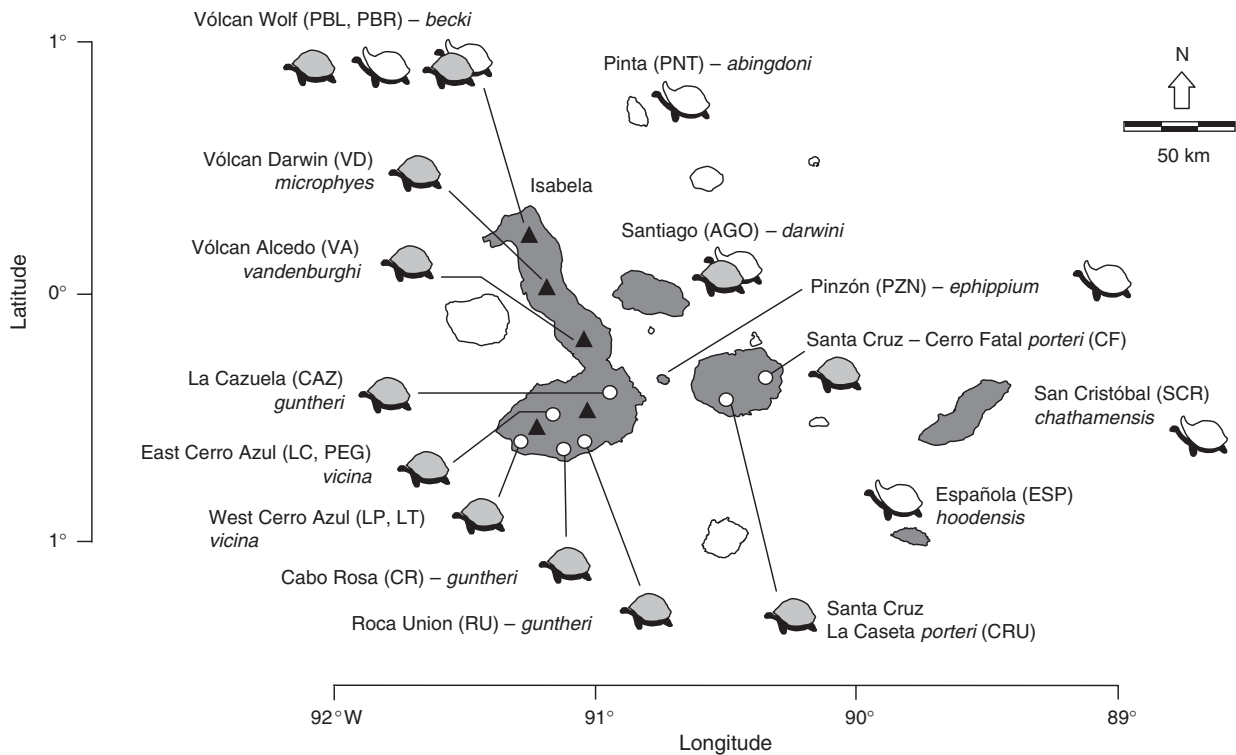


Figure 1 Distribution of giant tortoises throughout the Galápagos archipelago. Shaded islands indicate the presence of extant tortoise populations and italicized names represent current subspecific designations. Island names are capitalized, with triangles representing volcanoes on the island of Isabela. Distinct populations are indicated by name (e.g. Vólcan Wolf) and specific sampling site [e.g. Piedras Blancas on Vólcan Wolf (PBL), Puerto Bravo on Vólcan Wolf (PBR)]. Shaded tortoise caricatures indicate 'domed,' unshaded caricatures indicate 'saddleback' and overlapped caricatures indicate 'intermediate' carapace morphologies. Figure modified from fig. 1 in Burns *et al.* (2003).

Rannala & Mountain (1997) and Pritchard *et al.* (2000) exhibited a high degree of overlap, yielding consistent assignments for 87.8% of the individuals sampled. Overall, the genotypic assignments corroborated the results obtained from the mtDNA analyses, with 83.7% of individuals consistently assigned to the same locality by both datasets.

Individuals exhibiting a discrepancy of assignments according to mtDNA and nuclear microsatellites may be due to a number of possibilities including the shallow phylogenetic proximity of many of these taxa as well as the potential for mixed ancestry in captive settings. Ten of the 16 such individuals were assigned by the mtDNA and microsatellite to alternative populations on the same island of Isabela, which hosts a population complex known to have close phylogenetic ties, particularly in the southern region (Ciofi *et al.*, 2006). Of these ten, four were assigned by both datasets to different taxa localized in northern Isabela with the other six assigned alternatively to populations on the northern and southern sides of this largest of islands in Galápagos (Table 1). The remaining individuals were all assigned to the La Caseta *G. n. porteri* population of Santa Cruz by way of mtDNA, with alternative origins on the island of Isabela, primarily to a northern population in Piedras Blancas on Vólcan Wolf (PBL), according to their multi-locus genotypes. Within a phylogenetic context, La Caseta *G. n. porteri* and the *Geochelone nigra becki* of

northern Isabela are relatively distinct (Caccone *et al.*, 2002), suggesting that the discrepancy in assignment is likely due to mixed ancestry over the course of their breeding programs of origin. More specifically, these individuals of purported mixed ancestry spread out across the CABG, ECU-ESPOL, and Floreana collections were likely the progeny of a female La Caseta *G. n. porteri* with one of a number of Isabela males (*G. n. becki*, *G. n. guntheri*, or *Geochelone nigra microphyes*).

Discussion and conclusions

The genetic identification of source populations for individuals of unknown ancestry has important implications for conservation, especially for morphologically similar, yet genetically distinct taxa such as those currently described for the threatened Galápagos tortoise. The current study constitutes the single largest survey of captive *G. nigra* worldwide, investigating the origin of individuals residing in collections located on three continents. Moreover, for the first time, multi-locus genotypes of captive unknowns could be compared relative with historical population allele frequency data for the critically endangered *G. n. abingdoni* from Pinta, whose sole known survivor, Lonesome George, awaits a mate for propagating the future of this taxon.

Once the revelation emerged that Lonesome George may be the last surviving member of the Pinta population, colloquial evidence materialized suggesting that other related individuals may persist in collections outside of Galápagos. A Prague Zoo tortoise (PRZ01) was one such individual; however, the results of the mitochondrial and microsatellite analyses unequivocally assigned him to the *G. n. ephippium* taxon on Pinzón (Table 1). Pinzón *G. n. ephippium*, like the population formerly found on Pinta, exhibits an extreme saddleback morphology that may have led to the misclassification. Although not as critical as the current situation on Pinta, the finding of a *G. n. ephippium* individual in captivity may be useful within a conservation context, as the population on Pinzón experienced an extreme bottleneck in the early 20th century. Fortunately, the *G. n. ephippium* population is currently in recovery with the help of a highly successful head-start program carried out by the Galápagos National Park Service in collaboration with the CDRS (e.g. eggs from natural nests are brought to the CDRS and reared to a sufficient size to allow them to avoid predation by introduced rats before release). Despite these efforts, predation by introduced black rats remains a major threat of extinction for the *G. n. ephippium* lineage as the population size remains small ($n \approx 150\text{--}200$) and recruitment severely limited (Beheregaray *et al.*, 2003). Interestingly, PRZ01 exhibits two novel alleles never before sampled in *G. n. ephippium*, and also possesses two additional alleles found at very low frequencies (≤ 0.06) in the wild population. If deemed necessary in the future, inclusion of PRZ01 in a *G. n. ephippium* breeding program may be appropriate for reintroducing novel gene variants back into the population.

Another related issue involves identifying the appropriate lineage by which to hybridize Lonesome George in an attempt to preserve a portion of the *G. n. abingdoni* genetic legacy. Before the emergence of DNA-based evidence for identifying the closest living relative to the Pinta *G. n. abingdoni*, two females from the Volcán Wolf population on northern Isabela (CDRS106 and 107) were chosen to reside and potentially breed with Lonesome George based on their geographical proximity to Pinta and morphological similarity (e.g. extreme saddleback). In the current study, mtDNA haplotype data and the genotypic assignment method of Rannala & Mountain (1997) correctly assigned CDRS106 and 107 to the population in which they were collected in Puerto Bravo on the slopes of Volcán Wolf in northern Isabela (e.g. PBR). Interestingly, eight of the 28 individuals sampled from the PBR population exhibit 'alien' haplotypes most closely related to the single mtDNA control region haplotype recovered on the island of Española (Caccone *et al.*, 2002), one of which exhibits mixed ancestry with the Pinta population according to genotypic assignment tests (Russello *et al.*, in press). These findings are of particular interest as previous phylogenetic analyses have revealed that the *G. n. hoodensis* taxon on Española and northern Isabela 'aliens' collected on Volcán Wolf are the closest extant lineages to the Pinta *G. n. abingdoni* (Caccone *et al.*, 2002). Thus, in the absence of locating additional individuals of demonstrated Pinta origin, the two females

currently co-habiting with Lonesome George are among the most appropriate matches, at least from an evolutionary point of view.

Overall, it was not surprising that the majority of captive unknowns sampled were assigned to the La Caseta *G. n. porteri* population. This is the largest and most diverse population of *G. nigra*, residing primarily in a protected area on one of the most accessible sites within the archipelago. Similarly, the finding of multiple hybrids across collections is somewhat common among captive Galápagos tortoises, especially in combinations involving La Caseta *G. n. porteri* and northern/southern Isabela taxa (*G. n. becki*, *G. n. guntheri* or *G. n. microphyes*), the most-well represented lineages *ex situ*. The proportion of hybrids detected here is not indicative of their level within captive collections worldwide, as only individuals of purportedly pure bloodlines were targeted for sampling. Molecular approaches, such as those used in the current study, represent important tools for minimizing hybridization and guiding management programs for preserving the distinctiveness of *G. nigra* taxa in captivity.

All *G. nigra* captive management programs should strive to identify the origin of their captive unknowns in order to maintain the distinctiveness of the extant named taxa and locate individuals of unique heritage. Critically endangered taxa such as *G. n. abingdoni*, *G. n. ephippium* and *G. n. hoodensis* might benefit from the influx of novel genetic variation once endemic to their respective islands of origin. The combination of mtDNA and microsatellite analyses coupled with large reference databases of field-collected individuals provides an integrative approach for identifying the lineages of captive unknowns and assessing their immediate conservation value. Future studies will carry on this work in order to facilitate ongoing interactive *in situ* and *ex situ* management programs for the Galápagos tortoise, a conservation flagship and an enduring symbol of the birth of evolutionary theory.

Acknowledgements

The authors are grateful to Oliver Ryder at the San Diego Zoo, Sam Furrer at the Zurich Zoo, William Porter at the Caloosahatchee Aviary and Botanical Garden and all the participating institutions on mainland Ecuador for providing samples of their tortoises of unknown ancestry. Howard Snell and Thomas Fritts offered background information regarding the females housed with Lonesome George. Henry Nicholls facilitated the acquisition of samples in Europe and Miles Farmer aided in data collection. Financial support was provided by the Bay Foundation and the Yale Institute for Biospherics Studies through their ECOSAVE program directed by Elisabeth Vrba.

References

- Beheregaraya, L.B., Ciofi, C., Caccone, A., Gibbs, J.P. & Powell, J.R. (2003). Genetic divergence, phylogeography

- and conservation units of giant tortoises from Santa Cruz and Pinzon, Galápagos Islands. *Conserv. Genet.* **4**, 31–46.
- Burns, C.E., Ciofi, C., Beheregaray, L.B., Fritts, T.H., Gibbs, J.P., Marquez, C., Milinkovitch, M.C., Powell, J.R. & Caccone, A. (2003). The origin of captive Galápagos tortoises based on DNA analysis: implications for the management of natural populations. *Anim. Conserv.* **6**, 329–337.
- Caccone, A., Gentile, G., Gibbs, J.P., Fritts, T.H., Snell, H.L., Betts, J. & Powell, J.R. (2002). Phylogeography and history of giant Galápagos tortoises. *Evolution* **56**, 2052–2066.
- Caccone, A., Gibbs, J.P., Ketmaier, V., Suatoni, E. & Powell, J.R. (1999). Origin and evolutionary relationships of giant Galápagos tortoises. *Proc. Natl. Acad. Sci.* **96**, 13223–13228.
- Ciofi, C., Milinkovitch, M.C., Gibbs, J.P., Caccone, A. & Powell, J.R. (2002). Microsatellite analysis of genetic divergence among populations of giant Galápagos tortoises. *Mol. Ecol.* **11**, 2265–2283.
- Ciofi, C., Wilson, G.A., Beheregaray, L.B., Marquez, C., Gibbs, J.P., Tapia, W., Snell, H.L., Caccone, A. & Powell, J.R. (2006). Phylogeographic history and gene flow among giant Galápagos tortoises on southern Isabela Island. *Genetics* **172**, 1727–1744.
- Clement, M., Posada, D. & Crandall, K.A. (2000). TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* **9**, 1657–1659.
- Cornuet, J.M., Piry, S., Luikart, G., Estoup, A. & Solignac, M. (1999). New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics* **153**, 1989–2000.
- DeSalle, R. & Amato, G. (2004). The expansion of conservation genetics. *Nat. Rev. Genet.* **5**, 702–712.
- Doyle, R.W., Perez-Enriquez, R., Takagi, M. & Taniguchi, N. (2001). Selective recovery of founder genetic diversity in aquacultural broodstocks and captive, endangered fish populations. *Genetica* **111**, 291–304.
- IUCN (2006). 2006 IUCN Red List of threatened species. <<http://www.iucnredlist.org>>. Downloaded on 12 June 2006.
- Jones, K.L., Glenn, T.C., Lacy, R.C., Pierce, J.R., Unruh, N., Mirande, C.M. & Chavez-Ramirez, F. (2002). Refining the Whooping Crane studbook by incorporating microsatellite DNA and leg-banding analyses. *Conserv. Biol.* **16**, 789–799.
- Kaiser, J. (2001). Galápagos takes aim at alien invaders. *Science* **293**, 590–592.
- MacFarland, C.G., Villa, J. & Basilio, T. (1974). The Galápagos giant tortoises *Geochelone elephantopus* part 1: status of the surviving populations. *Biol. Conserv.* **6**, 118–133.
- Milinkovitch, M.C., Monteyne, D., Gibbs, J.P., Fritts, T.H., Tapia, W., Snell, H.L., Tiedemann, R., Caccone, A. & Powell, J.R. (2004). Genetic analysis of a successful repatriation programme: giant Galápagos tortoises. *Proc. Roy. Soc. Lond. Ser. B – Biol. Sci.* **271**, 341–345.
- Milinkovitch, M.C., Monteyne, D., Russello, M.A., Gibbs, J.P., Snell, H.L., Tapia, W., Marquez, C., Caccone, A. & Powell, J.R. (2007). Giant Galápagos tortoises: molecular genetic analysis reveals contamination in a repatriation program of an endangered taxon. *BMC Evol. Biol.* **7**, 2.
- Norton, J.E. & Ashley, M.V. (2004). Genetic variability and population differentiation in captive Baird's tapirs (*Tapirus bairdii*). *Zoo Biol.* **23**, 521–531.
- Powell, J.R. & Gibbs, J.P. (1995). A report from Galápagos. *Trends Ecol. Evol.* **10**, 351–354.
- Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.
- Pritchard, P.C.H. (1996). *The Galápagos tortoises: nomenclatural and survival status. Chelonian research monographs. 1*. Lunenburg, MA: Chelonian Research Foundation.
- Rannala, B. & Mountain, J.L. (1997). Detecting immigration by using multilocus genotypes. *Proc. Nat. Acad. Sci. USA* **94**, 9197–9201.
- Rodriguez-Clark, K.M. & Sanchez-Mercado, A. (2006). Population management of threatened taxa in captivity within their natural ranges: lessons from Andean bears (*Tremarctos ornatus*) in Venezuela. *Biol. Conserv.* **129**, 134–148.
- Russello, M.A. & Amato, G. (2004). *Ex situ* population management in the absence of pedigree information. *Mol. Ecol.* **13**, 2829–2840.
- Russello, M.A., Beheregaray, L.B., Gibbs, J.P., Fritts, T., Havill, N., Powell, J.R. & Caccone, A. (in press). Lone-George is not alone among Galápagos tortoises. *Curr. Biol.*
- Russello, M.A., Glaberman, S., Gibbs, J.P., Marquez, C., Powell, J.R. & Caccone, A. (2005). A cryptic taxon of Galápagos tortoise in conservation peril. *Biol. Lett.* **1**, 287–290.
- Snell, H.M., Stone, P.A. & Snell, H.L. (1996). A summary of geographical characteristics of the Galápagos islands. *J. Biogeogr.* **23**, 619–624.
- Townsend, C.H. (1931). Growth and age in the giant tortoise of Galápagos. *Zoologica* **4**, 459–474.