

TECHNICAL REPORT

Lineage Identification and Genealogical Relationships Among Captive Galápagos Tortoises

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Genetic tools have become a critical complement to traditional approaches for meeting short- and long-term goals of ex situ conservation programs. The San Diego Zoo (SDZ) harbors a collection of wild-born and captive-born Galápagos giant tortoises ($n = 22$) of uncertain species designation and unknown genealogical relationships. Here, we used mitochondrial DNA haplotypic data and nuclear microsatellite genotypic data to identify the evolutionary lineage of wild-born and captive-born tortoises of unknown ancestry, to infer levels of relatedness among founders and captive-born tortoises, and assess putative pedigree relationships assigned by the SDZ studbook. Assignment tests revealed that 12 wild-born and five captive-born tortoises represent five different species from Isabela Island and one species from Santa Cruz Island, only five of which were consistent with current studbook designations. Three wild-born and one captive-born tortoise were of mixed ancestry. In addition, kinship analyses revealed two significant first-order relationship pairs between wild-born and captive-born tortoises, four second-order relationships (half-sibling) between wild-born and captive tortoises (full-sibs or parent-offspring), and one second-order relationship between two captive-born tortoises. Of particular note, we also reconstructed a first-order relationship between two wild-born individuals, violating the founder assumption. Overall, our results contribute to a worldwide effort in identifying genetically important Galápagos tortoises currently in

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captivity while revealing closely related founders, reconstructing genealogical relationships, and providing detailed management recommendations for the SDZ tortoises. *Zoo Biol* 31:107–120, 2012. © 2011 Wiley Periodicals, Inc.

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INTRODUCTION

The Galápagos giant tortoises (*Chelonoidis spp.*) represent an extraordinary example of evolutionary diversification in island environments. These charismatic animals have historically radiated into 15 species; however, direct human intervention has induced the extinction of four of them. The 11 surviving lineages have been subject to various conservation pressures and face different conservation challenges [Ciofi et al., 2009]. A conservative estimate suggests that 650 Galápagos tortoises were moved to other continents by scientific expeditions in recent times [MacFarland et al., 1974]. Consequently, adult tortoises have been regularly found in zoos and private collections. Many of these captive tortoises have unknown origin, the vast majority of which lack pedigree information [Burns et al., 2003; Russello et al., 2007a]. Previous studies have shown that animals kept in captivity may represent endangered [Burns et al., 2003; Russello et al., 2007a], extirpated [Russello et al., 2007b] or presumed extinct [Russello et al., 2010] species of Galápagos tortoises. Coupled with knowledge of relatedness among individuals of unknown pedigree, these historical data can greatly inform ex situ conservation strategies [Russello and Amato, 2007].

The San Diego Zoo (SDZ) harbors a collection of Galápagos tortoises ($n = 22$) obtained through donations and rescue of individuals kept in private collections. Because of their diverse origin, very little background information is available regarding species identification and genealogical relationships among these captive individuals. In this study, we used mitochondrial haplotypic and nuclear genotypic data to (1) identify the evolutionary lineage of wild- and captive-born tortoises of unknown ancestry; (2) infer levels of relatedness among founders and captive-born tortoises; (3) assess putative pedigree relationships assigned by the SDZ studbook; and (4) make management recommendations in light of research results.

MATERIALS AND METHODS

Sample Collection

We obtained blood samples from 22 tortoises acquired at different times by the SDZ. Of these individuals, 15 were reportedly wild-born and five of them have since deceased (Table 1). Seven of the 14 wild-born individuals were previously examined by Russello et al. [2007a], and here we add three new microsatellite loci to the original data set (see “Data Collection” below). Seven of the 22 sampled tortoises were captive born. These seven individuals may represent the offspring of mixed and/or pure lineages; however, none of these tortoises have any pedigree documentation that could be used to assign them to any category. As such, we made the distinction between wild and captive born to refer to these two groups. Further, we use the taxonomy proposed by Le et al. [2006] and followed by Russello et al. [2010], which

TABLE 1. Lineage Identification of Captive and Wild-Born Galápagos Tortoises of Unknown Ancestry Based on mtDNA Haplotypes and Microsatellite Data

Origin	ISIS no.	Studbook assignment	Genetic assignment ^a	TCS			Structure			GeneClass2						
				Sex	Haplotype	Population ^b	Island ^c	Population	Island	q	Population	Island	L_1	L_2		
Wild	000128 ⁶	Unknown ^d	<i>guntheri</i>	F	CAZ6	CAZ	IS	CAZ	IS	0.438	LP	IS	24.061	CR	IS	31.155
	000129	<i>porteri</i>	<i>porteri</i>	F	CDRS56	CRU	SC	CRU	SC	0.915	CRU	SC	30.736	PBL	IS	34.043
	000130 ⁷	<i>vandenburghi</i> ^d	<i>guntheri</i>	F	CAZ6	CRU	IS	CAZ	IS	0.548	CAZ	IS	29.218	LP	IS	22.219
	000131	<i>porteri</i>	<i>porteri</i>	F	New ^e	CRU	SC	CRU	SC	0.894	CRU	LP	20.947	LP	IS	27.547
	000132	<i>microphyes</i>	<i>vandenburghi</i>	F	VA17	VA	IS	VA	IS	0.610	CAZ	IS	19.342	LP	IS	21.583
	000133	Unknown	<i>vandenburghi</i>	F	New ^e	VA	IS	VA	IS	0.599	CAZ	IS	20.018	LP	IS	21.643
	000134	Unknown	<i>becki</i>	F	H48	CF	SC	PBL	IS	0.563	PBL	IS	33.06	CR	IS	34.65
	000137	<i>vandenburghi</i>	<i>vandenburghi</i>	M	VA17	VA	IS	VA	IS	0.488	VD	IS	24.295	VA	IS	26.158
	000139	<i>vicina</i>	<i>vicina</i>	M	LP57	LP	IS	LP	IS	0.610	CR	IS	21.893	LP	IS	23.689
	000141 ¹	Unknown	<i>becki</i>	M	PBL5	PBL	IS	PBL	IS	0.889	PBL	IS	22.371	AGO	AGO	31.979
	000142 ²	Unknown ^d	<i>guntheri</i>	M	CAZ60	CAZ	IS	CAZ	IS	0.500	CAZ	IS	18.36	CR	IS	21.577
	000147 ⁵	Unknown ^d	<i>guntheri</i>	n/a	CAZ60	CAZ	IS	CAZ	IS	0.581	CAZ	IS	27.037	LP	IS	28.749
	000148 ³	<i>microphyes</i>	<i>vicina</i>	M	LP2	LP	IS	LP	IS	0.685	LP	IS	18.257	CR	IS	24.51
	000149	Unknown ^d	<i>porteri</i>	M	New ^e	CRU	SC	CRU	SC	0.820	CRU	IS	24.410	CR	IS	33.720
	001060 ⁴	<i>microphyes</i>	<i>microphyes</i>	M	LP2	LP	IS	VD	IS	0.643	CAZ	IS	17.164	CR	IS	37.852
	000121	Unknown	<i>guntheri</i>	M	CAZ6	CAZ	IS	CAZ	IS	0.511	CAZ	IS	20.624	CR	IS	20.652
000150	Unknown	<i>guntheri</i>	n/a	CAZ6	CAZ	IS	CAZ	IS	0.529	CAZ	IS	24.692	LP	IS	25.613	
010030	<i>vandenburghi</i>	<i>guntheri</i>	M	CAZ60	CAZ	IS	CAZ	IS	0.404	CAZ	IS	30.697	PBL	IS	33.339	
001063	Unknown	<i>vandenburghi</i>	M	VA17	VA	IS	VA	IS	0.654	CAZ	IS	33.061	CR	IS	34.654	
010029	<i>darwini</i>	<i>becki</i> / <i>vandenburghi</i>	n/a	VA17	VA	IS	VA	IS	0.400	CAZ	IS	28.571	LP	IS	31.783	
000641	Unknown	<i>porteri</i>	n/a	CRU19	CRU	SC	CRU	SC	0.915	CRU	SC	27.248	PBL	IS	36.679	
901146	Unknown	<i>porteri</i>	n/a	CDRS56	CRU	SC	CRU	SC	0.914	CRU	SC	25.847	CR	IS	27.667	

¹ to ⁷ Individuals SDZ-1 to SDZ-7 in Russello et al. [2007a].

^aUnknown tortoises are assigned to a particular species based on the location of a shared mtDNA haplotype previously sampled in the wild [Russello et al., 2007a, 2010] and according to the microsatellite genotypic assignment tests of Rannala and Mountain [1997] and Pritchard et al. [2000], indicated by their corresponding likelihood values (L_1 and L_2) and membership coefficients (q), respectively.

^bPopulation names as in Figure 1.

^cIsland names are abbreviated as follows: IS, Isabela; SC, Santa Cruz. Other island names are as in Figure 1.

^dDeceased individual.

^ePreviously unreported haplotype.

validates the genus name *Chelonoidis* for the Galápagos tortoises and maintains the 11 extant taxa as full species.

Data Collection

Total genomic DNA was extracted from blood samples with the DNeasy Tissue Kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA).

We amplified a 696 bp fragment of the mitochondrial DNA (mtDNA) control region by way of polymerase chain reaction (PCR) using primers and conditions described in Caccone et al. [1999]. Sequences were edited and aligned using Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI), and manually adjusted where necessary. The sequences were compared to a pool of 88 reference haplotypes recovered from 847 individuals sampled throughout the Galápagos Archipelago previously used to identify the origin of captive tortoises [Russello et al., 2007a, 2010]. The reference data also include haplotypes sampled in the extinct or nearly extinct species from Floreana and Pinta, respectively [Russello et al., 2007b; Poulakakis et al., 2008].

Of the 12 microsatellite loci were assayed in this study, nine [GAL45, GAL50, GAL75, GAL94, GAL100, GAL127, GAL136, GAL159 and GAL263; Ciofi et al., 2002] were amplified individually in 12.5 μ L reactions containing 6.4 μ L dH₂O, 1.25 μ L 10 \times Applied Biosystems (ABI, Ipswich, MA) PCR Buffer, 1.0 μ L ABI MgCl₂ (25 mM), 1.0 μ L dNTP mix (10 mM), 0.25 μ L New England Biolabs (Ipswich, MA) bovine serum albumin (100 \times), 0.5 μ L each primer (10 μ M), 0.1 μ L ABI AmpliTaq Gold[®] (5 U/ μ L), and 1.5 μ L genomic DNA. PCR amplifications used a profile consisting of: 95°C 5 min initial denaturation (1 cycle), 95°C 25 sec, 63–54°C 20 sec, 72°C 30 sec (10 "touchdown" cycles, with a 1°C reduction in annealing temperature per cycle), 95°C 25 sec, 53°C 20 sec, 72°C 30 sec (35 cycles), and 72°C 15 min final extension (1 cycle). These nine loci were previously used for assignment of captive animals [Burns et al., 2003; Russello et al., 2007a, 2010]. In this study, three additional loci [GAL194 and GAL288, Ciofi et al., 2006; and AC063, Milinkovitch et al., 2004] were added to the reference dataset and screened for the 22 SDZ tortoises of unknown origin. These three loci were co-amplified in 10 μ L reactions containing 2.98 μ L dH₂O, 5.0 μ L Qiagen Type-it[™] Multiplex PCR Master Mix (2 \times), 0.17 μ L each primer (10 μ M), and 1.0 μ L genomic DNA, with the following profile: 95°C 5 min initial denaturation (1 cycle), 95°C 30 sec, 56°C 30 sec, 72°C 30 sec (30 cycles), and 60°C 30 min final extension (1 cycle). For each locus-specific primer pair, one primer was labeled with a 5'-fluorescent dye (FAM, HEX, or TET), and all PCRs were performed in an Eppendorf ep-gradient Mastercycler. Amplified fragments were then run with GeneScan[™] 500-ROX size standard on an ABI3730 following the manufacturer's recommendations. Genotypes were scored with the assistance of GENEMARKER v3.7 (ABI), with allele calls manually adjusted where necessary.

Genotypes and allele frequencies from captive tortoises were compared to a reference data set, which includes genotypes from 370 tortoises from 20 reference populations from all extant species, including genotypes from bones from 12 museum specimens from Pinta (*C. abingdoni*) plus the only survivor of this species, a male named Lonesome George. The dataset also includes 16 museum samples from the extinct species from the island of Floreana [*C. elephantopus*; Poulakakis et al., 2008]. Further details on the reference data set can be found in Appendix A.

Population Genetic Analyses

Genealogical relationships were reconstructed between captive individuals (wild- and captive-born) and natural populations in the form of mtDNA haplotype networks using statistical parsimony as implemented in TCS v1.6 [Clement et al., 2000]. Captive individuals of unknown ancestry were also assigned to island populations based on their multi-locus genotypes using two separate approaches. First, the Bayesian model-based clustering method of Pritchard et al. [2000] for inferring population structure and assigning individuals to populations was used as implemented in structure v2.3. Membership coefficients (q) of the captive unknowns in one or more of the reference populations were estimated following a Markov chain Monte Carlo simulation (MCMC) of 500,000 steps following an initial “burn-in” of 100,000 steps. Consistency in the results was assured through reiterating each K value ($K = 14\text{--}16$) four times. Given the large body of research directed toward reconstructing population structure and genetic distinctiveness of extant and extinct *Chelonoidis* species [Caccone et al., 2002; Ciofi et al., 2002; Beheregaray et al., 2003; Russello et al., 2007b; Poulakakis et al., 2008], analyses were run using a model that utilized prior population information, as recommended by Hubisz et al. [2009] and Pritchard et al. [2000]. The structure harvester v.0.6.4 online application (http://taylor0.biology.ucla.edu/struct_harvest/) was used to summarize results obtained with structure. Second, the exclusion–simulation test of the partial Bayesian method of Rannala and Mountain [1997] was used to assign captive 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 v2 [Piry et al., 2004]. We used an exclusion threshold of 0.05 relative to a distribution estimated from 10,000 randomly generated genotypes.

Relationships among individuals were determined based on a combination of allele-sharing analysis, pairwise relatedness estimates, and full sibship reconstruction (FSR). First, straight allele sharing was used to identify potential parent–offspring relationships, requiring pairs of individuals to share at least one allele at all sampled loci following Mendelian expectations. Second, the pairwise relatedness estimator (r_{xy}) of Queller and Goodnight [1989] was calculated among different subsets of individuals as implemented in irel [Goncalves da Silva and Russello, 2010]. Specifically, pairwise relatedness estimates were calculated separately among individuals assigned to the same lineage based on the analyses described above, using the corresponding reference population allele frequencies. For example, to calculate relatedness among ISIS000129, ISIS000131, ISIS000149, ISIS000641, and ISIS901146 (all assigned to *C. porteri* from La Caseta, Results section), we used the population allele frequency distribution from that species and population (*C. porteri*, La Caseta). This approach was used to minimize bias, as marker-based pairwise relatedness estimates critically depend upon accurate estimates of the population allele frequency distribution [Suarez et al., 2008]. The r_{xy} values were used to apply the “cut-off” values method of Blouin et al. [1996] to classify pairs into relationship categories [i.e., unrelated individuals, half-sibs, first-order (full-sibs or parent-offspring)], with empirical cut-off values calculated in irel [Goncalves da Silva and Russello, 2010]. Finally, FSR was conducted using the “descent ratio” algorithm as implemented in kingroup v2 [Konovalov et al., 2004].

RESULTS

Haplotypic and Genotypic Assignment

Fifteen of the 22 individuals sampled ex situ were classified as wild-born in the SDZ studbook (Table 1). These tortoises corresponded to individuals acquired by the SDZ around the year 1931 from various sources. Haplotype sequences from these wild-born tortoises were compared to extant and extinct mitochondrial haplotypes [Caccone et al., 2002; Ciofi et al., 2002; Beheregaray et al., 2003; Poulakakis et al., 2008; Russello et al., 2010]. We recovered 11 haplotypes among the 15 wild-born tortoises. These haplotypes are nested within haploclades from two different islands: Santa Cruz ($n = 4$) and Isabela ($n = 7$) (data not shown). Three haplotypes from Santa Cruz Island are included in the same haplotype group characteristic of *C. porteri*, while one tortoise (ISIS000134) belongs to the haplogroup restricted to the Cerro Fatal population (Fig. 1), a highly divergent lineage likely to be a distinct species [Russello et al., 2005]. Seven haplotypes are included in haplogroups from three species on the island of Isabela: *C. becki* ($n = 1$), *C. vandenburghi* ($n = 2$), *C. guntheri* ($n = 2$), and *C. vicina* ($n = 2$).

We recovered three previously unrecorded haplotypes. Tortoises ISIS000131 and ISIS000149 show haplotypes that are one and five mutational steps away, respectively, from haplotype CDRS56 restricted to La Caseta *C. porteri* (Santa Cruz Island).

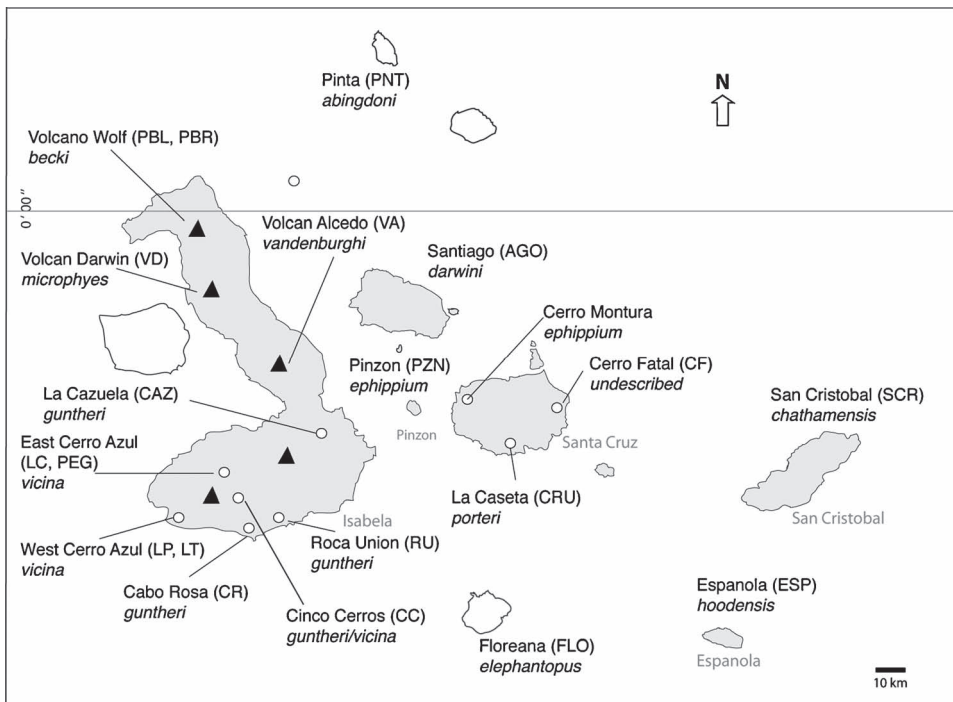


Fig. 1. Geographic distribution of *Chelonoidis* tortoises throughout the Galapagos Archipelago. Shaded islands indicate presence of extant populations of tortoises and associated sampling sites. Extinct tortoise populations are shown in nonshaded islands. Triangles represent volcanoes on Isabela Island and circles show sampling localities.

Tortoise ISIS000133 shows a new haplotype one mutational step away from haplotype VA17 associated with *C. vanderburghi* on Volcano Alcedo (Isabela Island).

The multi-locus genotype assignments of wild-born tortoises corroborated to a large extent the results obtained from mtDNA haplotypes, with the majority of individuals consistently assigned to the same locality by both genomic markers (Table 1). Samples originally assigned to La Caseta *C. porteri* (Santa Cruz Island; ISIS000129, ISIS000131, and ISIS000149) based on mtDNA were also assigned to La Caseta according to the microsatellite-based structure analyses. Similarly, 10 of the 11 individuals with mitochondrial haplotypes from Isabela Island were assigned to the same population and species by structure (Table 1). The results of the geneClass2 assignment tests were partially concordant with those from structure; 9 out of 15 wild-born individuals were assigned to the same population by both approaches (Table 1). The difference in population assignment between these two methods for the remaining six individuals (ISIS000128, ISIS 000132, ISIS 000133, ISIS00137, ISIS 000139, and ISIS 001060) was limited to individuals from the southern Isabela populations of *C. vicina* and *C. guntheri* (Table 1). This discordance is not surprising, as the two southern Isabela species are closely related with evidence for gene flow between them [Ciofi et al., 2006].

Two wild-born tortoises (ISIS001060 and ISIS000134) displayed mtDNA geographic origins that did not coincide with the results of the analyses based on multi-locus genotypic data. The tortoise ISIS001060 exhibited an mtDNA haplotype found previously only in Las Pampas (*C. vicina*) yet assigned to the Volcano Darwin lineage (*C. microphyes*) by way of multi-locus genotypic data. Tortoise ISIS000134 was assigned to the same mtDNA haplogroup found on San Cristobal Island (*C. chathamensis*) and closely associated with the cryptic species from Cerro Fatal on Santa Cruz Island, but assigned to *C. becki* from Volcano Wolf on northern Isabela Island by way of genotypic data.

The taxonomic assignments of wild-born tortoises performed on the basis of multi-locus genotypes were concordant with the SDZ studbook in 5 out of 15 individuals (ISIS000129, ISIS000131, ISIS00137, ISIS000139, and ISIS001060; Table 1). Here, we provide taxonomic assignment for seven wild-born individuals without documented origin (ISIS000128, ISIS000133, ISIS000134, ISIS000141, ISIS000142, ISIS000147, and ISIS000149; Table 1). For the remaining wild-born tortoises, genetic assignments and studbook records showed conflicting assignments. The tortoise ISIS000130 was assigned by studbook records to *C. vanderburghi* (Volcano Alcedo) and here we assign them to *C. guntheri* (La Cazuela). Similarly, tortoises ISIS000132 and ISIS000148 are *C. vandenburghi* and *C. vicina*, respectively, while the studbook record assigned them to *C. microphyes* (Table 1).

Seven of the 22 individuals sampled ex situ were born in captivity (Table 1), and limited or no pedigree information was available for these individuals. Our analysis revealed that six of them have completely concordant population assignments based on mtDNA and multi-locus genotypic data, suggesting low probability of a hybrid origin (Table 1). Two of these individuals (ISIS000641 and ISIS901146) were assigned to *C. porteri* (La Caseta population, Santa Cruz Island), while the other four were assigned to *C. vicina* (La Cazuela population, Isabela Island; ISIS000121, ISIS000150, ISIS010030) and *C. vanderburghi* (Volcano Alcedo population, Isabela Island; ISIS001063; Fig. 1 and Table 1). Tortoise ISIS010029 showed signatures of mixed ancestry, assigned to Volcano Alcedo (*C. vandenburghi*)

according to its mtDNA haplotype, yet exhibiting substantial genotypic contributions from both *C. vandenburghi* ($q = 0.400$) and *C. becki* ($q = 0.290$), the latter endemic to the northernmost volcano (Volcano Wolf) on Isabela Island.

Relatedness Between Wild- and Captive-Born Tortoises

Parentage and relatedness analyses identified a number of closely related individuals within this captive population (Table 2). Three pairs of individuals exhibited evidence for first-order relatedness, consistent with a parent–offspring relationship. Specifically, ISIS000128/000121, ISIS000129/901146, and ISIS000128/000142 each shared at least one allele at all 12 loci, exhibited pairwise relatedness values greater than the empirically determined cut-off value (>0.37), and parent–offspring relationships were inferred based on FSR hypothesis testing (Table 2). Of particular interest, ISIS000128 and ISI-000142 are both characterized as wild-caught, yet one is a putative parent of the other.

Five other pairs in the captive population are putatively of second-order relationship (half-sibling), two of which were identified by all three approaches. In particular, ISIS000134/010029 and ISIS000132/000150 shared at least one allele at $\geq 75\%$ of loci, exhibited pairwise relatedness values greater than the empirically determined cut-off value (>0.13), and second-order relationships were inferred based on FSR hypothesis testing (Table 2). Given that each pair includes a wild-caught and a captive-born individual, ISIS000134 and ISIS000132 are the likely grandparents of ISIS010029 and ISIS000150, respectively. The relationship between ISIS000134 and ISIS010029 was consistent with the results from the lineage identification analyses that suggested *C. darwini* and *C. becki* mixed ancestry for ISIS010029. Similar grandparent–grandchild relationships likely exist for ISIS000130/000121 and ISIS000132/001063, yet this inferred relationship was not significant according to the FSR analysis (Table 2). Finally, ISIS000150 and

TABLE 2. Inferred Kinship and Relationships Between Pairs of Nonpedigreed Wild- and Captive-Born Tortoises

ISIS #		Allele-sharing ^a	Pairwise relatedness ^b	Inferred relationship ^c
Individual 1	Individual 2			
000128 ^d	000121	12/12	0.41	First-order ^e (Parent-offspring)
000129	901146	12/12	0.53	First-order ^e
000128	000142 ^d	12/12	0.34	First-order ^e
000134	010029	09/12	0.26	Second-order ^e (half-sibling)
000130 ^d	000121	07/09	0.16	Second-order
000132	000150	09/11	0.18	Second-order ^e
000132	001063	08/11	0.17	Second-order
000150	001063	08/12	0.14	Second-order

Bolded specimen numbers are captive-born.

^aProportion of loci at which individuals shared one or more alleles.

^bPairwise relatedness according to the Queller and Goodnight [1989] estimator.

^cInferred relationship based on full sibship reconstruction.

^dDeceased individual.

^eInferred relationship significant ($\alpha < 0.05$) based on hypothesis testing and likelihood ratios.

ISIS001063 were inferred to be half-siblings according to all three approaches, yet the FSR analysis results were not significant.

DISCUSSION

The main goals of ex situ conservation programs are to manage self-sustaining populations that maintain the integrity of distinct lineages while minimizing the loss of genetic diversity by inbreeding and adaptation to captive environments [Frankham, 2008]. Most captive populations, however, were not established based on these goals and detailed records of the origin of wild-born tortoises or the ancestry of animals born in captivity are not always available. Uncertainty about lineage origin and/or levels of relatedness among captive individuals can have important genetic consequences for ex situ breeding programs, contributing to unintentional inbreeding among closely related individuals or hybridization of otherwise distinct taxa. To this effect, the combined information of genetic data and zoological records has been successfully used to prevent inbreeding, and mixing of founder lineages of unknown origin [Gautschi et al., 2003; Fowler et al., 2009; Kozfkay et al., 2008].

In this study we unambiguously assigned 15 wild-born tortoises to six distinct species from two different islands. Some of these individuals are now deceased (ISIS000128, ISIS000130, ISIS000142, ISIS000147, and ISIS000149; Table 1). Two individuals among the surviving wild-born tortoises (ISIS001060 and ISIS000134) were remarkable because of the mismatch between mitochondrial haplotype variation and the results of the nuclear assignment tests. Individual ISIS001060 showed a mtDNA haplotype from the coastal population of Las Pegas on southern Isabela Island (*C. vicina*; Fig. 1), yet the studbook record assigns it to *C. darwini*, a species found on the central part of Isabela on Volcano Darwin (Table 1). There is evidence that these two (and other neighboring) localities have been connected by gene flow in the recent past [Poulakakis et al., 2008; Fig. 3], thus ISIS001060 could represent a naturally occurring hybrid.

The discordance between marker assignments for the wild-born tortoise ISIS000134 is another reminder of the dynamic history of Galápagos tortoises. Volcano Wolf tortoises are characterized by the almost unique attribute of harboring two types of Galápagos tortoise carapace morphology (domed and saddleback) in one population. This morphological diversity correlates with multiple genetic lineages present in Volcano Wolf occurring together with the endemic species, *C. becki*. The presence of low frequency non-native haplotypes in this location has been linked to human intervention, which accounts for complete extirpations but also human-induced relocations on other islands [Townsend, 1925; Caccone et al., 2002; Russello et al., 2007b; Poulakakis et al., 2008]. The haplotype found in ISIS000134 is closely related to the haplogroup from San Cristobal Island (*C. chathamensis*) and the still undescribed species from Cerro Fatal in Santa Cruz Island. Notably, a haplotype from the same haplogroup has been previously found in Volcano Wolf on Isabela Island [Caccone et al., unpublished]. This suggests that ISIS000134, a native of Volcano Wolf, may possess mixed ancestry due to hybridization between tortoises belonging to different species in this highly admixed population [Caccone et al., 2002; Russello et al., 2007a; Poulakakis et al., 2008].

The results of the kinship analyses revealed two significant first-order relationship pairs between wild- and captive-born tortoises, one significant first-order relationship between wild-born captives, four second-order relationships between wild and captive tortoises, and one second-order relationship between two captive-born tortoises (Table 2). Our analyses also confirmed the hybrid origin of one captive-born individual (ISIS010029). Although some of the individuals included in our sample are deceased (ISIS000128, 000130, and 000142), the reconstructed relationships and origins are nevertheless important to establish pedigree and relationships within the group.

One particularly noteworthy result was the inference of a first-order relationship between two founder individuals (ISIS000128 and ISIS000142) of the SDZ captive population (Table 2). In general, wild-born individuals are assumed to be noninbred and equally unrelated to all nondescendant animals within the captive population [Ballou and Lacy, 1995]. Other studies have also found unexpectedly related wild-born individuals in captivity [Ivy et al., 2009; Rudnick and Lacy, 2008], suggesting that the generally made “founder assumption” (i.e., considering founder individuals as unrelated individuals) should be routinely tested before commencing captive breeding programs [Russello and Amato, 2004; Rudnick and Lacy, 2008; Goncalves da Silva et al., 2010].

Overall, our results contribute to a worldwide effort in identifying genetically important Galápagos tortoises currently in captivity [Burns et al., 2003; Russello et al., 2007a, 2010], while revealing closely related founders, reconstructing genealogical relationships, and providing detailed management recommendations (Appendix B) for the SDZ tortoises.

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APPENDIX A

List of species and populations are given in Table A1.

APPENDIX B: MANAGEMENT RECOMMENDATIONS

Here, we provide management recommendations based on the results of genetic analyses considered in tandem with current studbook records, if available. In general, wild-born tortoises belong to one species with the two exceptions discussed in the main text (Table 1). Captive-born tortoises do show cases of mixed origin but also significant and nonsignificant indices of first and second-order relatedness (Table 2).

Recommendation 1. Recognize currently unassigned tortoises ISIS000128, ISIS000142, and ISIS000147 as *C. guntheri* from La Cazuella—Isabela Island, ISIS000133 and ISIS000137 as *C. vandenburghi* from Volcano Alcedo—Isabela Island, and ISIS000141 as *C. becki* from Volcano Wolf—northern Isabela Island.

TABLE A1. List of Species and Populations Composing the Microsatellite Reference Database Used in This Study [Expanded from Poulakakis et al., 2008]

Population name	Island	<i>N</i>	Sample ID	Species
Espanola	Espanola	15	ESP01 ESP02 ESP03 ESP04 ESP05 ESP06 ESP07 ESP08 ESP09 ESP10 ESP11 ESP12 ESP13 ESP14 ESP15	<i>C. hoodensis</i>
San Cristobal	San Cristobal	19	SCR02 SCR03 SCR04 SCR05 SCR06 SCR07 SCR08 SCR10 SCR11 SCR13 SCR14 SCR15 SCR16 SCR19 SCR20 SCR21 SCR22 SCR23 SCR24	<i>C. chathamensis</i>
Cerro Fatal	Santa Cruz	30	CF01 CF02 CF03 CF04 CF05 CF06 CF07 CF08 CF09 CF10 CF11 CF12 CF14 CF15 CF16 CF17 F0913 F0914 F0930 F0933 F1652 F1653 F1660 F1661 F1668 F1669 F1670 F1676 F1677 F1684	Undescribed
La Caseta	Santa Cruz	25	CRU02 CRU04 CRU05 CRU06 CRU12 CRU13 CRU14 CRU16 CRU18 CRU19 CRU20 CRU21 CRU22 CRU23 CRU24 CRU34 CRU38 CRU41 CRU42 CRU43 CRU54 CRU60 CRU61 CRU64 CRU66	<i>C. porteri</i>
Pinzon	Pinzon	27	PZ03 PZ04 PZ05 PZ06 PZ08 PZ09 PZ10 PZ13 PZ14 PZ15 PZ16 PZ22 PZ28 PZ37 PZ40 PZ56 PZ57 PZ67 PZ70 PZ74 PZ77 PZ78 PZ80 PZ81 PZ82 Pz15192 Pz15193	<i>C. ephippium</i>
Santiago	Santiago	25	AGO02 AGO04 AGO08 AGO09 AGO11 AGO12 AGO14 AGO15 AGO17 AGO18 AGO19 AGO20 AGO21 AGO23 AGO24 AGO29 AGO31 AGO32 AGO34 AGO35 AGO41 AGO43 AGO44 AGO50 AGO51	<i>C. darwini</i>
Volcano Darwin	Isabela	25	VD01 VD02 VD03 VD04 VD05 VD06 VD07 VD08 VD09 VD10 VD11 VD12 VD13 VD14 VD15 VD16 VD17 VD19 VD20 VD21 VD22 VD23 VD24 VD25	<i>C. microphyes</i>
Volcano Alcedo	Isabela	24	VA01 VA02 VA03 VA04 VA05 VA06 VA07 VA08 VA09 VA10 VA365 VA373 VA378 VA379 VA385 VA387 VA423 VA682 VA935 VA984 VA993 VA1038 VA1082 VA1098	<i>C. vandenburghi</i>

TABLE A1. Continued

Population name	Island	<i>N</i>	Sample ID	Species
La Cazuela	Isabela	10	CAZ01 CAZ04 CAZ05 CAZ06 CAZ07 CAZ11 CAZ13 CAZ14 CAZ15 CAZ19	<i>C. guntheri</i>
Roca Union	Isabela	19	RU01 RU02 RU05 RU08 RU09 RU12 RU13 RU14 RU16 RU17 RU21 RU29 RU37 RU39 RU44 RU47 RU48 RU63 RU66	<i>C. guntheri</i>
Cabo Rosa	Isabela	10	CR01 CR02 CR03 CR04 CR05 CR06 CR07 CR08 CR09 CR11	<i>C. guntheri</i>
Cinco Cerros	Isabela	13	CC15 CC21 CC22 CC32 CC45 CC74 CC100 CC295 CC325 CC326 CC333 CC336 CC363	<i>guntheri/vicina</i>
Los Crateres	Isabela	10	LC01 LC02 LC03 LC04 LC05 LC06 LC08 LC09 LC11 LC32	<i>C. vicina</i>
Las Pegas	Isabela	10	P10 P11 P12 P13 P14 P34 P35 P36 P37 P38	<i>C. vicina</i>
Las Tablas	Isabela	10	LT01 LT02 LT03 LT04 LT05 LT06 LT07 LT10 LT11 LT12	<i>C. vicina</i>
Las Pegas	Isabela	10	LP01 LP02 LP03 LP06 LP07 LP08 LP11 LP21 LP31 LP34	<i>C. vicina</i>
Pinta	Pinta	12	PNT01 PNT02 PNT03 PNT04 PNT12 PNT13 PNT14 PNT15 PNTLG PNT8110 PNT8113 PNTV870	<i>C. abingdoni</i>
Floreana	Floreana	16	FLO1905 FLO11069 FLO29998 FLO32590 FLO4476 FLO45756 FLO46403 FLO46404 FLO46405 FLO46406 FLO46407 FLO46408 FLO46412 FLO46413 FLO46425 FLO46606	<i>C. elephantopus</i>
Piedras Blancas	Isabela	34	PBL02 PBL05 PBL06 PBL07 PBL09 PBL10 PBL11 PBL13 PBL14 PBL15 PBL16 PBL17 PBL18 PBL19 PBL20 PBL21 PBL25 PBL27 PBL29 PBL37 PBL41 PBL42 PBL43 PBL44 PBL45 PBL46 PBL47 PBL48 PBL49 PBL50 PBL51 PBL52 PBL53 PBL54	<i>C. becki</i>
Puerto Bravo	Isabela	26	PBR01 PBR02 PBR03 PBR04 PBR05 PBR06 PBR07 PBR08 PBR09 PBR10 PBR11 PBR12 PBR13 PBR15 PBR16 PBR17 PBR18 PBR19 PBR20 PBR21 PBR22 PBR23 PBR24 PBR25 PBR26 PBR28	<i>C. becki</i>

Recommendation 2. Retain tortoises ISIS000129, ISIS000131, and ISIS000149 as *C. porteri* from La Caseta—Santa Cruz Island and ISIS000139 as *C. vicina*—southern Isabela Island.

Recommendation 3. Consider tortoises ISIS000134 and ISIS0001060 as *C. becki* and *C. microphyes*, respectively, recognizing that they carry non-native haplotypes and thus are individuals that should not be included in the breeding program.

Recommendation 4. Reassign tortoises ISIS000130 (previously *C. vanderburghi*) to *C. guntheri* from La Cazueta—Isabela Island, ISIS000132 (previously *C. microphyes*) to *C. vandenburghi* from Volcano Alcedo—Isabela Island, and ISIS000148 (previously *C. microphyes*) to *C. vicina*—southern Isabela Island.

Recommendation 5. Classify ISIS000128 and ISIS000142, both founders of the SDZ captive population, as parent–offspring.

Recommendation 6. Classify the following pairs as parent–offspring: ISIS000128/ISIS000121 and ISIS000129/ISIS901146.

Recommendation 7. Classify the following pairs as second-order related: ISIS000150/ISIS001063, ISIS000132/ISIS000150, ISIS000132/ISIS001063, and ISIS000130/ISIS00012.

Recommendation 8. Classify the ISIS010029 as a hybrid of *C. becki* and *C. vandenburghi*.

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