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RESEARCH ARTICLE

Cryptic structure and niche divergence within threatened Galápagos giant tortoises from southern Isabela Island

D. L. Edwards · R. C. Garrick · W. Tapia · A. Caccone

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Abstract Although Galápagos giant tortoises are an icon for both human-mediated biodiversity losses and conservation management successes, populations of two species on southern Isabela Island (*Chelonoidis guntheri*, and *C. vicina*) remain threatened by hunting and persistence of feral animals. Conservation management of these tortoises has been hampered by lack of clarity regarding their taxonomy, ecological and morphological diversity, and the spatial distribution of evolutionarily significant units that may exist. Analyses of 16 microsatellite loci did not group samples according to current taxonomy. Instead, three (rather than two) genetic clusters were revealed. We show that the three regions of southern Isabela associated with these genetic clusters are significantly different in their ecological niches, which could suggest that ecological divergence may have shaped patterns of genetic differentiation in these tortoises. Furthermore, results suggest limited recent gene flow among sampled localities and between each of the three regions associated with genetic clusters. We discuss the need for further research on the ecological factors shaping the genetic and morphological diversity of southern Isabela tortoises. We suggest that current strategies whereby populations are managed separately are warranted pending further study, but due to mixed ancestry we recommend that Cerro Paloma tortoises be excluded from management programs.

Keywords *Chelonoidis* · Conservation · Management · Ecological divergence · Evolutionarily significant unit · Galápagos tortoise · Genetic divergence

Introduction

Galápagos giant tortoises not only represent a classic evolutionary radiation (Darwin 1839; Fritts 1984), but also are icons for conservation management as well as clear examples of human-induced population declines (Nicholls 2006). Galápagos giant tortoises (*Chelonoidis* spp.) are an integral part of Galápagos ecosystem balance and among those fauna most heavily impacted by hunting since the discovery of the archipelago in 1535 (Pritchard 1996; Cayot 2008). Tortoises were once numerous on at least nine islands within the Galápagos Archipelago. However, the number of tortoises taken by American whaling and military vessels alone has been estimated at >100,000, excluding those taken by other nations' whaling, military and pirate vessels throughout late eighteenth to early twentieth centuries (Townsend 1925). The introduction of feral animals (i.e., goats, donkeys, rats and pigs) during this period exacerbated population declines on many islands (Pritchard 1996; Phillips et al. 2012). This legacy of interactions with humans left most islands with severely depleted tortoise populations, and three islands without an endemic tortoise species (Pritchard 1996; Cayot 2008).

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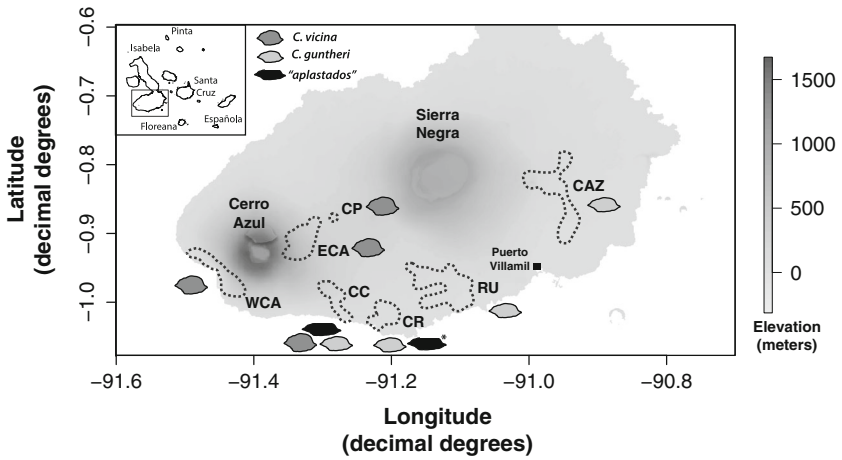

Fig. 1 Distribution of genetic sampling for southern Isabela Galápagos giant tortoises, with a map of the Galápagos Archipelago and geographic focus inset. The geographic extent of discrete tortoise populations is derived from long-term survey records and shown in *black dotted lines* overlaid on a digital elevation model (http://srtm.csi.cgiar.org/), showing the Cerro Azul and Sierra Negra volcanoes. The names of each sampling locality are abbreviated as in Table 1. Also shown are the putative phenotypic forms which have been recorded at each location, including the *C. guntheri*-type [Cazuela (CAZ), Roca Unión (RU), Cabo Rosa (CR), Cinco Cerros (CC)], *C. vicina*-type [CC, Eastern Cerro Azul (ECA), Cerro Paloma (CP), western Cerro Azul (WCA)], and the "aplastados"-type (CC, CR) which co-occurs with one (CR) or both (CC) nominate species phenotypes, but may have gone extinct in CR (*asterisks*)

This had dramatic ecological consequences, as tortoises are the primary herbivore (Gibbs et al. 2010), seed disperser (Blake et al. 2012a), and ecosystem engineer (Gibbs et al. 2008)in the Galápagos Islands.

Through targeted management efforts, Galápagos giant tortoises also represent a conservation success story (Cayot 2008; Hennessy 2013). The implementation of policies, informed by genetic and ecological analyses, has led to population growth and brought several species back from the brink of extinction (Cayot 2008). Genetic techniques have assisted conservation management by establishing the evolutionary significance of currently recognized lineages (Caccone et al. 2002; Poulakakis et al. 2012), identifying individuals of high conservation value in captive (Burns et al. 2003; Russello et al. 2010; Benavides et al. 2012) and wild tortoise populations (Russello et al. 2007; Poulakakis et al. 2008; Garrick et al. 2012; Edwards et al. 2013), as well as allowing monitoring of genetic diversity in repatriation programs (Milinkovitch et al. 2004; 2013).

While conservation success has been achieved for many Galápagos giant tortoise species, managers still face challenges. The tortoises from southern Isabela Island are an ongoing management concern. Formally described as two species (*Chelonoidis guntheri* and *C. vicina*; Van Denburgh 1914),these tortoises were historically continuously distributed across the southern and interceding slopes of two volcanoes, Cerro Azul and Sierra Negra (Fig. 1) with population sizes described as historically "enormous" (Pritchard 1996) and "the largest … in the islands" (Beck 1903). However, they were also heavily exploited (Pritchard 1996) as an oil source, with continuous harvesting operations based at Puerto Villamil, the only human settlement on Isabela Island, throughout the late nineteenth to mid twentieth centuries (Beck 1903; MacFarland et al. 1974). Following this initial depletion, hunting during the past 80 years has left the southern slopes of the Sierra Negra and surrounding Puerto Villamil without tortoises (Fig. 1). Within the last 40 years, continued poaching also has led to the local extinction of tortoises from Cerro Paloma (Cayot and Snell 1996), the extermination of most adults from Cazuela (Cayot and Lewis 1994; Tapia 1997; Márquez et al. 2007; Fig. 1), and contributed to the continued decline of remaining populations. Hunting, mostly for local consumption, and predation by feral animals (e.g., wild pigs) has prompted calls for management agencies to bolster recruitment and eliminate hunting (Cayot and Lewis 1994).

Efforts to implement conservation management strategies on southern Isabela are complicated by the lack of clarity of the patterns of morphological, ecological and genetic diversity of its tortoises. This knowledge is critical for evaluating how many evolutionarily significant units (ESUs) exist. ESUs can be considered geographically isolated, genetically divergent, or differentially locally adapted populations within a species (Ryder 1986; Crandall et al. 2000). The description of two species, *C. guntheri* (four local populations: Cazuela, Roca Unión, Cabo Rosa, Cerro Paloma) and *C. vicina* [eastern and western Cerro Azul (WCA)], geographically overlapping at Cinco Cerros (Fig. 1; Van Denburgh 1914; Porter 1976) might suggest the existence of two phenotypically divergent forms. However, southern Isabela tortoises have been described as

comprising a gradient in morphotypes whose distribution is thought to be associated with clinal environmental variation (Colinvaux 1972; Fritts 1984). Additionally, a third morphotype, with a characteristically flattened carapace ("aplastados"; Cayot and Lewis 1994; Tapia 1997),has also been reported at Cinco Cerros and up until the early 1900s in Cabo Rosa (Fig. 1; Pritchard 1996).

Genetic evidence for distinct ESUs has not been explicitly tested despite previous genetic studies (Ciofi et al. 2002, 2006). The two described species, *C. guntheri* and *C. vicina*, are the most recently diverged species within the Galápagos giant tortoise radiation (<470,000 years ago; Poulakakis et al. 2012), complicating efforts to understand the distribution of spatial genetic variation and divergence. This is likely correlated with the young geological age of Cerro Azul and Sierra Negra, which emerged <800,000 years (Geist et al. 2014), with Sierra Negra being slightly older than Cerro Azul (Naumann and Geist 2000). Genetic data indicate that tortoises colonized this region sometime after its emergence from the neighboring older islands of Santa Cruz and/or Floreana (Beheregaray et al. 2004). On southern Isabela, tortoises colonized WCA from Sierra Negra, with little recent gene flow between localities (Ciofi et al. 2006).

Despite efforts to understand the evolutionary history of tortoise populations on southern Isabela we will address several key questions for effective conservation management that remain unanswered. Namely, what is the spatial distribution of ESUs within southern Isabela Galápagos giant tortoise species? Do genetically differentiated populations occupy different niches?

Materials and Methods

Sampling and genetic data collection

Blood samples were collected from 172 individuals as described in Ciofi et al. (2002) and included samples from seven geographic localities(Table 1; Fig. 1) covering the distribution of two species, *C. vicina* and *C. guntheri*. An additional population, reported to contain hybrid individuals (Cinco Cerros) based on morphological assessment (Van Denburgh 1914; Porter 1976),was also included. These samples represent a randomly chosen subset of those analyzed by Ciofi et al. (2006), with the addition of representatives from Cero Paloma and eight additional individuals from WCA, all kept in captivity in the Parque Nacional de Galápagos (PNG) in Puerto Villamil. The Cerro Paloma tortoises are the last remaining individuals from this location, hunted to extinction in the wild within the last 40 years (Márquez et al. 2007). Approximate spatial data (see below), but no morphological data, were available for these samples.

Table 1 Data summary statistics calculated from microsatellite data for each of seven sampling localities across southern Isabela

Sampling Site	Summary statistic						
	N	H_O	H_E	F_{IS}	HWE *P* value	A_R	*Ne* 95 % CI
WCA	29	0.69	0.69	−0.01	0.25	4.60	31–49
Cerro Paloma (CP)	7	0.62	0.64	−0.02	0.31	4.25	7–Inf
ECA	30	0.75	0.72	−0.04	0.64	5.23	32–49
Cinco Cerros (CC)	26	0.74	0.75	0.02	0.31	5.74	372–Inf
Cabo Rosa (CR)	22	0.74	0.74	−0.01	0.41	5.05	17–28
Rocca Union (RU)	27	0.75	0.73	−0.04	0.08	5.27	28–44
Cazuela (CAZ)	23	0.65	0.62	−0.06	0.29	4.22	19–37

Shown is the number of individuals genotyped (N), mean observed (H_O) and expected (H_E) heterozygosity, mean Weir and Cockeram's (1984) F_{IS} coefficient, Hardy–Weinberg Equilibrium *P* values (HWE *P* value), rarefaction-corrected allelic richness (A_R), and effective population size (*Ne*). All statistics are averaged across 16 microsatellite loci

Genomic DNA extraction followed the procedures outlined in Benavides et al. (2012). Polymerase Chain Reaction (PCR) conditions for 20 microsatellite loci (Gal45, Gal50, Gal75, Gal94, Gal100, Gal127, Gal136, Gal159, Gal263, Gal194, Gal288, AC063, Gal158, AC247, AC251, Gal21, Gal39, Gal149, AC190, AC111) were as described in previous studies (Milinkovitch et al. 2004; Ciofi et al. 2006; Benavides et al. 2012). Amplified fragments were run on an ABI PRISM 3730 Genetic Analyzer together with a ROX-500 size standard. Allele peak size determination was performed using GeneMapper v3.7 (Applied Biosystems) and checked by eye. Microsatellite allele sizes were binned with Tandem v1.09 (Matschiner and Salzburger 2009), using default settings from raw allele size information for consistency (Idury and Cardon 1997). Loci were developed from an archipelago-wide reference dataset, comprised of ~350 tortoises that represent all extant (and extinct) species and chosen to be the most variable in all species (Milinkovitch et al. 2004; Ciofi et al. 2006; Benavides et al. 2012).

Tests of genetic diversity, effective population size (N_e) and population genetic structure

Microsatellite data from each locality were screened for homozygote excess, stuttering effects and null alleles using MICROCHECKER (Van Oosterhout et al. 2004). Further

assessment of microsatellite loci was performed using exact tests of Hardy–Weinberg Equilibrium (HWE) in GENEPOP v4.0.10 (Raymond and Rousset 1995), which resulted in the exclusion of four loci (Gal100, Gal158, AC251, and AC111). The remaining 16 loci were used in subsequent analyses. The average allelic richness (corrected by rarefaction) per locality was calculated in the hierfstat R package (Goudet 2013) using the R statistical software package (http://www.R-project.org/). For each locality, observed and expected heterozygosity were calculated using Arlequin v3.5.1.3 (Excoffier and Lischer 2010),and Weir and Cockerham's (1984) estimate of F_{IS} was calculated using GENEPOP v4.0.10 (Raymond and Rousset 1995). The LD bias correction method (Waples 2006), implemented in LDNe (Waples and Do 2008), was used to estimate the effective population size (*Ne*) of each sampling location. We ran the analysis using a lowest allele frequency of 0.001.

Bayesian clustering of genotypic data, implemented in STRUCTURE v2.3.3 (Pritchard et al. 2000), was used to determine the number of genetic clusters and the assignment of each individual to a given cluster. Values of $K = 1$–8 were examined, with 40 replicates per K. For each run, search settings were: 'admixture' and 'correlated allele frequencies' models, 1×10^5 MCMC iterations burn-in and 1×10^6 main iterations, with all other parameters as default. Replicates were run with and without a location prior. The best-fit number of genetic clusters from STRUCTURE analyses was determined using the ΔK method (Evanno et al. 2005). STRUCTURE results were processed using all replicates through Structure Harvester (Earl and von Holdt 2011), CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004).

Discriminant Analysis of Principal Components (DAPC) was also carried out using the dapc function of adegenet package (Jombart 2008). DAPC is analogous to discriminant function analyses, but measure the ability to differentiate groups (in this case the defined structure groups) in genetic space. For DAPC analyses, K was selected using the find.clusters function and Bayesian Information Criterion (BIC) and the number of principal components were optimized using the optim.a.score function with 9,999 replicates. The number of genetic clusters (K) was determined using the consensus results of the ΔK and BIC methods.

We calculated Jost's D (Jost 2008) estimates of genetic differentiation, more accurate than F_{ST}, between locations associated with genetic clusters and between all sampling localities using the divPart function of the diveRsity R package with 1,000 replicates (Keenan et al. 2013). To assess if isolation by distance plays a role in differentiation, we used Mantel tests for correlations between geographic distances and genetic differentiation (Jost's D). Centroid points for each locality were used to calculate the geographic distances [using the earth.dist function of the fossil R package; Vavrek 2011)] between sampling locations, which were log transformed prior to analysis. Mantel tests were undertaken using the mantel.rtest function of the ade4 R package (Dray and Dufour 2007) and 9,999 permutations for testing significance, and carried out both with, and without the Cazuela location.

Tests of migration and gene flow between localities and genetic clusters

The identification of first-generation (F_0) migrants and their population of origin provide insights into present-day rates and directionality of gene flow. To assess this we first identified F_0 migrants and then characterized the patterns of recent gene flow among localities and regions associated with genetic clusters. To detect F_0 migrants between pairs of either sampling locations ($N = 7$) or three regions associated with genetic clusters ($N = 3$), we used GENECLASS v2.0 (Piry et al. 2004) with the following settings: L_{home}/L_{max} likelihood computation (Paetkau et al. 2004), frequencies-based criteria for likelihood computation (Paetkau et al. 1995) with default missing allele frequency set at 0.01, and the probability computation Monte-Carlo re-sampling method (Paetkau et al. 2004) with 1,000 simulated individuals and significance assessed at the $\alpha = 0.05$ level.

BAYESASSv3.0.2 (Wilson and Rannala 2003) was used to estimate recent historical gene flow, with runs of 30×10^6 iterations, sampling every 10,000th iteration, with the first 3×10^6 iterations discarded as burnin. Setting the mixing parameters for migration rates, allele frequencies, and inbreeding to 0.80, 0.80, and 0.80 respectively in analyses between sampling locations consistently provided acceptance rates of 20–40 %. Similarly, setting the mixing parameters for migration rates, allele frequencies, and inbreeding to 0.20, 0.30, and 0.30 respectively in analyses between the three regions associated with genetic clusters consistently provided acceptance rates of 20–40 %. Convergence between runs was assessed by confirming the similarity of results from multiple runs with different seeds, while convergence within individual runs was assessed using trace files (i.e., ESS > 100).

Occurrence points and environmental data

Occurrence data for southern Isabela tortoises were obtained from spatial records associated with long-term survey data (2001–2011) collected by the PNG and curated by the Charles Darwin Foundation (CDF) across six of the seven sampling localities. No spatial data for the genetic samples used were available. The Cerro Paloma locality was excluded from these analyses given difficulties in assignment to a specific genetic cluster (see "Results"

section) and because data on their exact capture location is unavailable. Table S1 contains the spatial points used for this study.

Climate data were derived from the WorldClim global climate database (including 19 climatic variables available from Hijmans et al. (2005) for current conditions (1950–2000). To describe physical habitat components, satellite derived variables from the NASA-MODIS Terra datasets were obtained through the Land Processes Distributed Archive Center Data portal (https://lpdaac.usgs.gov/get_data/data_pool). The 2001–2011 Normalized Difference Vegetation Index (NDVI – MODIS 13Q1) 16-day interval datasets were used to derive an average annual mean and standard deviation (a measure of seasonality) over this period. This provided two layers describing average annual and seasonal variation in vegetation greenness or photosynthetic capacity (Goward et al. 1991). As a proxy for biomass productivity we used the Leaf Area Index (LAI) from MODIS 15A2 8-day interval product that describes canopy structure and is related to functional process rates of energy and mass exchange (Myneni et al. 1997). Both average annual mean and standard deviation (as a measure of seasonality) were calculated for LAI using the 2001–2011 datasets. Average annual percentage tree cover (TREE) was calculated using the MODIS44B annual data from 2002 to 2010. Elevation data were derived from a Global 90 m Digital Elevation Model from Diva-GIS (http://srtm.csi.cgiar.org/).

To improve the biological interpretation of niche axes, climate data were tested for autocorrelation prior to analysis. All vegetation variables, elevation, and seven climatic variables with a correlation coefficient $r < 0.95$ were retained (McCormack et al. 2010): Bio2 (mean diurnal temperature range), Bio3 (isothermality—comparison between diurnal temperature range and seasonal temperature range), Bio4 (temperature seasonality), Bio7 (temperature annual range), Bio9 (Temperature of the driest 3 months), Bio12 (annual precipitation) and Bio15 (precipitation seasonality). Environmental data were sampled for the six sampling localities using the extract function of the raster R package (Hijmans and van Etten 2013). First subtracting the mean and dividing by the standard deviation of each variable for subsequent analyses standardized all environmental data.

Tests of niche divergence between genetic clusters

We used McCormack et al.'s (2010)multivariate method to test for niche divergence between the three regions associated with genetic clusters in southern Isabela tortoises. This approach is ideal for detecting niche divergence between allopatric taxa, and provides added power over alternative approaches (e.g., Warren et al. 2008), in that it can detect divergence in niche aspects that do not explain large amounts of variation, but are nevertheless important contributors to niche divergence between closely related taxa (McCormack et al. 2010). Spatial occurrence data from long-term surveys were used to define the spatial distribution of each collection locality, which could be linked directly to each of three defined distinct genetic clusters, one of which was predominant at each of these sampling localities (see "Results" section). Polygons were drawn around the distributions of occurrence records in ArcMap 10 (Fig. 1), and these were used to generate 1000 random background points as jackknife replicates. Sampled environmental data from these background points was used to generate a null environmental distribution for each of three regions associated with genetic clusters, while the occurrence records were used as observed environmental samples.

All environmental data were extracted using the extract function of the raster R package (Hijmans and van Etten 2013). Principal components analyses (PCA) were undertaken in the dudi.pca R function of the vegan package (Oksanen et al. 2013) on both the observed and background environmental data. Together, the first six PC axes described ~95 % of the environmental variation, and were retained for tests of niche divergence/conservation. These six axes described a modest proportion of the variation (>3 %) following the methods outlined in McCormack et al. (2010). We use the loadings of these PC axes to assign a biological interpretation to each axis (Table 2).

Niche conservation/divergence was determined by calculating the difference between observed niche overlap values (D_{obs}) between regions associated with genetic clusters with the null distribution of niche overlap values (D_{null}) between regions associated with genetic clusters. Niche overlap for null and observed distributions was calculated as the difference between the null and observed PC axes for each comparison. Divergence between lineage niches was inferred when $D_{obs} > D_{null}$, while niche conservation was inferred when $D_{obs} < D_{null}$. Significance of niche conservation was inferred if D_{obs} was outside the 95 % confidence limits of D_{null}. To infer niche divergence significance required both an observed niche difference outside the 95 % confidence interval (CI) of D_{null} and that paired *t*-tests showed significant differences between both the observed and null distributions in paired lineage comparisons. Significance for paired *t*-tests was corrected using Benjamini–Yekutieli correction for multiple comparisons (Narum 2006).

Results

Tests of genetic diversity, effective population size (N_e) and population genetic structure

Summary statistic results for each of the sampling localities are shown in Table 1. None of the sampling localities

Table 2 PCA Loadings of environmental variables from observed data used in tests of niche divergence (McCormack et al. 2010) and cumulative percentage variation accounted for by each of the six PC axes

Environmental variables (% variance explained)	PC1 (44.45 %)	PC2 (19.82 %)	PC3 (14.13 %)	PC4 (8.26 %)	PC5 (4.43 %)	PC6 (3.75 %)
Bio2: avg. diurnal temp. range	**−0.92**	−0.22	0.22	0.13	−0.10	−0.05
Bio3: temp. isothermality	0.04	−0.33	**0.62**	**0.65**	−0.15	−0.17
Bio4: temp. seasonality	−0.77	0.03	−0.42	−0.33	−0.14	−0.02
Bio7: temp. annual range	**−0.97**	−0.09	−0.09	−0.15	−0.05	0.00
Bio9: avg. dry temp.	**0.96**	−0.11	0.05	−0.02	0.09	0.02
Bio12: annual prec.	**−0.98**	−0.03	0.04	0.04	−0.05	−0.05
Bio15: prec. seasonality	**0.96**	0.02	−0.14	−0.03	0.05	−0.00
Elevation	0.70	−0.13	−0.38	−0.13	**−0.43**	**−0.32**
LAI	−0.14	**0.63**	−0.44	**0.53**	−0.03	**0.28**
NDVI	−0.08	0.46	**0.74**	−0.35	0.16	−0.18
sdLAI	0.22	**−0.64**	0.40	−0.22	**−0.38**	**0.42**
sdNDVI	0.02	**0.87**	0.12	0.05	**−0.38**	−0.11
TREE	0.18	**0.79**	0.38	−0.17	−0.11	0.22
% Cumulative variance explained	44.45 %	64.27 %	78.40 %	86.66 %	91.09 %	94.84 %
PC correlation with latitude	$R = -0.04^{n.s.}$	$R = -0.50^{***}$	$R = -0.20^{**}$	$R = -0.09^{n.s.}$	$R = -0.24^{***}$	$R = 0.12^{*}$
PC correlation with longitude	$R = 0.\ 50^{***}$	$R = -0.17^{**}$	$R = -0.51^{***}$	$R = -0.13^{*}$	$R = -0.30^{***}$	$R = -0.01^{n.s.}$
Biological interpretation	Temperature and precipitation	Vegetation	Temperature and vegetation seasonality	Temperature seasonality and biomass	Elevation and Leaf Area Index	Vegetation and elevation

Environmental variables shown in bold contribute to the most variation within axes and are used for biological interpretations from each axis (last row). The Spearman correlation coefficients (R) between each PC axis and latitude and longitude are also shown to indicate the spatial autocorrelation associated with each niche axis. *LAI* Leaf Area Index, *NDVI* Normalized Difference Vegetation Index, *sdLAI* standard deviation LAI, *sdNDVI* standard deviation NDVI, and TREE

$^{n.s.}$ $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

deviated significantly from HWE expectations. Most locations had moderate levels of allelic richness (corrected by rarefaction), with the Cazuela and Cerro Paloma locations having the lowest estimates of allelic richness (Table 1). Effective population estimates were generally low across all locations, excepting Cinco Cerros, with the lowest *Ne* estimates for the Cazuela, Cabo Rosa and Cerro Paloma locations. The latter is likely inaccurate given the small sample size (England et al. 2006; Table 1).

The best-fit value of K, used to refer to the optimal number of genetic clusters given the data, ranged between $K = 3$ and $K = 4$ using DAPC and ΔK from STRUCTURE runs (Fig. S1). The value of K inferred from ΔK for STRUCTURE runs that did not explicitly use a location prior (Figs. 2a, S1B) suggest $K = 4$. However, the results were more ambiguous when STRUCTURE analyses were run with a location prior (Fig. S1A) and suggest K could either be 3 or 4. This ambiguity is also reflected in the DAPC results based on the lowest values of BIC (Fig. S1C). Individual assignment to a specific genetic cluster is more stable for $K = 3$ compared to $K = 4$, regardless of the method used (Fig. S1). As $K = 3$ also represents the smallest value that captures the major structure in the data (Pritchard et al. 2000),we therefore used $K = 3$ in subsequent analyses.

Assignment of individuals into the three genetic clusters largely represented three geographically separate groups constituting a western (WCA), central (eastern Cerro Azul, Cinco Cerros, Cabo Rosa, Roca Unión),and eastern cluster (Cazuela—Fig. 2). As individuals from the Cerro Paloma location were assigned with high probability ($Q > 0.94$) to one of two different clusters (western $N = 5$; central $N = 2$; Fig. 2a), these individuals were removed from subsequent analyses involving comparisons between the regions associated with genetic clusters. The majority of individuals within eastern, central and western regions could be assigned correctly to their home region, and therefore the genetic cluster specific to the region, using

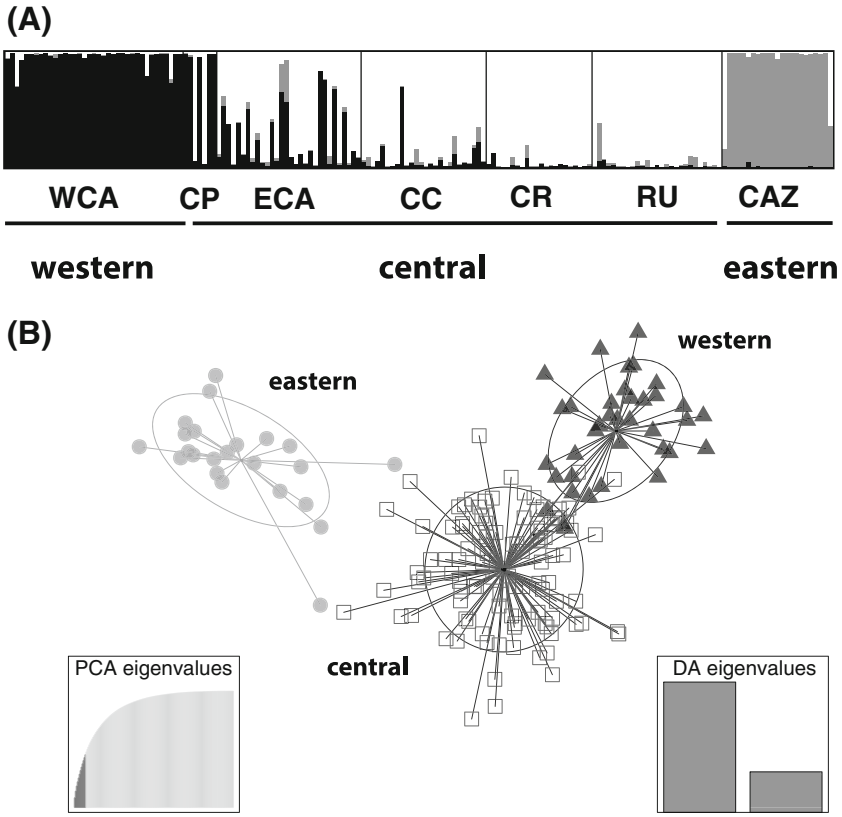

Fig. 2 Results of STRUCTURE (**a**) and DAPC (**b**) analyses of southern Isabela tortoises showing clear discrimination between three regions associated with genetic clusters where the Cazuela (CAZ) and WCA sites form distinct clusters, in addition to a central cluster encompassing several sampling locations. **a** Genetic clustering results (not using a location prior), where *bars* represent single individuals at each sampling location. *Colors* in each bar represent the proportion of assignment to three genetic clusters from western (*black*), central (*white*) and eastern (*grey*) regions of southern Isabela (see map inset). **b** DAPC results highlight the genetic distinctiveness of the western (*closed triangles*), central (*open squares*) and eastern (*closed circles*) regions associated with three genetic clusters. The graph plots discriminant axis 1 (x-axis) against discriminant axis 2 (y-axis) with the proportional variation explained by each show in the *bottom right panel*. Each point represents an individual. *Lines* link each individual to the centroid for each region and *circles* around the centroid encompass the 95 % CI around the centroid value. DAPC analyses were undertaken using the first 19 principal components (*bottom left panel*)

Q values (>90 %) with extremely high accuracy (eastern—91 % or 2 individuals not assigned; central—98 % or 2 individuals not assigned; western—100 %).

Differentiation measures support the distinction of the geographic regions as genetic clusters with moderate values of Jost's D (eastern vs. central: Jost's $D = 0.27$; eastern *vs* western: Jost's D 0.40; central vs. western: Jost's $D = 0.15$). Table S2 contains the pairwise estimates of genetic differentiation (Jost's D) between sampling locations. Mantel tests including the Cazuela location showed no evidence of significant isolation by distance ($r = 0.51$; $P > 0.05$), despite a moderate correlation coefficient. An effect possibly due to the large distance between Cazuela and remaining locations created by local extinction driven by hunting, as tests excluding Cazuela show little correlation between genetic differentiation and geographic distance ($r = 0.16$; $P > 0.05$).

Tests of migration and gene flow between localities and genetic clusters

Using GENECLASS the number of F_0 migrants ($N = 5$) among the three regions associated with genetic clusters was very low. There were two putative F_0 migrants sourced from the central cluster and sampled in the eastern cluster, one migrant from the central into the western cluster, and one putative F_0 migrant from the western into central cluster. Similarly low rates of recent gene flow were inferred from BAYESASS analyses (Fig. 3). The majority of gene flow comes from the western region into the central region, with some migration in the opposite direction and from the central region into the eastern region. Further confirming the distinctiveness of the three regions associated with genetic clusters, self-replenishment rates were 91–98 % suggesting the tortoise populations in these

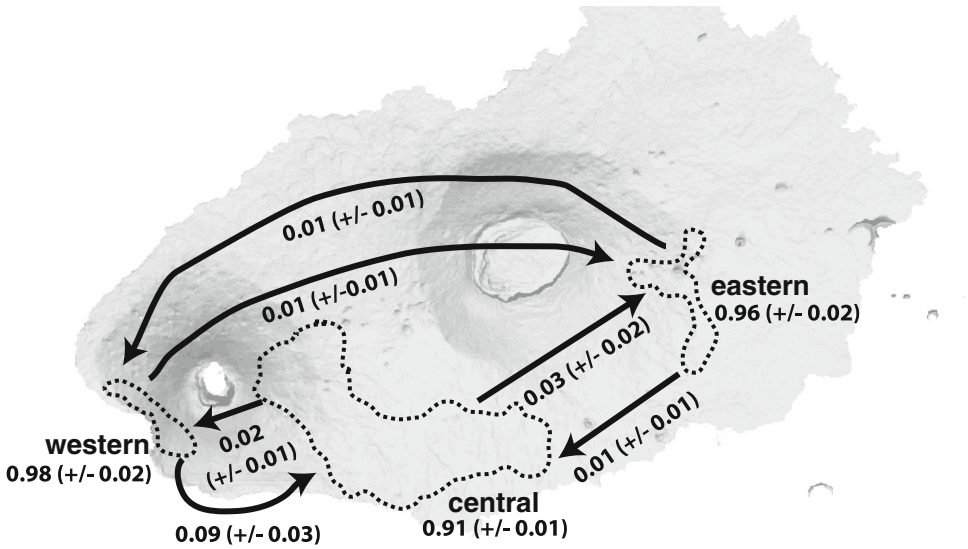

Fig. 3 BAYESASS estimated rates of recent gene flow between the three regions associated with genetic clusters inferred from STRUCTURE analyses. Mean estimates are shown for each direction below the *arrow* (95 % CI in *brackets*) and overlaid on top of a digital elevation model (http://srtm.csi.cgiar.org/) of southern Isabela Island. Below each region name self-recruitment rates are shown (95 % CI in *brackets*). *Dotted lines* circumscribe the spatial extent of the three regions housing putative ESU's identified in this study (western, central, and eastern)

regions are maintained by recruitment rather than migration (Fig. 3).

Analyses performed at level of sampling localities ($N = 7$) provided insights on migrant exchanges within and between each of the three major clusters above. GENECLASS results amongst localities showed low levels of F_0 migrants within localities (Table S3). The BAYESASS estimates of recent gene flow (Table S4) did not differ significantly from those reported by Ciofi et al. (2006), even with the inclusion of an additional sampling locality. Results show that the majority of recent gene flow between locations involves gene flow within the central region, specifically into the Cinco Cerros locality from the neighboring Cabo Rosa and Roca Unión localities. Additionally, there is evidence of recent gene flow between the western and central regions, specifically from WCA into eastern Cerro Azul and Cinco Cerros (Table S4). Our results also show that self-recruitment within sampling localities exceeds migration with self-recruitment rates of 68–94 % (Table S4).

Tests of niche divergence between genetic clusters

Environmental PCA across southern Isabela tortoises show that the first six PC axes explain ~95 % of the environmental variation (Table 2). The major determinants underlying ecological divergence amongst the three regions associated with genetic clusters are related to vegetation (i.e. Tree Cover: PC2, NDVI: PC4 and 6, LAI: PC5–6) and seasonality (LAI: PC2 and 5, Isothermality: PC3–4; Table 3). Although the most ecologically divergent regions are at the extremes of the tortoise distribution (i.e., the western and eastern regions; PC2–6), geographically proximate genetic clusters also show evidence of significant niche divergence [i.e., the central region compared with the eastern (PC2) and western (PC3 and 6) regions respectively]. Vegetation niche axes (including Tree Cover, LAI, and NDVI), particularly those describing seasonality in LAI and NDVI, show significant divergence across most comparisons, while temperature and precipitation niches are largely conserved (except isothermality; Table 3).

Discussion

Genetic diversity, migration, gene flow and effective population size (N_e)

Genetic diversity of populations is often correlated with fitness (Reed and Frankham 2003) and therefore linked with the health and evolutionary potential of populations under conservation management (Schwartz et al. 2007; Mills 2012). Relative to other lineages of Galápagos giant tortoise using the same molecular loci, southern Isabela populations have moderate genetic diversity (Table 1). The Cerro Fatal ($H_E = 0.54 \pm 0.06$) and Española ($H_E = 0.55 \pm 0.07$)lineages have the lowest genetic diversity with remaining species having equal or much higher heterozygosity relative to southern Isabela populations ($H_E = 0.68$–0.83; Ciofi et al. 2002). Cerro Fatal tortoises are most likely threatened by conversion of native habitat to farmland (Russello et al. 2005), and Española tortoises were reduced to just 15 individuals through over exploitation and hunting (Milinkovitch et al. 2013). Within southern Isabela, allelic richness and/or low *Ne* estimates may be associated with varying levels of exploitation, particularly comparing the Cazuela, Cerro Paloma, and to a

Table 3 Patterns of multivariate niche divergence and conservation (McCormack et al. 2010) amongst the three regions associated with genetic clusters across southern Isabela Island

Pairwise comparison	PC1		PC2		PC3		PC4		PC5		PC6	
	Difference	Inference	Difference	Inference	Difference	Inference	Difference	Inference	Difference	Inference	Difference	Inference
Eastern vs. central	1.09 (0.98–1.31)	N[#]	1.71 (1.37–1.65)	**D**[#]	0.37 (0.59–0.78)	**C**[#]	0.14 (0.04–0.11)	D	0.11 (0.57–0.67)	C	0.21 (0.24–0.37)	C
Eastern vs. western	1.98 (3.35–3.66)	**C**[#]	1.37 (1.19–1.38)	N[#]	2.28 (1.42–1.64)	**D**[#]	0.56 (0.32–0.51)	**D**[#]	0.65 (0.24–0.35)	**D**[#]	0.16 (0.01–0.12)	**D**
Central vs. western	0.89 (2.19–2.54)	**C**[#]	0.33 (0.08–0.37)	N	1.91 (0.74–0.95)	**D**[#]	0.42 (0.35–0.54)	N[#]	0.53 (0.86–0.97)	**C**[#]	0.37 (0.21–0.30)	**D**[#]
Important variables	(Annual prec.)		*LAI seasonality*		NDVI seasonality		Isothermality		(Elevation)		Av. LAI	
	(Annual temp. range)		*Tree cover*		Isothermality		Av. NDVI		(Av. LAI)		(Elevation)	
	(Diurnal temp. range)		(Av. LAI)						(LAI Seasonality)			
	Prec. seasonality		Av. NDVI									
	Av. dry temp.											
Biological interpretation	Temperature and rainfall		Vegetation		Seasonality		Temperature seasonality and biomass		LAI		Vegetation and elevation	

For each of the six niche axes (PC1–6), the average difference between observed and the null distribution (brackets) for each pairwise region(eastern, central, and western) comparison is reported together with the inference for either niche conservation (C) or divergence (D) as opposed to no difference (N). Bolded values indicate statistical significance ($P < 0.05$). Where niche divergence is also inferred from *t* tests (with significant Benjamini–Yekutieli corrected *P* values)

[#] Significant niche divergence is only inferred where *t* tests were significant. The "Important Variables" row lists the variables with high loadings used in the biological interpretation (last row) for each niche axis (Table 2)

certain extent Cabo Rosa locations vs. all other localities (Table 1; Cayot and Lewis 1994). Hunting may be associated with low allelic richness and *Ne* in the Cazuela population, and low *Ne* estimates in the Cabo Rosa and Cerro Paloma populations. Reports suggest that almost the entire adult tortoise population was extirpated from Cazuela, leaving mostly juveniles (Cayot and Lewis 1994). This is concerning given the low levels of gene flow into this area estimated from our data (Fig. 3). However, the existence of a large captive population in the PNG breeding center improves the conservation prospects of this population(Cayot and Lewis 1994). Large hunting events have also been reported in the Cabo Rosa locality and have been linked to the extirpation of a local phenotype (Pritchard 1996).

The results recovered for the Cerro Paloma tortoises are more complex to explain. STRUCTURE analyses (Fig. 1a) suggest that Cerro Paloma may comprise purebred individuals genetically assigned to both the genetic clusters associated with western and central regions. One likely explanation may be that the Cerro Paloma individuals taken into captivity were not endemic to this location, but were migrants recently arrived into this area after it was voided of tortoises through persistent heavy hunting (MacFarland et al. 1974; Cayot and Lewis 1994). This would also explain their low genetic diversity (Le Corre and Kremer 1998). If true, the native Cerro Paloma tortoises went extinct well before action was taken to preserve them.

ESUs within southern Isabela tortoises

Establishing the geographic extent of ESUs(Fraser and Bernatchez 2001) is a critical component of determining appropriate conservation management strategies (Lande 1988; Moritz 2002; Schwartz et al. 2007; Mills 2012). From a morphological perspective, two formal species are described for southern Isabela, suggesting the existence of at least two ESUs (Fig. 1). However, Pritchard (1996) presented the possibility of three tortoise lineages on southern Isabela, located at Cerro Azul (including Cinco Cerros), Sierra Negra (Cazuela), and on the lowland slopes between these two volcanoes (Cabo Rosa and Roca Unión; Fig. 1). While our genetic analyses also identify three ESUs, their distributions do not match the spatial distribution of the three units proposed by Pritchard (1996), nor do they mirror the two morphological species, as classified under the current taxonomy (Van Denburgh 1914).

A number of evolutionary scenarios could explain the existence and inferred distribution of the three genetic clusters, and the fact that the regions they occupy have divergent ecological niches (Tables 2, 3). Vegetation niche axes (including Tree Cover, LAI, and NDVI), particularly those describing seasonality in LAI and NDVI, show significant divergence across most comparisons, while temperature and precipitation niches are largely conserved (except isothermality; Table 3). Fritts (1984) suggested that different morphotypes on southern Isabela represent locally adapted phenotypes evolving in response to altitudinal and moisture gradients. We find some differences in the niches of the three regions associated with altitude and temperature seasonality. However, the tortoises in the three regions associated with genetic clusters on southern Isabela occupy significantly divergent habitats with respect to average vegetation, vegetation and temperature seasonality, and altitude (Table 3). This could support Fritts' hypothesis of local adaptation—albeit due to slightly different factors. Little gene flow between the regions occupied by the three genetic clusters (Fig. 3) may also support this hypothesis. However, as we do not have exact locality data and detailed morphology data for our genetic samples, we are unable to conclusively test if the evidence supports adaptive variation (i.e., Schoville et al. 2012).

An alternative explanation for the existence of three ESUs on southern Isabela is that they could represent the result of separate colonization events Isabela, possibly from Santa Cruz and/or Floreana islands (Beheregaray et al. 2004; Poulakakis et al. 2012). Equally plausible evolutionary explanations could also relate to ongoing isolation of populations by recent lava flows (Pritchard 1996; Beheregaray et al. 2003), or isolation by distance (Wright 1943) that has been interrupted by exploitation and subsequent local extinction of tortoises in the areas surrounding Puerto Villamil (Cayot and Lewis 1994; Tapia 1997; Márquez et al. 2007). Isolation by distance has not yet been observed in Galápagos giant tortoises, and we do not find evidence that isolation by distance is responsible for the patterns of population structure observed here. Furthermore, there is increasing evidence that tortoises have the ability to migrate large distances seasonally (Blake et al. 2012b).

Implications for conservation management of southern Isabela tortoises

Our results suggest that southern Isabela populations display moderate genetic diversity levels relative to other species across the Galápagos Archipelago. The success of management efforts to resurrect other giant Galápagos tortoise species with much lower levels of genetic diversity, such as *C. hoodensis* from Española (Milinkovitch et al. 2004; 2013), suggest moderate diversity will not impede recovery prospects for southern Isabela tortoises. Of more concern is ongoing hunting, and little to no recruitment in many locations across southern Isabela, as a result of nest predation by pigs and fire ants (Tapia 1997;

Causton et al. 2006; Edwards D.L. pers. obs). All are processes likely to continue to erode genetic diversity. However, if the Cerro Paloma tortoises in captivity are indeed migrants, we have some indirect evidence that migration, although rare, can help re-colonization of tortoises in depleted areas. Breeding programs have been established for southern Isabela tortoises, as they have been for other Islands in the archipelago (Cayot et al. 1994; Milinkovitch et al. 2004; 2013). However, ongoing hunting pressure has prevented repopulation efforts. Furthermore, captive breeding is a costly approach with limited long-term viability if the causes of population declines are not addressed.

The existence of three genetic groups associated with morphological complexity and ecological niche differences across southern Isabela, suggests that the PNG's strategy of managing southern Isabela tortoises from different locations as separate units is prudent and should be continued in the short-term. However, considering that tortoises from Cerro Paloma may not represent a subset of the native population but recent migrants from other areas, these individuals should not be part of any breeding program until further understanding of their evolutionary origin is achieved.

Conclusions

This data highlight the need for more information on the genetic and ecological complexity of the southern Isabela tortoise populations, as our results show cryptic genetic structure across southern Isabela, and that the locations occupied by these clusters are significantly ecologically divergent. While we cannot exclude alternative evolutionary hypotheses, the existence of niche divergence amongst regions occupied by three distinct genetic clusters provides support for Fritts' hypothesis (Fritts 1984) that adaptive processes may be involved in the evolution of southern Isabela tortoises. Further work is needed to understand the evolutionary history of the three clusters, their relationship to both extinct and currently un-sampled populations, and the association between genetic, environmental, and morphological diversity, as the possibility of local adaptation has consequences for the implementation of conservation programs (Allendorf et al. 2010). We are currently exploring the use of genome-wide markers to improve inferences, which, combined with a denser spatial sampling of the interior regions of southern Isabela and collection of morphometric and ecological data to link to genetic data, will likely provide additional insights to reconstruct the evolutionary history of these tortoises and the role of local adaptation in shaping the current genetic patterns.

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