Electronic Supplementary Material

Hybridization masks speciation in the evolutionary history of the Galápagos marine iguana

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Extended methods

This section provides additional details on field and laboratory protocols as well as statistical analysis, complementing information in the main Methods section.

Field sampling

Marine iguanas from San Cristóbal were newly sampled in 2011-2014 at 17 sites (Table S1). We obtained blood samples from 460 marine iguanas from this island between 2011 and 2013, and used these in addition to samples collected for earlier studies from Punta Pitt (1993; n = 22) and Loberia (1993; n = 31). Animals were captured using poles fitted with a lasso loop. From each individual ca. 0.1 ml of blood was collected from the caudal vein, with the individual in dorsal recumbency. Venipunction point was localized at one third of the distance between the cloacal opening and the tails' end, following the middle line. 24G (0.80 x 40 mm) needles were used for large males (>3kg body weight), 23G (0.60 x 25mm) needles for smaller adults, and 26G (0.45 x 25mm) for larger juveniles, while insulin needles were used for small juveniles. The needle was inserted with a 45°- 90° angle between the needle and the animal. Blood was stored in 75% ethanol or in a blood buffer (100 mM Tris, 100 mM EDTA, 2% SDS) and maintained at 4 – 7°C prior to extraction of genomic DNA.

Phylogenetic analysis and molecular dating: nuclear protein coding genes (dataset A)

To identify the closest relative of the Galápagos iguanas and date their origin on the archipelago, we selected four nuclear genes (RAG1, BDNF, R35 and NKTR) from a previous dataset [1] on the basis of their performance in reconstructing iguanid phylogeny. Sequences were newly determined for three marine iguanas, one land iguana (*Conolophus subcristatus*) and one individual each of *Cyclura cornata*, *Iguana iguana* and *Ctenosaura similis*, and combined with those of another 72 iguanians and other squamates from the dataset of Townsend et al. [1] to allow for the use of established time calibrations. DNA sequences were aligned taking into account their amino acid sequences, using the MAFFT [2] tool employed in Translator-X [3, 4]. Sequences were cleaned with GBlocks [5], using all three of the 'less-stringent' cleaning parameters available in Translator-X, resulting in a final sequence alignment of 3000 nucleotide positions which were used in analysis. Partitions and substitution models for analysis were identified using the Bayesian Information Criterion in Partition Finder [6], using the 'greedy search' scheme. The concatenated sequences were analysed using three approaches. (i) Partitioned Bayesian inference of phylogeny (BI) with MrBayes 3.2 [7], running two analyses of four chains for 20 million generations, sampling every 10,000 generation, and calculating a majority-rule consensus tree discarding the first 25% as burn-in. (ii) Simultaneous inference of phylogeny and divergence times was achieved using Beast 1.7.2 [8], with a set of 18 time constraints across squamates [1] and a Yule Speciation tree prior, running 200 million generations and calculating a maximum clade credibility tree with a burn-in of 25%. For this analysis we used an older Beast version (1.7.2) in order to strictly follow the previously published analysis [1] and to be able to use the same settings, with the goal of reaching fully comparable results. All Beast runs were given enough time to allow effective sample size values of all parameters to reach values well above 200.

(iii) Maximum parsimony bootstrapping was undertaken in Paup 4b2 [9], with 2000 heuristic search replicates under tree-bisection-reconnection, and with random addition sequence (10 replicates). Convergence of chains, mixing of parameters and appropriateness of burn-in settings of all Bayesian analyses was confirmed with AWTY [10].

Phylogenetic analysis and molecular dating: mitochondrial (mt) genes (dataset B)
On the basis of an earlier mitochondrial CR haplotype network [11] a representative selection of marine iguana samples were chosen from across the archipelago for analysis of mitochondrial phylogeny. Sampling was adjusted to represent the most divergent mtDNA phylogroups as well as the two San Cristóbal lineages (PP and LO). In total, up to 5557 bp were sequenced from seven mitochondrial genes (plus 3 adjacent tRNAs) in 20 *Amblyrhynchus*, six individuals of three species of *Conolophus* (including *C. marthae*), and one individual of *Ctenosaura similis*. Sequences of the *I. iguana* mt genome (GenBank accession number AJ278511) were used as outgroup. Sequences were edited using CodonCode Aligner (CodonCode Corporation) and aligned using Clustal W [12], as employed in MEGA (V6.0; [13]). Only single indels were present in the alignment (mainly in the outgroup) and all sites were therefore included in the analysis. Identification of partitions and substitution models, as well as phylogenetic analysis was undertaken as in dataset A, except for the "timetree" analysis with Beast. Here, we specified a coalescent uniform tree prior, a secondary time constraint with an uniform prior (2.76-6.67 mya) for the *Amblyrhynchus-Conolophus* split based on the estimate for this split obtained from analysis of dataset A (credibility intervals), and modified substitution rate priors to allow variation over a wide range. Posterior values did not stabilize with site models suggested by Partition Finder, probably due to overparametrization, and we therefore specified a simple HKY model for all partitions. Analyses with alternative priors and models (different coalescent or speciation tree priors; more complex substitution models; no phylogenetic constraints) were, however, congruent in divergence time estimates, in all cases recovering an age of basal *Amblyrhynchus* splits <0.3 mya.

Phylogenetic analysis and molecular dating: DNA sequences from RADSeq (dataset G)
See main Methods section for details of RADSeq analysis and inference of phylogeny based on SNP data. For molecular dating of splits within *Amblyrhynchus* we used a 4-taxon subset of dataset F (Table S2), containing one land iguana outgroup (Santa Fe, sample LSF06) and three marine iguana specimens (Santa Fe, FES01; San Cristóbal, SRIL10 and SRS19), representing one of the deepest split within marine iguanas and the PP/LO split. We excluded in Paup all sites with missing or ambiguous data in one or several taxa, resulting in a final matrix of 1,793,845 sites, of which 33,452 were variable, and only 330 were variable among the three marine iguanas. The dataset was analysed in Beast 2.0 under a coalescent tree prior (constant growth), time-calibrating the root MRCA according to analysis of dataset A at 4.6 mya (normal prior, standard deviation 0.3). We used a GTR substitution model selected by the Akaike Information Criterion (AIC) in MrModeltest [14]. We ran 2 billion generations, sampled every 100,000th generation, discarded the first 50% trees as burn-in after examination of parameters in Tracer, and ascertained that all ESS values were >200. Alternative explorative runs with the HKY model (selected by AIC for the dataset of three marine iguana samples only) did not result in relevant differences in time estimates.

Mitochondrial differentiation of marine iguanas (dataset C)
An alignment of the complete mitochondrial control region (CR) sequences, 1181 bp in length, was used to analyse archipelago-wide mitochondrial phylogeography of marine iguanas, and mitochondrial differentiation among PP and LO lineages. This gene segment

was newly sequenced from 310 individuals of marine iguanas from San Cristóbal and 34 so far unused samples from Darwin, Wolf, Rocca Redonda and Seymour Norte islands, using methods from a previous study [11], and leading to a final dataset of 1491 sequences. One individual from each of the three species of Galápagos land iguana were also sequenced as outgroups. Sequences were edited and aligned as described above for dataset B.
We visualized CR variation in haplotype networks reconstructed using information from phylogenetic trees as implemented in Haploviewer [15]. Maximum likelihood trees were estimated with PHYML [16] using the best-fitting substitution model (GTR +I +G) as identified by the Akaike information criterion [17] in J-Modeltest [18].

Microsatellite loci genotyping (datasets D and E)
As a basis for the archipelago-wide comparison of population structure (dataset D) we used microsatellite loci which had largely been determined in previous studies [11, 19], in this study, 12 loci were used due to omission of locus E17 which had a high failure rate. This available dataset was extended with some newly genotyped samples from other islands, collected during previous fieldwork, and with all newly collected San Cristóbal samples. DNA from previously genotyped samples were genotyped along with the newly obtained samples, in order to calibrate alleles between different sequencing machines and to ensure that alleles were correctly scored. This resulted in an available pool of almost 1500 samples that were all genotyped at 12 loci. All San Cristóbal samples (dataset E; Table S1, S2) were genotyped for the same 12 loci as the archipelago-wide dataset, plus an additional 6 loci, yielding data for 18 microsatellite loci described previously [20] excluding locus E17. Primers, multiplexes, and PCR parameters are detailed elsewhere [20]. Scoring of alleles was performed with Genemarker (version 1.95; Applied Biosystems).

Population structure analysis (datasets D and E)
A model-based Bayesian clustering method (Structure, v. 2.3.3; [21]) was used to infer population structure from microsatellite loci data. As this method requires no *a priori* sampling information, it is particularly useful for revealing cryptic population structure. For within-island analysis on San Cristóbal (dataset E), prior number of inferred populations (K) ranged from 1-5, and for analysis of samples across the archipelago (dataset D), K ranged from 1-20; both with 10 iterations for each K. Each run used 100,000 MCMC replicates following a burn-in period of one million replicates. An admixture model was employed, and the model parameter ‘alpha’ was inferred from the data in combination with correlated allele frequencies. Inferred number of populations was obtained [22] via the Structure Harvester application [23]. Results were permuted using CLUMPP [24] and visualized using Distruct [25].

Population structure analysis: sample selection for dataset D
Variation in animal density and sampling effort across the archipelago (dataset D; 12 microsatellite loci) mean that within this dataset, certain populations were strongly overrepresented with respect to others. Since this can lead to artefacts within population structure analysis [26, 27], prior to analysis, the sample sizes for each island were standardized to closely match the smallest sample size available for any island (around 50; Table S2). Any islands where sample size was well below 20 were not considered in this analysis. However, this applied only to very small islands (e.g. Rabida); located nearby larger (included) islands and were not found to be harbouring genetically distinct clusters in earlier studies [11, 19]. This standardization procedure was done by pooling samples from all sampling locations and occasions on each island, and randomly selecting 50 individuals, except in the cases of Santiago and Floreana islands where all available samples (47 and 43

respectively) were used. Data from San Cristóbal Island were treated slightly differently, because of the two strongly divergent populations identified previously [11, 19]. Here, 2 sets of n=50 were created, representing the PP and LO lineages, in addition to a third set collected from a previously non-sampled area of the island, where the genetic identity of individuals was unknown. In total, 614 individuals from all islands were included in this analysis (dataset E).

Population structure analysis: sample selection for dataset E

For analysis within San Cristóbal (dataset E; 18 microsatellite loci) our goal was to assign populations and specimens to the two lineages (LO and PP), and to identify possible hybrids among these two lineages. Therefore, in this analysis, we excluded all individuals that were identified as migrants or possible hybrids with other islands in the previous Structure analysis (dataset D) or assignment tests (see text section below). Additionally, any individuals demonstrating a mitochondrial haplotype private to other islands were removed for the same reason. Further, to exclude artefacts arising from missing data, individuals with missing data for more than one allele were excluded; this necessitated only a small reduction in sample size. Additionally, any resampled individuals were identified using Genalex [28] and removed. In total, 454 individuals were included in the San Cristóbal within-island dataset.

Detection of hybridization on San Cristóbal: datasets C, D and E

Hybrids or migrants from other islands on San Cristóbal were initially identified from the Structure analysis of dataset D as individuals who demonstrated a lower probability of belonging to either LO or PP than a predefined threshold (80%). Furthermore, several individuals had mitochondrial haplotypes (dataset C) predominantly found on other islands. All of these individuals were submitted to assignment tests [29] based on their microsatellite loci genotypes, using GENECLASS [30]. This method was chosen as it produced results, which were in close agreement to the islands associated with mitochondrial haplotypes. We considered as hybrids or migrants from other islands all individuals that did not assign with a probability of >90% [31] to either of the two San Cristóbal populations, and/or if they had a mitochondrial haplotype private to another island. For the assignment tests, a 12-loci reference dataset was created to include LO (N=151) and PP (N=286), and any other cluster where San Cristóbal individuals had been assigned to in the earlier analysis with Structure (dataset D): Española/Floreana (N=128) and Santa Cruz (N=116).

Isolation by distance (IBD) analysis (dataset E)

To test the extent to which isolation by distance (IBD) shaped the genetic diversification on each of the two San Cristóbal clusters, we estimated the geographic isolation among localities measuring in GoogleEarth™, the pairwise coastline distances (in km) between marine iguana populations of LO (5 localities) and PP (8 localities). The genetic differentiation between localities was estimated using pairwise R_{ST} values, an analogue of F_{ST} specifically developed for microsatellite loci [32]. For these calculations we considered a sampling locality to be a place where more than two individuals have been genotyped. Suspected hybrids and migrants (see previous section) were not included in the dataset. We evaluated the association between the two distance matrices with a Mantel test, as implemented in the R package vegan [33], evaluating statistical significance with 10000 permutations.

Demographic history of PP and LO on San Cristóbal (dataset E)

Two approaches were used to examine the demographic history of the LO and PP lineages based on 18 microsatellite loci data (dataset E). Firstly, the software Bottleneck [34] was used to determine the presence of heterozygote excess in each of the populations using the standardized difference test and the Wilcoxon-ranked test. We carried out 1,000 simulations

to assess the tests' significance under the two phased mutation model, assuming 30% of the mutations were multistep with a variance in mutation size of 30. Secondly, the software MsVar 1.3 [35] was used to characterize the recent demographic history of the LO and PP lineages. The method implemented in MsVar uses coalescent simulations to estimate the current effective population size (N_0), the ancestral population size (N_t) and the time at which a demographic change (t) may have occurred (i.e. an expansion after a bottleneck) following an exponential model of effective population size. The simulations carried out in MsVar were conditioned with various *a priori* combinations for the model parameters, so that stable scenarios, bottlenecks and expansion were considered, as well as variation in the mutation rate across microsatellite loci (Table S5). Each MsVar run consisted of 1,010 iterations of the MCMC algorithm, discarding the initial 20% of the coalescent simulations as burn-in. Convergence of the chains was assessed with Gelman & Rubi's diagnostic [36] calculated on the basis of the seven runs performed each for the PP and LO population with different priors. Gelman and Rubin's diagnostic was carried out using the CODA library [37].
We further tested whether the LO and PP lineages have evolved under a model with or without gene flow, using simulations performed using the software 2mod [38]. We performed three independent replicates with a total of 1,000,000 coalescent simulations, each using the MCMC algorithm implemented in 2mod. For each simulation, the likelihood of the drift or migration model is estimated on the basis of the allele counts in the data. After discarding the first 20% of the simulations as burn-in of the MCMC, we estimated the posterior probability of each model as the proportion of simulations that supported each scenario (i.e. drift or migration).

Morphological characterization of PP and LO

To analyse whether specimens of the two San Cristóbal lineages, PP and LO, are also morphologically differentiated, we scored morphometric variables and scale counts from 139 *Amblyrhynchus* specimens collected from San Cristóbal. These individuals were also genotyped at 18 microsatellite loci, and all specimens identified as hybrids or migrants from other islands were disregarded. Specimens were assigned to four different age classes (1: juveniles below 1 year of age; 2: larger juveniles estimated between 1 and 2-3 years; 3: sub-adults approaching adult size but lacking well developed adult morphology, and 4: adults of breeding age based on body size and development of external features such as elevated tubercular scales positioned dorsally on head, femoral pores, and dorsal crests. We sexed only specimens in age class 4, and considered specimens with obvious male characteristics such as enlarged dorsal crests, comparatively larger body size, and well-developed femoral pores as males, and those lacking these features as females. Measurements taken from living specimens in the field include snout-vent length (SVL) from snout tip to cloaca and total length (TL) from snout tip to tail tip to the nearest 10 mm, head width (HW; taken at point of maximum width of head), head length (HL) taken from the snout tip to the edge of the furthest elevated tubercular scale dorsally on head, maximum head height (HH), and length of the 4th toe, taken with a calliper to the nearest 0.1 mm, and weight (to the nearest 0.01 kg). Scale counts were taken from detailed photographs of each of the specimens.
In an initial search for potentially diagnostic differences, we took the following scale counts from 20 specimens of each genetic lineage: lamellae under the third and fourth toe on hindlimb (LAM3T, LAM4T), supralabials (SUPL), series of scales above supralabials (suprasupralabials, SUPSUPL), infralabials (INFL), series of scales below infralabials (infrainfralabials, INFINFL), minimum transversal count of large scales dorsally on head (TRHSC), minimum longitudinal count of large scales dorsally on head (LOHSC), innermost series of scales on upper and lower eyelid (EYELUI, EYELLI), second series of scales on upper and lower eyelid (EYELUO, EYLLO), femoral pores (males only; FP), number of

scales between large dorsal head scales and start of dorsal crest spines (HCSP), number of dorsal crest spines in anteriormost part of dorsal crest (DORSC1). All bilateral counts (LAM3T, LAM4T, SUPL, SUPSUPL, INFL, INFINFL, EYELUI, EYELLI, EYELUO, EYELLO) were done on both sides of the body whenever possible, the average between both values was calculated per specimen and used for further analysis. In cases where reliable counts could only be obtained from either the left or right side of the body, the respective value was used for analysis. In numerous cases, reliable counts could not be obtained due to injuries of the specimens (e.g. partly mutilated toes), scales being difficult to distinguish due to skin shedding, or poor quality of photographs. In all such cases, counts were disregarded. Our goal here is not to provide a full morphometric analysis of variation in these lizards, but to instead highlight morphological differentiation among the lineages. We therefore limited detailed comparisons to a set of variables in which such differentiation was clearly apparent. We compared the values of the initially analysed 40 specimens with non-parametrical Mann-Whitney U-tests for pairwise comparisons. Based on this initial evaluation, we identified the variables SVL, HL, LAM3T, LAM4T, SUPL, SUPSUPL, INFL, INFINFL, and HCSP as most clearly differentiated between the two genetically differentiated lineages, and therefore assessed these variables in the total number of 139 individuals. We used U-tests for final comparisons with the maximum number of available data for each variable. We further performed multivariate analyses of variance (ANOVAs) with Tukey's post-hoc tests for single variables. All statistical tests were done in Statistica 7.1 (StatSoft Inc.). As ANOVAs cannot deal with missing data, these analyses included a reduced number of specimens (i.e. those with complete sets of data for the variables compared). Morphometric measurements were size-corrected by linear regression against SVL, and the residuals used for further analysis.

Extended Results and Discussion

This section provides additional details on the results, as well as statistical analysis and discussion, complementing information in the main Results and Discussion parts of the paper.

Data Sharing

Single genes from datasets A, B and C are deposited in Genbank (www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: NKTR: KR350691 - KR350697, R35: KR350698 - KR350704, RAG1: KR350705 - KR350711, BDNF: KR350712 - KR350718, ND4: KR350719 - KR350742, ND2: KR350743 - KR350767, Cytochrome-B: KR350768 - KR350787, COI: KR350788 - KR350813, 12S: KR350814 - KR350838, 16S: KR350839 - KR350861, and control region: KR350862 - KR351205. Other data are deposited in Dryad (datadryad.org) under doi:10.5061/dryad.pp6bm. Brief information about the files is given here, with Dryad file names shown in bold. Aligned and concatenated nuclear sequences (including tRNA sequences not deposited in Genbank) were deposited as **dataset_A**, aligned mitochondrial genes are found in **Dataset_B_Mitochondrial**, and complete aligned mitochondrial control region sequences are provided in **Dataset_C**. Microsatellite genotypes representing marine iguanas from across the archipelago are provided in **Dataset_D_Microsat**s, genotypes from San Cristobal *only* are given in **Dataset_E_Microsat** and a subset of these samples used in MsVar and Bottleneck analysis are given in **Dataset_E_Microsat**. Data from RAD sequencing are given in **Datasets_F_G_H** with further notes in the **README.doc**. Morphometrics used in comparison between two populations of marine iguanas are given in the file: **Morphometry_Amblyrhynchus**.

Divergence time estimates

Our timetree, based on four nuclear genes (dataset A; Fig. S1), predicted a younger age for the split between Galápagos marine and land iguanas than a previous study had [39]. As such, our study reconciles the age of this endemic clade with the age of the currently extant islands. We consider our timetree to be more reliable than the one of the previous study, which was undertaken at a time when sophisticated molecular dating methods were not yet available, and is based only on mitochondrial genes, which are prone to overestimating divergence times [40]. Furthermore, this estimate was based on a possibly inadequate strict clock approach, with a relatively fast substitution rate (2% per my for the mitochondrial cytochrome b gene) which was derived from data on mammals. Conversely, our estimates are based on single-copy protein-coding nuclear genes, using multiple squamate calibrations, and relaxed clock models, which together are known to provide more realistic divergence time estimates.

Demographic history of LO and PP

The two approaches (i.e. bottleneck and MsVar) to examine the demographic history of LO and PP detected bottlenecks in the recent history of these lineages (Fig. S3). Both, the standardized difference test and the Wilcoxon-ranked test identified a significant excess in heterozygosity, consistent with the hypothesis of a recent bottleneck in the past history of PP and LO (Table S4). The MsVar approach identified that the bottleneck experienced by both lineages took place approximately 1,800-3,000 years ago and consisted of a reduction in the effective population size of ~100,000 individuals to ~1,000 individuals (see Table S6 for exact data and corresponding confidence intervals).

The demographic analysis with 2mod aiming at determining whether the LO and PP lineages evolved under a simple model of drift versus a model that included migration between populations found consistently across replicates of the analysis that the gene flow model was supported with a posterior probability of ~1.

Morphological analysis

As our dataset contains a large number of missing data and furthermore differences in body size imply the need for in-depth analysis of possible allometric effects, a simultaneous analysis of all data in a single multivariate model would either result in very low sample sizes (if full variable coverage was needed for each individual) or potential artefacts (if missing data were replaced by mean values). We therefore opted for a mixed strategy, first analysing pholidotic characters along with body size (SVL), and second, analysing the morphometric measurements. Rather than adopting a strict Bonferroni correction for multiple testing, we considered results as robust if they were consistently suggested by separate analyses, i.e. of independent datasets (males *versus* females) or of subsets of data (i.e. after excluding large and small specimens which might be overrepresented in either cluster).

(i) Body size. Both non-parametric Mann-Whitney U-tests (Tables: S9, S11 & S13; Fig. S7) and ANOVA coupled with Tukey's post-hoc tests (Tables: S10, S12 & S14) suggested statistically significant differences in body size between LO and PP, with PP specimens being smaller. This result remained significant in separate analyses of adult male and female specimens, despite wide overlap in sizes between the two clusters. Although p-values in U-tests would be insufficient under Bonferroni correction, the fact that statistical significance is maintained in the ANOVA post-hoc tests increases our confidence in the biological meaning of this pattern.

(ii) Pholidotic characters. Univariate pairwise U-tests flagged a large numbers of variables as statistically different between the two clusters (Tables: S9-S14; Fig. S7), but in many cases (LAM3T, LAM4T, SUPSUPL, EYELUO) the significance values were between 0.01-0.05,

and would not remain significant following Bonferroni correction for multiple testing. However, three variables remained significantly different after Bonferroni correction, remained so in Tukey's post-hoc tests, and showed similar trends in males and females. These were: INFL, INFINFL, and DORSC1. We therefore conclude that there is robust evidence for PP specimens having on average a larger numbers of infralabials, infra-infralabials, and spines in first part of dorsal crest than specimens of the LO cluster.
(iii) Morphometric characters. Morphometric measurements are as a rule strongly size-dependent, and this relationship is often near-linear in adult animals. However, many body proportions are subject to non-linear allometric growth in the transition from juveniles to adults. For instance in lizards, young specimens have proportionally larger heads, and may also have proportionally longer limbs than adults. In the case of marine iguanas, LO specimens grow to larger sizes than PP specimens. If growth of head and limbs would still be slightly allometric in the subadult or young adult stage, then between-cluster differences in related measurements could potentially be caused by their different body sizes. An initial comparison of all adult specimens (males and females) revealed statistically significant differences in HW, HL and TOEL residuals between the two clusters. Considering separate tests of males and females, and tests excluding small specimens (<20 cm SVL) as well as large specimens (>36 cm SVL), only the between-cluster differences in HL residuals were consistently retrieved, and as such, can be considered robust (Tables S15-S18). Therefore, we conclude that PP specimens have proportionally longer heads than LO specimens.

Hybridization
Given the large number of highly polymorphic loci used and the strong level of differentiation between clusters, it is likely that the analysis would have adequately identified hybrids [30]. Although results from New Hybrids did not unambiguously assign hybrid individuals to hybrid classes (e.g. F1, F2 etc.; data not shown), the disparate results between D-loop haplotypes and microsatellite loci clusters indicate that extensive backcrossing has occurred. For instance, several individuals demonstrated high assignment probabilities for being PP individuals based on microsatellite loci, whilst harbouring a D-loop haplotype private to the Espanola/Floreana cluster (Table S3; Fig. S2). This indicates that the hybridization event occurred several generations ago, but since then, most of the genetic signature indicative for Espanola/Floreana cluster in the microsatellite loci has been lost.

Isolation by distance (IBD) analysis
Genetic differentiation scales with distance between subpopulations within the Loberia population but not within the Punta Pitt population, indicating a more recent expansion within the PP lineage (Fig. S4). Within both clusters, observed maximum geographically separated subpopulations display a lower genetic distance than geographically closest subpopulations of different clusters (Tables: S7 & S8), but in the PP cluster no significant IBD is seen, suggesting that gene flow is maintained over the maximum coastal distance of 30 km.

Supplementary Material References

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Table S1. Locations of sampling and number of marine iguana blood samples collected from San Cristóbal Island. Asterisk denotes samples collected by K. Rassmann, all others collected by the authors.

Name	Code	Latitude	Longitude	Number of blood samples per year						Data available per location		
										Number genotyped		Number sequenced for D loop
				1991/3*	2004	2011	2012	2013	2014	12 loci only	18 loci	
Punta Carola	SRCA	0°53'22.01"S	89°36'45.04"W					15			13	10
La Loberia	SRL	0°55'19.80"S	89°37'15.04"W	31	39		30	40	27	35	111	133
Playa Ochoa	SRO	0°51'50.04"S	89°34'16.06"W				6	1			7	1
Isla Lobos	SRIL	0°51'20.04"S	89°34'5.04"W				7	15	15		33	11
Cerro Brujo	SRCB	0°45'50.10"S	89°27'31.20"W				6	19	3		14	5
Bahía Sardina	SRBS	0°42'11.90"S	89°21'52.30"W				1	1			2	1
La Galapaguera	SRG	0°41'34.90"S	89°18'10.90"W				20	35	8		66	42
Las Salinas	SRS	0°41'50.09"S	89°16'15.01"W				20	21	1		42	21
Playa Blanca	SRPB	0°41'42.04"S	89°15'27.08"W	22		13	15	50	10		78	72
Islote Pitt	SRIP	0°42'11.01"S	89°14'50.01"W			6	10	10	10		36	9
Playa Café	SRPC	0°42'51.04"S	89°14'30.09"W			5	15	22	22		87	29
Puerto Chino (East coast)	SRCH	0°55'32.52"S	89°25'33.10"W					1			1	1
East coast A	SRECA	0°51'20.60"S	89°21'55.20"W						8		8	8
East coast B	SRECB	0°47'9.50"S	89°17'53.00"W						5		5	5
East coast C	SRECC	0°45'43.40"S	89°16'38.70"W						2		2	2
East coast D	SRECD	0°44'55.10"S	89°16'5.30"W						6		6	6
East coast E	SRECE	0°43'44.90"S	89°15'3.60"W						2		2	2
Total counts				53	52	24	130	230	119	35	513	369

Table S2. Locations and number of marine iguana blood samples collected from across the Galapagos archipelago utilised in the various molecular datasets (A-H) used throughout the study.

	Island	Sample size per dataset							
		A	B	C	D	E	F	G	H
Amblyrhynchus cristatus	Darwin			3			1		1
	Rocca Redonda			9			1		1
	Wolf			12			2		2
	Fernandina			179	50		2	1	2
	Isabela	1	5	138	50		2		2
	Pinzon			12					
	Rabida			11					
	Pinta			93	50		2		2
	Marchena			78	50		2		2
	Genovesa			81	50		2		2
	Santiago			72	47		2		2
	Santa Cruz		5	116	50		2		2
	Seymour Norte			10					
	Santa Fé			159	50		2		2
	Floreana			60	50		2		2
	Española			98	43		2		2
	San Cristóbal: Loberia	1	5	166	50	157	4	1	4
	San Cristóbal: Punta Pitt	1	5	171	50	306	5	1	5
	San Cristóbal: East coast			23	24	11			
Outgroup	*Conolophus subcristatus*	1	4				2		
	Conolophus pallidus		1				2	1	
	Conolophus marthae								
	Ctenosaura similis	1					2		
	Ctenosaura pectinata		1						
	Iguana iguana	1	1						
	Other squamate species	73					2		
	Total: *A. cristatus*	3	20	1491	614	474	33	3	33
	Total: all	79	27	1491	614	474	41	4	33

Table S3. List of putative hybrids and migrants identified on Santa Cruz Island. D-loop haplotype numbers are as in a previous study (S11) and used in Fig. 2. Assignment tests were carried out with GENECLASS.

Loc. type	Individual and gender/size	Size Class	D loop hap #	Island(s) associated with haplotype	Microsat. assignment	probability of assignment	Conclusion	Weight	TL	SVL
East coast	SRECA_01	M4	4	Española	Santa Cruz	62	Inconclusive, possible hybrid: East coast population (Española origin) / Santa Cruz	4.6	117	45
	SRECA_02	F4	4	Española	Floreana/Española	96	Pure East coast population of Española origin	2.35	97	38
	SRECA_03	M4	4	Española	Floreana/ Española	100	Pure East coast population of Española origin	4.08	105	41
	SRECA_04	U2	4	Española	Floreana/ Española	100	Pure East coast population of Española origin	0.57	47	18
	SRECA_05	U2	6	Española	Floreana/ Española	100	Pure East coast population of Española origin	0.62	58	26
	SRECA_06	U2	4	Española	Punta Pitt	76	Hybrid: Punta Pitt/East coast population (Española origin)	0.68	55	21
	SRECA_07	M4	6	Española	Floreana/ Española	100	Pure Española (EC pop)	5.65	123	52
	SRECA_08	M4	4	Española	Loberia	100	Hybrid: Loberia / East coast population (Española origin)	6.46	123	50
	SRECB_03	M4	3	Española	Punta Pitt	100	Hybrid: Punta Pitt / East coast population (Española origin)	4.2	106	37
	SRECB_0	F4	3	Española	Punta Pitt	99	Hybrid: Punta Pitt / East coast population (Española origin)	2.39	81	32
	SRECC_01	F4	3	Española	Punta Pitt	100	Hybrid: Punta Pitt / East coast population (Española origin)	17.3	71	26
Punta Pitt	SRG12_06	M4	unavailable		Floreana/ Española	63	Inconclusive. Probable genotype signatures of Española origin	3.6	95	35
	SRG13_08	M4	67	Santa Cruz	Floreana/ Española	76	Inconclusive, possible hybrid: East coast population (Española origin) / Santa Cruz	5.2	108	40
	SRG13_11	F4	81	San Cristóbal: Punta Pitt	Loberia	61	Hybrid: Loberia/Punta Pitt	1.58	69	26
	SRG13_34	M4	67	Santa Cruz	Santa Cruz	83	Santa Cruz vagrant	3.04	91	34
	SRS13_05	M3	81	San Cristóbal: Punta Pitt	Loberia	90	Hybrid: Loberia/Punta Pitt	0.72	57	21
Loberia	SRL13_22	M3	67	Santa Cruz	Santa Cruz	100	Santa Cruz vagrant	2.2	81	31
	SRL13_24	M4	20	Santiago, Santa Fe	Santa Cruz	100	Probable Santa Cruz vagrant	5.12	108	40
	SRL14_02	F4	unavailable		Santa Cruz	100	Probable Santa Cruz vagrant	2.62	36	90
	SRIL13_06	M4	68	San Cristóbal: Loberia	Santa Cruz	89	Hybrid: Loberia/Santa Cruz	1.76	78	33

Table S4. Results from the Bottleneck Analysis. P-values for the standardized difference and Wilcoxon tests are shown. In bold is the non-significant p-value after Bonferroni correction.

Test	**Loberia**	**Punta Pitt**
Standardized Difference test	**0.049**	0.0009
Wilcoxon test	0.017	0.0002

Table S5. Prior distributions for the MsVar analysis. Each row in the table shows the prior distribution of the parameters of the MsVar analysis for each of the seven scenarios tested. The parameters are N_0= Current Effective Population Size, N_t= Ancestral Effective Population Size, u= mutation rate, t= time at which the change in effective population size took place. The value under each parameter is the log mean of the distribution and under the column parameter Var is the variance of the distribution as used in MsVar. The variance of the means and of the variances in the hyperpriors of all parameters were in all simulations 0 and 0.5, respectively. Each of these seven models were tested in each population.

Scenario	**N_0**	**N_0 Var**	**N_t**	**N_t Var**	**u**	**u Var**	**t**	**t Var**
1	4	2	4	2	-4	1	5	2
2	5	2	5	2	-4	2	6	2
3	5	2	3	2	-4	2	5	1
4	4	1	5	1	-4	1	5	2
5	3	1	5	1	-4	1	6	1
6	3	2	5	3	-4	3	5	3
7	4	1	5	1	-4	1	3	2

Table S6. Posterior distribution from the MsVar analysis. Data are given as log10.

	No	**HPD 95%**
LO	1000	96–2139
PP	1039	282–3236
	NA	**HPD 95% LB**
LO	141254	44868–568351
PP	100000	32643–386843
	t	**HPD 95% LB**
LO	1848	178–3561
PP	3043	857–9755

Table S7. Genetic (below diagonal, in R_{ST} values) and geographic (above diagonal, in km) distances between localities included in the Isolation by Distance analysis of the LO cluster. Locality codes as presented in Fig. 3.

	SRL	SRPA	SRO	SRIL	SRCB
SRL		6.5	13.41	15.02	41.52
SRPA	0.020		6.91	8.52	35.02
SRO	0.020	0.021		1.61	28.11
SRIL	0.036	0.007	0.004		26.5
SRCB	0.062	0.051	0.033	0.043	

Table S8. Genetic (below diagonal, in R_{ST} values) and geographic (above diagonal, in km) distances between localities included in the IBD analysis of the PP cluster. Locality codes as presented in Fig. 3.

	SRBS	SRG	SRS	SRPB	SRIP	SRPC	SRECD	SRECC
SRBS		10.5	15.53	17.33	19.41	21.43	26.8	30.88
SRG	0.00		5.03	6.83	8.91	10.93	16.3	20.38
SRS	0.02	0.01		1.8	3.88	5.9	11.27	15.35
SRPB	0.01	0.01	0.00		2.08	4.1	9.47	13.55
SRIP	0.08	0.03	0.02	0.02		3.05	8.42	12.5
SRPC	0.00	0.01	0.01	0.01	0.03		5.37	9.45
SRECD	0.00	0.01	0.02	0.01	0.03	0.00		4.08
SRECC	0.01	0.05	0.05	0.06	0.05	0.02	0.00	

Table S9. Results of Mann-Whitney U-tests for SVL and scale counts, based on the pooled dataset of all age classes and sexes. Note that variables in the second part of the tables were only assessed in a limited number of specimens (see Valid N columns). Group 1 refers to specimens genetically assigned to LO and Group 2 to PP. Hybrids were not considered in the analysis. Asterisks mark variables with significant differences between PP and LO. Note that under Bonferroni correction only p values <0.0032 would remain significant at the 0.05 level, i.e., only INFL, INFINFL, and DORSCI.

	Rank Sum Group 1	**Rank Sum Group 2**	**U**	**Z**	**p-level**	**Z adjusted**	**p-level**	**Valid N Group 1**	**Valid N Group 2**	**2*1sided exact p**
*SVL	5550.000	4180.000	1902.000	2.14978	0.031574	2.15168	0.031423	72	67	0.031437
*LAM3T	3418.000	3603.000	1338.000	-2.10666	0.035148	-2.12153	0.033878	64	54	0.035039
*LAM4T	3089.500	3465.500	1259.500	-2.04585	0.040772	-2.05537	0.039844	60	54	0.040449
SUPL	3915.500	3959.500	1569.500	-1.82663	0.067756	-1.88981	0.058784	68	57	0.067641
*SUPSUPL	3905.500	3969.500	1490.500	-2.19188	0.028389	-2.20484	0.027466	69	56	0.028033
*INFL	3591.500	3911.500	1245.500	-3.04373	0.002337	-3.10726	0.001888	68	54	0.002149
*INFINFL	2820.000	3508.000	929.000	-3.66035	0.000252	-3.68227	0.000231	61	51	0.000205
*DORSC1	1602.500	2957.500	699.500	-3.09862	0.001944	-3.14417	0.001666	42	53	0.001726
TRHSC	97.000	113.000	47.000	0.18993	0.849361	0.19506	0.845345	9	11	0.881984
LOHSC	157.000	143.000	52.000	1.12976	0.258577	1.17702	0.239186	11	13	0.276668
EYELUI	36.000	100.000	22.000	0.24254	0.808365	0.24470	0.806685	4	12	0.861538
EYELLI	158.000	248.000	67.000	-1.40499	0.160024	-1.41707	0.156464	13	15	0.169623
*EYELUO	227.000	179.000	43.000	2.46046	0.013876	2.47576	0.013296	12	16	0.013016
EYLLO	230.500	234.500	63.500	1.88384	0.059588	1.89997	0.057439	12	18	0.058779
FP	104.000	247.000	68.000	-0.22222	0.824141	-0.22283	0.823666	8	18	0.849089
HCSP	85.500	145.500	40.500	-0.95940	0.337356	-0.98065	0.326767	9	12	0.345103

Table S10. ANOVA results of test including 8 variables and all age classes and all sexes. Asterisks mark variables with significant differences between PP and LO.

	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.002862	2395.123	8	55	0.000000
SPNR	Wilks	0.663590	3.485	8	55	0.002547
Tukey's Post hoc test:						
*SVL	0.042853		SUPSUPL	0.113464		
LAM3T	0.104814		*INFL	0.022578		
LAM4T	0.090530		*INFINFL	0.002007		
SUPL	0.063207		*DORSC1	0.000460		

Table S11. Results of Mann-Whitney U-tests for SVL and scale counts, based on males in age class 4 only. Group 1 refers to specimens genetically assigned to LO and Group 2 to PP. Hybrids were not considered in the analysis. Asterisks mark variables with significant differences between PP and LO.

	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
*SVL	1589.500	688.5000	282.5000	3.34970	0.000809	3.36051	0.000778	39	28	0.000623
LAM3T	999.000	597.0000	366.0000	0.02539	0.979747	0.02555	0.979619	35	21	0.986616
LAM4T	944.000	709.0000	278.0000	-1.65431	0.098066	-1.65929	0.097059	36	21	0.100053
SUPL	1212.500	678.5000	378.5000	0.96701	0.333541	1.02850	0.303717	37	24	0.336203
SUPSUP	1069.000	822.0000	328.0000	-1.62205	0.104793	-1.63600	0.101841	38	23	0.106775
INFL	1052.000	718.0000	349.0000	-0.90914	0.363277	-0.94115	0.346630	37	22	0.370393
INFINF	950.000	646.0000	284.0000	-1.29957	0.193748	-1.30751	0.191042	36	20	0.198540
*DORSC1	281.500	498.5000	110.5000	-2.21149	0.027003	-2.31631	0.020542	18	21	0.025801

Table S12. ANOVA results of test including 8 variables and males of age class 4 only. Asterisks mark variables with significant differences between PP and LO.

	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.001500	1414.137	8	17	0.000000
SPNR	Wilks	0.382688	3.428	8	17	0.015616
Tukey's Post hoc test:						
*SVL	0.005099		SUPSUPL	0.503060		
LAM3T	0.506114		INFL	0.284878		
LAM4T	0.298264		INFINFL	0.223681		
SUPL	0.968222		*DORSC1	0.039254		

Table S13. Results of Mann-Whitney U-tests for SVL and scale counts, based on females in age class 4 only. Group 1 refers to specimens genetically assigned to LO and Group 2 to PP. Hybrids were not considered in the analysis. Asterisks mark variables with significant differences between PP and LO. Note that under Bonferroni correction only p values <0.0063 would remain significant at the 0.05 level, i.e., none of the variables would show significant differences.

	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
*SVL	95.50000	204.5000	14.50000	2.34570	0.018992	2.35752	0.018398	5	19	0.015246
LAM3T	44.50000	231.5000	29.50000	-1.15530	0.247968	-1.18773	0.234942	5	18	0.257066
LAM4T	47.00000	206.0000	32.00000	-0.82263	0.410717	-0.83644	0.402906	5	17	0.445584
SUPL	52.00000	179.0000	37.00000	-0.24772	0.804354	-0.25407	0.799445	5	16	0.841712
SUPSUP	56.50000	174.5000	38.50000	0.12386	0.901427	0.12496	0.900556	5	16	0.904713
INFL	34.50000	175.5000	19.50000	-1.57117	0.116144	-1.61103	0.107174	5	15	0.118550
INFINF	35.00000	155.0000	25.00000	-0.50000	0.617075	-0.50310	0.614895	4	15	0.664603
*DORSC1	25.00000	206.0000	10.00000	-2.47717	0.013243	-2.50083	0.012391	5	16	0.011106

Table S14. ANOVA results of test including 8 variables and females of age class 4 only. Asterisks mark variables with significant differences between PP and LO.

	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.000355	2464.067	8	7	0.000000
SPNR	Wilks	0.405860	1.281	8	7	0.378523

Tukey's Post hoc test:			
*SVL	0.016457	SUPSUPL	0.962186
LAM3T	0.194163	INFL	0.380532
LAM4T	0.479982	INFINFL	0.469247
SUPL	0.855120	DORSC1	0.096383

Table S15. Results of Mann-Whitney U-tests for residuals (after regression against SVL) of four morphometric measurements, based on all age classes and both sexes. Group 1 refers to specimens genetically assigned to LO and Group 2 to PP. Asterisks mark variables with significant differences between PP and LO. Hybrids were not considered in the analysis.

Residue	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
*HW	3997.500	3628.500	1417.500	2.35082	0.018732	2.35085	0.018731	57	66	0.018357
*HL	2853.000	4773.000	1200.000	-3.45396	0.000552	-3.45398	0.000552	57	66	0.000480
HH	3771.000	3855.000	1644.000	1.20204	0.229349	1.20206	0.229342	57	66	0.231237
*TOEL	3069.500	4556.500	1416.500	-2.35590	0.018479	-2.35591	0.018478	57	66	0.018103

Table S16. ANOVA results for four morphometric measurements, based on all age classes and both sexes. Asterisks mark variables with significant differences between PP and LO.

	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.996937	0.090650	4	118	0.985233
SPNR	Wilks	0.808862	6.971001	4	118	0.000045
Tukey's Post hoc test:						
*HW	0.040320		HH	0.332132		
*HL	0.002088		*TOEL	0.009745		

Table S17. Results of Mann-Whitney U-tests for residuals (after regression against SVL) of four morphometric measurements, based on all age classes and both sexes, but excluding all specimens <20 mm and >36 mm. Group 1 refers to specimens genetically assigned to LO and Group 2 to PP. Hybrids were not considered in the analysis. Asterisks mark variables with significant differences between PP and LO.

Residue	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
HW	1204.500	2116.500	631.5000	0.97681	0.328665	0.97684	0.328648	27	54	0.330836
*HL	831.000	2490.000	453.0000	-2.76512	0.005691	-2.76517	0.005690	27	54	0.005277
HH	1076.500	2244.500	698.5000	-0.30557	0.759935	-0.30558	0.759926	27	54	0.761407
TOEL	920.500	2400.500	542.5000	-1.86846	0.061699	-1.86851	0.061692	27	54	0.061413

Table S18. ANOVA results for four morphometric measurements, based on all age classes and both sexes, but excluding all specimens <20 mm and >36 mm. Asterisks mark variables with significant differences between PP and LO.

	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.996937	0.090650	4	118	0.985233
SPNR	Wilks	0.808862	6.971001	4	118	0.000045
Tukey's Post hoc test:						
HW	0.529953		HH	0.757532		
*HL	0.034761		*TOEL	0.034362		

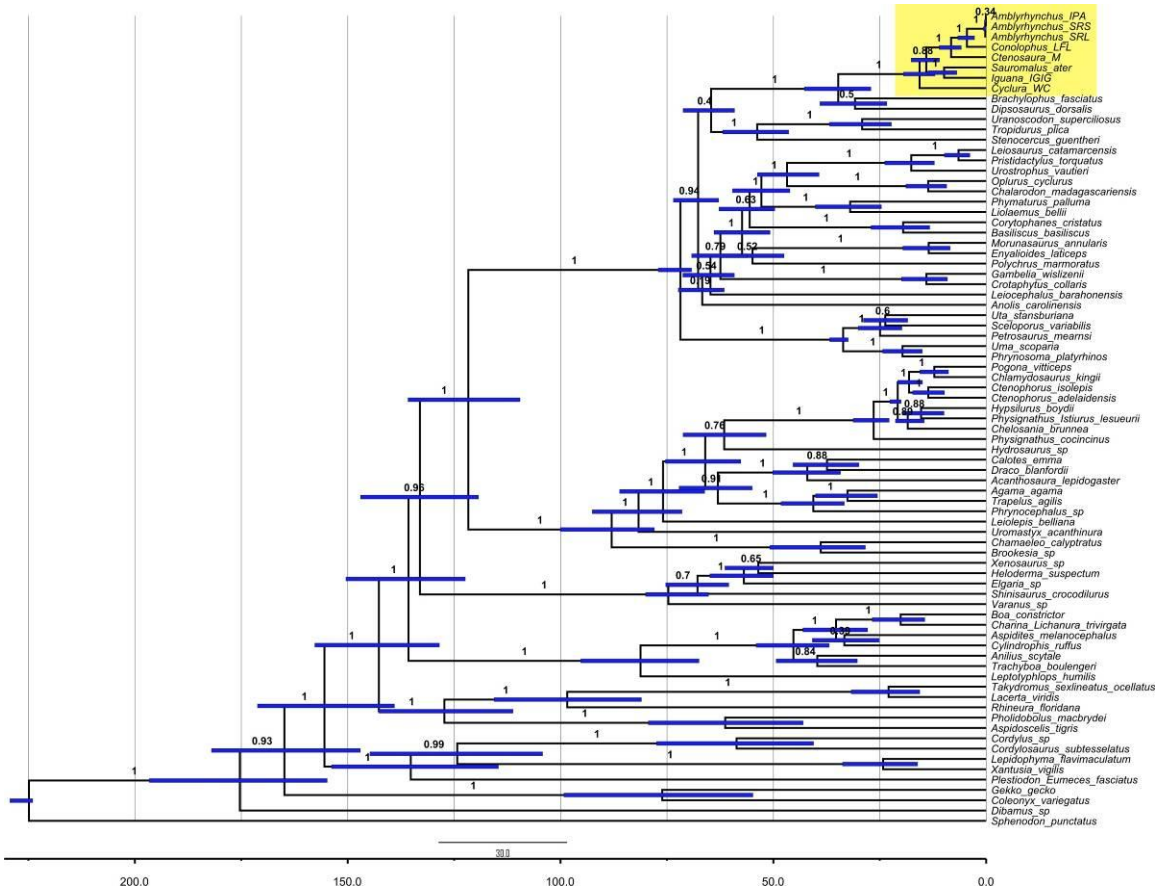

Figure S1. Timetree showing iguanine lizards (yellow box) among squamates. Calibrations and settings used are as in a previous study [1]

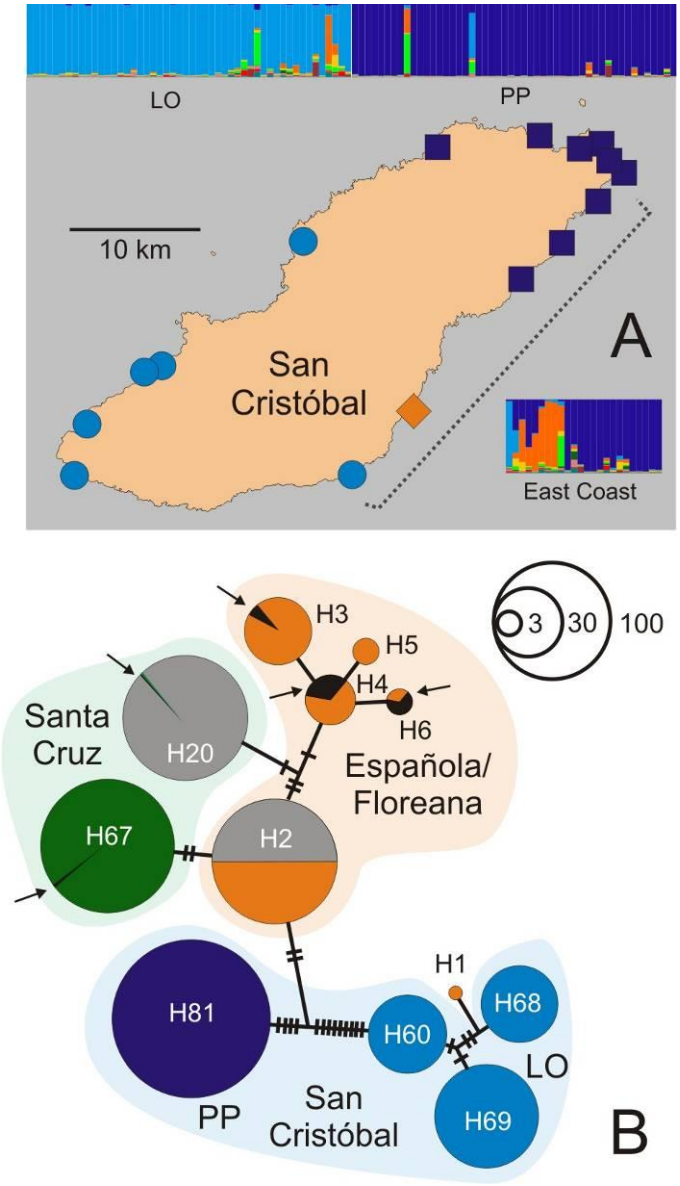

Figure S2. (A) Details of Structure assignment (based on 12 microsatellite loci) of specimens from San Cristóbal Island (extracted from Fig. 2 in main paper), showing a low incidence of gene flow between the two island-endemic lineages LO and PP, but several instances of migrants and hybrids from Española and Santa Cruz Islands. (B) Haplotype network of DNA sequences from the mt control region incorporating haplotypes and data from a previous study [11] with newly sequenced samples from San Cristóbal Island (Table S1). Only haplotypes found in iguanas from San Cristóbal are shown, thus the presence of private Española and Santa Cruz haplotypes confirms the presence of migrants from these islands on San Cristóbal, which also is suggested by the microsatellite data. Light grey shows individuals from Española and Santa Cruz, whilst individuals from San Cristóbal with haplotypes typical for Española or Santa Cruz are black and marked with small arrows.

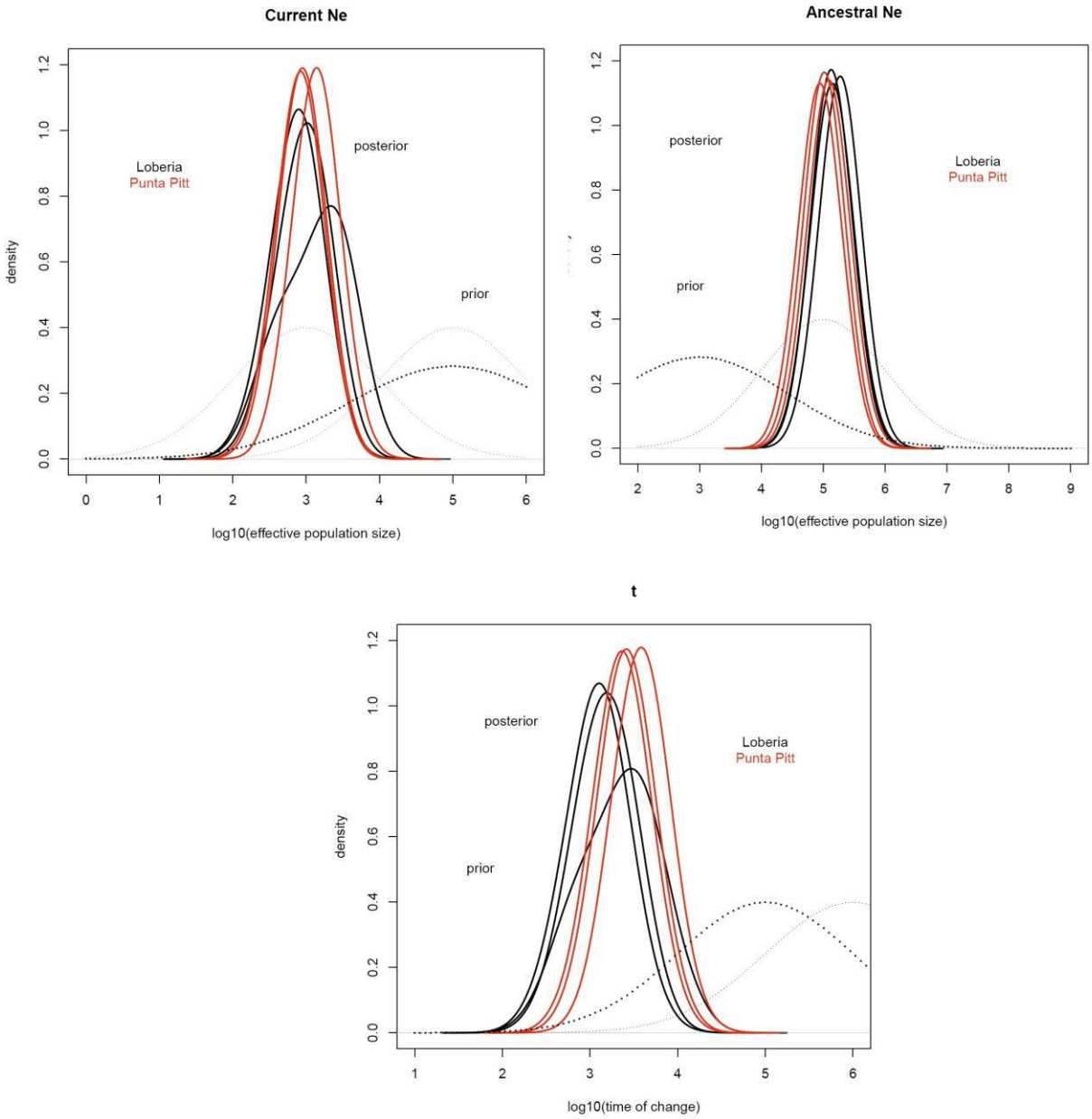

Figure S3. Changes from ancestral to current effective population size (Ne), and time of inferred change, in PP and LO clusters, based on simulations with MsVar software and based on data from 18 microsatellite loci.

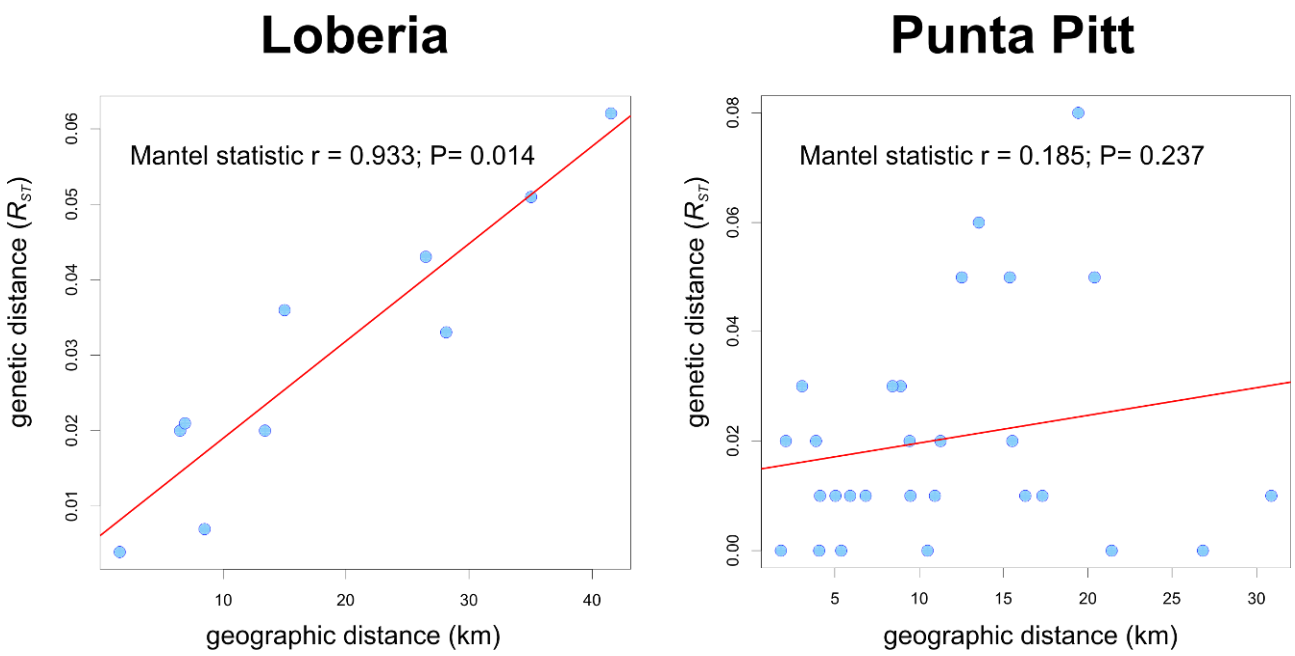

Figure S4. Isolation by distance (IBD) analysis for subpopulations of the LO and PP clusters. Significant genetic differentiation between subpopulations is found for the Loberia, but not the Punta Pitt, cluster, indicating a more recent expansion within the PP lineage.

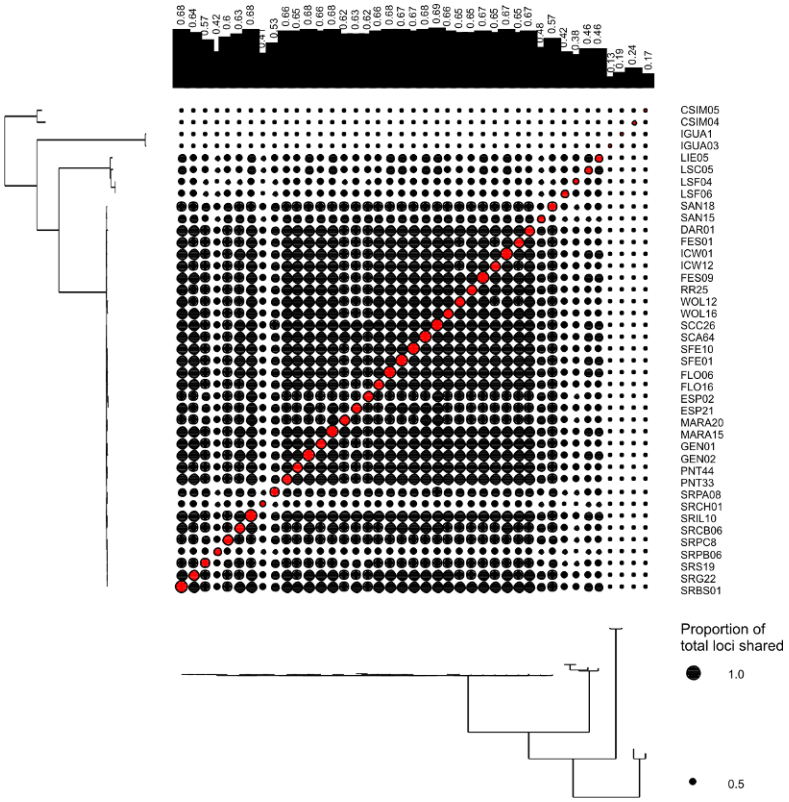

Figure S5. Proportion of RAD-Seq derived loci shared among individuals. Loci shared between individuals (black circles, off-diagonal cells) or successfully amplifying within a single individual (red circles along the diagonal) are expressed as the proportion from 0 to 1 of all 60,396 loci scored in this study.

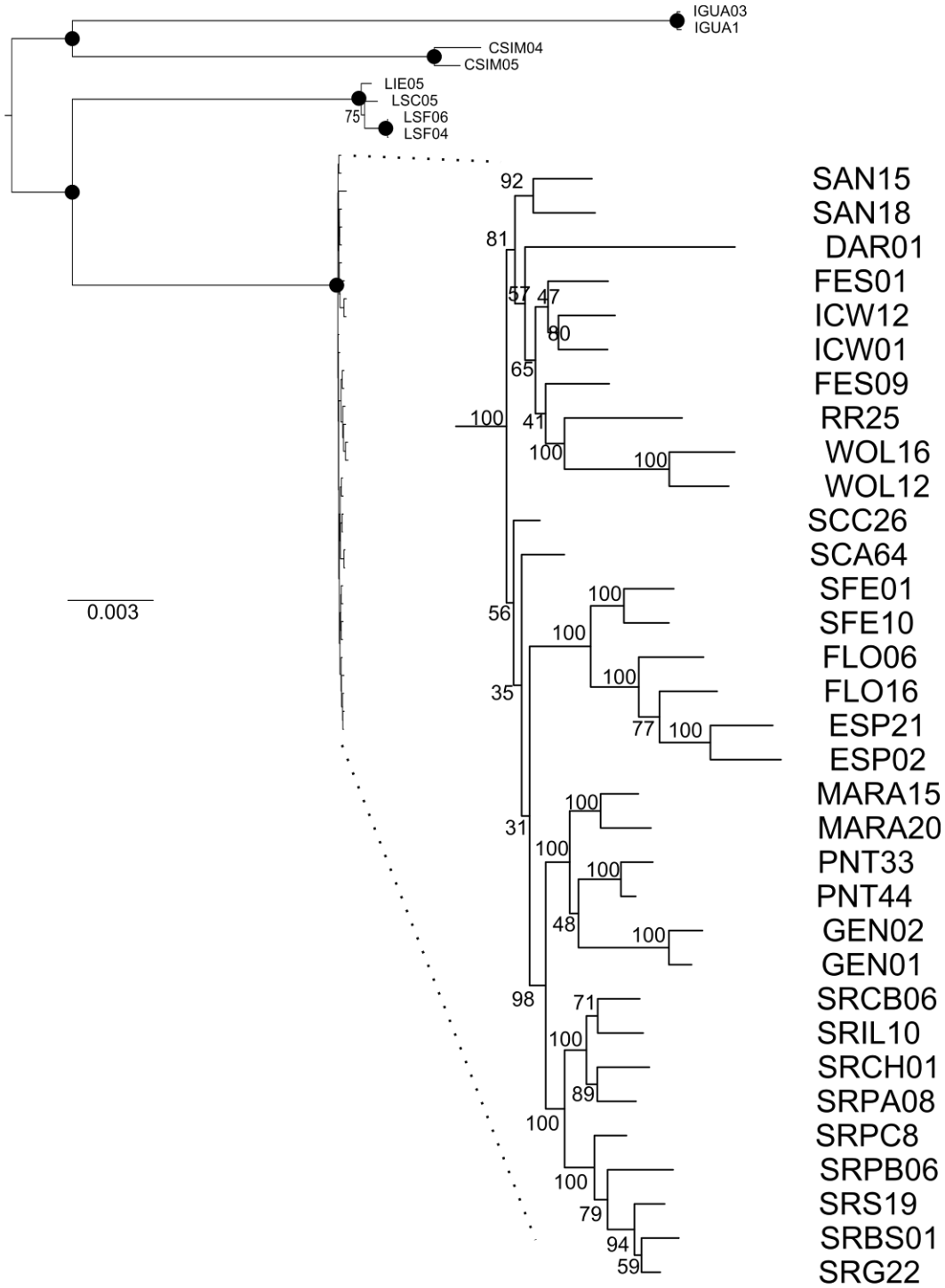

Figure S6. Maximum likelihood tree of the sparse matrix of RADSeq-derived DNA sequences. The tree was obtained in RAxML using a GTR + Γ model and an alignment of 5,082,978 bp, branch support was assessed by 100 bootstrap replicates.

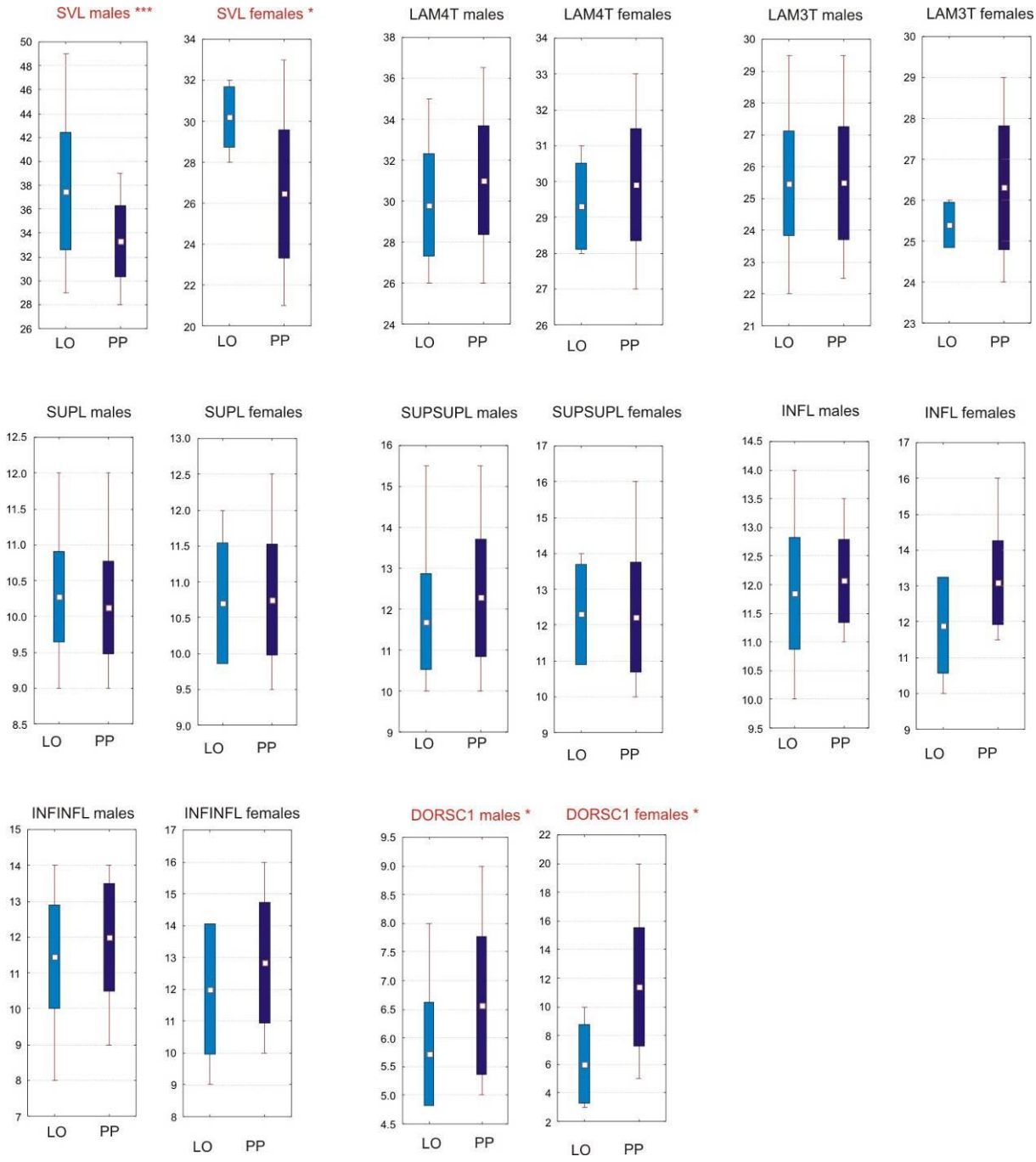

Figure S7. Box-Whisker plots comparing specimens of LO and PP iguanas for SVL and 15 scale count variables. All age classes and both sexes included. Asterisks indicate significance in Univariate Mann-Whitney U tests, * P<0.05, ** P < 0.01, ** P<0.001. Note that under Bonferroni correction only p values <0.0063 would remain significant at the 0.05 level, i.e., none of the variables would show significant differences. See Tables S9-S18 for detailed statistical results.