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Genomic insights into the biogeography and evolution of Galápagos iguanas

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ABSTRACT

Galápagos iguanas are a monophyletic group endemic to the Galápagos archipelago, comprising the marine iguana *Amblyrhynchus cristatus* and three species of land iguanas: *Conolophus subcristatus*, *C. pallidus* and *C. marthae*. The biogeographic history of the land species in relation to their current distributions remains uncertain, in particular the origins of *C. marthae*, which is restricted to a small area of the northern part of Isabela Island. The classification of *C. pallidus* as a separate species has also been debated.

We analyzed DNA sequences (RADseq) to reconstruct demographic histories of selected local populations of all Galápagos iguana species and estimate their divergence times within a multispecies coalescent framework. Our results indicate an early date for the colonization of Galápagos by iguanas, relative to island formation, at *ca.* 10 Mya, and support a recent split of *C. marthae* via allopatric speciation, after the emergence of Isabela Island, at *ca.* 0.57 Mya. We find contrasting demographic histories in *C. marthae* and the syntopic population of *C. subcristatus*, suggesting competitive interaction between these species. We also confirm that the divergence of *C. pallidus* from *C. subcristatus* is recent (0.09 Mya) and close in time to the split between populations of *C. subcristatus* from different islands. Our genetic data support recent census estimates indicating a relatively small current effective population size (N_e) in all the studied populations. Our findings shed light on the evolutionary history of Galápagos iguanas and emphasize the need for targeted conservation strategies.

1. Introduction

Island ecosystems harbor unique evolutionary processes, constrained by limitations to dispersal of terrestrial organisms and their population size (MacArthur & Wilson, 2001). On the one hand, these constraints control functional and taxonomic diversity of island communities which, in turn, favor adaptive radiation (Grant, 2013). On the other hand, they promote non-adaptive speciation by allopatric processes of vicariance and dispersal in both continental and oceanic island systems (Gentile et al., 2010; Presgraves & Glor, 2010).

Located in the eastern Equatorial Pacific, the Galápagos archipelago hosts a rich assemblage of land-bound vertebrates. This is despite the relatively recent geological origin of its present islands (≤ 4 Million years ago–Mya), the considerable distance from the nearest continent

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(*ca.* 930 km to mainland South America), and its small total area of 8,010 km^2 (Ali & Fritz, 2021). The biodiversity and endemism of these volcanic islands have captured the interest of scientists for nearly two centuries (Darwin, 1839). Reptiles, in particular, display remarkable and unique diversity on these islands (Ali & Aitchison, 2014), including several charismatic flagship species, such as Galápagos tortoises and iguanas (Losos & Ricklefs, 2009; Gentile et al., 2016).

Galápagos iguanas are a monophyletic group endemic to the Galápagos archipelago (Sudhaus, 2004; Hernández-Hernández, 2019), comprising the highly divergent marine iguana (*Amblyrhynchus cristatus*) and three species of land iguanas: the common land iguana (*Conolophus subcristatus*, hereafter yellow iguana*)*, the Barrington land iguana (*C. pallidus)*, and the recently described pink land iguana (*C. marthae*) (Tzika et al., 2008; Gentile et al., 2009; Gentile & Snell, 2009). Whereas the marine iguana occupies coastal habitats throughout most of the archipelago (MacLeod et al., 2020), the yellow land iguana is distributed across seven islands, namely Santa Cruz, Santiago (reintroduced), Baltra (repatriated), Seymour Norte (introduced), Plaza Sur, Isabela, and Fernandina (Kumar et al., 2020). The Barrington land iguana is endemic to the small Island of Santa Fe (Gentile et al., 2020), and the pink iguana *C. marthae* is only found in an extremely small area (*ca.* 38 km^2) on the northern slope of Wolf Volcano, at the northern end of Isabela Island (Garizio et al., 2024) (Fig. 1). The pink land iguana is syntopic with a population of *C. subcristatus* throughout its small range (Gentile et al., 2009). No evidence suggests that hybridization between the two species occurs at present (Di Giambattista et al., 2018), but potential interspecific competitive interactions may exist (Gargano et al., 2022). Indeed, the very limited distribution of *C. marthae* within the Island of Isabela (Fig. 1) is itself suggestive of a negative demographic interaction between this species and the more widely distributed *C. subcristatus*.

While *A. cristatus, C. subcristatus* and *C. pallidus* are classified as Vulnerable (VU) by the IUCN (MacLeod et al., 2019; Gentile & Grant, 2020; Kumar et al., 2020), *C. marthae* has been listed as Critically Endangered (CR) due to its extremely low population size and restricted geographic range (Gentile et al., 2012). A recent estimate based on long-term capture-mark-recapture data indicated a population of *ca.* 200 adult individuals (95 % CI 150–270) for *C. marthae* (Garizio et al., 2024).

Phylogenetic reconstructions to date have employed relatively small sets of mitochondrial and nuclear DNA sequence data, and have confirmed the monophyly of Galápagos iguanas. MacLeod et al. (2015) identified *Ctenosaura similis* as the closest mainland relative of the Galápagos iguana clade and estimated that *Amblyrhynchus* and *Conolophus* diverged from mainland iguanas approximately 8.2 Million year ago (Mya). However, Malone et al. (2017) later clarified that the resurrected genus *Cachryx*, which was not considered by MacLeod et al., (2015), represents the actual sister taxon of the Galápagos clade. Despite this correction, Malone et al., (2017) reported a similar divergence time estimate, suggesting that Galápagos iguanas split from their mainland relatives around 8.6 Mya. The lineage leading to the genus *Ctenosaura* (including *C. similis*, analyzed by MacLeod et al., 2015) is thought to have diverged from the *Cachryx*-Galápagos clade lineage much earlier, approximately 13.5 Mya. Marine and terrestrial lineages of Galápagos diverged from their common ancestor *ca.* 4.5 Mya, according to MacLeod and colleagues (2015), and 5.5 Mya, according to Malone et al. (2017). These estimates are consistent with the colonization of the archipelago by the common ancestors of Galápagos iguanas well after the date of the first available evidence of emergent volcanic islands in the region, *ca.* 9.1 Mya (Christie et al., 1992). As both *Cachryx* and *Ctenosaura* (as are all species of Iguanids but *Iguana iguana*) are absent from continental South America, Malone et al. (2017) concluded that the ancestors of Galápagos iguanas came from Central America, a relatively

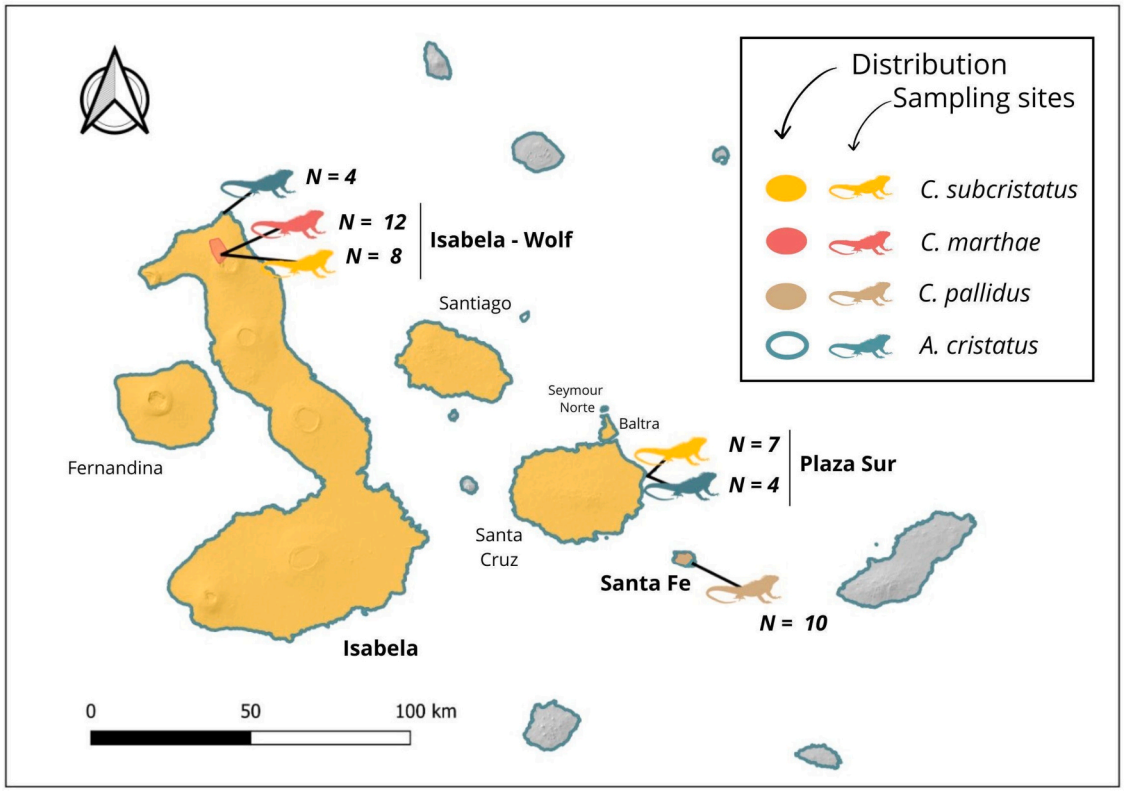


Fig. 1. Approximate geographical distribution of Galápagos Iguanas across the archipelago and overview of the samples used in this study. In yellow the islands where *Conolophus subcristatus* occurs. *C. marthae* only occurs in a very small area on Wolf Volcano, Isabela Island (pink area) in syntopy with a population of *C. subcristatus*. *C. pallidus* is endemic to Santa Fe (brown). The marine iguana, *Amblyrhynchus cristatus,* occurs along the coasts of most islands (blue outlines). Sampling sites are represented by iguana icons, with colors indicating the species, and respective sample sizes (*N*). Iguana silhouettes are from www.canva.com.

rare, but not unique case among other Galápagos clades. Thus, while most of the Galápagos terrestrial fauna apparently diversified in parallel to the geological formation of the islands (Parent et al., 2008), current evidence suggests this may not be the case for land iguanas. Despite Santa Fe being the oldest among the existing islands inhabited by this taxon (*ca.* 2.9 Ma, Geist et al., 2014), the Santa Fe endemic *C. pallidus*, seems to represent a recent lineage diverging from *C. subcristatus ca.* 300 kya (MacLeod et al., 2015). These findings further raise the question of whether *C. pallidus* should actually be recognized as a separate species from *C. subcristatus* (see also Rassmann et al., 2004; Snell et al., 1984).

Even more surprisingly, estimates based on mtDNA indicated that the lineage that gave rise to the pink iguana, *C. marthae*, endemic to the young Island of Isabela, split from other *Conolophus* species earlier than the emergence of the island it inhabits (0.8–0.5 Mya, Geist et al., 2014). MacLeod et al. (2015) estimated a divergence time of 1.5 Mya using indirect fossil calibration, while Gentile et al. (2009) proposed an even older split at 5.7 Mya (before any of the current islands existed, Geist et al., 2014) based on a biogeographic calibration. These split dates imply that the pink iguana's lineage started its independent evolution on now submerged islands. None of these authors, however, suggested an explicit biogeographic model accounting for the current distribution of the three *Conolophus* species.

Among terrestrial reptiles in the Galápagos, land iguanas are the least widely distributed, being limited to the central-western islands that were potentially connected by land bridges during late Pleistocene marine regressions (defined as 'core' islands, according to Ali & Aitchison, 2014). This distribution suggests that *trans*-marine dispersal has been extremely rare in this taxon. According to Ali & Aitchison (2014) the combined effect of eustatic sea level changes, subsidence of volcanic edifices and loading/unloading of sea floors determined repeated cycles of connection and isolation between Santa Cruz, Santa Fe, Santiago and other minor islands throughout the last million years. These islands, which have all existed for at least 1 Mya and for as much as 2.9 Mya, may have thus effectively acted as a single structured platform for the evolution of land iguanas, before becoming separated as recently as *ca.* 20 kya (Ali & Aitchison, 2014). Isabela may also have been part of this network of land connections, but, if this ever happened, it was for a shorter time, as this island gradually emerged starting *ca.* 0.5 to 0.8 Mya (Geist et al., 2014). In this scenario, the recent split between *C. pallidus* and *C. subcristatus* would naturally reflect the late Pleistocene loss of contact between Santa Cruz and Santa Fe. The combination of apparently early divergence and endemic distribution on a young island for *C. marthae*, however, represents a biogeographic conundrum. In principle, five main evolutionary scenarios could be considered for this species. We briefly present these hypotheses in Fig. 2 and provide further details in the Supplementary Material.

In this study, we use a RAD-seq (restriction site associated DNA markers sequencing; Davey & Blaxter, 2010) dataset to i) assess the genetic structure of Galápagos iguanas and test for potential hybridization and past introgression, ii) reconstruct the divergence process among species and local populations within the clade and their past demographic trends, iii) evaluate predictions of alternative biogeographic scenarios that may explain how Galápagos Iguanas attained their current distribution throughout the archipelago.

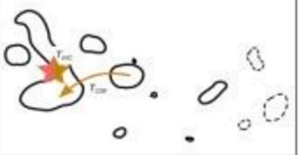
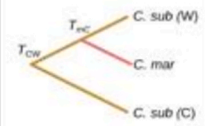
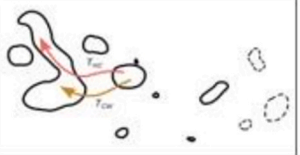
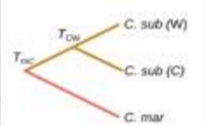
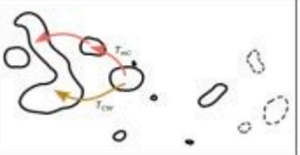
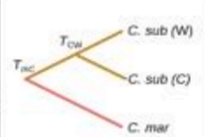
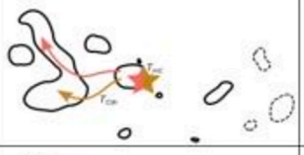
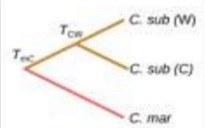
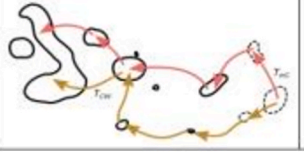
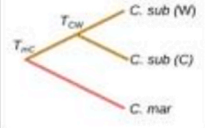
	Hypotheses		Dispersal Events	Speciation	Predictions	Predicted tree topology
H1	*C. marthae* speciation within Isabela after a single colonization of the island by a common ancestor of *C. marthae* and the western populations of *C. subcristatus* (from Isabela and Fernandina)		1	sympatric	$T_{mC} < 0.8$ Ma $T_{CW} < 0.8$ Ma $T_{mC} \leq T_{CW}$ Unlikely opposite demography	
H2	Allopatric speciation following colonization of Isabela by the ancestors of *C. marthae*, with *C. subcristatus* reaching Isabela later on		2	allopatric	$T_{mC} < 0.8$ Ma $T_{CW} < 0.8$ Ma $T_{mC} > T_{CW}$ Likely opposite demography	
H3	Allopatric speciation following colonization of an island different from Isabela (probably Santiago) by the ancestors of *C. marthae*, which later colonized Isabela and became extinct on this original island. *C. subcristatus* reached Isabela later		3	allopatric	$T_{mC} < 1.4$ Ma $T_{CW} < 0.8$ Ma $T_{mC} > T_{CW}$ Likely opposite demography	
H4	Speciation within the same island within the central 'core', followed by independent colonizations of Isabela by *C. marthae* and *C. subcristatus*		2	sympatric	$T_{mC} < 2.3$ Ma $T_{CW} < 0.8$ Ma Unlikely opposite demography	
H5	Speciation on currently submerged island(s) prior to the emergence of extant islands. *C. marthae* and *C. subcristatus* would have both survived by island-hopping for several million years		> 4	Allopatric / sympatric	$T_{mC} >> 0.8$ Ma Unlikely opposite demography	

Fig. 2. Five biogeographic hypotheses accounting for the current distribution of *C. marthae* and *C. subcristatus* on Isabela. T_{CW}: divergence time between Western (Isabela and Fernandina) and central (S. Cruz) populations of *C. subcristatus*; T_{mC}: divergence time between *C. marthae* and the central population of *C. subcristatus*, which in H2-H5 coincides with the divergence of *C. marthae* and the Western population of *C. subcristatus*. In the graphical depictions of hypotheses, continuous black lines represent approximate coastlines of current islands, dotted black lines represent islands that are now submerged, colored arrows represent dispersal events among islands (pink for *C. marthae* or its ancestors, yellow for *C. subcristatus* or its ancestors), while stars stand for sympatric speciation events.

2. Materials and methods

2.1. Samples

DNA from 43 individuals representing all four Galápagos iguana species and collected at four different locations (Fig. 1) between 2003 and 2014 was extracted and used to generate restriction site associated DNA sequence libraries (RAD-seq). Details on sampling and sample storage in the field are described elsewhere (Gentile et al., 2009; Gentile & Snell, 2009; Di Giambattista et al., 2018). See Table S1 for a more detailed summary of the study samples.

2.2. DNA extraction and sequencing

Genomic DNA was extracted from blood samples using the CTAB protocol (Doyle and Doyle, 1987). For each individual sample, 300 ng of genomic DNA were digested with high-fidelity SbfI restriction enzyme (New England Biolabs). The resulting DNA fragments were ligated to 150 nmol barcoded Illumina P1 adaptors (Microsynth), and after shearing and size selection, pooled into 3 libraries, each containing 10–18 individuals. Next, DNA was sheared using a Bioruptor™ sonicator (Diagenode) and concentrated using MinElute™ Columns (QIAGEN). The sheared DNA was size-selected at 300–500 bp on agarose gel and extracted using a QIAquick™ Gel Extraction Kit (QIAGEN). Subsequent steps involved repairing blunt ends using a Quick Blunting™ Kit (New England Biolabs), purification using MinElute™ Columns, and addition of 3′-dA overhangs with Klenow Fragment 3′→5′ (New England Biolabs). Illumina P2 adaptors were added, and a final amplification of 22 cycles was performed on 15 μL of each of the three libraries using 50 μL Phusion High-fidelity Master Mix (New England Biolabs) in a total volume of 100 μL. The final RAD libraries were then combined and sequenced on a single lane of an Illumina 2500 HiSeq platform at the Norwegian Sequencing Centre (Oslo, Norway).

2.3. Data processing

Data processing was performed on the PBS High Performance Computer (University of Leeds) and on SLURM High Performance Computer (Department of Biology, Mississippi State University). All raw sequence reads are available on NCBI (BioProject ID PRJNA1160247). Raw reads quality was assessed using the software FastQC (Andrews, 2010). We first filtered out reads lacking the complete SbfI recognition site or with more than 5 % of nucleotides with Phred quality score lower than 30. Libraries were demultiplexed using the software pipeline Stacks 2.55 with the program *process_radtags* (Catchen et al., 2013). We performed the trimming of the Illumina sequencing adapters with the software Trimmomatic (Bolger et al., 2014).

Reads were mapped onto the reference genome of *C. marthae,* produced and assembled by the Iguana Genome Consortium (López-Delgado, 2024) using BWA *mem* (Li & Durbin, 2009). We used programs *gstacks* and *populations* from the Stacks 2.55 package (Catchen et al., 2013) to perform variant calling and filtering, following the optimization procedure described by Rochette & Catchen (2017). In detail, *gstacks* was run with default parameters (*--model marukilow and --var-alpha 0.05*) to create a catalog of Single Nucleotide Polymorphisms (SNPs) across our sample set as a single population. We ran the program *populations* with the following parameters: --min-*samples-per-pop 0.80*, *--max_obs_het 0.70*, *--min_maf 0.05* and the *--write-single-snp* flag, which select only the first SNP of each locus, in order to minimize linkage disequilibrium effect and increase the statistical power of analyses. We made sure to exclude multi-allelic sites and missing sites from our dataset with the software bcftools (Danecek et al., 2021).

An R script from Wright et al. (2019) was used to extract the genotypes and associated metadata to further filter the SNP set on average allelic depth and coverage difference. We established a minimum average read depth of 2.5x for both the reference allele and the alternative allele. We determined the coverage difference by calculating the percentage difference in read depth between the reference and alternative alleles at each SNP, and we applied a cut-off threshold of 80 % or less for this difference (Wright et al., 2019). This helps ensure that the read depth is relatively balanced between the two alleles, which improves the reliability of the SNP calls. We excluded from the analyses several individuals with a coverage lower than 10x, one from the population of *A. cristatus* Isabela, three from *C. subcristatus* Plaza Sur and a last one from Wolf population of *C. subcristatus* (Rochette and Catchen, 2017). The filtered dataset in vcf format was re-processed through Stacks 2.55 populations −V to compute summary statistics and obtain input files for downstream analyses.

2.4. Analyses of genetic structure

We computed pairwise F_{ST} among populations using the *populations* module in Stacks 2.55, which estimates differentiation based on allele frequencies derived from filtered data. Filtering criteria included a minimum locus presence in 80 % of individuals per population and a minor allele frequency threshold of 0.01 to exclude rare alleles. We visualized patterns of genetic differentiation by plotting a heatmap of the pairwise F_{ST} matrix using the *pheatmap* R package(Kolde, 2018) (Fig. S1).

We explored the structure of the whole dataset by Principal Component Analysis, performed with the R (R Core Team, 2021) package *adegenet* (Jombart, 2008). The vcf obtained by Stacks 2.55 populations (*--write-single-snps*) was imported into the R environment with the package *vcfR* (Knaus & Grünwald, 2017) and transformed into a *genlight* object with the function *vcfR2genlight*. The PCA was performed with *glPca*, where missing values are replaced by the means of available observations (*i.e.,* mean allele frequency) (Jombart, 2008).

Second, we applied the clustering approach implemented in fastSTRUCTURE (Raj et al., 2014). This software was run on the PBS High Performance Computing Cluster (University of Leeds). fastSTRUCTURE performs inferences using a model of genetic admixture with unlinked loci. Since two of the species, *A. cristatus* and *C. subcristatus,* were represented by two different populations, we evaluated values of K (number of genetic clusters) from $K = 2$ to $K = 6$. The algorithm *chooseK.py* (Raj et al., 2014) built inside fastSTRUCTURE and based on cross-validation likelihood estimation and model evidence calculation, was employed to determine the optimal K value for our dataset. We ran fastSTRUCTURE for each different combination of K values and datasets using a hierarchical approach, sequentially subsetting our dataset to visualize the genetic structure both between and within species. Specifically, we conducted successive runs of fastSTRUCTURE by gradually excluding the most divergent samples based on PCA results (*i.e.*, *A. cristatus*, followed by *C. marthae*). This approach is aimed at enhancing the resolution of population substructure analysis by mitigating the effect of highly divergent clusters.

2.5. Phylogenomic inference and divergence time estimates

We initially assessed the topology of the phylogenomic tree including our species with the software IQ-TREE2 (Minh et al., 2020) (Fig. S3).

We then used the multispecies coalescent Bayesian approach implemented in the SNAPP program (Bryant et al., 2012), included in the BEAST2 package (Bouckaert et al., 2019), to reconstruct the divergence history of the sampled populations. SNAPP uses Monte Carlo

Markov Chains to sample from the posterior distribution of species trees starting from a set of single nucleotide biallelic or invariant loci that are assumed to be unlinked. The SNAPP model allows for different constant effective population sizes for each branch of the species tree and assumes no gene flow among branches. We run SNAPP on a Slurm High Performance Computer (Department of Biology, Mississippi State University). Filtered data were converted into a vcf containing both variant and invariant sites using Stacks 2.55 *populations (−-write-single-snp* and *−-vcf-all)*. We used the software bcftools (Danecek et al., 2021) to exclude multi-allelic sites, sites with missing data, and sites with low-confidence genotypes. As SNAPP can only reasonably handle a few thousands of biallelic sites, the resulting vcf was further subsetted, initially by randomly sampling *ca.* 100,000 sites with the software vcflib and algorithm *vcfrandomsample* (Garrison et al., 2022) and the option −r 0.062, which gives the sampling probability per locus. We then used the R package *SNPfiltR* (DeRaad, 2022) with option *distance_thin* and min. *distance = 5000* to retain only sites mapping on different scaffolds of the reference genome or sites > 5000 bp apart on the same scaffold, thus minimizing linkage among input sites. To ensure consistency, we performed two independent site selections to verify if both yielded the same results. We obtained a filtered vcf with 17,806 sites, of which 297 were SNPs, that was converted to nexus format using vcftools (option *−-012*) (Danecek et al., 2011). To create the xml input file for the SNAPP analysis, we used the software *beauti* (Drummond et al., 2012) implemented in BEAST2. The 43 individual genotypic profiles were divided into six current populations, based on species and sampling location (1, 5). We set the λ prior (speciation rate) following Leaché et al., 2014, who had a similar study system to ours in terms of expected divergence depths ($\alpha = 2.0$, $\beta = 200.0$). We set values for the common gamma prior distribution of the θ parameters (effective population size scaled by mutation rate, $\alpha = 2.0$, $\beta = 4000.0$) based on the observed heterozygosities of the current local populations. We ran SNAPP with two independent replicates of $1x10^6$ MCMC generations each, sampling every 10^3 generations, and applied a 10 % burn-in. To obtain rough estimates of the absolute divergence times in years, we scaled species trees by a mutation rate of $7.7x10^{-10}$ site^{-1} year^{-1}, computed as the average mutation rate for lizards by Perry et al. (2018). To obtain estimates of effective population sizes, we assumed a generation time of 10 years for the whole Galapagos iguana clade, following estimates from the literature (Fabiani et al., 2011; Garizio et al., 2024). Fabiani and colleagues (2011) considered 5 and 10 years as plausible generation times for Galápagos *C. subcristatus*. However, recently published mark-recapture data (Garizio et al., 2024) indicate that the survival rate of *C. marthae* over a period of approximately ten years is quite high, suggesting that 5 years might be an underestimate of actual mean generation times. Of course, it is hard to assess how much this information may be extrapolated to the whole clade throughout its long evolutionary path. However, unaccounted variation in generation times should only significantly affect our absolute estimates of effective population sizes, rather than population divergence estimates (which are based on a per-year mutation rate, see above) or demographic trends and timings of demographic events (see section 2.6). At any rate, to provide a rough measure of uncertainties related to generation time, we also provided conversion factors for population sizes based on reasonable generation times of 5 and 15 years (see section 3.4).

2.6. Demographic reconstruction

We assessed changes in effective population size (N_e) through time using the software Stairway Plot 2 (Liu & Fu, 2020). Stairway Plot 2 takes as input the Site Frequency Spectrum (SFS) of a sample of individuals to infer a multi-epoch demographic model assuming a closed population. We obtained the unfolded SFS for five local populations of each species (Fig. 4) by a custom R script starting from the summary statistics file (populations.sumstats.tsv) produced by Stacks 2.55 *populations (−-write-single-snp −-fstats)*. The ancestral/derived state of alleles within each population was determined by setting *Amblyrhynchus* as an outgroup for *Conolophus* samples and *vice versa*. The very rare SNPs that were polymorphic in samples from both genera were excluded. We run Stairway Plot 2 with default settings. A total of 37 individuals from five populations were analyzed (Fig. 5). The population of *A. cristatus* from Isabela was excluded because of small sample size, after filtering (Fig. 1). The demographic reconstructions were tentatively time-calibrated by a mutation rate of $7.7x10^{-10}$ site^{-1} year^{-1} (Perry et al., 2018). Three generation times (5, 10, 15 years, Fabiani et al., 2011; Garizio et al., 2024) were considered to translate estimates of the θ parameter into diploid effective population sizes (Fig. S2, S4).

3. Results

3.1. Genomic dataset

We obtained a total of 313,417,902 paired-end sequence reads from our 42 genotyped specimens. A total of 1,127,966 sites unambiguously mapped onto the reference genome, with a mean effective per-sample depth of 30.5x (range 14.3–49.6, sd = 9.3). After trimming and several filtering steps, we obtained 30,672 SNPs over 14,890 loci. Nucleotide diversity (π) was calculated for all six populations to assess genetic variation. The results revealed consistently low levels of nucleotide diversity across all populations, with values ranging from π = 0.00002 in *C. pallidus* to π = 0.00007 in *C. subcristatus* − Wolf. Intermediate levels of diversity were observed in *C. marthae* (π = 0.00005), *C. subcristatus* − Plaza Sur (π = 0.00004), and *A. cristatus* − Plaza Sur (π = 0.00005).

3.2. Between-population divergence: Genetic structure analyses

Pairwise F_{ST} values were calculated to assess the level of genetic differentiation among the six populations of our dataset (Fig. S1). Moderate differentiation was observed between *C. subcristatus* − Wolf and *C. subcristatus* − Plaza Sur (F_{ST} = 0.31). F_{ST} values between *C. pallidus* and the two populations of *C. subcristatus* were slightly higher (0.42 and 0.52). Differentiation between *C. marthae* and other *Conolophus* samples ranged between 0.65 (*C. subcristatus* − Wolf) and 0.76 (*C. pallidus*), confirming the genetic distinctiveness of this species. The lowest, although still substantial differentiation was observed between the two samples of *A. cristatus* from Isabela and Plaza Sur (F_{ST} = 0.15) which were, instead, very strongly differentiated from all *Conolophus* samples (0.90—0.94).

The first three principal components of PCA revealed the expected grouping of individuals according to the recognized species and clear clustering of *C. subcristatus* from the two geographic areas (Fig. 3).

The *chooseK.py* (Raj et al., 2014) program supported $K = 3$ as the best fit to the complete dataset in the fastSTRUCTURE analysis. Samples of *A. cristatus* and *C. marthae* were assigned to separate genetic units, while *C. pallidus* clustered together with *C. subcristatus*, indicating relatively strong genetic affinity between these species (Fig. 4a). *C. pallidus* and *C. subcristatus* were clearly assigned to separate clusters in the downstream run from which *A. cristatus* and *C. marthae* samples were excluded (Fig. 4c). The fastSTRUCTURE approach did not separate the

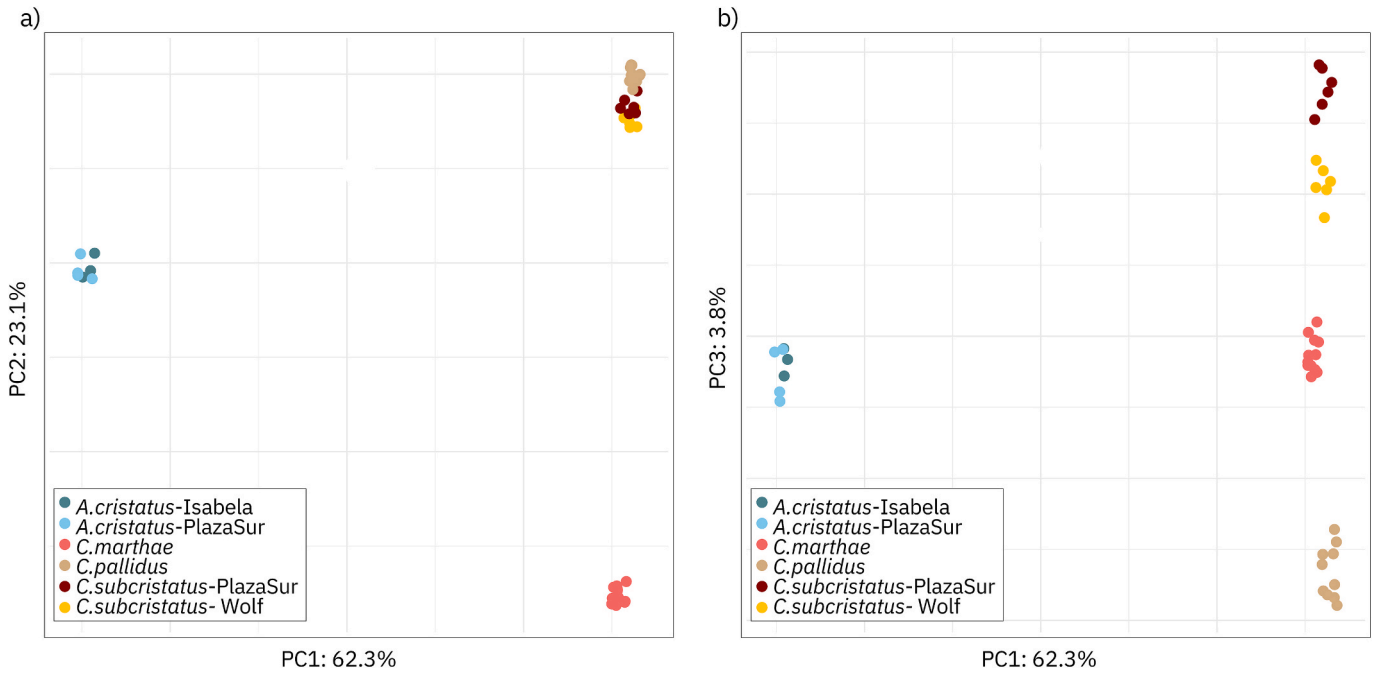


Fig. 3. Principal components analysis (PCA) of six populations of Galápagos iguanas. Color codes are shown in the inset legend. a) Scatterplot for the first and second principal components (PC1, PC2) b) Scatterplot for the first (PC1) and third (PC3) principal components.

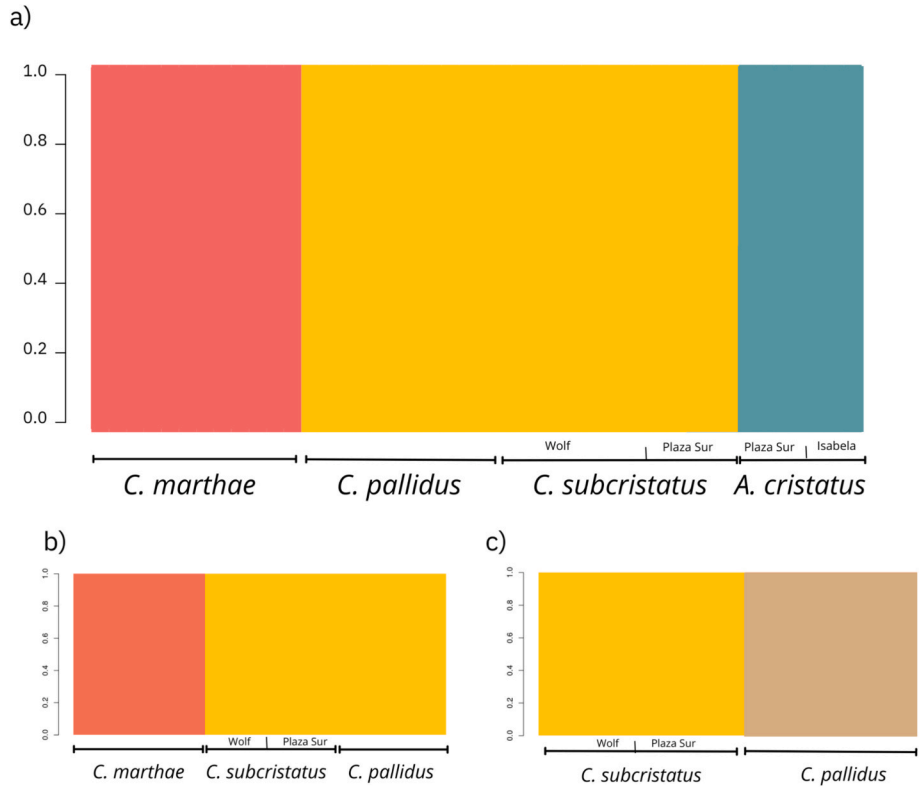


Fig. 4. Model-based Bayesian clustering performed with the software fastSTRUCTURE 4a) Output of fastSTRUCTURE, with K = 3 as best fit for the data, using all the four Galápagos iguanas species. 4b) fastSTRUCTURE analysis, with K = 3, excluding *A. cristatus* and the individuals with mixed ancestry 4c) fastSTRUCTURE analysis, with K = 2, excluding *A. cristatus,* the individuals with mixed ancestry and *C. marthae.*

two populations of *C. subcristatus* and *A. cristatus*. No sign of introgression between *C. marthae* and *C. subcristatus* (or any species pair) was detected (Fig. 4).

3.3. Phylogenomic analyses and divergence time estimates

The posterior sample from the SNAPP analysis contained three topologies, which only differed regarding the relative divergence order of

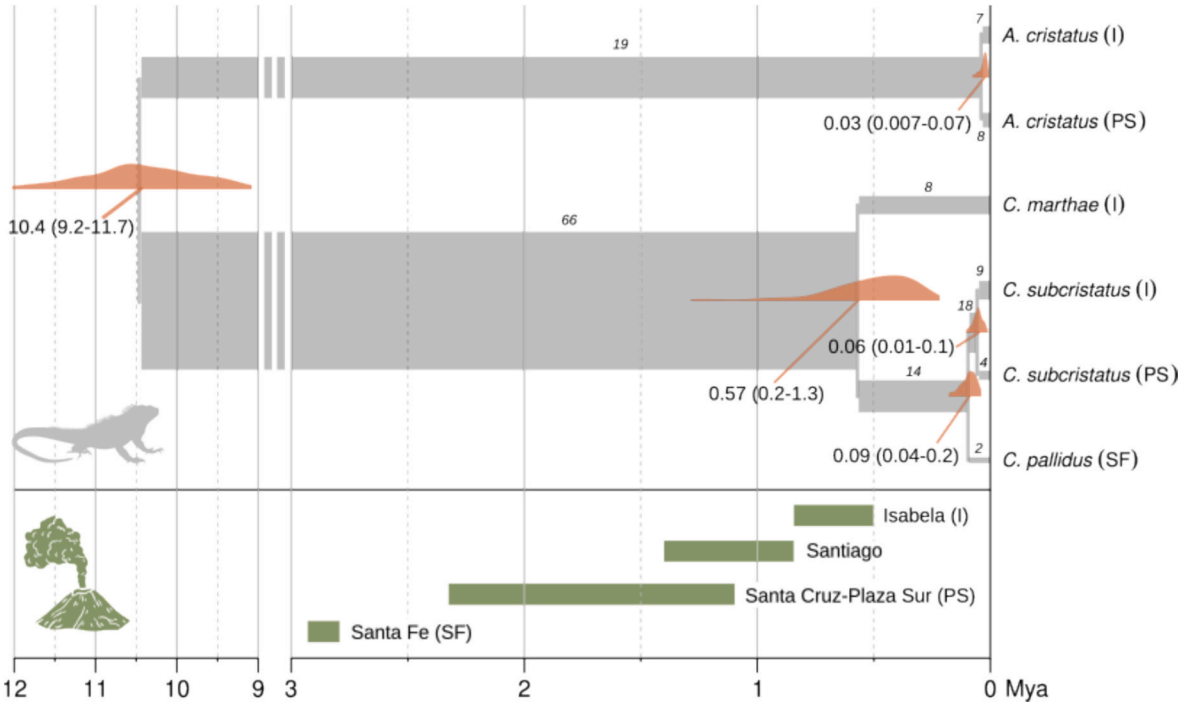


Fig. 5. The upper panel shows the time-calibrated species trees obtained from RAD-seq data with the SNAPP package of the software BEAST2. The most frequent topology in the posterior sample is shown. All nodes have a posterior probability of 1, with the exception of nodes connecting C. pallidus with the two populations of C. subcristatus (see section 3.3). Branch width is proportional to the estimated effective population size, which is also indicated (in thousands of individuals) by italicized numbers above branches. The posterior probability density distribution of node ages is shown (brown) and mean values (in million years) with 95 % Cis are indicated at nodes. The tree has been scaled considering a neutral genome-wide mutation rate of $7.7x10^{-10}$ site^{-1} year^{-1}, computed for lizards by Perry et al. (2018) and population size estimates assume a generation time of 10 years (see section 3.3 for details). The lower panel shows the ranges for the date of emergence of the four main islands occupied by Galápagos land iguanas as reported by Geist et al. (2018). Iguana and volcano silhouettes are from www.canva.com.

C. pallidus and the two *C. subcristatus* populations. The topology in which the divergence of *C. pallidus* predates the separation between *C. subcristatus* from Santa Cruz and Isabela islands (shown in Fig. 5) was recovered in 51.9 % of the SNAPP posterior sample, with the remaining 48.1 % being split between topologies with *C. pallidus* most closely related to *C. subcristatus* from Santa Cruz (30.8 %) or Isabela (17.3 %). By tentatively calibrating the SNAPP tree by the mutation rate of Perry et al. (2018), we obtained a divergence time between *Amblyrhynchus* and *Conolophus* of 10.4 (95 % CI 9.2–11.7) Mya. The split between *C. marthae* and the other Galápagos land iguana species could be estimated at 0.57 (95 % CI 0.2–1.3) Mya. The most recent divergence, between *C. subcristatus* and *C. pallidus*, is suggested to have occurred approximately 0.09 (95 % CI 0.04–0.2) Mya. Mean estimates of N_e for the tree branches computed by assuming a generation time of 10 years span between 2,000 for the terminal branch leading to the current population of *C. pallidus* and 66,000 for the very long branch corresponding to the ancestral population of all *Conolophus* iguanas (Fig. 5).

3.4. Historical demography

Demographic reconstructions with Stairway Plot 2 (Fig. 6) indicate all effective population sizes (N_e) to have been relatively small in the recent evolutionary past, particularly for the terrestrial *Conolophus* species. By calibrating the plots by a generation time of 10 years, all estimates range between *ca.* 50 (current N_e for *C. marthae*) and *ca.* 6,000 (current N_e for *A. cristatus* from Plaza Sur). Values of current N_e scaled by the different generation time used can be found in Table S2. Considering a plausible range of generation times from 5 to 15 years, all these estimates would be scaled by a factor from 2 to 0.66 (Fig. S2). The small size of all populations implies that Stairway Plot 2 reconstructions only extend into the last few hundreds of generations (*ca.* 5,000 to 20,000 years).

The population of *C. subcristatus* on Wolf Volcano shows a recent increase in population size, in rough temporal coincidence with a decrease in N_e for *C. marthae.* The *C. subcristatus* population of Plaza Sur went through a recent decline and shows a low current N_e estimate, while *C. pallidus* shows evidence of a relatively recent recovery from a strong bottleneck where N_e had been around 100. We inferred the demographic reconstructions using different generation times (5, 10, 15 years) (Fig. S4).

4. Discussion

4.1. Early colonization of Galápagos by iguanas

The Galápagos plume system has existed since at least 20 Mya (Hauff et al., 2000). Direct evidence of subaerial erosion in the Galápagos platform ranging from 9.1 My (Christie et al., 1992), to 16 My (Orellana-Rovirosa et al., 2018), indicate that the area could have hosted land organisms from at least the late Miocene, well before the emergence of the existing islands (< 4 Mya, Geist et al., 2014). Our divergence time analysis (Fig. 5), assuming a substitution rate calculated by Perry et al. (2018) for autosomal 4-fold degenerate sites in lizards ($7.7x10^{-10}$ site^{-1} year^{-1}), estimated the split between land (*Conolophus*) and marine (*Amblyrhynchus*) iguanas at 10.4 (95 % CI 9.2–11.7) Mya. As the monophyly of Galápagos iguanas strongly argues against independent colonization events by the ancestors of marine and land iguanas, our result implies that the common ancestors of the Galápagos clade entered the region soon after land became available, initially inhabiting now submerged islands. Previous molecular studies have reported divergence times within other endemic terrestrial vertebrate taxa endemic to the Galápagos archipelago predating the emergence of current islands (Ali and Fritz, 2021). This seems to definitely be the case for the first colonization event by *Phyllodactylus* geckos (Torres-Carvajal et al.,

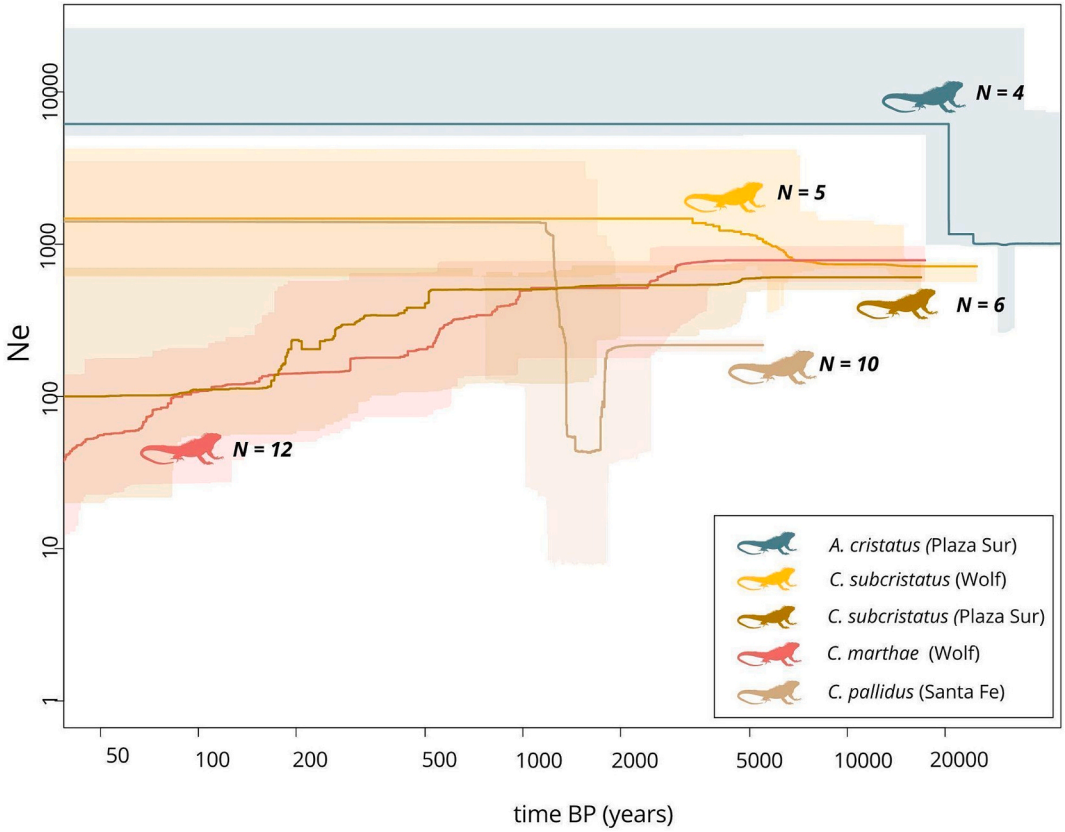


Fig. 6. Stairway Plot 2 results showing the recent demographic history of five local populations of Galápagos Iguanas. Panels show median effective population sizes (N_e) over time (*y* and *x* axes in logarithmic scale). Demographic reconstructions are shown for a generation time of 10 years. In the figure is indicated the number of individuals retained for the analysis for each population. We used 3575 variant sites over 58,536,589 invariant sites for *A. cristatus* – Plaza Sur, 841 variant sites over 56,641,024 invariant sites for *C. pallidus,* 2090 variant sites over 54,918,174 invariant sites for *C. marthae*, 2681 variant sites over 58,320,325 invariant sites for *C. subcristatus* – Wolf and 1906 variant sites over 55,692,067 invariant sites for *C. subcristatus* – Plaza Sur. Iguana silhouettes from www.canva.com.

2016) and, possibly, for the racer snakes (*Pseudalsophis*) (Zaher et al., 2018). Our estimate for the initial divergence within the Galápagos iguanas clade aligns with results by Rassmann (1997), who estimated the earliest split within the Galápagos iguana lineage at 10.5 Mya, but is older than more recent estimates by MacLeod et al. (2015) (*ca.* 4.5 Mya) and Malone et al. (2017) (*ca.* 5.5 Mya), which, anyway, also imply that iguanas landed on islands that are now underwater.

A potential issue with our estimates is the lack of internal calibration points. The volcanic nature of the archipelago does not allow for preservation of fossils useful for calibration of the Galápagos iguanas phylogeny (Ali & Fritz, 2021). The dates of fossils of stem iguanids (*ca.* 40 My old) and more distant relatives of Galápagos iguanas have been previously employed to estimate divergence times within this taxon using a small set of genetic loci (Malone et al., 2017; MacLeod et al., 2015). However, very distant calibration points hold relatively little information about the age of more recent nodes (*e.g.*, within *Conolophus*). Additionally, coalescent analysis on thousands of RAD loci from multiple individuals of multiple species would be inefficient because of the small number of shared loci among distantly related taxa that would be retained (Ivanov et al., 2021). On the other hand, biogeographic calibration points derived from estimated island ages, may provide upper bounds for the divergence times of endemic clades only if one assumes these clades split from their closest relatives after the emergence of their home islands. This approach was used by Gentile and coll. (2009), who assumed that the origin of western islands clade of *C. subcristatus* started after the colonization of Isabela Island, upon its emergence. Given the observed large divergence between mtDNA of *C. marthae* and the other land iguana species, the approach used in Gentile et al. (2009) indicated that the split of the pink iguana lineage would predate the emergence of Isabela Island (Geist et al., 2014). Here, our phylogenetic analysis aims to investigate a broad scenario including, for example, if the split of *C. marthae* follows the emergence of Isabela. Thus, we chose to rely on a "benchmark" mutation rate (Perry et al., 2018), and to consider the reciprocal implications between biogeographic scenarios and mutation rates.

Our estimate for the *Conolophus-Amblyrhynchus* split is, indeed, older than dates proposed by both MacLeod et al. (2015) (*ca.* 4.5 Mya) and Malone et al. (2017) (*ca.* 5.5 Mya). However, our estimates for splits within *Conolophus* are consistent with or younger than those inferred by these authors, so that a systematic upward bias due to an underestimation of the mutation rate can be ruled out. Perry et al. (2018) showed that nucleotide substitution rates at 4-fold degenerate sites are remarkably constant in squamate reptiles (and amniotes, in general). However, our RADseq dataset does not comprise only 4-fold degenerate sites and may also contain a portion of sites under negative selection (and a few sites under positive selection). RAD sequencing data may also capture more conserved regions of the genome compared to those containing 4-fold degenerate sites. Therefore, for all these reasons, it is more likely that Perry et al. (2018)'s rate, represents an overestimation, rather than an underestimation, of the actual substitution rate for our dataset. Moreover, simulation studies have shown that the SNAPP method does not tend to overestimate the age of deep divergences (Stange et al., 2018; Yan et al., 2023), while analyses of concatenated nuclear loci (such as from previous studies) tend to overestimate the divergence time between young species (Stange et al., 2018). Moreover, molecular clock analyses by both MacLeod et al. (2015) and Malone et al. (2017) ultimately rely on work conducted by Townsend et al. (2011). The latter authors used a calibration scheme with the bulk of the prior probability

for the age of nodes placed very close to the estimated age of fossils descending from the node itself, while the fossil age is a hard bound for the minimum age of the node. Such a scheme may favor an underestimation of node ages. We therefore argue that an early colonization of the Galápagos area by iguana ancestors about 9–10 Mya, as suggested by our SNAPP result, is the most plausible scenario given the available evidence.

4.2. Biogeography of C. marthae

When scaled by our "benchmark" mutation rate (Perry et al., 2018), our SNAPP analysis suggests a split of *C. marthae* from the *C. subcristatus* + *C. pallidus* lineage at 0.57 Mya (0.58 Ma, 95 % CI 0.2–1.3 Mya) (Fig. 5). Since Isabela Island, where *C. marthae* exclusively occurs, is estimated to have emerged *ca.* 0.5–0.8 Mya (Parent et al., 2008; Geist et al., 2014), this divergence time is consistent with allopatric speciation following the colonization of Isabela by the ancestors of the pink iguana (H2, Fig. 2). Similarly, Poulakakis et al. (2020) estimated that the colonization of southern Isabela Island from Santa Cruz by the ancestor of four species of tortoises in the genus *Chelonoidis* occurred approximately 0.41 (CI 0.24 – 0.57) Mya.

As mentioned above, we may expect our RAD loci to evolve at a somewhat slower rate than estimated by Perry et al. (2018) on 4-fold degenerate sites. Indeed, by setting the split of *C. marthae* at the oldest estimate for the emergence of Isabela (0.8 Mya), our SNAPP model still implies a reasonable genome-wide substitution rate of $5.7x10^{-10}$ site^{-1} year^{-1}. Gentile et al. (2009), estimated the mitochondrial time to the most recent common ancestor (*t*MRCA) of *C. marthae* and *C. subcristatus* + *C. pallidus* at 5.7 Mya. This date implied that *C. marthae* diverged from other *Conolophus* iguanas much earlier than the emergence of the Island of Isabela, to which the species is currently endemic, and, indeed, of all currently existing islands in the archipelago (H5, Fig. 2). Other instances of insular endemism whose divergence apparently predates the emergence of their homeland have been reported in reptiles (Salvi et al., 2021). However, the distribution of Galápagos land iguanas suggests that, contrary to Galápagos tortoises or marine iguanas (MacLeod et al., 2015; Poulakakis et al., 2020) *trans*-marine dispersal is very rare in this taxon, and a historical process involving several *trans*-marine dispersal events (H5, Fig. 2) appears unlikely due to the more complex chain of circumstances required. This seems to be true for other species inhabiting the Archipelago, such as Galápagos lava lizards (*Microlophus* spp.), that show limited ability to disperse across seawater (Jordan & Snell, 2008). Moreover, our Stairway Plot 2 reconstruction is consistent with negative demographic interaction between *C. subcristatus* and *C. marthae*, which is not predicted by H5. More importantly, the molecular clock calibration by Gentile et al. (2009) relies on the assumption that the mtDNA clade of *C. subcristatus* that occupies the western islands of Isabela and Fernandina originated *ca.* 0.5 Mya (about the time of Isabela's emergence, see Fig. 5). While plausible, this scenario does not represent a robust calibration, as the colonization of the western islands may have happened anytime after 0.5 Mya (more likely before *ca.* 0.1 Mya, according to the reconstructions of sea level changes by Ali & Aitchison, 2014). Therefore, we suggest that the hypothesis of an older divergence (> 4 Mya) of *C. marthae* (H5) may not be well-supported based on our data.

MacLeod et al. (2015) estimated the mitochondrial *t*MRCA of *C. marthae* and *C. subcristatus* + *C. pallidus* at 1.5 (95 % CI 0.9–2.2) Mya. While this estimate is also older than the emergence of Isabela, our SNAPP model suggests that it is still largely compatible with a split of *C. marthae* from the two other *Conolophus* species occurring *ca.* 0.8 Mya (the emergence date of Isabela Island). Indeed, by assuming a divergence time of 0.8 My, (and, hence, a mutation rate of $5.7x10^{-10}$ site^{-1} year^{-1}, see above), the estimated effective population size (N_e) for the ancestral population of all *Conolophus* scales to a mean estimate of *ca.* 87 k individuals. Although this may appear as a large number for Galápagos land iguanas, it actually represents a simplified parameter capturing the coalescent rate of a population that existed for several million years, most likely as a deeply structured metapopulation, probably scattered over different islands with alternating episodes of connection and isolation (Ali & Aitchinson, 2014). For such a population, long-term N_e would largely surpass census population size (Mazet et al., 2016). Based on coalescent theory, it is possible to compute the probability of observing a difference between the divergence time between a pair of species (which is estimated by the SNAPP approach) and the *t*MRCA of two mitochondrial gene copies sampled from each of the two species (which was estimated by McLeod et al, 2015). As the mtDNA effective population size is ¼ that of autosomal nuclear loci and the expected mean for the coalescence time of a pair of gene copies in a haploid (mtDNA) population is N_e generations, assuming a generation time of 10 years, the expected (mean) coalescence time of two haploid, maternally inherited, mtDNA lineages in a population of 87,000 diploid individuals is *ca.* 0.22 My, which implies an expected *t*MRCA of 0.8 + 0.22 ≈ 1 My. Moreover, the distribution of coalescence times for a pair of genes within the ancestral population follows a geometric distribution, therefore a mitochondrial coalescence time of *ca.* 65,000 generations (650,000 years), corresponding to a *t*MRCA of 1.45 Mya, can be computed as P_{geom} (tMRCA > 65,000, $p = 1/N_e = 1/22000$), which is still reasonably likely ($p > 0.05$). These values only become marginally lower for a mutation rate of $7.7x10^{-10}$ site^{-1} year^{-1} (Perry et al. 2018) (*t*MRCA = 1.1 My at p ≈ 0.04). Results by MacLeod et al. (2015) should not, therefore, be considered as evidence against the biologically plausible hypothesis of allopatric divergence of *C. marthae* after the emergence of Isabela (H2).

Hypothesis H3, by which *C. marthae* would have diverged allopatrically in a different island than Isabela, cannot be ruled out by the available evidence, as it is consistent with our multispecies coalescent tree (SNAPP, Fig. 5) and MacLeod et al. (2015)'s results, and it also predicts the negative demographic interactions observed in the Stairway Plot 2 output. However, it appears as unnecessarily complicated and cannot be considered as a parsimonious interpretation of available evidence. Similarly, we deem H4 as a less likely scenario based on its prior plausibility (despite speciation within the same island has been proposed to have occurred in Galápagos tortoises; Gaughran et al., 2023), with the addition that it does not predict the opposed demographic trajectories of sympatric *C. marthae* and *C. subcristatus* observed in the Stairway Plot 2 reconstruction.

4.3. Possible competition and lack of hybridization between C. marthae and C. subcristatus

The scaling of SNAPP parameters by the benchmark mutation rate indicates that the colonization of Isabela by *C. subcristatus* may have happened within the last *ca.* 100 ky (the divergence time of the Santa Cruz and Isabela populations of the species was estimated at 0.06 95 % CI 0.01–0.1, Fig. 5). Our demographic reconstruction revealed a decline of the effective population size (N_e) in *C. marthae* and a concurrent increase of N_e in the sympatric population of *C. subcristatus* from Wolf Volcano, Isabela (Fig. 6). Together with the small range of *C. marthae* and the much broader distribution of *C. subcristatus*, this result could suggests that *C. subcristatus* may have gradually out-competed the pink iguana on Isabela, limiting its distribution to the summit of Wolf Volcano. The decline of N_e in *C. marthae* is relatively recent, starting *ca.* 5–10 kya and resulting in a current N_e estimate around 100–50, consistent with the census population size estimates of *ca.* 200 adults by Garizio et al. (2024). The estimated split time between Santa Cruz and Isabela population of *C. subcristatus* (Fig. 5) suggests that this species may have colonized Isabela before the observed start of the demographic decline in *C. marthae*. However, it is reasonable that the site frequency spectrum of the current populations reflects local demographic dynamics, rather than processes involving the whole Island of Isabela. The current distribution of *C. marthae*, as well as the bathymetry of the ocean around the Galápagos islands (Ali & Aitchison,

2014), could be consistent with *C. subcristatus* landing in the south-eastern portion of Isabela, *ca.* 100 km from the Wolf volcano. The newly arrived species may have taken thousands of years to populate such a relatively large and topographically complex island. Of course, at the moment, this remains a hypothetical scenario, which could be tested in future studies including an expanded sample of *C. subcristatus* across Isabela.

Nevertheless, recently published nitrogen and carbon stable isotope analyses evaluated the trophic competition and overlap between *C. marthae* and *C. subcristatus* on Wolf Volcano (Gargano et al., 2022). These analyses hinted at some differentiation between the trophic niches of the two species, which suggest potential for coexistence, but, at the same time, highlighted a strong isotopic overlap, raising concern over the potential impact of interspecific competition on the small *C. marthae* population (Gargano et al., 2022). Although our observation of opposed demographic trajectories in sympatric populations of *C. marthae* and *C. subcristatus* might be merely coincidental, the collective evidence hints at competition with *C. subcristatus* as a likely determinant of the pink iguana's low numbers and severely restricted range – an enduring historical and ongoing competition with *C. subcristatus*.

Using microsatellite data, Di Giambattista et al. (2018) did not identify any obvious hybrids between *C. marthae* and *C. subcristatus* in their area of sympatry. However, they observed that *C. marthae* displayed a slightly lower average genetic differentiation from a sample of sympatric *C. subcristatus* than from samples of *C. subcristatus* from more distant locations on Isabela and suggested that this observation might hint at past events of hybridization between the two species. A more recent work based on the analysis of over 100 chemical compounds identified via gas chromatography and mass spectrometry of a waxy exudate secreted from iguanas' femoral pores suggests that the two sympatric species on Wolf Volcano may rely on different chemical cues to reinforce a mechanism of prezygotic isolation (Colosimo et al., 2020). While our analyses explicitly attempted to identify past rare admixture events, we did not retrieve any evidence for admixed individuals in the fastSTRUCTURE (Fig. 4), and PCA (Fig. 3) analyses do not indicate genetic admixture in any of our samples.

4.4. The status of C. pallidus as a separate species and its divergence from C. subcristatus

The low level of morphological differentiation and lack of coexistence in sympatry questions whether *C. pallidus* should be considered as a separate species endemic to Santa Fe Island or, rather, a local population of *C. subcristatus* (Rassmann, 2004). Our genetic structure analyses confirmed the existence of four main lineages of Galápagos Iguanas, corresponding to the recognized species (Fig. 4), thus corroborating previous results based on microsatellite and mtDNA (e.g., Tzika et al., 2008). Although the fastSTRUCTURE analysis using the full dataset did not identify *C. pallidus* as a separate cluster (Fig. 4a), its distinction emerged upon excluding the highly divergent species *A. cristatus* and *C. marthae* (Fig. 4b-4c), which were likely masking the smaller genetic differentiation of *C. pallidus* from *C. subcristatus*. Notably, both PCA and fastSTRUCTURE analyses highlighted a greater differentiation between *C. pallidus* and the two *C. subcristatus* populations (Plaza Sur and Isabela-Wolf) than between the two C. *subcristatus* populations. However, our phylogenetic analyses (Fig. 5) do not suggest that this difference is due to a longer divergence time, but rather to a stronger genetic drift effect, as our multispecies coalescent reconstruction (Fig. 5) did not fully resolve the splits among the two populations of *C. subcristatus* and the population of *C. pallidus*. We estimated the split between *C. subcristatus* and *C. pallidus* to have occurred approximately 0.09 (95 % CI 0.04–0.2) Mya, when scaled by a benchmark neutral mutation rate (Perry et al., 2018) or 0.12 (95 % CI 0.05–0.24) Mya, under the assumption of *C. marthae* diverging immediately after the emergence of Isabela (0.8 Mya, see above). Our estimate is only slightly lower, and compatible with the date suggested by MacLeod et al. (2015) based on secondary fossil calibration of mtDNA sequence data. Our model of population divergence is consistent with the ancestors of *C. pallidus* and *C. subcristatus* gradually colonizing several islands of the archipelago, with a more distinct form, *C. pallidus*, differentiating by vicariance on Santa Fe Island after the loss of a land connection with Santa Cruz in the late Pleistocene (Ali & Aitchison, 2014). Interestingly, while the split of *C. pallidus* is not significantly older than the split between the two *C. subcristatus* populations, SNAPP analysis and Stairway Plot 2 reconstructions indicate the historical population size of *C. pallidus* as the smallest in our reconstruction, thus suggesting an important role for genetic drift in the differentiation of the Santa Fe land iguana. The role of genetic drift in shaping the evolution of species inhabiting the Galápagos Archipelago has been well-documented, including in the lava lizard species complex (*Microlophus* spp.) (Jordan & Snell, 2008). These lizards appear to be similar to iguanas in having limited ability to disperse across seawater, which creates significant barriers to gene flow among populations. The younger age of Isabela compared to the eastern islands of Santa Cruz and Santa Fe suggests that the ancestral populations of *Conolophus* lived on one of the latter, while the small population size of *C. pallidus* (corresponding to the small size of Santa Fe) would argue in favor of Santa Cruz as the most likely homeland for the ancestors of the extant Galápagos land iguanas. On the other hand, the relatively large size estimated by SNAPP for this ancestral population suggests that it may have existed as a structured collection of local demes, possibly inhabiting distinct islets or ecologically isolated portions of the central 'core' islands (Ali & Aitchison, 2014).

4.5. Demography and conservation

Effective population size (N_e) is a reflection of the genetic variability of a population and is a pivotal parameter for conservation of endangered species, as it provides insights into a population's overall fitness and adaptive potential (Charlesworth, 2009). Our estimate of recent effective population size shows that all effective population sizes (N_e) have been small to very small ($100 < N_e < 10{,}000$), in the recent evolutionary past of Galápagos iguanas, especially so for the terrestrial species in genus *Conolophus* (Fig. 6) and for *C. marthae,* in particular, with a N_e *ca.* 50 in the most recent generations. Small population size could be an inherent feature of land iguana evolution in general, and not only characterizing the recent evolutionary past, as frequently observed in insular populations of terrestrial species (Leroy et al., 2021; Fernández-Palacios et al., 2021).

C. marthae showed the smallest N_e estimate among the studied populations, as expected, given its small range. Our estimate for the most recent generations is between *ca.* 20 and 150 (mean = 56) is consistent with recent estimates of census size based on mark-recapture experiments (Garizio et al., 2024) and supports the IUCN classification of this species as Critically Endangered (Gentile & Snell, 2009). As mentioned above, *C. marthae*'s demographic decline could be, at least in part, linked to competition from *C. subcristatus*. More recent and additional threats could include the introduction of invasive species in the Galápagos archipelago, such as rats (*Rattus rattus*) and cats (*Felis catus*) (Gentile & Snell, 2009), which are known to prey on juveniles and may pose a significant threat to population recruitment in *C. marthae* (Rueda et al., 2023).

In the past, it was hypothesized that introgression from *C. subcristatus* could also threaten survival of *C. marthae* as a distinct species. Our data, however, strongly support that hybridization between the two species is absent or extremely rare, despite range overlap.

Though spread over a relatively large range, *C. subcristatus* underwent a dramatic decline in the last centuries, becoming extinct in large portions of its historical range, including the entire Island of Santiago, where it has later been reintroduced, and most of the large Island of Santa Cruz. The most recent IUCN assessment (Kumar et al., 2020) reports an estimate for the total population of *C. subcristatus* at 10,216

(8,618–18,117) adult individuals distributed over several islands. Anthropogenic activities and, especially, introduced species led to a significant decline for this species since the 1700 s (Tzika et al., 2008; Fabiani et al., 2011). This is reflected in our Stairway Plot 2 reconstruction for the population of Plaza Sur, which shows a strong decline between *ca.* 500 and *ca.* 100 years ago (Fig. 6). Our N_e estimate for most recent generations in this small island (0.14 km^2) a few km off the coast of Santa Cruz is *ca.* 100 (Fig. 6), with a relatively stable trend, which is consistent with the IUCN census estimate of 380 individuals and apparently successful conservation efforts, including the eradication of feral goats (Kumar et al., 2020). The *C. subcristatus* sample from Wolf Volcano (in the North of Isabela Island) revealed a stable recent effective population size of *ca.* 1,500 over the last *ca.* 3,000 years, following a growth phase starting less than 10000 years ago (Fig. 6). As we have previously discussed, this growth phase may be linked to the expansion of the species in the north of Isabela, which is likely one of the last territories to have been colonized by this widespread species. According to Kumar et al. (2020), no estimates of the *C. subcristatus* population size are available for Isabela. However, the relatively large and stable N_e that we estimated for this population, joint with its higher genetic diversity compared to the Plaza Sur population (see section 3.1), suggests that the North of Isabela may currently represent an important stronghold for the species.

Our analysis indicates an increase in N_e for *C. pallidus* about 1,500 years ago, starting from a very small effective population (N_e *ca.* 120) and resulting in a recent, stable size of *ca.* 1,500. We do not place emphasis on the population bottleneck indicated by the median Stairway Plot estimate (thick beige line in Fig. 6), as the associated wide confidence interval allows for a simpler two-epoch demographic model. We cannot identify any obvious cause behind such a recent growth, however, the very small earlier size is consistent with the population of this species showing the lowest genetic variation in our dataset and with the strong drift suggested by our multispecies coalescent analysis (Fig. 5). A systematic survey completed in 2005 used the Petersen mark-recapture method to estimate a total census population of 5,016 individuals (range: 4,500–5,800) for *C. pallidus* (Márquez et al., 2010). This figure is consistent with our estimate of recent N_e. It has been long assumed that the presence of invasive species had strongly impacted the population of *C. pallidus* in the past (Gentile & Grant, 2020). However, no formal estimation of the population size had been performed prior to *ca.* 20 years ago (Márquez et al., 2010) and it is possible that the anthropogenic impact on this species has not been as severe as previously thought.

The current census estimates of the total number of individuals of *A. cristatus* range widely, between 19,800 and 210,000 (MacLeod et al., 2020). Recent calculations indicate significant variability in effective population sizes among its different subspecies (MacLeod & Steinfartz, 2016) and a general decline of the population has been suggested, largely due to the spread of invasive species across the archipelago (MacLeod et al., 2019). Recent genetic estimates (MacLeod & Steinfartz, 2016) indicated an N_e of 2,388 for the northern population of Isabela, which belongs to the subspecies *A. cristatus cristatus* (Miralles et al., 2017). The low number of samples did not allow us to compute a Stairway Plot 2 for the Isabela population. No previous N_e estimates exist for the population of Plaza Sur, for which we estimated a relatively high effective population size, at approximately 6,000, with a stable recent trend (Fig. 6). The estimate seems unexpectedly high for a small island like Plaza Sur and might reflect the influence of gene flow from the nearby island Santa Cruz. Our SNAPP analysis returned a mean estimate for the ancestral N_e of the Plaza Sur and Isabela samples at *ca.* 19,000, relatively low, as compared to *Conolophus* (*ca.* 65,000). This, along with the very recent divergence of the two considered populations (0.03 Mya, Fig. 5), is consistent with recent and ongoing gene flow among different *A. cristatus* populations, facilitated by the species' high mobility (MacLeod et al., 2019), and with *Conolophus* ancestral population presenting a stronger and prolonged population structure. Additionally, the marine iguana may have experienced historical phases of demographic fluctuations with severe population decline due to unpredictable yet recurring famine (during El Niño) and feast (during La Niña) events (MacLeod et al., 2019).

5. Conclusions

This study revealed that the ancestors of all Galápagos iguanas most likely reached the archipelago approximately 10 million years ago, shortly after the emergence of the land masses located in proximity to the current islands. We demonstrated that, in contrast to previous indications, our results are consistent with the divergence of *C. marthae* occurring after the emergence of Isabela Island. Site frequency spectra indicate opposing demographic trends in *C. marthae* and in the population of *C. subcristatus* living in sympatry with it in the last *ca.* 10 ky. We propose a model by which *C. marthae* diverged allopatrically from *C. subcristatus* following an early colonization of Isabela *ca.* 800–500 kya and later, gradually, shrunk its range possibly due to competition from *C. subcristatus*, which reached Isabela less than 100 kya. No evidence of past introgression or ongoing hybridization between *C. marthae* and *C. subcristatus* was detected in any of our samples. Furthermore, we confirmed the recent divergence of *C. pallidus* from *C. subcristatus*, attributing the former's relatively pronounced genetic and morphological differentiation to intense genetic drift in a historically small population. Our genetic data support recent census estimates indicating a very small current effective population size for *C. marthae* (*ca.* 50 individuals). Genetic diversity is also extremely low in *C. pallidus*, but our reconstructions suggest this to be due to a relatively recent bottleneck after which the reproductive population may have recovered. Interestingly, we estimated a relatively high effective population size for the Isabela population of *C. subcristatus*, indicating that this island may currently represent an important stronghold for this species, which has undergone local extinction throughout large portions of its historical range.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT to improve the readability and language of the manuscript. This tool was employed specifically to ensure the correctness of the English, as the first authors are not native English speakers. After using this tool/service, the authors thoroughly reviewed and edited the content as needed and take full responsibility for the content of the published article.

Ethical approval

Animal manipulation and blood sampling were performed according to a protocol that minimized animal stress, in accordance with the European Community guidelines and with the approval of the Galápagos National Park Directorate. Samples were exported and imported under the CITES permits 023/VS 2005; 024/VS 2005; IT/IM/2005/MCE/01620; 101/BG and IT/IM/2015/ MCE/01711 granted to GG.

CRediT authorship contribution statement

Cecilia Paradiso: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Paolo Gratton:** Writing – review & editing, Supervision, Conceptualization. **Emiliano Trucchi:** Writing – review & editing, Investigation, Data curation. **Julia López-Delgado:** Writing – review & editing, Resources. **Marco Gargano:** Writing – review & editing, Resources. **Lorenzo Garizio:** Writing – review & editing, Resources. **Ian M. Carr:** Resources. **Giuliano Colosimo:** Writing – review & editing, Resources, Investigation. **Christian Sevilla:** Resources. **Mark E. Welch:** Writing – review & editing, Supervision, Resources. **Mohd Firdaus-Raih:** Writing – review & editing, Resources. **Mohd Noor Mat-Isa:** . **Simon J. Goodman:** Writing – review & editing, Supervision, Resources. **Gabriele Gentile:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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