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1 # The impact of serial translocations on the genetic diversity of
2 # Anegada iguanas (*Cyclura pinguis*) in the British Virgin Islands.

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Abstract

Animal translocations are becoming increasingly popular as a tool for conservationists. Demographic factors can be crucial determinants dictating translocation viability in the short term. Translocated populations pass through artificial bottlenecks and can suffer from founder effects. Reduction in genetic variation relative to their source populations is likely, limiting their adaptive potential. Founder events can increase frequencies of deleterious alleles due to elevated rates of inbreeding and inbreeding depression. Here, we describe the effects of human-driven, serial population translocations on the genetic diversity of critically endangered Anegada iguanas (*Cyclura pinguis*) in the British Virgin Islands. Though founding populations were extremely small (N=8, N=4), the census sizes of translocated iguana populations increased dramatically over the first twenty years. This implies that these translocations were successful from a demographic perspective despite the small number of animals used, indicating a genetic paradox. To quantify genetic signatures in these bottlenecked populations, blood samples were collected from the source population and two translocated populations and genotyped at 21 microsatellite loci. We found that allele frequencies in translocated populations differed significantly from those of the source, with the translocated populations having less genetic diversity. However, common methods for estimating presence of genetic bottlenecks were non-significant. Estimates of internal relatedness by age class suggest that inbreeding depression may be elevated after translocation, likely reflecting the small initial population sizes associated with these translocation events. Anecdotally, our work shows that translocations may result in subtle genetic erosion that has long-term population viability impacts, even when census size indicates success.

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68 # Introduction

69 Conservation translocation is the human-driven movement of organisms from one location to another for
70 the benefit of that species' survival and of the ecosystem it naturally inhabits (IUCN/SSC 2013). Conservation
71 translocations may involve adding individuals to an existing population (supplementation or reinforcement), moving
72 individuals back to their indigenous range after extirpation (reintroduction), and moving individuals outside of their
73 native range for the benefit of the species as a whole (assisted colonization) or to fill an extinct ecological function
74 (ecological replacement) (IUCN/SSC 2013). Besides supplementation, which essentially acts as assisted migration,
75 all other translocation methods establish a new population of individuals.

76 Animal translocation has become an increasingly useful tool for the management and conservation of
77 wildlife imperiled by extinction, though results have varied (Berger-Tal et al. 2019; Morris et al. 2021; Evans et al.
78 2023). The founding of a population in a new habitat is usually smaller than the source population, thus leading to
79 different selection pressures, fitness outcomes, and allele frequency changes from genetic drift (Santos et al. 2012).
80 For a translocation to be considered successful in the long-term, the new populations must be both viable and
81 adaptable. Viability requires that the founders survive, reproduce, and their offspring successfully recruit to the
82 breeding population (Forsman 2014; Szűcs et al. 2014). Long-term success may not be achieved if the nascent
83 population is established with too few individuals, thereby increasing extinction risk. For example, stochasticity with
84 demographic rates, disease, and climactic events have larger impacts on recent colonization due to small population
85 size (Rajakaruna et al. 2015). Ecological mismatch between the source environment and the new environment also
86 may prevent successful colonization due to different abiotic and biotic factors (Pintar & Resetarits 2021, Cardador et
87 al. 2022).

88 Moving too few organisms can have detrimental genetic effects on the nascent population because it passes
89 through an artificial bottleneck, a reduction in genetic variability as a consequence of reduced population size
90 following translocation (Nei et al. 1975; Templeton 1980; Allendorf 1986; Santos et al. 2012; Hartl 2020). Although
91 bottlenecks are a central focus for conservation biologists, they are not necessarily viewed with the same concern by
92 invasion biologists. Species naturally colonizing new areas and expanding their original habitat via propagule
93 pressure are often able to overcome the hindrances of new populations founded by only a handful of individuals
94 (Kekkonen and Brommer, 2015). This apparent contradiction between conservation and invasion biology gave rise
95 to the so-called "genetic paradox" of invasive and colonizing species (i.e., how newly founded populations
96 overcome low genetic diversity and evolutionary potential to persist in the colonized environment; Allendorf &
97 Lundquist, 2003). Currently this paradox is considered resolved or, rather, difficult to prove. To truly be considered
98 a genetic paradox, in fact, the newly founded population must *i)* have lower genetic variation than the source
99 population, *ii)* not be demographically impacted by said lower genetic variation, and *iii)* adapt well to the new
100 environment it has been placed in (Estoup et al., 2016). However, many supposed genetic paradoxes have
101 comparable or increased genetic variation compared to the native population from multiple introduction events and

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102 admixture among founders (Uller & Leimu 2011, Estoup et al., 2016). Moreover, even in the case of documented
103 genetic impoverishment, newly established populations often do not show any significant adaptive challenge and
104 can reproduce successfully if the environment is benign or similar to that of the source population (Estoup et al.,
105 2016). Colonization might also be facilitated by preadaptation or exaptation, the co-option of characteristics that
106 contribute to fitness in a novel environment (Hufbauer et al., 2012).

107 Regardless of the actual occurrence of a genetic paradox, conservation biologists should prudently monitor
108 the genetics of a population involved in an assisted migration since the dispersal capacities of the species target of a
109 translocation are, by definition, insufficient to colonize new environments, and the translocated population cannot
110 naturally rely on further propagules to augment its genetic variability (Kekkonen and Brommer, 2015). Endangered
111 species, operating as small populations, are limited in their genetic variation (Frankham 2005). The genetic
112 hindrances of a translocation can further be aggravated if multiple translocations are seeded using individuals from a
113 previous translocation event. This chain of multiple bottlenecks can amplify the loss of genetic diversity and may
114 result in population decline, the insurgence of physical abnormalities, and an increase in extinction probability
115 (Gautschi et al. 2002; Leberg and Firmin 2008).

116 In the present study we explore genetic variation in three populations of Anegada iguanas (*Cyclura pinguis*)
117 to assess the genetic impact of serial bottlenecks resulting from conservation translocations in the British Virgin
118 Islands (BVI). This system is especially valuable since there is a record of source population genetic diversity, when
119 and how many founders were translocated, and no known immigration between islands prior to, or after,
120 translocation. We first reconstruct the history of two translocation events that occurred between 1984 and 1995 (see
121 Materials and Methods for details). We then use microsatellite data to compare genetic variation in the natural and
122 translocated populations. Current estimates of population census size of these translocations indicate successful
123 establishment from a demographic perspective. This could indicate a genetic paradox where the iguana populations
124 have overcome founder effects and preserved their evolution potential. Given the low number of individuals used to
125 seed the two translocation events, however, we predicted that there would be significant differences in allele
126 frequencies between the three populations, with the source population having the greatest genetic diversity. We also
127 evaluated common genetic bottleneck statistical tests and approaches for estimating effective population size to see
128 how these approaches compare to the species' known demographic history. As more translocations are used to
129 prevent local extinctions and initiate recolonization of species in their natural habitat, we use this case as an example
130 to provide some indication of how this practice may affect the survival of translocated populations.
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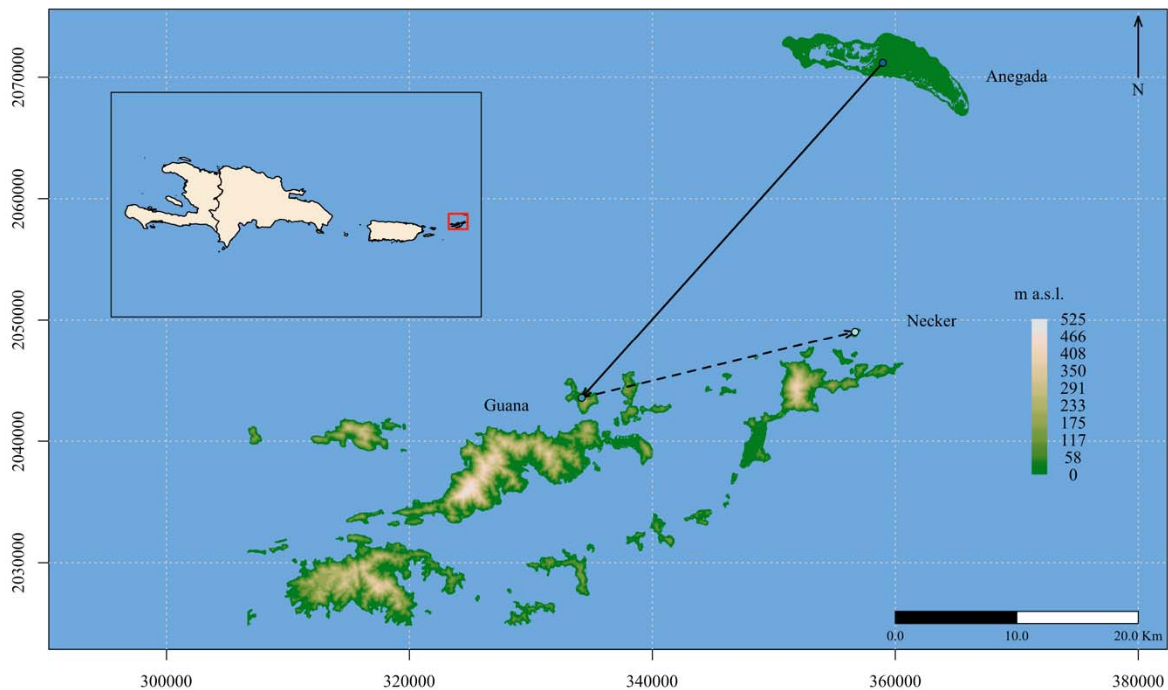
142 # Materials and Methods

143 ## Study system

144 Anegada iguanas, *Cyclura pinguis*, are one of 10 recognized species of rock iguanas (genus *Cyclura*)
145 (Iguana Taxonomy Working Group–ITWG 2016). This species, endemic to the BVI, is listed as Critically
146 Endangered (CR) under the International Union for the Conservation of Nature (IUCN) Red List of Threatened
147 Species™ (Bradley and Grant 2020). It represents the most basal and unique rock iguana lineage, which originated
148 10–20 million years ago (Reynolds et al. 2022). Thus, the conservation of Anegada iguanas could help maintain
149 more genetic diversity than the conservation of any other single rock iguana species. *C. pinguis* is sexually
150 dimorphic with males larger than females and individuals usually reach sexual maturity at 6 to 7 years and about 29
151 cm Snout-Vent-Length (SVL) for males and 36 cm SVL for females (Bradley and Grant 2020).



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153 **Fig. 1** Topographic map of the British Virgin Islands (BVI) with elevation expressed as meters above sea level (m
154 a.s.l.). The relative location of the BVI in the Greater Antilles is highlighted with a red square in the inset map. The
155 main map shows the first (solid line) and second (dashed line) order translocations between Anegada, Guana and
156 Necker islands. Between 1984 and 1986, eight individuals were moved from Anegada to Guana. In 1995, four

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157 hatchlings were translocated from Guana to Necker Island. The Guana and Necker populations of iguanas both
158 currently number in the hundreds
159
160 Historic evidence suggests that the range of *C. pinguis* once extended across the Greater Puerto Rican Bank
161 and numbered over 4000 animals (Carey 1975). Major population declines are thought to have occurred from
162 climatic fluctuations, leaving the island of Anegada as the sole habitat refugium for *C. pinguis* (Perry and Gerber
163 2006; Figure 1). Further declines began in the late 1960s when development on Anegada led to the proliferation of
164 introduced species and desertification. Goats, sheep, donkeys, and cattle compete with iguanas for food resources
165 alongside feral cats, rats and dogs that have a direct impact on the survivorship of young age classes (Goodyear and
166 Lazell 1994; Mitchell 1999). Extant iguanas on Anegada were reduced to approximately 160 individuals by the early
167 1990s (Mitchell 1999).
168 Conservation efforts to prevent further declines in the species include three principal strategies. The first is
169 directed at controlling the feral cat population and other invasive mammals on the island. The second focuses on
170 increasing recruitment on Anegada through a headstart and release program that raises juvenile iguanas away from
171 cats until they are large enough to better avoid predation (Perry and Gerber 2006). The third implements
172 translocations by moving animals from Anegada to other islands. The mammal eradication efforts and headstart
173 program are not within the scope of this study, but the slow progress in controlling exotic mammals is being
174 temporarily held in check by successes in the headstart program. However, headstarting juveniles is a temporary
175 solution to the problems faced by this species, and the long-term viability of the species is still very precarious
176 (Bradley and Gerber 2006). Therefore, strategies implying population restoration may serve as a critical tool in
177 managing this species (IUCN/SSC 2013).
178 Prior to the current conservation actions under consideration, translocations of *C. pinguis* took place. The
179 first translocation occurred between 1984 and 1986 and consisted of eight individuals brought from Anegada to
180 Guana Island (Figure 1). This translocation consisted of three males and five females (224-509 mm SVL), two of
181 which were palpably gravid at the time of release (Goodyear and Lazell 1994). The translocation led to a rapid
182 population increase on the island with an estimated 100 individuals by 2002 (Perry and Mitchell 2002). More
183 recently, this population is estimated to be 300 animals or more (Gerber, pers. obs.). In 1995, nine years after the
184 first translocation, four hatchlings (two females, two males) were moved from Guana Island to Necker Island
185 (Figure 1) and cage reared in captivity until their release the following year (Lazell 2002). The same author reported
186 the survival of all four animals and the emergence of new hatchlings by 1999. As on Guana, the population on
187 Necker has increased expeditiously and currently is in the hundreds (Gerber and Colosimo, pers. obs.). Within the
188 last several decades, translocations of *C. pinguis* to other small private islands have occurred, but little information
189 exists regarding the circumstances of these translocations or their success (Perry and Gerber 2006).
190

191 **Data collection and genotyping**

192 A total of 269 individuals were originally considered for this study. Upon capture by either noose or net,
193 each individual was sampled for morphological features, including snout-vent length (SVL), tail length (TL), and

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194 mass, as well as sex and age class. We considered hatchlings to have <100 mm SVL while adults have >250 mm
195 SVL. A passive integrated transponder (PIT, Trovan™, United Kingdom) was applied subcutaneously for long term
196 identification. Additionally, ca. 0.5 mL of blood was collected from the caudal vein using heparinized 1 mL
197 syringes. Blood was stored in EDTA buffer (Longmire et al. 1997) at environmental temperature while in the field
198 prior to long term storage in -80□ freezers.

199 Samples were scaled down to 174 as we excluded known hatchling clutch mates in an effort to reduce
200 biases while estimating the distribution of genetic variation. Of the samples considered for this study, 73 individuals
201 were sampled on Anegada, 63 on Guana Island, and 38 on Necker Island (see Table 1 for details). The Anegada
202 specimens were collected between 1999 and 2005. Samples from Guana and Necker were collected between 2006
203 and 2015. Genomic DNA was extracted from whole blood with Qiagen QIAamp DNA Mini Kit and accompanying
204 handbook protocols. A total of 21 loci were amplified using primers previously developed for *C. pinguis* (Lau et al.
205 2009, Supplementary Table 1) and following PCR conditions as indicated by Lau and colleagues (2009). Fragment
206 length was determined using an ABI 3100 or 3130xl Genetic Analyzer™ and GENEMAPPER™ Software (Applied
207 Biosystems).

208

209 Data analysis

210 Genetic diversity

211 We first investigated the presence of scoring mistakes and/or null alleles in our dataset. The
212 misinterpretation of electropherogram peaks during microsatellite genotyping and the presence of null alleles or
213 large allele dropouts can inflate the frequency of homozygous genotypes and bias estimates of overall population
214 diversity and differentiation. We used the software Micro-Checker 2.2.3 (van Oosterhout et al. 2004), which not
215 only evaluates the frequency of null alleles and short allele dominance, but also helps in the detection of scoring
216 mistakes due to microsatellite stuttering and/or other genotyping errors (van Oosterhout et al. 2004). We used all
217 methods for calculating null allele estimates in the software (Chakraborty et al. 1992, Brookfield 1996) We then
218 used the software FreeNA (Chapuis and Estoup 2007; Chapuis et al. 2008), which calculates the frequency of null
219 alleles using the Expectation Maximization (EM) algorithm described in Dempster et al. (1977). We repeated this
220 approach separately for samples from each island.

221 Unless otherwise specified we used R version 4.3.2 (R Core Team 2019) for the rest of the analyses. For
222 each locus in each island population, we then calculated the number of individuals typed, the number of alleles (A,
223 the raw count of alleles per locus), allelic richness (A_r, following the extrapolation algorithm of Foulley and Ollivier
224 (2006) as implemented in the *allelicrichness()* function from the R package pegas v. 1.3 (Paradis 2010)), observed
225 and expected heterozygosity (H_o, estimated as the number of heterozygous individuals compared to the total number
226 of typed individuals, and H_e, estimated using $\frac{2n}{2n-1} \times \left(1 - \sum_{i=1}^{k} p_i^2\right)$ following Nei (1978)). We then tested if loci
227 were in Hardy-Weinberg equilibrium (HWE) using a χ^2 test and a permutation test based on 10,000 computer
228 randomizations of genotypes resampled from the observed data. We summarized population-wise information over
229 all loci using GenAlEx 6.5 (Peakall and Smouse 2012). Count of private alleles unbiased for sample size was
230 calculated for each population using the formula $□' = □ \times \frac{n'}{n}$, where A is the number of private alleles in a

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231 population, *n'* is the rarefied (minimum) sample size out of all populations, and *n* is the actual sample size of the
232 population (Kalinowski 2004). We estimated rarefied allelic richness and pairwise F_{ST} between sampled islands
233 using Weir and Cockerham (1984) as implemented in the *pairwise.WCfst()* function from the R package hierfstat v.
234 0.5–11 (Goudet 2005).

235 We investigated how abrupt reductions in population size may affect multi-locus heterozygosity (MLH) by
236 calculating internal relatedness (IR; Amos et al. 2001). IR compares loci that are homozygous for rare alleles,
237 assumed to be more likely associated with identity by descent, with loci that are homozygous for common alleles
238 (Amos et al. 2001). We used IR instead of other indices to estimate MLH across the genome because IR operates
239 well when average heterozygosity is low (Aparicio et al. 2006). We used the R package Rhh v. 1.0.2 (Alho et al.
240 2010) to calculate IR. Comparisons of IR across islands and age class (adults and hatchlings) were conducted using
241 an Analysis of Variance (ANOVA) and a Tukey HSD test.

242 **Bottleneck event signatures**

243 Bottleneck detection is often difficult due to varying levels of pre-bottleneck genetic diversity, length and
244 magnitude of the bottleneck, and immigration (Williamson-Natesan 2005). Nevertheless, populations that have
245 recently undergone a bottleneck event present a specific distribution of allele frequencies such that only few alleles
246 with frequencies < 0.1 are left in the population (Luikart and Cornuet 1998). Luikart and colleagues (1998) suggest
247 that this particular signature can be detected using 5 to 20 loci and as little as 30 individuals. This signature can be
248 detected for up to 80 generations after the bottleneck event (Cornuet and Luikart 1996; Luikart and Cornuet 1998).
249 We used the software BOTTLENECK v1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999) to estimate observed and
250 expected heterozygosity excess across loci as a signature for genetic bottlenecks (Luikart and Cornuet 1998). We
251 conducted 1000 iterations under the Infinite Allele Model (IAM) (Maruyama and Fuerst, 1985), the Stepwise
252 Mutation Model (SMM) (Cornuet and Luikart 1996) and the Two-Phase Model (TPM) (Rienzo et al. 1994). TPM is
253 considered an intermediate mutation model less extreme than IAM and SMM. We ran the TPM twice with separate
254 parameter recommendations. The first was 90% of microsatellite mutations as stepwise, 10% as multi-step, and
255 assuming a variance σ_g^2 of 12 (Rienzo et al. 1994; Garza and Williamson 2001). The second was 78% of
256 microsatellite mutations as stepwise, 22% as multi-step, and assuming a variance σ_g^2 of 12, which better represents
257 vertebrate microsatellite mutation rates (Peery et al. 2012). Significant deviations from expected heterozygosity
258 excess were determined with a Wilcoxon signed rank test and the L-shape distribution of allele frequency
259 distribution (Cornuet and Luikart 1996; Piry et al. 1999). We also calculated the M-ratio (Garza and Williamson
260 2001) for each island using the R package strataG (Archer et al. 2017). The M-ratio uses the number of alleles and
261 range of allele sizes at a locus to estimate the genetic bottleneck effect (Garza and Williamson 2001).

262 We performed an individual assignment test using the Bayesian algorithm implemented in STRUCTURE
263 (Pritchard et al. 2000) to determine how the two serial bottleneck events have reshaped the distribution of genetic
264 variation. We ran the analysis with a flat prior for the admixed parameter and we allowed the allele frequencies to be
265 correlated among sampling sites. We tested values of K (number of genetic clusters) ranging from 1 to 4 and
266 performed 20 replicates of the analysis for each value of K (Gilbert et al. 2012). STRUCTURE ran 10^6 Markov
267 Chain Monte Carlo (MCMC) in each analysis while discarding the first 10^5 as burn-in. We used the command line

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268 back-end of STRUCTURE and the R package ParallelStructure v. 1.0 (Besnier and Glover 2013) to run the analysis.
269 The output of this analysis is used to calculate the second order of differences in the likelihood function of K [ΔK]
270 using the online tool Structure-Harvester (Evanno et al. 2005; Earl and VonHoldt 2012).

271 We further investigated the genetic structure of *C. pinguis* with a discriminant analysis of principal
272 components (DAPC) (Jombart et al. 2010). As DAPC does not rely on any priors or model assumptions, unlike
273 STRUCTURE, we can consider this approach as complementary to the Bayesian algorithm. We ran the analysis
274 using the R package adegenet v. 2.1.10 (Jombart et al. 2008). We followed the author guidelines as implemented by
275 others (Jombart et al. 2008, 2010; Welch et al. 2017; Pasachnik et al. 2020) to identify variables extrapolated from
276 allele frequencies (principal components) and to cluster individuals, maximizing between group variation while
277 minimizing variation within groups (Jombart et al. 2010).

278 Another consequence of population size fluctuation is the change in the degree of association between
279 alleles at any two or more loci (*i.e.*, linkage disequilibrium or LD). In general, in small populations a higher
280 correlation between alleles is expected, while little to no correlation is expected in large populations at equilibrium.
281 We first estimated LD using the correlation coefficient, $\underline{r}_d$, developed by Agapow and Burt (2001) and implemented
282 in the *ia()* and *pair.ia()* functions from the R package poppr 2.9.6 (Kamvar et al. 2014; Kamvar et al. 2015). We then
283 attempted to infer effective population size (N_e) with NeEstimator V2.1 (Do et al. 2014). The software uses four
284 different algorithms to infer N_e: one based on linkage disequilibrium (Waples and Do 2008), one based on excess
285 heterozygosity (Zhdanova and Pudovkin 2008), one based on molecular coancestry (Nomura 2008), and one based
286 on the temporal variation in allele frequency, considering a lowest allele frequency of 0.02 (Nei and Tajima 1981;
287 Pollak 1983; Jorde and Ryman 2007).
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289 # Results

290 ## Genetic diversity

291 We found no clear evidence of null alleles in our data set, nor consistent evidence of genotyping mistakes
292 across loci and populations (van Oosterhout et al. 2004, Supplementary 2). While locus D137 showed an excess of
293 homozygosity in the Anegada population, this was not true for the Guana or Necker populations. Loci D114, D130
294 and D135 showed excess homozygosity in individuals sampled from Guana Island while no loci showed any sign of
295 genotyping errors from null alleles in Necker Island individuals (Supplementary Table 2). The algorithm used by
296 FreeNA did not identify loci with a frequency of null alleles greater than 0.2 (max. value 0.118 for locus D137 in
297 Anegada population, Supplementary Table 2), the threshold level considered for significance (Chapuis and Estoup
298 2007; Chapuis et al. 2008). Only 13 (ca. 19%) estimations were greater than 0.05, representing moderate null allele
299 frequencies. D114 appears to have increased in null allele frequency by 10-fold after the first bottleneck event
300 (Anegada: 0.010, Guana: 0.115, Necker: 0.097; Supplementary Table 2). There were no deviations from HWE both
301 by loci and by population (Supplementary Table 3). We therefore decided to maintain all loci in the rest of the
302 analyses.

303

304 **Table 1** Sampled Island (Population), number of individuals collected on each site (N), average number of different
305 alleles per locus (N_A), rarefied allelic richness (A_R), rarefied private allelic richness ($Private_R$), observed
306 heterozygosity (H_o), and expected heterozygosity (H_e). These statistics were calculated using GenAlEx 6.5 (Peakall
307 and Smouse, 2012).

Population	N	N_A	A_R	$Private_R$	H_o	H_e
Anegada	73	4.095	3.871	7.808	0.550	0.564
Guana	63	3.286	3.129	0.603	0.482	0.503
Necker	38	3.143	3.056	1.000	0.454	0.444

308

309 Overall, the Anegada population showed greater diversity than Guana and Necker. The summary statistics
310 calculated over all loci indicated a greater number of alleles as well as greater values of observed and expected
311 heterozygosity in the source population as compared to the two sequentially translocated populations (Table 1). In
312 particular, the level of genetic diversity (as expressed by expected heterozygosity) is 10.8% lower after the first
313 translocation event, and an additional 10.3% is lost with the following translocation with an overall loss of diversity
314 of 21% (Table 1).
315 We found increased population differentiation based on the variance in allele frequencies consistent with
316 the sequential bottleneck events. Weir and Cockerham's (1984) calculation of F_{ST} increased from 0.07 between
317 Anegada and Guana to 0.13 between Anegada and Necker (Table 2).

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322 **Table 2** Population pairwise comparison of F_{ST} values as calculated following Weir and Cockerham (1984).

	Anegada	**Guana**	**Necker**
Anegada	NA		
Guana	0.068	NA	
Necker	0.133	0.092	NA

323

324 Anegada internal relatedness values were significantly different from the Necker population (ANOVA, p-
325 val = 0.034). IR increased sequentially after each bottleneck event, though not significantly (Figure 2). However,
326 when looking only at the adults, there are no significant differences across populations (Figure 3A, ANOVA, p-val >
327 0.668). In hatchlings, there were significant differences between Anegada and Necker (Figure 3B, ANOVA, p-val =
328 0.013), but Guana did not significantly differ from either population. Hatchlings and adults from the same island did
329 not significantly differ in IR from each other (Tukey HSD test, p-val > 0.10), though in general hatchlings had
330 higher IR values than adults.

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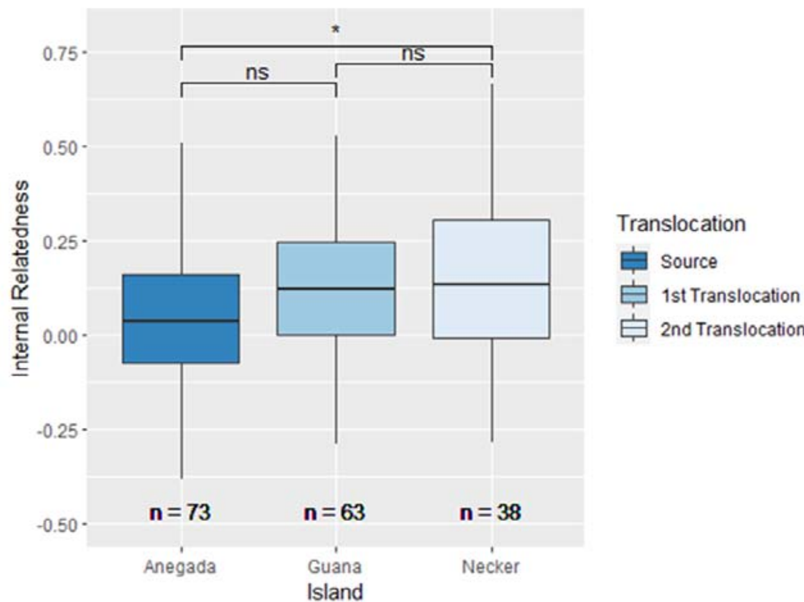

332

333 **Fig. 2** Internal relatedness values for Anegada, Guana, and Necker *Cyclura pinguis* populations. Sample sizes are
334 below each box plot. * denotes p-values < 0.05 based on Tukey's HSD, ns = not significant

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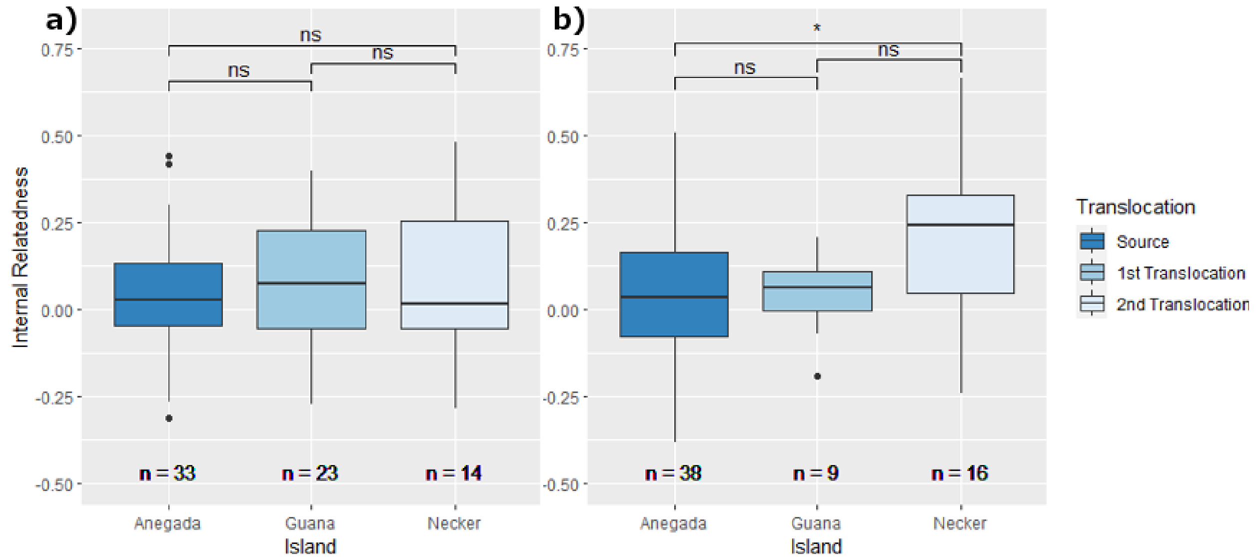

336
337 **Fig. 3** Internal relatedness values for Anegada, Guana, and Necker *Cyclura pinguis* populations split by age class, a)
338 adults and b) hatchlings. Sample sizes are below each box plot. * denotes p < 0.05 based on Tukey's HSD, ns = not
339 significant
340
341 **Bottleneck event signatures**
342 All tests under the Infinite Allele Model were statistically significant (p-val << 0.01) while none of the tests
343 using the SMM were significant (Supplementary Table 4). Tests under the TPM were statistically significant (p-val
344 < 0.05) for Guana but not for Necker, indicating only Guana had significant heterozygosity excess across loci after
345 translocation events (Table 3A and 3B). Interestingly, following the parametrization proposed by Peery et al. (2012),
346 Anegada also was found to have significant heterozygosity excess across loci (Table 3B). M-ratio on all three
347 islands was in the range of populations that have not undergone a demographic bottleneck (M > 0.650) rather than
348 those that have (M < 0.650) (Garza and Williamson 2001; Table 4).
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361 **Table 3 a)** BOTTLENECK output from a Two-Phase Model (TPM) using a variance of 12, 10% step-wise
362 mutations, and 1000 iterations based on Garza and Williamson 2001. **b)** BOTTLENECK output from a Two-Phase
363 Model (TPM) using a variance of 12, 22% step-wise mutations, and 1000 iterations based on Peery et al. 2012.
364 Significant values are bolded and in red.

	Island	Sign Test	1-tail Wilcoxon	2-tail Wilcoxon
a)	Anegada	0.519	0.108	0.216
	Guana	0.051	**0.013**	**0.026**
	Necker	0.335	0.226	0.452
b)	Anegada	0.456	**0.015**	**0.029**
	Guana	**0.044**	**0.003**	**0.007**
	Necker	0.091	0.084	0.168

365
366 **Table 4** Values of Garza and Williamson (2001) M-ratio for Anegada, Guana, and Necker *Cylcura pinguis*
367 populations. In general, populations that have undergone a genetic bottleneck have M < 0.650.

Island	M-Ratio	Variance
Anegada	0.857	0.048
Guana	0.863	0.046
Necker	0.814	0.071

368
369 The second order of differences in the likelihood function of K [ΔK] indicated K = 3 as the most likely
370 number of genetic clusters (Table 5). Individuals were grouped largely according to their sampling location (Figure
371 4). However, there was one individual from Guana whose genotype seemed to match the Necker cluster and two
372 individuals from Necker whose genotypes matched more to the Guana cluster (Figure 4).
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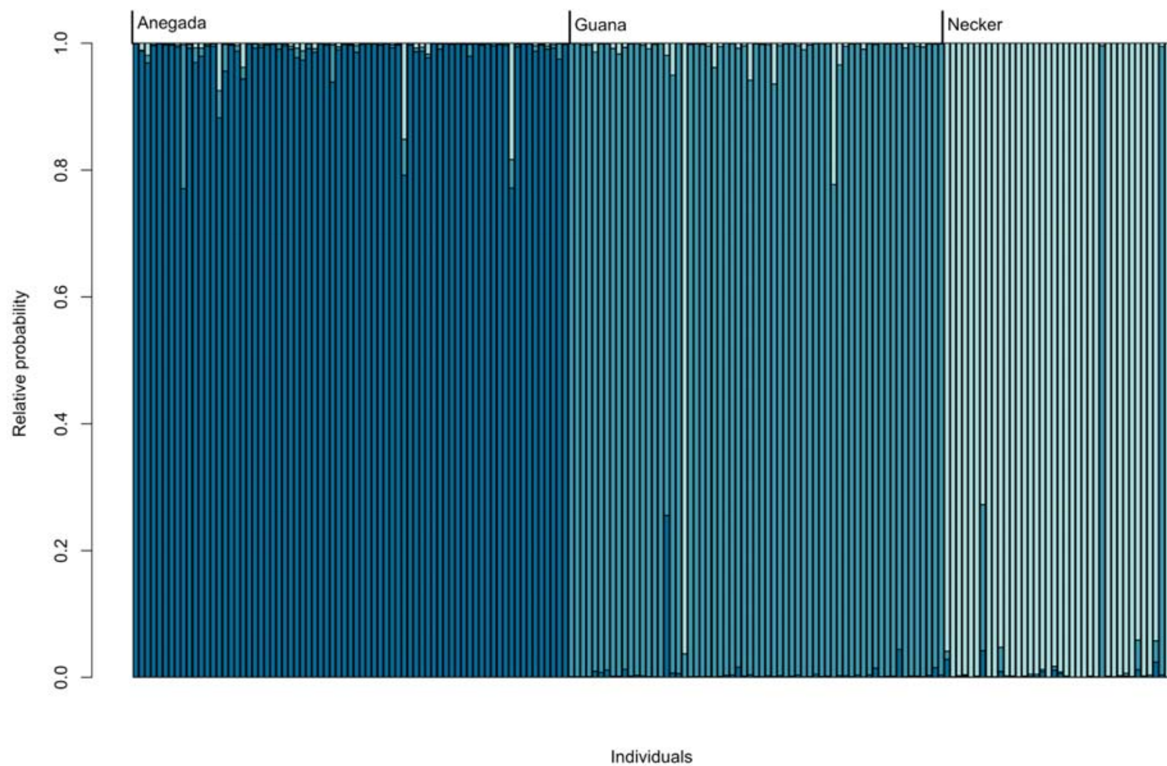
378 **Table 5** Table of the Evanno method output. Values highlighted in red show the largest value in the Delta K
379 column, indicating that the number of K groups to best explain the data is 3.

K	nRep	Mean LnP (K)	Stdev LnP (K)	Ln' (K)	|Ln'' (K)|	Delta K
1	20	-7277.925	0.242	NA	NA	NA
2	20	-6926.830	0.819	351.095	93.350	113.992
3	**20**	**-6669.085**	**0.936**	**257.745**	**200.745**	**214.473**
4	20	-6612.085	0.153	57.000	NA	NA

380



381
382 **Fig. 4** STRUCTURE results presented as a barplot. Individuals, represented as bars, are ordered by sampling site.
383 The software groups them using colors based on the relative probability that, genetically, the individual belongs to a
384 specific cluster
385
386 A very similar result was obtained using the DAPC analysis. Figure 5 shows how individuals are clustered
387 together along the two main principal components describing the differences in allele frequency between
388 populations.

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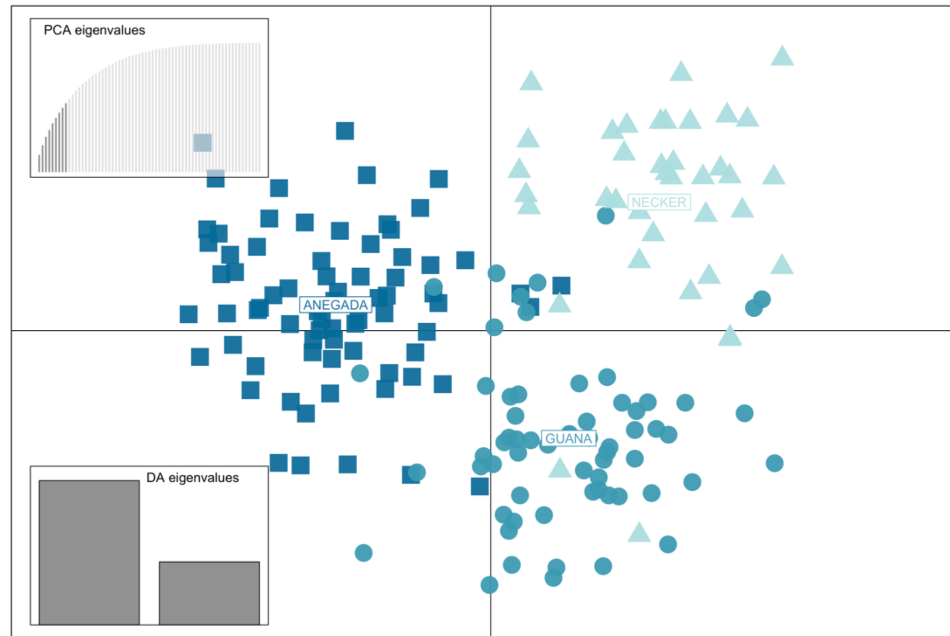

Fig. 5 Discriminant Analysis of Principal Components. The scatterplot shows the first two principal components using sampling site as prior clusters, with samples from Anegada represented as squares, samples from Guana represented as circles, and samples from Necker represented as triangles. The two smaller plots display the Principal Component Analysis eigenvalues and the Discriminant Analysis eigenvalues, respectively

Estimates of linkage disequilibrium as expressed by the correlation coefficient $_{}_{d}$ were highest among individuals sampled on Necker Island ($_{}_{d} = 0.0360$, p-val << 0.01). The second highest value was recorded from individuals sampled on Anegada ($_{}_{d} = 0.0113$, p-val < 0.01) while Guana individuals showed the lowest, but still significant, indication of linkage disequilibrium ($_{}_{d} = 0.0111$, p-val < 0.01; Table 6 and Supplementary Figures 1-3).

Table 6 Analysis of linkage-disequilibrium. Sampling island, unbiased estimator of correlation among loci ($_{}_{d}$), results of significance test after 1000 permutations (p-val).

Island	**$_{}_{d}$**	**p-val**
Anegada	0.011	0.002
Guana	0.011	0.005
Necker	0.036	0.001

Our estimates of effective population size using the LD and heterozygote-excess methods are reported in Table 7. The results of these approaches are different from the demographic history we know through translocations. A better approximation of what we know has happened demographically is provided by the other two estimates. The molecular coancestry method estimated an infinite effective population size on Anegada, N_e of 5.8 (3.4–8.9) on

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402 Guana, and 3.1 (1.3–5.7) on Necker. Finally, Nei and Tajima (1981)'s temporal method estimated two values of
403 effective population size: N_e = 6.9 (4.6–10.1) on Guana and N_e = 5.1 (3.2–7.7) on Necker.
404
405 **Table 7** Comparison of four methods (Linkage Disequilibrium–LD, Heterozygous Excess–Het. Exc., Coancestry
406 and Temporal as implemented in the program NeEstimator) of calculating effective population size in *Cyclura*
407 *pinguis*. Values of N_e are reported with the associated 95% confidence intervals.

Island	**LD**	**Het. Exc.**	**Coancestry**	**Temporal**
Anegada	29.5 (25.2–34.8)	34.6 (29.3–41.2)	Inf.	N/A
Guana	36.8 (28.6–48.7)	38.2 (29.6–50.9)	5.8 (3.4–8.9)	6.9 (4.6–10.1)
Necker	25.6 (18.3–37.8)	21.8 (16.3–30.3)	3.1 (1.3–5.7)	5.1 (3.2–7.7)

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409 **Discussion**

410 In this manuscript we describe the genetic signature of two translocation events, with the second one
411 seeded using individuals from the first. While we found evidence consistent with the occurrence of a genetic
412 bottleneck and with the potential of an ongoing genetic paradox in both populations of *C. pinguis* established by
413 translocation, we currently cannot ascertain the long-term adaptive repercussion of such genetic depletion.

414 Due to the small number of individuals moved during the two translocations, we anticipated that the genetic
415 variation would be unevenly distributed across islands, with a progressive loss of variability as the translocations
416 occurred. The first line of evidence supporting our prediction is provided by the documented decrease of average
417 number of different alleles, observed, and expected heterozygosity from the source (Anegada), to the first (Guana)
418 and the second (Necker) translocation. In particular, our results indicate that iguanas on Necker Island have
419 experienced an overall loss of diversity, as measured by expected heterozygosity, greater than 20% when compared
420 to the source population on Anegada (Table 1). This pattern is consistent with findings in other studies of
421 translocations using small founding populations (Ewing et al. 2008; Jamieson 2011). The recommendation for
422 maintaining an effective population size of 50 or more was established because theory indicates this should be
423 sufficient to prevent a loss of genetic diversity of more than 1% in the first 100 generations (Franklin 1980;
424 Frankham 1995). It is further projected that 20 founder genome equivalents should be sufficient for the maintenance
425 of 90% of genetic variation (Soulé et al. 1986). Larger founding populations and supplementation have shown more
426 success in maintaining genetic diversity (Jamieson 2011; White et al. 2018; White et al. 2020). However, each
427 translocation is unique and species' life history traits, current habitat characteristics, environmental stochasticity,
428 and management goals should be modeled to ensure population success (White et al. 2020). The data presented here,
429 together with the historical reconstruction of the translocations involving only a handful of individuals, provide a
430 well corroborated explanation of the rapid loss of variability experienced throughout the *C. pinguis* translocation
431 process.

432 Both individual assignment tests (STRUCTURE and DAPC, Fig. 4 and Fig. 5) indicate that the standing
433 genetic variation of the translocated population has drifted enough from that of Anegada, that individuals can now
434 be assigned to different islands based solely on their genotypes. Only three individuals were clustered in a different
435 genetic cluster despite their geographic provenience (Figure 4). We can speculate that the two individuals collected
436 on Necker Island and clustered as genetically belonging to the Guana group could have been two of the 4 original
437 founders moved in 1995. Similarly, the one individual sampled on Guana and genetically clustered with individuals
438 from Necker could retain alleles that are now predominant on Necker Island.

439 Further evidence corroborating our genetic diversity expectations is provided by the increase in IR values
440 from 0.04 measured in samples from Anegada, to 0.147 measured in samples from Necker (Figure 2). Specifically,
441 we found that hatchling internal relatedness increased significantly after translocation events while adult internal
442 relatedness did not change (Figure 3). When samples were pooled, internal relatedness increased after translocation
443 events, seemingly driven by the hatchlings. Though non-significant, hatchlings had higher internal relatedness
444 values when compared to adults from the same island (data not shown). Increased levels of homozygosity in
445 hatchlings relative to adults may indicate that hard selection (density-independent selection favoring advantageous

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446 traits in the current environment) or soft selection (density-dependent selection on a population's relative fitness in a
447 specific habitat) is acting on inbred individuals (Christiansen 1975; Whitlock 2002). An external factor in these
448 environments may be preventing homozygous hatchlings from reaching adulthood, favoring outbreeding. Due to the
449 relative recentness of both translocations, carrying capacity has not been reached and therefore intraspecific
450 competition (soft selection) is unlikely to be yet occurring (Ho and Agrawal, 2012), suggesting that inbreeding
451 depression may be driven by the expression of deleterious alleles as expressed in the mating between related
452 individuals (hard selection). This is different from other Caribbean rock iguanas, which show more support for soft
453 selection driving inbreeding depression (Berk 2013; Colosimo 2016; Moss et al. 2019). While this is an interesting
454 and important result from a conservation perspective, we are aware that the difference between age classes in the
455 detected IR values could also be explained by a lack of statistical power due to small sample sizes, and a more
456 detailed analysis aimed at disentangling hard versus soft selection using a more appropriate sample size could be
457 necessary.
458 Though demographic bottlenecks via translocation occurred twice in these *C. pinguis* populations, we were
459 unable to pick up significant genetic bottleneck signatures using heterozygosity excess tests. Other studies have also
460 generated non-significant results from well-documented, human-driven demographic bottlenecks (Aguilar et al.
461 2008; Henry et al. 2009), even in reptiles (Davy and Murphy, 2014; Bradke et al. 2021). The intermediate mutation
462 model (TPM) showed significant heterozygosity excess across loci on Guana and in one instance Anegada, but not
463 on Necker. Heterozygosity excess on Anegada may result from the ongoing demographic bottleneck that was
464 initiated in the 20th century. Garza and Williamson's M-ratio also did not indicate evidence of bottlenecks. Similar
465 results were found with a translocated, insular population of elk (Hundertmark and Van Daele, 2010). The authors
466 posit that the M-ratio, reliant on allele size, would lag behind genetic diversity loss and thus only pick up on
467 bottleneck events multiple generations ago. Because our populations are 3-5 generations separated from
468 translocation and sampling events, the genetic bottleneck may be too recent for the M-ratio to detect (Hundertmark
469 and Van Daele, 2010).
470 Many factors, such as associative overdominance, influence distributions of alleles thereby impacting
471 heterozygosity excess tests (Gilligan et al. 2005). Single-sample population genetic methods assume mutation-drift
472 equilibrium (Peery et al. 2012). Due to the demographic history and small population sizes of these three iguana
473 populations, it is highly unlikely that mutation is adding genetic variation as much as genetic drift is decreasing it
474 (Whitlock 2000). Heterozygosity excess tests may lose statistical power if population size increases rapidly and
475 panmixia increases (Hundertmark and Van Daele, 2010). Though this may in part explain our non-significant results
476 for Necker, heterozygosity excess tests have been criticized for being sensitive to the starting parameters of the two-
477 phase mutation model (Spong and Hellbord, 2002; Peery et al., 2012). We see this in our own results with only one
478 of two sets of parameters showing heterozygosity excess on Anegada. Microsatellite mutation rates across
479 vertebrates vary significantly but are difficult to measure for every study species (Peery et al. 2012). Researchers are
480 forced to use parameter values recommended by the software's authors or use averages that may be very different
481 from actual rates (Peery et al. 2012). Bayesian approaches using coalescent theory and MCMC may be more
482 accurate at indicating genetic signatures after a bottleneck (Peery et al. 2012).

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483 In most cases, the estimates of effective population sizes generated by the various methodologies exceeded
484 census numbers of the translocated populations at their founding (Table 7). The least accurate was the
485 heterozygosity excess method estimating an infinite N_e for all three islands. This technique has been shown to be
486 imprecise due to the function's need for >20 breeders, a large sample size, and its sensitivity to deviations from
487 random mating (Luikart and Cornuet, 1999; Wang et al. 2016). The one-sample linkage disequilibrium method has
488 been shown to detect changes in N_e only 1-2 generations after abrupt population size changes, like bottlenecks,
489 better than the two-sample temporal method (Antao et al. 2011, Waples 2024). Surprisingly, the LD method
490 generated estimates inconsistent with the known history of these translocations. This may be due to the lack of
491 linkage disequilibrium after the first translocation, which we also saw with the r_d values (Table 6). Internal
492 relatedness and heterozygosity excess results suggest that there are more heterozygous individuals in our dataset
493 than expected. This can decrease linkage disequilibrium estimates (Sabatti and Risch, 2002). Both the two-sample
494 temporal method and the one-sample molecular coancestry method estimated effective population sizes similar to
495 the founding population on the translocated islands. These calculations are less impacted by genetic drift and
496 bottlenecks than other methods, and are theorized to be less affected by small, inbred populations (Nomura 2008).
497 The temporal method is robust to non-random mating, population structure, age structure, and overlapping
498 generations (Wang et al. 2016). We believe this serves as an anecdote highlighting the accuracy of the molecular
499 coancestry method over other one-sample methods for estimating effective population size. However, it is
500 recommended to factor in multiple, independent estimates of N_e to get a more complete picture of the study
501 population (Luikart and Cornuet, 1999).
502 Both translocated populations of *C. pinguis* studied here meet at least two of the criteria for a genetic
503 paradox set by Estoup et al. (2016). First, we found ample evidence supporting a loss of genetic variability in the
504 translocated populations compared to that in the source population. Second, estimates of census population size on
505 both Guana and Necker are in the hundreds. This indicates rapid population growth and establishment following
506 introductions of very few animals. However, it is more difficult to assess whether these populations are meeting the
507 third criterion and adapting to novel environmental conditions in their introduced range. It is possible that adaptation
508 has not yet happened but is actively occurring (Meek et al. 2023). It might also be argued that *C. pinguis* were
509 preadapted to their translocated island habitat. Fossil evidence from Puerto Rico implies that the entire Puerto Rican
510 Bank may have been home to *C. pinguis* or its ancestral species (MacLean 1982). Even so, there are no reports of *C.*
511 *pinguis* naturally occurring on Guana or Necker. If these *C. pinguis* populations successfully navigate potential
512 pitfalls, they serve as a good example of a genetic paradox, and this may bode well for the future of translocation as
513 a conservation strategy.
514 We conclude that the iguana populations of Guana and Necker are genetically depauperate and should not
515 be considered as source populations for future conservation translocations. Though currently impacted by
516 anthropogenic disturbances, the naturally occurring population of *C. pinguis* on Anegada remains the most
517 appropriate genetic stock for future translocations. However, supplementing the populations on Guana and Necker
518 with iguanas from Anegada may instigate genetic rescue (eg. Pimm et al. 2006). The decrease in genetic diversity on
519 Guana and Necker is directly tied to the small number of founders translocated to each island (N=8 and N=4,

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520 respectively). The specific number of individuals translocated for conservation purposes depends heavily on the
521 species, habitat, and other external factors (Furlan et al. 2020). However, larger founding populations decrease the
522 impacts of genetic drift and prevent inbreeding depression, thereby preserving genetic diversity over multiple
523 generations (Frankham et al. 2014). Though considered successfully colonized from a demographic perspective, we
524 posit the populations of Guana and Necker represent a genetic paradox, though it is too early to comment on
525 population fitness and evolution potential. Preventing inbreeding depression should be a high priority for wildlife
526 managers considering conservation translocations to address long-term goals, such as population sustainability
527 without human intervention. Conservation translocations can protect genetic diversity, if done thoughtfully, to
528 ensure long-term resiliency through evolutionary time, especially in the face of a stochastic and rapidly changing
529 world.
530

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531 **Data Availability**

532 R script and microsatellite genotypes associated with this project are available upon request.

533 **Competing Interests**

534 The authors have no relevant interests to disclose.

535 **Compliance with Ethical Standards**

536 All animal capture, handling, and sampling was performed following the American Society of Ichthyologists and
537 Herpetologists (ASIH) guidelines for use of reptiles and amphibians in research and all methods were approved
538 under the authors' IACUC permits.

539 **Author Contributions**

540 GC, MW and GPG designed the research; GP, ZTH, and GPG collected the samples; ZTH performed lab work; GC
541 and ZD performed data analysis with guidance from MW, GG, and GPG; GC and ZD wrote the primary manuscript;
542 all authors edited and revised the manuscript.

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549 staff for sample collection and ongoing support of iguana conservation efforts.

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