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Contributed Paper

Genetically informed captive breeding of hybrids of an extinct species of Galapagos tortoise

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Abstract: *Hybridization poses a major challenge for species conservation because it threatens both genetic integrity and adaptive potential. Yet, hybridization can occasionally offer unprecedented opportunity for species recovery if the genome of an extinct taxon is present among living hybrids such that selective breeding could recapture it. We explored the design elements for establishing a captive-breeding program for Galapagos tortoises (*Chelonoidis *spp.) built around individuals with admixed ancestry involving an extinct species. The target individuals were hybrids between the extinct species from Floreana Island,* C. niger*, and an extant species,* C. becki*, which were recently found in the endemic range of* C. becki*, from Wolf Volcano on Isabela Island. We combined genotypic data from 35 tortoises with high ancestry from* C. niger *with forward-in-time simulations to explore captive breeding strategies that maximized overall genetic diversity and ancestry from* C. niger *while accommodating resource constraints, species biology, and the urgency to return tortoises to Floreana Island for facilitating ecosystem restoration. Overall genetic diversity was maximized when in the simulation tortoises were organized in relatively small breeding groups. Substantial amounts of the* C. niger *genome were captured despite limited resources available for selectively breeding tortoises in captivity. Genetic diversity was maximized when captive-bred offspring were released to the wild rather than being used as additional breeders. Our results provide genetic-based and practical guidance on the inclusion of hybrids with genomic representation from extinct taxa into species restoration programs and informs the ongoing debate on the value of hybrids in biodiversity conservation.*

Keywords: *Chelonoidis niger*, ex situ population management, Floreana Island, forward-in-time simulations, genetic ancestry, genetic relatedness, hybrid conservation value, museum samples

Reproducción en Cautiverio Informada Genéticamente de Híbridos de una Especie Extinta de Tortuga de las Galápagos

Resumen: *La hibridación representa un obstáculo importante para la conservación de especies ya que amenaza tanto a la integridad genética como al potencial adaptativo. Aun así, la hibridación ocasionalmente puede ofrecer una oportunidad sin precedentes para la recuperación de una especie si el genoma de un taxón extinto está presente entre los híbridos vivientes de tal manera que la reproducción selectiva pudiera recuperarlo. Exploramos los elementos de diseño para el establecimiento de un programa de reproducción en cautiverio de la tortuga de las Galápagos (Chelonoidis spp.) construido en torno a los individuos con linajes*

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mixtos que incluyeran una especie extinta. Los individuos fueron los híbridos de la especie extinta en la Isla Floreana, C. niger, y la especie viviente C. becki, encontrados recientemente en la distribución geográfica endémica de la segunda especie en el Volcán Wolf (Isla Isabela). Combinamos los datos genotípicos de 35 tortugas con un linaje cargado de C. niger usando simulaciones futuras de la descendencia generada por el programa para explorar las estrategias de reproducción en cautiverio que maximizaran en general la diversidad genética y el linaje de C. niger a la vez que se ajustaba a las restricciones de recursos, la biología de la especie y la urgencia por regresar las tortugas a la Isla Floreana para facilitar la restauración del ecosistema. En general, la diversidad genética se maximizó cuando en la simulación las tortugas estuvieron organizadas en grupos de reproducción relativamente pequeños y cuando cantidades sustanciales del genoma de C. niger fueron capturados con base en los recursos disponibles para reproducir selectivamente a las tortugas en cautiverio. La diversidad genética se vio especialmente maximizada cuando las crías reproducidas en cautiverio fueron liberadas en lugar de ser utilizadas como reproductoras adicionales. Nuestros resultados proporcionan una guía práctica y basada en la genética para la inclusión de híbridos con representación genómica de un taxón extinto en los programas de restauración de especies. Cuando incorporamos a los híbridos con diversidad genética que previamente se creía perdida en los programas con el propósito de la reintroducción de especies, nuestro estudio informa al debate continuo sobre el valor de los híbridos para la conservación de la biodiversidad.

Palabras Clave: *Chlenoidis niger*, Isla Floreana, linaje genético, manejo poblacional ex situ, muestras de museos, relación genética, simulación futura, valor de conservación de híbridos

摘要: *杂交对物种保护提出了重大挑战, 因为它威胁着物种的遗传完整性和适应性潜力。然而, 如果一个已灭绝类群的基因组存在于现存的杂交种中, 那么杂交也可以为物种恢复提供前所未有的机遇, 通过选择性育种重新获得灭绝物种的基因组。本研究探讨了如何利用含有已灭绝物种基因组的混合血统个体, 为加拉帕戈斯陆龟(*Chelonoidis *spp.) 建立圈养繁殖计划。我们的目标个体是弗洛里安娜岛灭绝物种* C. niger *与近期在其分布范围内伊莎贝拉岛沃尔夫火山发现的现存物种* C. becki *的杂交后代。我们将 35 只含有较高比例* C. niger *血统的乌龟的基因型数据与对该计划获得后代的前进式模拟相结合, 在满足总体遗传多样性和 C. niger 血统最大化, 且考虑资源限制、物种生物学特性和弗洛里安娜岛急需重引入乌龟来促进生态系统恢复的情况下, 分析了可能的圈养繁殖策略。在模拟实验中, 建立相对较小的乌龟繁殖群时的总体遗传多样性最高, 且根据已有资源对圈养乌龟进行选择性育种可以捕获大量的* C. niger *基因组。若将圈养繁殖的后代释放到野外而不是继续用于繁殖, 则遗传多样性会特别高。我们的研究结果为将携带已灭绝类群的代表性基因组的杂交种纳入物种恢复计划提供了基于遗传学的实践指导。我们的研究还表明, 可以在物种重引入项目中纳入携带了被认为已经丢失的遗传多样性的杂交种, 这也为目前杂交种在生物多样性保护中的价值的争论提供了信息。* **【翻译: 胡怡思; 审校: 聂永刚】**

关键词: 迁地种群管理, 杂种保护价值, *Chelonoidis niger*, 弗洛里安娜岛, 博物馆标本, 遗传相关性, 遗传血统, 前进式模拟

Introduction

Invasive exotic species are a major threat to biodiversity (Perrings et al. 2005; Meyerson & Mooney 2007; Johnson et al. 2018). Hybridization between native and nonnative species is an insidious form of invasion that can erode the genetic identity and integrity of native species and introduce maladaptive variation into local populations (Rhymer & Simberloff 1996; Todesco et al. 2016). In rare situations, however, hybridization can create opportunities for species recovery (e.g., Stelkens et al. 2014). An unusual case concerns the existence of genomic material from extinct taxa within living hybrids. Rescuing genome remnants from such hybrids could lead to partial species de-extinction and ultimately reintroduction of a formerly extinct species to the wild. We explored such a situation presented by the recent discovery of wild individuals with mixed ancestry between an extant and an extinct species of Galapagos tortoises.

Galapagos tortoises (*Chelonoidis* spp.) include 15 named species that together represent an iconic example of a rapid species radiation (Fritts 1984; Beheregaray et al. 2004). Most of the larger Galapagos islands host one endemic species of tortoises with the exception of the 2 largest islands of Santa Cruz and Isabela (Fig. 1), which host 2 and 5 species, respectively (Rhodin et al. 2017). Giant tortoises, as the native megaherbivore of the archipelago, play important ecological roles, including seed dispersal and habitat disturbance (Gibbs et al. 2010; Blake et al. 2012). Unfortunately, Galapagos tortoises have been decimated by human exploitation, habitat destruction, and introduced invasive species (Townsend 1925; Harper & Carrion 2011). The species of Galapagos tortoise endemic to Floreana Island, *Chelonoidis niger* (previously *Chelonoidis elephantopus* [Rhodin et al. 2017]), is a case in point. This taxon is considered to have gone extinct by the 1840s (IUCN 2016). However, our previous genetic work (Poulakakis et al. 2008; Russello et al. 2010; Garrick et al. 2012)

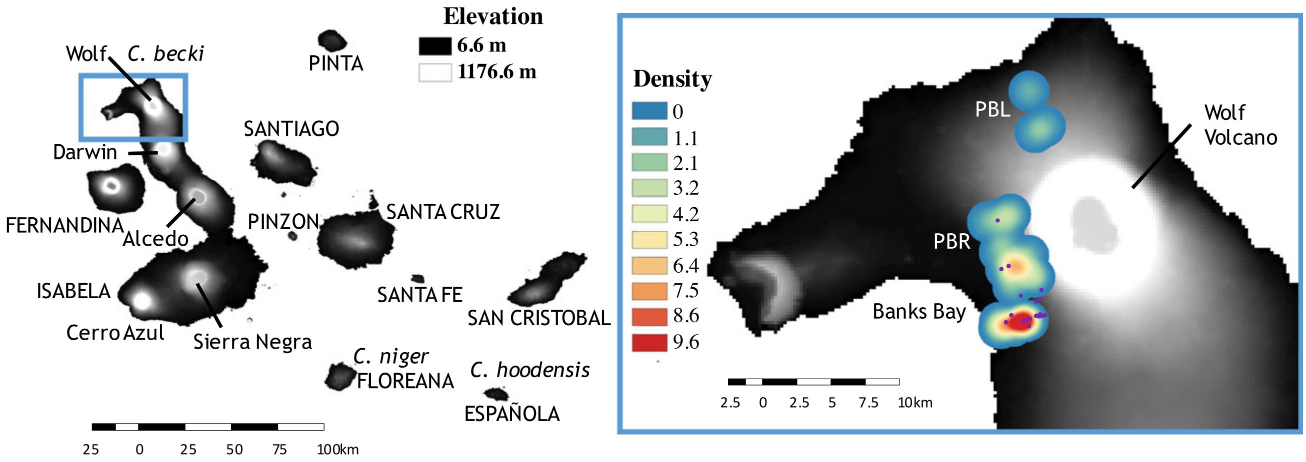

Figure 1. Galapagos archipelago (uppercase, main islands; lowercase, volcanos; scientific names, distribution of particular tortoise; rectangle, Wolf Volcano) and density (individuals per square kilometer) (right) of individuals with C. niger *ancestry identified prior to 2015 (Garrick et al. 2012) in 2 distinct areas on the western slopes of Wolf Volcano: Piedras Blancas (PBL) and Puerto Bravo (PBR) (purple dots, individuals sampled in 2015).*

identified genetically admixed tortoises (hybrids) with genomic representation from this extinct species (*C. niger* ancestry) now living on Wolf Volcano (northern Isabela Island), where they co-occur with tortoises of the endemic species, *Chelonoidis becki* (Fig. 1) (Poulakakis et al. 2008). The hybrids on Wolf Volcano share the same distinctive carapace morphology, known as saddle-backed, with the extinct species *C. niger*, whereas the endemic *C. becki* have a domed carapace morphology (Garrick et al. 2012). In general, the saddle-backed carapace morphology is typical of tortoises living in dry conditions (Fritts 1984). Notably, the hybrids are found on the lower slopes of Wolf Volcano (Fig. 1), which suggests preference for arid habitats (Supporting Information). These interspecies hybrids, a likely outcome of human-mediated translocations (Poulakakis et al. 2008), provide a novel opportunity for restoring the *C. niger* lineage in some form.

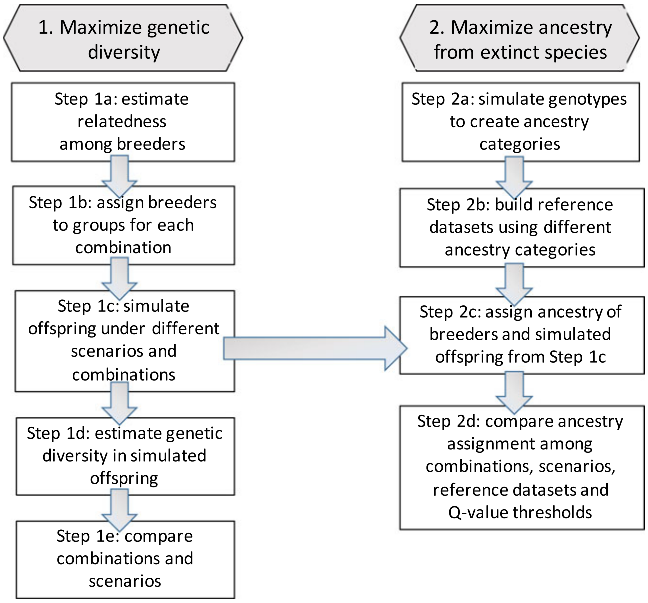

Figure 2. Methodological process to identify the best breeding strategy to maximize genetic diversity (steps 1a–e) and ancestry from the extinct species (steps 2a–d) in captive-bred tortoise offspring. Methodological details are in Supporting Information.

An expedition to the sector of Wolf Volcano where the hybrid tortoises occur was conducted in 2015 and revealed that 127 of the 144 individuals sampled with the saddle-backed morphology characteristic of *C. niger* had mixed ancestry with the extinct species *C. niger* (Miller et al. 2017). Eighteen of these individuals were brought to the Galapagos National Park Directorate (GNPD) breeding center on Santa Cruz Island and combined with 4 other individuals with high genetic assignment to *C. niger* already in captivity (Russello et al. 2010) to initiate a captive-breeding program. The program is novel in that the species to be reintroduced is nominally considered extinct (van Dijk et al. 2017). The program also poses major challenges. Some are conventional, such as the need to maximize the amount of genetic diversity in the resulting offspring (Witzenberger & Hochkirch 2011; Fienieg & Galbusera 2013; Attard et al. 2016). Others are unique, such as the objective to maximize the retention of *C. niger* ancestry present in the breeders. Moreover, these genetic considerations need to be balanced against the pressing need to introduce tortoises on Floreana Island to help restore the ecological services they provide as "ecosystem engineers" (Gibbs et al. 2010). This is especially true given the species' life history (age of first breeding of ∼20 years; Marquez et al. 1991) (and hence

Table 1. Number of saddle-backed hybrid tortoises screened to identify those with *C. niger* ancestry for the breeding program.

Locality	*Total number of individuals*	*Breeders*
GNPD	38	22 (13 F, 9 M)
Wolf Volcano	118	13 (4 F, 7 M, 2 J)
Total	156	35 (17 F, 16 M, 2J)

One hundred fifty-six tortoises were located at the breeding center (GNPD) or in the wild (Wolf Volcano, first column). A subset of these was selected as potential breeders (third column) in this study because they harbor high C. niger ancestry: 33 adults and 2 juveniles. Abbreviations: M, male; F, female; J, juvenile.

expected lag time in reestablishing an ecologically effective population) and limited resources available for captive rearing giant tortoises.

We examined the unusual challenges and opportunities presented by building captive-breeding programs for species recovery that rely on hybrid individuals. Our primary goal was to evaluate strategies to maintain the highest breadth of genetic diversity while maximizing the genetic representation of *C. niger* present in the breeders. We used genetic data from living hybrids and a forward-in-time, simulation-based framework to identify optimal groups of breeders (Fig. 2). Our study is the first assessment for any taxon of the practical steps required to capture the genome of a lost species and is intended as a case study for future captive-breeding programs in other taxa with a similar situation. Given the present rates of human-mediated species translocations and habitat perturbations, we expect the need for captive management of hybrid individuals and the utility of guidelines we developed will increase.

Methods

Samples and Genetic Markers

Saddle-backed tortoises previously identified as having high ancestry assignments to *C. niger* (Miller et al. 2017) are the 35 breeders considered in this study. Twenty-two of these breeders are in captivity at the GNPD breeding center. At least 13 hybrid individuals still reside on Wolf Volcano and could be integrated into the program (Table 1 & Supporting Information).

We used 21 microsatellite loci to estimate relatedness among breeders and genetic diversity in their simulated offspring (Supporting Information). We carried out genetic ancestry assignments based on 12 of these loci (previously used to identify the hybrid individuals [Miller et al. 2017]) because of inconsistent polymerase chain reaction amplification of the remaining 9 loci for the *C. niger* museum specimens. We also evaluated the impact of missing data on ancestry assignments based on a subset of 7 loci with <45% missing data for the *C. niger* museum samples.

Process Steps

To identify the best breeding strategy to maximize both genetic diversity and genetic ancestry from the extinct species, we compared different numbers and organizations of the breeders (hereafter combinations) under different breeding-program duration and actions (hereafter scenarios) (glossary in Supporting Information). We focused on a set of 5 combinations and 3 types of scenarios, which are the most realistic given the species biology and logistical constraints (Fig. 3a & Supporting Information). These constraints include the current availability of only 4 enclosures (corrals), while exploring the implications of adding a new one, with each corral hosting ≤7 individuals with ≤3 males to limit aggressive behavior (W.T., personal observation).

Combinations 1–5 were used to evaluate the impact of different numbers of individuals and groups in each combination based on either actual or actual and simulated genotypes (Fig. 3a). The strategy that maximized genetic diversity was evaluated by simulating the offspring from each combination under 3 types of scenarios (A–C), which differed in the number of the reproductive cycles (or years given that females oviposit annually) and breeder organization (Fig. 3b & Supporting Information). Scenarios A and B were run for 10 and 50 reproductive cycles, respectively. With the B scenarios, we investigated the effect on the retained diversity of reorganizing the breeder pool into different sets of males and females half way through the total breeding period: after 5 and 25 years in scenarios B10 and B50, respectively. Because the number of individuals in each combination pool was larger than needed to form the groups, some breeders that were not included in the first round of cycles may be included in the following round. The C scenarios were similar to B but differed in that they included the offspring contribution of F1s introduced on Floreana after 20 years when they reached sexual maturity (Fig. 3b). In C scenarios, the frequency of rotations was increased to every 5 years (scenario C5–50) or kept to only once after 25 years (C25–50). Reorganizing breeders every 5 years (scenario C5–50) allowed for a more diverse F1 offspring to be produced earlier on; thus, a more diverse F1 offspring starts to contribute after the initial ~20 years. For combination 5, where F1 were introduced as breeders in the captive program, we ran simulations only for the C scenario, changing group composition once after 25 years, because the F1 needed to reach sexual maturity before being added to the breeder pool (scenario C25–50). Combination 5 was the only one in which some (20 individuals) of the F1s were added to the actual breeding pool in captivity once they reached sexual maturity.

To identify the strategy that maximized *C. niger* ancestry in the offspring, we calculated and compared ancestry assignment (*Q* value) in the breeders and their

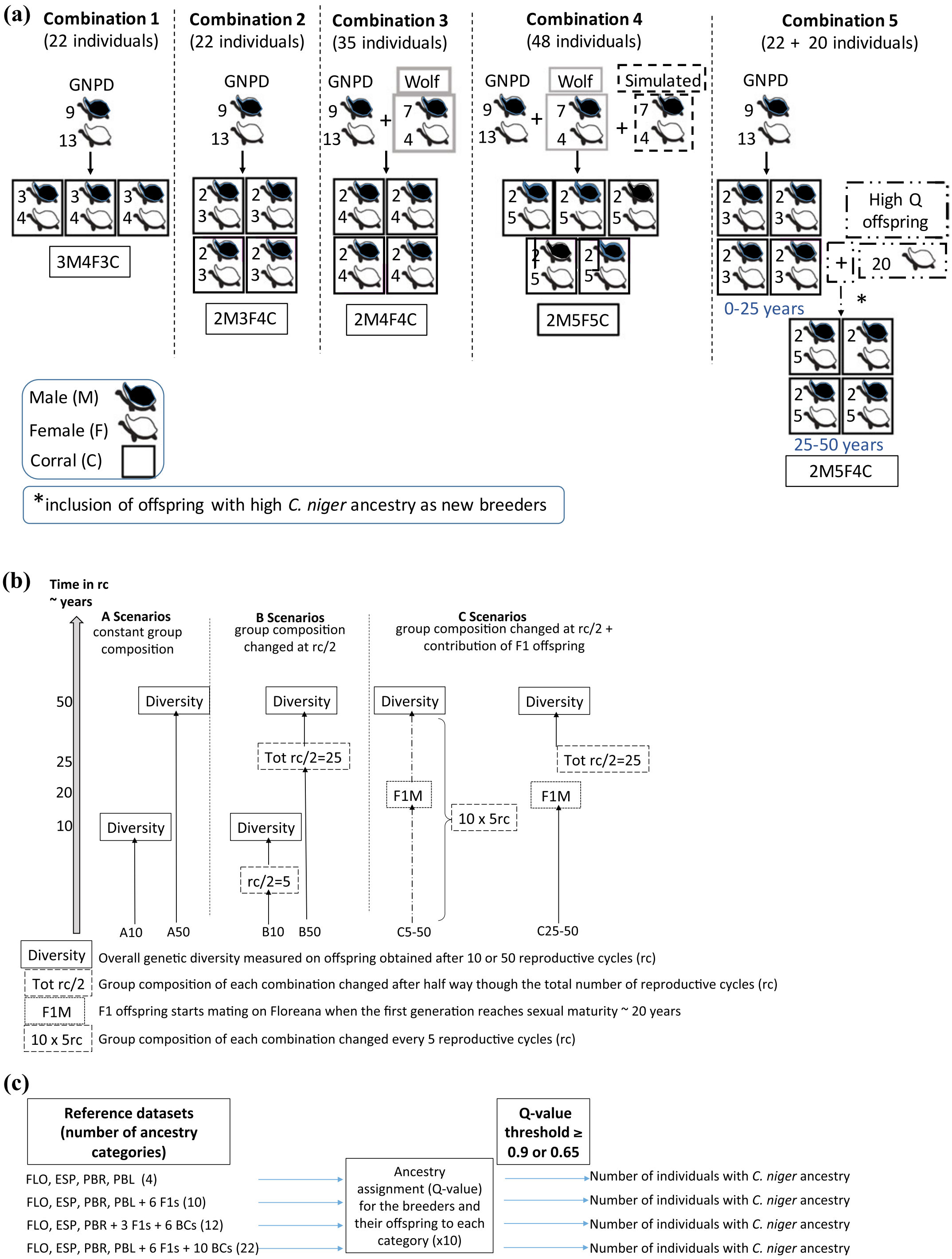

Figure 3. (a) Combinations of tortoise breeders in 3–5 corrals with 5–7 individuals per corral (parentheses, total number of individuals available to build each combination; numbers next to silhouettes, number of males and females; codes at bottom of each combination block are read, for example, as 3 male, 4 female, 3 corrals; GNPD, breeding center tortoises; solid rectangle, tortoises on Wolf Volcano; dashed rectangle, simulated tortoises based on known genotypes from the breeders; black rectangles, corrals; combinations 1–4, individuals at GNPD; combination 5, individuals from 22 breeders at the GNPD plus at least 6 of their offspring with high ancestry assignment to C. niger *[rectangles with dashed and dotted lines] to reach maximum capacity in each corral [Supporting Information]). (b) Relative levels of genetic diversity was measured in simulated tortoise offspring after multiple reproductive cycles (rc) for 3 scenarios (A–C). (c) Procedure followed for ancestry assignment (Q values) with 4 reference data sets and 2 Q-value thresholds (FLO,* C. niger*; ESP,* C. hoodensis*).*

offspring under combinations and scenarios as above. We tested the robustness of the results with different reference data sets, number of loci, and Q-value thresholds (Fig. 3c & Supporting Information). The reference data sets differed in the number of ancestry categories: groups of genotypes characterizing the ancestry of individuals from a given cross. Given that 4 taxa contribute to the genetic makeup of the hybrids found on Wolf Volcano (Russello et al. 2007; Miller et al. 2017), we created 22 ancestry categories: 4 pure and 18 hybrid, including first-generation hybrids (F1) and backcrosses (BC). The 4 pure categories included the 2 genetically distinct populations of the endemic species of Wolf Volcano (*C. becki*, Piedras Blancas [PBL] and Puerto Bravo [PBR] in Fig. 1), *C. niger* (FLO), and the species endemic to Española Island, *Chelonoidis hoodensis* (ESP). Using different combinations of the 22 ancestry categories, we created 4 reference data sets (Fig. 3c; breakdown of ancestry categories in Supporting Information). For these 4 reference data sets, we used 2 sets of loci (12 and 7 loci) to calculate Q values and 2 Q-value thresholds, relaxed ($Q \geq 0.65$, as in Miller et al. 2017) and a stringent ($Q \geq 0.9$), to identify simulated offspring individuals with high *C. niger* ancestry.

We used a multistep process to identify the breeding strategy that maximizes the retention of genetic diversity and ancestry from *C. niger* in the offspring (Fig. 2) (detailed methods in Supporting Information). Steps 1a–e were used to maximize genetic diversity. We first selected the relatedness index that performed best with our data set (step 1a) based on all 21 microsatellite loci (Supporting Information). Using these best-preforming indices, we measured relatedness among breeders to assign them to groups designed to minimize relatedness among breeders to reduce risks of genetic diversity loss and inbreeding expected under random mating in a finite population over multiple generations (step 1b). We then used simulations to create the F1 genotypes for 5 combinations and 3 types of scenarios (A–C; step 1c), estimated F1 genetic diversity (step 1d; Supporting Information), and compared these estimates across combinations and scenarios (step 1e). Steps 2a–d were used to maximize genetic ancestry from the extinct species. We simulated genotypes for the 22 ancestry categories (step 2a: Supporting Information). Using both empirical and simulated genotypes, we used 4 reference data sets (step 2b; Supporting Information) to define Q values for the simulated F1 offspring from step 1c (step 2c). We then compared Q values among the combinations and scenarios (step 2d).

We calculated differences in ancestry assignment based on the average and range of Q values from 10 simulations randomly selected among 1000 for the 2 Q-value thresholds and for the 4 different reference data sets (Supporting Information). Because the choice of reference data set, number of loci, and Q-value threshold can affect ancestry assignments and given that combinations started with different numbers of breeders, we explored the robustness of these assignments by comparing the proportion of breeders and offspring with high *C. niger* ancestry among these different treatments.

Results

In terms of genetic diversity, observed heterozygosity (H_o) estimates across combinations and scenarios showed that F1 offspring from combinations 1–4 retained >84% of the diversity in the breeders, whereas this estimate dropped to 51% for combination 5, where few F1 offspring were included as breeders (Fig. 4). Results based on the QG89 (Queller & Goodnight 1989) relatedness index, which performed best, indicated that the 35 breeders were not related and allowed us to identify the 2 best group compositions for the 5 combinations of breeders (Supporting Information). Comprehensive genetic diversity estimates (H_o and expected heterozygosity [H_e] and Shannon index of allelic diversity [SAR]) were broadly corroborative (Supporting Information).

For combinations 1–3 in the A scenarios (group composition remains constant), H_o decreased as the number of reproductive cycles increased (A50 vs. A10). This unexpected pattern is likely due to the random variation in reproductive output among breeders so that the effective population size of the breeders varies over time. In combination 4, more new breeders were added, and they did not carry new alleles because their genotypes were simulated from real ones (see above). Thus, because in combination 4 we further increased effective population size (i.e., more groups and larger groups) but only increased the number of copies of each allele and not the number of alleles, we likely decreased the effect of reproductive skewness. In B scenarios, where we rotated breeders among groups and replaced some of them half way through the breeding period, we also increased effective population size, which often lessened diversity loss (i.e., A50 vs. B50).

In general, genetic diversity increased as the number of groups within each combination increased, even when we accounted for the F1 contributions (C scenarios across combinations 1–4). Across combinations H_o decreased significantly when the F1 contribution was included (C vs. A–B scenarios). For instance, for combination 3 (the one retaining the highest H_o in the A and B scenarios) H_o decreased by up to 9% between scenarios C5–50 and B50 (Fig. 4 & Supporting Information). This result was even more drastic for combination 5, where F1s with high *C. niger* ancestry were added as breeders in captivity, because H_o decreased by ~40% (Fig. 4 & Supporting Information). The consistent drop in heterozygosity across all C scenarios was an expected result because related individuals (F1) contributed to the gene pool. Although most of these comparisons were

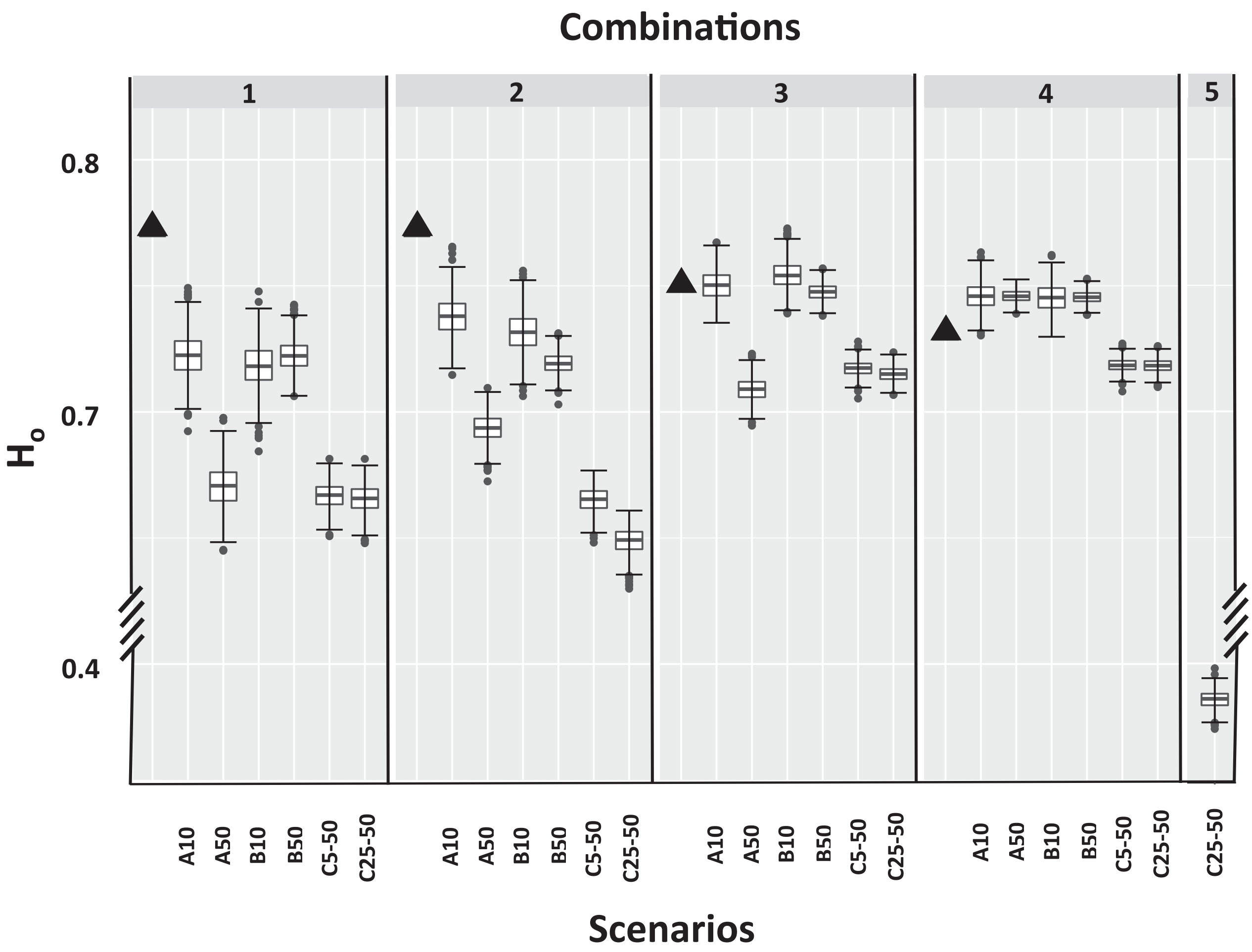

Figure 4. Comparison of observed heterozygosity (H_O) for the breeders (base of black triangles) and their offspring for different breeder combinations and scenarios (x-axis) (horizontal line across boxes, median; box first and third quartiles, 25th and 75th percentiles, respectively; vertical lines, degree of dispersion [spread] and skewness; dots, outliers; diagonal lines, truncation of y-axis to improve presentation). Diversity estimates for the offspring calculated from 1000 replicates of simulated offspring for combinations 1–4 simulated under scenarios running for 10 years without (A10) and with (B10) changing group composition after 5 years, running for 50 years without (A50) and with (B50) changing group composition after 25 years, or running for 50 years but also including the contribution of the F1 offspring and changing group composition every 5 years (C5–50) or once after 25 years (C25–50). Combination 5 was simulated only under scenario C25–50 because we changed only the group composition to incorporate the F1 individuals once they reached sexual maturity (Supporting Information).

highly significant statistically (Supporting Information), most differences were small (for instance, <1% for H_O); thus, their biological significance should be interpreted with caution.

Assignments with the stringent *Q*-value threshold ($Q \geq 0.9$) and the reference data set of 4 ancestry categories and 12 loci showed that combination 3, which retained the most genetic diversity, had the highest number of offspring and founders assigned to *C. niger*, ~12–14% more offspring and 10% more founders assigned to *C. niger* than combinations 2 and 5 (Fig. 5 & Supporting Information). Overall, we observed similar patterns regardless of the number of ancestry categories in the reference data sets, the number of loci, the *Q*-value threshold, and the scenario. If a combination had fewer founders assigned to *C. niger*, it also had fewer offspring assigned to *C. niger* (Fig. 5 & Supporting Information).

Discussion

Genetic Diversity and Relatedness

Although many reintroductions of captive-bred individuals into the wild have been successful, such as for the European bison (*Bison bonasus*) (Tokarska et al. 2011) and the red wolf (*Canis rufus*) (Soorae 2013),

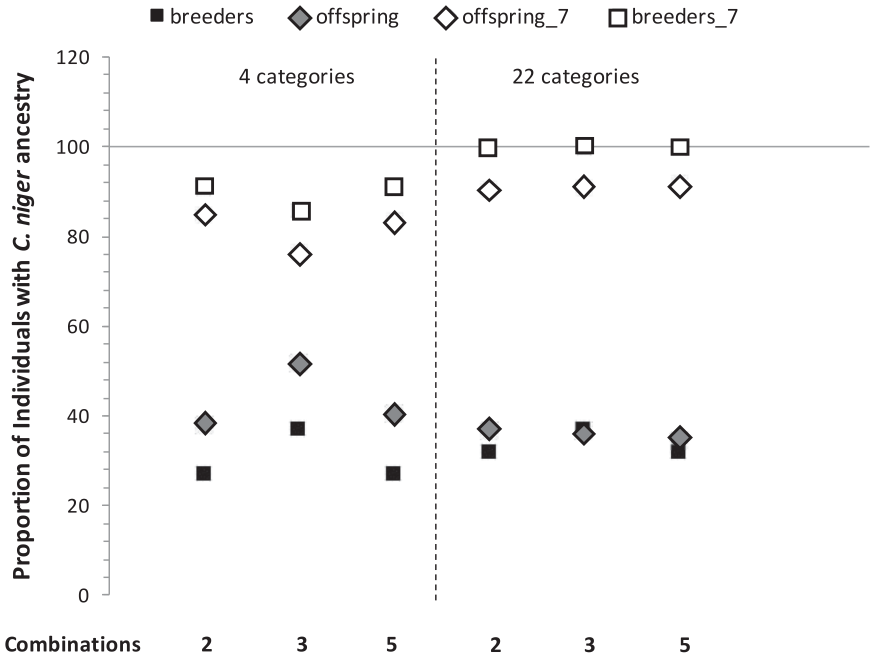

Figure 5. Proportions of individuals displaying C. niger *ancestry (*Q ≥ *0.9) among the breeders (squares) and their offspring (diamonds) with the reference data set of 4 and 22 ancestry categories (horizontal line, highest possible proportion of individuals assigned to* C. niger*; dashed vertical line, reference data sets on either side [4 or 22 ancestry categories]). The* Q *values were estimated for the 3 breeder combinations (2, 3, and 5) with 4 corrals with either 12 or 7 loci (filled or empty symbols, respectively).*

many have failed (Fischer & Lindenmayer 2000). Some of these failures may be due to lack of genetically informed management (e.g., Attard et al. 2016; Russello & Jensen 2018). Such management aims to ensure that genetic diversity is efficiently and evenly transmitted from parents to offspring, thereby minimizing the influence of genetic drift and inbreeding on the small populations typically found in captive-breeding programs (Williams & Hoffman 2009; Witzenberger & Hochkirch 2011; Willoughby et al. 2015). This can be achieved by maximizing the genetic diversity in the breeders, prioritizing mating between unrelated individuals to limit inbreeding, and minimizing time in captivity to reduce adaptation to captive conditions (Ballou & Gilpin 1995; Seddon et al. 2007; Robert 2009).

With regard to the impact of group combinations on the retention of genetic diversity in Floreana Island giant tortoises, using more groups with fewer individuals retained more genetic diversity in the offspring. This strategy may also have the added benefit of increasing the breeding population effective size by limiting reproductive skewness (see below) among breeders. In general, both combinations 3 and 4 perform better than combinations 1 and 2. Although the difference in H_O estimates between combinations 3 and 4 was small, combination 3 retained higher genetic diversity levels than combination 4, even though the breeder pool of combination 4 was larger than that of combination 3 (48 vs. 35) (Fig. 3a). This unexpected result was likely due to the fact that adding 13 individuals in combination 3 added new alleles in the breeder gene pool, whereas adding 13 individuals made up of simulated genotypes based on the genotypic makeup of the 35 real potential breeders (Fig. 3a; Supporting Information) in combination 4 did not. These results together with the logistical and cost implications of adding a fifth breeding group argue against expanding breeder numbers. Similarly, although adding breeders to the captive breeding population is expected to increase aggregate genetic diversity, our results showed that adding F1 individuals with high *C. niger* ancestry as breeders (combination 5) resulted in a large drop of H_O (Fig. 4), suggesting that the best strategy is to add unrelated individuals with high *C. niger* ancestry rather than the offspring of the current breeders because offspring only bring more copies of preexisting alleles. Thus, we identified a strategy to maximize the retention of genetic diversity with 4 corrals and the current resources. However, if more hybrids with high *C. niger* ancestry and harboring variants not yet present in the current pool of breeders are discovered on Wolf Volcano in coming years, it will be appropriate to revisit our conclusion.

Rotating individuals among breeding groups often led to higher genetic diversity retention in offspring (B scenarios, Fig. 4, & Supporting Information). This increase was even higher when changing groups more frequently (e.g., every 5 years instead of once after 25 years, as in C scenarios). Frequently changing group composition may also have the added value of breaking up group hierarchies, which can lead to skewed reproductive success among breeders. Reproductive skewness has important implications on population persistence in the wild (Lande & Barrowclough 1987) because it translates into an uneven breeder's contribution (e.g., Williams & Hoffman 2009; Asa et al. 2011), thus reducing effective population sizes. This is of particular concern in this case study because reproductive skewness occurred in the pilot phase of this work (Miller et al. 2018) and in a breeding program for another species of Galapagos tortoises, *C. hoodensis*

(Milinkovitch et al. 2007). We simulated reproductive skewness as an uneven contribution of each breeder to each reproductive cycle based on biological parameters such as hatchling success and number of eggs per clutch (Supporting Information). The drop in observed heterozygosity over time for some combinations under the A scenarios (breeders left in the same group over time) likely resulted from this type of reproductive skewness.

In general, rotating breeders regularly among groups and increasing the number of breeders reduced the negative effects of reproductive skewness. However, if tortoises form stable pairings through mate choice, rotating mates may reduce the overall reproductive output by breaking up pairs. As limited data are available on the mating behavior of Galapagos tortoises, the reproductive success and mating preferences of the breeders need to be monitored and management dynamically adjusted to minimize reproductive skewness.

Preserving the Genetic Ancestry of the Extinct *Chelonoidis niger*

Overall, our results indicated that a captive-breeding program starting with founders with high genetic representation of *C. niger* will preserve high *C. niger* ancestry in the offspring population (Fig. 5). This goal could be best achieved by adding to the program individuals from the wild rather than F1 offspring of the breeders (Fig. 5). The new unrelated individuals would add novel genetic variation not present in the original breeder pool. We are aware that the diversity captured in the breeder pool is likely not representative of the entire species' original genetic complement. However, previous work showed that the hybrids present on Wolf Volcano harbor a wealth of genetic diversity from the extinct species (Garrick et al. 2012), which bodes well for the potential of the introduced population to adapt in the wild.

Additional challenges to this program are the complexity of the genomic makeup of the hybrids and the low number of genetic markers available for assessment. The presence in the hybrids of genomic material assigned to more than 2 species (*C. becki*, *C. niger*, and *C. hoodensis* [Garrick et al. 2012; Miller et al. 2017]) complicates the determination of *C. niger* ancestry. This challenge is compounded by technical limitations because the reference data set of *C. niger*, based only on museum samples, is not as complete as that of the extant species. Having multiple taxa represented in the hybrids and using a reference data set that has missing data make ancestry assignment (*Q* value) unstable and thus difficult to interpret in absolute terms.

For these reasons, we explored the robustness of ancestry assignments based on various reference data sets with different groups of ancestry categories and different numbers of loci. Although the overall trends and recommendations for the breeding program were similar when we used 7 and 12 microsatellite loci, the *Q* values of the breeders and their offspring for the extinct species were ~50% higher when we used the 7 rather than 12 loci in the data set (Supporting Information & Fig. 5). This suggests that, although missing data and loci number affected our ability to estimate exact levels of *C. niger* ancestry, we were still able to consistently identify *C. niger* ancestry in the breeders and their offspring with the current markers. Nevertheless, to improve our estimates of *C. niger* ancestry and facilitate future monitoring of the program, we plan to switch from microsatellite to single nucleotide polymorphism markers.

Although more-refined genetic assays may alter conclusions, given existing data we recommend using 4 corrals with 6 individuals, selected among 35 breeders (22 in captivity and 13 still in the wild). We also recommend rotating breeders among groups every 5 years, assessing the genetic composition of the offspring regularly, and releasing all offspring on Floreana Island. We stress the need for adaptive program management with a long-term genetic management perspective to identify potential weaknesses or improvements based on newly acquired data (e.g., detection of reproductive skewness and discovery of new hybrids).

Conservation Value of Hybrids with Ancestry from an Extinct Species

Lessons learned from this case study are relevant to 2 general areas of conservation biology: the status and the utility of hybrids and their use for de-extinction programs. The role of hybrids in conservation management is a topic of active debate (e.g., Piett et al. 2015). Hybrids have previously been relegated to low conservation status due to their genetically mixed state that makes their legal status and utility for species recovery programs problematic. More recently, the focus of this ongoing debate has shifted to a case-by-case type of approach, acknowledging the importance of including ecological and ethical considerations in the decision-making process (Jackiw et al. 2015; Wayne & Shaffer 2016). Regardless of their natural or human-assisted origin, hybrid individuals may be either considered harmful to the so-called genetically pure species to which they are affiliated (e.g., FitzSimmons et al. 2002) or as a source of genetic diversity to bolster a genetically affiliated taxon's survival, as in the cases of cheetahs (*Acinonyx jubatus*) (Marker-Kraus & Grisham 1993) and the Norfolk Island Boobook Owl (*Ninox novaeseelandiae undulata*) (Garnett et al. 2011).

In general, our results showed that hybrids can be used in a breeding program to reach 2 apparently contrasting genetic goals: maximize genetic diversity and retain a large genetic complement from a single given taxon. This work, the first of its type, also brings to the forefront the challenges and unique opportunities of building such breeding programs based on hybrids with an extinct species. Given increasing species extinctions rates (Ceballos et al. 2015) and the ease of detecting individuals with mixed ancestry with genomic data, even from archival samples (Shapiro & Hofreiter 2014; Hofman et al. 2015), we anticipate such hybrids will be identified in other species as well. Our work also contributes to the ongoing discussion of the role of hybrid individuals in conservation, stressing their importance as genomic archives of potentially unique and valuable diversity. Such individuals may, in fact, play an influential role in enhancing species survival and, in some cases, facilitating de-extinction. Although the goals of the current program do not include *C. niger* de-extinction, given the complexities described above, our work highlights how a genetically informed captive breeding could potentially lead to species de-extinction in other systems if warranted.

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Supporting Information

Details on the methodological process (Appendix S1), glossary explaining the components of the breeding program (Appendix S2), conditions and primers used to amplify the microsatellite markers (Appendix S3), raw genotypes for 91 individuals and the 9 additional microsatellite loci (Appendix S4), observed genetic diversity for the 21 microsatellite makers (Appendices S5 and S6, respectively), relatedness measures used in SWINGER (Sandoval-Castillo et al. 2017) analyses (Appendix S7), detailed results (Appendix S8), performance of relatedness assignment with 5 different relatedness indices (Appendix S9), distribution of pairwise relatedness for simulated and observed dyads (Appendix S10), compositions of breeding groups (Appendix S11), diversity estimates and associated t test p values (Appendix S12), an example of DAPC result (Appendix S13), numbers of offspring individuals with high *C. niger* ancestry for different reference data sets in table format (Appendix S14) and plotted for different combinations (Appendix S15), results of the Mann-Whitney Wilcoxon statistical test on different ancestry assignment analyses (Appendix S16), and proportions of offspring with high *C. niger* ancestry for different combinations (Appendix S17) are available online. The authors are solely responsible for the content and functionality of these materials. Queries should be directed to the corresponding author.

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