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Genetic analysis of a successful repatriation programme: giant Galápagos tortoises

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As natural populations of endangered species dwindle to precarious levels, remaining members are sometimes brought into captivity, allowed to breed and their offspring returned to the natural habitat. One goal of such repatriation programmes is to retain as much of the genetic variation of the species as possible. A taxon of giant Galápagos tortoises on the island of Española has been the subject of a captive breeding–repatriation programme for 33 years. Core breeders, consisting of 12 females and three males, have produced more than 1200 offspring that have been released on Española where *in situ* reproduction has recently been observed. Using microsatellite DNA markers, we have determined the maternity and paternity of 132 repatriated offspring. Contributions of the breeders are highly skewed. This has led to a further loss of genetic variation that is detrimental to the long-term survival of the population. Modifications to the breeding programme could alleviate this problem.

Keywords: *Geochelone*; giant Galápagos tortoise; breeding programme; Española; conservation genetics; microsatellites

1. INTRODUCTION

When natural populations of endangered species shrink to the point of no longer being self-sustaining, as a last resort to avoid outright extinction, remaining individuals may be brought into captivity. If the species is able to reproduce in captive settings and if the factor(s) causing the population decline in nature can be ameliorated, subsequent release of offspring can potentially restore a self-sustaining population in the original natural habitat. Such ambitious programmes have been undertaken for a number of plants and animals (Griffith *et al.* 1989; Falk & Olwell 1992). In an attempt to maximize the genetic variation of the repatriated population, consideration is sometimes given to the mating structure of the breeders (Haig *et al.* 1990; Geyer *et al.* 1993; Jones *et al.* 2002). Rarely has it been documented what proportion of the genetic diversity of the breeders is actually represented in the repatriated offspring in nature. Because captive breeding and population restoration underpin many conservation efforts around the world, detailed assessments are needed of how genetic diversity changes through each of the phases of near extinction, captive breeding and population restoration (Jones *et al.* 2002).

Española Island in the Galápagos Archipelago is the site of one of the most successful, but least heralded, species reintroduction efforts ever attempted (Cayot *et al.* 1994; Fritts *et al.* 2000). Once numbering at least 3000 (Pritchard 1996), tortoises on Española (*Geochelone hoodensis*, sometimes considered a subspecies of *G. nigra*) had been reduced, by 1965, to just 14 individuals by hunters from sealing, whaling and pirate ships (Townsend 1925). Española is one of the flattest and most accessible islands in the Galápagos Archipelago and its tortoise population was among the first to be depleted. Whalers also introduced goats to the island, which over the course of 200 years dramatically increased in numbers and converted a densely vegetated island to one of open thorn scrub.

With no apparent tortoise reproduction taking place by the mid-1960s, the remaining 14 tortoises (two males and 12 females) were transferred to the Breeding Centre of the Charles Darwin Research Station and Galápagos National Park on Isla Santa Cruz (MacFarland *et al.* 1974). During the 1970s, goats were eliminated from Española as the result of an intense campaign by the Galápagos National Park Service, setting the stage for ecological restoration of the island. The first tortoises, whose parents originated from Española, hatched in 1971 and were subsequently repatriated in 1975 (Townsend 1925; Pritchard 1996). In 1977, the number of males was augmented by the arrival of a third adult male from the San Diego Zoo, increasing

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the breeding group to 15 tortoises. This core population continues to reproduce in captivity at the Breeding Centre in Santa Cruz and by August 2002 had generated 1200 repatriated offspring. Much of the vegetation has recuperated rapidly on Española since the elimination of feral goats, although some key species, such as the island's endemic *Opuntia* cactus, have not recovered well.

A notable landmark in the Española tortoise repatriation programme was the discovery that repatriated tortoises are now reproducing *in situ*. In 1988 tortoise nests were observed on Española, and offspring hatched in the wild were documented in 1994. Reproduction by the released individuals is a good indication that this repatriation programme may be on its way to achieving a healthy self-sustaining population in the original rehabilitated habitat. However, because little effort was made to match pairings of male and female breeders, little is known about how much of the genetic diversity of the 15 breeders is represented in the repatriated population. We used microsatellite DNA markers to determine the maternity and paternity of 134 repatriated offspring and to estimate the impact of the current breeding-programme settings on the genetic effective population size (N_e), probably a major determinant of the long-term survival of the repatriated population.

2. MATERIAL AND METHODS

In 1994, we collected blood samples from 134 out of an estimated 347 surviving released tortoises on Española (556 had been repatriated at that time). Thus, our sample represents 39% of the population surviving in 1994. Each sample was collected from the brachial vein of one of the front legs of the tortoise, preserved in a lysis buffer containing 0.1 M of Tris–HCl, 0.1 M of ethylenediaminetetraacetic acid (EDTA), 0.2 M of NaCl and 1% sodium dodecyl sulphate (SDS) at a pH of 8.0, and subsequently stored at 4 °C. About 200 µl of each blood sample was digested at 37 °C overnight in a buffer (100 mM of Tris–HCl, 100 mM of NaCl, 5 mM of EDTA, 0.5% SDS) containing 200 µg of proteinase K. Genomic DNA was isolated following standard phenol–chloroform extraction procedures (Hillis *et al.* 1996). DNA was resuspended in Tris-EDTA (TE) buffer (10 mM of Tris–HCl and 1 mM of EDTA at a pH of 7.2) and stored at −20 °C.

Isolation of Galápagos tortoise microsatellites was performed as follows: genomic DNA was partly digested with *Sau*3AI and a fraction of the resulting DNA fragments ranging from 500 bp to 2000 bp was isolated after electrophoresis in an agarose gel. The fragments corresponding to the selected fraction were purified, ligated to a Zero Background vector and transformed into DH5α competent cells (Invitrogen). Clones were hybridized with oligonucleotide probes specific to di-, tri- and tetranucleotide repeats. Recombinant DNA molecules were isolated and sequences of inserted genomic DNA fragments were obtained by cycle sequencing. Sequenced products were purified and separated by electrophoresis using an ABI Prism 377 DNA sequencer (Applied Biosystems). The full sequence of each positive clone was fed into a β-version of an in-house program (D. Van Belle and M. C. Milinkovitch, unpublished data) that both identifies any string of $([2]...[N])_j$ nucleotides (where $j > 3$) through the use of a finite state automaton, and designs optimal (i.e. minimizing dimmer and hairpin interactions and maximizing specificity) primers flanking the repeated sequence. We screened the 15 breeders for variation at 69 microsatellite loci using fluorescent nucleotides (Applied Biosystems) and selected the 15 loci (table 1) most informative for maternity and paternity analyses.

Genotyping was performed using PCR carried out in a total volume of 25 µl containing 10–100 µg of genomic DNA, 1 × PCR buffer, 2 mM of $MgCl_2$, 0.25 mM of each dNTP, 15 pm of each primer (one with fluorescence labelling, the other with a GTTTCTT tail in 5′ to force +A alleles) and 0.7 units of FastTaq DNA polymerase (Roche). Thermal profiles consisted of an initial denaturation step at 95 °C for 4 min, followed by 35 cycles of 30 s at 95 °C, 30 s at the annealing temperature (table 1) and 30 s at 72 °C, with a final extension step of 60 min at 72 °C (to force the formation of +A alleles). PCR products were separated by electrophoresis using an ABI 3100 capillary sequencer.

Deterministic and probabilistic parentage analyses were performed with the program PAPA v. 1.1 (Duchesne *et al.* 2002). Effective population sizes of females and males (N_{ef} and N_{em}) were computed taking into account the observed variance in reproductive success with the following equations (Lande & Barrowclough 1987):

$$N_{ef} = \frac{N_f\overline{k_f} - 1}{\overline{k_f} - 1 + V_{kf}/\overline{k_f}}; \quad N_{em} = \frac{N_m\overline{k_m} - 1}{\overline{k_m} - 1 + V_{km}/\overline{k_m}},$$

where N_f and N_m are census numbers of females and males, respectively, $\overline{k_f}$ and $\overline{k_m}$ are the means of offspring number per female and male breeder, respectively, and V_{kf} and V_{km} are the variances in offspring number per female and male breeder, respectively. Second, the total (male plus female) effective population size (N_e) was computed taking into account the biased sex ratio as (Wright 1931):

$$N_e = \frac{4N_mN_f}{N_m + N_f}.$$

Expected heterozygosity (H_e) was calculated for population (rather than sample) allele frequencies (eqn 8.1 in Nei (1987)). Average H_e values of breeders and offspring were compared locus by locus and evaluated with a *t*-test for paired observations (Nei 1987). Pairwise genetic identities among breeders were calculated as the percentage of identical alleles across all loci (Nei 1987).

3. RESULTS AND DISCUSSION

We extracted DNA and screened the Española breeders for variation at 69 microsatellite loci to find 15 loci informative for maternity and paternity analysis. This population is exceptionally low in genetic variation, having: (i) a single maternal lineage (as assessed by mitochondrial DNA; Caccone *et al.* 2002); (ii) an average of 2.7 alleles per microsatellite locus; and (iii) a mean expected heterozygosity (H_e) per locus of 0.537. These numbers are much lower than those exhibited by other Galápagos tortoise populations (Ciofi *et al.* 2002). Note that our conclusion of low variation in the Española parental population is conservative as we selected the 15 most variable loci out of the 69 tested. Out of the 134 offspring analysed, 118 could be deterministically assigned to parents and an additional 14 were probabilistically assigned (with maximum likelihood), so that only two remained unassigned. The per-locus average H_e in the surviving F_1 population, as estimated from our sample of 134

Table 1. The microsatellite loci used in this study. The letters in the locus name identify the type of repeat. (Tm, melting (annealing) temperature; *N*, number of alleles in the parental population; range, range of allele sizes observed in the parental population; dye, type of fluorescent dye (Applied Biosystems) used for the labelled primer (indicated with an asterisk). A GTTTCTT tail (underlined) is added in 5′ of the unlabelled primer to force +A alleles and, hence, improve binning of alleles.)

locus	forward primer sequence (5′–3′)	reverse primer sequence (5′–3′)	Tm	*N*	range (bp)	dye
AC039	GTTTCTTCCATTGCCACCCCTATTCCTTCC	GAGAAGTGTGTGAGTGGGGGCA*	62	3	94–98	6-Fam
AC063	GTTTCTTGGGAGGGGGCTGAATCTTGAT	CTGGAAGCAGGAGACAAAAGGGAG*	62	2	78–98	Ned
AC149	GTTTCTTATCAGGGCTCAGTTGTGCTTGAC	TACTGTAAGCTCTTTGGGACAGGG*	62	3	105–113	Hex
AC111	GTTTCTTTCTCTTTGTCTCTTGCCCTCCCA	GGAGCTGCAGAGCACTCTC*	62	3	87–134	Ned
AC263	GTTTCTTGCTGAGGCTAGCTAATTTTTATGT	GGGAAAGTACTATTTCCAGAGCTGG*	62	4	82–110	Hex
AC045	GTTTCTTTATCTCCTTCCACACGGAGATGGG	CCCCAAAGTAAAGTTAGCTCTCTCA*	62	2	95–107	Hex
AC190	GTTTCTTCACTGTTTGTTATTTGCTTTCTTA	AGCTGCTTCCATTAAATAAAAAGA*	59	2	104–106	Hex
AC127	GTTTCTTAGTGTCATCTGCATATGCTTT	GTAACTATAAACATCAACTGGCAGA*	57	4	95–143	6-Fam
AC075	GTTTCTTGTTACCATAGCATTCCTGATTATAG	GAAGCCATTTACCACAAACTTATT*	57	3	93–97	Hex
AC247	GTTTCTTATTAACTGATTTGAGCAGTCATCCA	TGCTGTGAATAGTAACTGAGC*	57	3	84–113	6-Fam
AC100	GTTTCTTTCTTAATAAATTCCATGAGTTGAGCT	AGGGTGATTTCATAAACAAACAGAA*	57	2	103–118	6-Fam
AC251	GTTTCTTCTTATCTGCCTGGCTGCA	TGAAGTGCTACCACTAAGC*	57	3	96–113	6-Fam
GGA45	GTTTCTTACATCAACATTCTAACTGAGCAATA	GAATAAAAGCACCAGCTAAATAA*	59	2	75–85	Ned
AGG68	GTTTCTTAGGATGAGGACGAGGATGAT	AATTAATGTAAGAGGATGCTGAAC*	59	2	73–82	6-Fam
ATGT70	GTTTCTTATGTGTGTAGGTATAGTGTGTA	ACTATAAGGAAACAAGGC*	52	2	81–85	Hex

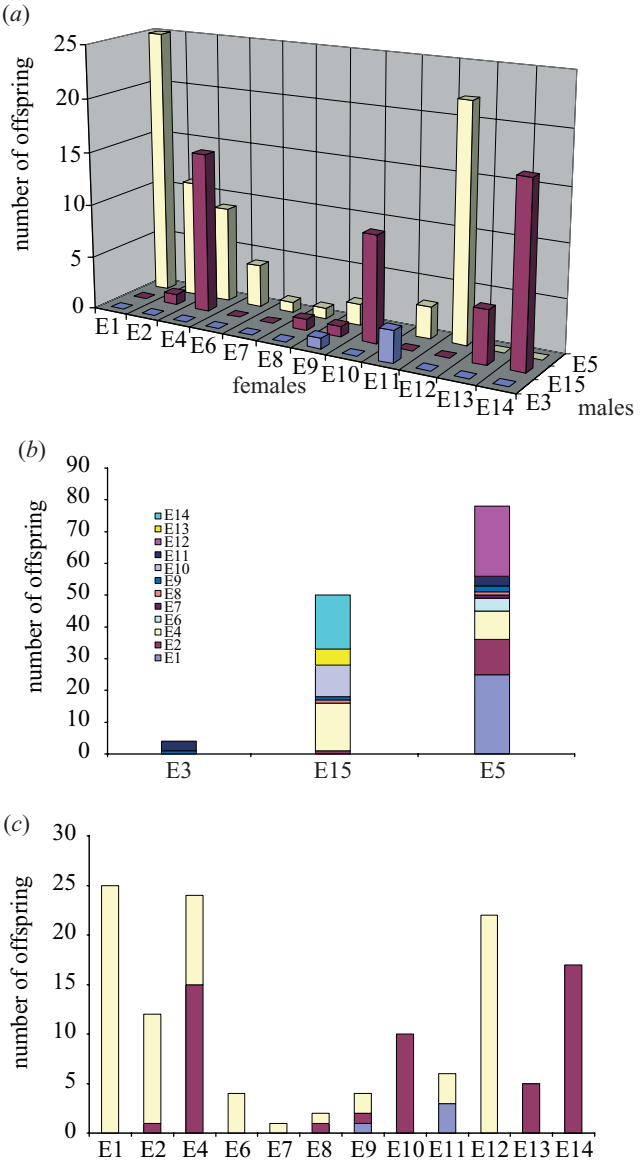

Figure 1. (*a*) Distribution of offspring assigned to breeding couples (yellow bars, male E5; red bars, male E15; blue bars, male E3); (*b*) relative success of each male (see figure for female identities); and (*c*) relative success of each female (yellow bars, male E5; red bars, male E15; blue bars, male E3).

individuals, is 0.519. This decrease in H_e from the breeders to the offspring population shows a tendency towards significance ($p < 0.077$), with an average decrease per locus of 0.018 ± 0.012 (s.e.).

Figure 1*a* shows the distribution of surviving offspring with regard to each possible set of parents, while figure 1*b*,*c* shows the relative success of each male and female individual, respectively. Clearly, the contribution of the breeders is highly skewed (the null hypothesis of randomly distributed differences in reproductive success is rejected; $p < 0.01$). Among males, E3 has contributed very little to the repatriated population. Five female breeders have five or fewer offspring attributed to them, while three have more than 20. Particular pairs are also unevenly distributed. For example, females E1 and E12 have contributed many offspring with male E5, but none with the other two males. Females E10, E13 and E14 have contributed well

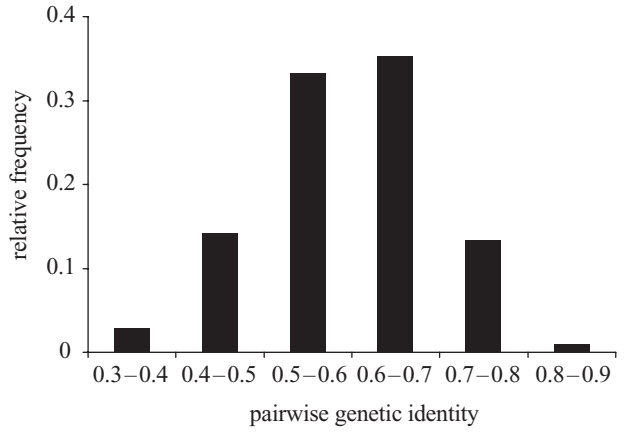

Figure 2. Relative frequencies of pairwise genetic identities among breeders.

with male E15, but not with the other two males. E15 is the male returned from the San Diego Zoo; clearly his presence in the breeding programme has been important.

The unequal sex ratio of the breeding population along with the unequal contributions lead to a very reduced genetic effective population size (N_e). Using established procedures (see § 2) we estimate that the N_es for females and males are 7.6 and 1.8, respectively, leading to an overall N_e of 5.7. Because the long-term N_e is the harmonic mean across generations (Wright 1931), this very low N_e in the breeding population will have a long-term effect on the N_e of the repatriated population. For example, even if the F_1 repatriated population had an N_e of 1000, the N_e over the two generations would be 11.3; if N_e remains at 1000 for 10 generations, long-term N_e reaches only about 59. Rectifying the low N_e in the parents would have a long-term effect on the repatriated population. The exact mating structure that would best achieve this goal depends on the pairwise genetic relatedness among the founders. Figure 2 shows the relative frequency of pairwise genetic identities among the breeders (mean of 0.58) based on our microsatellite data. The unimodal distribution does not indicate that there are particularly closely related individuals (e.g. parent–offspring; full-sibs) among the breeders, although this possibility cannot be ruled out with certainty, given the high background genetic identity in the population. The mean identities between each of the three males (E3, E5 and E15) and all females are 0.572, 0.611 and 0.619, respectively. Clearly, a finer assessment of the genetic relatedness among breeders would require much additional data. Nevertheless, an easy way to increase variability in the repatriated population is to equalize reproductive success among breeders. Indeed, although this breeding scheme might not fully maximize diversity (because it assumes that breeders are 'unrelated'), it will certainly yield a higher genetic diversity than that generated under the strongly skewed distribution observed here.

Unequal contributions among parent individuals could be the result of four factors or, most likely, a combination of them. First, not all pairs had equal opportunity to mate. In the breeding programme, males and females were moved infrequently between two pens, so not all males had equal access to all females. Second, there may be non-random mating occurring; given the limited movement of tortoises and the potential for social dominance in pens with more than one male, it is not clear how to disentangle this from the first point. Third, fertility may vary greatly. For example, male E3's low contribution may reflect low fertility; likewise female E7 may be less fecund than the other females. On the other hand, females E1, E10, E12 and E14 are clearly highly fecund, but are producing repatriated survivors with only one male. Finally, the unevenness of genetic representation may reflect differential survivorship. Unfortunately, the importance of this factor is difficult to assess because no records were kept of the parents of the offspring at the time of release and our sample in 1994 included individuals hatched up to 23 years previously. In any case, increasing the reproductive success of individuals currently contributing little to the repatriated gene pool should probably be encouraged because: (i) even if some of their genes impart low fitness, genetic variation at other unlinked loci might be beneficial for individuals in following generations; and (ii) high reproductive success in captivity is not necessarily correlated with fitness in the wild.

It remains controversial whether genetic considerations are of primary importance in efforts to preserve dwindling populations (Lande 1988; Soulé & Mills 1988; Caughley 1994; Frankham 1995). One could argue that this is a case in point: despite the uneven genetic contribution by the breeders leading to a very low N_e, this programme can be deemed a 'success'. The need for greater genetic variation in populations is often cited as a *sine qua non* for future evolutionary adaptation. However, such processes occur on a time-scale of thousands to millions of years, and conservation biologists' concerns are much more immediate. On the time-scale of concern, the low N_e raises the spectre of severe inbreeding depression (Madsen *et al.* 1999). In addition, there is ample evidence that more genetically variable populations can better withstand disease epidemics than can less variable populations (Lively *et al.* 1990; Coltman *et al.* 1999; Meagher 1999). Tortoises are known to be subject to infectious diseases that have reduced species to very low levels (Jacobson *et al.* 1991). Thus, while successful reproduction for one generation is a first indication of success, the probability of longer-term survival will almost certainly be increased by efforts to restore as much genetic variation as possible in the founders of the newly repatriated population. The results presented here can be used as a guide to modify the Española tortoise breeding programme to achieve this goal.

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