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RESEARCH ARTICLE

Genetic rescue of an inbred captive population of the critically endangered Puerto Rican crested toad (*Peltophryne lemur*) by mixing lineages

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Abstract The Puerto Rican crested toad (*Peltophryne lemur*) is currently composed of a single wild population on the south coast of Puerto Rico and two captive populations founded by animals from the northern and southern coasts. The main factors contributing to its decline are habitat loss, inundation of breeding ponds during storms, and impacts of invasive species. Recovery efforts have been extensive, involving captive breeding and reintroductions, habitat restoration, construction of breeding ponds, and public education. To guide future conservation efforts, genetic variation and differentiation were assessed for the two captive colonies and the remaining wild population using the mitochondrial control region and six novel microsatellite loci. Only two moderately divergent mitochondrial haplotypes were found, with one fixed in each of the southern and northern lineages. Moderate genetic variation exists for microsatellite loci in all three groups. The captive southern population has not diverged substantially from the wild population at microsatellite loci ($F_{ST} = 0.03$), whereas there is little allelic overlap between the northern and southern lineages at five of six loci ($F_{ST} > 0.3$). Despite this differentiation, they are no more divergent than many populations of other amphibian species. As the northern breeding colony may not remain viable due to its small size and inbred nature, it is recommended that a third breeding colony be established in which northern and southern individuals are combined. This will preserve any northern adaptive traits that may exist, and provide animals for release in the event that the pure northern lineage becomes extirpated.

Keywords *Peltophryne lemur* · Microsatellite loci · Mitochondrial DNA · Conservation genetics · Captive breeding · Reintroduction

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Introduction

The evaluation of current taxonomy and identification of genetic lineages within threatened species are vital to developing efficient and optimal recovery programs. For example, the use of morphological traits to designate taxonomic entities often does not reflect patterns of genetic variation (Burbrink et al. 2000; Culver et al. 2000; Nittinger et al. 2007), which can waste limited resources on groups that are not distinct and may have little value to the conservation of the species as a whole (Avise and Nelson 1989; Zink et al. 2000). A lack of distinct lineages can also facilitate recovery, as it may increase the number of source populations for efforts such as captive breeding or translocation between isolated populations (Eizirik et al. 2001; Godoy et al. 2004). In contrast, early detection of multiple lineages may enable intervention before an evolutionarily significant lineage becomes irreversibly imperiled (Nielson et al. 2001; Shaffer et al. 2004a; Vredenburg et al. 2007), as occurred for the tuatara (Daugherty et al. 1990). Knowledge of the genetic structure of a species can identify the most closely related population or subspecies for use in reintroductions following extirpation (Drew et al. 2003; Kraaijeveld-Smit et al. 2005), while individuals from divergent populations may be incorporated to maximise

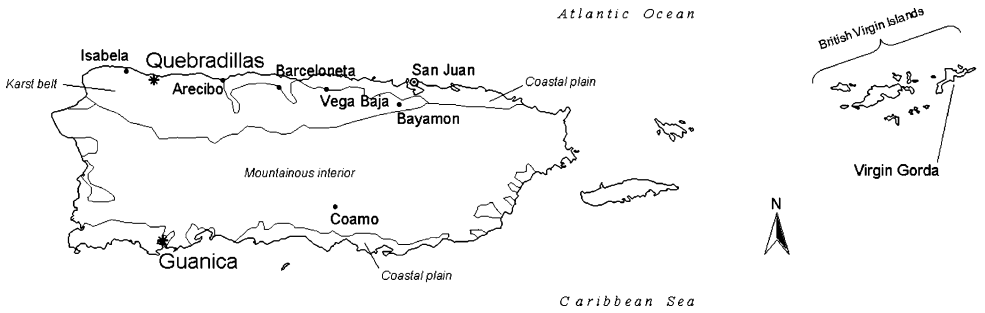

Fig. 1 Map of Puerto Rico and the British Virgin Islands showing historical locations of *Peltophryne lemur* (●) and sites represented by captive zoo programs (*). San Juan (⊙) is the capital of Puerto Rico and is shown for reference only. The three main physiographic regions of Puerto Rico are indicated: the karst belt in the northeast, the mountainous interior, and the discontinuous coastal plain (after Monroe 1976)

genetic variation (Madsen et al. 1999; Land et al. 2004). Finally, assessment of the genetic composition of existing captive programs can determine whether animals are suitable for further breeding (Brock and White 1992; Ruokonen et al. 2000) or reintroduction (Garcia-Moreno et al. 1996; Wyner et al. 1999).

The Puerto Rican crested toad (*Peltophryne lemur*) is a highly endangered bufonid for which two separate lineages are currently maintained in captivity. Captive breeding was initiated prior to the frequent use of molecular markers to identify genetic structure within species. In this study, mitochondrial DNA sequences and six novel microsatellite loci are used to investigate the genetic variation and differentiation of the two *P. lemur* captive breeding colonies, as well as the sole remaining wild population. This information will be used to determine the optimal future management actions to ensure the persistence of *P. lemur* in the wild.

Biology and conservation history of *Peltophryne lemur*

The only toad endemic to Puerto Rico, *P. lemur* has been collected from at least eight sites across Puerto Rico and one on Virgin Gorda of the British Virgin Islands (Fig. 1). It generally occupies low-elevation (<200 m), arid or semi-arid coastal regions and is often associated with limestone karst formations that provide fissures into which it retreats during the day (Rivero et al. 1980; Miller 1985). Breeding is sporadic, and occurs in temporary pools formed by heavy rains (USFWS 1992). *Peltophryne lemur* has not been seen on Virgin Gorda for over 40 years, and was thought to be extinct on Puerto Rico until 1965 (USFWS 1992). Populations have since been found on both the northern (Quebradillas and Isabela) and southern (Guánica State Forest Reserve) coasts of Puerto Rico (Rivero et al. 1980; USFWS 1992), although no toads have been seen in the north since 1988 (Barber 2007). It is therefore probable that Guánica, which may have numbered as few as 80 individuals in 2003 (Hedges et al. 2004), is the only remaining wild population. *P. lemur* is listed as critically endangered by the International Union for Conservation of Nature. The primary cause of decline is habitat loss and alteration, including drainage of breeding sites and spraying of surrounding areas with pesticides (Johnson 1990; USFWS 1992). Predation and/or competition by introduced species (e.g., cane toad, *Bufo marinus*; mongoose, *Herpestes javanicus*; feral dogs and cats) may have contributed to declines (Rivero et al. 1980; USFWS 1992). The small size and isolation of the remaining population in Guánica indicates that it is also vulnerable to stochastic environmental (e.g., hurricanes Johnson 1990) and demographic events.

Recovery efforts for *P. lemur* have been extensive. The Guánica breeding pond is closed for three weeks during the breeding season and is no longer drained for parking (Johnson 1990). Toads have been radiotracked to monitor movements, and habitat characteristics have been evaluated (Johnson 1990; Matos-Torres 2006). Wild individuals were collected during the 1980s to establish two breeding colonies representing the northern (four founders) and southern (32 founders) populations. To date, more than 110,000 southern tadpoles and toads have been released into constructed ponds in Guánica Forest, and releases of northern animals began at Arecibo in 2006 (Barber 2007). In 2003 and 2005, captive-born adult toads returned to the southern release site to breed (PRCT SSP 2006). Additional property is being secured and several ponds constructed in both regions, while public outreach and education are a continuing component of the recovery program (Barber 2007).

In order to maintain the optimal recovery strategy for *P. lemur*, several important issues must be resolved using genetic data. On average, a founding population of 20 individuals will capture >95% of heterozygosity and ~87% of allelic diversity (Frankham et al. 2002). While

the southern captive colony may have sampled sufficient genetic variation from the wild to maintain a healthy population for many generations, the northern colony likely had low variation from its initiation. The potential for increased homozygosity due to inbreeding and loss of genetic variation through drift are factors that must also be considered in the breeding populations. This is especially applicable to the northern colony, as the 123 individuals currently in captivity and used for breeding are the product of four siblings that themselves are descended from two first cousins. In addition, the isolation of the captive colonies from each other and the wild population may have led to genetic or morphological divergence between them (Rowe et al. 1998; Shuster et al. 2005; Hakansson et al. 2007). The degree of genetic differentiation between the northern and southern populations also requires assessment. Fossil evidence suggests that *P. lemur* was widespread throughout Puerto Rico during its history (Pregill 1981); however, it is currently restricted to low-elevation areas on opposite coasts, separated by the Cordillera Central mountain range. Previous allozyme assays suggested that the two populations may be divergent (Lacy and Foster 1987). It thus remains unclear whether the northern and southern populations have been separated throughout their history, or if population declines and habitat fragmentation have isolated them more recently.

The genetic data obtained here will be used to address three main questions: (1) do the captive populations suffer reduced genetic diversity relative to the wild population; (2) has the captive southern population diverged genetically from the wild southern population; and (3) what is the level of differentiation between the populations from the northern and southern coasts? Given the precarious state of the northern breeding colony and its probable extirpation in the near future, the long-term reintroduction of purely northern individuals is not realistic. The information from this study will thus be used to discriminate between the few remaining options for this lineage: (1) continue breeding the northern and southern populations separately. If the northern colony becomes extirpated, terminate reintroductions in this region; (2) continue breeding the northern and southern populations separately. If the northern colony becomes extirpated, reintroduce individuals from the southern lineage to the northern region; or (3) create an additional breeding colony in which northern and southern individuals are mixed. If adaptive differences exist between the northern and southern populations, this approach will facilitate the preservation of these traits. In the event that the pure northern colony becomes extirpated, these mixed individuals can be reintroduced to the north and may experience greater survival over purely southern animals due to the presence of some northern adaptations.

Methods

Sample collection and DNA extraction

Muscle or organs (heart, kidney, liver) were collected post-mortem from individuals of the northern and southern breeding colonies at the Toronto Zoo and Audubon Nature Institute in New Orleans. In total, four individuals from the northern colony and 58 from the southern colony were sampled. During a breeding of wild toads in 2003 at Tamarindo Pond in Guánica State Forest, Puerto Rico, an additional 43 tadpoles that had died were collected opportunistically. The southern breeding colony was founded by individuals collected from several breeding ponds, including Tamarindo, during the 1980s. Differential mortality of tadpoles may reflect the genotype at the major histocompatibility complex (Barribeau et al. 2008), suggesting that the targeted collection of dead individuals may not constitute a random genetic sample. However, the perilous state of *P. lemur* in the wild mandates that sampling impacts be minimised. Furthermore, the high mortality rate of amphibian larvae, particularly in temporary ponds such as those used by *P. lemur* (Duellman and Trueb 1986), indicates that assumptions of genotype interactions may be premature without supporting information. Hereafter, the southern breeding colony will be called Guánica, the northern breeding colony will be called Quebradillas, and the wild southern population will be called Tamarindo.

All 105 samples were genotyped at the six microsatellite loci to ensure that no rare alleles were missed. However, maternal inheritance of the mitochondrial genome means that all offspring of a female possess the same haplotype; thus, mitochondrial DNA was sequenced for only a subset of samples from the breeding colonies. According to the *P. lemur* studbook maintained by the Toronto Zoo, the 58 Guánica samples employed in this study trace their lineage to six wild females and 14 wild males. Thirty captive-bred individuals representing these six matrilineal lineages were sequenced, as were five male founders that could potentially harbour different mitochondrial lineages. Due to the small sample size for Quebradillas, all samples were sequenced. Because the genealogy of the Tamarindo individuals is unknown, all 44 samples were sequenced.

Although the number of samples used for the northern colony is small, it must be emphasised that a very limited gene pool is available for profiling. Only four wild individuals (two male and two female) were used to establish the colony, and all current breeding individuals are descendants of two cousins. Two of the samples profiled here are direct offspring of these cousins, and the other two are the product of a mating between two of these offspring (i.e. between full siblings). Tissue samples of earlier

generations are not available. Thus, a maximum of only one mitochondrial lineage and four alleles for each microsatellite locus are possible in any combination of samples from this population. Additional sampling would therefore not be informative, and the four samples analysed here are representative of the variation present in the northern colony.

Samples were prepared for extraction by dissolving 10 mg of tissue in 500 µl of 1× lysis buffer (2 M urea, 0.1 M NaCl, 0.25% *n*-lauroyl sarcosine, 5 mM CDTA, 0.05 M Tris–HCl pH 8), and digesting with 50 µl of proteinase K. Total genomic DNA was extracted with the DNeasy Tissue Kit (Qiagen Inc.) and quantified with the PicoGreen Quantitation Kit (Molecular Probes) on a FLUOStar Galaxy (BMG Labtech).

Mitochondrial control region sequencing

A fragment of the control region was amplified with the primers CytbA-L and ControlP-H (Goebel et al. 1999) in a 25 µl reaction containing 1–5 ng DNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 mg/ml BSA, 0.2 µM each primer, and 2 U Taq polymerase (Invitrogen). Thermal cycling used an MJ Research PTC-225 thermal cycler with the following conditions: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension of 72°C for 10 min. PCR product was visualised with ethidium bromide on 1% agarose gels, and 5 µl was purified with ExoSAP-IT (USB). Both strands were sequenced using the PCR primers with the DYEnamic ET-Terminator cycle sequencing kit on a MegaBACE 1000 DNA Analysis System (GE Healthcare).

Microsatellite development and profiling

Tetranucleotide microsatellites were isolated according to the protocol of Beauclerc et al. (2007). In total, 115 clones were sequenced, of which 83 were positive for a tetranucleotide repeat, with five duplicate clones found. Primer pairs were designed manually for 20 positive clones that possessed sufficient flanking sequence. Individual loci were amplified in 10 µl volumes containing 5 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.3 µM each primer, and 0.5 U Taq polymerase using the following thermal cycling conditions: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperature (see Table 1) for 1 min, and extension at 72°C for 1 min, with a final extension of 60°C for 45 min. Nine and three individuals from Guánica and Quebradillas, respectively, were screened for polymorphism by visualising PCR products on 4% agarose gels stained with SYBR Green I (Molecular Probes). Six loci amplified reliably and were polymorphic. To profile all samples, each locus was amplified individually and pooled into two multiplex reactions prior to desalting (Table 1). All PCR product was desalted using Sephadex G50, profiled on a MegaBACE 1000 DNA Analysis System, and scored using GENETIC PROFILER v.2.0 (GE Healthcare).

Data analysis

Sequences were aligned in CLUSTALX v.1.81 (Thompson et al. 1997) and edited by eye using BIOEDIT v.7.0.533 (Hall 1999). Pairwise sequence divergence was calculated using MEGA v.3.1 (Kumar et al. 2004). Microsatellite diversity indices (number and frequencies of alleles, observed and expected heterozygosity) were calculated using GENEPOP v.3.4 (Raymond and Rousset 1995), and conformation to Hardy–Weinberg equilibrium was tested by estimation of exact *P* values with a Markov chain method (Guo and Thompson 1992). Allelic richness (number of alleles per population) and private allelic richness were calculated by standardisation to the smallest sample size ($N = 4$) using rarefaction in the program HP-RARE v.1.0 (Kalinowski 2005).

Population differentiation was estimated between all pairs of populations for all loci with Nei's genetic distance D_S (1972) and the genotype likelihood ratio distance D_{LR} (Paetkau et al. 1997) using software from http://www2.biology.ualberta.ca/jbrzusto/ (J. Brzustowski, unpublished). These distance measures are calculated in very different manners, and Paetkau et al. (1997) recommended their use to provide independent estimates of genetic distance between populations. Population structure was evaluated with θ_W (Weir and Cockerham 1984), the unbiased estimator of Wright's (1951) F_{ST}, using ARLEQUIN v.3.1 (Excoffier et al. 2005). Significance was determined by a permutation test of 1,000 iterations. Genetic differentiation was further evaluated using the clustering program STRUCTURE v.2.0 (Pritchard et al. 2000). The likelihood that the samples represented between one and five genetic clusters ($K = 1$–5) was determined, using the following model: admixture, correlated allele frequencies, burn-in of 500,000 steps, and 1,000,000 iterations of the Markov Chain Monte Carlo algorithm. The model was run four times for each value of *K* to ensure consistency between runs. The largest value of ΔK (Evanno et al. 2005) was used to determine the most likely number of populations.

Results

Mitochondrial sequencing

Quality sequence was obtained for 963 bp of the mitochondrial control region, yielding two haplotypes (GenBank accession numbers EU149946 and EU149947): PleCR-1

Table 1 Primers and amplification conditions for six microsatellite loci isolated from *Peltophryne lemur*

Locus	Primer sequence (5′–3′)[a]	Repeat motif [b]	T_a (°C)[c]	Primer (μM)[d]	Pooling volume (μl)	GenBank accession #
Multiplex 1 (T_a = 55°C)						
PleMs-4	F: TGCCACTGAGAAAGATTTGG (H)	$(GATA)_9$	55–60	0.2	5	EU149940
	R: CCTGAAAAAAACTGAGAGATGG					
PleMs-8	F: ATGGGTGAATAAAGACCTCC (F)	$(GATA)_{18}$	55–65	0.3	10	EU149942
	R: CCCAGGGTACTGCAACTCG					
PleMs-14	F: CGTACCAGAAACTAATCTCAACTGG	$(GATA)_{12}$ imperfect	50–60	0.6	10	EU149944
	R: TCAGTTCCTATGCACTGAGC (H)					
Multiplex 2 (T_a = 55°C)						
PleMs-3	F: GACTATGTATGTGTGTGTAGC (H)	$(GATA)_{13}$	55–60	0.3	2.5	EU149941
	R: CAGGTTTTGAGAAGAGTTCC					
PleMs-10	F: GGGAAACTGGAGCAAATACC	$(GATA)_{36}$ imperfect	55	0.6	10	EU149945
	R: TTCTGTAAGGTCTGGCTGC (H)					
PleMs-11	F: TCCATTACCTTCTCAGTGTTGC (F)	$(CATA)_3$ $(GATA)_{12}$	55–60	0.3	5	EU149943
	R: AGTTGTGACTGCTGTGACC					

Loci were amplified individually and pooled into multiplexes prior to desalting

[a] Fluorescently labeled primers are indicated by the letters *H*, *F*, and *N*, which refer to HEX, 6-FAM and NED, respectively

[b] Repeat motif is based on sequence from the original clone. "Imperfect" indicates that one or more base pairs interrupted the repeat motif. See GenBank for complete sequences

[c] Annealing temperature is the range of temperatures at which the single locus amplified successfully and cleanly, based on agarose gels stained with SYBR Green I

[d] Primer concentration is for both the forward and reverse primers in the reaction

Table 2 Number of alleles, allelic richness when rarefied to $N = 4$, and size range of alleles for six microsatellite loci in three populations of *Peltophryne lemur*

Locus	Allele size		No. alleles (no. private alleles)				Allelic richness (private allelic richness)		
	Min	Max	Total	Guánica	Tamarindo	Quebradillas	Guánica	Tamarindo	Quebradillas
PleMs-3	143	173	10	7 (2)	7 (2)	3 (1)	4.28 (1.73)	3.48 (0.88)	3.00 (1.01)
PleMs-4	87	128	6	3 (0)	3 (0)	3 (3)	2.23 (0.13)	2.59 (0.49)	3.00 (3.00)
PleMs-8	147	201	9	5 (2)	4 (1)	3 (3)	3.08 (0.92)	2.76 (0.60)	3.00 (3.00)
PleMs-10	311	426	9	6 (1)	5 (0)	4 (3)	3.65 (0.89)	3.29 (0.52)	4.00 (3.00)
PleMs-11	129	157	8	7 (1)	6 (0)	2 (1)	3.37 (1.23)	3.14 (1.00)	2.00 (1.00)
PleMs-14	282	298	4	3 (0)	3 (0)	2 (1)	2.65 (0.07)	2.11 (0.03)	2.00 (1.26)
Total			46	31 (6)	28 (3)	17 (12)	19.26 (4.97)	17.37 (3.52)	17.00 (12.27)
Average			7.67	5.17	4.67	2.83	5.51 (1.42)	4.96 (1.00)	4.85 (3.51)

Corresponding indices for private alleles are shown in parentheses

occurred only in Guánica and Tamarindo, and PleCR-2 was exclusive to Quebradillas. Sixteen variable sites were found: 13 transitions, one transversion, and two indels of one base pair. Pairwise sequence divergence between the haplotypes was 0.015.

Microsatellite profiling

The total number of alleles for the six microsatellite loci was 46, with a range from 4 to 10 and an average of 7.7 alleles per locus (Table 2). Guánica possessed the largest number of alleles; however, the disparate sample size between groups clearly contributed to this outcome. When standardised by rarefaction, allelic richness was nearly equal for all three populations. Each population also had several private alleles (Fig. 2; Table 2): Guánica possessed six, Tamarindo three, and Quebradillas twelve. The sampling of amphibian tadpoles from a single location is typically discouraged as it may consist of a group of siblings and yield biased data. However, the observation of seven alleles at locus PleMs-3 in the Tamarindo samples indicates that this group is descended from at least four parents, and individuals are therefore not all full siblings.

Observed and expected heterozygosities ranged from 0.41 to 1.00 and 0.43 to 0.84, respectively (Table 3). Following sequential Bonferroni correction, all loci were in Hardy–Weinberg equilibrium and linkage equilibrium in Quebradillas and Tamarindo. In Guánica, however, loci PleMs-8 and PleMs-14 continued to deviate significantly from Hardy–Weinberg equilibrium due to heterozygote excess, and significant linkage disequilibrium remained between loci PleMs-14/PleMs-10, PleMs-14/PleMs-3, PleMs-10/PleMs-11 and PleMs-10/PleMs-3. It is probable that the disequilibrium in Guánica is due to the managed breeding of this closed population. The fact that all loci are in equilibrium in the wild population, which presumably experiences random mating, suggests that the loci are not linked or under selection and are therefore suitable for use in further analyses.

Guánica and Tamarindo shared the greatest number of microsatellite alleles, 25 out of 46 total alleles (54%; Fig. 2). When excluding alleles found only in Quebradillas, Guánica and Tamarindo shared 74% of their alleles (25 out of 34). Very few alleles were common between Quebradillas and the southern populations: none were shared for loci PleMs-4 and PleMs-8, and only one was shared at loci PleMs-10, PleMs-11, and PleMs-14. In total, Quebradillas shared only five alleles (11%) with both Guánica and Tamarindo; its remaining 12 alleles were unique. Both Guánica and Tamarindo are much more distant from Quebradillas than they are from each other based on the pairwise genetic distance measures D_S and D_{LR} (Table 4). F_{ST} values were significant at $P = 0.01$ between all pairs of populations, but values were much larger between either of the southern populations and Quebradillas (Table 4).

The average log probability of K [ln Pr($X|K$)] increased with the number of clusters (data not shown). However, the rate of change of the log probability of K (ΔK) was largest for $K = 3$ ($\Delta K_2 = 2.6$; $\Delta K_3 = 551.5$; $\Delta K_4 = 117.1$). This was therefore chosen as the number of groups most likely represented by the microsatellite data. Guánica and Tamarindo individuals were both assigned in similar proportions and nearly equally to clusters 1 and 2, with no individuals assigned to cluster 3 at >2% of their genome (data not shown). All Quebradillas individuals were strongly assigned to cluster 3 (>98% of each genome).

Discussion

Genetic diversity within populations

The control region is the most variable section of the mitochondrial genome (Moritz et al. 1987), making the

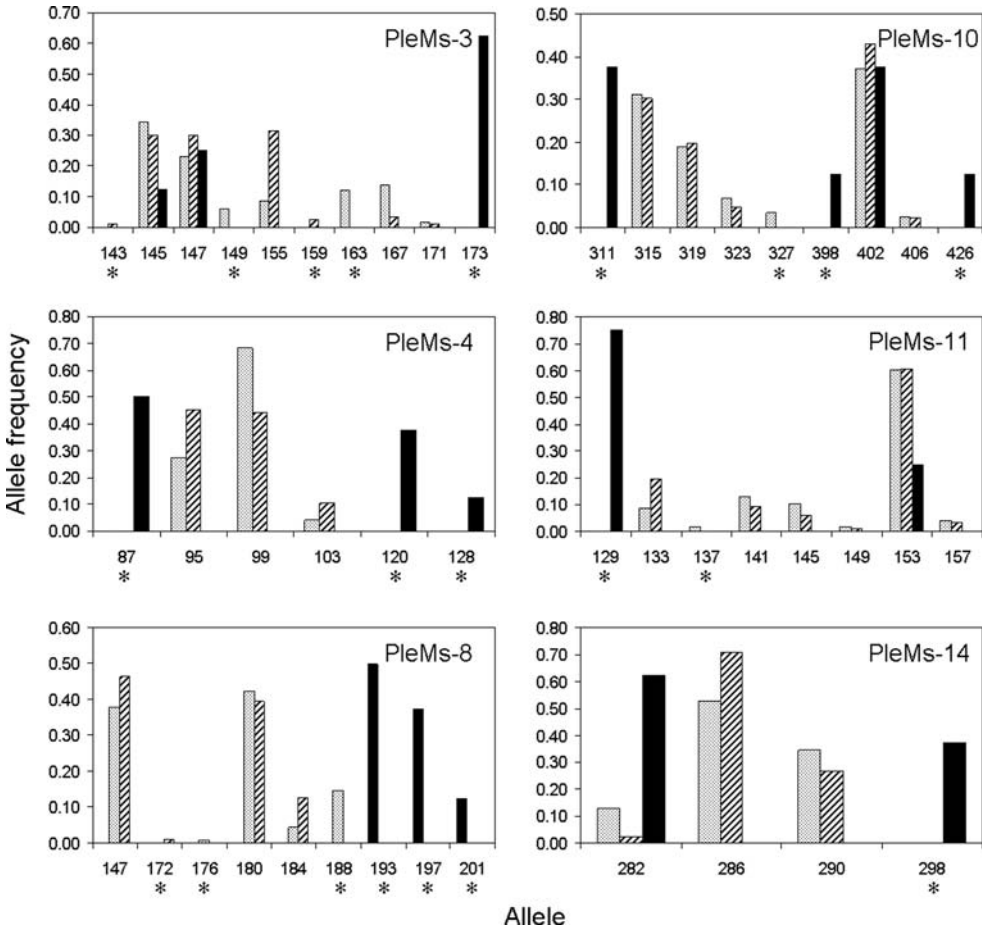

Fig. 2 Allele frequencies at six microsatellite loci for three populations of *Peltophryne lemur*: Guánica (*shaded*), Tamarindo (*hatched*) and Quebradillas (*solid*). Private alleles are indicated by *asterisks*

Table 3 Observed heterozygosity (H_O), expected heterozygosity (H_E), and exact P values (P) of Hardy–Weinberg equilibrium for six microsatellite loci in three populations of *Peltophryne lemur*

Locus	Guánica			Tamarindo			Quebradillas		
	H_E	H_O	P	H_E	H_O	P	H_E	H_O	P
PleMs-3	0.79	0.81	0.13	0.73	0.84	0.44	0.61	0.50	0.43
PleMs-4	0.46	0.41	0.23	0.60	0.49	0.34	0.68	1.00	0.31
PleMs-8	0.66	0.71	0.0002	0.62	0.56	0.18	0.68	0.75	1.00
PleMs-10	0.73	0.72	0.06	0.69	0.67	0.94	0.79	1.00	1.00
PleMs-11	0.60	0.71	0.16	0.59	0.60	0.80	0.43	0.50	1.00
PleMs-14	0.59	0.78	0.02	0.43	0.51	0.25	0.54	0.75	1.00
Overall	0.64		0	0.62		0.57	0.61		0.98

Table 4 Pairwise values of F_{ST} (calculated as θ_W), Nei's genetic distance (D_S), and the genotype likelihood ratio distance (D_{LR}) using six microsatellite loci for three populations of *Peltophryne lemur*

Population pair	θ_W	D_S	D_{LR}
Guánica/Tamarindo	0.03	0.06	1.07
Guánica/Quebradillas	0.31	1.65	16.02
Tamarindo/Quebradillas	0.34	1.76	16.16

All θ_W values were significant at $P < 0.01$

very low haplotypic diversity of *P. lemur* somewhat surprising. This bufonid has one of the lowest mitochondrial diversities among a number of amphibian species: multiple haplotypes typically exist for this and less variable regions (Bos and Sites 2001; Austin et al. 2002; Smith and Green 2004), and often over relatively small scales (Shaffer et al. 2004b; Dever 2007). However, Rowe et al. (2006) found that all British and Irish individuals of *Bufo calamita* possessed a single control region haplotype.

Peltophryne lemur is moderately variable at the six microsatellite loci. Within-population genetic diversity (H_E) is similar to or greater than that found for microsatellite loci in several other amphibians (Rowe et al. 1998; Palo et al. 2003; Brede and Beebee 2004; Martinez-Solano et al. 2005; Arens et al. 2006), while only a few have been found to have substantially greater H_E (Austin et al. 2003; Burns et al. 2004; Hoffman et al. 2004). Allelic diversity for *P. lemur* is comparable to several species examined over similar and larger geographical scales, despite much larger sample sizes for some (Rowe et al. 1998; Palo et al. 2003; Brede and Beebee 2004; Kraaijeveld-Smit et al. 2005; Arens et al. 2006). This diversity is surprising, considering the small sample sizes, relatedness of individuals, and limited variation in mtDNA. It thus appears

that neither the captive nor wild populations of *P. lemur* have suffered a severe reduction in heterozygosity due to small population sizes and prolonged isolation. In fact, the Guánica captive colony had *greater* heterozygosity than expected at two loci. This likely reflects the minimisation of kinship management system used to designate pairs for breeding (Johnson 1990; Ballou and Lacy 1995), which reduces the tendency to homozygosity via inbreeding and the loss of alleles through genetic drift (Montgomery et al. 1997). By comparison, random mating in the wild Tamarindo population, combined with a possible bottleneck of 80 individuals (Hedges et al. 2004), has allowed these processes to reduce heterozygosity below that of Guánica for several loci.

Comparison of populations

There appears to be very little difference in the amount of genetic variation found in Guánica and Tamarindo. Both populations possess similar numbers of alleles and H_E estimates for most loci. There is also little divergence between them: a single mitochondrial haplotype exists in all individuals, there are very few unshared alleles, and the measures of genetic distance and differentiation are very low. Comparable estimates of these measures have been found over similar distances in several other amphibian species (Newman and Squire 2001; Brede and Beebee 2004; Burns et al. 2004; Funk et al. 2005; Arens et al. 2006). In addition, the STRUCTURE algorithm could not distinguish individuals from these two groups. The genetic similarity of these populations is not surprising, as the Guánica captive colony is recently descended from the wild Tamarindo population. Although gene flow between captive-bred individuals released into Guánica State Forest and wild individuals located at Tamarindo pond cannot be completely excluded, it is highly unlikely as *P. lemur* has been shown to move only up to 2 km (Johnson 1999) and these sites are separated by 20 km of migratory distance.

Despite the fact that only a small number of related, inbred individuals were sampled, moderate genetic variability exists at nuclear loci in Quebradillas. It is probable that most alleles present in the northern population were detected during this analysis, as 50–100% of the maximum number of possible alleles (four) was observed for all loci. Thus, more extensive sampling would likely have revealed little additional variation. Although it possesses fewer alleles than Guánica or Tamarindo based on direct counts, it is nearly equal when all groups are standardised to $N = 4$. As well, H_E is very similar to both southern populations. Quebradillas is moderately divergent from the southern populations at mtDNA, with 1.5% sequence divergence between the two haplotypes. A comparison with other amphibian mitochondrial studies shows that many species possess deeper divergences in the control region (Shaffer et al. 2004b) and more conserved regions (Lougheed et al. 1999; Austin et al. 2002; Vredenburg et al. 2007). In contrast to the mtDNA data, Quebradillas is quite differentiated from the southern populations at nuclear microsatellite loci. It possesses far more private alleles than either Guánica or Tamarindo, and shares more than a single allele with them at only one of the six loci. This abundance of private alleles in Quebradillas is likely not an artifact of sample size, as one would expect the populations with larger sample sizes (i.e. Guánica or Tamarindo) to detect a greater number of rare or unique alleles. Furthermore, STRUCTURE clearly distinguished the northern individuals, and the measures of genetic distance and differentiation are much greater between Quebradillas and both southern populations than between the two southern populations. These F_{ST} values of >0.3 are quite large compared to some other amphibian microsatellite studies (Newman and Squire 2001; Brede and Beebee 2004; Johansson et al. 2005), particularly given the small distance separating the two populations ($\sim$55 km). However, some studies have found similar or greater values over very short geographic distances (Kraaijeveld-Smit et al. 2005; Arens et al. 2006), and several report values >0.4 across the distribution of a single species (Rowe et al. 1998; Burns et al. 2004; Funk et al. 2005).

For small animals with low vagility such as amphibians, it is often not the geographic distance that determines levels of gene flow between populations, but rather the ecological and/or geographical barriers that separate them such as mountain ridges (Funk et al. 2005; Kraaijeveld-Smit et al. 2005; Martinez-Solano et al. 2005), unsuitable habitat (Johansson et al. 2005), or even ancient geological features that are no longer apparent (Lougheed et al. 1999). The Cordillera Central mountain range, with a maximum elevation >1,300 m (Pico 1974), extends across the interior of Puerto Rico in an east-west direction (Fig. 1). The strong differentiation between northern and southern populations of *P. lemur* is thus not unexpected, as this species is restricted to low-elevation coastal areas (Rivero et al. 1980). The genetic data, combined with *P. lemur*'s historical distribution and probable inability to traverse the mountainous interior, suggests that northern and southern populations have likely not experienced gene flow for a substantial time.

Conservation and management recommendations

An important finding of this study is that the breeding of *P. lemur* in AZA (Association of Zoos and Aquariums) institutions has been very successful in terms of genetic management: the captive Guánica population has not suffered reduced genetic diversity relative to the wild

Tamarindo population, nor has it diverged substantially during its isolation. Although only neutral molecular markers were evaluated, this suggests that adaptation to captivity has been minimal (e.g., Ford 2002) and that the captive southern population is suitable for continued reintroduction to the south coast of Puerto Rico. Additional supplementation of the captive colony by wild individuals does not appear to be immediately necessary for genetic purposes.

The future of *P. lemur* in northern Puerto Rico, however, is in jeopardy. Although the Quebradillas breeding colony possesses adequate variation at this time, it likely cannot be sustained much longer as the 123 individuals currently in captivity are descended from four inbred siblings. It was previously believed that the colony had reached reproductive senility as no clutches had been obtained for some time (Bloxam and Tonge 1995). While this is likely in part due to the age of the individuals, it is also possible that inbreeding avoidance (Waldman et al. 1992) and/or depression have contributed to lower fitness. Successful breeding of northern animals has occurred recently, with releases of tadpoles on the north coast of Puerto Rico. However, it is unlikely that individuals have become established since it required nearly 20 years and over 100,000 released individuals before breeding animals were observed in the south. This indicates that there will be limited opportunities to restock the captive northern colony should it become depleted. The prospects for the northern lineage of *P. lemur* are therefore dire and it is no longer sufficient to maintain separate populations in captivity; in order to ensure the persistence of *P. lemur* in the north, less conventional management options must be explored.

The presence of an exclusive mitochondrial haplotype and many unique microsatellite alleles in Quebradillas suggests that the northern and southern populations of *P. lemur* are quite distinct and have not experienced gene flow for some time. This isolation may have enabled unique, adaptive characteristics to evolve in the north, facilitated by differences in habitat and climatic conditions: Guánica is located in an arid coastal plain, receiving $\sim$30–35 inches of rainfall annually and possessing dry limestone vegetation, while Quebradillas receives 40–60 inches of annual rainfall and consists of limestone hills with humid limestone vegetation (Pico 1974). Any unique adaptations that northern individuals may possess have the potential to greatly assist with the establishment of reintroduced individuals to this region and should be preserved. It is therefore recommended that whilst the Quebradillas breeding colony is successful in producing offspring for release, the Guánica and Quebradillas colonies should continue to be maintained separately and animals released into their respective regions. In order to ensure the continuation of the northern lineage, however, a third breeding colony consisting of both northern and southern individuals should be created. This will increase the genetic diversity and number of breeding individuals for the Quebradillas colony, thereby increasing its health and longevity. Initially, this would serve as an experimental colony to determine whether mixed individuals possess greater or diminished fitness prior to their release into the wild. In the event that the pure Quebradillas lineage becomes extirpated, these animals could then be used for release to the northern coast of Puerto Rico. This strategy would preserve the most overall genetic diversity, including any unique genetic adaptations that may be present in the northern lineage. The alternative option to release pure Guánica individuals into the north should the Quebradillas colony become extirpated is less desirable, as they would lack any potentially adaptive traits that may increase their survival in the conditions found in northern Puerto Rico.

The mixing of distinct lineages is generally avoided because of the possibility of reduced fitness due to outbreeding depression. However, it "should not be dismissed lightly as a management option, especially where there is evidence that remnant populations have reduced viability because of inbreeding depression" (Moritz 1999). Inbreeding depression is generally considered a more serious problem than outbreeding depression, and the negative impacts of outbreeding depression may be overemphasised while the benefits of mixing lineages are overlooked (Frankham et al. 2002). "Genetic rescue" (e.g., Tallmon et al. 2004) by mixing distinct lineages has successfully reduced inbreeding depression and increased fitness for several threatened species (Westemeier et al. 1998; Madsen et al. 1999; Land et al. 2004) and continues to be recommended (Soltis and Gitzendanner 1999; Wyner et al. 1999; Godoy et al. 2004). Experimentally inbred populations of plants (Richards 2000; Newman and Tallmon 2001) and animals (Bryant et al. 1999; Ball et al. 2000) also experienced increased fitness when immigrants were introduced. Very low levels of migration (e.g., one individual per generation) may be sufficient for successful genetic rescue, which minimises genetic swamping and allows local adaptation to proceed (Hedrick 1995; Newman and Tallmon 2001; Palo et al. 2003; Vila et al. 2003). Despite the considerable divergence of northern and southern *P. lemur* populations, they do not appear to warrant classification as separate subspecies. Many other amphibian species are much more differentiated at mtDNA, and the F_{ST} estimates fall within those for several other amphibians. Furthermore, husbandry protocols are the same for the northern and southern captive colonies, suggesting that critical reproductive and rearing aspects do not differ (Lentini 2000). Although the potential for reduced fitness due to outbreeding depression cannot be completely excluded, these factors indicate that it should

be minimal. In contrast, it is known that the northern colony of *P. lemur* is highly inbred. While the extent of inbreeding depression has not been thoroughly evaluated and can vary within a single lineage (Pray and Goodnight 1995), it was suggested over a decade ago that the northern colony may be suffering from reduced fitness (Bloxam and Tonge 1995). Given the available evidence, inbreeding depression is a much greater concern for the future of the northern lineage than should be outbreeding depression in a mixed population.

The establishment of a third breeding colony of mixed northern and southern *P. lemur* lineages can be viewed as insurance for securing some northern traits, as it is probable that the pure northern colony will become extirpated due to demographic stochasticity or inbreeding depression. Because outbreeding depression is due primarily to the breakup of coadapted gene complexes between divergent lineages, it is probable that negative effects would not manifest until the F_3 generation when such complexes have undergone recombination (Tallmon et al. 2004). The mixed colony should therefore be created immediately so that the fitness of mixed individuals can be monitored for several generations prior to release into the wild (e.g., Moritz 1999). Although fitness in captivity cannot necessarily be extrapolated to the wild, monitoring should provide an indication of the overall health and reproductive capacity of the interbred colony relative to the pure northern colony. Sufficient individuals are generally produced in captivity that these efforts should not affect the existing breeding colonies or release efforts. Its initiation would also not require a large investment of resources, as several facilities currently house *P. lemur* and much is known about its husbandry (Miller 1985; Lentini 2000). Future work should develop models to determine the minimum number of southern individuals necessary to effect genetic rescue of the northern population while minimising genetic swamping, as was done for the Florida panther (Hedrick 1995).

Not only will a mixed breeding colony facilitate the establishment of *P. lemur* populations in northern Puerto Rico by preserving adaptive traits, its experimental nature enables these recovery efforts to contribute to the development of reintroduction biology as a science (sensu Seddon et al. 2007). It can also provide insight into the actual repercussions of outbreeding depression, allowing future conservation biologists to assess the potential for adverse effects on a given species. Finally, this study demonstrates that it is not always possible to follow generalised conservation guidelines for all species; rather, each species must be evaluated individually and decisions made to maximise the retention of genetic diversity, thereby maximising the species' prospects for recovery.

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