DETERMINING THE ORIGIN OF THE NEW POPULATION OF THE CRITICALLY ENDANGERED PUERTO RICAN CRESTED TOAD

by

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ABSTRACT

The Puerto Rican Crested Toad (*Peltophryne lemur)* is the only native toad in Puerto Rico. It was once found in great abundance along the north and south coastal areas of Puerto Rico. The toads' populations have become severely depleted due to the loss of habitat and the introduction of the marine toad *Bufo marinus*, to control the sugar cane beetle in the 1920s. Currently, there is a single wild population in Southern Puerto Rico and there are also captive populations in the south and north of Puerto Rico, being maintained by the Fort Worth Zoo (through captive breeding and reintroductions) as a hedge against extinction. Recently, another population in southern Puerto Rico has been discovered with unknown population origins about 3 km away from the original southern population. We extracted DNA from 47 individuals and genotyped them with six microsatellite loci and sequenced the mitochondrial control region to determine the genetic diversity of the population and if it was similar or different from the original southern population. The new population had genetic diversity levels that were similar to the original population, the mitochondrial haplotypes matched the southern population's haplotypes, and the microsatellite allele frequencies were statistically similar to the southern population, indicating little divergence. These data suggest that this new population was the result of a colonization event from the original southern population. Currently listed as critically endangered by the IUCN, our conclusion that this new population was a colonization from the known population gives hope to the species' ability to survive and expand their range.

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INTRODUCTION

Genetic markers, such as microsatellite DNA and the mitochondrial control region, can help conservation efforts by delineating populations and to estimating the degree of dispersal between populations (Zschokke et al. 2011, Putman 2014). These same genetic markers can also be used to determine levels of genetic diversity within populations and determine if they may have experienced a genetic bottleneck resulting in low genetic diversity and are at an increased risk of inbreeding depression. Understanding a species population structure can help managers determine if a species has highly differentiated populations that could be candidates for different conservation units and management strategies (Frankham et al. 2009). Dispersal (gene flow) between populations can also be estimated and could potentially lead to the construction of dispersal corridors if dispersal is very low or non-existent or to an understanding of colonization dynamics. The discovery of a new population in a species of conservation concern raises several questions. Is this new population a previously undetected isolated population that might represent a new conservation unit or does it represent a colonization event by an already established known population? By taking a naturalized or newly found population of a species and assessing the genetic markers, you can discover which of the known populations it originated from (Hedmark and Ellegren 2007, Epps et al. 2010, Millar 2012). In addition, determining the genetic diversity of the new population can tell if there was a limited colonization (i.e. a founder event) or if it was the result of a colonization event and there is ongoing gene flow (Epps et al. 2010, Millar 2012).

The Puerto Rican Crested Toad, *Peltophryne lemur,* has been collected in 8 areas of Puerto Rico and one site in the British Virgin Islands. It mostly inhabits areas of low elevation with limestone fissures, where it escapes to during the day. *Peltophryne lemur* does not have regular breeding patterns, but rather, breeds sporadically in small, temporary pools after rains (USFWS 1992, White 2010). Puerto Rico's only endemic toad disappeared from the Virgin Islands and Puerto Rico and was thought to be extinct for over 40 years. In 1965, two populations were found on the northern and southern coasts, known as Quebradillas and Guanica, respectively (USFWS 1992). It was found that the northern, Quebradillas, did demonstrate differentiation from the southern population at 6 microsatellite loci due to geographic isolation and the unlikelihood of these populations experiencing gene flow recently (Beauclere et al. 2009). Tamarindo is a site in the south, near Guanica, where the only known wild population of *Peltophryne lemur* lives today. The Puerto Rican Crested Toad has declined mainly from habitat loss, invasive species, and pesticide contamination (USFWS 1992).

Since the 1980s, both of these populations have been bred in captivity with some success in sustaining population genetic diversity, considering the number of founders for each population (Miller 1985, Barber 2007). Captive breeding programs have been quite successful in terms of genetic management as the captive, Guanica, has not strayed from the wild, Tamarindo, in terms of genetic diversity. It is great news that there has been little adaptation to captivity and that reintroductions have been successful.

Recently, a new population of *Peltophryne lemur*, Ventana, has been discovered some distance (~4 km) from the known location of Tamarindo, along the southern coast of Puerto Rico. This new appearance suggests two possibilities: 1) a group of

Peltophryne lemur from the wild Tamarindo population may have colonized this new area or 2) this is a previously unknown, isolated population of Puerto Rican Crested Toad. If this is a new colonization event, then one would expect little genetic differentiation between this site and the Tamarindo site at the six microsatellite loci and in the mitochondrial control regions. If this was a new population, then one would expect some divergence and variation in the six alleles and in the control regions. Finding a new population is very exciting because if it is a colonization, then it shows that the toad is capable of relocation, migration and sustainability on its own. If it is an unfound population, then it would be very helpful to introduce even more genetic variation, traits and adaptations to the breeding pools to help with the genetic rescue of the Puerto Rican Crested Toad.

METHODS

DNA Sampling:

Between the winter of 2008 and spring of 2013, the Fort Worth Zoo took mouth swab samples from 30 individuals collected from the newly discovered population of *Peltophryne lemur* and 18 individuals from the captive zoo population taken from Tamarindo. All of the samples were done by employees of the Fort Worth Zoo and sent to our lab at Texas Christian University.

DNA Extraction:

The swabbed tissue, containing cells from the toads, were placed in tubes containing lysis buffer and Proteinase K and were left to incubate overnight at 55˚C in order to breakdown the tissue. Half of a volume of 7.5 M ammonium acetate was added to precipitate proteins, which were then frozen, thawed and centrifuged for 15 minutes to

pellet. Next, 0.7 volume 100% isopropanol was added to the supernatant to precipitate the DNA and it was frozen and centrifuged for 15 minutes to pellet the DNA. The DNA pellet was washed with 70% ethanol and allowed to dry before resuspending in 100 µL 10mM Tris-HCl pH 8.5.

Genotyping:

The samples were genotyped at 6 microsatellite loci developed for *Peltophryne lemur* (Beauclere 2009). The six loci were amplified in 2 multiplex sets with 3 microsatellite loci in each set. The Multiplex 1 contained the microsatellites loci: PleMs-4, PleMs-8, and PleMs-14. The Multiplex 2 contained the microsatellite loci: PleMs-3, PleMs-10, and PleMs-11. Polymerase chain reactions (PCR) (10µL) contained 10-50 ng DNA, 0.5 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM $MgCl_2$ pH8.7, and dNTPs.. Reactions were cycled in an ABI 2720 thermal cycler with an initial 15 minute denaturation period (94^{o}C), followed by 30 cycles of 30 seconds of denaturation (94 ^{o}C), 1.5 minutes of annealing (55 ^{o}C/60 ^{o}C) and one minute of elongation (72 ^{o}C). Reactions ended with a 30 minute elongation period (60 ^{o}C). The amplified PCR products were diluted 10 times with deionized water. To analyze genotypes of the samples, 0.5 µL of the diluted PCR product from each individual was loaded with 10 µL HIDI formamide with 0.1 µL Liz-500 size standard and electrophoresed on the ABI 3130XL Genetic Analyzer. The genotypes for each individual were scored using Genemapper v5.0. We used GenAlEx v.6.5 to calculate allele frequencies, genetic diversity measures and genetic differentiation (Peakall and Smouse 2012).

Table 1. Microsatellite Loci used for the two multiplex sets. This table illustrates the sequences, repeats, annealing temperature, size range (base pairs) and the GenBank accession numbers for each locus.

Locus	Primer Sequence (5'-3')	Repeat in Original Clone	Annealing T (˚C)	Size Range (bp)	GenBank accession #
Multiplex 1					
PleMs-4	F: TGCCACTGAGAAAGATTTGG R:CCTGAAAAAAACTGAGAGATGG	(GATA) - 9	55-60	95-103	EUI149940
PleMs-8	F: ATGGGTGAATAAAGACCTCC R: CCCAGGGTACTGCAACTCG	(GATA) - 18	55-60	145-186	EUI149942
PleMs-14	F:CGTACCAGAAACTAATCTCAACTGG R: TCAGTTCCTATGCACTGAGC	(GATA) - 12	50	280-288	EUI149944
Multiplex 2					
PleMs-3	F:GACTATGTATGTGTGTGTAGC R:CAGGTTTTGAGAAGAGTTCC	(GATA) – 13	55-60	141-171	EUI149941
PleMs-10	F:GGGAAACTGGAGCAAATACC R :TTCTGTAAGGTCTGGCTGC	(GATA) – 36	55-60	314-402	EUI149945
PleMs-11	F: TCCATTACCTTCTCAGTGTTGC R: AGTTGTGACTGCTGTGACC	(CATA) -3 (GATA) - 12	55-60	131-156	EUI149943

Mitochondrial DNA control region sequencing:

The control region was amplified in a PCR (10µL) containing 10-50 ng DNA, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM MgCl2 pH8.7, and dNTPs, 0.5 µM of each primer, CytbA-L and ControlP-H (Goebel 1999) for the control region. Reactions were cycled in an ABI 2720 thermalcycler with an initial 15 minute denaturation period (94^{o}C), followed by 30 cycles of 30 seconds of denaturation (94 ^{o}C), 1.5 minutes of annealing (55 ^{o}C) and one minute of elongation (72 ^{o}C). Reactions ended with a 30 minute elongation period (60 ^{o}C). PCR products were

then cleaned of unused primers and nucleotides with Exo I and Shrimp Antarctic phosphatase (New England Biolabs). Products were sequenced using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Life Technologies) using the PCR primers. Sequences were electrophoresed on an ABI 3130XL Genetic Analyzer (Life Technologies); edited, contiged, and trimmed using Sequencher v5.0 (Gene Codes USA); and then aligned in MEGA 6.0 (Tamura et al. 2013) using Muscle (Edgar 2004). The individual sequences were then compared to the two known haplotypes of the northern and southern populations on GenBank (GenBank accession numbers EU149946 and EU149947).

RESULTS

After genotyping the 30 Ventana samples and 18 Ventana samples, we charted and looked at the allele frequencies of the two populations for the 6 microsatellite loci in the 2 multiplexes that were scored. Each locus had both homozygous and heterozygous individuals and they turned out very similar in the distribution of alleles between populations (Fig 1).

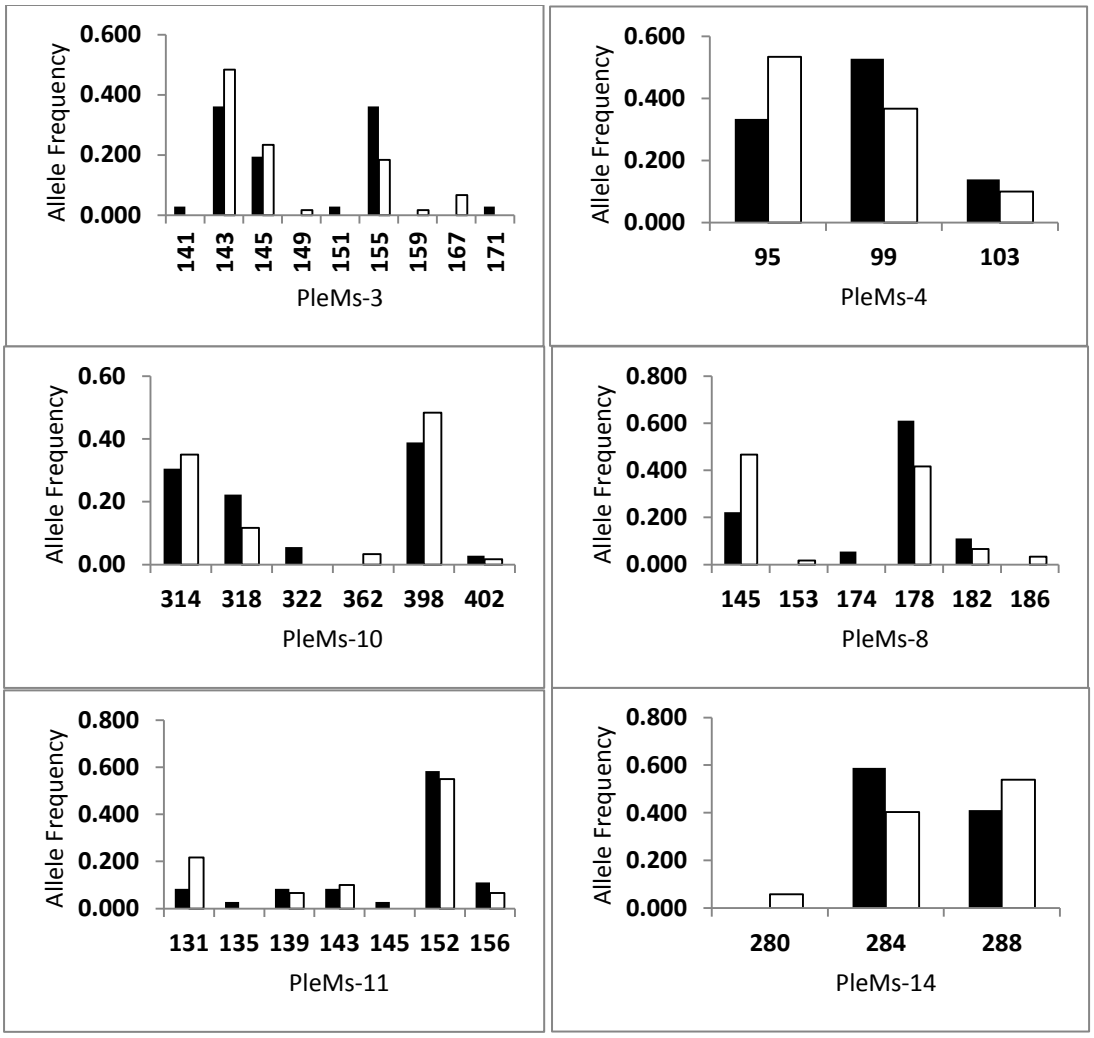

Fig 1. Allele frequencies for the two multiplex sets. The first multiplex set is on the left and the second multiplex set is the column on the right. These frequencies compare the two populations, Ventana (white bars) and Tamarindo (black bars) at each microsatellite locus.

Private alleles (alleles found in only one population) were present in both populations at relatively low frequencies. In the Tamarindo population, there were 7 private alleles among the 6 loci ranging from frequencies of 2.8% to 5.6%. The Ventana population had 7 private alleles among the 6 loci ranging in frequency from 1.7% to 6.7%.

Table 2. Genetic Diversity of microsatellite loci in *Peltophryne lemur*. The number of individuals, (N) alleles (Na),observed heterozygosity (Ho), expected heterozygosity (HE), and the inbreeding coefficient (F).

Population	Locus	N	Na	Ho	H_E	F
Tamarindo	PleMs-10	18	5	0.67	0.72	0.05
Tamarindo	PleMs-11	18	7	0.72	0.64	-0.16
Tamarindo	PleMs-14	17	2	0.47	0.50	0.03
Tamarindo	PleMs-3	18	6	0.78	0.72	-0.11
Tamarindo	PleMs-4	18	3	0.50	0.61	0.15
Tamarindo	PleMs-8	18	4	0.61	0.58	-0.09
Ventana	PleMs-10	30	5	0.57	0.64	0.10
Ventana	PleMs-11	30	5	0.80	0.64	-0.27
Ventana	PleMs-14	26	3	0.38	0.55	0.29
Ventana	PleMs-3	30	6	0.60	0.68	0.11
Ventana	PleMs-4	30	3	0.47	0.58	0.18
Ventana	PleMs-8	30	5	0.63	0.61	-0.05

The observed heterozygosity and the expected heterozygosity were similar for most of the loci(Table 2). Observed heterozygosity was significantly lower than expected heterozygosity for the PleMs-14 locus in Ventana suggesting the presence of a null allele. It should be noted that for the PleMs-14 locus, we had 4 individuals in the Ventana population and 1 in the Tamarindo population that, despite multiple attempts, would not amplify at this locus suggesting the presence of null homozygotes. This is likely due to the specific primer for that locus encountering a mutation at the 3' end keeping it from

annealing to the template. Fortunately, the number of affected genotypes was low and does not have a significant effect on our data.

The average heterozygosities of the two populations were very similar with Tamarindo having a value of 0.625 ± 0.050 and Ventana having a value of 0.575 ± 0.059 with (Fig 3). In addition, the two populations shared the same number of average alleles per locus with 4.5±0.76alleles in Tamarindo and 4.5±0.5 alleles in Ventana (Fig 2).

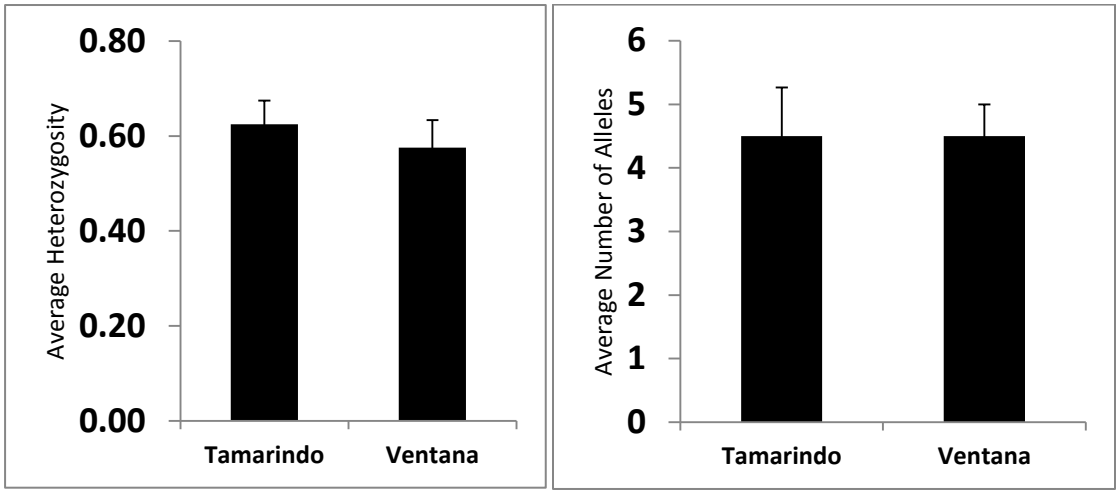

Fig 2. Average heterozygosity and average number of alleles for both Tamarindo and Ventana.

We conducted an analysis of molecular variance and found that 90% of the variance was within individuals, 8% was among individuals and 2% was between populations (Fig 3). The F_{ST} value was 0.019 and the P value for the data was 0.056.

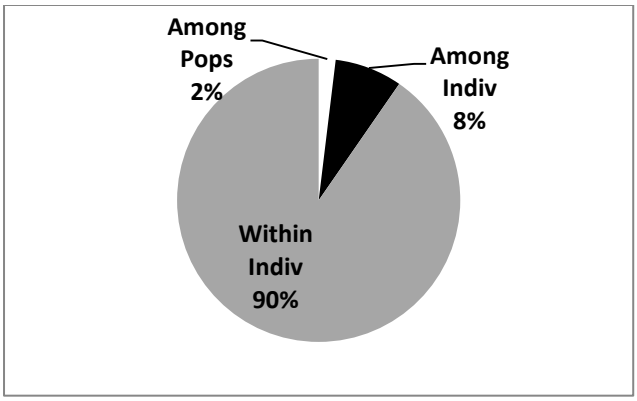

Fig 3. Pie Graph illustrating the molecular variance within individuals, among individuals and among populations.

The mitochondrial control region sequenced for all of the individuals, both Tamarindo and Ventana, matched the single known haplotype of the southern population: Guanica and Tamrindo. This is expected as there are only two haplotypes known: the northern, Quebradillas, and the southern populations, Tamarindo and Guanica.

DISCUSSION

The allelic frequencies were very similar between the Tamarindo and Ventana populations. Some of the variation we observed between the populations may have been due in part to the small sample size available for Tamarindo which can potentially skew allele frequencies. The small sample size for Tamarindo may also explain some of the private alleles that were found. For instance, some of the low frequency private alleles present in Ventana may simply not have been sampled in Tamarindo because only a few individuals were genotyped in that population. I examined the results from Beauclere et. al. 2009, and visually compared the alleles present in Guanica, Tamarindo, and Quebradillas to my data. The alleles and their frequencies found in my study are very similar to what Beauclere et al. 2009 found for the Tamarindo site. This strengthens the conclusion that the Ventana population was very clearly linked to the Tamarindo

population, rather than the two other known populations. When separating the molecular variance of all of the individuals combined, most of the variation is within individuals and not between populations, and the level of divergence between the two populations was very low (~2%) and non-significant, strengthening the claim even more. If Ventana was an isolated and undiscovered population, I would have also expected to see a similar level of divergence between Ventana and Tamarindo as Beauclere et al. 2009 found between Tamarindo and Guanica (the captive population).

The observed heterozygosity and number of alleles in the Ventana and Tamarindo populations were the same.. The similarity in allele frequencies and also heterozygosity and numbers of alleles suggests that the two populations are linked by ongoing dispersal. If Ventana had simply been colonized by a few individuals from Tamarindo then we would expect to see genetic evidence of a founder effect which would be similar to a genetic bottleneck. In this scenario Ventana would have had a lower genetic diversity than Tamarindo and a small subset of the alleles found in Tamarindo, neither of which was observed in this study.

We suggest the data strongly support that Tamarindo colonized the Ventana area and it is probable the two populations experience regular gene flow. . It is also likely that Ventana has been established now for multiple generations and this population is set up to sustain itself.

There is a need to understand *P. lemur's* population structure and dynamics in order to orchestrate an effective rehabilitation plan and continue to manage the species survival in the future. The toad has been decimated in the past due to a number of factors and thus, efforts to ensure future generations are incredibly fragile as it would be tragic to

lose the low amount of diversity that exists. However, the toads appear to have accomplished the colonization of new habitat 4 km away which is 2X as far as the longest previously recorded dispersal event (2 km) (CBSG 2006). This brings a positive hope that one day the species may be able to retake the island of Puerto Rico and dominate as the country's most thriving toad by radiating out from pond to pond. We hope that our results will prove to make an effective impact in its role in the grander scheme to save the future of the Puerto Rican Crested Toad.

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