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Strong Founder Effects and Low Genetic Diversity in Introduced Populations of Coqui Frogs

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Strong founder effects and low genetic diversity in introduced populations of Coqui frogs

Running title: Invasion dynamics of the Coqui frog

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Abstract

The success of non-native species may depend on the genetic resources maintained through the invasion process. The Coqui (*Eleutherodactylus coqui*), a frog endemic to Puerto Rico, was introduced to Hawaii in the late 1980s via the horticulture trade, and has become an aggressive invader. To explore whether genetic diversity and population structure changed with the introduction, we assessed individuals from 15 populations across the Hawaiian Islands and 13 populations across Puerto Rico using six to nine polymorphic microsatellite loci and five dorsolateral color patterns. Allelic richness (R_T) and gene diversity were significantly higher in Puerto Rico than in Hawaii populations. Hawaii also had fewer color patterns (2 vs. 3-5 per population) than Puerto Rico. We found no isolation by distance in the introduced range even though it exists in the native range. Results suggest extensive mixing among frog populations across Hawaii, and that their spread has been facilitated by humans. Like previous research, our results suggest that Hawaiian Coquis were founded by individuals from sites around San Juan, but unlike previous research the color pattern and molecular genetic data (nuclear and mtDNA) support two separate introductions, one on the island of Hawaii and one on Maui. Coquis are successful invaders in Hawaii despite the loss of genetic variation. Future introductions may increase genetic variation and potentially its range.

Introduction

Genetic resources, which can be an important factor in determining whether an introduced species becomes invasive, are affected by both the size and number of introductions (Lee, 2002; Lockwood *et al.*, 2005; Sakai *et al.*, 2001). When there have been few introductions and founding population sizes are small, genetic diversity of a species is often decreased in the invasive range (Dlugosch, Parker, 2008). Successive, nested founder events can also result in cumulative losses of genetic diversity (Amsellem *et al.*, 2000).

Alternatively, non-natives which arrive in repeated immigrations can have even greater genetic diversity than that observed in the native range, especially if the introductions are from different parts of a genetically-structured native range (i.e., Kang *et al.*, 2007; Kolbe *et al.*, 2004; Rius *et al.*, 2008). It is often assumed that greater genetic diversity may lead to rapid evolutionary response to selection pressure and increase invasiveness (Dlugosch, Parker, 2008). However, there are multiple examples of successful invaders with reduced genetic diversity (e.g., Dlugosch, Parker, 2008; Le Roux *et al.*, 2008), and in some cases low levels of genetic diversity have even increased invasiveness, as in the Argentine ant (Tsutsui *et al.*, 2000). Determining the relationship between genetic diversity and invasive success can elucidate the relative importance of genetic variability in the invasion process.

In addition to genetic resources, population genetic structure, and size and origin of founder populations can be important to determine. As an example, they may allow researchers to study the fate of populations that differ in genetic diversity and origin. Knowledge of how a species is introduced and spreads could inform more effective control measures. For example, eradication of local populations in highly structured population networks is likely to be more effective than in situations where gene flow among populations is high.

The nocturnal, terrestrial frog, the Coqui (*Eleutherodactylus coqui*), is endemic to Puerto Rico and was introduced to the Hawaiian Islands, USA in the late 1980s via nursery plants (Kraus, Campbell, 2002; Kraus *et al.*, 1999). Since its introduction, Coquis have continued to spread rapidly. In 1998, there were five documented populations on the island of Hawaii and three on Maui (Kraus *et al.*, 1999). By 2001, there were over 200 populations on the island of Hawaii, 36 on Maui, 14 on Oahu, and two on Kauai (Kraus, Campbell, 2002). In part due to major efforts to eradicate and control Coquis only a handful of populations outside of nurseries remain on Maui, Oahu, and Kauai at present (Beard *et al.*, 2009). However, the Coqui continues to expand its range on the island of Hawaii, with new populations discovered monthly; its range expanded from 2800 ha in 2006 to over 8000 ha in 2007 (HISC, 2007).

Because the Coqui can attain extremely high densities (up to 90,000 frogs ha^{-1}) (Beard *et al.*, 2008; Woolbright *et al.*, 2006), and consume an estimated 700,000 prey items ha^{-1} $night^{-1}$ (Beard *et al.*, 2008), the invasion threatens endemic invertebrates and associated ecosystem processes (Beard, 2007; Sin *et al.*, 2008). Earlier research has shown that Coquis can alter patterns of nutrient cycling, which in turn may benefit the invasion of non-native plants (Beard, Pitt, 2005; Sin *et al.*, 2008). From an anthropogenic perspective, the primary reason the Coqui is considered an invasive pest is because of its loud mating call (85-90 dB at 0.5 m), which exceeds levels set to minimize interference with the "enjoyment of life" (70 dB, Department of Health, Hawai'i Revised Statutes Section 324F-1).

While Coquis are assumed to be spreading primarily through nursery plants (Kraus *et al.*, 1999), and are thought to have little genetic diversity in the introduced range (Velo-Antón *et al.*, 2007), no study has reconstructed the history of the introduction across the Hawaii Islands, nor investigated nuclear genetic or phenotypic variation in Hawaii. In this study, we expand on the

existing knowledge of the introduction by first quantifying color pattern variation of the Coqui across the Hawaiian Islands. Coquis exhibit a wide range of color patterns in Puerto Rico (Woolbright, 2005; Woolbright, Stewart, 2008), which have been shown to be genetically determined, following a single autosomal locus, five-allele model with unstriped recessive to all other codominant patterns (O'Neill, Beard, in review). Second, mtDNA cytochrome b haplotype data for three populations on the island of Hawaii reveal little genetic diversity and suggest the Hawaiian populations originate from populations near San Juan in Puerto Rico (Velo-Antón *et al.*, 2007). We expanded on this study by investigating populations across all four main islands (Hawaii, Oahu, Kauai, and Maui) using known nuclear genetic markers (Peters *et al.*, 2008) and by sequencing additional individuals at cytochrome b from Maui (Velo-Antón *et al.* (2007).

In summary, we used phenotypic variation in color patterns, multilocus nuclear microsatellite, and mtDNA cytochrome b haplotype data to examine the entry and spread of Coqui frogs across the Hawaiian Islands. Specifically, we used these data to determine (1) the level of genetic diversity in these populations; (2) the distribution of this diversity within and among populations; (3) similarities in genetic diversity between Hawaiian and Puerto Rican populations; and (4) the extent to which microsatellite allele frequencies and mtDNA haplotype diversity in Hawaii match potential donor populations in the native range.

METHODS

Field sampling and color patterns

Frogs were sampled from study sites in Puerto Rico (n = 13) and Hawaii (n = 15) that were chosen to maximize the geographic and elevational coverage within each range (Table 1). Plots were generally located in closed canopy forests with moderate to heavy understory of herbaceous

and/or woody vegetation. In Hawaii, our sampling focused on the island of Hawaii where the majority of populations occur, but also included two populations from Maui, two from Oahu, and one from Kauai (Beard, Pitt, 2005). At the time of the study, few wild populations remained on Maui, but the largest, Maliko Gulch (MMG), was sampled. Wild populations had been eradicated on Oahu and therefore we were restricted to including frogs collected in nurseries. The one wild population left on Kauai was included here.

To collect frogs at each site, we established $20 \times 20m^2$ plots with four, 5m strips, following Woolbright (2005). Beginning at dusk, around 1900 h, two people surveyed each transect and hand-captured all adult frogs (> 25mm) for 15 min, not including handling time, for a minimum total of 120 person-min per plot. If necessary to collect at least 20 individuals, after surveying each plot, we searched adjacent areas, up to 50m in each direction, and returned subsequent nights. We did not collect pre-adults to reduce the possibility of collecting siblings. For each frog captured, we collected tissue samples in the form of toe-clips (in 95% ethanol). We scored dorsal patterns at the time of capture using the four striped and unstriped patterns described by Woolbright (2005). The only frogs that were not collected in this manner were those from Oahu and Kauai. These frogs were frozen and overnight mailed to Utah State University by managers working on those islands, and were scored and toe-clipped upon arrival.

DNA Extraction, Molecular Markers and PCR amplification

DNA was extracted from 664 frogs from 26 locations [12 in Puerto Rico (no samples were available from GL for genetic analysis) and 14 in Hawaii (no samples were available from KP for genetic analysis)] using a Chelex-100® extraction method (Walsh *et al.*, 1991) with some modifications. One toe from each individual was placed in 200µl of a digestion buffer (0.2%

129 Sodium Doecyl Sulphate, 10mM Tris pH=8, 0.5mM EDTA pH=8) and 40mg proteinase K added
130 to each sample. Samples were allowed to digest at least 24 h at room temperature. Fifty mg of
131 Chelex-100® resin (Biorad) was then added to each sample and allowed to digest at 65°C with
132 agitation for 1-2 h. Following digestion, samples were placed at -20°C overnight before
133 centrifugation at 3000 x g for 2 min. Supernatant containing DNA was subsequently diluted to
134 ~40ng with TLE (10mM Tris pH=8, 0.1mM EDTA).
135 We used nine microsatellite loci that had been developed from Coqui frog enriched
136 genomic libraries for the Hawaii populations and six of these loci for the Puerto Rico populations
137 (Peters *et al.*, 2008). We chose loci that did not have systematic patterns of null alleles, allelic
138 dropout, or linkage disequilibrium across all sampling locations (Coq10, Coq20, Coq27, Coq 28,
139 Coq31, Coq203, Coq219, Coq 221, and Coq224, MICROCHECKER version 2.2.3, van
140 Oosterhout *et al.*, 2004). Because Coq28, 219 and 221 were out of Hardy-Weinberg equilibrium
141 (HWE) in many of the Puerto Rico populations we did not use these loci for the Puerto Rico
142 analyses. Specific PCR conditions and characteristics of the nine microsatellite loci are
143 described in Peters *et al.* (2008). Using a three primer reaction, each locus was tagged with a
144 complementary fluorescently labeled primer: Fam (Integrated DNA Technologies), Vic, or Ned
145 (Applied Biosystems). Following PCR, loci tagged with three different labels were combined
146 and run on an ABI-3130xl automated sequencer using Naurox size standard. Results were
147 analyzed in Genemapper version 4.0 (Applied Biosystems).
148
149 **Statistical Analyses**
150 *Genetic Variation*
151 We used FSTAT (version 2.9.3.2; Goudet, 1995) and Genepop (Genepop v3.4; Raymond,

Rousset, 1995) to assess deviations from HWE (F_{IS}), calculate expected (H_E) heterozygosity and allelic richness (R_S) per locus per sampling location and per locus across populations (R_T). We also conducted a Mantel test to test for an isolation-by-distance pattern across the Hawaiian Islands and Puerto Rico using the ISOLDE option in Genepop (v3.4) and $F_{ST}/(1-F_{ST})$ as the dependent variable.

Population Genetic Structure

To assess genetic population structure, we used a Bayesian genotype clustering methodology (STRUCTURE, version 2.0, Pritchard *et al.*, 2000). The grouping criteria based upon multilocus genotypes includes HWE and gametic phase equilibrium between loci within groups. In STRUCTURE, we used an admixture model where individuals with novel genotypes can be identified and assigned to a genotype cluster. We specified an initial range of potential genotype clusters (k) based upon the number of sampling locations. We specified a 50,000 iteration burn-in period followed by ten 100,000 Markov Chain Monte Carlo replicates per k to approximate posterior allelic distributions. Individual multilocus genotypes were then compared to the posterior allelic distributions and assigned to a cluster according to the HWE criteria (Pritchard *et al.*, 2000). We used the Δk method of Evanno *et al.* (2005) to determine the optimal number of genotype clusters for Hawaii and Puerto Rico separately. This method calculates the largest change in the LnP(D) between each any pair of k and k-1 for all tests of k. Evanno *et al.* (2005) demonstrated through simulation modeling that Δk (defined as the second order rate of change of the likelihood function with respect to k) shows a clear peak at the true value of k.

Determining Source Populations in Puerto Rico for Hawaiian Island Coqui Frogs

We conducted two analyses to determine the most likely source populations for Coquis introduced to Hawaii from Puerto Rico. First, we conducted a phylogenetic analysis using the microsatellite data, Cavalli-Sforza genetic distance and neighbor-joining tree-building algorithm (POPULATIONS, version 1.2.26, http://www.cnrs-gif.fr/pge) as follows: (1) Puerto Rico sites grouped according to Bayesian genotype cluster analysis and mtDNA cytochrome b clade membership (Velo-Antón *et al.* 2007) and Hawaiian sites grouped by sampling location, and (2) Puerto Rico sites grouped as above with Hawaiian sites grouped by island. Phylogenetic trees were visualized using the program TREEVIEW, version 1.6 (http://taxonomy.zoology/gla.ac.uk/rod/rod.html). We used the six microsatellite loci used in the Puerto Rico analyses only to build these phylogenies.

Second, because frogs from the Maui MMG site were phenotypically distinct (see results), we compared sequence data for the mtDNA cytochrome b gene from Velo-Antón *et al.* (2007) to sequence data generated for 12 individuals collected from the Maui MMG population. PCR was used to amplify 646 bp fragment from the cytochrome b gene using the MVZ15 and MVZ16 primers (Velo-Antón *et al.*, 2007). PCRs were done in a 25µl reaction volume containing 0.2µl Titanium taq, 1x Titanium buffer with 3.5mM MgCl2, 0.25mM each primer, 0.2mM DNTPs, and molecular grade water. The reaction included 33 cycles of 94^{o}C for 30s, 55^{o}C for 60s, and 72^{o}C for 30s, followed by a 10 minute extension at 72^{o}C. PCR products were cleaned up using QIAquick PCR Purification Kit (QIAGEN) and product was sequenced at the Nevada Genomics Center on an Applied Biosystem 3730 DNA Analyzer. Sequences were imported into Geneious Pro 3.8.5 and aligned and edited to a 646bp segment corresponding to the sequences reported in the Velo-Antón *et al.* (2007) study. Sequences were then aligned and compared for point mutations.

198

199 **RESULTS**

200 *Phenotypic Variation*

201 Hawaii populations had no more than two color patterns [2.0 (± 0.0 SE) alleles per population]
202 and Puerto Rico populations had three to five color patterns [4.4 (± 0.2 SE) alleles per
203 population] (Table 2).

204

205 *Genetic Variation*

206 Deviations from HWE were observed at multiple loci and sampling locations in Puerto Rico, but
207 few deviations were observed in Hawaii. There were no systematic deviations for loci across all
208 populations or at all loci within populations (Table 3). The total number of alleles varied from 3-
209 20 in Hawaii and from 5-41 in Puerto Rico at the same loci. Overall gene diversity (H_T) per
210 locus was significantly lower in Hawaii (0.513) than in Puerto Rico (0.750) (t = 5.913,
211 Bonferonni adjusted $P = 0.000$; Table 4). The lowest levels of expected heterozygosity were
212 recorded for the two Maui sampling locations (MMG and MKN), 0.385 and 0.283, respectively.
213 Allelic richness (R_T) per locus was also significantly lower in Hawaii than Puerto Rico (t =
214 5.178, Bonferonni adjusted $P = 0.001$). The MMG site had alleles at three loci that were not
215 found in any other population sampled in the Hawaiian Islands (Coq20, *236*; Coq31, *269*;
216 Coq219, *292* and *296*). The only other location the Coq219, *296* allele was found was in the San
217 Juan (SJ) lowlands population in Puerto Rico.

218

219 *Population Genetic Structure*

220 Puerto Rico. Two genotype clusters was the best fit of the data for the 12 sampling locations in

Puerto Rico with k=4 the next best fit (Fig. 1). Individuals from the sampling locations MAL-TNH, which corresponds to the Central-Western mountainous region as described by Velo-Antón *et al.* (2007), assign almost exclusively to one of the two genotype clusters in the k=2 analysis (Fig. 2). While individuals from the TNL site south of the Central-Western mountainous region and sites found in the headwaters and east of the Rio Grande de Loiza River, assign primarily to the other genotype cluster. Individuals from the SJ site in the lowland area are a mixture of individuals that assign to each of two genotype clusters. When the data are constrained to fit k=4, the MAL-TNH sampling sites continue to assign to a single distinct genotype cluster while the TNL-EYL sites found south of the Central-Western mountainous region, the headwaters and east of the Rio Grande de Loiza River assign to two genotype clusters. The proportional membership in these two clusters changes as you move down the watershed to the coast. The SJ site assigns to the fourth genotype cluster to which very few individuals from the other sampling locations assign. This pattern corresponds with the three major groupings of cytochrome b haplotypes from the Velo-Antón *et al.* (2007) study. We also observed a significant pattern of isolation-by-distance across the island (Mantel test, P = 0.002; Fig. 3a).

Hawaii. The best fit of the data for the Hawaiian island sampling sites was two genotype clusters (k=2). We also present the results for k=3 for which there is statistically support based upon the Δk analysis (see Fig. 1). Overall the pattern of cluster membership suggests extensive mixing of gene pools across the sampling sites within and among islands with the exception of the MMG site (Fig. 4). Concomitantly, we did not observe a significant pattern of isolation-by-distance across the islands (Mantel test, P = 0.25; see Fig. 3b).

244

245 *Phylogenetic Relationships*

246 The phylogeny for the Hawaiian Island sites based upon sampling location was largely
247 unresolved due to very low bootstrap support. When grouped by island, the Hawaiian Islands
248 formed a single monophyletic clade, which then grouped with the lowland site SJ in Puerto Rico
249 (Fig. 5). The RAL, TNL, and CH Puerto Rico sites, which had been not included in Velo-Antón
250 *et al.* (2007), were kept separate in the analysis and grouped with spatially proximate
251 populations.

252

253 *Mitochondrial Sequence Comparisons*

254 There was a single mtDNA cytochrome b haplotype for all 12 samples from Maui MMG site.
255 The Maui sequence differed from the island of Hawaii sequence (IL) by a single nucleotide
256 substitution (Table 6). Both Hawaiian Island haplotypes (IL and Maui) were most similar to the
257 XLV haplotype from the lowlands area of Puerto Rico (Velo-Antón *et al.*, 2007) differing from
258 this haplotype by only two nucleotide substitutions. The IL haplotype differs from both the Maui
259 and XLV Puerto Rico haplotype at the same single nucleotide while the Maui haplotype differed
260 from both the IL and XLV at an additional nucleotide.

261

262 **DISCUSSION**

263 Both the color pattern and genetic data (nuclear and mitochondrial) support that the Hawaiian
264 populations are significantly less variable in terms of average heterozygosity and more
265 importantly allelic richness when compared to populations sampled in the native range of Puerto
266 Rico. The Coqui frog shows a wide variety of color patterns across its native range; up to five

unique stripe patterns and combinations of stripes were observed in most populations, but only two strip variants were observed in all of the Hawaiian populations. Clearly the invasion success of this species does not appear to be dependent on high levels of genetic variation, although we did not measure variation at adaptive traits. The results of this study suggest that very small populations in nursery plants can establish large, stable demographic populations that can eventually spread over large areas.

Although low bootstrap support for the tree topology of the Puerto Rico clades (57) makes us unable to clearly resolve the relationship among these clades using microsatellite data, the internal nodes have much higher bootstrap support including the node grouping the Hawaii sites with the San Juan site. However, even this bootstrap value (77) is still relatively low, which can be partially attributed to the small sample size from the San Juan site (N=22) (but see below). Overall, the concordance between the cytochrome b haplotype (Velo-Antón *et al.* 2007) and microsatellite phylogenetic trees does allow for robust conclusions concerning the origin of the Hawaiian Coqui populations.

The phylogenetic analyses, mtDNA sequence data, and color pattern polymorphism support two separate introductions into the Hawaiian Islands: one on the island of Hawaii and one on Maui. The Maui MMG site has a number of alleles that are not found in any of the other Hawaii populations and one allele at the Coq219 locus that was found in only one other location, the San Juan site in Puerto Rico. We also report a previously undescribed mtDNA cytochrome b haplotype found in the MMG population, a site not sampled by Velo-Antón *et al.* (2007). Finally, every population in Hawaii except MMG had only the single narrow stripe morph (L) or the unstriped morph (U), whereas MMG had the interoccular bar (B) phenotype, strongly supporting the idea that MMG was a separate introduction. The marked differences in

290 morphology and genetic data suggest that populations in the lowlands area around San Juan
291 could be spatially structured. More specifically, it would appear that these two introductions
292 were derived from two separate populations near San Juan. Sampling for genetic analyses was
293 confined to a single site around San Juan, so we cannot test the spatial structure hypothesis here.
294 It had been unclear from the available data whether Coquis were first introduced to the
295 island of Hawaii or Maui (Kraus *et al.*, 1999). The genetic data for all sites sampled (except
296 MMG) reveal extensive gene flow suggesting that enough time has passed for frogs to become
297 established on all islands via human-mediated movements. Conversely, the Maui site is very
298 distinct genetically from the other collection sites in Hawaii and shows no evidence of mixing
299 with any other population sampled on the islands at this time. As a result, we believe these data
300 support the hypothesis that the island of Hawaii was the first introduction site.
301 Bayesian genotype clustering results and few significant inbreeding values (F_{IS}) suggest
302 there was widespread mixing of frogs once they were transplanted to Hawaii. However, the
303 genetic signature of bottleneck or founder events can remain in populations long after increases
304 in demographic census size (Caro, Laurenson, 1994; Weber *et al.*, 2000), which is reflected here
305 in the low levels of genetic variation in the Hawaiian populations. It is likely Coqui populations
306 grew quickly once they arrived in Hawaii because they are prolific breeders; breeding year-
307 round, with relatively large clutches, and require less than a year to reach sexual maturity
308 (Stewart, Woolbright, 1996; Townsend, Stewart, 1994).
309 The extensive mixing among frog populations in Hawaii is in contrast with the
310 population genetic structure found in the native range in Puerto Rico, which shows a significant
311 isolation-by-distance pattern across the island and an overall site specific pattern of membership
312 for individual genotype clusters. The widespread occurrence of Coqui frogs in the Hawaiian

Islands, particularly on the island of Hawaii, has clearly been facilitated by movement of frogs by humans. We know that some of this spread has occurred through the sale and movement of nursery plants. Some movement is known to have been intentionally done by humans [e.g., Akaka Falls State Park (AK) and Manuka Natural Area Reserve (MP); W. Pitt, pers. comm.], but some of this spread might be occurring through other means (such as vehicular movement or natural dispersion).

Broader Implications

Ecosystems worldwide have been highly disrupted by the introduction of non-native taxa with island ecosystems being particularly vulnerable (Englund, 2008; Parker *et al.*, 1999). When Coquis were introduced into the Hawaiian Islands, 23 of the 71 known endemic Hawaiian birds were already extinct while 30 of the remaining 48 species and subspecies were listed as endangered or threatened under the U. S. Endangered Species Act (Boyer, 2008; Leonard, 2008). Today approximately half of all organisms in Hawaii are non-native (Wagner *et al.*, 1999) and the majority of intact native ecosystems are found at high elevations in alpine and subalpine habitats (The Nature Conservancy, http://www.hawaiiecoregionplan.info/ecoregion.html).

Prior to the introduction of multiple frog and toad species over the past century, there were no amphibians on the Hawaiian Islands (Kraus, 2003). Removal of well entrenched non-native populations represents one of the biggest challenges to recovery of threatened and endangered species (Peacock, Kirchoff, 2004; Vander Zanden *et al.*, 2004). There has been a tremendous effort to removal and control Coqui frogs from the Hawaiian Islands, primarily with the use of chemical pesticides. The cost to public agencies for Coqui management exceeded $4 million in 2007 and on the island of Hawaii alone was $2.8 million (HISC, 2007). At the time of

writing, in part due to these efforts, Maui has only a handful of populations and one relatively large population (MMG site); Oahu has only one wild population and Kauai has only one small population that is close to eradication. Island-wide eradication is thought possible on these islands. On the island of Hawaii, where Coquis are not considered eradicable, control efforts are focused on treating small isolated populations. The results of our study suggest that with the amount of movement occurring between populations keeping areas free of Coquis requires vigilance.

The pronounced genetic distinctiveness of the Maui MMG site strongly suggests that this is a very recent introduction. The lack of gene flow among sites within Maui also suggests there could be undescribed barriers to movement either natural or human mediated into and out of the MMG population. This possibility deserves further exploration. The Maui MMG Coqui frog introduction offers an opportunity to track the spread of frogs into and out of this population by characterizing dispersal patterns, both human-mediated and natural movements, in order to identify dispersal rate, direction, and corridors. The unique mtDNA cytochrome b haplotype and microsatellite alleles in this population permit tracking of individuals across the landscape via genetic assignment tests. These data can then be used to develop realistic control programs aimed at keeping frogs out of undisturbed habitats based upon how frogs move or are moved on the landscape. Genetic monitoring is now being used to track movements and population size of threatened and endangered species (Janečka *et al.*, 2008; Kang *et al.*, 2008). Here we suggest this same approach can be used to (1) track the spread of Coqui frog from this discrete second introduction, and (2) as an effectiveness monitoring tool for control strategies.

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366
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Table 1. Populations of *Eleutherodactylus coqui* sampled including sample sizes, mtDNA cytochrome b haplotype clade association, elevation, and date sampled.

Site	Island	Site Abbrev	Color Pattern (n)	Msat Analysis (n)	Clade*	Elevation (m)	Date Sampled
Cayey High	Puerto Rico	CH	28	32	E	865	May-06
Cayey Low	Puerto Rico	CL	28	25	E	232	May-06
El Yunque High	Puerto Rico	EYH	58	25	E	714	May-06
El Yunque Low	Puerto Rico	EYL	84	25	E	198	May-06
Guilarte High	Puerto Rico	GH	59	23	W	995	May-06
Guilarte Low	Puerto Rico	GL	16	0	W	150	May-06
Los Piedras	Puerto Rico	LP	36	24	E	117	May-06
Maricao	Puerto Rico	MA	42	29	W	315	Mar-06
Rio Abajo High	Puerto Rico	RAH	51	25	W	714	May-06
Rio Abajo Low	Puerto Rico	RAL	64	26	W	80	May-06
San Juan	Puerto Rico	SJ	0	25	E	29	Mar-07
Toro Negro High	Puerto Rico	TNH	32	25	W	978	May-06
Toro Negro Low	Puerto Rico	TNL	29	25	W	241	May-06
Akaka Falls State Park	Hawaii	AK	99	26	-	405	Nov-06
Glenwood	Hawaii	GW	119	26	E	766	Jun-06
Humane Society	Hawaii	HS	232	25	-	135	Nov-06
Kalopa State Park	Hawaii	KP	57	0	-	610	Oct-05
Kona High	Hawaii	KH	53	38	-	952	Jun-06
Kona Low	Hawaii	KL	47	30	-	265	Jun-06
Lava Tree State Park	Hawaii	LT	372	28	E	192	Jun-06
Manuka State Park	Hawaii	MP	35	27	E	560	Nov-06
Puainako	Hawaii	PK	322	24	-	45	Nov-06
Waipio Overlook	Hawaii	OL	130	25	-	300	Nov-06
Lawai	Kauai	LW	32	24	-	130	Jun-07
Kihei Nursery	Maui	MKN	43	23	-	15	Aug-04
Maliko Gulch	Maui	MMG	113	26	-	440	Aug-04
Hawaii Hai Nursery	Oahu	LH	22	19	-	30	Feb-07
Waimanalo Nursery	Oahu	CAL	24	14	-	15	Feb-07

*Clade based on geographic overlap with eastern and western clades in Velo-Antón *et al.*, 2007.

475 Table 2. Color patterns observed at each study site. B = interoccular bar; L = dorsolateral
476 stripes; N = narrow middorsal stripe; U = unstriped; and W = wide middorsal stripe.

Site	Island	Color pattern
Cayey High	Puerto Rico	L, N, U
Cayey Low	Puerto Rico	L, N, U
El Yunque High	Puerto Rico	B, L, N, U, W
El Yunque Low	Puerto Rico	B, L, N, U, W
Guilarte High	Puerto Rico	B, L, N, U, W
Guilarte Low	Puerto Rico	L, N, U, W
Los Piedras	Puerto Rico	B, L, N, U, W
Maricao	Puerto Rico	B, L, N, U, W
Rio Abajo High	Puerto Rico	B, L, N, U, W
Rio Abajo Low	Puerto Rico	B, L, N, U
Toro Negro High	Puerto Rico	B, L, N, U, W
Toro Negro Low	Puerto Rico	B, L, N, U
Akaka Falls State Park	Hawaii	N,U
Glenwood	Hawaii	N,U
Humane Society	Hawaii	N,U
Kalopa State Park	Hawaii	N,U
Kona High	Hawaii	N,U
Kona Low	Hawaii	N,U
Lava Tree State Park	Hawaii	N,U
Manuka State Park	Hawaii	N,U
Puainako	Hawaii	N,U
Waipio Overlook	Hawaii	N,U
Lawai	Kauai	N,U
Kihei Nursery	Maui	N,U
Maliko Gulch	Maui	**B**,U
Hawaii Hai Nursery	Oahu	N,U
Waimanalo Nursery	Oahu	N,U

Table 3. F_{IS} values per locus per (A) sampling site in Puerto Rico and (B) sampling sites grouped per island in Hawaii. Italicized and bolded values are significant at $P = 0.00069$ (adjusted for multiple comparisons obtained after 1440 randomizations). NA = not available. Coq28, 221, and 224 were not used in the Puerto Rico analyses due to deviations from HWE at most sampling locations.

A.	Central-Western					Eastern					Lowlands
	MA	GH	RAH	TNH	RAL	TNL	CH	CL	LP	EYH	EYL	SJ
Coq10	0.152	-0.003	-0.008	0.247	0.001	0.323	0.018	0.41	0.5	0.425	-0.021	***0.404***
Coq20	0.213	-0.046	-0.139	0.107	-0.064	-0.064	-0.063	-0.008	-0.097	0.422	0.028	***0.601***
Coq27	-0.051	0.239	-0.496	-0.29	0.054	0.166	***0.544***	0.269	0.59	0.47	0.385	***0.826***
Coq31	0.053	-0.234	-0.094	***0.514***	0.015	0.271	0.051	0.066	-0.108	0.25	0.16	0.027
Coq203	-0.303	-0.097	0.255	0.377	0.172	0.347	0.005	0.578	0.454	0.472	-0.215	0.292
Coq224	0.084	0.114	0.067	0.04	-0.224	0.481	-0.047	***-0.043***	***0***	-0.043	-0.846	0

B.	Hawaiian Islands				
	Hawaii	Maui		Oahu	Kauai
		MKN	MMG		
Coq10	0.127	-0.011	-0.176	-0.052	0.326
Coq20	0.085	0	0	-0.28	0.181
Coq27	-0.102	0.254	-0.228	-0.093	-0.294
Coq28	0.037	0.318	NA	0.084	0.011
Coq31	***0.165***	0.342	0.621	-0.259	0.862
Coq203	-0.029	0	-0.064	-0.103	0.489
Coq219	-0.013	-0.094	***0.677***	-0.157	-0.144
Coq221	0.038	0.058	-0.063	-0.105	-0.094
Coq224	-0.065	NA	-0.087	-0.07	NA

Table 4. Gene diversity (expected H_E) and allelic Richness (R_S) per locus and sampling location and per locus across all populations (R_T) for both Puerto Rico and the Hawaiian Islands populations. Gene diversity of the two Maui populations (MKN and MMG) are the lowest of all populations sampled (highlighted).

Gene Diversity

	Puerto Rico											
N	*29*	*23*	*25*	*26*	*25*	*25*	*32*	*25*	*24*	*25*	*25*	*25*
Locus	MA	GH	RAH	RAL	TNH	TNL	CH	CL	LP	EYH	EYL	SJ
Coq10	0.879	0.781	0.863	0.871	0.476	0.763	0.445	0.511	0.082	0.449	0.118	0.907
Coq20	0.697	0.541	0.704	0.688	0.804	0.640	0.471	0.516	0.571	0.549	0.452	0.822
Coq27	0.702	0.739	0.675	0.802	0.718	0.797	0.747	0.870	0.573	0.856	0.775	0.745
Coq28	0.628	0.600	0.614	0.152	0.792	0.833	0.768	0.662	0.674	0.790	0.616	0.737
Coq31	0.785	0.725	0.660	0.703	0.815	0.853	0.877	0.856	0.669	0.783	0.807	0.595
Coq203	0.631	0.706	0.612	0.556	0.255	0.669	0.722	0.656	0.755	0.600	0.761	0.765
Coq219	0.512	0.800	0.958	0.874	0.332	0.938	0.950	0.388	0.889	0.953	0.962	0.888
Coq221	0.926	0.934	0.947	0.931	0.922	0.958	0.930	0.931	0.926	0.950	0.943	0.806
Coq224	0.585	0.294	0.471	0.631	0.458	0.153	0.149	0.115	0.042	0.115	0.507	0.040
Average	**0.703**	**0.748**	**0.757**	**0.667**	**0.737**	**0.698**	**0.743**	**0.683**	**0.650**	**0.732**	**0.722**	**0.716**

	Hawaii													
N	*24*	*14*	*19*	*26*	*23*	*25*	*26*	*24*	*25*	*28*	*26*	*27*	*30*	*38*
Locus	LW	CAL	LH	MMG	MKN	OL	AK	PK	HS	LTK	GW	MP	KL	KH
Coq10	0.307	0.206	0.152	0.558	0.086	0.117	0.113	0	0	0.137	0.212	0.073	0.066	0
Coq20	0.507	0.452	0.273	0.038	0.043	0.301	0.510	0.422	0.246	0.299	0.038	0.230	0.503	0.478
Coq27	0.372	0.415	0.428	0.566	0.348	0.675	0.474	0.601	0.554	0.620	0.180	0.371	0.437	0.569
Coq28	0.606	0.542	0.568	0	0.127	0.656	0.654	0.386	0.597	0.670	0.597	0.686	0.654	0.619
Coq31	0.782	0.669	0.770	0.744	0.733	0.492	0.601	0.597	0.668	0.549	0.401	0.694	0.729	0.646
Coq203	0.403	0.474	0.363	0.145	0.043	0.316	0.540	0.573	0.500	0.556	0.271	0.108	0.693	0.641
Coq219	0.496	0.754	0.733	0.587	0.677	0.683	0.673	0.351	0.602	0.678	0.629	0.682	0.749	0.745
Coq221	0.623	0.731	0.686	0.646	0.493	0.813	0.651	0.717	0.760	0.741	0.684	0.737	0.759	0.727
Coq224	0	0.204	0.149	0.177	0	0.187	0.265	0.042	0	0.169	0.280	0.112	0.066	0.149
Average	**0.455**	**0.494**	**0.458**	**0.385**	**0.283**	**0.471**	**0.498**	**0.410**	**0.436**	**0.491**	**0.366**	**0.410**	**0.517**	**0.508**

Allele Richness (based on minimum sample size of 12 diploid individuals)

	Puerto Rico												
Locus	MA	GH	RAH	RAL	TNH	TNL	CH	CL	LP	EYH	EYL	SJ	R_T
Coq10	9.556	6.585	7.864	8.556	5.487	6.340	4.282	6.239	2.000	5.329	2.440	13.18	8.344
Coq20	4.913	4.718	3.995	4.691	6.321	4.439	4.707	3.449	3.932	5.483	6.217	9.620	6.455
Coq27	4.649	4.477	4.344	7.760	6.177	6.583	5.884	9.554	5.813	8.087	6.776	5.929	8.500
Coq28	5.069	2.980	4.638	1.957	6.205	5.000	5.183	3.895	4.749	5.812	5.432	5.361	5.615
Coq31	5.490	6.531	4.664	4.771	6.505	8.237	7.741	7.352	5.274	6.049	7.176	7.124	7.486
Coq203	4.057	5.258	5.485	5.197	2.929	5.017	5.597	3.934	5.700	4.855	5.641	6.965	7.089
Coq219	5.038	9.786	13.856	9.793	4.349	12.009	13.552	4.722	9.288	14.349	14.871	11.625	13.023
Coq221	11.525	12.786	13.732	13.089	11.094	15.042	13.062	12.081	11.899	14.867	13.927	10.400	15.075
Coq224	3.467	1.999	2.48	3.833	2.000	2.347	2.376	1.867	1.500	1.867	2.000	1.480	3.222

	Hawaii														
	LW	CAL	LH	MMG	MKN	OL	AK	PK	HS	LTK	GW	MP	KL	KH	R_T
Coq10	4.643	3.786	2.590	2.999	2.13	2.295	2.500	1.000	1.000	2.318	2.475	1.963	1.683	1.000	3.225
Coq20	2.000	2.000	2.000	1.500	1.565	1.999	2.000	2.000	1.997	1.999	1.500	1.993	2.000	2.000	2.037
Coq27	2.000	2.997	2.974	3.637	2.000	3.895	2.995	3.519	3.512	3.463	2.445	2.867	2.433	3.269	3.282
Coq28	3.000	3.000	2.983	1.000	2.382	4.081	3.755	2.953	2.980	3.928	2.995	3.963	3.000	3.000	3.296
Coq31	6.067	4.414	5.791	4.847	3.981	3.415	3.883	3.386	3.000	2.986	2.000	3.632	3.997	3.785	5.500
Coq203	2.000	2.929	2.972	1.945	1.565	2.888	2.755	3.519	3.511	3.182	2.495	2.217	3.949	3.566	3.312
Coq219	2.925	4.000	3.998	4.998	4.616	4.51	4.857	2.773	3.934	3.717	3.495	4.346	4.429	4.813	4.797
Coq221	3.000	4.929	4.702	3.000	3.616	5.993	4.524	3.954	5.291	5.952	3.500	3.998	5.243	4.343	6.603
Coq224	1.000	2.926	1.974	1.975	1.000	2.474	1.998	1.542	1.000	2.571	3.327	2.255	1.683	2.227	2.228

Table 5. Maui MMG Cytochrome b sequence (Genbank Accession # GQ267467) compared to IL haplotype from the Big Island Hawaii (Velo-Antón *et al.* 2007) and the XLV haplotype from the lowland area in Puerto Rico (Velo-Antón *et al.* 2007). There is one base pair difference between the Maui and Big Island haplotypes and two base pair differences between the Maui and the Puerto Rico haplotype (outlined).

Coqui Maui 1	gaaactttggctccttattaggcatctgccttattacccaagtcgccacaggactcttcc
ef637002.xlv	gaaactttggctccttattaggcatctgccttattacccaagtcgccacaggactcttcc
ef637028.il	gaaactttggctccttattaggcatctgccttattacccaagtcgccacaggactcttcc
Coqui Maui 1	tggctatacactactctgcagacacatcactagccttctcatccatcgctcatatctgcc
ef637002.xlv	tggctatacactactctgcagacacatcactagccttctcatccatcgctcatatctgcc
ef637028.il	tggctatacactactctgcagacacatctctagccttctcatccatcgctcatatctgcc
Coqui Maui 1	gagacgttaataacggatggcttctgcgtaatcttcacgcaaacggcgcctcatttttt
ef637002.xlv	gagacgttaataacggatggcttctgcgtaatcttcacgcaaacggcgcctcatttttt
ef637028.il	gagacgttaataacggatggcttctgcgtaatcttcacgcaaacggcgcctcatttttt
Coqui Maui 1	ttatctgtatttaccttcacattggacggggtctttactatggctccttcttatttctag
ef637002.xlv	ttatctgtatttaccttcacattggacggggtctttactatggctccttcttatttctag
ef637028.il	ttatctgtatttaccttcacattggacggggtctttactatggctccttcttatttctag
Coqui Maui 1	aaacctgaaatattggagttattctgctactcctcaccatagccacagcatttgtggggt
ef637002.xlv	aaacctgaaatattggagttattctactactcctcaccatagccacagcatttgtggggt
ef637028.il	aaacctgaaatattggagttattctgctactcctcaccatagccacagcatttgtggggt
Coqui Maui 1	atgtcctcccatgaggacagatatcattttgaggtgccacagtcattaccaatcttctat
ef637002.xlv	atgtcctcccatgaggacagatatcattttgaggtgccacagtcattaccaatcttctat
ef637028.il	atgtcctcccatgaggacagatatcattttgaggtgccacagtcattaccaatcttctat
Coqui Maui 1	ctgccgcaccctatattggaacaaatttagtacaatggatttgaggcggattttctgtag
ef637002.xlv	ctgccgcaccctatattggaacaaatttagtacaatggatttgaggcggattttctgtag
ef637028.il	ctgccgcaccctatattggaacaaatttagtacaatggatttgaggcggattttctgtag
Coqui Maui 1	acaatgctaccctcacccgatttttcacctttcacttcattctcccatttgttattattg
ef637002.xlv	acaatgctaccctcacccgatttttcacctttcacttcattctcccatttgttattattg
ef637028.il	acaatgctaccctcacccgatttttcacctttcacttcattctcccatttgttattattg
Coqui Maui 1	gggcaaccgctctccacctgctttttctacacgaaacaggatcttccaaccccacaggac
ef637002.xlv	gggcaaccgctctccacctgctttttctacacgaaacaggatcttccaaccccacaggac
ef637028.il	gggcaaccgctctccacctgctttttctacacgaaacaggatcttccaaccccacaggac
Coqui Maui 1	ttaactctaacctagacaaagttcaatttcacaccttcttttcctacaaagatatccttg
ef637002.xlv	ttaactctaacctagacaaagttcaatttcacaccttcttttcctacaaagatatccttg
ef637028.il	ttaactctaacctagacaaagttcaatttcacaccttcttttcctacaaagatatccttg
Coqui Maui 1	gatttgccattctcttcaccctcctatccttagtttccacattttt

71	ef637002.xlv	gatttgccatttcttcaccctcctatccttagtttccacattttt
72	ef637028.il	gatttgccattctcttcaccctcctatccttagtttccacattttt

FIGURE LEGENDS

Fig. 1. (A) The mean LnP(D) and SD of 10 runs conducted for each *k* for the multilocus genotype data generated for the Puerto Rico sampling sites. Although the standard deviations are plotted for each mean they are too small to be observed at the scale necessary to plot the LnP(D). (B) The mean LnP(D) and SD of 10 runs conducted for each *k* for the multilocus genotype data generated for the Hawaii sampling sites. (C) Delta *k* (Δk) as per Evanno *et al.* (2005) plotted against number of genotype clusters (*k*) (see text), where *k*=2 is the best fit of the data followed by *k*=4 for the Puerto Rico sampling sites. (D) Delta *k* (Δk) as per Evanno *et al.* (2005) plotted against number of genotype clusters (*k*) (see text), where *k*=2 is the best fit of the data followed by *k*=3 for the Hawaii sampling sites.

Fig. 2. (A) Map of Puerto Rico with all sampling locations indicated and showing the location of the Rio Grande de Loiza River. No samples were available from site GL for genetic analysis. (B) Bayesian genotype clustering results for *k*=2 showing clear geographic structure among sampling locations grouped by landscape features and for *k*=4 showing the lowlands SJ site as a genetically distinct breeding group (see text).

Fig. 3. Results of the isolation-by-distance analysis for (A) Puerto Rico and (B) Hawaii. The pattern was significant for Puerto Rico [$P = 0.002$, $R^2 = 0.416$, $Y = 0.0732X - 0.1359$)], but not for Hawaii ($P = 0.25$, $R^2 = 0.0204$, $y = 0.0254x + 0.0562$).

Fig. 4. (A) Map of Hawaii with all sampling locations indicated. No samples were available from site KP for genetic analysis. (B) Bayesian genotype clustering results for *k*=2 and for *k*=3 the next best fit of the data. The first shows MMG to be genetically distinct from all other sampling locations in Hawaii, which collectively represent a single genotype cluster. The second shows again shows MMG as distinct and individuals from sites MKN (Maui) and Lawai (Kawai) as assigning primarily to single genotype clusters.

Fig. 5. Neighbor-joining phylogenetic tree constructed using multilocus microsatellite data and a Cavalli-Sforza genetic distance metric. The Puerto Rico populations were grouped according to Bayesian genotype clustering results and mtDNA clade membership for cytochrome b haplotype data generated by Velo-Antón *et al.* (2007). The Hawaii samples were grouped by island. CH, RAL, and TNL represent sampling locations on Puerto Rico that were not included in the Velo-Antón *et al.* (2007) study. The placement of these populations on this tree reflects their spatial proximity to the populations included in the earlier mtDNA cytochrome b haplotype analysis of Velo-Antón *et al.* (2007).