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EVIDENCE FOR MORPHOLOGICAL AND GENETIC DIVERSIFICATION OF TÚNGARA FROG POPULATIONS ON ISLANDS

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***Abstract.*—Despite the ongoing debate about the mechanisms involved in speciation processes, evolutionary biologists agree that isolation is a key factor in promoting the evolution of different species. Islands provide natural models for the study of isolated populations. Populations on islands are usually small and may also be subject to intense directional selection or genetic drift. In this study we investigated island populations of the Túngara Frog, *Physalaemus* (*Engystomops*) *pustulosus,* and documented their level of morphological, genetic, and behavioral divergence. We also compared our results to a mainland population. We found that larger islands and/or islands characterized by more human traffic with the mainland were more likely to harbor populations of Túngara Frogs than smaller islands. All island populations differed significantly from the mainland population and from each other at the genetic and, to a slightly lesser degree, morphological levels. Male mating calls had also diverged but did not show a clear pattern. We conclude that isolated populations of Túngara Frogs diverge very rapidly. They might therefore provide useful models for the investigation of evolution in small populations and potentially of speciation processes.**

Key Words.—amphibians; dispersal; mating call; microsatellite markers; morphology; *Physalaemus (Engystomops) pustulosus*; population genetics, speciation

INTRODUCTION

Early in the history of biology it was noted that islands often hold species with unique characteristics. Subsequently, island biodiversity and ecology have been studied extensively to unravel the mystery of speciation processes (Coyne and Orr 2004; Adams 2009). Despite an ongoing debate about the relative importance of alternative mechanisms of speciation, researchers agree that geographic isolation is the most frequent factor that interrupts gene flow and may eventually lead to speciation (Mayr 1963; White 1978; Templeton 1981; Coyne and Orr 2004; Ritchie 2007). Depending on dispersal ability of species, islands may effectively isolate conspecific population groups; a well know example for this phenomenon are the Galapagos finches (Grant 2003). Islands therefore provide naturally occurring laboratories for the study of evolution, and the effects of population isolation can be studied at short time scales and in manageable geographic areas (MacArthur and Wilson 1967; Grant 1998; Grant 2003).

Island populations may undergo rapid speciation because populations are reproductively isolated, usually small, and may be derived from only very few founder individuals (Mayr 1963). Small population size and founder effects have been proposed to facilitate speciation and radiation processes, especially compared to a relative evolutionary stasis in large and well connected populations (Mayr 1963; Carson 1975; Templeton 1981). In addition, selection pressures on island populations might be strong due to limited space and altered environmental conditions (Whittaker 1998; Losos and Ricklefs 2009). Natural selection pressures might be altered compared to the mainland due to a variety of factors such as differing predation pressure or food availability. Sexual selection might also act differently on islands compared to the mainland. Due to genetic drift and the potentially different selection pressures, the divergence of mate recognition signals might occur faster on islands than on the mainland, which in turn can greatly influence the evolution of reproductive isolation between populations (Grant and Grant 2010; Millien 2011). As mate recognition signals and mate preferences are critical components in sexual reproduction (West-Eberhard 1979; Anderson 1994; Ryan and Rand 2003a), the rapid divergence of these signals could promote behavioral isolation among populations and, ultimately, the formation of new species (West-Eberhard 1979; Lande 1981; Ryan et al. 1996; Ritchie 2007). Consequently, responses to selection might be rapid on islands and quickly lead to population divergence, adaptation, and speciation.

Islands may therefore facilitate the origin of new species.

This study focuses on *Physalaemus* (*Engystomops*) *pustulosus* (Cope 1864; Ron et al. 2006), the Túngara Frog. Túngara Frogs are a member of the family Leiuperidae and are abundant in a variety of habitat types throughout Middle America and northern South America (Ryan 1985). They have become a model system for the study of sexual selection and the evolution of communication because (as in many frog species) female mate choice is clearly based on the characteristics of the male advertisement call (Ryan 1985, 1998; Ryan and Rand 2003a; Ryan 2010, 2011). Population genetic studies at large and small geographic scales revealed high levels of genetic differentiation between mainland populations (Ryan et al. 1996; Lampert et al. 2003; Weigt et al. 2005; Pröhl et al. 2006; Pröhl et al. 2010). Island populations of Túngara Frogs have been the topic of a preliminary study (Lampert et al. 2007), which found that population colonization history is the best predictor of population relatedness and morphology.

In this study we expanded the previous data collection by Lampert et al. (2007) and analyzed morphological, molecular, and mating call differentiation in *P. pustulosus* from six different Panamanian islands and a mainland population. We were interested in populations' divergence in morphology, genetics, and calls in an attempt to characterize the diversification process. We found that there were profound morphological and genetic differences between island and between island and mainland populations. Mating calls, however, did not diverge quite as obviously between isolated island populations. We conclude that genetic divergence occurs rapidly in isolated Túngara Frog populations, followed by mating signal divergence, which might in the long term lead to speciation.

MATERIAL AND METHODS

Field sites and sampling.—We studied populations on nine islands (Fig. 1). Eight islands were located on the Pacific side of Panama: Contadora, Pedro Gonzales, Del Rey, Coiba, Coibita, Cebaco, Gobernadora, and Taboga. One island, Isla Grande, was located on the Caribbean side. Contadora (N 8° 37′, W 79° 02′) and Pedro Gonzales (N 8° 27′, W 79° 09′) are part of the Archipelago Las Perlas, which consists of hundreds of small islands. All islands are continental and were connected to the Panamanian mainland until 12,000 to 10,000 y ago (Castroviejo and Ibañez 2001). The presence of some of the island frog populations was already known. For example, the populations of Túngara Frogs on Isla Del Rey and Isla Taboga were reported by Weigt et al. (2005). Isla Taboga (N 8° 48′ W 79° 33′) lies close to the coast and is a popular tourist destination. Ferries run twice a day between Panama,

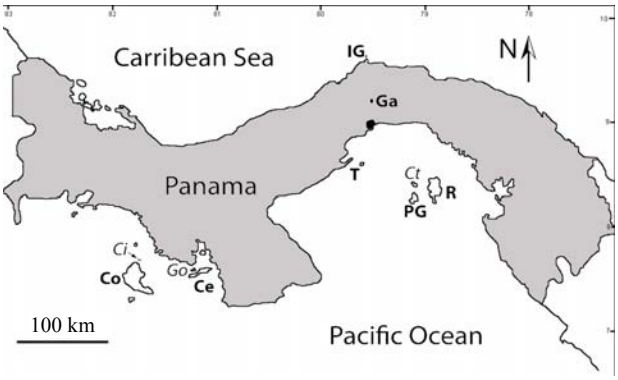

FIGURE 1. Map of Panama and sampling localities: Contadora (*Ct*), Pedro Gonzales (PG), Del Rey (R), Coiba (Co), Coibita (*Ci*), Cebaco (Ce), Gobernadora (*Go*), Taboga (T), and Isla Grande (IG). Islands that were not inhabited by Túngara Frogs are shown in italic typeface. The population chosen to represent the mainland is Gamboa (Ga).

City and the island, which could facilitate gene flow between mainland and this island. Coiba (N 7° 28′,W 81° 44′), the second largest Panamanian island, is a national park and additionally protected due to its isolated location and its past as a prison island. Frogs on the smaller islands Coibita (N 7° 39′, W 81° 42′) and Gobernadora (N 7° 33′, W 81° 12′), and the larger Isla Cebaco (N 7° 32′, W 81° 08′), have not been studied. Isla Grande (N 9° 37′, W 79° 34′), the only Caribbean island that was studied, is a small island close to the mainland (500 m) and, like Taboga, a popular tourist destination. The geographic distances between the islands and the mainland and among the islands were measured using Google Earth (Available from http://www.google.de/earth/ [Accessed 18 October 2011]). All islands differed in their distance to the mainland and in size; hence, different levels of isolation, habitat availability, and consequently differentiation of the frog populations were expected. We compared the island populations to data from a mainland population, Gamboa (N 9° 07′, W 79° 42′).

Between 13 June and 20 July 2007, we digitally recorded male frog calls (Ryan and Rand 2003b), measured snout-vent-length (SVL) using digital calipers to the nearest 0.01 mm, and took toe clips (two toes per individual) to prevent re-recording and for molecular analyses. We released all animals immediately at the point of capture. We visually inspected capture sites and adjacent areas for predator presence and fresh water availability.

Molecular analyses.—We stored toe clips in a 20% ethylenediaminetetraacetic (EDTA)/sarcosyl buffer and processed in the laboratory at the University of Texas in Austin. We extracted DNA using Chelex (20%) following a standard protocol (Altschmied et al. 1997). We amplified alleles at six microsatellite loci (CA120, CA298, A#3.11, A#19.11, C#30.11, ATG159; Pröhl et

al. 2002) following a slightly adapted protocol to enhance PCR yield by reducing the annealing temperature to 57 °C for all amplifications and increasing the number of cycles to 39. One primer of each pair was labeled with fluorescent dye. We determined fragment sizes on an ABI Prism®3100 capillary sequencer (Lampert et al. 2003) using the ROX size standard in every sample as recommended by the manufacturer.

Call analyses.—We analyzed call recordings using the program SIGNAL RTS (Engineering Design, Belmont, Massachusetts, USA). In SIGNAL the call was digitally partitioned into two components; the whine and the chuck (Ryan 2011). We analyzed several call parameters from the whine (maximum frequency [Hz], initial frequency, time from the whine's onset to its mid-frequency, final frequency, duration, rise time, time from the whine's onset to its half-rise time, fall-time, time from the whine's peak amplitude to its half-fall time, dominant frequency) and the chuck (duration and dominant frequency, Fig. 2; Ryan and Rand 2003a) and we compared the resulting means and variations between populations.

Data analyses.—To calculate differences in body size between populations and to analyze differences between call parameters, we used Kruskal-Wallis tests followed by a *post hoc* Scheffé test using the program STATISTICA version 9.1 (StatSoft, Inc., Tulsa, Oklahoma, USA). For genetic analyses we used the GENEMARKER software (Applied Biosystems/PE Biosystems, Foster City, California, USA) to determine individual genotypes. We used MICRO-CHECKER (van Oosterhout et al. 2004) to determine the presence of genotyping errors due to large allele dropout, stutter bands, or null alleles. No evidence for potential genotyping errors were found in any of the loci analyzed.

We used ARLEQUIN version 2.000 (Schneider et al. 2000) to calculate allele frequencies, deviation from Hardy-Weinberg equilibrium, linkage disequilibrium, and for assessing population differentiation (F_{ST}). In addition, we used FSTAT (Goudet, J. 2001. Fstat. Available from http://www2.unil.ch/popgen/softwares/fstat.htm Accessed 5 February 2013]) to determine levels of inbreeding (F_{IS}) and to calculate allelic richness. We performed a STRUCTURE version 2.2 analysis to assign individuals to different genetic clusters (Pritchard et al. 2000). We determined the number of clusters present in the dataset using the criteria proposed by Evanno et al. (2005).

We analyzed population differentiation with a principal component analysis that was performed using the program PCAgen (Goudet, J. 1999. PCAgen. Available from http://www2.unil.ch/popgensoftwares/pcagen.htm [Accessed 5 February 2013]). We used Microsatellite Analyzer (MSA; Dieringer and Schlötterer 2003) to calculate individual genetic distances (Cavalli-Sforza and Edward's chord distance). We used a Mantel test (i.e., linear correlation between variables, same scales within matrices, and independent observation pairs) with 1000 iterations to test for a correlation between genetic and geographic distance as well as for a potential correlation between call parameter and geographic distance (Liedloff, A.C. 1999. Mantel version 2.0 Mantel nonparametric test calculator. Available from http://www.terc.csiro.au/mantel.htm [Accessed 5 February 2013]).

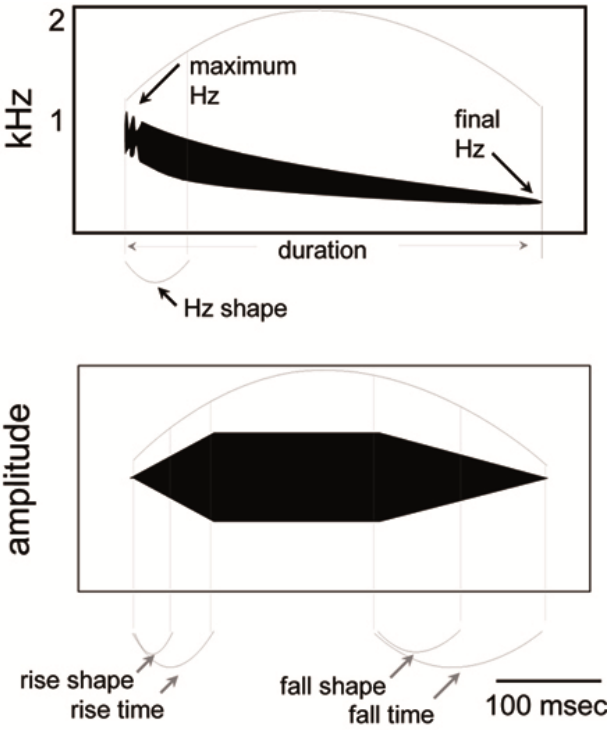

FIGURE 2. Schematic of analyzed call parameters. Above: Sonogram showing how call frequency changes over time. Below: Oscillogram showing how the call amplitude changes over time. Call parameters that are important for mate choice are indicated in the graph.

RESULTS

Even though we chose the islands for their potential to sustain Túngara Frog populations (abundant vegetation and availability of fresh water), Túngara Frogs were only found on six of the nine islands. Based on call activity, population sizes on the islands seemed smaller than on the mainland. We were able to analyze 108 frogs (Table 1). Sample sizes per island varied between three (Del Rey) and 23 (Coiba). We found no Túngara Frogs on Contadora, Coibita, or Gobernadora (Fig. 1). There was no correlation between presence of Túngara Frogs and island

TABLE 1. Allelic diversity and inbreeding coefficients (F_{IS}) for all island populations plus Gamboa. N(i) = Number of individuals, N(a) = mean number of alleles over all loci. For comparison, allelic richness (Ar) was calculated for each population standardized for one diploid individual (the maximum possible value would be 2). IG = Isla Grande, PG = Pedro Gonzales, R = Del Rey.

	Cebaco	Coiba	IG	PG	R	Taboga	Gamboa
N(i)	7	23	10	7	3	10	48
N(a)	3.83	11.0	3.33	4.33	3.00	6.67	18.17
Ar	1.72	1.69	1.47	1.54	1.59	1.74	1.81
F_{IS}	0.14	0.35	0.18	0.18	0.26	0.26	0.15

size, presence of Túngara Frogs, or distance of the island to the mainland, or between presence of Túngara Frogs and environmental conditions (i.e., presence of predators or water availability). Our inability to locate Túngara Frogs during relatively short visits does not prove their absence.

Body size of calling males differed significantly between populations (H = 82.75, P < 0.05). All island frogs were significantly larger than frogs from the mainland population (P = 0.001; Fig. 3). There were also striking differences among the island populations. Frogs from Coiba were significantly larger than frogs on Cebaco (P = 0.041), Del Rey (P < 0.001), and Taboga (P = 0.01).

Genetic variability.—We used six microsatellite loci in the study. Amplification success varied between populations but each individual included in this study could be genotyped for at least five of the six loci. Mean allelic richness was quite high for all populations investigated, with 21 to 59 alleles per locus (Table 1). No linkage disequilibrium among loci or significant deviations from Hardy-Weinberg equilibrium within loci was found in any population. Positive inbreeding coefficients (F_{IS} > 0) were found in most island populations (Table 1).

Although sample sizes were rather small, almost all island populations were significantly differentiated from each other genetically, as well as from the mainland population. The only exception was Del Rey, which did not differ significantly from any of the other islands or the mainland (Table 2). This result was confirmed by the STRUCTURE analysis (Fig. 4) that found six clusters to be the most likely number of subdivisions of the sample (Evanno et al. 2005). The clustering analysis clearly assigned individuals to their populations of origin, the only exception being individuals from Pedro Gonzales and Del Rey that were identified as one cluster. The principal component analysis based on the individual genotypes (Fig. 5), however, clearly separated Del Rey and Pedro Gonzales along the second axis of variation. The first two PCA-axes explained 74% of the genetic variability among populations.

We found only a marginally significant correlation of geographic and genetic distance (Mantel test: G = 1.70, Z = 253.8, r = 0.45, P = 0.07). The geographic distance between the capturing sites did therefore not explain the genetic distance between the populations (Table 2). Calculations of individual genetic distances revealed that

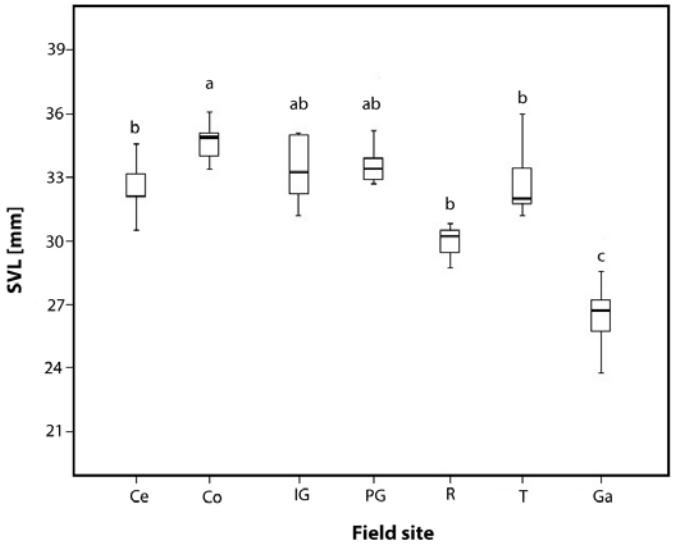

FIGURE 3. Male sizes (SVL [mm]) in all Túngara Frog populations. Given are the median (horizontal line), inter-quartile range (box), and 95% confidence intervals. Groups that differed significantly in SVL are marked with different letters (a, b, c).

the frogs within one island population were more closely related to each other than to individuals on other islands or the mainland (Fig. 6). Individual genetic distances between Cebaco and Gamboa (mainland; $P = 0.034$), in the dominant frequency of the chuck differed between Cebaco and Pedro Gonzales ($P < 0.001$), in whine duration between Coiba and Pedro Gonzales ($P = 0.001$) were very low within the populations of Isla Grande and Cebaco, while individuals from Coiba, Del Rey, and Taboga showed higher intra-populational genetic distances. Not surprisingly, genetic distances within sampling localities were lower than among sampling localities (Fig. 6).

Call diversity.—We plotted call parameters in a multivariate space (Fig. 7). A separate call parameter analysis clarified the difference between populations (Appendix 1). Differences were found in the shape of the whine's frequency sweep (time from the whine's onset to its mid-frequency) between Cebaco and Pedro Gonzales ($P < 0.001$), in the final frequency of the whine and between Coiba and Taboga ($P = 0.049$), in the whine's fall time between Coiba and Pedro Gonzales ($P = 0.002$), and in its half-fall time between Taboga and Gamboa (mainland; $P = 0.041$). Whine duration ($P = 0.017$) and fall time ($P = 0.035$) were the only call parameters significantly correlated with body size. Call differences were also correlated with geographic distance ($r = 0.59$, $P = 0.03$) but not with genetic differences ($r = 0.29$, $P = 0.36$).

TABLE 2. Genetic distances (F_{ST}, below the diagonal) and geographic distances (km, above the diagonal) between the islands and the mainland (Gamboa). Significant levels of genetic differentiation ($P < 5\%$) are marked in bold typeface. IG = Isla Grande, PG = Pedro Gonzales, R = Del Rey. (n = Number of individuals analyzed in the population)

	Cebaco (n = 7)	*Coiba (n = 23)*	*IG (n = 10)*	*PG (n = 7)*	*R (n = 3)*	*Taboga (n = 10)*	*Gamboa (n = 48)*
Cebaco	-	54.18	295.31	252.26	270.26	229.63	244.05
Coiba	0.007	-	331.36	302.35	321.05	275.89	283.54
IG	**0.081**	**0.058**	-	143.82	146.26	93.32	58.29
PG	**0.044**	**0.024**	**0.060**	-	18.01	65.24	101.72
R	0.037	0.021	0.054	0.000	-	77.15	111.01
Taboga	**0.025**	**0.016**	**0.047**	**0.011**	0.000	-	39.56
Gamboa	**0.031**	**0.018**	**0.045**	**0.011**	0.001	0.000	-

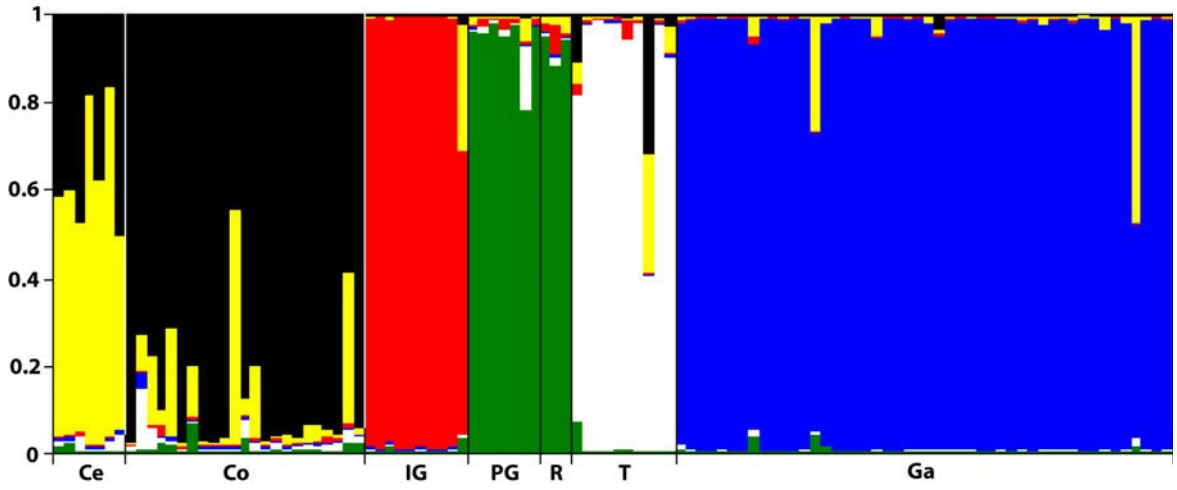

FIGURE 4. Result of the genetic assignment analysis for K = 6 clusters. Each column represents an individual and the y-axis shows the fraction of each cluster (different colors). Islands are: Ce = Cebaco, Co = Coiba, IG = Isla Grande, PG = Pedro Gonzales, R = Del Rey, T = Taboga, Ga = Gamboa. Clusters match the geographic distribution except for the green cluster that includes all individuals from Pedro Gonzales as well as all individuals from Del Rey.

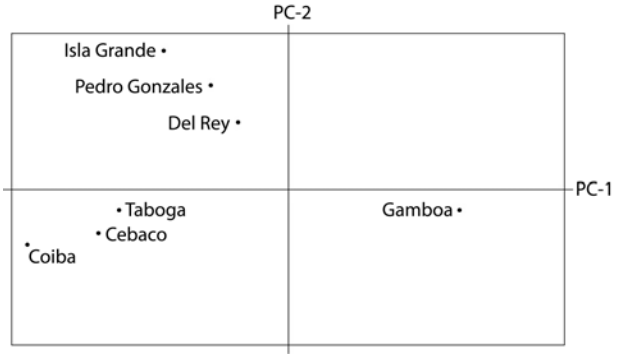

FIGURE 5. Principal component analysis of individual genotypes. The x- and the y-axis represent the first and second principal component, respectively. Both axes (PC-1 and PC-2) were found to be significant ($P(x)$ = 0.01; $P(y)$ = 0.002) and together explained 74% of the variation.

DISCUSSION

Coastal islands may have one of two possible geological origins, and their origin may play a determining factor in what evolutionary processes influences an island population. If an island is oceanic (usually formed by volcanic activity) it starts as a 'blank slate' where species have to colonize the island. Oceanic island populations are usually formed by a small group of founders and the genetic variety therefore might be low (Mayr 1963; Carson 1975; Templeton 1981). Continental islands, on the other hand, are formed from land that was formerly connected to the mainland that becomes isolated by rising sea levels. Populations in these environments might have a high genetic variation to start with, but the strong geographical isolation causes them to evolve independently than those from the mainland (Thornton 2007). The islands investigated in this study were all of continental origin and part of the Panamanian mainland until approximately 12,000 years ago (Smith and Bermingham 2005).

Nevertheless not all islands presently harbor Túngara Frog populations. This observation might be explained by extinction events which are likely to happen more frequently on small islands with small frog populations. The probability that these islands might be re-colonized by Túngara Frogs would depend on the distance to the mainland and the closest Túngara Frog population. Like other amphibians, Túngara Frogs cannot readily disperse across salt water and therefore can only reach islands by natural floats (e.g., floating vegetation) or human transport (e.g., in building materials). Therefore the island's geographic distance to the closest Túngara Frog population either on the mainland or another island, as well as water currents and the island's exposure to human impact should greatly influence the probability of Túngara Frog populations being present on an island (MacArthur and Wilson 1967). Interestingly, however, we found no Túngara Frog populations on the islands Contadora, Coibita, and Gobernadora, even though these islands lie relatively close to the mainland shore and other islands where Túngara Frog populations were found. Coiba, Del Rey, and Cebaco, however, which are much further from the mainland and also far from other island populations of Túngara frogs did harbor large populations of Túngara Frogs. One explanation could be that compared to the uninhabited islands (Contadora, Coibita, and Gobernadora) the islands inhabited by Túngara Frogs (Coiba, Del Rey, and Cebaco) are rather large and might therefore harbor remnant populations from before the islands separation from the mainland. Size could influence population survival as large islands might offer more possibilities for retreat when environmental conditions deteriorate and prevent or delay inbreeding and mutation meltdown. Habitat heterogeneity on larger islands could influence interspecific and intraspecific competition over resources (food, breeding sizes, partners). This could have had an effect on the population size and structure of these islands (MacArthur and Wilson 1967). Small islands such as Taboga and Isla Grande, however, were also inhabited by Túngara Frogs. These islands, on the other hand, are very popular tourist destinations and might be re-colonized by Túngara Frogs at regular intervals. In summary, large islands seem to have a higher probability of being inhabited by Túngara Frogs, which is consistent with the theoretical predictions from island biogeography which explains species diversity (MacArthur and Wilson 1967; Whittaker 1998). In addition, a high frequency of human visitors might also enhance the probability that an island becomes inhabited by Túngara Frogs.

Another explanation for the absence of Túngara Frogs on some islands, however, might be enhanced levels of predation. While predation pressures have been generally known to be weaker on islands than on the mainland because islands usually support fewer predator species (Li et al. 2011), predator populations were thriving on some islands. Especially large numbers of marine toads and crabs, both known predators of Túngara Frogs (Ryan 1985), were found in potential breeding areas of the Túngara Frog on the uninhabited islands Contadora, Coibita, and Gobernadora. Larger water bodies might have also held fish that potentially prey on Túngara Frog eggs and prevent colonization. Because these islands are all rather small compared to the other islands, it is possible that the Túngara Frog populations were too small to maintain themselves under such a high predator rate.

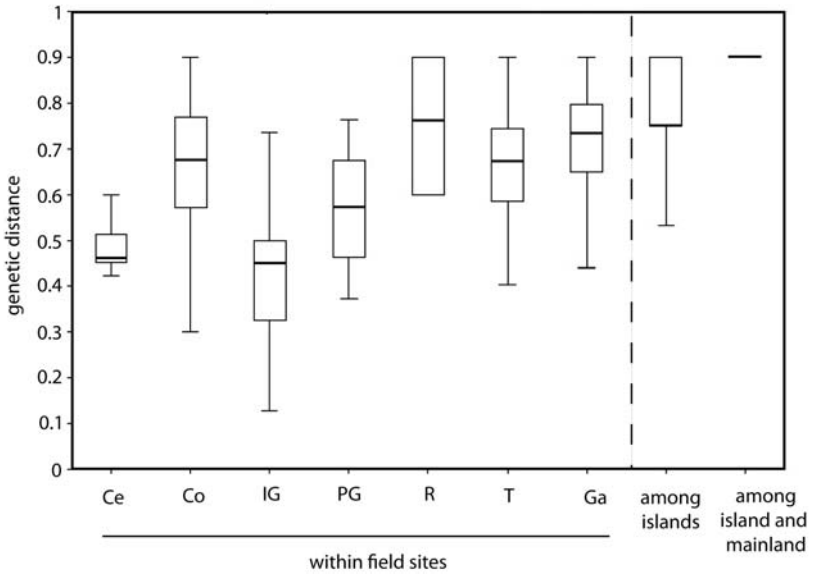

FIGURE 6. Mean individual genetic distances (Cavalli-Sforza and Edwards' chord distances) within and among field sites. Box plots show the median (horizontal line), inter-quartile range (box), and 95% confident intervals.

Natural selection pressures (predation, food availability) in general might not only influence the presence or absence of species but might select for phenotypes that differ from the mainland. In addition, selection pressures on island populations can be stronger due to limited space and altered environmental conditions (Whittaker 1998). We found quite distinct phenotypic differences between the island and the mainland populations. Body size of island frogs was significantly larger than that of the mainland frogs, which is consistent with 'island gigantism' described in many species (Ryan 1982; Castellano and Giacoma 1998; Grant 2001; Robinson-Wolrath and Owens 2003; Clegg et al. 2008; Sota and Nagata 2008). The 'island rule' (Van Valen 1965) states that on islands small species tend to become larger and large species tend to become smaller. These size differences can be explained by four primary factors: predation pressure, resource availability, inter-specific competition, and immigrant selection (Li et al. 2011). On the islands where we found no predators, the frogs were significantly larger than on the mainland. Frogs were largest in Coiba, which is consistent with earlier results from Lampert et al. (2007). Comparatively large body sizes were also found on Isla Grande. Relatively rapid changes in body size have been reported for other species as well. Clegg et al. (2008) and Li et al. (2011) found that reduced predation pressure (lower number of predatory species and individuals) were the main factor driving body gigantism in a Rice Frog (*Rana limnocharis*) and this negative relationship between body size and predators has also been reported in insular lizards and rats (Case 1978; Angerbjorn 1986; Smith 1992; Michaux et al. 2002). Other explanations could be that due to the lack of high predation pressure the frogs live to an older age, or increase foraging time, both of which could result in a larger size. A population with a higher expected survival rate could increase its fitness by maturing later and at a larger size, which could be an evolutionary (genetic) response over a long time period (Adler and Levins 1994; Palkovacs 2003). Another potential explanation for island gigantism might be food availability. Food abundance and quality might differ on islands. It would be interesting to perform common garden experiments to determine if the observed differences in body size found in this present study can indeed be explained genetically (Herczeg et al. 2009; Tanaka 2011).

Differences between the islands were not only present in morphology but also in genetic diversity. All six microsatellite loci investigated were highly variable with many alleles (21 to 59) present in all individuals investigated ($n_{(total)} = 108$). We found large variation in allelic diversities and frequencies at all microsatellite loci among the islands and between the islands and the mainland. Some islands, however, showed extraordinary patterns of allelic diversity independent of sample size, for example, the Túngara Frogs on Isla Grande. In a sample size of 10, a maximum number of four alleles per locus was found while on other islands with a similar sample size many more alleles were detected; this is a sign that this population was likely founded by only a few individuals. Even Del Rey, with a sample size of only three individuals, showed up to four alleles per microsatellite locus. The exceptionally low allelic variability on islands confirms an earlier study (Lampert

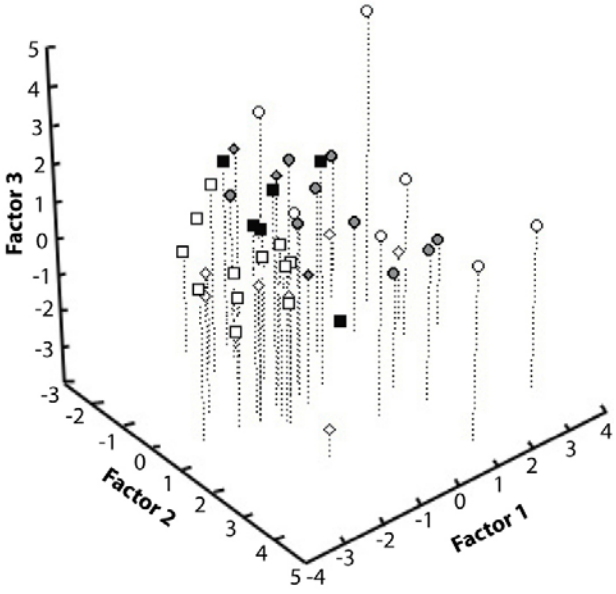

FIGURE 7. Individual male mating calls plotted in a three dimensional space using the three most informative factors found in a main component analysis. Field sites are designated by symbols: Cebaco = white diamonds, Coiba = white squares, Pedro Gonzales = white circles, Del Rey = grey diamonds, Taboga = grey circles, Gamboa = black squares.

et al. 2007).

All of our molecular analyses showed that island populations were clearly distinct genetically. The only exception was Del Rey, which was not genetically differentiated from the other groups, but this lack of statistically significant differentiation may have been an artifact due to low sample size. While this study found genetic divergence among the islands, no clear geographic pattern emerged (i.e., no significant isolation by distance effect was found). This might also be due to low sample sizes in some of the populations and/or to the notion that the isolation-by-distance model even though it is based on the island model normally applies to continuous landscapes (Wright 1943). Isolation-by-distance on large and small scales has already been documented for Túngara Frogs (Lampert et al. 2003; Pröhl et al. 2006). Even though the correlation of genetic and geographic distance was marginally significant, only 21% of the genetic difference could be explained by the geographic distance among the field sites. The remaining genetic variability is likely due to founder effects or related stochastic processes. The most closely related individuals were found on Isla Grande, which confirms earlier findings and speculation (Lampert et al. 2007) that this population was probably founded rather recently and possibly by a single clutch of eggs.

The microsatellite markers used in this study have been shown to be highly variable and therefore informative on individual relatedness (Pröhl et al. 2002; Lampert et al. 2003; Lampert et al. 2006; Pröhl et al. 2006). The low level of diversity detected on Isla Grande was therefore not due to a limited ability of the markers to resolve genetic diversity. Instead, as bottlenecks have much more severe effects on allelic diversity than on heterozygosity (Leberg 1992; Spencer et al. 2000; Williamson-Natesan 2005), the low allelic diversity in the Isla Grande population is likely due to a severe recent bottleneck (e.g., a very small founder population).

Túngara Frog calls recorded on islands differed from calls recorded on the mainland. While call parameters varied among individuals, no clear pattern emerged distinguishing the island populations. Island populations showed overlapping ranges even in multivariate acoustic space despite their genetic distinctiveness. Interestingly, this seems to be different from certain bird species where song seems to be the first characteristic to diverge between species (Mirsky 1976; Ritchie and Phillips 1998; Parker et al. 2012). Call variation seemed higher in island populations compared to the mainland. While the Gamboa mainland calls were all quite similar, calls recorded on Pedro Gonzales varied widely in their characteristics. In this study the call parameters mirrored the results from the genetic study: populations that were more closely related also had more similar calls. Further studies also found a correlation of geographic and call divergence at the population level (Ryan et al. 1996), while study on the individual level did not find a correlation between call similarity and genetic relatedness (Lampert et al. 2006). Additionally, two call parameters were related to body size (fall time and duration), which are the parameters that are critical components for the attractiveness of the call to females (Ryan and Rand 2003a). As the island frogs were clearly larger than the mainland frogs, we would expect mating calls from island frogs to be more attractive to females. Even though this could be a mechanism of speciation and needs to be investigated in more detail, an earlier study on Túngara Frogs did not find a clear preference for local male mating calls compared to conspecific foreign calls (Pröhl et al. 2006; Ryan et al. 2007).

Túngara Frogs inhabit several Panamanian continental islands. These island populations differ significantly at the genetic level as well as in body length and show differences in male mating calls. Isolated populations of Túngara Frogs diverge rapidly and might therefore be useful models to investigate speciation processes.

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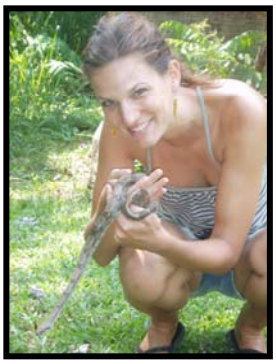

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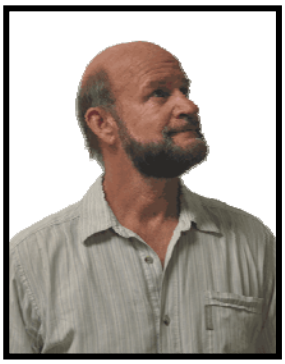

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APPENDIX 1.

Box plots (median [horizontal line], inter-quartile range [box], and 95% confidence intervals) showing the average values of the whine component of male mating calls in the different populations. Significant differences between sites are marked with asterisks according to significance level (* < 0.05, *** < 0.001).