GEOGRAPHIC VARIATION OF GENETIC AND BEHAVIORAL TRAITS IN NORTHERN AND SOUTHERN TÚNGARA FROGS

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Abstract.—We use a combination of microsatellite marker analysis and mate-choice behavior experiments to assess patterns of reproductive isolation of the túngara frog *Physalaemus pustulosus* along a 550-km transect of 25 populations in Costa Rica and Panama. Earlier studies using allozymes and mitochondrial DNA defined two genetic groups of túngara frogs, one ranging from Mexico to northern Costa Rica (northern group), the second ranging from Panama to northern South America (southern group). Our more fine-scale survey also shows that the northern and southern túngara frogs are genetically different and geographically separated by a gap in the distribution in central Pacific Costa Rica. Genetic differences among populations are highly correlated with geographic distances. Temporal call parameters differed among populations as well as between genetic groups. Differences in calls were explained better by geographic distance than by genetic distance. Phonotaxis experiments showed that females preferred calls of males from their own populations over calls of males from other populations in about two-thirds to three-fourths of the contrasts tested. In mating experiments, females and males from the same group and females from the north with males from the south produced nests and tadpoles. In contrast, females from the south did not produce nests or tadpoles with males from the north. Thus, northern and southern túngara frogs have diverged both genetically and bioacoustically. There is evidence for some prezygotic isolation due to differences in mate recognition and fertilization success, but such isolation is hardly complete. Our results support the general observation that significant differences in sexual signals are often not correlated with strong genetic differentiation.

Key words.—Bioacoustic variation, genetic variation, microsatellites, *Physalaemus pustulosus*, reproductive isolation, túngara frogs.

Divergence in sexual traits between populations can result from drift, adaptation to environmental conditions, or sexual selection (Panhuis et al. 2001; Coyne and Orr 2004). Sexual selection can lead to rapid and often arbitrary divergence of traits involved in mate recognition and thus generate prezygotic reproductive isolation between lineages. Several examples demonstrate divergence in acoustic signals between closely related species (Mendelson and Shaw 2005) or populations within a species (Claridge and Morgan 1993). Associated female preferences for the acoustic signals of their own lineage relative to the signals of other lineages reduce the probability of mating and thus genetic exchange between lineages (Claridge and Vrijer 1993; Gerhardt and Schwartz 1995; Coyne and Orr 2004). Ecological factors can generate directional selection on mating signals and mating preferences (Schluter 2001; Coyne and Orr 2004). For instance, sexual signals might evolve to maximize transmission distance in their specific habitat (Ryan et al. 1990a), to maximize the signal's localization accuracy (Forrest 1994), or to enhance avoidance of predators and parasites (Endler and Houde 1995; Simmons et al. 2001). Although arbitrary divergence due to sexual selection or directional selection due to ecological selection are expected to operate between populations, stabilizing selection on parameter involved in species and mate recognition is expected within populations (Gerhardt and Huber 2002).

Prezygotic isolation is often coupled with postzygotic isolation (Gerhardt and Schwartz 1995; Coyne and Orr 2004). In these cases behavioral (or other) isolation results when conspecifics do not mate with heterospecifics, which may result in inviable, sterile, unfit, or sexually unattractive off-spring (Littlejohn and Watson 1985; Pfennig 2000; Tregenza 2002; Höbel and Gerhardt 2003). In other cases prezygotic isolation and postzygotic isolation evolved independently (Littlejohn and Watson 1985; Szymura 1993; Wu et al. 1995).

The divergence in mate recognition differences and genetic distance between populations have been compared with the expectation that they should covary. The results of these studies are inconsistent: some found no correlation between mate recognition and genetic distance (Tilley et al. 1990; Lougheed and Handford 1992; Gleason and Ritchie 1998; Soha et al. 2004) while others do (Balaban 1988; MacDougall-Shackleton and MacDougall-Shackleton 2001; Christianson et al. 2005). Likewise, isolation-by-distance effects, that is, correlations between genetical or behavioral divergence with geographic distance, were detected in some studies (Tilley et al. 1990; Rowe et al. 2000; Newman and Squire 2001; Vos et al. 2001; Stenson et al. 2002; Lampert et al. 2003; Soha et al. 2004), but not in others (Wake and Yaney 1986; Seppä and Laurila 1999; Leblois et al. 2000).

The emerging picture indicates a complex relationship between genetic and geographic distances, signal and receiver divergence, and the strength of pre- and postzygotic isolation, and such relationships seem to vary among taxa (Sasa et al. 1998; Gabor and Ryan 2001; Edmands 2002). Unfortunately, most studies do not focus on more than two or three of these phenomena and do not include geographic variation in reproductive patterns and their respective outcomes (but see Tilley et al. 1990). Since geographic variation in communication systems is important for the process of speciation we use a widespread species, the túngara frog *Physalaemus pustulosus*, to measure the possible correlations between geo-

graphic and genetic distances and differences in sexual signals, as well as geographic variation in the mate recognition system, and we discuss them in light of reproductive isolation between diverged lineages.

The System

Túngara frogs are a model system for studying sexual selection, communication, and breeding biology (Ryan 1985) with emphasis on species and mate recognition (Ryan 1980, 1998; Ryan et al. 1990b; Ryan and Rand 1993). These frogs are abundant inhabitants of the dry and wet lowland forests from northern Mexico to the Caribbean coast of northern South America. In Costa Rica and Panama their distribution is limited to the Pacific coast. The mating system can be described as a lek system with males calling in small choruses from temporary puddles, pools, and ditches during the wet season (Ryan 1985).

The advertisement call of the túngara frog is unusually complex and consists of a frequency-modulated whine that can be produced alone or followed by up to seven chucks (Rand and Ryan 1981). Phonotaxis experiments revealed that the addition of chucks enhances the attractiveness to females (Ryan 1980, 1983, 1985) but that the whine alone is sufficient for mate recognition (Ryan et al. 1990b; Ryan and Rand 1995).

Ryan et al. (1996) examined mating call variation in relation to allozyme variation and geographic distances among 30 populations along a 5000-km transect spanning the species' range. The study revealed two genetically different lineages of túngara frogs: a northern group ranging from Mexico to northern Costa Rica and a southern group ranging from western Panama to northern South America. This geographic/genetic pattern was supported by a phylogenetic analysis based on mitochondrial cytochrome oxidase I sequence data (Weigt et al. 2005). This investigation also found limited introgression at two (of 14) allozyme loci with typical northern gene products in two West Panamanian populations. Based on phylogenetic reconstruction, estimate of divergence times, and evidence for introgression the authors concluded that the two genetic groups were separated before the final closure of the Panamanian isthmus in the Pliocene (>3 million years ago) and must have reestablished contact afterwards. None of these studies analyzed frogs in central or southern Costa Rica, the area between the northern and southern genetic group. Bioacoustic analyses showed that call variables differed significantly among populations: some call variables exhibited clinal variation, whereas most others differed between the two genetic groups (Ryan et al. 1996). The same study also found that differences in calls among populations were better predicted by geographic distance than by allozyme dissimilarity (Ryan et al. 1996). A recent study examined population-based preferences of females in central Panama, far from the area of contact between the two groups, for calls throughout the species' range. Those results showed that there can be local mate preferences in about one-third of the population comparisons, but there was no evidence for preferences sorting among the genetic groups (M. J. Ryan, X. E. Bernal, and A. S. Rand, unpubl. ms.). Our study is a geographically finer-scale analysis of populations in the un-

studied area between the previously identified genetic groups. Our main goal is to understand patterns of divergence between the two genetic groups of túngara frogs in this critical region of their distribution. Toward that goal we examined (1) patterns in genetic variation; (2) aspects of prezygotic reproductive barriers: bioacoustic mating signals, female preferences for mating signals, and nesting success; and (3) a postzygotic reproductive barrier, fertilization success. We studied 25 populations of túngara frogs along a transect from northern Costa Rica to west and central Panama in the south and east (Fig. 1). First, we documented genetic differentiation of túngara frogs among populations using polymorphic microsatellites as genetic markers. Second, we examined the correlation between geographic, genetic, and bioacoustic distances between these populations, and determined whether there was significant divergence of mating calls and mating call preferences between populations and the two genetic groups.

Materials and Methods

Field Collections

From July to October 2000, we collected tissue and recorded mating calls from 24 Costa Rican and Panamanian túngara frog populations along a transect from Santa Rosa National Park in the north (Costa Rica) to Santiago in the southwest (Panama), a straight-line distance of 565 km. In addition, we analyzed a population from Gamboa in central Panama which is 188 km from Santiago (Fig. 1). Most sites, with the exception of Gamboa, were within 20 to 30 km from the nearest site (see Appendix 1, available online only at http://dx.doi.org/10.1554/05-278.1.s1). The transect included eight populations in the northern group and 17 populations in the southern group as defined by Ryan et al. (1996) and Weigt et al. (2005). In total, we sampled calls and collected tissues from 455 males. At least 16 males were sampled from each population (mean $\pm$ SD = 19 $\pm$ 4) with the exception of Palma, Nicoya, OSA, and El Forastero (Appendix 2, available online only at http://dx.doi.org/10.1554/05-278.1.s2). We removed one toe tip from every male and stored it in a NaCl-saturated 20% DMSO/0.25M EDTA buffer at room temperature. We recorded several calls from each male using a Sony (Tokyo, Japan) Professional Walkman and a Sennheiser (Wedemark, Germany) ME-80 microphone. We also recorded the longitude and latitude of each sample site and calculated geographic distances between sample sites using a 12 channel GPS (Garmin, Taipei, Taiwan).

Call Analysis and Synthetic Calls

We analyzed up to six whines for every male using the Signal software program version 4 (Beemann 1996). Calls were digitized at a sampling rate of 50 kHz. The call characters analyzed were initial frequency of the whine, final frequency of the whine, duration of the entire call, rise and fall time of the whine (time from the onset of the call to its maximum amplitude; time from the call's maximum amplitude to the end of the call), whine shape (the proportion of the call's duration from the onset of the call to its midfrequency), rise shape (proportion of the call's duration from
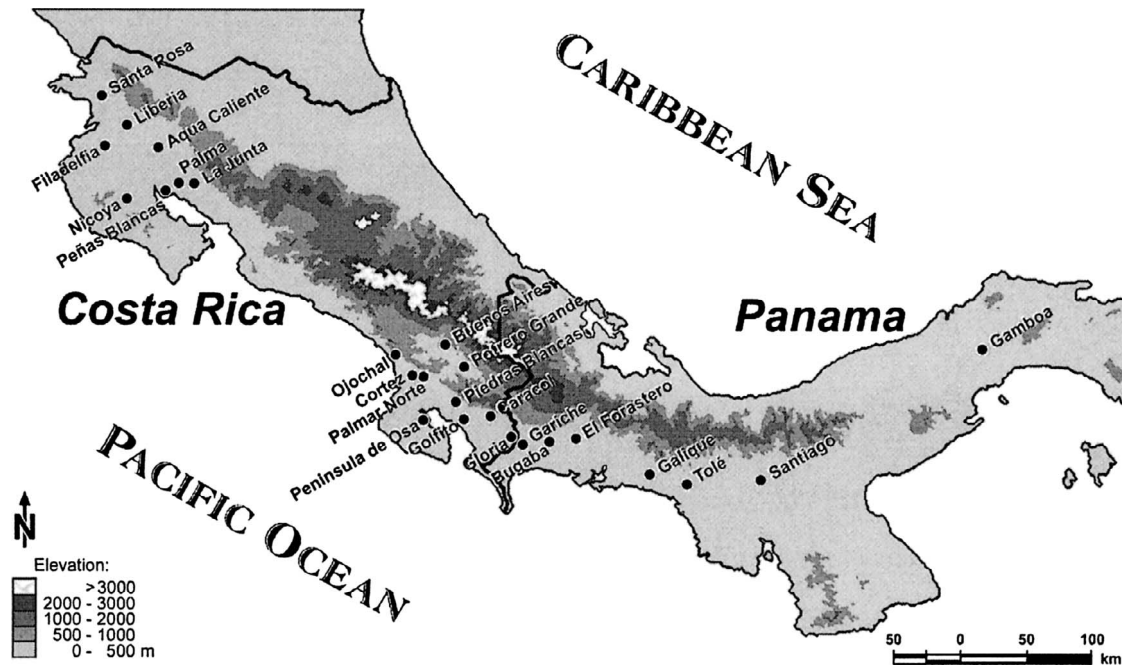
FIG. 1. Call and tissue sampling sites in 25 túngara frog populations in Costa Rica and Panama. We did not find túngara frogs in central Pacific Costa Rica.

the call onset to one-half maximum amplitude during the rise), and fall shape (the proportion of the call's duration from the maximum amplitude to one-half the maximum amplitude during the fall; see Ryan and Rand [1995] for further explanation of call variables and illustration). We used the population means of call variables to synthesize average whines for each population with synthesis program provided by J. Schwartz, Pace University, Pleasantville, NY.

Genetic Analysis

We extracted DNA with the DNeasy Tissue Kit (Qiagen, Valencia, CA) and subsequently amplified six microsatellites DNA loci with primers previously developed for túngara frogs (Pröhl et al. 2002). We did not uncover linkage disequilibrium for any loci pair in any population. Five loci exhibited a significant heterozygosity deficit in one to four populations. Expected heterozygosity was significantly higher than observed heterozygosity at locus A 19.11 in eight populations. Since variation at this locus did not deviate significantly from Hardy-Weinberg expectation in most populations, we included all six loci in our analysis. Polymerase chain reaction product sizes were analyzed on the ABI (Applied Biosystems, Foster City, CA) 3100 Genetic Analyzer and then scored using Gene Scan Analysis Version 3.5 (ABI) and Genotyper Version 3.6 NT (ABI) software.

Reproductive Isolation: Female Choice Experiments

In September–October 2001, August–October 2002, and September–October 2003 we conducted phonotaxis experiments with females from five túngara frog populations: Liberia (northern group), Ciudad Cortez, Golfito, Gariche, and Santiago (southern group; Fig. 1). The experiments in the northern genetic group remained unreplicated due to un-

availability of reproductively active females during the time of the field work.

We transported females to a portable test arena measuring 1.8 m × 1.5 m. The bottom of the test arena was gray plastic and the walls consisted of 15-cm thick and 30-cm high sections cut from a foam mattress. A red light was suspended 1.5 m above the floor and allowed us to observe the frog's movements within the arena. The light intensity was within the wide range of illumination that female frogs experience when they choose mates in the wild (total dark to quite bright full-moon light; Ryan 1985). We conducted phonotaxis tests between 2200 and 0400 h.

At the beginning of an experiment, we placed each female inside an inverted funnel in the center of the test arena. Two speakers (Radio Shack [Fort Worth, TX] Mini Speaker System, with a deviance in the sound level intensity of ±2 dB within the frequency range of the túngara frog call [400–5000 Hz], placed directly opposite to each other in the center of the shorter sides of the arena, broadcast alternately via a computer the synthetic average call from the female's own population and a synthetic average call from a foreign population. Test calls were broadcast at a peak intensity of 78 to 80 dB sound pressure level (relative to 20 µP) at the center of the arena and a rate of one call per second. After 2 min we lifted the funnel while broadcasting continued and the behavior of the female was observed. If the female moved within 10 cm of one speaker, the experiment was terminated and the result scored as a preference of the female for that call. A ''no response'' was noted if the female did not move for 5 min at any time during the experiment, or if she did not approach a speaker within 15 min. Females who did not show a preference for one or the other call were tested again the next day. We tested every female in five choices, all of which contrasted the local call against one of five different

foreign calls. The five foreign calls included two calls from the study transect: the call in the northern group that was most distinct from the local call, the call from the southern group that was most distinct from the local call; and the calls from Veracruz (Mexico), Colombia, and Carupano (Venezuela) as described in Ryan et al. (1996). For each experiment approximately 20 female responses were recorded. Sample sizes varied slightly among populations due to variation in both availability and responsiveness of females.

Reproductive Isolation: Mating Experiments

Females were placed in a plastic dish (diameter approximately 20 cm) filled with water after having shown a positive response in the phonotaxis experiments. We introduced a male to the female, and over the next two days we recorded whether the pair had built a foam nest. After successful fertilization, eggs hatched in two days. At this time the experiment was terminated, and we counted the number of normally developed tadpoles. Sires were either from the local population of the respective female or a foreign population. Foreign males were from the same or the other genetic group. Females from Panama were only tested with males from Panama and females from Costa Rica were tested only with Costa Rican males due to restrictions on transporting live frogs across international borders. Males were transported from their original site to the female population in plastic terraria and fed with termites every second day. All tested frogs were released at their site of origin after the experiments.

Statistical Analyses

We calculated allele frequencies, observed and expected heterozygosities, and deviations from linkage and Hardy-Weinberg equilibrium with Arlequin 2.0 (Schneider et al. 2000). Pairwise linkage disequilibrium (Slatkin and Excoffier 1996) between loci and departure from the Hardy-Weinberg equilibrium (Guo and Thompson 1992) were tested using a Markov Chain approximation with 100,000 steps. To analyze genetic population differentiation we calculated pairwise F_{ST} and R_{ST} values (Weir and Cockerham 1984; Slatkin 1995; review in Lowe et al. 2004) as well as D_A (Nei's net genetic distance, Nei and Li 1979) for every population pair (see Appendix 3, available online only at http://dx.doi.org/10.1554/05-278.1.s3). We used D_A instead of Nei standard genetic distance (D_S; Nei 1978, 1987) because D_A includes the difference in number of repeats between alleles at the same locus. The probability of nondifferentiation was estimated with over 3000 randomizations. We also calculated the global F_{ST} and R_{ST} values across all populations. Finally, we calculated F_{RT} and R_{RT} which measure the effect of population structure between northern and southern frogs (Hartl and Clark 1997).

Variation in calls between populations was calculated by using Euclidean distances from z-transformed averages of all measured call parameters using Systat (SPSS Inc., Chicago, IL) version 9 (see Appendix 1, available online). Multidimensional scaling (MDS) can be applied to any kind of distance matrix, and moves objects around in space defined by a requested number of dimensions by maximizing the goodness-of-fit to the observed distance matrix. We used MDS to arrange genetic and bioacoustic distances between population pairs in a two-dimensional space. We also used Mantel tests to calculate correlation and partial correlation coefficients between geographic distance, genetic distance (D_A), and bioacoustic distance (Euclidean distances) between populations (Mantel version 2.0, Liedloff 1999; Arlequin, Schneider et al. 2000). Mantel tests calculate correlation coefficients between similarity/dissimilarity matrices. The partial Mantel test calculates the relationship between two matrices after controlling for covariation with a third matrix (Smouse et al. 1986). One thousand permutations determined the statistical significance of the correlation coefficients. Since Gamboa is more distant geographically (Fig. 1) and might disproportionately influence these correlations, we also calculated Mantel tests excluding Gamboa. We used coefficients of variation (CV) and a nested ANOVA (populations nested in groups) to assess the variation in call parameters within and among populations and between the northern and southern genetic group.

We applied a repeated-measures logistic regression analysis using the general estimating equation (GEE analysis) for binary data with logit link function (SAS Online DocTM, ver. 8, p. 1452; SAS Institute, Cary, NC) to analyze several aspects of female preferences for the local versus nonlocal mating calls. An initial analysis included the overall effect of the female population, the genetic group of the nonlocal call with respect to the tested female (own or other genetic group) and the bioacoustic (Euclidean) distance between the local and nonlocal call employed in the phonotaxis experiment. A test of significance for the intercept of the logistic regression can be used to determine whether the degree of preference of the females is significantly different from 50/50 (which equals an odds ratio of 1). We used the intercept values to calculate, first, the overall probability and significance of whether females prefer the local call or not and, second, the probability and significance for each female population.

For mating experiments, we distinguished between males that came from the same population as the female (local population matings), males that came from another population but from the same group (foreign populations, same-group matings), and males from the other group (foreign population, different-group matings). We used a logistic regression to test the null hypothesis that construction of foam nests was not influenced by genetic distance between the male and female population used for mating experiments. We used a 2×3 contingency table (chi-squared test) to test the null hypothesis that successful construction of a foam nest was not influenced by male group. In frogs, the results of reciprocal crosses between diverged lineages are often asymmetric (Sasa et al. 1998). We tested for asymmetry by performing a 2×2 chi-squared test on the success of nest construction of crosses between northern females/southern males versus southern females/northern males. Finally, we calculated a Kruskal-Wallis analysis of variance to test the null hypothesis that the percentage of tadpoles that hatched from the eggs did not differ between male groups. All statistical procedures that include multiple testing on the same dataset (deviance from linkage disequilibrium, deviance from Hardy-Weinberg

TABLE 1. Comparison of pairwise genetic distance measurements (F_{ST}, R_{ST}, D_A) between populations of túngara frogs.

	Between populations of the northern group	Between populations of the southern group	Between northern and southern populations
F_{ST}	0.008–0.261	0.012–0.257	0.161–0.428
R_{ST}	0.006–0.170	0.002–0.447	0.496–0.907
D_A	0.014–3.194	0.080–22.22	27.58–62.18

equilibrium, Mantel tests) were Bonferroni corrected (Sokal and Rohlf 1995).

RESULTS

Distribution and Gap

During our survey we did not find túngara frogs in six localities between Ojochal in south Costa Rica and La Junta in north Costa Rica. Apparently there is a gap about 200 km long between the northern and southern túngara frog group, not published in corresponding literature when we started this research in 2000 but recently discussed by Savage (2002).

Molecular Genetic Variability and Population Differentiation

We found 199 alleles at the six loci examined. All loci were polymorphic showing one to eight alleles (ATG 263) or as many as six to 20 alleles (A19.11; Appendix 2 available online only) in a single population. Standard gene diversity (Nei 1987, p. 180) was very high (always >0.98) in all populations. No linkage disequilibrium (Slatkin and Excoffier 1996) was found among the six loci in any population (all $P > 0.0033$, 15 pairwise comparisons in every population). All loci were in linkage disequilibrium with all other loci when testing across all populations ($P < 0.0033$, 15 pairwise comparisons). These results suggest significant genetic structuring among túngara frog populations because nonrandom association between alleles of different loci is expected when gene flow is less between than within populations (Hartl and Clark 1997).

Pairwise F_{ST}, R_{ST}, and D_A were larger between the northern and southern populations than within the two groups (Table 1). The global F_{ST} value was 0.18 ($P < 0.0001$), and F_{RT} between the northern and the southern region was 0.17 ($P < 0.0001$). The respective global R_{ST} and R_{RT} values were 0.52 and 0.67 (both $P < 0.0001$).

Correlation between Geography, Genetics, and Call Distances

The first two dimensions extracted from the MDS model described nearly all the variation in genetic and bioacoustic divergence among túngara frogs populations (genetic divergence: $R^2 = 0.99$; bioacoustic divergence: $R^2 = 0.96$). Visual inspection of the genetic dissimilarities in a bidimensional MDS plot revealed that the northern group and the southern group of túngara frogs are clustered genetically (Fig. 2A). This pattern of genetic divergence corresponds exactly to the gap in the distribution in Central Pacific Costa Rica (Fig. 1). Gamboa did not cluster as tightly with the other populations,
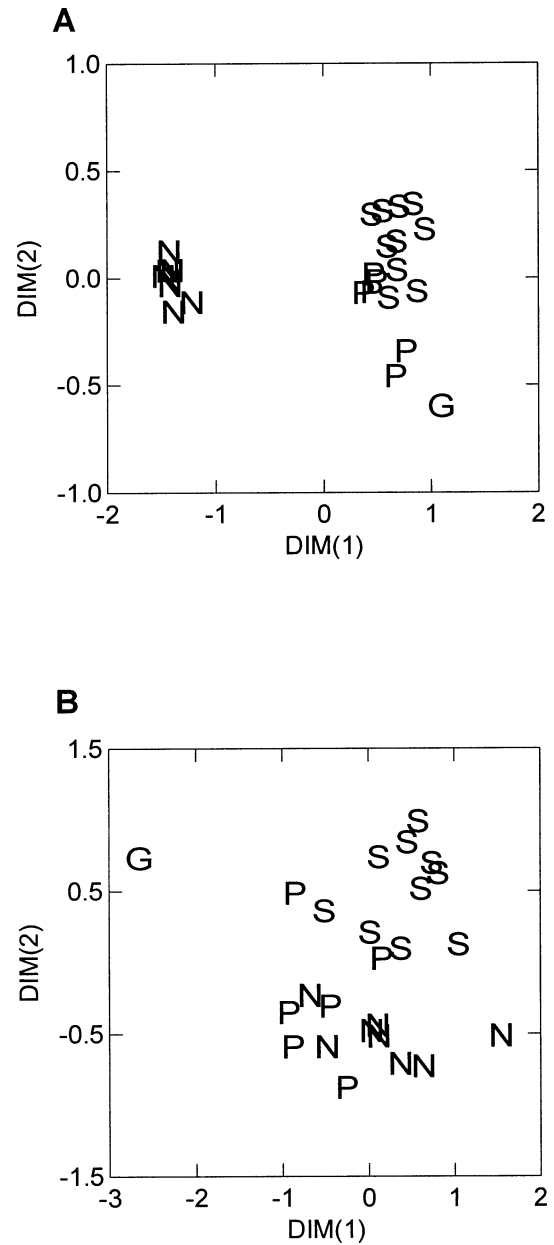

FIG. 2. Multidimensional scaling plot of (A) the genetic divergence (measured as D_A), and (B) bioacoustic distance (measured as Euclidean distances) between pairs of populations. N, northern Costa Rica; S, southern Costa Rica; P, Panama; G, Gamboa.

but there is also a distance of 188 km between Gamboa and the closest population surveyed. It is possible that sampling in these areas would have shown a more continuous distribution of genetic similarity between Gamboa and the other southern populations.

Unlike genetic differences, variation in the mating calls among populations did not cluster into northern and southern groups (Fig. 2B). Although northern and southern Costa Rican populations formed separate clusters in the two-dimensional MDS space, calls of some Panamanian populations resembled southern Costa Rican calls whereas others were more similar to northern calls (Fig. 2B).

TABLE 2. Association between geographic distance in km, genetic divergence (D_A) and bioacoustic Euclidean distances of male calls between 25 populations of túngara frogs in Costa Rica and Panama. Results are shown as correlation and partial correlation coefficients of Mantel tests. Results calculated without Gamboa are in parentheses. Due to Bonferroni correction within regions, $P < \alpha = 0.05/6 = 0.0083$ are significant.

Genetic group		r	P
Total range	Correlation		
	Geographic—genetic	0.79 (0.84)	<0.0001 (<0.0001)
	Geographic—bioacoustic	0.46 (0.22)	<0.0001 (0.0007)
	Genetic—bioacoustic	0.24 (0.24)	0.001 (0.004)
	Partial correlation		
	Geographic—genetic	0.78 (0.83)	<0.001 (<0.0001)
	Geographic—bioacoustic	0.46 (0.04)	0.009 (0.34)
	Genetic—bioacoustic	−0.23 (0.09)	0.99 (0.15)
Northern group	Correlation		
	Geographic—genetic	0.50	0.01
	Geographic—bioacoustic	0.05	0.33
	Genetic—bioacoustic	0.08	0.35
	Partial correlation		
	Geographic—genetic	0.50	0.018
	Geographic—bioacoustic	0.008	0.43
	Genetic—bioacoustic	0.06	0.36
Southern group	Correlation		
	Geographic—genetic	0.79 (0.64)	<0.0001 (<0.0001)
	Geographic—bioacoustic	0.85 (0.68)	<0.0001 (<0.0001)
	Genetic—bioacoustic	0.69 (0.49)	<0.0001 (0.0001)
	Partial correlation		
	Geographic—genetic	0.54 (0.47)	0.001 (0.001)
	Geographic—bioacoustic	0.69 (0.55)	<0.0001 (<0.0001)
	Genetic—bioacoustic	0.05 (0.09)	0.36 (0.17)

Genetic distances (D_A) inferred from distribution of microsatellite allele frequencies were highly correlated with geographic distances among all the populations sampled (Table 2). Also, the partial correlations coefficients across the total range and in the southern region were high and significant. The correlations between bioacoustic and geographic distances were not very strong but showed a similar trend. In contrast, genetic and bioacoustic distances were only weakly correlated and the corresponding partial correlation coefficients not significant. This general pattern did not change when Gamboa was excluded from the dataset (Table 2).

Differences in Bioacoustic Traits between Populations and Regions

Coefficients of variation indicate that the spectral parameters were the less variable parameter within and among populations as well as between groups. Rise shape and whine shape were the most variable parameters within and among populations (Table 3). Relative to the within-population variation, duration and fall time showed the largest effect among populations. There were significant differences in all bioacoustic traits measured between at least two populations within a region (nested ANOVA, Table 3). All call parameters, except the spectral parameters, exhibited significant differences between the northern and southern group. Especially the parameters describing the shape of the call (fall time, fall shape) differed between the groups. Figure 3 shows the distribution of call parameters for each population and allows a visual assessment of differences among them. Duration and fall time increased within the southern group but not within the northern group whereas whine shape decreased toward the south. It is noticeable that the differences in the population means between groups of fall time, fall shape, and rise time were extremely large in comparison with differences within the groups. Aside from some outliers, in fall time and fall shape the mean values between the northern populations

TABLE 3. Within-population coefficients of variation (CV; mean ± SD) and among-population CV for call parameters and results of nested analysis of variance (populations nested in genetic groups) testing for differences in individual call characters among populations and between the northern and the southern genetic group.

| Call parameter	CV_{within}	CV_{among}	Populations (df = 22)		Groups (df = 1)	
			F	P	F	P
Initial frequency	1.58 ± 0.22	2.89	3.05	<0.0001	1.80	0.18
Final frequency	2.37 ± 0.27	5.16	3.68	<0.0001	1.91	0.17
Duration	2.94 ± 0.71	12.9	22.12	<0.0001	14.2	0.0002
Whine shape	8.69 ± 2.05	21.2	6.24	<0.0001	6.92	0.0088
Rise time	8.02 ± 2.15	15.3	4.02	<0.0001	12.7	0.0004
Fall time	2.88 ± 0.65	14.6	25.5	<0.0001	116	<0.0001
Rise shape	11.9 ± 3.65	18.7	2.70	<0.0001	15.7	<0.0001
Fall shape	3.56 ± 0.51	9.74	4.50	<0.0001	87.4	<0.0001
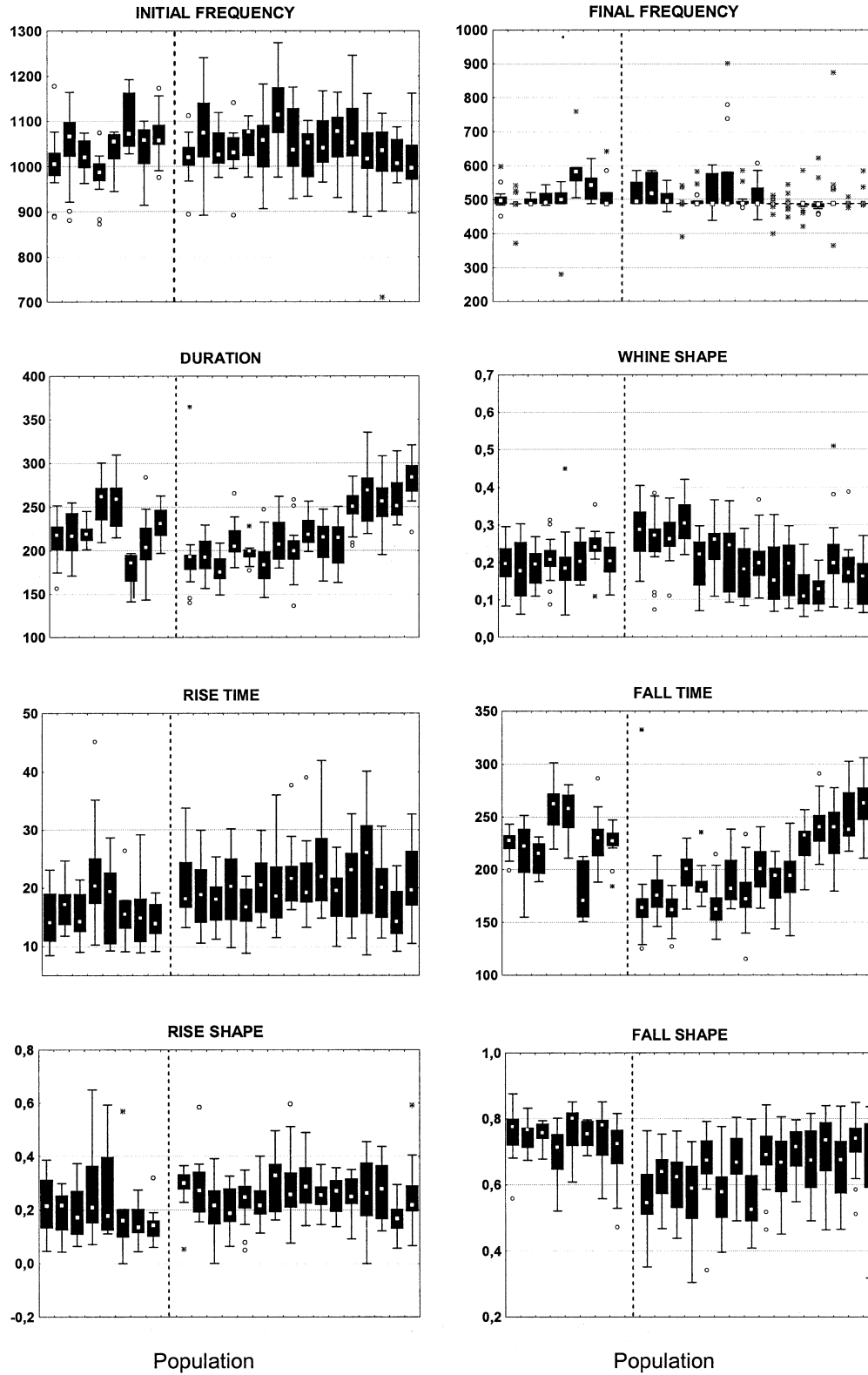
FIG. 3. Population variation in call variables presented as box plots. Populations are arranged from northwest (left) to southeast (right) in the same sequence as in the online Appendix tables. The gap is indicated by a dotted line.

TABLE 4. Results of phonotaxis experiments in five populations across a transect from northern Costa Rica to Central Panama. In every test population approximately 20 females (n) were tested for preferences for the average calls of their local population over calls from other populations. We used the intercept values of the logistic regression (GEE analysis) to estimate the probability and significance of the preference for the local call for each female population. N.Gr., northern group; S.Gr., southern group.

Test population	n (no. of tests)	No. of local choices	Probability for preferring the local call	P
Liberia (N.Gr)	110	72	0.66	0.0008
Cortez (S.Gr.)	99	46	0.45	0.32
Golfito (S.Gr.)	101	67	0.68	0.0003
Gariche (S.Gr.)	107	83	0.79	<0.0001
Santiago (S.Gr.)	83	64	0.77	<0.0001

were more similar to Panamanian populations than the population in south Costa Rica located close to the gap. A similar trend, although less pronounced, was observed in whine shape.

Female Choice Experiments

We tested phonotactic preferences for local versus foreign population calls in five populations (one north of the gap, four southeast of the gap; Table 4). Females exhibited statistically significant overall preferences for their local call to foreign calls (GEE analysis, probability that the intercept of the logistic regression for all females together equals an odds ratio of 1:$P < 0.0001$). The overall probability that a female preferred the local call was 0.67. When female populations were tested separately this preference was significant in all populations except Cortez (Table 4). The strength of preference was significantly different between female populations (GEE analysis: $P < 0.001$), but not between genetic groups of the call with respect to the females ($P = 0.32$) and was not influenced by the bioacoustic distance between local and nonlocal call ($P = 0.51$).

Mating Experiments

Eighty receptive females were mated with males in one of three treatment groups: local male; foreign male same group; foreign male different group. Seventy percent of the couples constructed a foam nest within two nights. The probability of nest construction was significantly influenced by genetic distance, measured as either D_A or F_{ST} between male and female population (logistic regression: $P < 0.01$). Furthermore, the construction of a foam nest was significantly dependent on male group (Table 5). Most females constructed a foam nest with local males (85%) and with foreign males in the same group (67%). Fewer females (41%) constructed a nest when their mate was from the other genetic group. In addition, the success of nest construction was asymmetric: although most northern females (67%) constructed nests with males from the south, only one female (12.5%) from southern Costa Rica constructed a foam nests with a male from the north ($\chi^2 = 5.1$, df $= 1$, $P < 0.025$). In this last case the eggs did not develop. Among nests where eggs developed there was no indication of a significant influence of group

TABLE 5. Results of mating experiments for 80 females, showing the sample size for successful or not successful nest construction with three different male groups. The difference between the male groups was significant ($\chi^2 = 10.6$; df $= 2$, $P < 0.005$).

Male group Nest	(1) Local males	(2) Foreign male same genetic group	(3) Foreign male other genetic group	n
No	6	8	10	24
Yes	33	16	7	56
n	39	24	17	Total $n = 80$

membership on hatching success (Kruskal Wallis ANOVA: $n = 56$, $h = 1.0$, df $= 2$, $P = 0.60$).

DISCUSSION

Population-genetic analyses and analyses of bioacoustic signals revealed significant variation among 25 populations of túngara frogs studied along a transect from Costa Rica to Panama. A gap of approximately 200 km in the distribution of frogs in central Pacific Costa Rica coincides with the genetic divergence between two groups of populations: one north of this gap and one south of it. Genetic differences among populations are also highly correlated with geographic distance over the entire range of the study as well as within each of two genetic groups. To the contrary, bioacoustic differences do not cluster into groups that are congruent with the genetic groups, and are more likely to be predicted by geographic distance than genetic distance. The correlation between geographic and bioacoustic distance was high and significant in the southern but not in the northern group, probably due to the limited geographic extension of the northern group. Call components describing the shape of the call, primarily duration, fall time, and fall shape differed more between groups and populations than did spectral components. There is a general and significant female preference for local over foreign calls, but this preference was not significantly influenced by the genetic group of the foreign call. Mating experiments provided no evidence for a reproductive barrier between male and female populations from the same genetic group. In contrast, we found some evidence for asymmetric isolation between females and males from different genetic groups.

Patterns of Population Structure

Earlier studies of genetic differences among túngara frog populations showed two genetic groups, a northern and southern group (Ryan et al. 1996; Weigt et al. 2005). The genetic differences indicated by allozyme variation and measured by Nei's standard genetic distance (Nei 1978) between the two groups ($D_S = 0.29$) were several times higher than D_S within the groups; a similar pattern was true for sequence divergence of mitochondrial DNA (between groups: 12.6%, within groups: 3.5% and 4.5%; Weigt et al. 2005). The results from our study show that both groups are separated geographically and further confirm the genetic divergence. Although the fixation index F_{RT} between the northern and southern groups is not that high (0.17), the R_{RT} value (0.67), as well as the multidimensional scaling results based on D_A, indicate a strik-

ing genetic division between the groups on both sides of the gap. Since F_{ST} tends to underestimate genetic differentiation when applied to microsatellite data, the R_{ST} statistic might be a better estimator for genetic divergence in our case (Slatkin 1995). Genetic distance (D_A) is highly correlated with geographic distance, implying that much of the variation across the transect can be explained by isolation by distance (Wright 1943; Slatkin 1993). The residual genetic variation can be explained by divergence in allopatry. Based on our results combined with the data from Weigt et al. (2005), we suggest that after the initial separation of the two groups in the Pliocene, túngara frogs from both groups might have reestablished contact and subsequently disrupted again. Because introgression is small and genetic divergence between north Costa Rican and south Costa Rican frogs is large, we assume that the reconnection was of short duration, a long time ago, or both.

Signal and Preference Variation

Male túngara frog calls differ between populations as does the strength of female preference for the calls of the local population. Overall, in about two-thirds of all phonotaxis experiments females preferred the local call over the foreign call. The fact that female preference strength varies among populations and is not absolute is consistent with other studies on túngara frog communication (M. J. Ryan et al., unpubl. ms.).

Signals used in animal communication are composed of multivariate traits (Zuk et al. 1992; Endler and Houde 1995; reviewed in Rowe 1999). A number of túngara frog studies suggested that females do not weight all signal features equally (Ryan and Rand 2001, 2003). Thus, the relative attraction of females to calls is not necessarily predicted by the overall acoustic similarity (measured as Euclidean distance) of the target call to the local call. Ryan and Rand (2003) showed that duration and fall time are critical call components for the attractiveness of the call to females within a population. Our study indicates stabilizing selection on duration and fall time within populations but a strong divergence among populations. Fall time and fall shape are also the most different parameters between the two genetic groups. Thus, it is possible that the females' ability to distinguish between local and foreign calls relies for the most part on these two or three components.

The patterns of call variation found in our study are largely consistent with the results from Ryan et al. (1996). Across the much longer transect from Mexico to northern South America, Ryan et al. (1996) found substantial variation in call characters among populations and between the two genetically different groups. They also found a significant but only modest geographic pattern of call variation (partial Mantel test: $r = 0.29$) and a still weaker correlation between allozyme divergence and call variation ($r = 0.13$). The authors suggested that the proportion of call variation not explained by geography might be driven by arbitrary sexual selection and pointed to the significance of arbitrary divergence of mate recognition signals for reproductive isolation and formation of new species (reviewed in Panhuis et al. 2001). For anurans there is little evidence that the environ-ment affects signal diversity (Kime et al. 2000; Gerhardt and Huber 2002; but see Ryan et al. 1990a). Parameters probably most important for females to distinguish between local and foreign males, duration and fall time, indeed show a clear geographic pattern. Moreover, there is some hint that character displacement in male calls might have occurred during the reestablishment of contact between both groups because the population means for fall time, fall shape, and whine shape deviate exceptionally between populations on both sides of the gap. Whether these parameters are particularly salient to females, and the additional possibility that there is character displacement in female preferences, should be explored in this same geographical region.

Reproductive Isolation

We conducted mating experiments between males and females from both sides of the gap to investigate postzygotic isolation in these frogs. Most female-male combinations produced nests and tadpoles, indicating no absolute reproductive isolation during the egg or tadpole stage. It is still possible, however, that frogs from different populations are reproductively isolated during later stages of their life; that is, adults may be sterile or the F_2 offspring may be inviable. Except for one case, females from southern Costa Rica did not construct foam nests with males from northern Costa Rica. Because the failure of nest production is prezygotic, our sample size for evidence on postzygotic isolation is reduced to one and should be confirmed in additional studies.

The possibility of asymmetric reproductive isolation in túngara frogs is intriguing but not new and is well documented in frogs (Sasa et al. 1998; Hoskin et al. 2005). The comprehensive survey of Sasa et al. (1998) found a positive relationship between genetic divergence (measured as D_S using allozymes) and degree of postzygotic isolation. According to Weigt et al. (2005), D_S between northern and southern túngara frogs is 0.29. This is nearly the value ($\sim D_S = 0.3$) that Sasa et al. extracted for being the limit of viability for hybrid offspring.

Gleason and Ritchie (1998) suggested that divergence in song in *Drosophila* is rather unrelated to genetic distance between and within species even though song is important for male mating success (Ritchie et al. 1998). They proposed that song is the first to diverge during speciation, followed by prezygotic isolation due to sexual selection for certain song types. Postzygotic isolation evolves when some prezygotic barriers are already established and increases with genetic distance until complete. The data from this study support this general pattern of relationship between signal variation, reproductive isolation, and genetic divergence: as in the preceding studies, túngara frog mating calls vary between populations but are not correlated with genetic distance. Behavioral isolation is widely present but discrimination against foreign calls is not restricted to calls from the other side of the gap or genetically very distant populations (for similar results in a species of salamander see Tilley et al. 1990). The question whether northern and southern túngara frogs belong to one species, two subspecies, or incipient separate species needs further analysis of pre- and postzygotic isolation patterns.

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