Patterns of mating call preferences in túngara frogs, *Physalaemus pustulosus*

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Abstract

We examine acoustic mating preferences of a focal population at four different scales of divergence: within the population, between populations in the same genetic group, between populations in different genetic groups and between different species. At all scales there is substantial genetic divergence, variation in mating signals and preferences are influenced by signal variation. There is, however, no support for the hypothesis that mating preferences accumulate predictably with genetic distance. Females preferred the local conspecific call to the foreign conspecific call in about one-third of the experiments, and preferred the local call to all of the heterospecific calls tested. But there was no significant relationship between the variation in the strength of preference and genetic distance either among conspecific populations, or among heterospecific species. Thus, in this study macroevolutionary patterns are not apparent at the microevolutionary scale.

Introduction

Mating preferences can have important effects on genetic structuring among individuals of a population, among populations and among species. The genetic effects of mating can, in turn, influence the evolution of preferences at these different scales. Little is known, however, as to how preferences vary across such scales. This is a relevant issue in understanding how species mate recognition evolves. For example, studies often examine mate preferences among conspecific populations to understand how population preferences can give rise to species preferences, whereas at the other end of the spectrum, studies examine preferences between well-defined species to gain insights into how these existing preferences arose from population-based preferences (Andersson, 1994; Coyne & Orr, 2004). There seems to be an implicit assumption, perhaps an incorrect one (e.g. Houde, 1993), that mating preferences scale predictably across different scales of evolution but this assumption has rarely been examined (but see Safi *et al.*, 2006).

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There is no doubt that mating preferences exist across different scales of evolution. The fact that females prefer to mate with some males in the population over others was suggested by Darwin (1871) and has been documented extensively since (Andersson, 1994). Sometimes such mating preferences are influenced by genetic relatedness of potential mates. Much of the evidence for such genetically based mating preferences document preferences based on MHC variation among males through olfactory cues (e.g. Potts *et al.*, 1991; Milinski *et al.*, 2005; Boehm & Zufall, 2006). Waldman (Waldman *et al.*, 1992; Waldman, 2001), in addition, has demonstrated mating preferences based on genetic relatedness mediated by acoustic signals in toads.

Differences in mating preferences can also be exhibited between populations, and this is an arena in which speciation and sexual selection can intersect (e.g. Boake, 2000; Panhuis *et al.*, 2001; Carson, 2003; Coyne & Orr, 2004; Boul *et al.*, 2007). Sexual selection can cause the rapid divergence of mate recognition among geographically proximate populations and thus contribute to speciation (Lande, 1981; West Eberhard, 1983). There are few studies, however, that have documented how mating preferences vary among populations as a function of genetic divergence (e.g. Tilley *et al.*, 1990; Butlin & Ritchie, 1991).

Mating preferences between species have been well documented since the modern evolutionary synthesis (Dobzhansky, 1940; Mayr, 1942). Preferences can maintain reproductive isolation between conspecifics and heterospecifics, and can contribute to speciation by generating prezygotic isolation between incipient taxa (Dobzhansky, 1940; Mayr, 1942, 1963; Shaw & Lugo, 2001; Coyne & Orr, 2004). Mating preferences between species can vary as a function of the genetic divergence between species. For example, Coyne & Orr (1989, 1997, 2004) showed that both prezygotic and post-zygotic isolation increases with genetic divergence between species of *Drosophila*. Mendelson (2003) showed similar results in her study of darters in the fish genus *Etheostoma*. Other studies have addressed post-zygotic, but not prezygotic, isolation in lepidoptera (Presgraves, 2002), frogs (Sasa *et al.*, 1998), fish (Russel, 2003) and birds (Price & Bouvier, 2002) and also show that isolation increases with genetic divergence.

Although mating preferences at different scales are well documented, there has been little attempt to relate these patterns empirically (Ryan & Rand, 1993; Boake *et al.*, 1997; Safi *et al.*, 2006). Our purpose was to determine how mating preferences covary with genetic differences between males in the same population, between males from different populations in the same genetic group, between different genetic groups in the same species and between different species.

Here, we document acoustic mating preferences in a focal population of the túngara frog, *Physalaemus pustulosus*. We determine the preference of these females for their local call vs. the calls of 27 foreign populations across the species' range. We then combine these data with previously published data from this same population on mating preferences between calls of individuals within the populations, and between conspecific and heterospecific calls. We then ask if genetic distance can predict patterns of mate choice within and between these levels. We further supplement these data with synthetic calls that are intermediate between species to gain some further insights into the dynamics of female preference evolution.

The system

The sexual communication system of the túngara frog has been studied in detail (reviewed in Ryan, 1985; Ryan & Rand, 2003a). Female mating preferences are mediated to a large degree by the conspecific mating call. Females attend to variation in a variety of call parameters when choosing a mate (e.g. Ryan, 1980; Ryan *et al.*, 1990; Wilczynski *et al.*, 1995; Bosch *et al.*, 2000a,b). They also exhibit phonotactic preferences between calls that differ in signal complexity (simple vs. complex; Rand & Ryan, 1981) that differ among individuals within the population (Ryan & Rand, 2003b), and they prefer conspecific

to heterospecific calls (Ryan & Rand, 1995, 1999; Ryan *et al.*, 2003).

Túngara frogs have an interesting phylogeographic pattern. The genus *Physalaemus* is South American in origin and *P. pustulosus* is the only species to extend its range north into Middle America. Túngara frogs range from just north of Veracruz, Mexico, southward through the Darien Gap of Panama and into the Magdalena Valley in north-eastern Colombia, and westward across the llanos of Venezuela and onto the Guyana Shield (Fig. 1).

Physalaemus pustulosus and its sister species, *P. petersi*, diverged from the rest of the *P. pustulosus* species group about 14 million years ago, and from each other about 12 million years ago (Fig. 1; Weigt *et al.*, 2005). There are two major genetic groups or clades within *P. pustulosus* (Ryan *et al.*, 1996; Weigt *et al.*, 2005). One group consists of populations from Mexico to northern and western Costa Rica. The other consists of populations from southern and eastern Costa Rica to South America. The two genetic groups diverged about 9 millions years ago, prior to the establishment of the Panamanian land bridge, which is thought to have formed between 2.8 and 3.1 million years ago (Fig. 1; Weigt *et al.*, 2005). The data suggest the initial invasion became the northern genetic group of *P. pustulosus* and the southern group resulted from at least one additional invasion over the land bridge. The only data on population-based preferences in túngara frogs is a study limited to one particular area in Costa Rica and western Panama, an area that included populations of both the northern and southern genetic groups (Pröhl *et al.*, 2006).

The rather long period of geographical isolation among populations offers an unusual context for analysing the divergence of female mating preferences in a well-studied communication system. When these data are combined with previous data on genetically based mate preferences within the population and preferences between conspecifics and heterospecfics, it provides an unprecedented examination of how mate preference varies across very different scales of genetic divergence: within the population, between populations in the same genetic group, between populations in different genetic groups and between different species.

Materials and methods

We present data on mating call preferences of female túngara frogs from Gamboa, Panama for local calls vs. foreign calls from 27 conspecific populations. In addition, we examine female preferences for local calls vs. calls that are intermediate between the local call and calls of two heterospecifics, *Physalaemus coloradorum* and *Physalaemus enesefae*. These data are presented along with published data from the same population in which we measured female preferences for calls of males within the
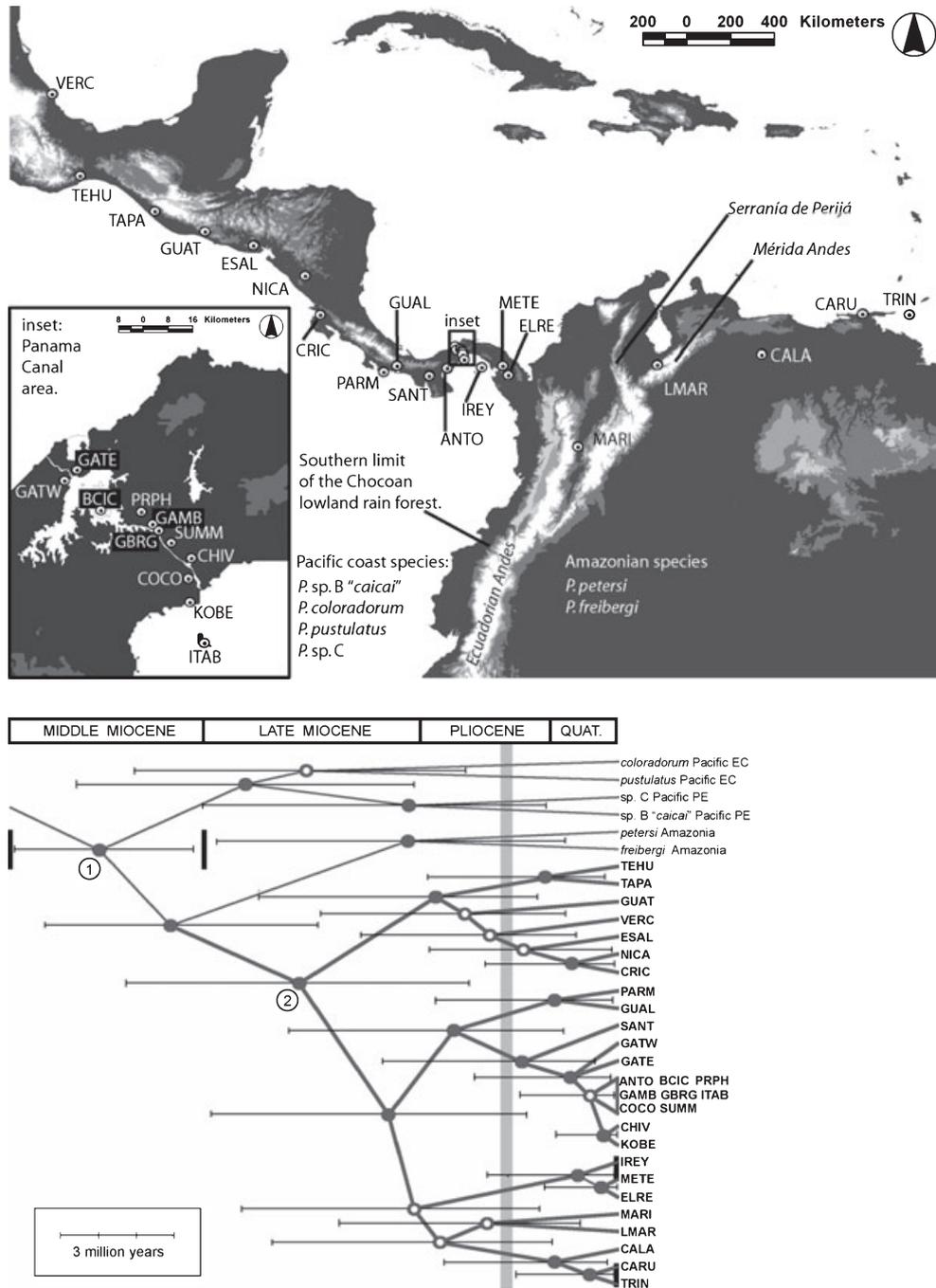
Fig. 1 (Top) Map showing collecting localities for *Physalaemus pustulosus* (circled dots). Each locality is labelled with the same four-letter code used in Table 1. A close-up map of the Panama Canal Zone localities (inset) is shown on the lower left. Elevation is indicated by five different shades, with the darkest shade representing 1–500 m and the lightest shade representing elevations above 2000 m. The general ranges of other species in the *P. pustulosus* species group are also indicated. (Bottom) Estimation of absolute divergence times for species of the species group and populations of *P. pustulosus*. Heavy black bars on either side of node (1) illustrate the assumption that the rise of the Ecuadorian Andes between 16.4 and 11.2 years ago precipitate the split at node 1. Nodes are placed according to their mean divergence time relative to the geological timescale indicated above (Quat., quaternary). Black nodes received higher statistical support than open nodes, and the error bars on each node denote the central 95% of the estimated posterior probability distribution of divergence time. The long thick vertical grey bar indicates the rise of the Isthmus of Panama between 3.1 and 2.8 million years ago. Node (2) represents the split between north-east and south-west lineages of *P. pustulosus* (modified from Weigt *et al.*, 2005).

same population who varied in their genetic relatedness to the female being tested (Lampert *et al.*, 2006), and female preferences for local calls vs. calls of heterospecifics (Ryan & Rand, 1995, 1999). Details of the phonotaxis studies are identical for the other studies, as is stimulus synthesis except where noted.

Collection sites and samples

Ryan *et al.* (1996) sampled call and genetic structure in 30 populations across the distribution of *P. pustulosus* (Fig. 1; see also Table 1). In this study, we conducted phonotaxis experiments with females from one site, Gamboa (GAMB), which has been the focus of a detailed analysis of female preferences over the last two decades. We also report on some phonotaxis experiments with females from a nearby population, Gamboa Bridge (GBRG). This population is less than a kilometre from Gamboa, but is on the other side of the Chagres River, which acts as an important barrier to gene flow (Lampert *et al.*, 2003). There is substantial genetic isolation between these two populations (population pairwise-genetic distance $F_{ST} = 0.031$; Lampert *et al.*, 2003).

Genetic distances

We used differences in COI sequence to estimate the maximum likelihood genetic distances (Weigt *et al.*, 2005) between GAMB (and in some cases GBRG) and other populations as a variable in predicting population-based preferences. COI distances between túngara frogs and other heterospecifics were also available (Ryan & Rand, 1995, 1999). Divergence in mitochondrial genes is not rapid enough to allow estimates of genetic relatedness between individuals in the populations, which is also a 'genetic distance' albeit at a finer scale. Those estimates were based on variation in microsatellites (Lampert *et al.*, 2006). Nevertheless, the results from that study lead to a clear interpretation in the context of our general goal, documenting how mate recognition diverges as a function of genetic distance.

Call analysis and stimulus synthesis

Male túngara frogs produce a two component call consisting of a whine followed by 0–7 chucks. The whine is both necessary and sufficient to elicit phonotaxis from females. Chucks can be added to the call, and calls with

Table 1 Code for localities (cf. Fig. 1), localities, their latitudes and longitudes, the geographic distance (kilometres) to Gamboa, the Euclidean distance of their call to the Gamboa call, the genetic distance based on mitochondrial COI sequence to Gamboa and the proportion of females from Gamboa preferring the local call to the call of each population ($N = 20$ in all cases).

Code	Locality	Latitude	Longitude	Geographic distance	Call distance	Genetic distance	Local preference
VERC	Veracruz, Mexico	19.73	96.43	2150.4	1.807	13.9	0.55
TEHU	Tehuantepec, Mexico	16.35	95.28	1869.9	1.682	14.1	0.75
TAPA	Tapachula, Mexico	14.86	92.22	1503.6	1.692	13.5	0.80
ESAL	San Miguel, El Salvador	14.49	88.18	1098.9	1.885	14.7	0.60
NICA	Tipitapa, Nicaragua	12.20	86.07	775.7	1.690	12.5	0.65
CRIC	Liberia, Costa Rica	10.61	85.45	651.0	1.962	13.1	0.70
PARM	Puerto Armuelles, Panama	8.27	82.86	360.2	1.292	4.6	0.55
GUAL	Gualaca, Panama	8.53	82.29	292.3	0.973	4.2	0.70
SANT	Santiago, Panama	8.13	80.98	178.1	1.310	2.0	0.85
ANTO	Anton, Panama	8.40	80.24	99.3	1.822	2.0	0.80
GATW	Gatun west, Panama	9.25	79.95	31.2	1.028	1.2	0.70
GATE	Gatun east, Panama	9.28	79.92	30.1	1.678	0.5	0.70
BCIC	BCI, Panama	9.17	79.85	17.3	0.875	0.2	0.65
PRPH	Pipe Line Rd, Panama	9.16	79.73	5.8	0.632	0.4	0.60
GAMB	Gamboa, Panama	9.12	79.70	0	0	0	Local call
GBRG	Gamboa Bridge, Panama	9.11	79.69	0.5	1.677	0.5	0.80
SUMM	Summit, Panama	9.07	79.65	7.9	0.874	0.5	0.50
CHIV	Chiva Chiva, Panama	9.02	79.59	15.7	1.516	0.4	0.75
COCO	Cocoli, Panama	8.97	79.59	19.8	1.096	0.4	0.65
KOBE	Kobbe, Panama	8.90	79.59	26.9	1.345	0.4	0.65
ITAB	Isla Toboga, Panama	8.80	78.45	141.8	1.021	0.2	0.75
IREY	Isla El Rey, Panama	8.45	78.85	119.2	1.655	7.9	0.65
METE	Metete, Panama	8.50	77.97	202.3	1.052	8.3	0.45
MARI	Mariquita, Colombia	5.18	74.90	686.7	1.314	8.3	0.85
LMAR	Lago Maracaibo, Venezuela	8.56	71.63	888.9	1.121	6.7	0.80
CALA	Calabozo, Venezuela	8.98	67.35	1356.1	0.810	6.3	0.70
CARU	Carupano, Venezuela	10.64	63.22	1812.6	1.046	7.3	0.30
TRIN	Trinidad	10.63	61.28	2024.0	1.285	7.0	0.50

chucks are more attractive than calls without chucks. Furthermore, the fundamental frequency of the whine is the component critical to elicit phonotaxis. A synthetic fundamental is more attractive than the other four harmonics combined, and adding upper harmonics to the fundamental does not increase its attractiveness (Rand *et al.*, 1992; Wilczynski *et al.*, 1999). Furthermore, a synthetic call with only the fundamental frequency is on average as attractive as natural calls. Because we are interested in female recognition and preferences among populations, we conducted phonotaxis experiments with these synthetic whines.

We synthesized calls based on the average of eight call parameters (Fig. 2) from each of the 27 populations surveyed by Ryan *et al.* (1996); two populations were not used (Table 1). This same approach was used to synthesize the calls of heterospecifics used in the study of Ryan & Rand (1995, 1999) and whose results are reported here. We also used several synthetic calls that were intermediate between *P. pustulosus*–*P. coloradorum* and *P. pustulosus*–*P. enesefae*. These calls varied in each acoustic parameter by fixed increments between the two species. We constructed intermediate calls by multiplying each call parameter by a fixed proportion of the difference between the conspecific and heterospecific call. Those proportions were: 0.06, 0.12, 0.25, 0.37 and 0.50. Thus, for the 0.06 call, for example, each of the call parameters deviated from the *P. pustulosus* call by 6% and from the heterospecific call by 94%, whereas the call parameters of the 0.50 call were equally similar to the call of both species (see also Ryan *et al.*, 2003). Because the *P. coloradorum* call is more similar to the *P. pustulosus* call than is the *P. enesefae* call, for any pair of intermediate calls of the same proportion (e.g. *P. coloradorum* – 0.37 vs. *P. enesefae* – 0.37), the *P. coloradorum* call is always more similar to the *P. pustulosus* call than is the *P. enesefae* call (see Ryan *et al.*, 2003).

The only exception in using synthetic calls in the phonotaxis experiments reported here is in the study by Lampert *et al.* (2006). They tested the hypothesis that females assessed acoustic cues to choose mates within a population based on degree of genetic relatedness. Natural calls of males were used in that study. As indicated above, however, females do not discriminate between synthetic calls and natural calls, thus these phonotaxis results are comparable to the ones using synthetic calls.

Phonotaxis experiments

Females from GAMB were tested in paired-choice tests in which they were presented with the local call and one of the other 27 foreign conspecific calls. In all cases 20 females were tested, for a total of 540 female choice tests.

We conducted a smaller set of phonotaxis experiments with females from the GBRG population. We selected eight foreign calls that bracketed the responses from
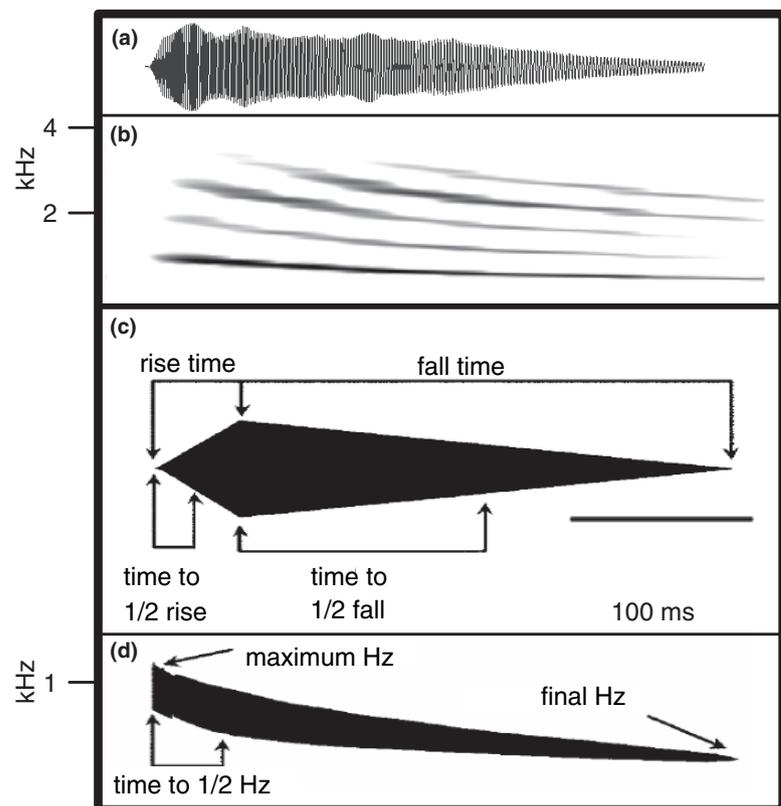

Fig. 2 Illustration (a) waveform and (b) spectrogram of a natural *Physalaemus pustulosus* whine call, and the call variables, measured in the (c) time and (d) spectral domain.

GAMB females; again all sample sizes were 20. We chose five calls that GAMB females found the most unattractive (CRIC, GUAL, SANT, MARI and CALA), one call that was similar in attractiveness to the GAMB call (SUMM) and one foreign call that was almost significantly preferred to the GAMB call (CARU). We also tested the preference of GBRG females to local calls of GAMB, and their preference for simple (whine) vs. complex (whine-chuck) calls which is used to screen for phonotactic females.

We conducted the phonotaxis experiments in an Acoustic Systems (Austin, TX, USA) sound attenuation chamber that measured 1.8 × 2.7 m. The female's behaviour was observed on a video monitor equipped with an infrared light source connected to a wide-lens video camera on the chamber's ceiling. We placed a female under a small cone in the centre of the chamber; the cone was raised remotely to initiate testing. We broadcast the test stimuli antiphonally from speakers in the centre of walls opposite to one another such that the peak amplitude of the whine of each test call at the centre of the arena was 82 dB SPL (re. 20 μPa). Calls were broadcast at a rate of one call per 2 s from each speaker, a typical calling rate for male túngara frogs. A positive phonotactic response was noted if a female approached within 10 cm of one of the speakers as long as this response did not result from the female following the chamber's walls. A 'no response' was recorded if a female remained motionless for the first 5 min or any subsequent 2 min of the trial, or did not exhibit phonotaxis after 15 min. Typically, female túngara frogs are only receptive to mating calls on the night they are collected in amplexus at the breeding site.

Statistics

For any single experiment our sample size of 20 will uncover large effects; at $\alpha = 0.05$ with an exact binomial probability test, a preference strength of 0.66 for either stimulus would be a statistically significant preference. We have only weak power to accept the null hypothesis when there is a trend slightly above $P = 0.05$ ($\beta = 0.308$). Although we note if a preference is statistically significant for each experiment, our main question is whether there are patterns of covariation of preference strength with genetic distance. For this analysis, the sample of 20 females is used to estimate preference strength for the calls of each of the 27 populations studied. As will be noted below, within the conspecific populations the strength of preferences varied by a factor of 2.83 and the genetic distances by a factor of 73.5.

Females were tested with more than one stimulus, but it was not possible to test females with all 27 stimuli during her receptive period (less than one night), thus a repeated measures design was not possible. On average, each female was tested and responded in 3.9 (SE = 0.23) of the 27 phonotaxis experiments. Previous studies,

however, have shown that female responses are independent among phonotaxis experiments, and individual females are not consistently different from one another when repeatability has been measured (Kime *et al.*, 1998; Ryan *et al.*, 2003).

Data were analysed with SYSTAT. Individual call variables were compared among populations and genetic groups with a nested analysis of variance (ANOVA) using a general linear model. Multivariate analyses were conducted with a principal component analysis (PCA) of the standardized call variables with varimax rotation. Discriminant function analyses were used to determine the accuracy with which call variation assigned populations to the northern or southern group.

We used call distance, genetic distance (based on COI sequence) and geographic distance as predictor variables of female preference among populations. Call distance is the Euclidean distance between the standardized call variables of GAMB and the other populations and species. The genetic distance measure is explained above and geographic distance is obvious. We measured the association of distance measures and female preference with a Pearson product-moment correlation. Variation in female preferences among populations was analysed with a nested ANOVA using a general linear model. As a general benchmark, the significance of each population contrast was ascertained with an exact binomial test. An analysis of covariance was used to compare preferences for the same set of foreign calls between populations using the acoustic similarity between foreign and each local call as a covariate. Multiple regression analysis was used to estimate predictability of multiple factors on call preference.

Results

Genetic divergence among populations and species

The genetic distance based on COI sequence variation between GAMB and all other conspecific populations and species tested ranged from 0.002 to 1.026 (mean = 0.187, SE = 0.048). The distance to all other conspecifics populations ranged from 0.002 to 0.147 (mean = 0.054, SE = 0.010). The genetic distances from GAMB to the other southern populations (range: 0.002–0.082, mean = 0.031, SE = 0.007) was substantially smaller and did not overlap the range of distances to the northern populations (0.125–0.147, mean = 0.136, SE = 0.003). The genetic distance to other species ranged from 0.492 to 1.026 (mean = 0.701, SE = 0.067).

Call variation among populations

Calls varied substantially among populations and they also sorted among the northern and southern genetic groups (Table 2, Fig. 3). A nested analysis showed significant variation in five of eight call variables between

Table 2 Mean of call variables from each population.

Code	Maximum Hz	Final Hz	HF shape	Rise time	Rise shape	Fall time	Fall shape	Duration	N
VERA	944	525	0.29	27	0.26	195	0.46	222	10
TEHU	920	523	0.23	22	0.33	222	0.55	243	7
TAPA	963	512	0.21	19	0.30	243	0.58	261	10
ESAL	938	492	0.24	18	0.28	245	0.74	263	11
NICA	954	483	0.25	17	0.28	235	0.66	252	13
CRIC	1018	510	0.21	19	0.29	225	0.69	244	11
PARM	1018	510	0.21	19	0.29	225	0.69	244	10
GUAL	950	484	0.27	46	0.17	216	0.34	262	4
SANT	954	511	0.26	61	0.29	184	0.18	245	25
ANTO	1006	569	0.29	25	0.23	228	0.58	254	11
GATW	938	500	0.25	21	0.26	235	0.53	256	12
GATE	938	500	0.25	21	0.26	235	0.53	256	11
BCIC	927	465	0.27	34	0.37	267	0.46	301	25
PRPH	871	472	0.26	49	0.25	264	0.24	313	9
GAMB*	1030	464	0.19	41	0.39	282	0.63	324	250
GBRG	964	506	0.24	37	0.25	233	0.24	270	10
SUMM	964	506	0.24	37	0.25	233	0.24	270	13
CHIV	942	513	0.26	28	0.29	259	0.50	287	8
COCO	971	522	0.25	46	0.22	253	0.47	300	9
KOBE	971	522	0.25	46	0.22	253	0.47	300	6
ITAB	859	454	0.27	40	0.29	258	0.18	298	5
IREY	973	543	0.31	41	0.34	303	0.42	344	5
METE	938	489	0.29	38	0.40	309	0.51	347	12
MARI	915	512	0.25	26	0.17	237	0.42	263	8
LMAR	938	489	0.29	38	0.40	309	0.51	347	6
CALA	875	495	0.32	64	0.29	191	0.11	255	4
CARU	827	429	0.28	33	0.31	223	0.38	257	9
TRIN	835	469	0.35	19	0.22	217	0.01	236	1
Clades	2.03	3.66	8.53	14.60	0.55	12.24	24.92	55.00	
P-value	0.15	0.06	< 0.01	< 0.01	0.46	< 0.01	< 0.01	< 0.01	
Populations (clades)	8.56	8.64	8.04	2.08	4.18	12.04	2.71	13.66	
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	

The bottom two rows present the results of a nested analysis of variance for each call variable comparing clades and with populations nested within clades. Within each cell the *F*-statistic is shown on top and the probability on bottom. The 'local' call of the focal population is GAMB and is starred.

groups, and of all call variables between populations within groups.

Principal component analysis also revealed that calls vary among populations and tend to sort into northern and southern groups (Fig. 3). A discriminant function analysis of the mean call variables also sorts calls among genetic groups. All six of the northern populations were correctly assigned to the northern group, whereas 18 of the 22 southern populations were correctly assigned to their group (Wilks' $\lambda_{7,1} = 0.379$, $F_{7,20} = 4.677$, $P = 0.003$).

We determined the degree that differences between the local call (GAMB) and each of the foreign calls is associated with geographic and genetic distance. Call differences were correlated with the geographic distance between GAMB and other populations ($r_{26} = 0.43$, $P = 0.025$), but not with genetic distance ($r_{26} = 0.018$, $P = 0.92$). Geographic and genetic distance are significantly correlated ($r_{26} = 0.751$, $P < 0.001$), but only 56% of the variation is accounted for, thus it is not surprising that

calls are significantly correlated with geographical but no genetic distance.

Preference variation among populations

Females showed preferences for local calls over foreign calls (i.e. exact binomial probability, $P \leq 0.05$) in nine of the 27 phonotaxis experiments (Fig. 4; Table 1). There were no preferences for a foreign call, although there was a strong trend for such in response to calls from Carupano, Venezuela (CARU, Table 1). There was significant variation in female preference for local vs. foreign calls among populations nested within genetic groups ($F_{25,513} = 1.62$, $P = 0.032$), but not between groups ($F_{1,513} = 0.074$, $P = 0.796$). Furthermore, the mean strength of preference for GAMB females vs. calls from populations pooled over smaller, although arbitrary, geographical regions are quite similar: 0.68 preference for local calls vs. calls from populations within 100 km of GAMB; 0.67 vs. the remainder of the popu-
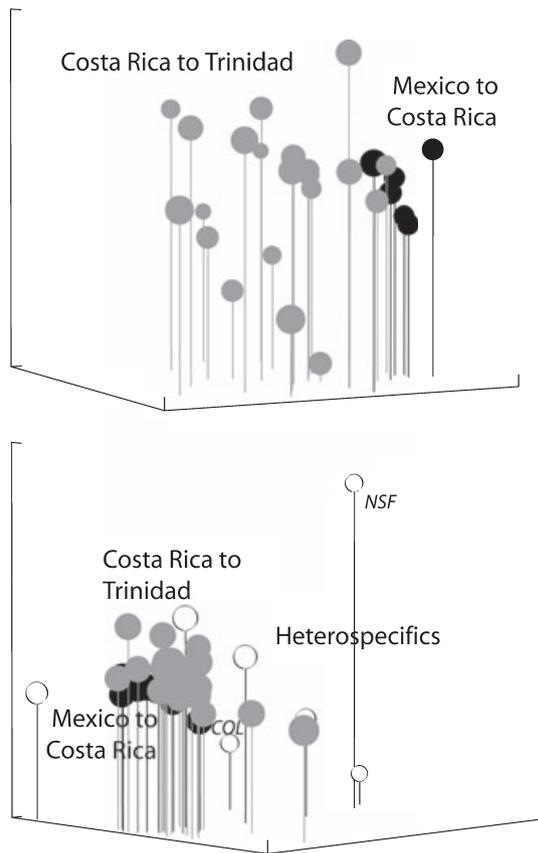
Fig. 3 Plots of first three principal components for measures of call variation (top) among populations of *Physalaemus pustulosus* and (bottom) *P. pustulosus* populations and the heterospecifics tested. Gray circles represent *P. pustulosus* populations in the southern group, filled circles the northern group and open circles the other species. 'NSF' is *Physalaemus enesefae*, and 'COL' is *Physalaemus coloradorum*, the two heterospecifics used in the synthesis of intermediate calls.

Fig. 4 The proportion of females that prefers the local call vs. the foreign call of each population. Order of populations is from Veracruz to Trinidad (see abbreviations in Table 2). The area on and above the upper horizontal dashed line indicates a significant preference for local call; on and below the lower horizontal dashed line is a preference for the foreign call (binomial $P < 0.05$). Vertical dashed line separates northern (left) and southern populations.

lations in Panama; 0.63 vs. populations in South America; 0.65 vs. the remainder of populations in Central America and 0.70 vs. the populations in Mexico. At any level of analysis there appears not to be variation in the strength of preference for local vs. foreign calls among different geographic regions.

Variation in female preferences, unlike calls, was not predicted by geographic or genetic distances. A multiple regression analysis in which the independent variables were geographic distance and genetic distance explained only 10% of the variation in preferences among populations ($F_{3,23} = 0.81$, $P = 0.50$; Fig. 5).

The acoustic (Euclidean) distance between the GAMB calls and foreign calls did not predict variation in preference ($r^2 = 0.02$, $F_{26} = 0.39$, $P = 0.54$). But the first three components of the PCA, which sorted calls into northern and southern groups, tended to predict variation in preferences, although the trend was not quite statistically significant ($r^2 = 0.274$, $F_{3,23} = 2.89$, $P = 0.057$). These
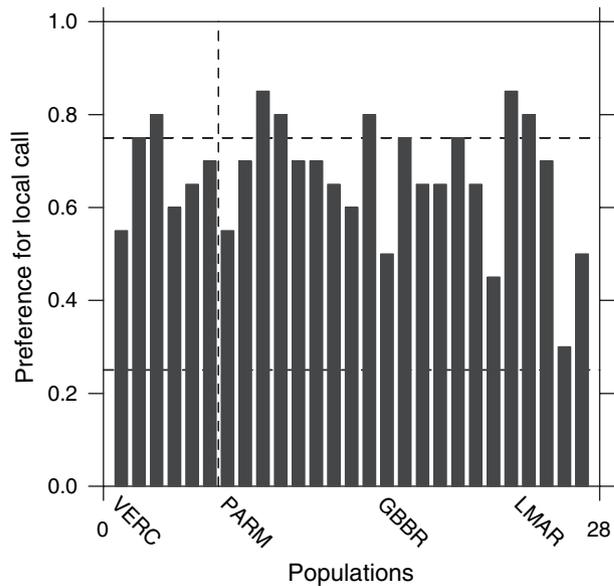
variables also sorted calls into northern and southern groups in the analysis presented above.

We also conducted some phonotaxis experiments with females from GBRG. First, we tested their preferences for complex calls vs. simple calls. We use this test to screen for phonotactic females, so each female is tested multiple times with this stimulus pair. There was a strong preference for complex calls to simple ones (63 vs. 10, $P < 0.001$). The strength of this preference for complex calls (0.863) is very similar to the strength of preference for complex calls in the GAMB population over a 20-year period (0.856; 3135 complex vs. 527 simple; Gridi-Papp *et al.*, 2006). Thus, for this preference the females from both populations are quite similar.

The populations were not very similar in their response to the same foreign calls ($r_7 = -0.63$, $P = 0.123$). The comparison in each experiment is not equivalent because females are presented the same foreign calls vs. their own local call. The two local calls are different (*z*-score of call Euclidean distance = 0.686), and the distance between each local call and the same foreign calls differs significantly between the populations (mean distance GBRG = 0.93, GAMB = 2.00, $t_7 = -5.41$, $P < 0.01$). There were no differences between the two populations in the adjusted mean preferences for the foreign calls when call distance was a covariate (GAMB, mean = 7.98, *SE* = 1.71; GBRG, mean = 8.02, *SE* = 1.71; $F_{1,11} < 0.001$, $P = 0.99$).
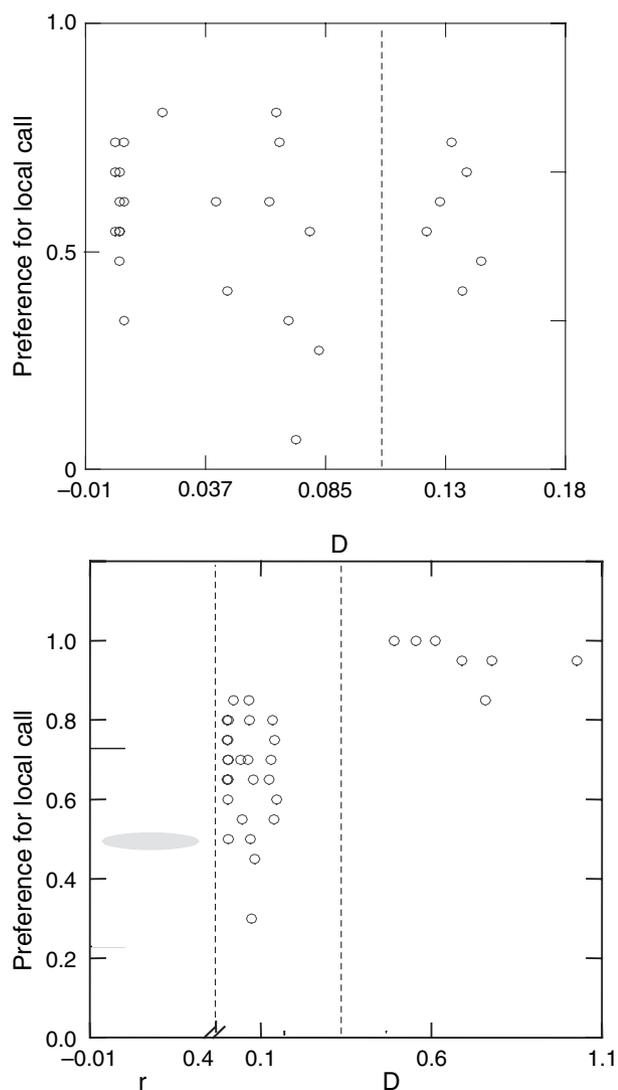
Fig. 5 (Top) The proportion of females showing preference for the local call vs. the foreign call as a function of genetic distance (D). Circles to the right of the dashed line represent northern populations. (Bottom) The same data as above with preferences for males within the population (far left section) and heterospecifics (far right section). Preferences for calls within the population are plotted against genetic relatedness (r) between the female and the call preferred. Actual data are not plotted but illustrate that there is no relationship between preference and r. Preferences among populations and species are plotted against sequence divergence (D).

Preferences within populations

Lampert *et al.* (2006) used highly variable microsatellites (Pröhl *et al.*, 2002) to estimate genetic relatedness of males and females in the GAMB population. Relatedness (r) between males in the population ranged from −0.378 to 0.630 and averaged 0.015. Differences in calls among males were not correlated with genetic relatedness.

Relatedness between males and females ranged from −0.402 to 0.563 with a mean of −0.015. The average genetic relatedness between a male and female of a mated pair (0.049) was not significantly different from a random sample of the population.

Other factors could circumvent mate choice, and the researches might not have measured relevant acoustic parameters that were informative of relatedness. This appears not to be the case, however. In phonotaxis experiments female call preference was not predicted by the genetic relatedness of the test female to the calls of each of the males with which she was tested. This was true when pairs of calls of males for testing were drawn from a random sample ($n = 20$, $P = 0.75$), or if males were intentionally selected to maximize the difference in genetic relatedness ($n = 20$, $P = 1.0$).

Preferences among species

Ryan & Rand (1995, 1999) tested preferences of GAMB females for the local call vs. the calls of seven other congeneric species, four in the species group. In all experiments there was a significant preference for the local call over the heterospecific call; strength of preference ranged from 0.85 to 1.0 (all $n = 20$, all $P < 0.05$; Fig. 5). Given both the small sample size of tests ($n = 7$) and the uniformly strong preferences for conspecifics calls, there was not a significant correlation between call preference and genetic distance ($r_6 = -0.49$, $P = 0.26$). When combining the data on preferences between populations and between species, however, there was a significant correlation between strength of local preference and genetic distance ($r_{33} = -0.683$, $P < 0.01$; Fig. 5). As there is not a significant correlation between preference and distance at either the population level or the species level, the significance of the entire data set result is spurious and results from an intergroup correlation (populations preferences vs. species preference).

Preferences for intermediate calls

There is a gap in both genetic distances (Fig. 5) and call distances (Fig. 3) between conspecific populations and heterospecifics. There is no ideal way to estimate how females would respond to calls of species that were intermediate in genetic distance between túngara frogs and the heterospecifics. Even F_1 hybrids and backcrosses would have their limitations. We did explore, however, how females would respond to calls that are intermediate between túngara frogs and *P. coloradorum* and *P. enesefae*. In both sets of five intermediate calls, females did not show a significant preference between the local call and the 0.06 intermediate call (the one most similar to the local call), they exhibited a trend in increasing preference for the local call as the intermediate call became less similar to it, and a significant preference for the local call to 0.50 call (Fig. 6). Combining the data there is a
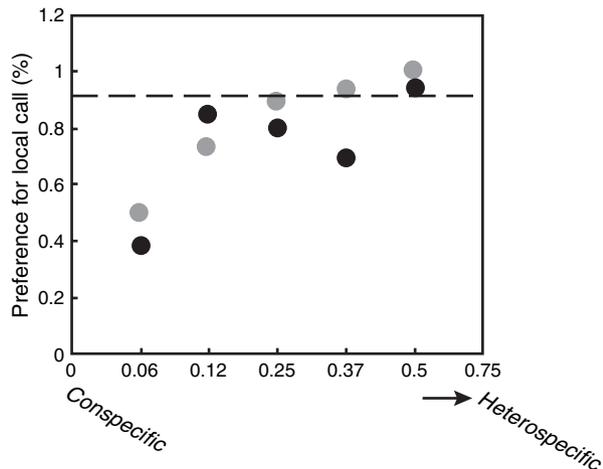
Fig. 6 The proportion of females showing preferences for the local call vs. the calls that are intermediate between *Physalaemus pustulosus* and both *Physalaemus enesefae* (grey) and *Physalaemus coloradorum* (black).

significant correlation between the strength of preference for the call and its acoustic distance relative to its heterospecific ($r_9 = 0.757$, $P = 0.01$; Fig. 6), as there is within the *P. enesafae* transect ($r_4 = 0.908$, $P = 0.03$) but not within the *P. coloradorum* transect ($r_4 = 0.649$, $P = 0.24$).

Discussion

In this study, we examine how female preferences for mating calls vary with genetic distances that range over a factor of more than 600, from within the population to between species. The general expectation is that divergence of mate recognition should increase with genetic distance.

Female mating call preferences are not predicted by genetic differences among males within the population (Lampert *et al.*, 2006). This is despite the fact that there is substantial genetic variation among males within the population, calls can vary markedly, and females are influenced by this call variation when choosing mates (Ryan & Rand, 2003b). Female choice in this species influences fertilization success (Ryan, 1985), and this direct benefit might outweigh any indirect benefits obtained from informed genetic mate choice (Kirkpatrick & Barton, 1997). Also, when mate choice is based on relatedness it is often based on olfactory cues, which may derive from MHC variation (Boehm & Zufall, 2006); perhaps information about relatedness is less likely to be encoded in acoustic cues. Regardless, this study shows that within the population there is no relationship between mate preference and genetic distance; the fact that we used microsatellites rather than mitochondrial genes to estimate genetic distance, as is

appropriate for this level of analysis, does not change our interpretation.

There is substantially more genetic variation and mating call variation among populations of túngara frogs than within populations. The call differences between GAMB and other populations are predicted by geographic distance but not by genetic distance. A similar result was found when Ryan *et al.* (1996) compared all pairwise distances between populations. There is, however, genetic structuring of call variation at the level of the two major genetic groups.

Preferences of female túngara frogs are influenced by call variation among populations. The preference of GAMB females for the local call over the foreign call ranged from 0.30 to 0.85, and females showed a statistically significant preference for the local call in one-third of the experiments conducted. Although the power to accept the null hypothesis of no preference is low, the large number of populations sampled should allow us to discern patterns of covariation of preference with either genetic or geographic distances between the local and foreign populations. Neither showed a significant pattern of covariation. There was a trend for the preferences to be predicted by the same principle components of call variation that sorts the populations into northern and southern groups. Even though females attend to these vectors of call variation, however, they do not show preferences based on genetic group. The lack of any influence of genetic distance on population preference is not restricted to GAMB. Females from a nearby but genetically divergent population, GBRG, show a similar pattern in response to local calls vs. their own call, and are remarkably similar in their preference for simple vs. complex calls. Thus, these patterns of preferences discerned in GAMB females appear similar to that exhibited by conspecific females in other populations.

The GAMB population is 696 km from the closest population in the northern genetic group of túngara frogs as delimited by Pröhl *et al.* (2006), and the two groups are separated by a gap of about 200 km (Savage, 2002; Pröhl *et al.*, 2006). It is possible that preferences might be stronger in the region in which the two genetic groups are the closest. Pröhl *et al.* (2006) found preferences for local calls in two-thirds of the populations tested in this area. There was, however, no evidence for an effect of genetic group on preference. Thus, although túngara frogs exhibit substantial genetic variation and mating call variation across their range, much of this genetic variation is partitioned into two genetic groups, and there can be population-based preferences, there is no evidence that prezygotic isolation from assortative mating has accrued with genetic distance with the species.

It is worth noting that the time of divergence between the two genetic groups of *P. pustulosus* is estimated to have occurred about 9 million years ago, and in other frogs such divergence times are often associated with

species status. Sasa *et al.* (1998) showed that complete post-zygotic isolation in frogs usually occurs between a Nei's *D* of 0.3 and 0.5. Based on Maxson & Maxson's (1979) calibration of Nei's *D* in salamanders ($D = 1 \approx 14$ million years), those distance would translate to approximately 4 and 7 million years.

These results of preferences between populations parallel to those of Tilley *et al.* (1990) with the salamander *Desmognathus ochrophaeus*. That species also shows substantial variation among populations and there is considerable ethological isolation among many population pairs. There is, however, no relationship between ethological isolation and genetic distance. In both studies the markers that are used to estimate divergence (allozymes in their study, COI sequence in ours) might not be totally accurate predictors of overall genetic divergence. This could explain the lack of a linear correlation between genetic distance and preference, but it is less likely to account for the lack of female preference between the two genetic groups as that deep divergence is apparent in studies of allozymes (Ryan *et al.*, 1996), DNA sequence (Weigt *et al.*, 2005) and microsatellites (Pröhl *et al.*, 2006), and is also consistent with the historical geography of the region and the phylogeography of codistributed species (see Discussion in Weigt *et al.*, 2005). As these two groups are separated by a substantial geographical gap there is probably no opportunity for reinforcement to contribute to divergence in mate recognition.

The lack of a correlation between call preference and population divergence in the face of substantial genetic differences in this species is in stark contrast to the situation in its sister species, *P. petersi* (Boul *et al.*, 2007). Sexual selection has apparently caused almost complete behavioural isolation between a pair of populations only 20 km apart. These two populations are separated by the Napo River in Amazonian Ecuador, but which have diverged genetically much less than the populations in the northern and southern groups of túngara frogs. The two *P. petersi* populations also occupy similar habitats, and a population genetic analysis shows no evidence of reinforcement having been important. It is a mystery why mate recognition systems have diverged in *P. petersi* but not in *P. pustulosus*.

When we compare preferences for calls of conspecifics vs. heterospecifics the results are rather different. In all cases there is a significant preference for the local call over the heterospecific call. The heterospecifics all differ from *P. pustulosus* by at least 0.5% sequence divergence, and their calls are all substantially different from the túngara frog calls (Fig. 3). Thus, as with many other studies of conspecific–heterospecific mating preferences (Andersson, 1994; Coyne & Orr, 2004), genetic distances are large, mate recognition signals are easily discernible and conspecific preferences are strong.

Our goal for this study was to ask if there were discernible patterns of covariation of mate recognition and genetic distance that spanned microevolutionary and macroevolutionary domains. Our results do show a significant relationship over the range of genetic distances. As Fig. 5 shows, however, this relationship is spurious and results from an intergroup correlation (populations preferences vs. species preference) and not from an overall correlation within both groups. Our results predict a threshold for preference vs. no-preference somewhere between 0.15% and 0.50% sequence divergence. Thus, our conclusion is that in this system macroevolutionary patterns are not just microevolutionary patterns writ large. There are several possible reasons why.

One possible explanation is that females have categorical perception of conspecifics calls vs. others (see Discussion in Ryan *et al.*, 2003). The fact that signals can vary continuously does not assure that perception of signals do as well (Harnad, 1987). Thus, even if there were no substantial gaps in call variation between the túngara frog and heterospecifics, it is possible that the same pattern of preference, a step function between no preference and strong preference, would be observed. We tested females with the intermediate calls to resolve that issue. The results reject the hypothesis that the difference in microevolutionary and macroevolutionary patterns we observe is due to female categorical perception of calls; the strength of preference varies continuously with call variation. Thus, if there were no gaps in call variation, and perhaps if there were no gaps in genetic distances, there might have been a stronger correlation between preference and genetic distance. This speculation does not, however, address the lack of such a correlation for preferences among the populations and among the species.

Few studies have investigated mating preferences across such scales of variation. Safi *et al.* (2006) show that preferences of female grasshoppers for calls within the species are best predicted by how different those calls are from sympatric heterospecific calls rather than how similar they are to the mean conspecific call. In their study, therefore, preferences at a larger scale predict preferences at a smaller scale. In this study, all taxa are allopatric with the target population, and we do not see any relationship between scales of mating preferences. The relationship between mating preferences within- and between-species in allopatry seems to require further data and thought. There is no question that preferences are stronger when genetic distance between sender and receiver are greater, but our analysis also shows clearly that macroevolutionary patterns are not apparent at the microevolutionary scale.

There are many possibilities as to why patterns of mating call preferences do not vary continuously from within-populations to among-species. The relevant information encoded in the call might vary when females are making different choices, for example, choosing larger mates is advantageous when choosing among males within a population but not when discerning species status. It is also possible that the tempo of signal/receiver evolution is not continuous. Changes in recognition

systems might be concentrated around speciation events, thus producing a pattern of punctuated equilibrium. Finally, given the intricacies of mate recognition and the different modalities in which it can occur, evolutionary patterns might be idiosyncratic among species. Nevertheless, we are left questioning the implicit assumptions of many studies that an analysis of mate recognition at one scale, e.g. sexual selection within a species, might always provide insights into mate recognition at another scale, e.g. the evolution of mate recognition between species (see also Houde, 1993; Boake *et al.*, 1997).

Acknowledgments

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