increased). ELISA of EGFR-immunoreactive material was performed with a Biodot microfiltration apparatus (Bio-Rad, Richmond, CA). Serial dilutions in 20 m*M* Hepes-NaOH, *p*H 7.2, of pooled extracts from three to five animals, were applied to nitrocellulose paper, immunoreacted with rabbit anti-EGFR (dilution 1/1000) and developed by the immunoperoxidase method with the immunoblot assay kit provided by Bio-Rad. The relative concentration of ERI was determined from the linear portion of serial dilutions (1 to 10 μg of extract protein). ERI content was also determined by using ^{125}I–protein A binding after incubation with the antibody. Assuming a 1/1 stoichiometry for the binding of antibody to ERI attached to nitrocellulose and to protein A, the ERI content of adult brain extracts was estimated as about 15 ng per milligram of protein.

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Coevolution of Sender and Receiver: Effect on Local Mate Preference in Cricket Frogs

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Mate recognition in frogs requires congruence of call characters, such as dominant frequency, and properties of the auditory system, such as frequency sensitivity of inner ear organs. Two neighboring populations of cricket frogs (*Acris crepitans*) exhibit statistically significant differences in the dominant frequency of the advertisement call and the frequency to which the basilar papilla of the inner ear is most sensitive. Call frequency and frequency sensitivity are matched within but differ between populations. These characters usually are negatively correlated with body size, and thus their congruence and coevolution often is explained by pleiotropic effects of size. However, within this species call frequency and frequency sensitivity of the basilar papilla evolved independent of body size, yielding local mate preferences that could contribute to genetic differentiation among neighboring populations.

IN MANY ANIMALS, MALES PRODUCE signals that are used by females for recognition of conspecific mates. Mate recognition requires congruence between the structure of the signal and the response properties of the sensory system that decodes the signal. This occurs in visual, olfactory, and electrosensory modalities and has been especially well documented in acoustic mate recognition systems (*1*). This congruence is necessary for efficient communication, and during evolution must be maintained by correlated changes in the signal and the receiver. By promoting assortative mating, these correlated differences in signal and receiver can restrict genetic exchange and promote genetic divergence among populations (*2*). Thus divergence in courtship signals can be an important component of the speciation process.

Studies of mate recognition systems usually are restricted to interspecific variation, but intraspecific variation in anuran advertisement calls has been investigated by Capranica *et al.* (*3*). Cricket frogs (*Acris crepitans*) from two localities 2500 km apart in the United States (New Jersey and South Dakota) showed correlated differences in both the dominant frequency of the advertisement call and the frequency sensitivity of the central auditory system. Within one population, females discriminated among synthetic calls that differed in frequency alone. However, these populations were at opposite and extreme ends of a cline in body size. Differences in body size can account for differences in many traits among animals, such as brain size, metabolic rate, and territory size (*4*). Body size can also influence strongly both the dominant frequency of a frog's advertisement call (*5*) and the tuning of the peripheral auditory system, especially the best excitatory frequency (BEF) of the basilar papilla (*6*), the inner ear organ that is used in the reception of the calls produced by this species (*7*); both are correlated negatively with size.

Large size in cricket frogs is advantageous in arid western environments because it decreases desiccation, and pleiotropic effects of body size on the signal and receiver might be the major factor accounting for variation in the communication system that results in local mating call preferences (*3*). Such local preferences for natural calls have also been demonstrated for some distantly separated populations (*3*). Because of the gradual cline of body size variation across longitude in this species and the great distances separating the populations investigated, it is difficult to interpret the effect of any differences in the communication system on reproductive interactions.

We investigated correlated changes in the communication system of this species on a more fine-grained geographic scale. First, we determined whether there were differences in the call frequency and the tuning of the basilar papilla between two geographically proximate populations. Second, we examined whether pleiotropic effects of body size changes alone could generate differences between populations and preserve the match between sender and receiver within populations. Third, we determined whether differences in the mate recognition system between neighboring populations could result in female mate preferences for local calls and thus potentially contribute to intraspecific genetic divergence.

Within its geographic range in Texas, *A. crepitans* exhibits two areas in which there is a large change in dominant frequency of the advertisement call over a relatively short geographic distance. One is in East Texas in the zone of parapatry of the two subspecies of *A. crepitans* (*A. c. crepitans* and *A. c. blanchardi*), and another in Central Texas, near Bastrop, within what is thought to be the continuous distribution of *A. c. blanchardi* (*3, 8*). We examined 300 advertisement calls from ten cricket frogs each in Austin and Bastrop (*9*), which are within 65

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km of one another in Central Texas but are found in different ecological settings, grassland and pine forest, respectively (*8*). We also determined the BEF of basilar papilla afferents for four individuals each from Austin and Bastrop (*10*).

Both the dominant frequency of the call and the BEF of the basilar papilla show statistically significant differences between the Austin and Bastrop populations (Table 1 and Fig. 1). However, within each population the call frequency and the BEF are matched. We then compared the call frequencies and the BEFs between and within populations after removing the effects of body size. The results are similar; the populations differ in both their call frequencies and BEFs but maintain congruence between these two characters within each population (Table 1 and Fig. 1).

Finally, we asked if these correlated differences in the mate attraction system could result in assortative mating and thus contribute to genetic divergence among populations. We tested females from Austin for preferential phonotaxis in response to stimuli that typified calls from Austin and Bastrop. Test stimuli were single calls from each population organized into call groups with a standard repetition rate of 10 Hz (*11*). Females showed a statistically significant preference for calls from their own population (12 versus 2, $\chi^2(1) = 7.14$, $P < 0.01$). Calls vary in other traits besides dominant frequency (*8*). Therefore, we repeated the experiments using synthetic calls that were identical in all parameters except dominant frequency. A synthetic call with a low dominant frequency (3.5 kHz) resembled the Austin calls, whereas a high dominant-frequency call (3.8 kHz) resembled calls from Bastrop (*11*). Again, Austin females showed an overwhelming preference for the call that more closely resembled the local call (10 versus 0, $\chi^2(1) = 10.0$, $P < 0.005$).

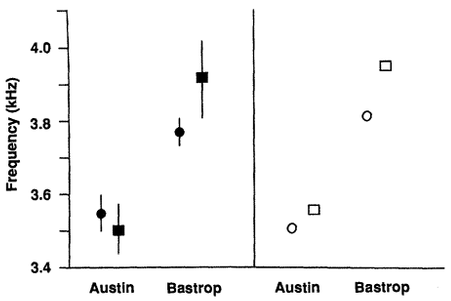

Fig. 1. The mean ± 1 SE of the dominant frequency of the call ($n = 10$, solid circles) and best excitatory frequency of the basilar papilla ($n = 4$, solid boxes) for cricket frogs from Austin and Bastrop. The open figures are the analogous means after being adjusted for body size (see Table 1).

These data show correlated differences in the call and the auditory system between two geographically proximate populations. Although the factors responsible for the divergence are not known, the data permit us to reject the hypothesis that these populational differences are due to differences in body size alone. Although such correlated changes due to pleiotropy of a third character under selection have been offered as a popular and parsimonious explanation for the evolution of communication (*12*), this explanation is inadequate for the system we studied. Rather, our data suggest coevolution of the communication system independent of body size. Moreover, the populations we studied are conspecific and are close enough geographically that considerations of reproductive interactions are biologically relevant. The phonotaxis experiments indicate that the differences in signal and receiver between populations could promote assortative mating and thus potentially reduce reproductive interactions between populations.

Our results are consistent with the hypothesis generated by studies of bird song dialects suggesting that divergence of communication systems can result in genetic structuring of populations (*13*). This hypothesis has proven controversial when applied to birds for a number of reasons, including the inability to document rigorously the correlated variation in the sensory system that should parallel variation in the song, and the difficulty in experimentally demonstrating that dialect differences influence female preferences (*14*). In the case of *A. crepitans* in Texas, however, variation in advertisement calls are correlated with sensory changes. This match between sender and receiver promotes local mate preferences; it could restrict gene flow and ultimately promote genetic divergence and speciation.

Table 1. The means and standard errors of the dominant frequency of the advertisement call and the best excitatory frequency (BEF) of the basilar papilla of male cricket frogs from Austin and Bastrop. An analysis of variance reveals statistically significant differences among the mean frequencies for each call ($F = 6.78$, df = 24 and 3, $P = 0.002$). A post hoc test (Duncan multiple range test, significance level $P = 0.05$) shows significant differences between the calls and the BEFs of each population, but not between the call and BEF within populations. Head width is used as the estimate of body size because it explains more variation in call frequency than other size estimates (*8*) and was used as the covariate in an analysis of covariance to remove the effect of body size. F is the statistic that tests the null hypothesis of no difference between the adjusted mean frequencies; P is the associated probability level.

Parameter	Austin	Bastrop	F	P
Call (kHz)	3.56 (0.16)	3.77 (0.11)	11.99	0.004
Head (cm)	0.79 (0.012)	0.73 (0.002)		
BEF (kHz)	3.52 (0.17)	3.94 (0.33)	13.94	0.014
Head (cm)	0.82 (0.011)	0.81 (0.021)		
F	0.01	1.85		
P	0.934	0.207		

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11. Natural calls were digitized and resynthesized in call

groups that consisted of ten calls repeated at a call rate of 10 Hz. Synthetic calls were constructed by digitally adding sine waves. Calls were broadcast antiphonally with a Sony TCD-5M stereo cassette recorder, a stereo amplifier, and two ADS L-200-C speakers. The female was placed equidistant from each speaker (1.1 m); sound pressure level (SPL; relative to 2×10^5 dynes/cm^2) was 75 dB SPL at this point. The female was released by a remote device, stimulus broadcast began, and a phonotactic response was recorded if she approached to within 10 cm of one of the speakers. Each female was tested only once with each stimulus pair.

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Production of Stable Rabbit-Mouse Hybridomas That Secrete Rabbit mAb of Defined Specificity

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Inclusion of normal rabbit serum (NRS) in culture medium after interspecific fusion of hyperimmunized rabbit spleen cells with murine SP2/0 myeloma cells produced 271 rabbit-mouse hybridomas (RMHs) that secreted rabbit immunoglobulin against group A *Streptococcus* (GAS). Continued use of NRS-supplemented medium during cloning yielded stabilized monoclonal RMH lines that have secreted GAS-specific rabbit antibody at concentrations similar to murine hybridomas (3 to 8 micrograms per 10^6 cells per 24 hours), for over 4 months of culture in vitro. The use of NRS as a medium supplement during initial culture, cloning, and stabilization of RMHs enables production of considerably more specific rabbit monoclonal antibody (mAb)–secreting RMHs than have previously been reported.

Hybridoma technology has now reached a level where it is possible to produce mAbs against most antigens. Some immunogens, however, stimulate poor antibody responses in mice, and consequently high-affinity murine mAbs with certain epitope specificities can be difficult to obtain. These problems are reflected by the small numbers of GAS-specific mAb-secreting murine hybridomas that have been reported (*1–3*).

Rabbits, in contrast, produce high titers of high-affinity antibody when hyperimmunized with most immunogens, including streptococcal carbohydrate (*4*). The ability to produce rabbit mAbs could therefore overcome many of the disadvantages of murine systems. Myelomas, however, are unknown in rabbits, and transformation of rabbit B cells in vitro with viruses has proved difficult (*5*).

RMHs have been produced (*6*) but, because of the instability of rabbit chromosomes in RMH cells, only cell lines that secrete a rabbit light (L) or heavy (H) chain, not an intact rabbit immunoglobulin, have been described. Kuo *et al.* (*7*), however, succeeded in producing RMH cell lines that secreted intact rabbit H and L chain immunoglobulins (Igs) but only obtained three stable clones from four different fusions.

One of us (T.J.G.R.) previously reported production of stable bovine-murine (*8*) and porcine-murine (*9*) hybridomas that secrete bovine or porcine mAb of defined specificities. We therefore applied these procedures in producing stable RMHs that secrete rabbit mAb specific to group A streptococcal carbohydrate.

A female New Zealand White rabbit was hyperimmunized with a nitrous acid extract (*10*) of group A *Streptococcus pyogenes* cells (American Type Culture Collection 19615) until its serum gave a high antibody titer in a GAS-specific enzyme-linked immunosorbent assay (ELISA) (*11*). We prepared RMHs by polyethylene glycol–mediated fusion (*12*) of spleen cells from this rabbit with murine SP2/0-Ag14 cells (*13*), using the method described by Gefter *et al.* (*14*). After fusion, cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) (*15*).

The RMH culture supernatants were screened for the presence of GAS-specific rabbit antibody by an ELISA (*11*). Selected positive culture wells were cloned by limiting dilution until stabilized, and resulting monoclonal hybridomas that secreted GAS-specific rabbit antibody were stored in liquid nitrogen. Rabbit mAbs selected for further study were produced by first adapting the appropriate RMH lines to grow in DMEM supplemented with fetal calf serum (FCS) [that is, in the absence of normal rabbit serum (NRS)]. Rabbit antibody was

Table 1. The effect of serum and feeder cell type on the outcome of fusion between hyperimmune rabbit spleen cells and murine SP2/0 plasmacytoma cells.

Feeder cells	Appearance of cultures on day 4 after fusion	Number of wells positive in initial ELISA*	Cell growth on day 17 after fusion
	Cell fusion A; 15% FCS		
Splenocytes and thymocytes (*18*)	Multiple RMH in every well, most at 16-cell stage	13% (40/300)	Massive fibroblastic cell growth, outgrowing hybridomas
Peritoneal exudate cells (*18*)	Multiple RMH in every well, most at 16-cell stage	30% (18/60)	Few areas of fibroblastic cell growth; good hybridoma growth
	Cell fusion B; 15% NRS		
Splenocytes and thymocytes	Many viable cells in every well, but no cell division	55% (159/288)	Minimal fibroblastic cell growth; good hybridoma growth easily observed
Peritoneal exudate cells	Many viable cells in every well, but no cell division	90% (54/60)	Minimal fibroblastic cell growth; good hybridoma growth easily observed

*Initial ELISA screening for GAS-reactive wells was performed on day 17 after fusion.

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