

- teichthytes: Osteoglossidae, Doradidae, Pimelodidae; Characidae: *Colossoma*; Callichthyidae; Reptilia: Chelonia: Pelomedusidae, Testudinidae; Crocodylia: Caviidae, Alligatoridae; Aves, Mammalia, Marsupialia, Edentata: Mylodontidae: *Stenodon campbelli*; Megalonychidae; Dasypodidae: Pamphathiinae; Rodentia: Erethizontidae; Dinomyidae: *Potomarchus murinus*, *Telcomys amazonensis*, *Tetrasylus*; Hydrochoeridae: *Kiyutherium orientalis*; Caviidae: Cardiomyinae, Neopibemidae, Dasypodidae; Echimyidae: Heteropsomyinae; Litopterna: Protheriidae, Macrauchenidae; Notoungulata, Astratheria, Sirenia: Trichechidae.
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Female Responses to Ancestral Advertisement Calls in Túngara Frogs

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Phylogenetic techniques were used to estimate and reconstruct advertisement calls at ancestral nodes. These calls were used to investigate the degree of preference of female túngara frogs (*Physalaemus pustulosus*) for both extant and ancestral calls. Females did not discriminate between calls of males of their own species and calls at their most recent ancestral node. They also recognized calls of three extant species and at four ancestral nodes as the signals of appropriate mates. Both shared ancestral history, and call convergence might differentially influence call preferences.

Differences in mate recognition systems behaviorally isolate species and are thought to be one of the most important causes of speciation; mate recognition, however, requires coordination of signal and receiver (1). The fact that such coordination characterizes many species is evidence that this functional association persists, but it tells us little of the historical patterns by which it arose. Species-specific mate recognition could evolve by the coordinated divergence of signal-receiver interactions of each incipient species, as has been suggested by some hybridization studies (2). Alternatively, the evolution of signals and receivers could occur in a more haphazard manner, in which stimuli that make signals more or less preferred are gained or lost through evolutionary history. In this case, other aspects of the calls that are perhaps irrelevant to the receiver preference could result from stochastic processes, constraints, or selection in other contexts, as has been argued from studies showing asymmetric mating preferences among species (3).

To truly understand how species recognition evolves, it is preferable to investigate directly the signals and receivers of ances-

tral species. The unique geological history of the Hawaiian islands and the amazing diversity of its indigenous fruit flies have

allowed such an approach to studies of mate recognition (3). In most studies, however, there are no clear ancestor-descendent relations among extant species. We have partially circumvented this problem by estimating and reconstructing the mate recognition signals at ancestral nodes; these nodes represent hypothesized ancestral species. We then conducted phonotaxis experiments to quantify female responsiveness to these signals. These experiments determined if the female's response was elicited only by the conspecific call, as would be suggested by the coordinated divergence of signal and receiver, or if ancestral calls contained key stimuli that effectively elicited a response, as is concluded from studies of mating asymmetries. If females were responsive to heterospecific calls, including those of both extant and hypothesized ancestral species, we also determined the degree to which female preferences were influenced by phylogenetic divergence and overall call similarity.

We examined the advertisement calls and phylogenetic relations of five species of frogs in the *Physalaemus pustulosus* species group and three of their close relatives (Fig. 1). The túngara frog, *P. pustulosus*, is sympatric with *P. enesefae* in Venezuela and allopatric with the other species. For 10 individuals of each species, 12 call parameters were measured and average calls were digitally synthesized with these variables (4) (Fig. 1). Phylogenetic relations were determined from an analysis of several morpho-

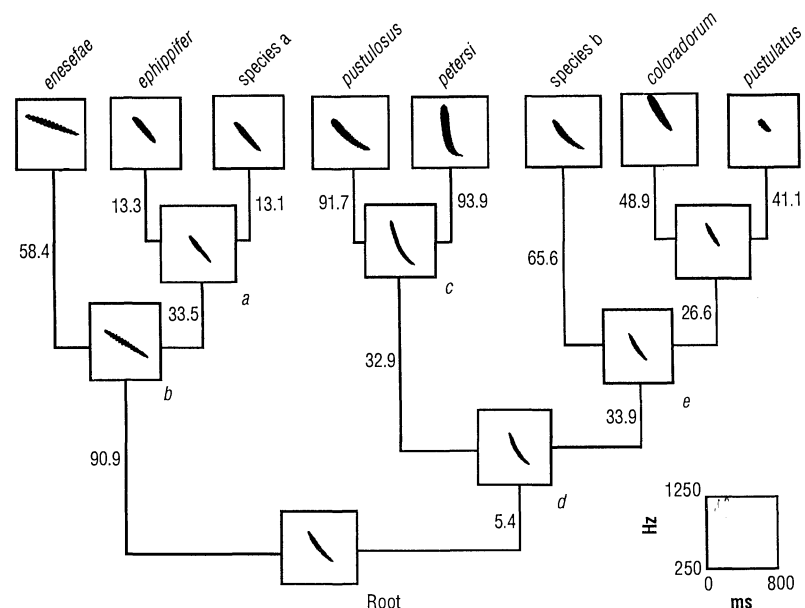


Fig. 1. The phylogenetic relations of frogs of the *P. pustulosus* species group and three closely related species (*P. enesefae*, species a, and *P. ephippifer*) (5). Species a and species b are undescribed. Shown are sonograms of the synthetic advertisement calls, which were determined from species' means for the extant (tip) species (4) or from phylogenetic estimates for calls at the ancestral nodes (7). Branch lengths are estimates of changes in mitochondrial DNA base sequences (7). A discriminant function analysis shows that 92% of the individuals of extant species, 10 per species, are assigned to the correct species by call alone. Numbers indicate branch lengths (7).

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logical characters, 27 allozymes, and 1200 base pairs of the 12S mitochondrial genome and its flanking regions; call characters were not used in the phylogenetic analysis (5) (Fig. 1). Bootstrap estimates showed strong statistical support for all of the nodes of the phylogeny (all $P < 0.05$) (5).

There are several phylogenetic procedures for deducing quantitative traits at ancestral nodes (6). Estimates of call characters for the seven ancestral nodes in the phylogeny were derived from two widely used methods, local squared-change parsimony and squared-change parsimony. Both

methods yielded similar results, on average within 5% of one another (7). We used the estimates of call traits derived from local squared-change parsimony to digitally synthesize the calls for each of the ancestral nodes (Fig. 1). These calls, together with the calls of extant species, were used in phonotaxis experiments, and we refer to both sets of calls as heterospecific.

We used phonotaxis experiments with female túngara frogs, *P. pustulosus*, to quantify two expressions of preference: discrimination and recognition (8). We operationally define discrimination as the degree to which female túngara frogs choose a conspecific signal over a heterospecific signal, and recognition as the degree to which female phonotaxis is exhibited to a signal in the absence of other signals; we realize that these definitions are arbitrary. In the recognition experiments, we used white noise as a control stimulus. Both discrimination and recognition assess biologically realistic tasks for females searching for mates.

For both sets of female responses, we also determined the degree to which phylogenetic distance among species and overall similarity among calls predict the responses of female túngara frogs. Call similarity was determined with the use of a principle component analysis and by measurement of the Euclidean distance between each of the heterospecific calls and the call of *P. pustulosus* (9). We determined the phylogenetic relatedness or distance by estimating the number of changes in the mitochondrial DNA sequence between *P. pustulosus* and each of the other species and nodes (9). Call similarity and phylogenetic distance were not significantly correlated ($r = 0.43$ and $P = 0.13$). The degree to which phylogenetic distance and call similarity predict female responses gives some indication of the relative influences of shared evolutionary history and convergence of signal-receiver systems.

Female túngara frogs showed strong discrimination between the conspecific call and most of the heterospecific calls (Fig. 2). The null hypothesis of a random response—that is, the inability to discriminate—was rejected in all cases but one; females did not discriminate between the conspecific call and the call at the immediate ancestral node (node c). These results suggest that although the calls of male túngara frogs and those of their immediate ancestor differ significantly (9), these differences do not influence female preference. Thus, the evolution of calls need not be in response to female preferences but could result from stochastic factors or selection related to other aspects of the call. This conclusion could not have been reached if we merely assessed the responses of female túngara frogs to the calls of extant species.

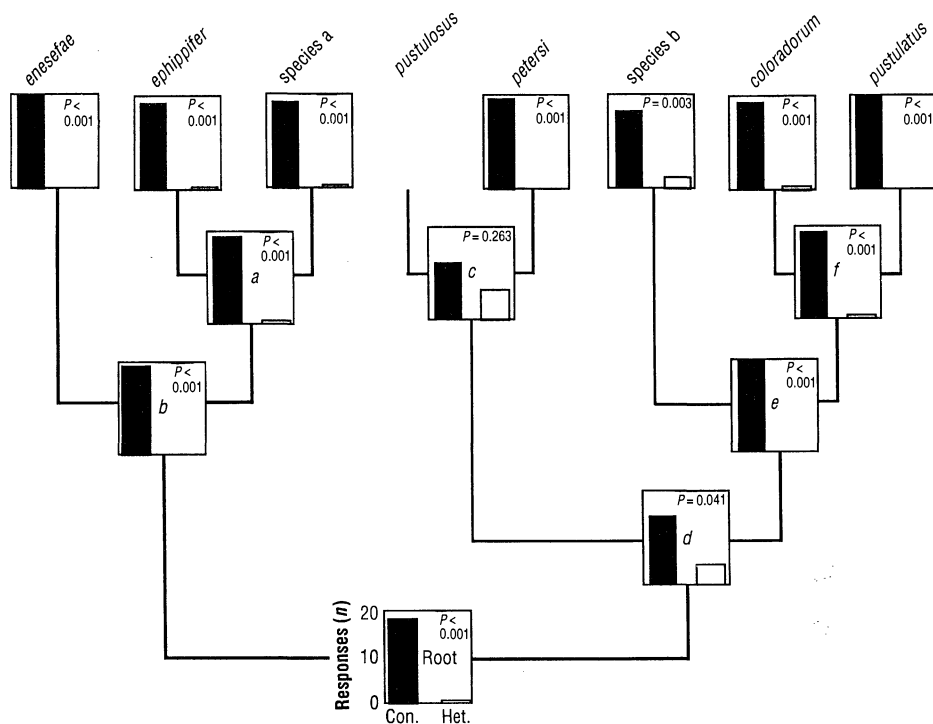


Fig. 2. Phonotactic responses of female túngara frogs (*P. pustulosus*) to conspecific (Con.) versus heterospecific (Het.) calls (8).

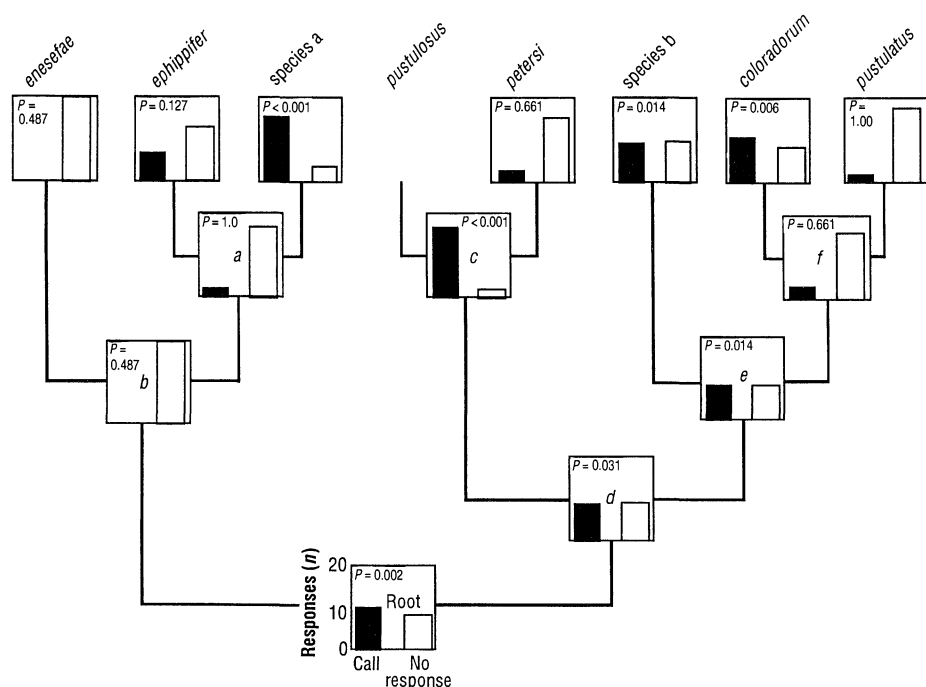


Fig. 3. Phonotactic responses of female túngara frogs (*P. pustulosus*) to heterospecific calls versus no response, which includes no phonotaxis and a response to white noise (8). In only four of the 280 tests did females exhibit phonotaxis to noise.

Although female túngara frogs usually preferred the conspecific call, they did respond to several heterospecific calls. The null hypothesis that heterospecific calls are not recognized (8) was rejected for the calls of three extant species and four ancestral nodes (Fig. 3). Calls of species a, species b, and *P. coloradum* elicited statistically significant phonotactic responses from female túngara frogs. These extant species are not the closest relatives of *Physalaemus pustulosus*; species a is not even a member of the *P. pustulosus* species group.

Túngara frog females also recognized the calls at four ancestral nodes. These four nodes are those most closely related to *P. pustulosus* (Fig. 3; root, nodes c, d, and e). Neither the calls at the node immediately ancestral to *P. coloradum* and *P. pustulosus* (node f) nor the two immediate ancestral nodes of the outgroup species elicited responses from female túngara frogs. Unlike the phonotaxis experiments testing preferences between conspecific and heterospecific calls in which females almost always preferred the conspecific call, these experiments showed that stimuli that elicit phonotaxis to advertisement calls do not arise de novo with each speciation event but instead are evident throughout the recent evolutionary history of the species.

Univariate analysis showed that phylogenetic distance was correlated with the females' degree of discrimination (number of responses to the heterospecific call) between conspecific and heterospecific calls ($r = -0.61$ and $P = 0.02$), but not with the females' recognition of heterospecific calls ($r = -0.45$ and $P = 0.11$). Call similarity predicted the females' responses in both experiments equally well (in both cases, $r = -0.57$ and $P = 0.03$). A stepwise multiple regression analysis showed that phylogenetic distance best predicts female discrimination between calls ($P = 0.02$); call similarity does not explain a significant portion of the residual variation ($P = 0.15$). In contrast, only call similarity predicts female recognition of heterospecific calls ($P = 0.03$); phylogenetic distance is not a significant predictor of female recognition ($P = 0.36$).

Our results do not indicate a pattern of tightly correlated signal-receiver evolution. The fact that females do not discriminate between the conspecific call and the call at the immediate ancestral node suggests uneven and thus uncoupled rates of signal and receiver evolution. Also, signal stimuli that elicit recognition are present throughout much of the history of the species as well as in several extant species. This also rejects the hypothesis of a tightly coevolved signal-receiver system. It appears that in these frogs there are call traits widely distributed throughout the species group, currently and ancestrally, that are sufficient for recogni-

tion. Discrimination in favor of conspecific calls, however, is more restricted. Thus, we suggest that in this system, stimuli that elicit recognition are widespread throughout the history of these frogs, but that speciation usually results in addition or fine-tuning of stimuli effecting discrimination in favor of conspecific calls relative to calls of close relatives.

The fact that phylogenetic distance better predicts female discrimination between conspecific and heterospecific calls and call similarity better predicts call recognition suggests the possibility of two perceptual processes being influenced differently during call evolution. This hypothesis could be more rigorously tested by experimental isolation of the precise features that elicit phonotaxis in túngara frogs for each heterospecific call and then the use of phylogenetic inference to determine if these characters are shared or are independently derived. Estimating and recreating ancestral characters may be an additional approach for studies of the evolution of multiple component systems.

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4. Twelve standard spectral and temporal call parameters were measured with a DATA 6000 digital wave form analyzer (Analogic, Peabody, MA) at a sampling rate of 20 kHz and a Uniscan sonograph (Multigen, Mt. Vernon, NY) (10). A combination of 8 of the 12 variables was used in call synthesis. Using the average values for extant species and the estimated values for the ancestral nodes, we synthesized calls with an Amiga 3000 computer at a sampling rate of 20 kHz with a synthesis program developed by J. Schwartz (University of Missouri at Columbia). Previous data have shown that female *Physalaemus pustulosus* do not discriminate between synthetic calls and some natural calls; thus, these call parameters include all acoustic stimuli relevant to female preferences (A. S. Rand and M. J. Ryan, unpublished data).
5. D. C. Cannatella, D. M. Hillis, P. Chippendale, A. S. Rand, M. J. Ryan, unpublished data. The consistency index for the proposed hypothesis of phylogenetic relations is 0.775; skewness, $g_1 = -0.96$ and $P < 0.01$, which indicate that there is a statistically significant phylogenetic signal [see D. M. Hillis and J. P. Huelsenbeck, *J. Hered.* **83**, 189 (1992)].
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7. We computed the values of call parameters at ancestral nodes using local squared-change parsimony and squared-change parsimony (6) as provided in programs discussed in (11). For both algorithms we used the options of gradual evolution; branch lengths were estimated by changes in mitochondrial DNA base sequences (5). A punctuated model of evolution, in which branch lengths are set to 1.0, produced similar results. Local squared-change parsimony tends to concentrate changes around the node, whereas squared-change parsimony distributes the changes more evenly across the entire tree. The different techniques make different biological assumptions, and although it is not clear which assumptions are more appropriate for this study, estimates from both approaches were similar; on average, parameter estimates were within 5% of one another. We used the values estimated by local squared-change parsimony. The values of call parameters for all the ancestral nodes were outside of the 95% confidence intervals for the same call parameters of *P. pustulosus*.
8. Phonotaxis experiments were conducted at facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama. Methods are detailed in A. S. Rand, W. Wilczynski, M. J. Ryan, *Am. Zool.* **32**, 81 (1992). The null hypothesis of no discrimination between conspecific and heterospecific calls was tested by an exact binomial probability. The null hypothesis for response to a heterospecific call versus noise was determined empirically. The number of times a female approaches a silent speaker estimates the number of random approaches to a speaker without reference to the stimulus being broadcast. Eighteen of 20 females showed no response (as defined below), and two came into contact with the silent speaker. Thus, if females ignore the heterospecific call as a communication signal, the null expectation is that they would still approach the call by chance in the ratio of 2:18. This expectation was compared to the actual responses with a Fisher's exact test. In testing female responsiveness to heterospecific calls, we paired the call with a white noise stimulus of similar duration and intensity. This control was necessary to eliminate the possibility that a female's response to a call was merely a more general approach to any sound. We used noise instead of calls of other frogs because we wanted to determine the female's recognition of the heterospecific call being tested, as opposed to her discrimination between two heterospecific calls. A "no response" was recorded either if the female approached the speaker broadcasting noise or if she did not exhibit phonotaxis after 15 min, but only if the female subsequently responded to the conspecific call. Thus, a "no response" indicates that lack of phonotaxis was due to lack of a meaningful stimulus rather than lack of motivation. Some of the results with extant species were previously reported, although with smaller sample sizes (10). They are included here for completeness.
9. Call similarity was measured by a principle component analysis on standardized call variables. The first three components, which explained 87% of the variation in calls among species and nodes, were retained, and the Euclidean distances between each call and the call of *P. pustulosus* were determined. Phylogenetic distance was estimated as the number of mitochondrial DNA changes among taxa and nodes from the average number of changes hypothesized for all the most parsimonious reconstructions of the single most parsimonious tree (5). A stepwise multiple regression analysis was used to determine the degree to which call similarity and phylogenetic distance predicted female responses.
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