

# Call degradation in diploid and tetraploid green toads

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According to the 'environmental selection' hypothesis, the physical characteristics of environments influence the evolution of long-range acoustic signals by favouring those properties that decrease sound attenuation and distortion with distance. Different environments could favour different acoustic properties and therefore contribute to the intra- and interspecific variation of calls. In the present paper, we investigate whether this hypothesis could explain the differences between the advertisement calls of three taxa of central-Asian green toads: lowland diploids, highland tetraploids and lowland tetraploids. The pattern of propagation of 12 natural calls (four for each taxon) was analysed in nine localities of Kyrgyzstan and Kazakstan. We broadcast the calls and recorded them along a transect at distances of 2, 4, 8, 16, and 32 m from the speaker, to estimate sound attenuation and propagation. Attenuation was quantified from the oscillogram (by directly calculating the SPL of calls) and from the power spectrum (by measuring the relative amplitude of the fundamental frequency), whereas degradation was estimated by cross-correlating spectrograms of the same call at different distances. Results show that: (1) the pattern of sound propagation significantly differs among localities in relation to the different vegetation and environmental noise; (2) in most localities, call attenuation and degradation differ significantly among the three taxa; (3) such differences are not consistent to those expected under the hypothesis of environmental selection: independent of altitude, lowland tetraploid calls fare worse than both diploid and highland tetraploid calls, whereas diploid and highland tetraploid calls show different patterns of propagation in a few localities only. © 2003 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2003, 78, 11–26.

**ADDITIONAL KEYWORDS:** advertisement call – *Bufo viridis* – cross-correlation – sound attenuation – sound degradation – sound transmission.

## INTRODUCTION

In many animals, acoustic signals are employed for long-range communication. The effectiveness of these signals depends on the distance at which they can be perceived and recognized by recipients (Brown, 1989). In order to be perceived, signals must have sufficient energy to elicit a response from the receiver, whereas to be recognized they must maintain unaltered temporal and spectral structures encoding information. Selective pressures acting on long-range signals to decrease both attenuation (energy content) and distortion (information content) with distance would depend not only upon the signal acoustic properties (that is, the biology of signal production), but also upon the

physical characteristics of the habitats where signals are broadcast (Bradbury & Veherencamp, 1998; Kime, Turner, Ryan, 2000; Wiley & Richards, 1978; Ryan & Kime, in press).

Since the pioneering works of Chappuis (1971) and Morton (1975), many researchers have investigated the role of the environment in influencing the evolution of signal structure. These researchers have adopted either a comparative or an experimental approach. In the first case, investigators compare calls of taxa within a phylogenetic framework to find out whether similarities are due to common descent or to convergent evolution and whether between-taxon differences are due to lack of phylogenetic affinity or to divergent selection (Cosens & Falls, 1984; Ryan & Brenowitz, 1985; Wiley, 1991). With the experimental approach, investigators directly test the transmission efficiency of calls broadcast in different habitats and

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analyse their pattern of degradation with distance (Brenowitz, Wilczynski & Zakon, 1984; Ryan & Sullivan, 1989; Ryan, Cocroft & Wilczynski, 1990; Penna & Solis, 1998; Kime *et al.*, 2000).

The comparative and experimental approaches complement each other. These methodologies have often provided congruent evidence of a direct role of environmental selection in shaping acoustic structure of calls of birds and primates (Brown, 1989; Gish & Morton, 1981; Brown & Gomez, 1992). However, a less clear picture has emerged from studies with anurans. Ryan & Sullivan (1989) found significant differences in the spatial pattern of degradation of call temporal structure in two species of toads. Furthermore, Ryan *et al.* (1990), by means of transmission experiments, provided evidence to attribute call differences between two subspecies of cricket frogs, *Acris crepitans*, to environmental selection for increased transmission efficiency. On the other hand, some recent studies on tropical and temperate frogs have failed to find differences in call structure consistent with the different acoustic environments where species typically call (Kime *et al.*, 2000; Penna & Solis, 1998). These studies have suggested that differences among the calls of anuran species inhabiting different habitats are related to phylogenetic and morphological constraints rather than to different selective pressures for enhancing transmission efficiency.

In the present paper, we show results of transmission experiments on green toad calls. Experiments were carried out to test the hypothesis that differences between advertisement calls of diploid and tetraploid green toads (*Bufo viridis* complex) have originated from different environmental pressures (Castellano *et al.*, 1998; Castellano, Giacoma & Dujsebayaeva, 2002).

#### THE ASIAN GREEN TOADS

The Green toad (*Bufo viridis* complex) is a widespread species extending as far west as Morocco and eastern France and as far east as north-western China and Mongolia. In central Asia diploid ( $2n = 22$ ) and tetraploid ( $2n = 44$ ) green toad populations coexist (Castellano, Giacoma & Dujsebayaeva, 2000; Kadyrova, Mazik & Toktosunov, 1976; Dujsebayaeva *et al.*, 1997; Stöck & Grosse, 1997). In some regions (i.e. Kyrgyzstan), diploids and tetraploids show an altitudinal segregation, such that tetraploids inhabit the mountains and diploids the lowlands. In other regions (i.e. eastern Kazakstan), however, tetraploids are also found in the lowlands (Borkin *et al.*, 1996; Castellano *et al.*, 2000; Borkin *et al.*, 2001).

To date there is no clear consensus as to the mechanism (auto- or allo-polyploidization) that led to the origin of tetraploid toads (Borkin *et al.*, 1986; Roth & Ráb, 1986; Mezhzherin & Pisanets, 1995), but cytolog-

ical data provide evidence for their relative ancient origin (Odierna *et al.*, 1995). The two taxa differ in their morphological and behavioural characters: diploids are larger than tetraploids and produce calls having lower frequencies and higher pulse-rates (Castellano *et al.*, 1998; Stöck, 1998). Differences in call frequency are not totally explained by differences in body size: although call frequency decreases with size, diploid toads call at frequencies lower than those of tetraploids of similar body size. Furthermore, in two-choice discrimination tests, diploid females significantly prefer a typical diploid call over a tetraploid alternative, showing that between-taxon call differences might function as a pre-mating isolation mechanism (Giacoma & Castellano, 2001).

In previous papers (Castellano *et al.*, 1998; Castellano *et al.*, 2002) we addressed the question of the evolutionary mechanisms responsible for the differences between diploid and tetraploid advertisement calls. In contrast with release calls (short-range signals mediating male–male interactions), advertisement calls do not vary congruently with the phylogeny of the group; in fact, the advertisement calls of Asian diploid and tetraploid populations differ from each other more than predicted by their genetic distances (Castellano *et al.*, 2002). Furthermore, triploid calls did not differ significantly from tetraploid calls (Castellano *et al.*, 1998), indicating that call differences are not the direct effect of polyploidization. Although these findings suggest that natural selection is responsible for call differences, it is not clear whether selection acts by favouring reproductive isolation in sympatry (reproductive character displacement) or by enhancing signal propagation in allopatry (environmental hypothesis). In the present paper we test the environmental hypothesis experimentally.

#### MATERIAL AND METHODS

##### STUDY SITES

Transmission experiments were carried out in May and June 1997 in nine localities of south-eastern Kazakstan (KZ) and north-eastern Kyrgyzstan (KY). Four localities were in the plains, at altitudes of up to 800 m above sea level (a.s.l.): (1) Kapchagai (KZ, latitude 43°57'98" N, 77°21'74" E, altitude 554 m a.s.l.); (2) Damba (KZ, latitude 44°13'54" N, 74°47'57" E, altitude 559 m a.s.l.); (3) Kopa (KZ, latitude 43°28'97" N, 75°46'87" E, altitude 648 m a.s.l.); (4) Tulek (KY, latitude 43°06'56" N, 74°05'51" E, altitude 758 m a.s.l.). The other five localities were in the mountains at altitudes above 1000 m: (5) Charin Cañon (KZ, latitude 43°15'61" N, 78°59'55" E, altitude 1225 m a.s.l.); (6) Kok-jar (KY, latitude 42°41'54" N, 74°39'40" E, altitude 1283 m a.s.l.); (7) Chon-Kurchak (KY, latitude 42°38'84" N, 74°37'63" E,

altitude 1920 m a.s.l.); (8) Big Lake (KZ, latitude 43°04'52" N, 76°59'05" E, altitude 2300 m a.s.l.); (9) Koi-Tash (KY, latitude 42°41'04" N, 74°40'22" E, altitude 1350 m a.s.l.). All sites were in open grasslands. For each locality we estimated the mean height of plants and the percentage of ground covered by vegetation. Five 1 m<sup>-2</sup> quadrats were sampled at regular distances along the experimental transect (see below). For each quadrat, we visually estimated the percent cover according to the classes defined by the Braun-Blanquet cover scale (class 0, cover 0–0.9%; class 1, cover 1–5%; class 2, cover 6–25%; class 3, cover 26–50%; class 4, cover 51–75%; class 5, cover 76–100%). To estimate the mean vegetation height, we grouped vegetation in five height classes (1, 1–10 cm; 2, 11–25 cm; 3, 26–50 cm; 4, 50–75 cm; 5, >75 cm) and ranked these categories according to their abundance in the quadrat. We calculated the mean vegetation height by weight-averaging the medians of the vegetation height categories (mean vegetation height =  $\Sigma(r_i \times x_i) / \Sigma r_i$ ; where  $x_i$  is the median of class (i) of vegetation height, and  $r_i$  is its rank of abundance).

#### EXPERIMENTAL PROCEDURE

We carried out the transmission experiments during night time, between 2100 and 0200 h, when these toads call. Experimental calls (see below), contained on a digital tape, were played back with a DAT recorder (Sony TCD-D8) and passed through a sound amplifier (Proton D230) and a loud speaker (JBL control-1), placed at ground level.

Before each transmission session we adjusted the volume of the tape recorder so that a 1.4 kHz pure tone had a fast-root mean square (RMS) of 90 SPL dB at 0.5 m in front of the loud speaker. We measured the sound pressure levels to the nearest dB by means of a sound pressure level meter REALISTIC (mod. 33–2050). After calibration, and during each recording session, we kept constant the output level of the broadcasting tape recorder.

We recorded the broadcast stimuli with a second Sony TCD-D8 DAT recorder and a Sennheiser K6 microphone. Each broadcast sound was subsequently recorded along a transect, at 2 m, 4 m, 8 m, 16 m, and 32 m from the source, with the microphone at a height of 10 cm above the ground. At each distance, we adjusted the microphone input level so that the broadcast sounds from stimulus tape could be recorded at the highest possible input without being overloaded. Once we set the microphone input level, we broadcast with a Marantz CP430 tape recorder a calibration tone near the microphone and read its sound pressure level into the tape recorder. This sound was employed as a calibration signal to calculate the absolute SPLs of the recorded sounds. Within each locality we repeated a

complete series of recordings three times, along the same transect, and during the same night. Before each recording session, we measured the relative humidity and the air temperature.

The tape employed in the transmission experiments contained 12 natural calls, the acoustic properties of which are shown in Appendix. These calls were selected to cover the whole natural variation of fundamental frequency and pulse rate observed in the taxa. Calls 1–4 were of diploids toads, calls 5–8 were of highland tetraploid toads, and calls 9–12 were of lowland tetraploid toads. Calls were previously normalized and therefore they had the same peak SPL. Since calls have different amplitude modulation rates, they also have different RMS SPLs. However, these differences were not statistically significant among the three taxa (Kruskal–Wallis = 3.515; d.f. = 2;  $P > 0.1$ ).

#### ANALYSES OF THE BROADCAST CALLS

We digitized recordings of transmitted calls at a sampling rate of 20 kHz using the software program Sound Forge v. 4.0 for PC. From the time wave we delineated all natural calls (Fig. 1) and 1 s of the three pure tones and saved them in separate files for successive analyses. Moreover, we saved in separate files 1 s of recorded environmental noise preceding each signal.

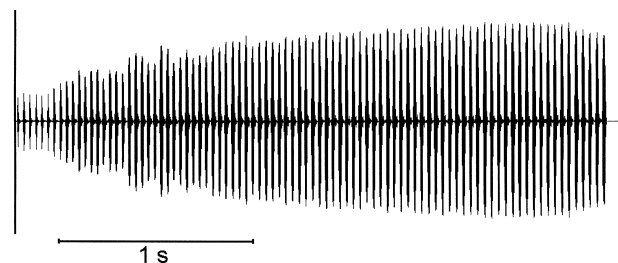
We analysed sound attenuation and studied sound degradation by carrying out cross-correlation analyses on the call spectrograms with the SIGNAL 2.2 software (Engineering Design, 1994).

#### Sound attenuation

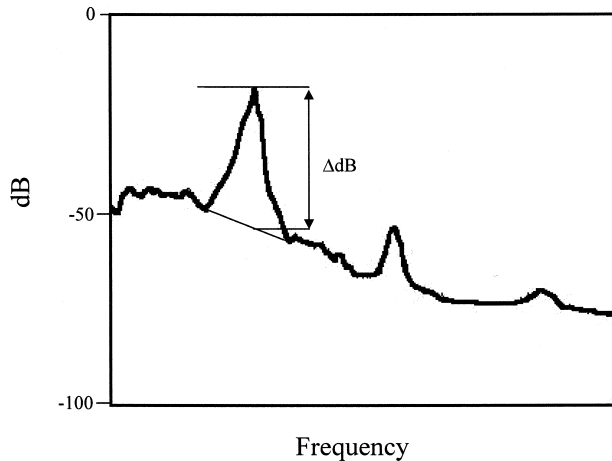
We calculated the absolute RMS SPL of both signals (plus noise) and environmental noise with the formula:

$$L_s = L_c - (R_c - R_s)$$

Where  $L_s$  = absolute RMS SPL of the signal (plus noise);  $L_c$  = absolute RMS SPL of the pure calibration tone  $c$  (measured with the sound pressure level meter);  $R_c$  = digital RMS of the pure calibration tone  $c$



**Figure 1.** Oscillogram of the green toad advertisement call.



**Figure 2.** Power spectrum of the green toad advertisement call. The relative amplitude of the fundamental frequency was employed to describe the pattern of call attenuation in the spectral domain.

(measured after digitalization of the signal);  $R_s$  = digital RMS of the signal  $s$ .

Since calls were longer than the maximum time interval over which the recorded RMS calculation was possible (1 s), for each call we calculated the mean amplitude over the same 1 s interval (precision at the nearest ms).

For signal-to-noise SPL differences higher than 7 dB, the contribution of the environmental noise to the total sound pressure level (that is, the signal plus noise SPL) was lower than the accuracy of the sound pressure meter we employed ( $\pm 1$  dB). However, for differences lower than 7 dB, the environmental noise had detectable effects. In these cases, therefore, we adjusted the signal SPL according to the formula (Magrab, 1975):

$$L'_s = L_s + 10 \text{ Log} (1 - 10^{-(L_s - L_n)/10})$$

where  $L_s$  = absolute RMS SPL of the signal (plus noise);  $L'_s$  = adjusted absolute RMS SPL of the signal;  $L_n$  = absolute RMS SPL of the 1 s noise preceding the signal.

The anuran auditory system operates as a band-pass filter, often tuned to the typical frequencies of the species-specific signals. Since in green toads most of the signal acoustic energy is concentrated in the fundamental frequency, we considered its amplitude as an index of how well the signal could be perceived. We also measured the relative amplitude of fundamental frequencies from the mean power spectrum (FFT = 2048; Sampling window = Hamming) as shown in Figure 2.

#### Sound degradation

Degradation affects the structure of a signal as it

propagates over distance. To analyse degradation, we carried out a cross correlation analysis on call spectrograms, using the software SIGNAL 2.2 (Engineering Design, 1994). Spectrogram correlation calculates a correlation function between two digital spectrograms by sliding one against the other and measuring the Pearson's correlation coefficient as a function of time offset between them (Kime *et al.*, 2000).

For each locality and trial, we cross-correlated calls recorded at the nearest distance (2 m) with those recorded at 4, 8, 16, and 32 m, and saved the maximum cross-correlation coefficients for statistical analyses. We restricted the sonogram comparison to the range of frequencies between 500 and 5000 Hz. All sonograms were obtained with a transform length of 128 points.

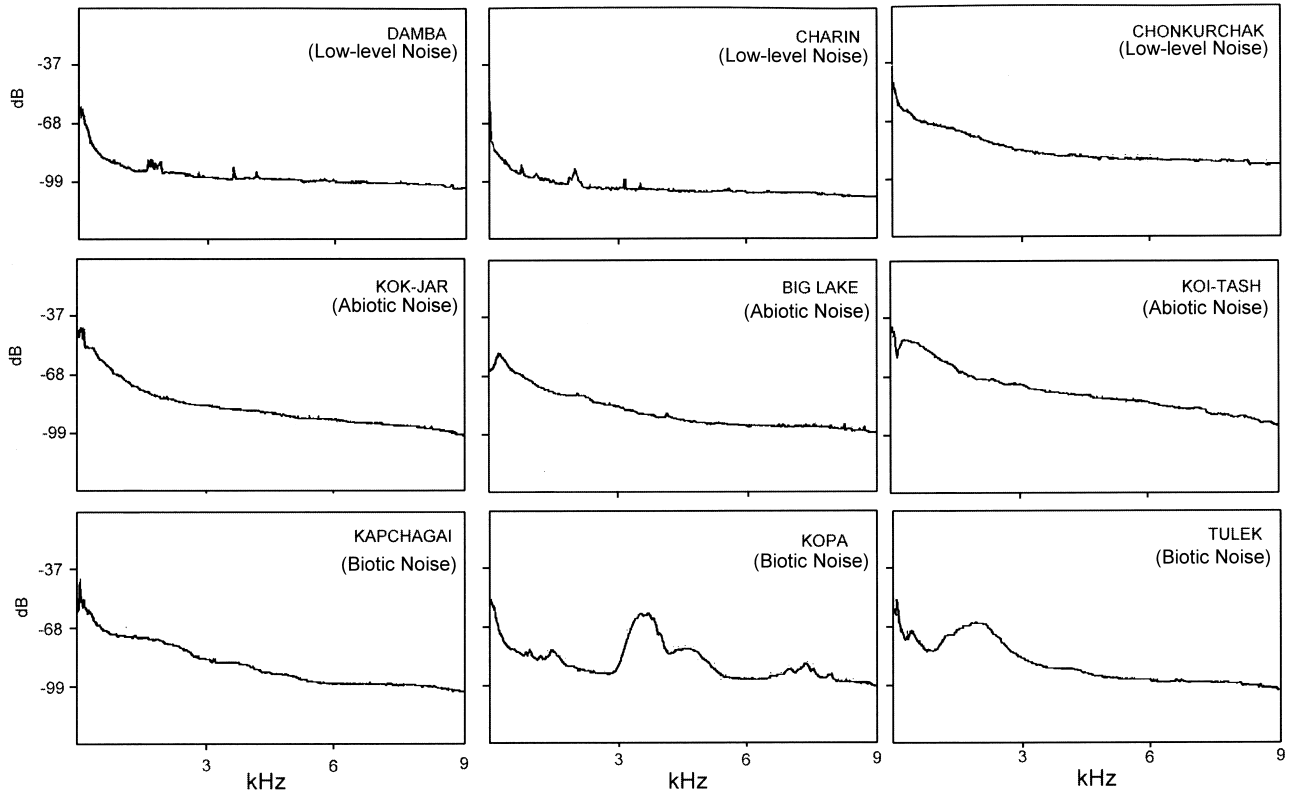
#### Environmental noise

To analyse the spectral properties of noise in each locality, we pooled in a file all the 1 s noise samples recorded at the 16 m and 32 m distances (when the recording level was as high as possible) and obtained a 90 s noise which we assumed to be representative of the local environmental noise. We performed a Fourier analysis of this sound so that we obtained a spectral representation of the local environmental noise.

#### STATISTICAL ANALYSES

We repeated the transmission experiments three times along the same transect for each locality, and calculated the average values in sound levels for each call and distance and employed them for successive analyses. To calculate averages (and standard deviations) we first converted dB levels to sound pressure values, then we computed the statistics, and finally we reconverted these values to decibels.

We used two- and three-way analyses of variance to analyse differences in call attenuation among localities and taxa. Because of the non-normal distribution and unequal variance, we estimated the tests' statistical significance by means of a randomization procedure. For this purpose we employed the RT-Anova program (v. 2.1) by B.J.F Manly (1997). This program first carries out a conventional ANOVA and, for all effects and for their interactions, calculates the correspondent  $F$ -ratios. The program then randomly allocates each value to a new effect combination, with the only constraint of maintaining the same number of cases for each category of effects. It computes the new  $F$ -ratios and compares them with those from the original set of data. After 10 000 randomizations, the program shows the percentage of permutations with  $F$ -ratios equal to or higher than those from the original matrix. This percentage is interpreted as the probability that the null-hypothesis is true, in other words,



**Figure 3.** Power spectra of the environmental noise in the nine localities where transmission experiments were carried out.

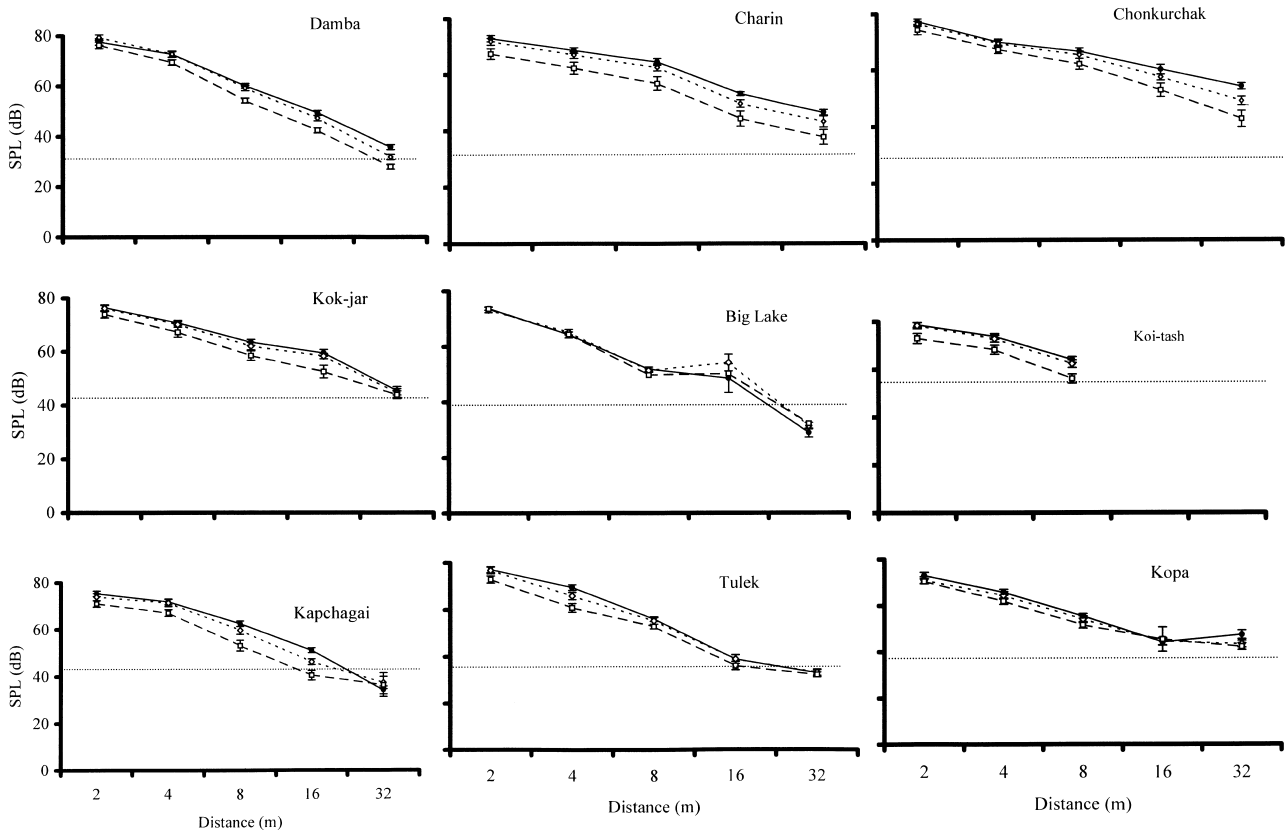
that the counts for each effect combination are random samples of the same distribution.

We carried out two-way ANOVAs, with distance and taxon as independent effects, to analyse differences among taxa for each locality. We also carried out three-way ANOVAs to test the environmental selection hypothesis in which distance, taxon, and the type of environment were considered as independent effects. We considered eight localities (Koi-tash was excluded because no recordings at 16 m and 32 m were conducted in this locality) and classified them according to two criteria: altitude and environmental noise. In the first case, we classified Damba, Kopa, Kapchagai, and Tulek as lowland localities, and Chonkurchak, Kok-jar, Charin and Big Lake as highland localities. In the second case, we distinguished three categories of environmental noise: (i) low noise (Chonkurchak, Charin and Damba); (ii) biological noise (Tulek, Kapchagai and Kopa); and (iii) atmospheric noise (Kok-jar and Big Lake). Since the randomization program we employed required the same number of replicates for each effect, and since the number of localities for the three categories of environmental noise were not the same, we carried out nine analyses of variance, comparing six localities at time, so that all possible combinations were considered.

## RESULTS

### LOCALITIES AND THEIR ENVIRONMENTAL NOISE

Figure 3 shows mean power spectra of the environmental noise recorded in the nine localities. Damba, Charin and Chonkurchak show low noise SPLs with acoustic energy steeply decreasing with frequency. Kok-jar and Big Lake show high noise SPLs due to atmospheric perturbations with most of acoustic energy at frequencies below 200 Hz. Koi-tash shows the highest noise SPLs due to nearby stream running water. As in Kok-jar and Big Lake, in Koi-tash acoustic energy is higher at low frequencies, but, in this locality, it decreases less steeply as frequencies increase. Three lowland localities (Kopa, Tulek and Kapchagai) show intermediate levels of environmental noise. The power spectrum of Kopa shows two peaks of energy between 3.0 and 5.0 kHz, corresponding to the call fundamental frequencies of two species of crickets. In Tulek, the peak at frequencies between 1.0 and 2.5 kHz, is due to the calls of water green frogs (*Rana ridibunda*). In Kapchagai, the calling of water green frogs is still a remarkable but less important component of the environmental noise, which is also influenced by low frequency atmospheric perturbations.



**Figure 4.** Call attenuation in the temporal domain: SPL variation with distance in the calls of the three taxa. The dotted lines show the environmental noise SPLs (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

#### AMONG-LOCALITY DIFFERENCES IN SOUND PROPAGATION

##### *Attenuation of calls*

Figure 4 shows, for each locality, the mean SPLs of the call of the three taxa at different distances from the source. Independent of taxon, we observed different patterns of attenuation with distance. We regressed the absolute SPLs of calls against distances and employed the absolute values of the regression coefficients as an estimate of the sound attenuation rate. By regressing these coefficients against the mean grass height we found a significant positive relationship ( $N = 9$ ;  $R = 0.756$ ;  $b = 0.166$ ;  $F_{1,7} = 9.355$ ;  $P = 0.018$ ): the taller the grasses the greater the sound attenuation. On the other hand, we did not find any significant relationships between attenuation and either the vegetation cover ( $N = 9$ ;  $R = 0.117$ ;  $b = 0.009$ ;  $F_{1,7} = 0.098$ ;  $P = \text{NS}$ ) or the level of background noise ( $N = 9$ ;  $R = 0.156$ ;  $b = -0.043$ ;  $F_{1,7} = 0.174$ ;  $P = \text{NS}$ ).

Diploid calls showed higher mean SPLs than both highland and lowland tetraploid calls in all localities but Kopa. Table 1 shows results of two-way ANOVAs,

with distance and taxon as independent effects. In all localities the distance significantly affected call SPL. However, the call taxon showed significant effects in only seven out of nine localities (in Kopa and Big Lake diploid, and highland and lowland tetraploid calls did not show significantly different patterns of propagation), and in none of them was the interaction between distance and taxon statistically significant.

Among the three taxa, lowland tetraploids showed the larger differences relative to the other two species. By considering only diploid and lowland tetraploid calls, we found significant differences in mean SPL in seven of nine localities: Kapchagai ( $F = 14.58$ ;  $P = 0.0008$ ); Koi-tash ( $F = 23.44$ ;  $P = 0.0001$ ); Chonkurchak ( $F = 27.70$ ;  $P = 0.0001$ ); Charin ( $F = 41.45$ ;  $P = 0.0001$ ); Kok-jar ( $F = 11.36$ ;  $P = 0.0022$ ); Damba ( $F = 22.11$ ;  $P = 0.0001$ ); and Tulek ( $F = 14.30$ ;  $P = 0.0008$ ). By comparing highland and lowland tetraploid calls, we found significant differences in mean SPL in six localities: Koi-tash ( $F = 14.50$ ;  $P = 0.0026$ ); Chonkurchak ( $F = 11.44$ ;  $P = 0.0018$ ); Charin ( $F = 17.01$ ;  $P = 0.0006$ ); Kok-jar ( $F = 9.49$ ;  $P = 0.0058$ ); Damba ( $F = 15.93$ ;  $P = 0.0004$ ); and Tulek ( $F = 9.87$ ;  $P = 0.0008$ ).

**Table 1.** Two-way ANOVAs on the call SPLs. Factors are distance (2 m, 4 m, 8 m, 16 m, 32 m) and taxon (diploids, highland tetraploids, lowland tetraploids)

	Distance		Taxon		Distance*Group	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Kapchagai	225.62	0.0001	8.25	0.0013	2.29	0.0371
Charin	143.77	0.0001	24.85	0.0001	0.21	0.9871
Kopa	90.45	0.0001	2.83	0.0715	0.36	0.9364
Kok-jar	187.13	0.0001	7.76	0.0015	0.83	0.5930
Koi-tash	80.51	0.0001	14.54	0.0002	0.21	0.9305
Chonkurchak	136.02	0.0001	17.11	0.0001	1.36	0.2363
Tulek	454.04	0.0001	7.11	0.0027	1.42	0.2174
Damba	450.79	0.0001	15.19	0.0001	0.9	0.5110
Big Lake	305.51	0.0001	0.81	0.4625	0.6	0.7731

**Table 2.** *F*-ratio from three-way ANOVAs on the signal sound pressure levels. The significance levels are estimated by means of a randomization procedure (\*\*\*  $P < 0.001$ ; \*  $P < 0.05$ ). To obtain the same number of replicates for the background-noise effect, two localities have been alternatively excluded from the analyses

Localities not included in the analysis	Effects					
	Background Noise	Distance	Taxon	Noise*Distance	Noise*Taxon	Distance*Taxon
Damba–Kapchagai	44.5 ***	518.5 ***	18.9 ***	11.6 ***	2.9 *	0.1
Damba–Tulek	19.4 ***	479.2 ***	18.7 ***	8.3 ***	2.9 *	0.6
Damba–Kopa	33.2 ***	573.9 ***	22.8 ***	13.5 ***	2.6 *	0.8
Chonkurchak–Kapchagai	9.5 ***	657.5 ***	20.9 ***	7.5 ***	2.1	1.0
Chonkurchak–Tulek	2.4 ***	557.8 ***	17.3 ***	6.6 ***	2.3	0.8
Chonkurchak–Kopa	9.5 ***	657.5 ***	20.9 ***	7.5 ***	2.1	1.0
Charin–Kapchagai	32.4 ***	503.0 ***	6.4 ***	12.8 ***	1.3	0.1
Charin–Tulek	12.9 ***	468.5 ***	12.6 ***	4.8 ***	1.3	0.5
Charin–Kopa	23.4 ***	551.4 ***	15.5 ***	6.2 ***	1.2	0.7

Main effects: background noise (low, biotic noise, abiotic noise); distance (2, 4, 8, 16, 32 m); taxon (diploids, lowland tetraploids, highland tetraploids).

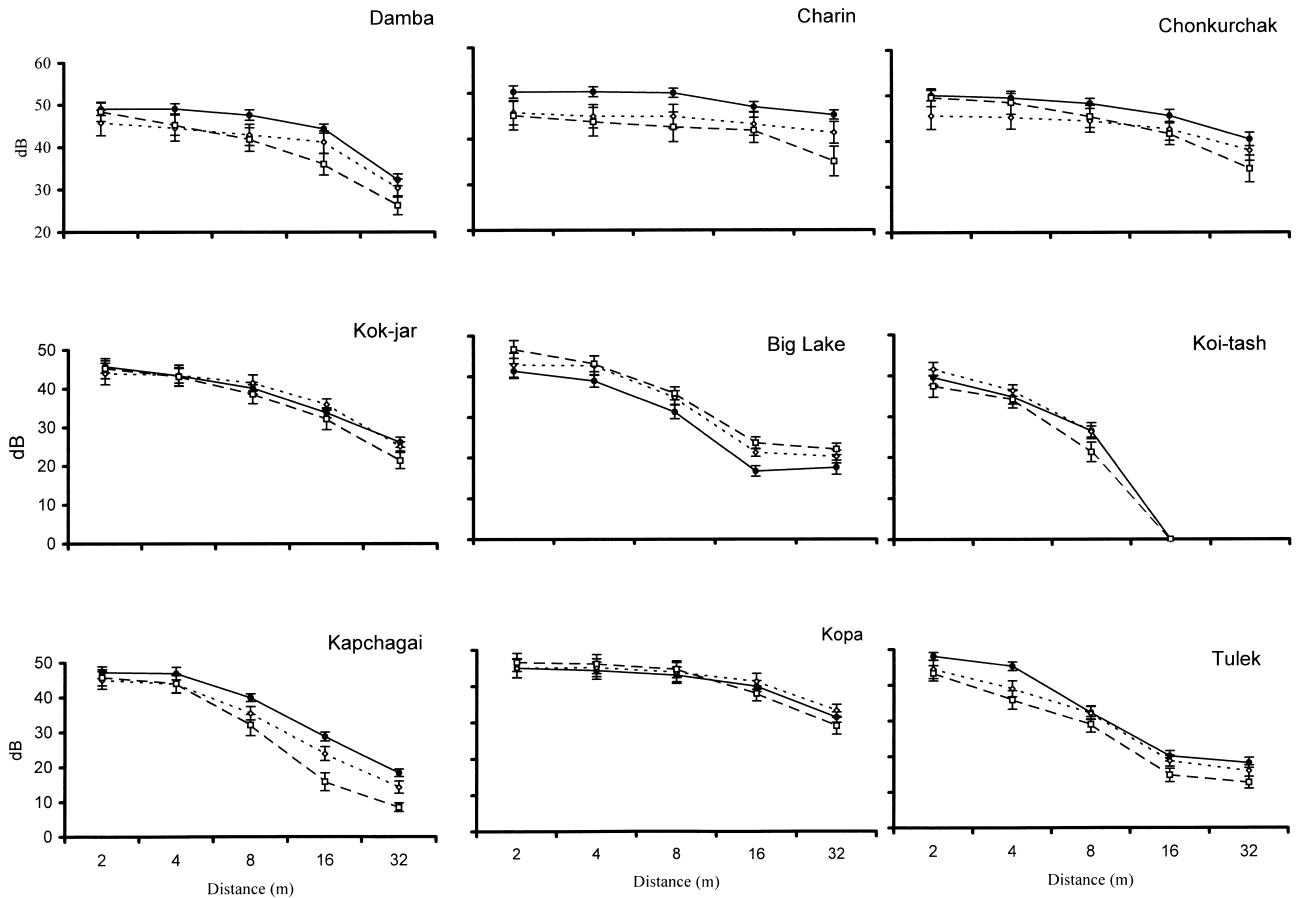
= 0.0041). Finally, when we compared diploids and highland tetraploids, we detected significant differences only in three localities: Kapchagai ( $F = 4.5$ ;  $P = 0.0429$ ), Chonkurchak ( $F = 6.54$ ;  $P = 0.0169$ ), where diploid calls broadcast better than tetraploid calls, and Kopa ( $F = 5.48$ ;  $P = 0.028$ ), where tetraploid calls broadcast better than diploid calls.

#### Attenuation of the fundamental frequency

We measured the relative amplitude of calls' fundamental frequency within each locality and distance (Fig. 5). Independent of the taxon, we estimated the rate of attenuation by regressing frequency amplitudes against distances. In simple regression models, the rate of attenuation (that is, the absolute values of

the regression coefficients) was not significantly correlated with either the vegetation height ( $N = 9$ ;  $R = 0.427$ ;  $b = 0.108$ ;  $F_{1,7} = 1.564$ ;  $P = 0.251$ ) or the vegetation cover ( $N = 9$ ;  $R = 0.042$ ;  $b = -0.004$ ;  $F_{1,7} = 0.013$ ;  $P = 0.914$ ) or the level of background noise ( $N = 9$ ;  $R = 0.554$ ;  $b = 0.173$ ;  $F_{1,7} = 3.107$ ;  $P = 0.121$ ), but it was significantly correlated with the coefficient of attenuation of the calls ( $b = 0.825$ ;  $P = 0.005$ ) and the level of background noise ( $b = 0.209$ ;  $P = 0.008$ ). These results show that the relative amplitude of the fundamental frequency depends on the absolute SPL of sounds but is affected also by the masking effects of background noise.

In Table 2 we show results of two-way ANOVAs. With the only exception of Charin ( $P = 0.05$ ), the relative



**Figure 5.** Call attenuation in the spectral domain: decreasing with distance of the fundamental frequency relative amplitude in the calls of the three taxa (diploids:●; highland tetraploids:◇; lowland tetraploids:□).

amplitude of fundamental frequency decreased significantly with distance ( $P < 0.001$ ). The taxon-call had significant effects in only five of nine localities: in Kapachagai, Tulek, Charin and Damba diploid calls had fundamental frequencies with higher peak amplitudes than both highland and lowland tetraploid calls; in contrast, in Big Lake tetraploid calls showed fundamental frequencies with higher peak amplitudes than diploid calls.

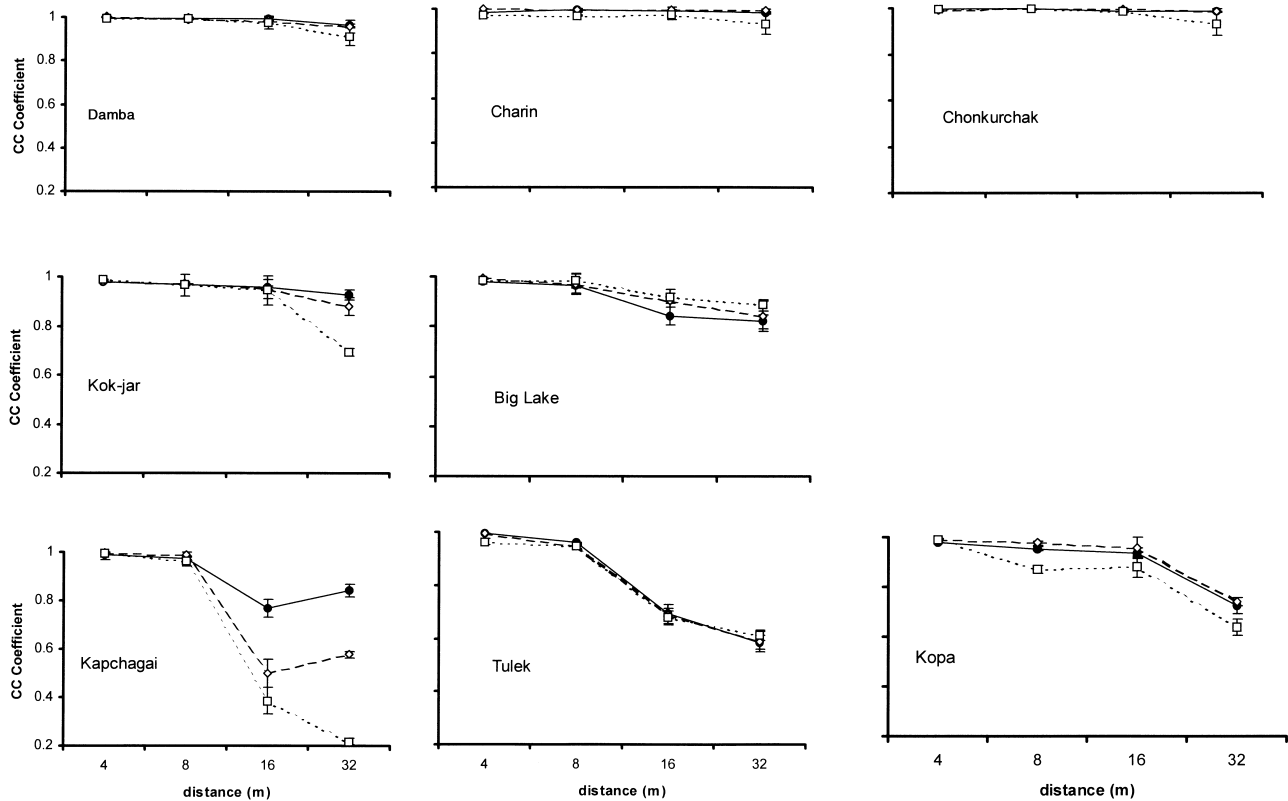
When we compared diploid calls with highland tetraploid calls, we found between-taxon significant differences in five localities: Charin ( $F = 9.51$ ;  $P = 0.0035$ ), Damba ( $F = 4.44$ ;  $P = 0.0422$ ), Chonkurchak ( $F = 5.68$ ;  $P = 0.0217$ ), Kapchagai ( $F = 6.82$ ;  $P = 0.0144$ ), where the fundamental frequencies of diploid calls attenuated relatively less than highland tetraploids, and Big Lake ( $F = 12.20$ ;  $P = 0.0020$ ), where fundamental frequencies of tetraploid calls attenuated less than diploids. By comparing diploid calls with lowland tetraploid calls, we obtained similar results: in Big Lake tetraploid calls transmitted better than diploid calls ( $F = 23.50$ ;  $P = 0.0001$ ); in Charin

( $F = 11.56$ ;  $P = 0.0015$ ), Kapchagai ( $F = 15.38$ ;  $P = 0.0008$ ), Damba ( $F = 7.11$ ;  $P = 0.011$ ) and Tulek ( $F = 10.13$ ;  $P = 0.0046$ ) diploid calls broadcast more efficiently than tetraploid calls, whereas in Kopa, Chonkurchak, Koi-tash and Kok-jar no significant differences were observed. Finally, when we compared lowland and highland tetraploid calls, none of the nine localities showed significant differences in the pattern of frequency attenuation.

#### Sound degradation

We cross-correlated sonograms of calls at different distances and employed the correlation coefficients as an overall estimate of sound degradation. For each locality, independent of the taxon, we estimated the rate of degradation by regressing cross-correlation coefficients against distances. The rate of degradation was not significantly correlated to the vegetation height ( $N = 8$ ;  $R = 0.425$ ;  $F_{1,6} = 1.323$ ;  $P = 0.294$ ), the vegetation cover ( $N = 8$ ;  $R = 0.106$ ;  $F_{1,6} = 0.068$ ;  $P = 0.803$ ), the level of background noise ( $N = 8$ ;  $R = 0.547$ ;  $F_{1,6} = 2.558$ ;  $P = 0.161$ ), or the rate of call attenuation





**Figure 6.** Variation with distance of cross correlation coefficients in eight localities (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

( $N = 8$ ;  $R = 0.409$ ;  $F_{1,6} = 1.208$ ;  $P = 0.314$ ), but it was significantly correlated with the attenuation rate of the fundamental frequency ( $N = 8$ ;  $R = 0.826$ ;  $F_{1,6} = 12.91$ ;  $P = 0.011$ ).

In Figure 6 we show results of the cross-correlation analyses on the calls of the three taxa. To analyse the effect of distance and taxon on the correlation coefficients, for each locality, we carried out a two-way ANOVA (Table 3).

Distance had highly significant effects in all localities except Charin. Similarly, the calls of the three taxa had significantly different cross-correlation coefficients in all localities except Tulek. The interaction between distance and taxon was statistically significant only in Kapchagai, Kok-jar, Chonkurchak and Damba, whereas in Charin, Kopa, Tulek and Big Lake the between-taxon differences in the correlation coefficients did not change significantly with the increase in distance.

Analyses of variance considering diploid and highland tetraploid calls only showed between-taxon significant differences in two localities: Charin (highland locality), where tetraploid calls had higher coefficients of correlation than diploid calls ( $F = 10.63$ ;  $P = 0.0039$ ), and Kapchagai (lowland locality) where diploid calls

showed higher coefficients of correlation than tetraploid calls ( $F = 42.89$ ;  $P = 0.0001$ ).

When we compared diploid calls with lowland tetraploid calls, we found significant differences in cross-correlation coefficients in all localities except Tulek: in Chonkurchak ( $F = 4.95$ ;  $P = 0.0136$ ), Damba ( $F = 10.14$ ;  $P = 0.0015$ ), Charin ( $F = 5.40$ ;  $P = 0.0087$ ), Kopa ( $F = 5.67$ ;  $P = 0.0257$ ), Kapchagai ( $F = 162.44$ ;  $P = 0.0001$ ) and Kok-jar ( $F = 4.32$ ;  $P = 0.0261$ ) diploid calls propagated better than tetraploid calls; in contrast, in Big Lake tetraploid calls broadcast better than diploid calls ( $F = 18.91$ ;  $P = 0.0007$ ).

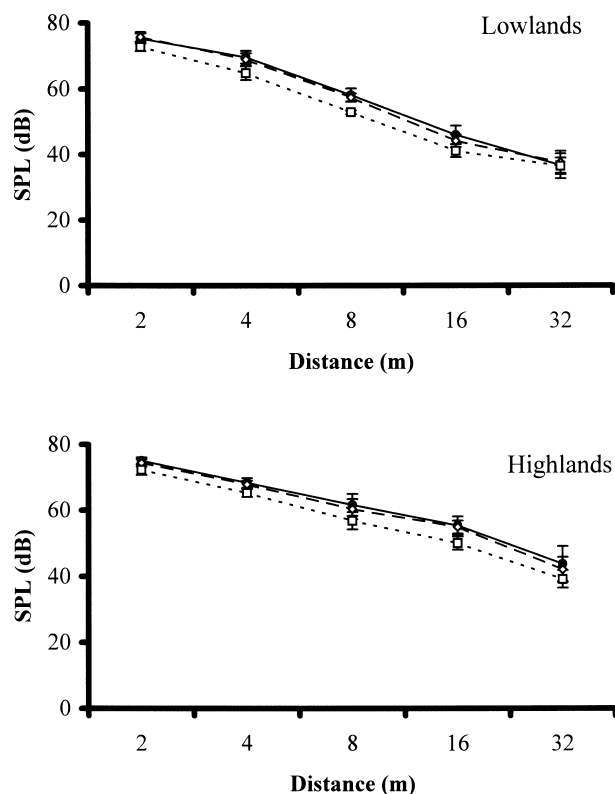
Highland tetraploid calls had higher cross-correlation coefficients than lowland tetraploid calls in six localities, both of low altitude (Damba,  $F = 4.27$ ;  $P = 0.0493$ ; Kopa,  $F = 8.90$ ;  $P = 0.0064$ ; Kapchagai,  $F = 28.49$ ;  $P = 0.0001$ ) and high altitude (Chonkurchak,  $F = 4.86$ ;  $P = 0.0205$ ; Charin,  $F = 10.76$ ;  $P = 0.0007$ ; Kok-jar,  $F = 4.80$ ;  $P = 0.0273$ ).

#### ALTITUDINAL EFFECTS

To test the effect of altitude on call transmission, we carried out three-way analyses of variance, in which the three independent factors were distance, taxon

**Table 3.** Two-way ANOVAs on the relative amplitude of call fundamental frequency. Factors are Distance (2 m, 4 m, 8 m, 16 m, 32 m) and taxon (diploids, highland tetraploids, lowland tetraploids)

Locality	Effects					
	Distance		Group		Distance*Group	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Big Lake	94.47	0.0001	11.97	0.0002	0.14	0.9963
Koi-Tash	32.50	0.0001	1.44	0.2567	0.28	0.8849
Kok-jar	33.95	0.0001	0.6	0.5664	0.29	0.9645
Charin	2.55	0.0500	6.37	0.0031	0.18	0.9912
Chonkurchak	9.32	0.0001	2.17	0.1283	0.44	0.8919
Damba	24.26	0.0001	3.46	0.0392	0.51	0.8445
Kapchagai	104.41	0.0001	8.36	0.0008	1.25	0.2938
Kopa	15.43	0.0001	0.89	0.4196	0.36	0.9373
Tulek	118.51	0.0001	5.25	0.0099	0.44	0.8843

**Figure 7.** Call attenuation in the temporal domain in the two altitudinal categories (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

and altitude (highlands vs. lowlands), and the dependent variables describing the pattern of sound attenuation were, respectively, (1) the absolute RMS sound pressure levels; (2) the fundamental

frequency amplitudes; and (3) the cross correlation coefficients.

(1) The sound pressure level differed significantly among the calls of the three taxa ( $F = 24.07$ ;  $P = 0.0001$ ), and between highland and lowland localities ( $F = 46.95$ ;  $P = 0.0001$ ), but the combined effect of altitude and taxon was not statistically significant ( $F = 0.15$ ;  $P = \text{NS}$ ). Between-taxon differences were mostly due to lowland tetraploid calls. In fact, when we limited the comparison to diploid and highland tetraploid calls we still found a significant effect of altitude ( $F = 7.89$ ;  $P = 0.013$ ), but we did not find a significant difference between taxa ( $F = 1.96$ ;  $P = 0.164$ ) nor a significant interaction between taxon and altitude ( $F = 0.25$ ;  $P = 0.6521$ ) (Fig. 7).

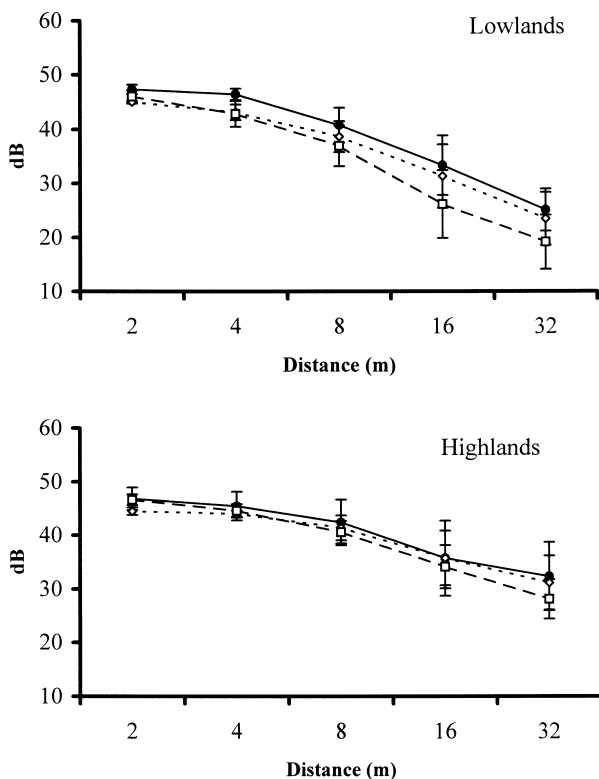
(2) Altitude significantly affected the pattern of attenuation of the call fundamental frequency ( $F = 19.69$ ;  $P = 0.0001$ ), but its effect did not differ significantly among diploid and the two tetraploid taxa ( $F = 0.89$ ;  $P = \text{NS}$ ) (Fig. 8).

(3) Degradation, as expressed by the cross-correlation coefficients, was significantly affected by all three independent factors (altitude,  $F = 69.80$ ;  $P = 0.0001$ ; distance,  $F = 59.40$ ;  $P = 0.0001$ ; taxon,  $F = 6.80$ ;  $P = 0.0007$ ). Moreover, we observed a marginally significant interaction between taxon and distance ( $F = 2.16$ ;  $P = 0.0432$ ), and between taxon and altitude ( $F = 3.03$ ;  $P = 0.0498$ ). However, these differences were mostly due to the lowland tetraploid calls. In fact, when we limited the comparison to diploid and highland tetraploid calls we found neither significant differences between the calls of the two taxa ( $F = 1.05$ ;  $P = 0.3138$ ) nor a significant interaction between altitude and taxon ( $F = 2.30$ ;  $P = 0.1167$ ).

**Table 4.** *F*-ratio from three-way ANOVAs on the relative amplitude of the call fundamental frequency. The significance levels are estimated by means of a randomization procedure (\*\*\*)  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ ). To obtain the same number of replicates for the background-noise effect, two localities have been alternatively excluded from the analyses

Localities not included in the analysis	Effects					
	Background Noise	Distance	Taxon	Noise*Distance	Noise*Taxon	Distance*Taxon
Damba–Kapchagai	83.5 ***	88.8 ***	0.9	6.7 ***	3.2 *	0.5
Damba–Tulek	81.4 ***	101.6 ***	1.6	8.0 ***	3.6 **	0.9
Damba–Kopa	202.9 ***	198.2 ***	6.0 ***	13.5 ***	2.6 *	0.8
Chonkurchak–Kapchagai	57.8 ***	94.7 ***	1.4	22.6 ***	7.0 ***	0.5
Chonkurchak–Tulek	55.3 ***	107.2 ***	2.3	5.1 ***	3.8 **	0.9
Chonkurchak–Kopa	147.2 ***	202.0 ***	7.0 **	16.6 ***	6.8 ***	0.9
Charin–Kapchagai	59.8 ***	106.6 ***	0.4	3.5 **	2.5 *	0.6
Charin–Tulek	57.8 ***	121.8 ***	0.9	4.1 ***	2.9 *	1.1
Charin–Kopa	156.8 ***	230.3 ***	4.3 **	15.3 ***	6.3 ***	1.1

Main effects: background noise (low, biotic noise, abiotic noise); distance (2, 4, 8, 16, 32 m); taxon (diploids; lowland tetraploids; highland tetraploids).



**Figure 8.** Call attenuation in the spectral domain in the two altitudinal categories (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

In conclusion, independently of altitude, diploid and highland tetraploid calls exhibited less attenuation and less degradation with distance than lowland tetraploid calls.

#### ENVIRONMENTAL NOISE EFFECTS

In Table 4 we show results of the nine three-way ANOVAs, in which we analysed the effect of distance, taxon and noise type on the call RMS sound pressure levels. All three independent factors significantly affected sound attenuation, and, in the three categories of environmental noise, sounds attenuated differently with distance. However, the taxon–noise interaction showed significant effects only in three out of nine analyses. This interaction became more evident when we considered the fundamental frequency amplitude (Table 5). In this case, we found a statistically significant interaction between the type of environmental noise and the taxon: tetraploid calls performed better in those environments where atmospheric perturbations produced low frequency noise, whereas diploid calls did better in habitats with low levels of environmental noise (Fig. 9).

Finally, we analysed the effects of distance, taxon and environmental noise on call degradation (Fig. 10 and Table 6). Independent of the localities considered, all factors showed significant effects. However, we found a statistically significant interaction between the type of environmental noise and the taxon only when Kapchagai was included in the analysis. In these cases, diploid calls performed better in habitats with low levels of environmental noise, whereas tetraploid calls did better in those environments with low frequency noise.

#### DISCUSSION

Our transmission experiments show that: (1) the pattern of sound propagation is affected by vegetation

**Table 5.** Two-way ANOVAs on the sonogram cross correlation coefficients. Factors are distance (2 m, 4 m, 8 m, 16 m, 32 m) and taxon (diploids, Highland tetraploids, lowland tetraploids)

	Effects					
	Distance		Taxon		Distance*Taxon	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Kapchagai	213.97	0.0001	72.51	0.0001	27.39	0.0001
Charin	1.24	0.3056	8.14	0.0001	0.81	0.6127
Kopa	53.59	0.0001	6.24	0.0064	0.83	0.5580
Kok-jar	20.38	0.0001	5.71	0.0037	5.28	0.0011
Koi-tash	–	–	–	–	–	–
Chonkurchak	6.20	0.0009	4.34	0.0123	3.28	0.0109
Tulek	220.66	0.0001	0.14	0.8708	0.41	0.8686
Damba	28.64	0.0001	6.18	0.0033	3.10	0.0131
Big Lake	46.00	0.0001	6.50	0.0040	1.48	0.2139

**Table 6.** *F*-ratio from three-way ANOVAs on the cross-correlation coefficients. The significance levels are estimated by means of a randomization procedure (\*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ ). To obtain the same number of replicates for the background-noise effect, two localities have been alternatively excluded from the analyses

Localities not included in the analysis	Effects					
	Background Noise	Distance	Taxon	Noise*Distance	Noise*Taxon	Distance*Taxon
Damba–Kapchagai	119.9 ***	107.9 ***	4.77 **	32.8 ***	0.4	1.1
Damba–Tulek	76.4 ***	62.3 ***	12.3 ***	22.2 ***	6.6 ***	4.5 ***
Damba–Kopa	204.4 ***	138.4 ***	14.6 ***	62.5 ***	7.6 ***	5.7 ***
Chonkurchak–Kapchagai	115.7 ***	113.0 ***	4.7 ***	30.5 ***	0.4	1.0
Chonkurchak–Tulek	74.2 ***	65.0 ***	12.4 ***	21.0 ***	6.6 ***	4.5 ***
Chonkurchak–Kopa	200.0 ***	143.1 ***	14.7 ***	60.4 ***	7.5 ***	5.7 ***
Charin–Kapchagai	121.0 ***	117.3 ***	3.8 *	29.8 ***	0.5	1.2
Charin–Tulek	76.6 ***	66.8 ***	11.6 ***	20.6 ***	6.8 ***	4.7 ***
Charin–Kopa	206.1 ***	147.4 ***	13.9 ***	60.0 ***	7.8 ***	6.0 ***

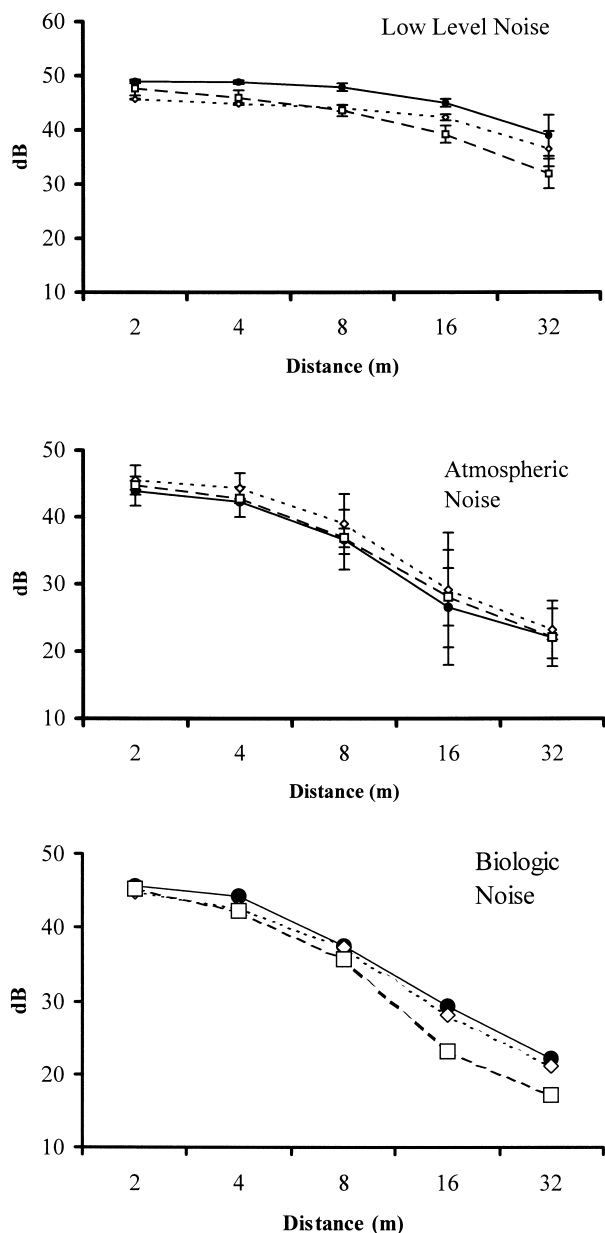
Main effects: background noise (low, biotic noise, abiotic noise); distance (2, 4, 8, 16, 32 m); taxon (diploids, lowland tetraploids, highland tetraploids).

height and significantly differs among localities in relation to their environmental noise; (2) in most localities, diploid and highland and lowland tetraploid calls show significantly different patterns of attenuation with distance: lowland tetraploid calls usually undergo the strongest attenuation, and diploid calls the weakest attenuation; (3) like attenuation, call degradation also differs significantly among localities and between diploid and tetraploid calls: lowland tetraploid calls degrade more than diploid and highland tetraploid calls; (4) attenuation and degradation show stronger effects at low than at high altitude; (5) at both high and low altitude, however, lowland tetrap-

loid calls fared worse than both diploid and highland tetraploid calls, whereas diploid and highland tetraploid calls show different patterns of propagation in a few localities only.

#### PATTERNS OF CALL ATTENUATION AND DEGRADATION

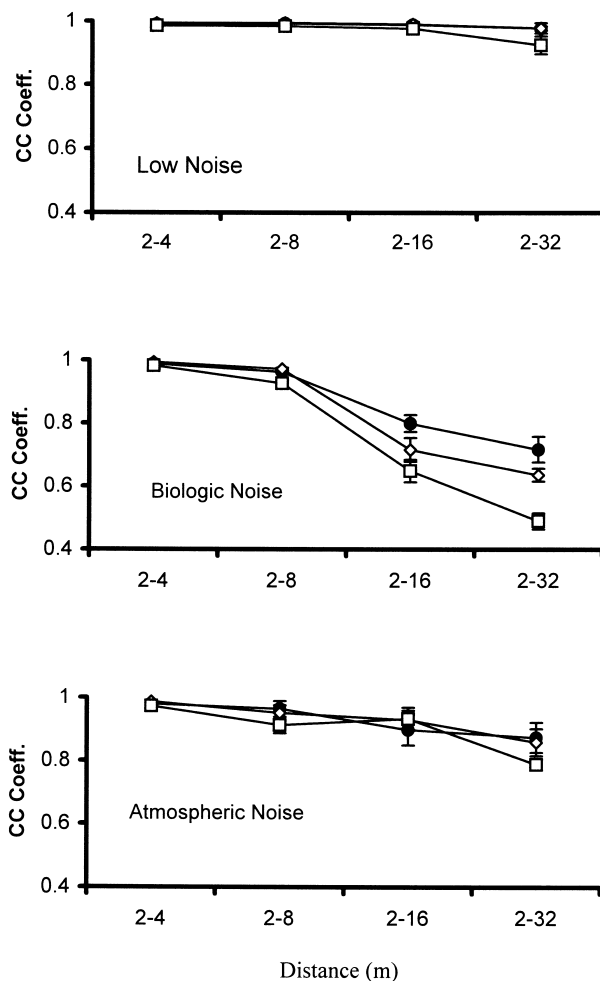
In the present work, we have tried to distinguish the effects of attenuation from those of degradation. Attenuation is a loss of overall acoustic energy, whilst degradation is a loss of fidelity of acoustic energy relative to time and frequency. Although the rate of degradation was not significantly correlated to the rate of



**Figure 9.** Call attenuation in the spectral domain in the three categories of environmental noise (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

attenuation of absolute SPL, it was correlated significantly with the rate of attenuation of the fundamental frequency. Therefore, attenuation did affect degradation, and the more noisy the habitat the stronger its effect.

The pattern of call propagation differed significantly not only between localities but also between the calls of the three taxa within the same locality. In most localities, lowland tetraploid calls suffered both a stronger attenuation and a stronger degradation than



**Figure 10.** Variation with distance of cross correlation coefficients in the three categories of environmental noise (diploids: ●; highland tetraploids: ◇; lowland tetraploids: □).

diploid and highland tetraploid calls. On the other hand, differences between diploid and highland tetraploid calls were mostly due to differences in attenuation rather than to differences in degradation.

The calls of the three taxa differed with respect to fundamental frequency and pulse rate: diploid toads call at lower frequencies and at faster pulse rates than both highland and lowland tetraploid toads; whereas highland tetraploids call at lower frequencies than lowland tetraploids, but with similar pulse rates. Fundamental frequency is known to play a relevant role in influencing call propagation (Marten & Marler, 1977; Marten, Quine & Marler, 1977; Brown & Gomez, 1992; Kime *et al.*, 2000): high frequencies tend to undergo stronger attenuation than lower frequencies. Few studies have shown that the temporal structure of calls could have significant effects on propagation. Ryan & Sullivan (1989) compared the pattern of call

degradation of two species of toads (*Bufo valliceps*, and *B. woodhousei*) with similar fundamental frequencies but markedly different pulse rates, and found that temporal degradation was stronger in *B. valliceps* calls (lower pulse rates) than in *B. woodhousei* calls (higher pulse rates).

Both temporal and spectral properties of diploid calls could therefore explain why they often propagated more efficiently than both highland and lowland tetraploid calls. Most surprising, perhaps, is that in some localities this was not the case, and diploid calls fared as well as or even worse than tetraploid calls. Kapchagai was the locality with the more accentuated between-taxon differences and where diploid calls fared much better than tetraploid calls. At the other extreme, Big Lake was the locality where tetraploid calls fared better than diploid calls. We suggest that differences in the spectral structure of the background noise could have an important role in determining the different propagation pattern between these localities: in Big Lake most of the acoustic energy of the environmental noise is at low frequencies and might produce a stronger masking effect on the diploid calls than on the tetraploid calls (which have higher fundamental frequencies than diploid calls); on the contrary, in Kapchagai, because of the calling of green frogs, the environmental noise had a second peak of energy at frequencies between 2 and 4 kHz, and might have a stronger masking effect on the high-pitched tetraploid calls (mostly, on the lowland tetraploid calls).

#### THE ROLE OF ENVIRONMENTAL SELECTION

A number of studies on bird and primate calls have found evidence consistent with the environmental selection hypothesis (Gish & Morton, 1981; Morton, 1975; Hunter & Krebs, 1979; Wiley, 1991). Similar studies on anurans, however, have led to different conclusions: Penna & Solis (1998) compared patterns of call propagation of several South American frogs, and Kime *et al.* (2000) carried out a similar study on 22 Central American frogs; both these researches failed to find evidence that differences among calls evolved to maximize habitat-specific sound propagation. In addition, Zimmerman (1983) showed that differences in call structure among Amazonian frogs are more likely to reflect differences in body size or phylogeny, than to reflect differences in their acoustic environments.

One of the few studies on frogs that provided evidence consistent with the environmental selection hypothesis was that of Ryan *et al.* (1990) on two subspecies of cricket frog, *Acris crepitans crepitans*, which inhabits forests, and *A. c. blanchardi*, which lives in open habitats. Transmission experiments showed that calls of both subspecies transmitted better in open

than in forest habitats, but in forests *A. c. crepitans* calls fared better than *A. c. blanchardi* calls, whereas in open habitats no differences were observed.

To provide evidence for the environmental selection hypothesis three conditions should be satisfied: (1) highland and lowland habitats should be acoustically different environments; (2) these acoustic differences should produce different effects on diploid and tetraploid calls; (3) so that calls tend to transmit better (either in absolute or relative terms) in the habitats where they are typically broadcast.

In our study, the first and second conditions are only partially satisfied. In fact, of the three categories of acoustic environments (low-level noise, biological noise, and atmospheric noise) only two were exclusive of either one of the altitudinal category (biological noise was exclusive of lowlands and atmospheric noise was exclusive of mountains), whereas one (low-level noise) was present at both low and high altitudes. The third condition is satisfied by the diploid calls (in the typical lowland habitats, they propagate better than tetraploid calls), partially satisfied by the highland tetraploid calls (they propagate as efficiently as or even better than diploid calls in atmospheric noise conditions, but they broadcast less efficiently in low noise environments), but it is not satisfied by the lowland tetraploid calls (toads live in the lowlands but they call relatively better in the highlands).

In the case of lowland tetraploid calls, we can reject the environmental selection hypothesis. The differences in their call acoustic properties are more likely to be the side-effect of their small body size. With respect to mountain tetraploids, lowland tetraploids are smaller not because they are younger but because they grow at a slower rate (Castellano *et al.*, 2000) and they grow slowly possibly because they must suspend their activity during the hot and dry lowland summers. In some way, therefore, the environment is responsible for the acoustic differences of lowland calls, not directly through selection for enhancing call propagation, but indirectly by constraining toad body size.

In the case of diploid and highland tetraploid advertisement calls, transmission experiments do not provide strong evidence either to support or to reject the environmental hypothesis. Differences in call propagation between diploid and mountain tetraploid calls are generally weak and they are statistically significant only rarely, but when they are significant they tend to be consistent with the environmental hypothesis. Although we cannot exclude that environmental selection might have played a role in call differentiation, such a role must have been marginal, and it seems unlikely it could explain the large-scale differences between diploid and tetraploid advertisement calls. Since neither phylogeny nor polyploidization can

explain these differences (Castellano *et al.*, 2002), the rejection of the environmental selection hypothesis further strengthens the alternative adaptive hypothesis of reproductive character displacement, according to which selection favoured call differentiation to enhance reproductive isolation between diploid and tetraploid green toads.

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## APPENDIX

Taxon	Duration (s)	Pulse rate (Hz)	Fundamental frequency (Hz)
Diploid	4.75	28	1116
Diploid	1.76	39	1115
Diploid	2.32	29	1292
Diploid	10.00	15	1115
Highland tetraploid	3.89	17	1429
Highland tetraploid	5.68	12	1370
Highland tetraploid	6.13	16	1292
Highland tetraploid	4.59	14	1370
Lowland tetraploid	5.86	15	1423
Lowland tetraploid	4.16	14	1686
Lowland tetraploid	2.24	22	1785
Lowland tetraploid	3.59	20	1507