



# Morphometrical and acoustical comparison between diploid and tetraploid green toads

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In the present paper we compare, on the basis of morphometrical characters and acoustical properties of the advertisement calls, a sample of 158 male green toads (*Bufo viridis* complex) collected in 12 breeding populations of south Kazakhstan and north Kyrgyzstan. The samples of three populations resulted in only diploid toads ( $2n=22$ ), those of eight populations in only tetraploid toads ( $2n=44$ ) whereas in one locality diploid, tetraploid and many triploid toads were collected. Diploid toads show significantly larger body size and proportionally larger head and shorter limbs than both tetraploids and triploids, whereas no evident morphometrical differences were observed between triploids and tetraploids. Diploid advertisement calls have spectral and temporal properties that significantly differ from those of both triploid and tetraploid advertisement calls. In particular, diploids produce significantly longer calls with higher pulse-rates and lower frequencies than those of tetraploids. We address the question of the factors that could be responsible for these differences and we discuss four hypotheses: (1) the direct effect of polyploid mutation, (2) genetic drift, (3) reproductive character displacement and (4) environmental selection.

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ADDITIONAL KEY WORDS: — anuran – polyploidy – acoustics – morphometry.

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## INTRODUCTION

Although common in plants, polyploidy is a relatively rare phenomenon in animals (Orr, 1990). Since polyploid animals are reproductively isolated from their ancestors, polyploidy is an example of instantaneous speciation. Among vertebrates a large number of polyploid species is found in anuran amphibians: polyploid taxa have been documented in 12 genera in nine families of frogs and toads (Kuramoto, 1990; Tymowska, 1991). The mechanism that has led to polyploid taxa is often controversial. Some species are thought to have arisen through hybridization (allopoloidy), even though the most common way to acquire a polyploid karyotype may be autopoloidy (Tymowska, 1991). In some cases polyploid taxa and their sister diploid species inhabit geographically close areas, and may sometimes exhibit a broad overlap of their ranges. A well known case is that of diploid-tetraploid grey tree frogs, *Hyla chrysoscelis* and *H. versicolor*. They are distributed in the central, mid-Atlantic and south-eastern regions of the U.S.A, and in a large part of this range they often share the same breeding site (Gerhardt, 1994a). Since hybrids between diploids and tetraploids are sterile (Johnson, 1963), strong selection should act upon individuals of both species to choose the correct species for mating. For this reason grey tree frogs have been profitably employed as a model for the study of the evolution of mate choice mechanisms in Anurans (review in Gerhardt, 1994b).

Tetraploid green toad populations were first described about 20 years ago from Kyrgyzstan, Central Asia, (Kadyrova, Mazik & Toktosunov, 1976; Mazik, Kadyrova & Toktosunov, 1976). Further studies have shown tetraploids to be distributed over a broad area from Turkmenistan through Tadzhikistan, Uzbekistan, Kyrgyzstan, Kazakhstan, as far as north-western China and Mongolia (Pisanetz, 1978a; Borkin *et al.*, 1986; Borkin & Kuzmin, 1986; Roth & Ráb, 1986; Dujsebajeva *et al.*, 1997). In most of these regions tetraploids preferentially inhabit mountain areas, often reaching altitudes as high as 4000 m (Pisanetz, pers. comm.), whereas the lowlands (from 200 m up to 800 m over the sea level) are preferentially inhabited by diploid populations. This altitudinal shift is not always the rule and some lowland tetraploid populations are known as well (Borkin *et al.*, 1986, 1996). Despite the scarce number of findings (no more than 100 populations over an area of more than 1 million square kilometres), due to the few studies carried out in these regions, sympatric populations have been occasionally found (Pisanetz, 1978a,b; Golubev, 1990).

The Central Asian diploid-tetraploid green toad complex is still controversial with respect to nomenclatural, systematic and phylogenetic issues. Pisanetz (1978a) described tetraploids as a new species and called them *B. danatensis*. Hemmer, Schmidtler & Boheme (1978), without carrying out any karyological examination,

distinguished central Asian green toads in two distinct groups: a *grossform* that they named *B. viridis turanensis*, and a *kleinform*, that was described as an independent species and named *B. latastii*. According to Pisanetz & Shcherlbak (1979) the so-called *kleinform* is actually a polyphyletic group, composed of three different *taxa*, one of which is diploid (*B. viridis asiomontanus*) and the other two tetraploid and that should correspond to *B. danatensis* (reviewed by Roth, 1986). Since a number of names are available for Central Asiatic toads and no general revision is possible at this stage, in this paper we will refer to all populations as *B. viridis* complex, only distinguishing Asian diploids and tetraploids. The mechanism that generated tetraploid green toads is another controversial issue. The allopolyploidy hypothesis was suggested by Mezhzherin & Pisanetz (1995) on the basis of the high level of heterozygosity of tetraploid populations from Turkmenistan and Tadzikistan. These results, however, were not confirmed by Borkin *et al.* (1986) and by the electrophoretic, cytogenetical and biomolecular analyses carried out on the populations that are the object of this study (Lattes, Bigoni & Castellano, 1995; Odierna *et al.*, 1995; Cervella, Delpero & Balletto, 1995). The low level of heterozygosity of tetraploid toads observed in these studies strongly supports an autopolyploid origin of the taxon. Pisanetz (1991) has also hypothesized that, as found in grey tree frog complex (Ptacek, Gerhardt & Sage, 1993), tetraploidy could have arisen independently more than once, and possibly by either mechanism.

The advanced level of diploidization observed in the tetraploid genome of Kyrgyz and Kazak populations (Roth & Ráb, 1986; Odierna *et al.*, 1995) suggests that polyploidization is a relatively ancient event. But electrophoretic analyses of Random Amplified Polymorphic DNAs (RAPDs) reveal a higher similarity between the Central Asian diploids and tetraploids than between them and the European green toads (Cervella, DelPero & Balletto, 1995), suggesting that Central Asiatic diploid green toads are phylogenetically closer to the tetraploids than to the Italian diploid green toads.

In the present paper we compare diploid and tetraploid green toads in Central Asia on the basis of morphometrical and acoustical parameters. Since in green toads advertisement calls produced by males play an important role in mate choice, particular attention is given to their differences. Finally, we will discuss some hypotheses of the mechanisms responsible for these differences: whether they are a direct consequence of polyploidy (Bogart & Wasserman, 1972) or, whether they should be seen as the result of an evolutionary process of diversification. In the latter case, we address the question of the evolutionary forces that have played the most relevant role in the diversification process. Can the differences in advertisement calls between diploid and tetraploid toads be seen as the result of random differentiation due to genetic isolation between *taxa* (genetic drift hypothesis)? Alternatively, should they be interpreted as the result of selective pressures that favour diversification either because of the reduced risks of hybridization (reproductive character displacement hypothesis) or because of the different habitat where the advertisement calls are transmitted (environmental selection hypothesis)?

To attempt to answer these questions, the acoustic differences between diploid and tetraploid toads will be analysed and compared with a sample of triploid specimens to verify the possible importance of ploidy level in determining the call structure (Bogart & Wasserman, 1972; Ralin, 1977). A diploid population of European green toads will be employed as an outgroup with respect to Asian diploid and tetraploid toads (Cervella, DelPero & Balletto, 1995) and their advertisement

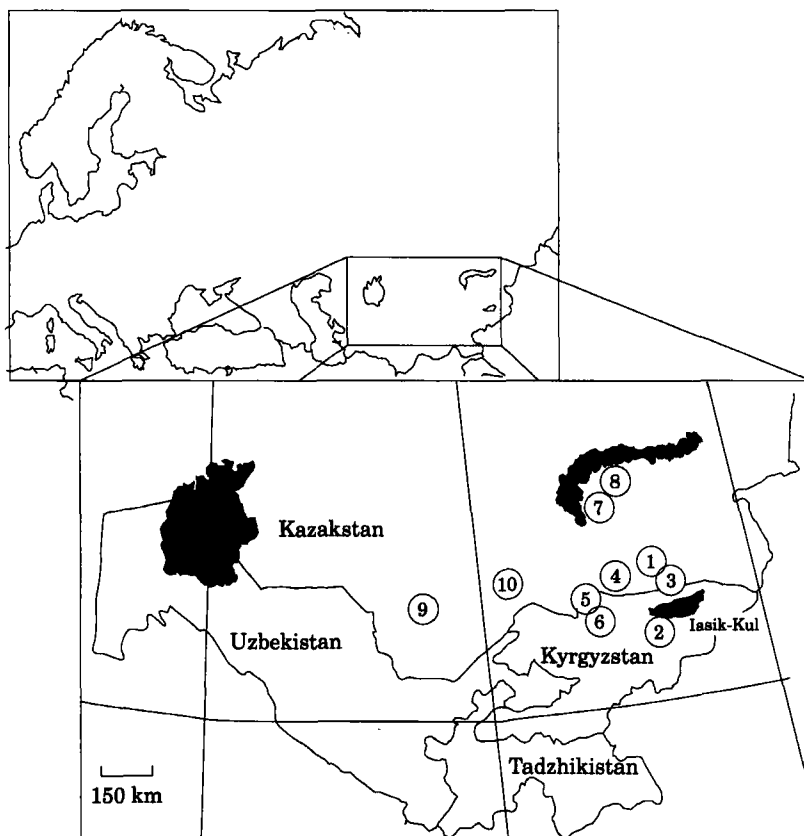


Figure 1. Map of the localities where toads were collected: 1, Almaty; 2, Issik-Kul; 3, Big Lake; 4, Kopa; 5 Tulek; 6, Kok-jar; 7, Ili-river; 8, Karaoj; 9, Kizil-Kum; 10, Zhabagli. In the localities of Almaty and Kok-jar animals were collected from two different breeding sites a few kilometres from each other.

calls will be compared in order to verify the plausibility of the genetic drift hypothesis. Finally, the reproductive character displacement hypothesis will be tested by comparing the advertisement calls of sympatric and allopatric populations.

## MATERIAL AND METHODS

### *The sample*

Samples of male and female green toads were collected in June 1994 and in May and June 1995 from 12 breeding populations, nine of which were in South Kazakhstan (see Dujsebayaeva *et al.*, 1997) and the remaining three in North Kyrgyzstan (see Figure 1). Breeding sites were visited in the first hours of the night when the reproductive activity reached its peak. Only adult specimens with well-developed secondary sexual characters were sampled. The advertisement calls of most of the males were recorded and their cloacal temperature was measured by means of a mercury thermometer calibrated to 0.5°C. Within 48 hours of being captured, all

specimens were anaesthetized in MS-222 (Sandoz), weighed, and 20 precisely defined body-proportion characters (Appendix 1) were measured with a digital calliper (Mitutoyo mod. 500–321) calibrated to 0.01 mm. Most of the animals were taken alive to our laboratories where a karyological analysis was carried out. In order to compare green toad advertisement calls over a broader geographic area, we recorded advertisement calls of a population from the westernmost part of the range of the species (Pellice, north-western Italy) (see Castellano, 1996)

### *Karyological analysis*

From each specimen 100–200 µl of venous blood were incubated for 4 days at 25°C in MEM (Minimal Eagle Medium, GIBCO) with 20% calf serum and 3% Phytohaemagglutinin M. Chromosome preparations were produced by conventional air-drying method, using KCl 0.075 M as hypotonic solution. Standard staining method was performed using Giemsa 5% in phosphate buffer pH 7.

### *Morphometric analysis*

Males were grouped according to their number of chromosomes, but independently from the populations where they were captured. Before applying univariate, bivariate and multivariate statistical techniques, all body-proportion characters and the body weight were log-transformed.

Multiple group principal component analysis (MGPCA) was employed to obtain a suitable body-size factor, corresponding to the first eigenvector of the pooled-within-group covariance matrix of morphometrical characters (Thorpe, 1981, 1983; Corti *et al.*, 1988). MGPCA was run using the program MULT written by Thorpe (University of Wales Bangor, UK).

In order to analyse the differences in body-shape between diploids and tetraploids, all body proportion characters were adjusted for body-size. Size adjusted morphometric characters were obtained by calculating the residuals of the simple linear regressions between the log-transformed morphometric parameters (dependent variables) and the body-size factor (independent variable).

Step-wise discriminant function analysis was carried out in order to define the minimum number of body-proportion characters for which the best discrimination between karyotypes was obtained.

### *Acoustical analysis*

Advertisement calls were recorded with a Marantz CP 230 tape-recorder and a Sennheiser K3U-ME 88 directional microphone. Each male calling activity was continuously recorded for at least three minutes in order to obtain a sufficient number of calls (minimum = 3, maximum = 20, average = 7). Sound analysis was carried out on an Apple Macintosh IICI personal computer. Recordings were digitized at a sample rate of 44100 Hz by means of an AD converter implemented in the Audiomedia card (Audiomedia II system). Sound Designer II and Canary 1.1

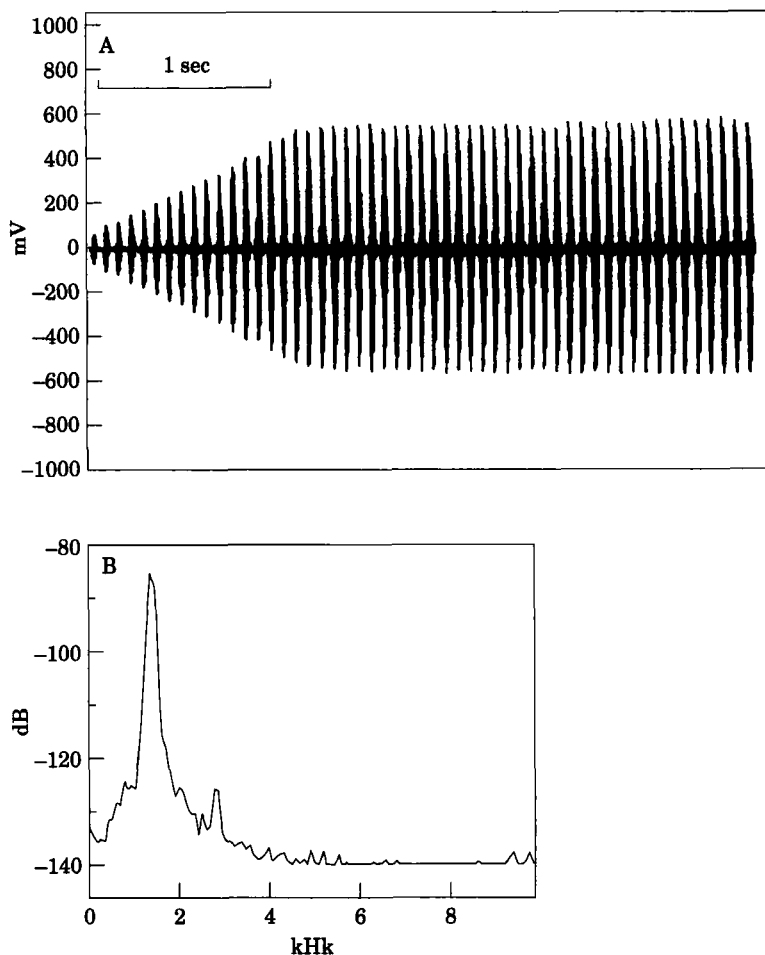


Figure 2. Oscillogram (A) and power-spectrum (B) of the green toad advertisement call. Power spectrum was calculated on a single pulse of the amplitude-stable phase.

(Charif, Mitchell & Clark, 1993) were used for the measurements of temporal and spectral parameters of the calls.

*Spectral parameters.* Because of the simple, tonic structure of the green toad's advertisement call, only fundamental frequency was considered. It was measured directly from the wave form as the inverse of the pulse average wave period ( $T$ ) obtained by dividing the pulse duration by the number of wave periods. This measurement is equivalent to that obtained from the averaged spectrum of single pulses.

*Temporal parameters.* Temporal parameters were collected directly on the wave form (oscillogram or time-wave) (Fig. 2). Call was defined as the entire sequence of pulses uttered at regular frequency (pulse-rate). Intercall was considered as the gap of time between two successive calls. A typical call shows an initial fast rise of amplitude, which we named *opening phase*, followed by an amplitude stable part (*stable phase*). Call duration, intercall duration and number of pulses per call were measured in

all the recorded calls, while pulse and inter-pulse durations were measured in a subsample of six pulses/interpulses per call, three of which were from the *opening phase* and three from the *stable phase* of the call.

The pulse-rate, defined as the number of pulses per second (Hz), was calculated by dividing the number of pulses per call by its duration. Moreover we considered the ratio between interpulse and pulse durations (interpulse-pulse ratio) and the ratio between the *opening phase* and the *stable phase* durations (*opening phase/stable phase* ratio).

*Temperature correction of call temporal parameters.* Since in Anurans the body temperature of vocalizing males significantly affects temporal structure of their calls (reviewed in Ryan, 1986, 1988; Gerhardt, 1994b), acoustical parameters were adjusted to the same body temperature by means of the within-group regression coefficients.

## RESULTS

With the exception of one diploid population (Kopa), all specimens from Kazakh populations had a tetraploid karyotype ( $2n=4x=44$ ) (see Dujsebayaeva *et al.*, 1997). One out of four populations from Kyrgyzstan consisted only of tetraploids (Issik-kul) and two had only diploids (Tulek and Kok-jar I), whilst at Kok-jar III diploid, tetraploid and triploid specimens were found. Since Kok-jar I and III were located in the same valley, a few kilometers from each other, even if at different altitudes (Kok-jar III is about 300 m higher than Kok-jar I), we have considered this region as an area of sympatry between diploid and tetraploid green toads, whereas all the other populations have been considered as coming from allopatric areas.

### *Morphometrical comparison between diploid and tetraploid green toads*

A sample of 158 adult male green toads was collected in the 1994 and 1995 expeditions. Since the main purpose of this paper is to analyse differences between diploid and tetraploid specimens, all the animals have been grouped according to their karyotype, but independently of the locality they came from. In Appendix 2 mean values and standard deviations of all the morphometric parameters are shown.

Table 1 shows the first MGPCA eigenvector coefficients, whose homogeneity in sign and magnitude supports the choice of this component as a multivariate expression of body size (because of the negative signs of the coefficients the body-size factor was defined as the first MGPCA component rotated 180°). As previously shown by univariate analysis, diploids have larger body size than tetraploids ( $F=167.537$ ;  $df=1, 138$ ;  $P<0.001$ ). Figure 3 shows the frequency distribution of body size factor of both taxa.

In order to analyse differences in body shape all the morphometric characters were adjusted by regressing out body size effects. The comparison between diploid and tetraploid males permits examination of significant differences in many of the size adjusted body proportion characters. Tetraploids have smaller mean values in all the size adjusted head characters, the difference is significant in the nostril-to-tip of the snout distance, NOSTIP<sup>adj</sup> ( $F=9.959$ ;  $df=1, 138$ ;  $P=0.003$ ), in the distance between the eyes and the nostrils, NOSEYE<sup>adj</sup> ( $F=4.294$ ;  $df=1, 138$ ;  $P=0.044$ ), in

TABLE 1. Coefficients of the first Multiple Group Principal Component (MGPC) eigenvector. The body size factor is obtained after a 180° rotation of this component

Body proportion characters	First eigenvector
Snout vent length (SVL)	-0.185
Length of the head (LHEAD)	-0.154
Width of the head (WHEAD)	-0.178
Inter nostril distance (INTNOS)	-0.167
Nostril tip of the snout distance (NOSTIP)	-0.169
Eye to nostril distance (NOSEYE)	-0.127
Eye to tympanum distance (EYETYM)	-0.203
Diameter of the eye (DEYE)	-0.162
Diameter of the tympanum (DTYM)	-0.211
Length of parotoids (LPAR)	-0.247
Elbow to elbow distance (WGRASP)	-0.187
Radioulna length (RADUL)	-0.190
Length of the hand (LHAND)	-0.162
Length of the 1st finger (L1FING)	-0.156
Length of the femur (LFEM)	-0.186
Length of the tibia (LTIB)	-0.173
Length of the tars (LTARS)	-0.177
Length of the foot (LFOOT)	-0.166
Length of the web (WEB)	-0.183
Length of the metatarsal tubercle (LMET)	-0.205
% of variance explained	75.82

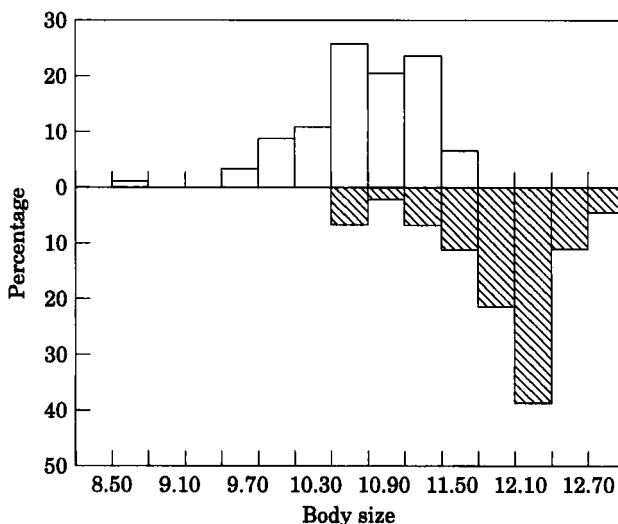


Figure 3. Frequency distribution of the body size factor in diploid (▨) and tetraploid (□) green toads. The body size is the first MGPCA component rotated to 180° (see Results).

the distance between the eyes and the tympanums,  $EYETYM^{adj}$  ( $F=6.642$ ;  $df=1$ , 138;  $P=0.011$ ) and particularly in the length of the parotoid glands,  $LPAR^{adj}$  ( $F=27.934$ ;  $df=1$ , 137;  $P<0.001$ ). The size-adjusted limb characters show even more relevant differences between groups: tetraploids have significantly higher value both in the forelimb characters ( $WGRASP^{adj}$ :  $F=7.732$ ;  $df=1$ , 138;  $P=0.006$ ) and in the



TABLE 2. Canonical loadings of the discriminant function on the morphometrical characters between diploid and tetraploid toads

Body production characters	Canonical loadings
Snout vent length (SVL)	0.377
Length of the head (LHEAD)	0.407
Inter nostril distance (INTNOS)	0.256
Nostril tip of the snout distance (NOSTIP)	0.445
Eye to nostril distance (NOSEYE)	0.411
Length of the parotoids (LPAR)	0.571
Elbow to elbow distance (WGRASP)	0.336
Radioulna length (RADUL)	0.351
Length of the femur (LFEM)	0.391
Length of the tibia (LTIB)	0.249
Length of the tars (LTARS)	0.241
Length of the foot (LFOOT)	0.224
Length of the web (WEB)	0.244
Length of the metatarsal tubercle (LMET)	0.349
Canonical correlation	0.940

hindlimb characters (LFEM<sup>adj</sup>:  $F=11.629$ ;  $df=1, 138$ ;  $P<0.001$ /LTIB<sup>adj</sup>:  $F=20.923$ ;  $df=1, 138$ ;  $P<0.001$ /LTARS<sup>adj</sup>:  $F=18.385$ ;  $df=1, 138$ ;  $P<0.001$ /LFOOT<sup>adj</sup>:  $F=33.157$ ;  $df=1, 138$ ;  $P<0.001$ /WEB<sup>adj</sup>:  $F=15.457$ ;  $df=1, 138$ ;  $P<0.001$ ).

Results of the discriminant function analysis confirm those obtained by univariate and bivariate analyses. Highly significant differences are found in morphometrical characters between diploid and tetraploid toads (Wilks'  $\Lambda=0.117$ ;  $df=14, 124$ ;  $F=66.903$ ;  $P<0.001$ ). Moreover, the calculation of the Mahalanobis distances on the discriminant function allows us to assign correctly all specimens to their own karyological group. All canonical loadings of the discriminant function (Table 2) show a positive sign and those referred to the head proportion characters have values higher than those referred to the limb proportion characters. It is possible to conclude, therefore, that the best discrimination between diploid and tetraploid toads is obtained by considering both their body size and the relative dimensions of their head.

#### *Bioacoustical comparison between diploid and tetraploid green toads*

##### *Temporal parameters*

We recorded the advertisement call of 51 diploid males and 91 tetraploid males. The body temperature of calling males ranges from 10°C to 28°C in diploids and from 10°C to 26°C in tetraploids. Mean values of temporal and spectral parameters of the calls are shown in Appendix 3, Pearson's  $r$ -correlation coefficients between acoustical characters are reported in Table 3. Pulse duration is shorter in the opening phase of the call than in the following stable phase (paired  $t$ -test = 10.131;  $df=135$ ;  $P<0.001$ ), while the inter-pulse duration shows an opposite trend (paired  $t$ -test = 6.799;  $df=135$ ;  $P<0.001$ ). Fundamental frequencies are about 55 Hz lower in the opening phase with respect to those calculated in the stable phase (paired  $t$ -test = 21.288;  $df=134$ ;  $P<0.001$ ). Because of the high correlation between acoustical

TABLE 3. Pearson's  $r$  correlation coefficients among the acoustic properties of the green toad advertisement calls

	Opening phase					Stable phase						
	Call duration (sec)	Pulse-rate (Hz)	Intercall duration (sec)	Opening - stable phase ratio	Duration (sec)	Interpulse duration (msec)	Pulse duration (msec)	Fundamental frequency (kHz)	Duration (sec)	Interpulse duration (msec)	Pulse duration (msec)	Fundamental frequency (kHz)
Pulse-rate (Hz)	-0.42											
Intercall duration (sec)	0.20	0.13										
Opening-Stable phase ratio	-0.25	-0.14	-0.04									
OPENING PHASE												
Duration (sec)	0.45	-0.46	0.05	0.55								
Interpulse duration (msec)	0.42	-0.88	-0.08	0.12	0.42							
Pulse duration (msec)	0.45	-0.86	-0.14	0.14	0.45	0.80						
Fundamental frequency (kHz)	-0.12	-0.43	-0.22	0.04	0.06	0.30	0.19					
STABLE PHASE												
Duration (sec)	0.83	-0.35	0.10	-0.48	0.33	0.34	0.33	-0.02				
Interpulse duration (msec)	0.42	-0.89	-0.10	0.12	0.43	0.97	0.84	0.29	0.35			
Pulse duration (msec)	0.45	-0.89	-0.11	0.20	0.51	0.87	0.95	0.19	0.31	0.87		
Fundamental frequency (kHz)	-0.12	-0.42	-0.20	0.06	0.07	0.27	0.17	0.99	-0.03	0.27	0.19	
Pulse-interpulse ratio	-0.07	0.43	0.04	-0.03	-0.11	-0.50	-0.17	-0.34	-0.11	-0.56	-0.16	-0.32

parameters in the opening and stable phases of the call only the parameters involved in the amplitude stable phase have been considered in subsequent analyses.

In order to analyse the effect of body size and body temperature on temporal call parameters multiple linear regression analyses were carried out on both diploid and tetraploid samples. Partial regression coefficients of SVL and body temperature and their significance levels are shown in Table 4. The analysis of tetraploid and diploid samples provides qualitatively similar results. With the exception of the intercall duration, body temperature strongly affects temporal structure of all the other parameters of the call. An increase in body temperature is associated with a decrease in call, pulse and inter-pulse duration and with an increase in pulse-rate. On the contrary, body size has only marginal effects on the call temporal structure: in both taxa larger males at similar body temperature tend to produce calls with slightly higher pulse-rates.

Covariance analyses were carried out to compare the between-taxon temperature-acoustical parameters relationships. Diploid and tetraploid call-duration/body-temperature linear relationships differ in the intercept ( $N=108$ ;  $F=7.964$ ;  $P=0.006$ ) but not in the slope ( $N=108$ ;  $F=0.371$ ;  $P=0.544$ ) (Fig. 4A). On the contrary, the linear relationship between pulse-rate and body temperature in tetraploids strongly differ from that calculated for diploids both in the slope ( $N=108$ ;  $F=56.906$ ;  $P<0.001$ ) and in the intercept ( $N=108$ ;  $F=321.365$ ;  $P<0.001$ ) (Fig. 4B). The two lines cross at the body temperature of about 8°C and diverge with diploids exhibiting the highest pulse-rate values.

To investigate further call differences between diploid and tetraploid green toads, call temporal parameters were adjusted at three different body temperatures (10°C; 15°C and 25°C) by using the within group regression parameters. Diploids at 10°C produce vocalizations that are, on average, 15.2% longer and with a 20.5% higher pulse-rate than those emitted by tetraploids. At 15°C and at 25°C the difference in call duration is proportionally the same, whereas pulse-rate differences proportionally increase in high thermic conditions. At 15°C diploid calls show pulse-rates 42% higher than those of tetraploids and 61% at 25°C.

Discriminant function analyses of the temporal parameters adjusted at the three thermic conditions were carried out. Even though the canonical functions always provide highly significant discrimination at the adjusted temperature of 10°C (Wilks'  $\lambda=0.490$ ;  $df=5, 102$ ;  $P<0.001$ ), 15°C (Wilks'  $\lambda=0.240$ ;  $df=5, 102$ ;  $P<0.001$ ) and 25°C (Wilks'  $\lambda=0.072$ ;  $df=5, 102$ ;  $P<0.001$ ), their classification efficiency improves with increasing body temperature. At 10°C, 86% of the calls were correctly attributed to their own group, at 15°C the percentage of correct classifications was 95% and reached a peak of 99% at 25°C. Moreover, as shown by the canonical loadings reported in Table 5, at different temperatures the adjusted temporal parameters play different roles in discrimination. At 25°C canonical function is determined by pulse-rate only, whereas at 10°C it is the expression of both call duration and pulse-rate.

### *Spectral parameters*

Table 4 shows partial regression coefficients of SVL and body temperature on call fundamental frequency. In contrast to what has been previously observed for temporal parameters, the fundamental frequency is strongly influenced by body size (Fig. 5) but not by body temperature (in tetraploids the fundamental frequency is

TABLE 4. Results of multiple regression analyses between call parameters and body size and temperature in diploids and tetraploid toads. Partial regression coefficients and their significance levels are shown. (O = opening phase; S = stable phase)

Dependent variables	Independent variables					
	Diploids			Tetraploids		
	SVL	P	Temperature	SVL	P	Temperature
	b		b	b		b
Call duration (sec)	0.033	0.216	-0.240	-0.026	0.078	-0.182
Pulse-rate (Hz)	0.201	0.001	1.682	0.122	0.000	0.926
Intercall duration (sec)	0.106	0.287	0.072	-0.031	0.831	-0.068
Opening phase (sec)	0.004	0.561	-0.052	0.003	0.718	-0.039
Stable phase (sec)	0.027	0.317	-0.198	0.017	0.362	-0.133
Interpulse duration [O] (msec)	-0.280	0.014	-1.576	-0.287	0.006	-1.995
Interpulse duration [S] (msec)	-0.180	0.053	-1.445	-0.224	0.013	-1.925
Pulse duration [O] (msec)	0.061	0.391	-1.161	-0.022	0.765	-1.155
Pulse duration [S] (msec)	-0.029	0.624	-1.296	-0.067	0.389	-1.274
Fundamental frequency [O] (kHz)	-0.006	0.000	-0.002	-0.013	0.000	0.011
Fundamental frequency [S] (kHz)	-0.006	0.000	-0.001	-0.013	0.000	0.011

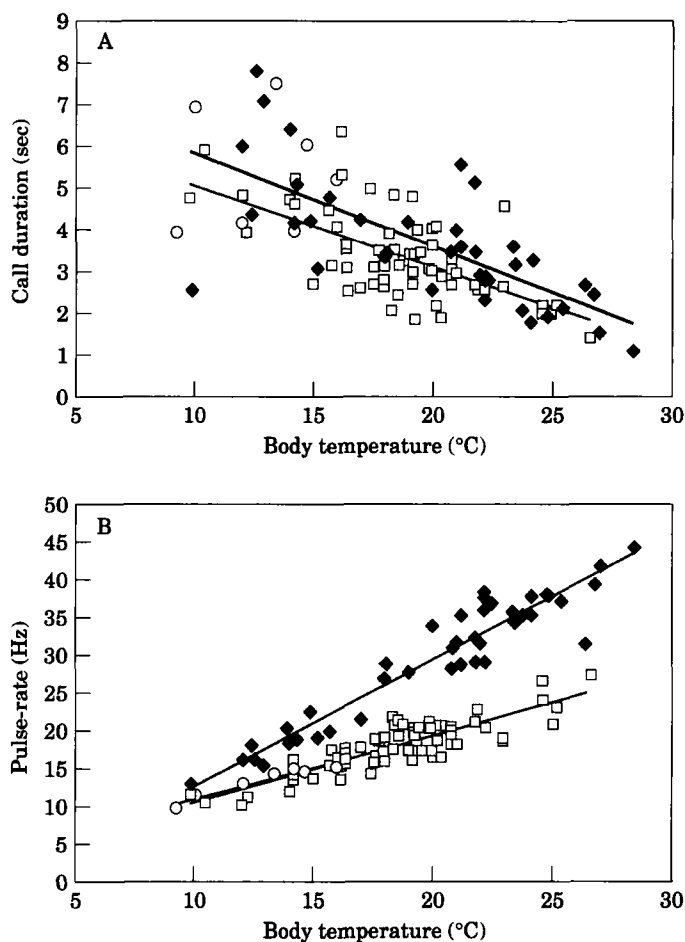


Figure 4. Analysis of the linear relationships between temporal properties of the call and body temperature of calling males in diploids (◆), tetraploids (□) and triploids (○). A, call duration. B, pulse-rate.

TABLE 5. Canonical loadings of the discriminant functions between diploid and tetraploid toads on the temporal acoustic properties of their calls. The analyses were carried out by adjusting the acoustic properties at three different thermic conditions, respectively at 10, 15 and 25°C

	(10°C)	Canonical loadings (15°C)	(25°C)
Call duration	-0.408	-0.200	-0.052
Pulse-rate	-0.488	-0.560	-0.912
Intercall duration	-0.075	-0.061	-0.050
Opening-stable phase ratio	0.180	0.102	0.024
Pulse-interpulse ratio	-0.018	-0.018	-0.022

only marginally correlated with temperature). Covariance analysis shows significant differences in slope between *taxa* frequency-SVL linear relationships ( $F=5.452$ ;  $P=0.022$ ).

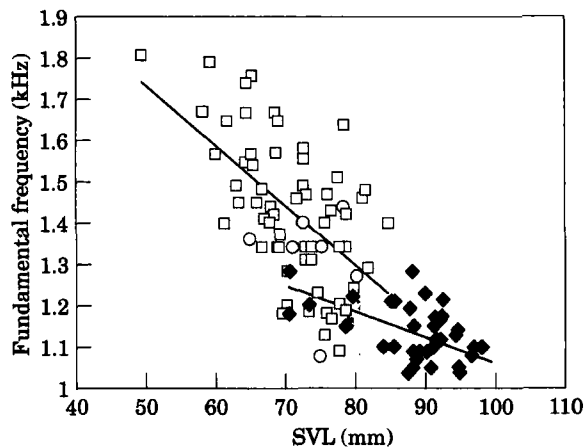


Figure 5. Analysis of the linear relationships between call fundamental frequency and the body length (SVL) of diploid (◆), tetraploid (□) and triploid (○) males.

### *Triploid males*

In 1994 and 1995 we collected a sample of 20 males from the breeding population of Kok-jar III in Kyrgyzstan. Most of the specimens (16) had a triploid karyotype ( $2n = 3x = 33$ ), three were tetraploid and two diploid. Triploids had normally developed secondary sexual characters and the advertisement call of seven of them was recorded.

### *Morphometrical analysis*

Univariate analyses shows significant differences between diploid and triploid males for all the body proportion characters and body weight (ANOVA:  $P < 0.001$ ), whereas triploids significantly differ from tetraploids only in the nostril-snout tip distance (NOSTIP) ( $F = 6.731$ ;  $df = 1, 107$ ;  $P = 0.001$ ), in the first finger length (L1FIN) ( $F = 5.310$ ;  $df = 1, 107$ ;  $P = 0.023$ ) and in the metatarsal tubercle length (LMET) ( $F = 5.055$ ;  $df = 1, 107$ ;  $P = 0.027$ ). The mean value of the multivariate body size factor of triploid males is 11.051 (SD = 0.267), significantly lower than that calculated for diploid males ( $F = 55.455$ ;  $P < 0.001$ ), but not significantly different from the average body size factor of tetraploid males ( $F = 1.993$ ;  $P = 0.161$ ).

Discriminant function analysis provides results consistent with those obtained by univariate analyses. The morphometrical comparison between diploid and triploid specimens shows highly significant differences (Wilks'  $\Lambda = 0.093$ ;  $F = 17.103$ ;  $df = 21, 37$ ;  $P < 0.001$ ). When Mahalanobis distances are calculated on the discriminant function to allocate each specimen to its closest group, all the animals are correctly attributed to their own *a priori* group.

Significant differences are also shown by discriminant function analysis applied to the triploid and tetraploid samples (Wilks'  $\Lambda = 0.695$ ;  $F = 2.253$ ;  $df = 20, 87$ ;  $P = 0.005$ ). In this case, however, the proportion of correct classifications, by means of the calculation of Mahalanobis distances, is significantly reduced: 17.2% of tetraploid toads and 25% of triploid toads are erroneously classified. Moreover, all the three tetraploid males from Kok-jar are classified as triploids. We conclude, therefore,

TABLE 6. Comparison of green toad advertisement calls between diploid–tetraploid sympatric and allopatric populations. All the temporal properties have been adjusted at the body temperature of 19°C, whereas the fundamental frequency has been adjusted at the SVL of 75 mm

	Allopatric				Sympatric			
	Diploids		Tetraploids		Diploids		Tetraploids	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Call duration (sec)	3.817	0.775	3.296	0.806	3.844	1.594	3.924	1.441
Pulse-rate (Hz)	27.994	2.931	18.293	1.754	26.965	1.551	18.163	1.191
Frequency (kHz)	1.221	0.067	1.344	0.053	1.208	0.143	1.282	0.104

that in the comparison between triploid and tetraploid specimens the morphometrical differences outlined by the discriminant function should be interpreted as geographic variation rather than as differences between karyotypes.

#### *Bioacoustical analysis*

We recorded the advertisement call of seven triploid males, whose body temperature ranged from 9°C to 15°C. Despite the small number of individuals a statistically significant linear relationship is found between pulse-rate and body temperature ( $R=0.965$ ;  $P<0.001$ ) (Fig. 4B). This linear relationship significantly differs from that observed in diploid advertisement calls ( $F=12.481$ ;  $P<0.001$ ), whereas no significant differences are found when the pulse-rate of triploids is compared with that typical of tetraploids ( $F=0.619$ ;  $P=0.433$ ).

Because of the small range of body size, no significant correlation between fundamental frequency and body length (SVL) is found in triploids ( $R=0.162$ ;  $F=0.135$ ;  $P=0.728$ ). Covariance analysis, however, shows significant differences in the frequency/SVL relationships with respect to diploid calls ( $F=6.508$ ;  $P=0.015$ ), but not with respect to tetraploid calls ( $F=1.459$ ;  $P=0.232$ ).

Both morphometrical and acoustical analyses concur that, despite the different chromosomes number, triploid and tetraploid males form a phenotypically homogeneous group clearly distinguished from that formed by diploid males.

#### *Acoustical differences among sympatric and allopatric populations*

In order to test the reproductive character displacement hypothesis on the differences between diploid and tetraploid advertisement calls a two-way analysis of variance was carried out. Diploid and tetraploid males of the two sympatric (one syntopic) populations of Kok-jar were distinguished from their 'conspecifics' from allopatric populations. Since no significant differences were found between tetraploid and triploid advertisement calls (see previous paragraph), triploid calls were analysed together with tetraploid calls. Three acoustical parameters were considered (Table 6): call duration, pulse-rate (both adjusted to a constant body temperature of 19°C) and fundamental frequency (adjusted to the average SVL of 75 mm). As previously shown, call duration ( $F=3.988$ ;  $P=0.048$ ), pulse-rate ( $F=562.25$ ;  $P<0.001$ ) and fundamental frequency ( $F=21.576$ ;  $P<0.001$ ) differ significantly between karyotypes, whereas no significant effect related to the sympatric *versus* allopatric condition is found for any of these parameters.

TABLE 7. Mean values and standard deviations of the temporal properties of the call in the three groups of green toads. All parameters have been adjusted at the body temperature of 15°C

	Italian diploids			Asian diploids			Asian tetraploids		
	N	mean	SD	N	mean	SD	N	mean	SD
Call duration (sec)	75	4.69	0.73	41	4.71	1.06	67	4.08	0.79
Pulse rate (Hz)	75	16.91	1.86	41	21.01	2.63	67	14.83	1.75
Intercall duration (sec)	71	14.38	8.53	41	9.50	3.93	67	8.15	6.88
Opening-stable ratio	74	0.40	0.13	41	0.37	0.19	67	0.51	0.28
Pulse-interpulse ratio	74	0.82	0.14	41	0.97	0.31	67	0.85	0.14

### *Acoustical comparison between Asian and Italian green toads*

In order to investigate advertisement call relationships among green toad populations over a broader geographic scale, diploid and tetraploid calls were compared with those recorded in a diploid population of *B. viridis viridis* from the westernmost part of its range (Pellice, North-West Italy). Seventy-five advertisement calls were analysed. Male body temperatures ranged from 13 to 23°C. When pulse-rate was regressed against body temperature and SVL a highly significant linear relationship was found for body temperature ( $b=0.874$ ;  $P<0.001$ ) but not for SVL ( $b=0.034$ ;  $P=0.627$ ). Similar results were obtained when the call duration was assumed as dependent variable (body temperature:  $b=-0.235$ ;  $P<0.001$ ; SVL:  $b=0.015$ ;  $P=0.581$ ). Neither the opening-stable phase ratio nor the interpulse-pulse ratio were significantly correlated with body temperature or SVL. Fundamental frequency was highly and negatively correlated with SVL ( $b=-0.011$ ;  $P=0.001$ ), but not with body temperature ( $b=0.009$ ;  $P=0.107$ ). To compare Asian diploid, tetraploid and European diploid green toad advertisement calls the temporal parameters (pulse-rate, call duration, opening-stable phase ratio and inter pulse-pulse ratio) were adjusted for the same body temperature of 15°C (about the mean value of body temperature of the total sample) using the within-group regression parameters. Mean values of the adjusted acoustical variables are shown in Table 7. One-way ANOVA shows between-group significant differences for all of them ( $P<0.01$ ). Canonical variate analysis confirms the results obtained from univariate tests (Wilk's  $\Lambda=0.270$ ;  $F=31.819$ ;  $df=10, 344$ ;  $P<0.001$ ). Figure 6 plots the animals onto the plane described by the two canonical functions: European green toads are placed between tetraploid and diploid specimens indicating that the differences in calls between Asian diploid and tetraploid toads are more accentuated with respect to those observed between each and the European toads.

### DISCUSSION

Asian diploid and tetraploid male green toads significantly differ both in their body proportion characters and in a number of parameters derived from their advertisement calls. With respect to tetraploids, diploids have larger body size, proportionally larger parotoid glands, and proportionally shorter limbs. Further, diploid advertisement calls show longer call duration, higher pulse-rate and lower fundamental frequency than tetraploid calls. Although strong associations are found



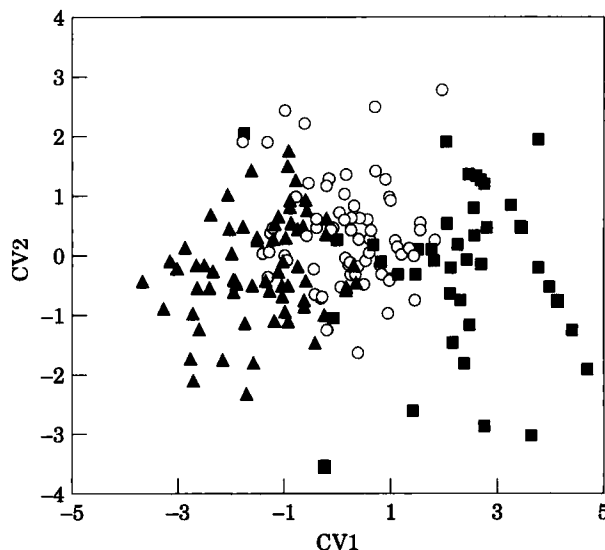


Figure 6. Canonical Variate Analysis on the temporal acoustic properties of the Asian diploid (■), Asian tetraploid (▲) and Italian diploid (○) green toad advertisement calls. Calls are described by the first and second canonical functions of CVA.

between some temporal and spectral call parameters and body size of the calling male, our analyses demonstrate that the observed differences in advertisement calls cannot be explained as the by-product of body size differentiation only.

In anurans both advertisement calls and body size are important factors that favour mating between individuals of the same species. As in many other species of toads and frogs (review in Gerhardt, 1988, 1994b; Ryan & Keddy-Hector, 1992), green toad gravid females are attracted by conspecific male vocalizations (Giacoma, Zugolaro & Beani, 1997; Castellano & Giacoma, in press). Playback experiments carried out on this species complex (Castellano & Giacoma, in press) have discovered a high discriminative capability of females with respect to many of the temporal and spectral parameters of the advertisement call. Since body size significantly influences the spectral parameters of the call, it may indirectly affect the phonotactic response of females. Berven (1982) found that in *Rana sylvatica* large males from high altitude populations avoid mating with proportionally smaller females from low altitude populations. Moreover, in those species where males fight to achieve and defend mating (scramble competition), body size may influence the outcome of struggles favouring syngamy through size assortative mating (Davies & Halliday, 1979).

On the basis of these considerations it is plausible to assume that the observed differences in size and in advertisement call between diploids and tetraploids can reduce the risk of hybridization in the areas of sympatry. Should these differences be seen as the result of selective pressures that directly favour diversification and the appearance of an effective pre-mating isolating mechanism, or should they rather be interpreted as a by-product of different evolutionary processes?

In order to answer these questions particular attention has been given to the analysis of advertisement calls. Two categories of explicative hypothesis are formulated and

discussed. First we consider the hypothesis that between-taxon differences are the direct consequence of the polyploidization event. Second, we discuss the hypotheses that explain the differences as the result of an evolutionary process influenced by one of the following evolutionary forces: (a) genetic drift; (b) environmental selection or (c) divergent selective pressure acting upon sympatric populations to avoid hybridization (reproductive character displacement hypothesis).

### *The direct effect of polyploid speciation*

When polyploid speciation arises through hybridization (allopolyploidy) most phenotypic characters of the new 'species' should be expected to be distinguishable from those of the parental species (Gerhardt, 1994b; Mable & Bogart, 1991). Advertisement calls of the European water frog (*Rana esculenta* complex) are intermediate with respect to the parental species *R. ridibunda* and *R. lessonae* (Brzoska, 1982). In anurans, however, autopolyploidy is thought to be the most common mechanism of polyploid speciation (Tymowska, 1991). In this case differences in phenotypic characters between diploid and polyploid species are less easily inferrable. A deeply studied case of autotetraploid frog is that of *Hyla versicolor* (Ptacek, Gerhardt & Sage, 1993). Although morphologically indistinguishable from diploid *H. chrysoscelis*, *H. versicolor* shows advertisement calls with a significantly lower pulse-rate (Gerhardt, 1978). A similar pattern is shown by tetraploid *Odontophrynus americanus* and its diploid counterpart (Bogart & Wasserman, 1972). Bogart & Wasserman (1972) and Ralin (1977) hypothesized that the reduction of the pulse-rate of polyploid species calls may be due to their larger cell dimensions or bigger tissue mass, a direct consequence of the doubled number of chromosomes. Laboratory produced autopolyploid specimens of *Hyla japonica* provide further evidences for a direct influence of the degree of ploidy on the pulse-rate of the advertisement call (Ueda, 1993).

Although not yet conclusive, most of the karyological, biochemical and genetical evidence supports the hypothesis of an autopolyploid origin of tetraploid green toads (Borkin *et al.*, 1986; Odierna *et al.*, 1995; Lattes, Bigoni & Castellano, 1995; Cervella, DelPero & Balletto, 1995). Since tetraploid advertisement calls have a lower pulse-rate than those of both European and Asiatic diploid toads, our results are apparently consistent with the hypothesis of a direct influence of the karyotype on the temporal parameters of the call. The capture of triploid specimens and the recordings of the advertisement calls of some of them make it possible to further analyse the effect of chromosome number on call characteristics. Triploid hybrids between tetraploid and diploid grey treefrogs (Mable & Bogart, 1991) had calls with a pulse-rate not significantly different from that of tetraploid calls but with a dominant frequency more similar to that of diploids. On the contrary, triploid green toads produce calls that differ neither in pulse-rate nor in frequency components from those of tetraploid calls.

If on the one hand the evidence begs the question about the origins of triploid toads (Odierna *et al.*, 1995; Cervella, DelPero & Balletto, 1995), it also suggests that the ploidy level by itself does not seem to affect conspicuously the call parameters. Our results, however, cannot exclude the hypothesis suggested by Gerhardt (1994b) according to which the increase in number of chromosomes may result in call

differentiation sufficient to serve as the basis for selection for assortative mating by ploidy, even if the ploidy change does not generate call differences of the magnitude observed at present.

### *The process of differentiation*

As suggested by Roth & Ráb (1986) and confirmed by the karyological study carried out on the specimens treated in the present work (Odierna *et al.*, 1995), polyploidy is a relatively ancient event. In fact, an almost complete diploidization is observed both in the meiotic (22 bivalents in meiotic cells) and in the somatic line (only two NOR bearing chromosomes are observed during the mitotic phases). The *sine qua non* condition for an historical explanation of the differences between diploid and tetraploid advertisement calls is therefore satisfied.

Asiatic calls were compared with those recorded in a diploid population of European green toads to test the hypothesis that between-taxa differences in advertisement calls are the by-product of a random process of genetic differentiation due to the interruption of the gene flow (*genetic drift hypothesis*). DNA investigations suggest the latter as an outgroup with respect to diploid and tetraploid Asiatic toads (Cervella, DelPero & Balletto, 1995). The more pronounced differences observed within Asiatic taxa relative to those observed between either diploid or tetraploid Asiatic toads and European toads should allow us to reject the genetic drift hypothesis.

The alternative hypothesis considers the morphological and acoustical differences between diploids and tetraploids as the result of selective pressures, due to either reproductive character displacement or different environmental selections. Brown & Wilson (1956) defined character displacement as the accentuation of phenotypic differences between sympatric populations of two closely related species relative to differences between allopatric populations. The authors distinguished between displacement in ecological characters and displacement in the premating isolating mechanisms and termed the latter *reinforcement of reproductive barrier*. Butlin (1987) proposed to restrict the term *reproductive character displacement* to interactions between two distinct species (between which there is not a relevant amount of gene flow) and proposed to employ the term character reinforcement only when it is referred to interactions between incipient taxa. By definition, character displacement can be assumed to play or have played an important role in the evolution of between taxa differences only if they overlap in a significant part of their range. Despite the lack of detailed information on the distribution of both species it seems rather clear that, at least in some regions (i.e. Kyrgyzstan), diploids and tetraploids inhabit different habitats. Diploids are found in lowlands whereas tetraploids prefer mountains habitats. Cases of syntopy are described by Pisanetz (1978a, b) in Turkmenistan and by Golubev (1990) (not supported by karyological analysis) in Eastern Kazakhstan. Only one out of 12 breeding sites visited during our expeditions resulted simultaneously colonized by diploids and tetraploids. Since morphometrical and acoustical differences between sympatric populations were not significantly greater than those observed between allopatric populations the hypothesis of character displacement is not supported. Waage (1979) argued that character displacement of signals structure need not to occur if the signals are discriminably different when sympatry is re-established and Gerhardt (1994b) found that diploid *Hyla chrysoscelis* females from sympatric populations with tetraploid *H. versicolor* show a significantly

higher selectivity with respect to pulse-rate and call duration than females from remote allopatric populations. In this case character displacement may have involved females selectivity rather than male's advertisement call.

Our data appear not to be consistent with the hypothesis that differences between diploid and tetraploid advertisement calls are the effect of reproductive character displacement. In order to reject this hypothesis, however, we think that further research on female selectivity from sympatric and allopatric population is still needed. Particular attention should be given to sympatric populations of lowland regions. We observed that at higher temperatures differences between diploid and tetraploid acoustic properties of the calls (mostly pulse-rate) are greater. Temperature, by constraining the expression of the acoustic characters, may also constrain the effect of selective pressures that favour differentiation of the characters. For this reason it may be worthwhile to analyse the advertisement calls of sympatric population inhabiting lowland regions where the temperature conditions, during the breeding season, are not as severe as in the mountain regions.

An alternative, but not necessarily exclusive, explanation of the ultimate causes of the differences between diploid and tetraploid advertisement calls is the environmental selection hypothesis, according to which divergence of the acoustic characters is the result of different selective pressures acting upon taxa that inhabit different environments.

It has been hypothesized (Nevo, 1972, 1973) that climate could influence morphometry favouring larger body size in the more arid areas, by indirectly affecting those parameters of the signals that are highly correlated with body size (i.e. dominant frequency). Since the pioneering researches by Morton (1975), much evidence have been found on the role of the environment in favouring different call types in different habitats for enhanced transmission efficiency (reviewed in Gerhardt, 1983). Investigations into the intra-specific geographic variation of the advertisement calls of some North American anurans have supported this hypothesis (Ryan, 1988; Ryan, Cocroft & Wilczynsky, 1990; Ryan & Sullivan, 1989; Ryan & Wilczynsky, 1991).

The known distribution of diploid and tetraploid toads suggests a clear differentiation of habitats. At least in Kazakstan and Kyrgyzstan no diploid populations have been found over 2000 m above sea level. Whereas tetraploid populations are mainly distributed in the mountain regions, sometimes they are also found at low altitudes. Lowland habitats are characterized by small amounts of rainfall, extremely hot summer and extremely low winter temperatures, whereas mountain habitats have a larger amount of rainfall and a less extreme annual temperature range (Walter & Lieth, 1967).

On the basis of the differences in distribution between diploid and tetraploid toads we may suggest that the larger body size and the proportionally shorter limbs of diploids with respect to tetraploids could be seen as the result of selective pressures that favour a reduction of desiccation risks.

Lowland habitats are also characterized by a more accentuated air turbulence arising from stronger temperature gradients or wind. The resulting interference should especially influence the low rate amplitude modulated signals (Wiley & Richards, 1978). The higher pulse-rate of diploid advertisement call relative to the typical tetraploid pulse-rate may be seen, therefore, as an adaptive response to reduce distortion in the propagation of signals in open windy habitats.

In conclusion, our data, although not conclusive, suggest that the differences in

advertisement call should not be interpreted as the direct result of polyploid mutation, as proposed in other diploid-tetraploid species complexes (Bogart & Wasserman, 1972; Gerhardt, 1994b), but should rather be seen as the result of a gradual process of differentiation, principally influenced by selective deterministic forces. The first selective hypothesis we have discussed—reproductive character displacement—is not supported by our data, although we argue that further research in different areas of sympatry is necessary to definitely reject it. The second hypothesis, involving different environmental selection pressures, may plausibly explain both the morphometrical and acoustical differences between tetraploid and diploid toads.

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## APPENDIX 1

## Body proportion characters

1. SVL (Snout-vent length): distance between the tip of the head and the cloaca
3. LHEAD: length of the head
4. WHEAD: width of the head
5. INTNOS: minimum distance between nostrils
6. NOSTIP: distance between nostril and the tip of snout
7. NOSEYE: minimum distance from nostril opening to anterior corner of eye
8. EYETYM: minimum distance from the eye to the tympanum
9. DEYE : Horizontal diameter of the eye
10. DTYM: Vertical diameter of the tympanum
11. LPAR: length of parotoid glands
12. WGRASP: distance between the elbows when animal is kept with humerus at 90° angle with respect to body axis
13. RADUL: length of radioulna
14. LHAND: length of the hand
15. LIFING: length of the first finger of the hand
16. LFEM: length of the femur
17. LTIB: length of the tibia
18. LTARS: length of the tars
19. LFOOT: length of the foot
20. WEB: minimum distance from the distal extremity of the inner metatarsal tubercle to the web between the third and fourth finger
21. LMET: length of metatarsal tubercle

## APPENDIX 2

Mean values and standard deviations of body proportion characters in diploid and tetraploid green toads

	Tetraploids			Diploids		
	N	Mean	SD	N	Mean	SD
Snout-vent length (SVL)	111	71.87	6.29	47	87.58	7.48
Body weight	106	29.69	7.72	43	56.67	13.64
Length of the head (LHEAD)	111	17.12	1.23	47	20.59	1.68
Width of the head (WHEAD)	111	23.45	1.89	47	28.81	2.52
Inter nostril distance (INTNOS)	111	4.18	0.46	47	4.94	0.56
Nostril tip of the snout distance (NOSTIP)	111	4.96	0.44	47	6.23	0.55
Eye to nostril distance (NOSEYE)	111	4.86	0.34	47	5.75	0.34
Eye to tympanum distance (EYETYM)	111	2.32	0.29	47	3.05	0.30
Diameter of the eye (DEYE)	111	6.45	0.50	47	7.81	0.71
Diameter of the tympanum (DTYM)	111	3.40	0.45	47	4.18	0.62
Length of parotoids (LPAR)	111	14.46	1.92	47	21.86	2.48
Elbos to elbow distance (WGRASP)	111	56.05	5.15	47	67.30	5.97
Radioulna length (RADUL)	111	17.07	1.63	47	20.78	1.90
Length of the hand (LHAND)	111	17.36	1.35	47	20.76	1.72
Length of the 1st finger (LIFING)	111	8.30	0.73	47	10.14	0.84
Length of the femur (LFEM)	111	26.83	2.58	47	31.73	2.84
Length of the tibia (LTIB)	111	22.62	2.15	47	25.87	2.30
Length of the tarsus (LTARS)	111	13.93	1.37	47	16.08	1.51
Length of the foot (LFOOT)	111	30.94	2.73	47	34.67	2.90
Length of the web (WEB)	111	18.83	1.74	47	21.58	2.06
Length of the metatarsal tubercle (LMET)	111	3.65	0.45	47	4.61	0.49



## APPENDIX 3

## Means and standard deviations of the advertisement call parameters

Call parameters	Diploids		Tetraploids	
	Mean	sd	Mean	sd
Call duration (sec)	3.539	1.414	3.464	1.149
Pulse-rate (Hz)	29.818	8.095	17.903	3.745
Intercall duration (sec)	9.854	4.010	7.960	6.516
Opening-stable phase ratio	0.375	0.182	0.511	0.306
OPENING PHASE				
Duration (sec)	0.887	0.330	1.209	0.567
Interpulse duration (msec)	16.889	8.274	31.238	9.608
Pulse duration (msec)	17.414	5.616	24.734	5.596
Fundamental frequency (kHz)	1.077	0.075	1.363	0.185
STABLE PHASE				
Duration (sec)	2.700	1.298	2.682	1.158
Interpulse duration (msec)	18.936	7.563	32.529	8.956
Pulse duration (msec)	18.413	6.015	27.442	6.252
Fundamental frequency (kHz)	1.133	0.078	1.418	0.191
Pulse-interpulse ratio	1.032	0.300	0.863	0.149