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Character convergence in advertisement call and mate choice in two genetically distinct water frog hybridogenetic lineages (*Rana kl esculenta*, *Rana kl grafi*)

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Abstract

Patterns of advertisement call were investigated in two genetically distinct water frog lineages (*Rana kl esculenta*, *Rana kl grafi*), which were identified by starch gel electrophoresis – the aim being to determinate the role of vocalization in the hybridogenetic process. Both hybrids displayed major modifications from the basic structure of the *Rana ridibunda* call. In *Rana kl grafi*, the call structure tended to correspond to that of *Rana perezi* in most of the studied parameters (frequency, duration, number of pulses) whereas the call of *Rana kl esculenta* tended to resemble that of *Rana lessonae*. The ascendant hierarchical classification clearly revealed such a convergence toward parental species and accounted for a divergence between hybrids. Changes in call patterns might result from both the expression of the non-*ridibunda* genome and the sexual selective pressure through female mate choice. Only few non-*ridibunda* females exhibited a preference for hybrid calls which, however, allowed some hybridogenetic males to obtain successful mates. Thus, hybridogenesis induced character convergence in courtship signal with the non-*ridibunda* species in hybrid zones. In any case, these changes in the courtship signal favoured the particular hybridogenetic process, which constitutes a quasi-parasitism of the non-*ridibunda* genome.

Key words: Allozymic variation – convergence – hybrid zones – hybridogenesis – *klepton* – mating call – water frogs

Introduction

Studies on the evolutionary biology of water frogs have revealed the coexistence of sexual and asexual forms. The water frog complex in western Palearctic included several species and numerous hybrids resulting from hemiclinal hybridogenetic gametogenesis (Tunner 1974; Uzzell et al. 1980; Berger 1983; Beerli et al. 1994; Dubois and Ohler 1994; Schmidt 1996). In each new generation, the non-*ridibunda* genome is excluded before meiosis and mating with the syntopic parental species restores the diploidy. In France, as well as the presence of different Mendelian species such as *Rana lessonae*, *Rana ridibunda*, *Rana perezi* and the hybridogenetic hybrid *Rana kl esculenta*, recent studies documented the occurrence of another natural hybrid *Rana kl grafi* (Graf et al. 1977; Uzzell and Tunner 1983; Crochet et al. 1995; Pagano et al. 2001). The hybrids exhibited few morphological differences and could only be identified by genetic analysis and electrophoresis (Dubois and Ohler 1994; Crochet et al. 1995; Pagano and Joly 1999; Lodé and Pagano 2000). In spite of their genetic difference, the hybrids behaved identically to *R. ridibunda*, especially for breeding, and performed a real parasitism of *lessonae* and *perezi* genomes that constituted a quasi gene robbery (*klepton*) (Dubois and Günther 1982). Generally, hybridization is regarded as having a restricted evolutionary effect because of the low fertility of hybrids (Milinski 1994). However, the expansion of hybridogenetic water frogs may prove that hybrids are able to found new evolutionary lineages. Because the hybrids are only partially fertile, the success of hybridogenesis chiefly depends on the ability of hybrid males to attract the parental females or of hybrid females preferentially to select males of the parental species.

However, in anurans the mating calls act both as mechanisms of specific pre-zygotic recognition and of sexual selection. Males produce specific calls as an attractive courtship signal (Schneider 1966; Crespo et al. 1989; Ryan and Wilczynski 1991; Schneider and Sinsch 1992) and in addition, other kinds of calls were emitted such as the territorial call (Radwan

and Schneider 1988; Schneider and Steinwarz 1990). Mating call is a determinant for attracting females and the characteristics of acoustic signal are selected through female choice (Gerhardt and Davis 1988; Telford et al. 1989; Gerhardt 1991; Márquez and Bosch 1997). In water frog hybrids such as *R. kl esculenta*, mating calls seem to display an intermediate pattern between the two parental calls (Brzoska 1982). Nevertheless, it would be expected that the calls should only manifest little deviation thus allowing the hybrid to attract the non-*ridibunda* female. The water frog hybrid zone provides conditions in which to study character change on sexual communication. Both hybridization and competition for mate could induce character shift in the hybrid zone. Character displacement is expected when several species come into secondary sympatry and corresponds to an increased difference between two closely related species (Gerhardt 1994; Robinson and Wilson 1994). By contrast, character convergence may favour the hybridization. Because the non-*ridibunda* female actively selected the male call, it could be predicted that the mating calls of hybrids resemble their non-*ridibunda* parental species, either *R. lessonae* or *R. perezi*, increasing their own divergence.

The aim of this work was: (1) to investigate the mating call characteristics in the two *kleptons* *R. kl esculenta* and *R. kl grafi* from genetically determined individuals; and (2) to assess the influence of different mating calls on mate choice in females of non-*ridibunda* parental species (*R. perezi* and *R. lessonae*). Considering that the two hybridogenetic lineages are clearly genetically identified, this study provides new information on the influence of genomes and on attractive characteristics which affect the mating behaviour in Ranidae.

Materials and methods

Water frogs were sampled during the pre-spawning period in June 1997 and June 1998 from six sites (24 *R. lessonae*, 34 *R. kl esculenta*, 20 *R. perezi* and 11 *R. kl grafi*). Frogs were observed at night and calling males were immediately recorded, hand caught and toe clipped. Single

calls were tape-recorded using a microphone (Emu 4535 electret with EM 700 condenser shot gun, 20–22 000 Hz at 8 dB, 600 Ω , sensitivity 3–27 dB, efficacy –69 dB, distortion < 0.3% (EMU-LEM Industries, Fesnes, France) and Digital Audio Tape Corder (DAT-Sony TCD-D8, sampling frequency 44.1 kHz, 20 Hz–20 kHz, 16 bit resolution (Sony, Londerzeel, Belgium)). A sample of six to 11 calls was obtained from each male and results were averaged for each individual. Because exogenous factors could influence call structure (Obert 1975; Kuhn and Schneider 1984), the recordings were performed during similar weather and temperature conditions (water temperature range: 19°0–20°6°C and precipitation < 70 mm/month). The toe was immediately frozen and stored at –22°C. Individuals of *R. perezi*, *R. kl esculenta*, *R. lessonae* and *R. kl grafi* were identified from allozymic variations. The tissues were homogenized and centrifuged. A starch gel electrophoresis using the TC6 buffer system was performed following Hotz's procedure (1983), but considering only allozymic variations at four presumptive gene structure loci that proved to be diagnostic between parental species and hybrids: (*CK-A* 2.7.3.2, *LDH-B* 1.1.1.27, *MPI* 5.3.1.8., *PGM-2* 5.4.2.2 (Graf et al. 1977). Alleles were designated by letters following the usual system (Graf et al. 1977; Hotz 1983).

Oscillograms and spectrograms were obtained using two PC programs of mathematical fast Fourier transforms (resolution 22 Hz, 1024 points) (Spectrogram 4.2 R.S. and Avisoft Saslab pro version, Berlin, Germany). Only calls from recordings of identified males were used. Calls were analysed for eight different acoustic parameters: call duration (ms); dominant frequency of the beginning of the call (Hz); dominant frequency at the end of the call (Hz); maximum dominant frequency (Hz); amplitude between these frequencies (Hz); number of pulses (n); call duration/number of pulses and mean duration of interval between pulses (ms). Additionally, four *R. ridibunda* males were recorded and analysed for comparison. To avoid possible auto-correlation, acoustic parameters were averaged for each individual and Kruskal–Wallis (KW) analysis of variance was calculated. An ascendant hierarchical classification (mean Euclidean distance) was performed to determine overall phenetic similarities among all taxa (Pcsm program (Pcm Software, meylan, France)).

To assess the influence of genomes on mate choice, 24 *R. perezi* females and 24 *R. lessonae* females were individually tested. Play-back tests were performed *in situ* with randomly selected females from the same populations. For most of the acoustic parameters (mean frequency, number of pulses and mean duration), data were obtained from six typical *R. kl esculenta* and *R. lessonae* calls to test *R. lessonae* females and from five *R. kl grafi* and *R. perezi* calls for *R. perezi* females. At night, each female was put in a 18 cm \times 18 cm box with two openings and placed apart at a distance of exactly 2 m between two hidden speakers. As soon as the female came out of the box, she was observed using a red light for a period of 5 min. The two-choice trial was considered positive when the female moved towards and reached one of the speakers. Three tests were conducted per female and the results were averaged.

Results

Electrophoretic determination

Animals from the four studied taxa clearly showed allozymic variation as revealed by the specific protein markers used (Table 1).

In *LDH-B*, the *e* allele characterized the *R. lessonae* western population, and the *d* allele the *R. perezi*, whereas the *a* allele was the *R. ridibunda* allele in the two hybrids. In *PGM-2*, the

c allele made it possible to discriminate *R. lessonae* and *R. esculenta* from the others. In *PMI*, *R. lessonae* was determined by the *h* allele, *R. perezi* by the *l* allele and the *a* allele was the *R. ridibunda* allele in hybrids. The *d* allele in *CK-A* allowed the identification of *R. perezi* but the *a* allele was not specific for *R. lessonae* (Hotz 1983).

Call pattern

In water frogs, the mating call consisted of distinct pulses repeated at regular intervals as revealed by the oscillograms (Fig. 1). The call exhibited a slight increase–decrease in dominant frequency on the spectrogram that was more pronounced in *R. perezi* than in *R. lessonae*.

Call duration varied significantly among groups (KW = 61.3, $p < 0.0001$, Table 2). The call had a low frequency in *R. lessonae* and a high frequency in *R. perezi* whereas the two hybrids produced intermediate frequencies. Although frequencies at the beginning of the call significantly varied among taxa (KW = 54.3, $p < 0.0001$), the mean frequency of *R. kl grafi* call was close to that of the *R. perezi* (mean differences 4.05 Hz) and the *R. kl esculenta* mean frequency was also relatively close to that of the *R. lessonae* (mean difference 190.8 Hz) whereas the two hybrids differed (mean difference 288.5 Hz). The end of the call had a higher frequency but varied significantly among taxa (KW = 22.4, $p < 0.0001$). Call amplitude averaged 309.3 Hz (SD = 122.2 Hz) but differed among taxa (KW = 16.1, $p < 0.002$).

The males produced series of pulses which varied from 6.8 pulses in *R. perezi* to 33 in *R. lessonae* (KW = 67.9, $p < 0.0001$) and the intervals between two pulses differed according to the taxon. (KW = 54.7, $p < 0.0001$). In fact, the ratio duration/number of pulses characterized the specific call (KW = 65.9, $p < 0.0001$). Diverging from *R. ridibunda*'s call structure (see Table 2), this ratio was relatively similar between *R. lessonae* and *R. esculenta* (mean difference 21.28) and between *R. grafi* and *R. perezi* (mean difference 2.46, Fig. 2).

A hierarchical ascendant classification illustrated the tendency among the call patterns. Considering all acoustic parameters, a first cluster associated *R. perezi* and *R. grafi* (mean Euclidean distance $D = 160.6$, Fig. 3) whereas *R. esculenta* was grouped with *R. lessonae* ($D = 526.7$).

Mate choice

All 48 females were moved towards one or the other speaker in less than 5 min and showed a clear preference for a call.

In *R. lessonae*, 70.8% of females exhibited a significant preference for the con-specific male call (goodness of fit $\chi^2 = 4.2$, $p < 0.05$) whereas only 29.2% preferred *R. kl esculenta* call (Fig. 4). On the other hand, no significant preference was found in *R. perezi* ($\chi^2 = 0.67$, $p > 0.05$) although 58.3% of females moved towards the con-specific call whereas only 41.7% moved towards the hybrid call.

Discussion

In water frogs, the hybridization process was widely favoured by modifications in the courtship call patterns. Both hybrids exhibited patterns that were rather similar to call characteristics described in *R. ridibunda* by Schneider and Joermann (1988) and Schneider and Sinsch (1992). Nonetheless, the calls of each hybrid type resembled the call patterns of their syntopic parental species. The call in *R. kl grafi* resembled that

Table 1. Electrophoretic alleles found in water frogs from western France by starch gel electrophoresis

	<i>CK-A</i>	<i>LDH-B</i>	<i>PMI</i>	<i>PGM-2</i>
<i>R. lessonae</i>	<i>a, b</i>	<i>e, b</i>	<i>h</i>	<i>c</i>
<i>R. kl esculenta</i>	<i>a, b</i>	<i>ae</i> or <i>ec</i>	<i>ah</i>	<i>d</i>
<i>R. perezi</i>	<i>d</i>	<i>i</i> or <i>d</i>	<i>l</i>	<i>d</i>
<i>R. kl grafi</i>	<i>a, d</i>	<i>ad</i> or <i>cd</i>	<i>al</i>	<i>d</i>

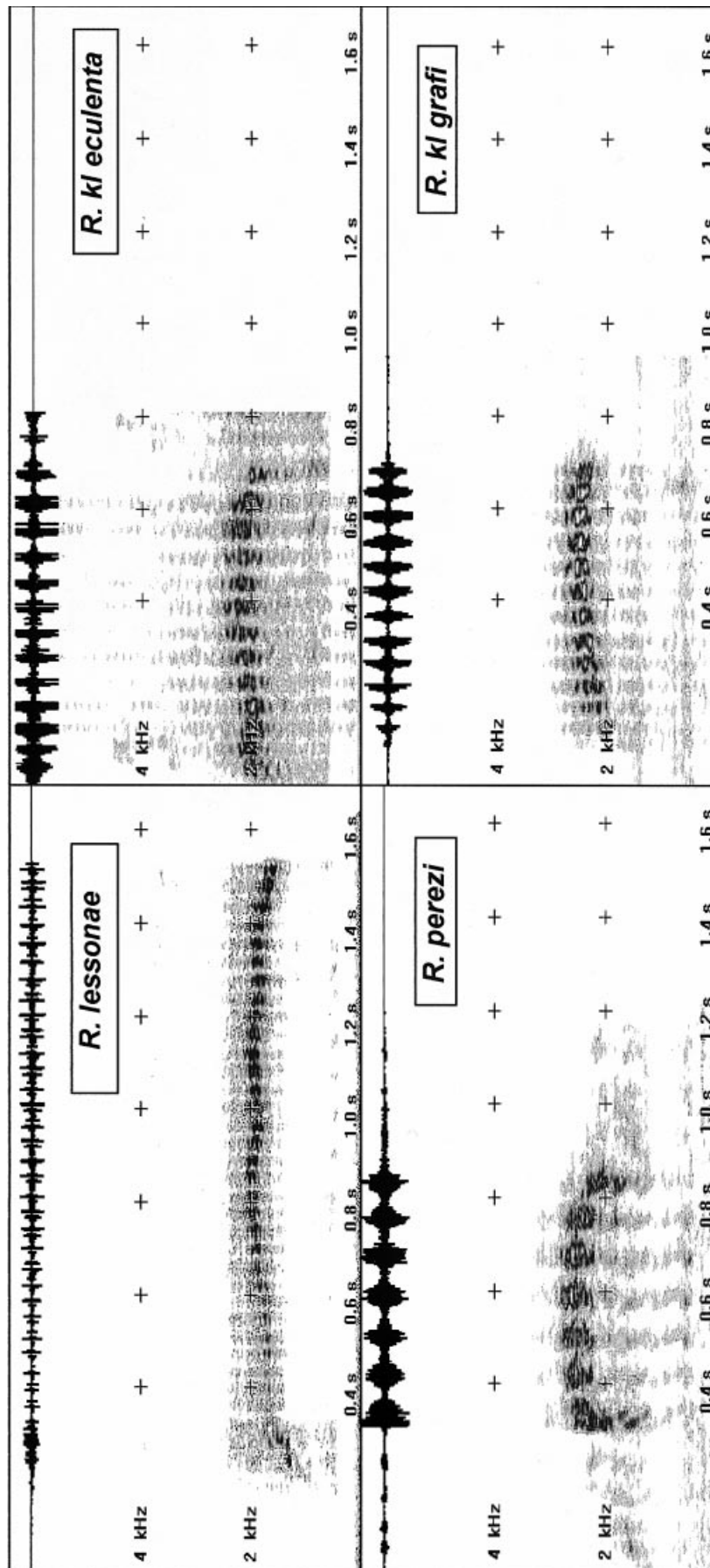


Fig. 1. Courtship call patterns of two Mendelian species *Rana lessonae* and *Rana perezi* and two hybridogenetic water frogs *Rana kl esculenta* and *Rana kl grafi* from France

Table 2. Mean characteristics of acoustic parameters studied in water frogs at 19–20.6°C

	Dur	FreqB	FreqE	Max	Ampl	Npul	Ratio	Interv	n
<i>R. lessonae</i>	1121.29	1601.6522	1723.8696	1943.8696	342.22	33	33.9013	7.4	24
SD	253.077	191.2175	170.0771	100.1741	176.46	7.3188	1.72202	0.9	
<i>R. kl esculenta</i>	880.18	1792.5	1926.9706	2129.2647	329.07	15.941	55.1774	19.2	34
SD	183.573	89.01592	88.04871	109.585	85.159	2.9638	4.60756	5.3	
<i>R. kl grafi</i>	533.55	2081	2021.2727	2379.3636	298.36	8.8182	61.7337	26.9	11
SD	125.921	221.4753	358.8263	298.7368	120.3	3.5637	7.61245	6.5	
<i>R. perezi</i>	429.95	2085.05	1941.35	2315.75	230.7	6.55	65.739	24.2	20
SD	81.3598	217.4027	146.0614	184.3986	65.936	1.099	7.57152	4.2	
<i>R. ridibunda</i>	1162.5	1712	1906	2509	797	8	145.31	68.5	4
SD	14.85	304.05	60.81	57.98	246.1	0	1.86	9.9	

Dur, call duration (ms); FreqB: frequency at the call beginning (Hz); FreqE, frequency at the call end (Hz); Max, maximum frequency (Hz); Ampl, amplitude (Hz); Npul, number of pulses; Ratio, call duration/number of pulses; Interv, duration of intervals (ms); SD, standard deviation.

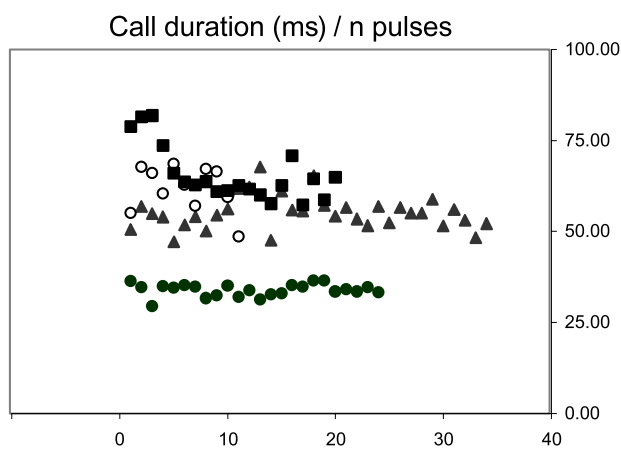


Fig. 2. Distribution of the ratio call duration/number of pulses considering the different Mendelian species and the two kleptons of water frog from western France. ●, *Rana lessonae*; ▲, *Rana 'esculenta'*; ○, *Rana 'grafi'*; ■, *Rana perezi*

of *R. perezi* either by the duration of emissions, the number of pulses or by the frequency, whereas the call of *R. kl esculenta* displayed a longer emission with numerous pulses at a low frequency and was more similar to that of *R. lessonae*.

A courtship signal could be selected through the female mate choice (Ryan and Keddy-Hector 1992; Bush and Bell 1997) and *Alytes obstetricans* females showed a strong preference for larger males (Márquez 1995). These noticeable patterns in the mating calls of water frogs might result from such a sexual selection mechanism. Different selection constraints may also influence phenotypic variances. Nevertheless, the non-*ridibunda* part of the hybrid genome should facilitate the expression of these characteristics because the traits could not be inherited

additively. Although speciation through hybridization was regarded as a common phenomenon in plants, hybridization remained much rarer in animals (Bullini and Nascetti 1990; Sites et al. 1990; Grant and Grant 1994). However, when successful, the hybrids were generally more variable than the parental species and their intermediate traits were heritable (Grant and Grant 1989, 1994). The mechanism of hemiclinal hybridogenesis constituted an evolutionary challenge because the hybrid was not able to survive without use the non-*ridibunda* genotype. Hybrid × hybrid matings only produce *ridibunda* offspring that are generally not viable. In order to breed and survive, hybrid males should attract and mate with non-*ridibunda* females whereas *R. kl esculenta* females tended to prefer *R. lessonae* males (Abt and Reyer 1993). In anurans, mating calls of hybrids usually showed intermediate but variable characteristics (Zweifel 1968; Schneider and Eichelberg 1974; Littlejohn and Watson 1976; Green 1982). In the *Litoria* hybrid zone, calls from close allopatry differed from those from distant allopatry, mainly in the number of pulses (Littlejohn 1976; Littlejohn and Watson 1983). Here the hybrid call structure was not only intermediate between parental species but also tended to resemble the non-*ridibunda* pattern. The change in courtship signal was a determinant trait for successful breeding in hybridogenetic frogs. By parasitizing the non-*ridibunda* species, the *ridibunda* genome could exploit a broader ecological niche (Pagano et al. 2001). The maintenance of the hybridogenetic strategy suggests that the advantage is to allow the colonization of new habitats from which one of the parental species is missing or which is marginally occupied.

The colonization of a new environment depends upon the intrinsic variability of a species and the source of genetic diversity mainly resulted from endogenous mutation and recombination (Ridley 1996). The water frog hybrids use the exogenous contribution of new genes but do not transmit them

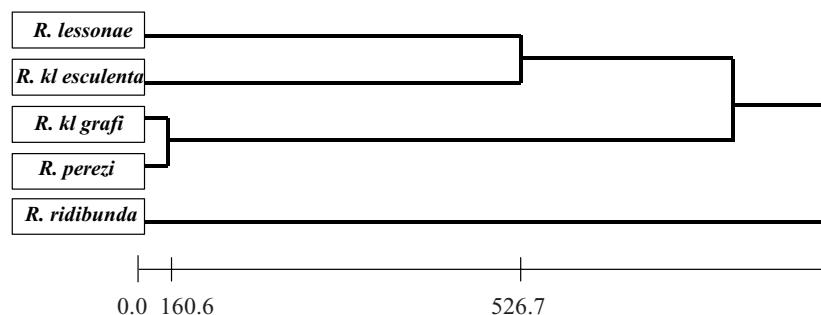


Fig. 3. Dendrogram resulting from the cluster analysis of call pattern in three water frogs Mendelian species and their two distinct hybridogenetic lineages. (analysis of eight acoustic parameters, mean Euclidean distances)

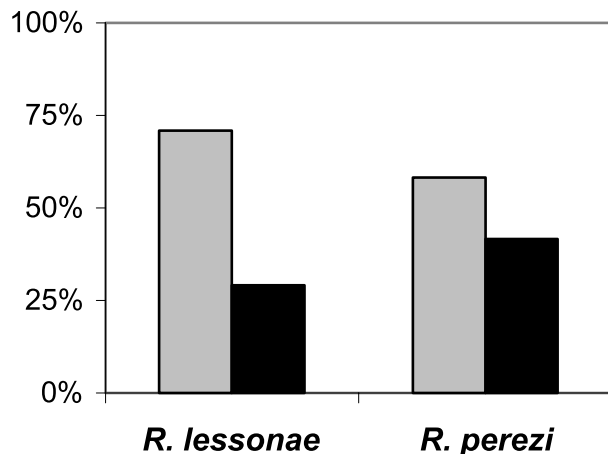


Fig. 4. Female preferences for specific (shaded bars) or hybrid calls (black bars) as revealed by play-back tests

to their lineages (Tunner 1974; Uzzell et al. 1980). Nevertheless, recombination without exclusion of parental genome has also been described (Uzzell et al. 1975; Hotz and Uzzell 1983; Guerrini et al. 1997). Hybridization generally raised several linked concerns such as genome incompatibility and reproductive barriers. Using locally adapted genomes of parental species, different hemiclones of hybridogenetic frogs might adjust to environmental constraints (Semlitsch et al. 1996). Both hybrids eliminated almost all of the non-*ridibunda* genome during the gametogenesis and showed changes in call patterns which produced an attractive courtship signal rather similar to that of their syntopic parental species. The hybridogenesis may be assumed to lead to a dead end, because Muller's ratchet predicted an increase of deleterious mutations (Milinski 1994). Conversely, this natural form of hybridization was also demonstrated to be able to establish new evolutionary lineages because hybrids may possess heterotic advantages (Semlitsch et al. 1997; Hotz et al. 1999) and introgression events may limit Muller's ratchet (Schmidt 1996; Guerrini et al. 1997; Lesbarrères et al. 1999; Pagano and Schmeller 1999).

Modifications, which affected the call structure from the *ridibunda* pattern, favoured the hybridogenesis parasitism strategy by attracting and mating with the non-*ridibunda* species and reduced the risks that the deception would be detected. Frogs newly arrived at breeding ponds could often be discriminated in numerous species such as *Rana clamitans* (Owen and Perrill 1998) and habituation to familiar vocalizations allowed the avoidance of costly aggressive interactions. Abt and Reyer (1993) found that *R. kl esculenta* females showed a strong preference for *R. lessonae* males. However, *R. lessonae* females were also able to distinguish between hybrids and their own species and exhibited a strong preference for *R. lessonae* males whereas *R. perezi* females did not clearly differentiate between hybrids and *R. perezi*. The observation that hybrids displayed only a little attractive call suggests that either a best fitness in the hybrid or the existence of an alternative mating strategy favours the hybridogenesis. Nevertheless, the convergence between the call pattern of non-*ridibunda* species and hybrids emphasized the importance of the courtship signal in sexual selection. Although, the call might be less efficient than that of non-*ridibunda* males, the characteristics of the call structure should however, allow some hybrids to breed successfully. Despite the fact that only a few

non-*ridibunda* females were attracted by the hybrid calls, these changes in call pattern were sufficient to obtain some successful mates, thus, maintaining the long-term persistence of mixed populations. As in the case of hybridization, hybridogenesis induced character shift on courtship signal in hybrid zones leading to convergence with the non-*ridibunda* species. Character convergence concerns here a behaviour that is directly involved in breeding success. This convergence towards the non-*ridibunda* patterns constituted an original example of an adaptation favouring the co-existence of mixed populations of new lineages of hybrids and parental species and, in the long term, influencing the evolutionary history of water frogs.

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Zusammenfassung

Merkmalskonvergenz beim Lockruf und bei der Partnerwahl von zwei genetisch distinkten hybridogenen Linien des Wasserfrosches (Rana kl esculenta, Rana kl grafi)

Die Muster der Paarungsrufe wurden bei zwei genetisch durch Stärkeelektrophorese eindeutig unterscheidbaren Linien (*Rana kl esculenta* und *Rana kl grafi*) in der Nachkommenschaft von Wasserfroschpopulationen untersucht. Ziel der Studie war es, die Rolle des Gesanges beim Prozess der Hybridogenese zu bestimmen. Beim Vergleich der Grundstruktur des Gesangs mit der von *Rana ridibunda* weisen die Hybriden wichtige Änderungen auf. Bei *Rana kl grafi* scheinen mehrere Parameter der Struktur des Gesangs (Frequenz, Dauer, Anzahl der Pulse) denen von *R. perezi* angeglichen zu sein, während die Rufe von *R. esculenta* eher denen von *R. lessonae* ähnlich sind. Ein hierarchisch aufsteigende Klassifikation lässt eine deutliche Konvergenz mit den Elternarten erkennen, was zu den Unterschieden bei den Hybriden geführt hat. Die Modifikationen im Gesangsmuster dürften auf die Wirkung des nicht-*ridibunda* Genoms und den sexuellen Selektionsdruck durch die weibliche Partnerwahl zurückgehen. Obgleich nur wenige Hybridweibchen eine Präferenz für die Hybridrufe zeigen, ermöglichen diese Unterschiede im Paarungsruf doch einigen hybridogenen Männchen eine erfolgreiche Paarung, so dass durch die Charakterkonvergenz im Werbungsruf bei nicht-*ridibunda* Arten die Hybridzonen entstehen. In jedem Fall begünstigen die Modifikationen des Paarungssignals den besonderen Hybridierungsprozess, der einen Quasi-Parasitismus der nicht-*ridibunda* Genome begründet.

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