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**Ecology / Écologie**

# Variations in call and morphology in male water frogs: taxonomic and evolutionary implications

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**Abstract** – In Anurans, the specific mate recognition system (SMRS), which contributes to prezygotic isolation, is mainly based on morphological and call features. This is why such criteria are used by zoologists for taxonomic identification. In hybridogenetic water frogs (*Rana ridibunda*, *R. lessonae*, *R. perezi*, *R. kl. grafi* and *R. kl. esculenta*), hybridization opens up the question of the efficiency of these criteria for taxon identification and of the specific recognition system in this context. Variation in both morphological and call features revealed both significant mean differences among taxa but also large overlapping among individuals belonging to different taxa. Thus, using call or morphometrical features in order to identify water frogs may be hazardous. Moreover, species differ poorly in their specific mate recognition system, facilitating hybridization events, and therefore suggesting that postzygotic mechanisms may be prevalent over prezygotic mechanisms. © 2000 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

**specific recognition / hybridization / prezygotic isolation / postzygotic isolation**

**Résumé – Variations du chant et de la morphologie des mâles de grenouilles vertes : implications taxinomiques et évolutives.** Chez les Anoures, le système de reconnaissance spécifique (*specific mate recognition system*; SMRS), qui contribue à l'isolement prézygotique, repose largement sur des critères morphologiques et des caractéristiques du chant. C'est pourquoi ces critères sont utilisés pour l'identification taxonomique. Dans les complexes hybridogénétiques de grenouilles vertes (*Rana ridibunda, R. lessonae, R. perezi, R. kl. grafi,* et *R. kl. esculenta*), l'hybridation pose la question de la validité de ces critères taxinomiques et interroge sur le SMRS. L'analyse de la morphologie et de la structure du chant de ces grenouilles montre que, en moyenne, les différences entre taxons sont significatives mais que les nuages de points se chevauchent. L'utilisation à des fins d'identification taxinomique des critères morphométriques et de chant est donc hasardeuse. Par ailleurs, comme les espèces diffèrent peu dans leur SMRS, des hybridations sont possibles. Cela suggère que les mécanismes d'isolation postzygotiques peuvent prévaloir sur les mécanismes prézygotiques. © 2000 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

**reconnaissance spécifique / hybridation / isolation prézygotique / isolation postzygotique**

## Version abrégée

D'après Paterson, le système de reconnaissance spécifique (*specific mate recognition system*; SMRS) contribue fortement à l'isolation prézygotique. Chez les Anoures, ce système repose largement sur des critères morphologiques et des caractéristiques du chant. C'est pour cette raison que ces critères sont utilisés par les

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zoologistes pour l'identification taxonomique. Dans les complexes d'hybridation, l'hybride a souvent des caractéristiques intermédiaires (par rapport aux deux espèces parentales), ce qui rend souvent difficile leur identification par les biologistes. D'autre part, de tels complexes soulèvent la question du SMRS des espèces impliquées dans l'hybridation. Dans les complexes hybridogénétiques de grenouilles vertes (*Rana ridibunda, R. lessonae, R. perezi, R. kl. grafi,* and *R. kl. esculenta*), le maintien des lignées hybrides implique le parasitisme sexuel d'une espèce parentale par l'hybride. Aussi, nous faisons l'hypothèse 1) que les hybrides présentent une grande variabilité de leurs traits (morphologie et chant) pour pouvoir ressembler à l'espèce hôte et 2) que la variabilité intraspécifique doit être grande de telle sorte que si les espèces diffèrent en moyenne, des individus de ces espèces peuvent se ressembler (donc potentiellement s'hybrider).

L'analyse de variables morphométriques et du chant montre que, en moyenne, les différences entre taxons sont significatives mais la variabilité reste grande y compris au niveau intra-taxonomique. Sur les graphiques, le chevauchement des nuages de points met en évidence que des individus de taxons différents ont des traits morphologiques et/ou de chant semblables. De fait, l'utilisation à des fins d'identification taxonomique des critères morphométriques et de chant est donc hasardeuse pour les grenouilles vertes. Par ailleurs, comme les espèces diffèrent peu dans leur caractéristiques de chant et morphologiques, leur SMRS n'est pas strictement fermé par les mécanismes d'isolement prézygotique. Des hybridations sont possibles. Cela suggère que contrairement au modèle de Paterson, les mécanismes d'isolation postzygotiques peuvent prévaloir sur les mécanismes d'isolement prézygotique.

## 1. Introduction

Mate recognition mechanisms could significantly contribute to prezygotic isolation, and Paterson [1] proposed that species can be characterized as a set of individuals with a common way of recognizing mates, the specific mate recognition system. It is emphasized that the evolutionary event for speciation first originated from a new mate recognition system while other mechanisms reducing interbreeding were incidental consequences. In most species of Anurans, mechanisms of specific prezygotic recognition chiefly depend upon some morphological features and courtship calls. Males compete for mating, calling in large choruses, and produce courtship calls as attractive acoustic signals [2, 3, 4, 5, 6]. Female mate choice is mainly based on some particular morphological traits and on the peculiar characteristics of the call pattern [7, 8, 9]. Moreover, reproductive success is often related to morphometrical features [10, 11, 12, 13]. Both the Fisherian process and the Zahavi's handicap theory predict that particular male traits result in best fitness, even if the peculiar trait may represent a handicap for survival, because sexual selection, through female choice, favours males with such traits [14, 15]. The sexual selection, resulting both from male competition and female preference, influences the maintenance of male characteristics among species. Thus, zoologists have often used these morphometrical characteristics and courtship calls as tools for species identification.

Nevertheless, some species are able to hybridize and this ability has often been associated with similarities for several traits such as morphology and/or call patterns. Influenced by the genetic pool of their two parental species, hybrids often exhibit numerous intermediate characteristics. The water frog complex includes some Mendelian species and many hybrids stemming from hemiclonal gametogenesis, called hybridogenesis [16, 17]. Before meiosis, the hybrid eliminates the non-*ridibunda* genome and, acting as a sexual parasite, re-establishes diploidy by mating with the non-*ridibunda* parental host species (*figure 1*). At least five taxa exist in France and are connected with two hybridogenetic complexes: 1) *Rana lessonae*, *R. ridibunda* and their *hybrid R. kl esculenta*; 2) *R. ridibunda*, *R. perezi* and their hybrid *R. kl grafi* [18–21].

The fact that the hybrids exhibit numerous intermediate traits has a taxonomic consequence, making their identification difficult for biologists. However, several methods have been proposed to distinguish the different taxa. Such identification is based on a morphometric ratio considering both variables reflecting size (body length, tibia length and toe length) and shape (length and height of metatarsal tubercle [22, 23]). Schneider et al. [24] and Sinsch and Schneider [25] also suggested that taxonomic identification might be based on call patterns.

However, the intermediate hybrid features also open up the question of the specific mate recognition system. Indeed, the hybridogenetic process requires that hybrids mate with a parental non-*ridibunda* species. Therefore, they should possess some characteristics allowing them to be recognized by the parental species in order to breed and to ensure their lineages. Thus, we hypothesized that 1) water frog hybrids should maintain a large variability both in their morphological traits and in their courtship call characteristics in order to resemble their host parental species, and, 2) water frog species may have a great intra-specific variation. Therefore, even if mean morphological features and call patterns of distinct species are different, several individuals may have fewer differences and thus allowing opportunities for hybridization.

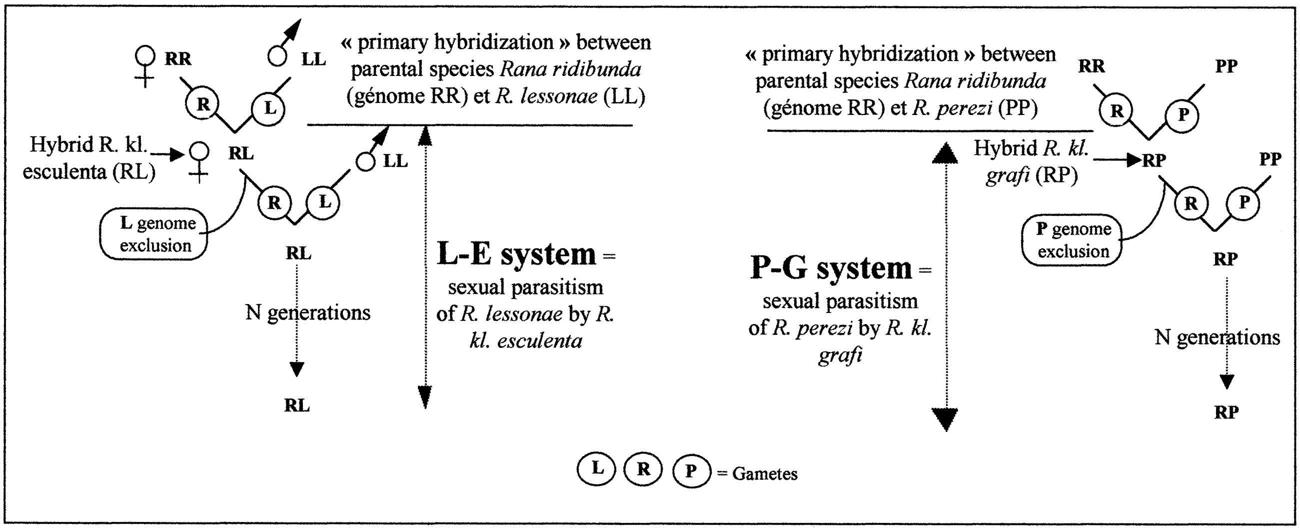

**Figure 1**. The hybridogenetic model in the L-E and P-G system.
In hybrid germinal cells, the non-*ridibunda* genome is excluded before meiosis. Thus, hybrids produce gametes with a *ridibunda* genome of clonal origin. Hybrid persistence is possible through sexual parasitism of the non-*ridibunda* parental species.

Therefore, our study aimed at documenting courtship calls and morphometry in water frog males from the two different hybridogenetic complexes sympatrically occurring in southern and western Europe [26, 27]. Using factorial maps of multivariate analysis, we examined both inter-taxon and intra-taxon variations considering genetically identified individuals. Regarding characteristics implied in specific mate recognition, such as courtship call patterns and morphometric traits, this study assesses variation in order to specify whether hybrids and parental species could be discriminated or whether they exhibit too variable intermediate traits to allow such a distinction. Evolutionary implications of our results are also discussed.

## 2. Materials and methods

Water frog males ($n = 151$) were sampled during the pre-spawning period in June 1995, 1997 and 1998. Studied sites were located in eastern France (near Lyon; *R. kl. esculenta* and *R. ridibunda*), in western France (near Nantes; *R. lessonae* and *R. kl. esculenta*), in central France (near Limoges; *R. lessonae* and *R. kl. esculenta*) in south-western France (near Bayonne; *R. perezi + R. kl. grafi*), in southern France (near Nimes; *R. perezi + R. kl. grafi*) and in northern Spain (near Zaragosa; *R. perezi + R. kl. grafi*). Morphometrical data were collected on 141 frogs while 97 where call-recorded. All 151 frogs were genetically identified

### 2.1. Frog identification

Individual taxon identification was inferred from allozymic variations (*table I*). A starch gel electrophoresis was carried out on toe tissues using the TC6 buffer system. Slices were stained following Hotz's procedure [28], considering allozymic variations at four to six 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, α-*GDH* 1.1.1.8, *AHH* 3.3.1.1). Alleles were designated by letters following the usual system [18, 28].

### 2.2. Call records

Courtship calls were tape-recorded using microphone (Emu 4535 electret with EM 700 condenser shot gun, 20–22 000 Hz at 8 dB, 600 W, sensibility 3–27dB, efficacy –69 dB, distortion < 0.3 %) and digital audio tape recorder (DAT TCD-D8, sampling frequency 44.1 kH, 20 Hz–20 kHz, 16 bit resolution). For each male, we sampled six to eleven different calls. Oscillograms and

**Table I**. Allozymic and genotypic variation detected among the five water frog taxa*.

| LDH-B | MPI | PGM-2 | α-GDH | CK-A | AHH | Species |
|---|---|---|---|---|---|---|
| a, c | a, c | b, d | fast | a | fast | *R. ridibunda* |
| e, rarely b | h | c | fast | a | fast | *R. lessonae* |
| ae, rarely ab, bc, ce | ah, ch | cd | fast | a, b, ab | fast | *R. kl. esculenta* |
| ad, rarely cd | al | dd | fast–slow | ad | fast–slow | *R. kl. grafi* |
| d | l, m | d | slow | d | slow | *R. perezi* |

* One letter = allozyme, two letters = genotype.

spectrograms were obtained from two different software through mathematics fast Fourier transform (resolution 22 Hz, 1 024 points) (Spectrogram 4.2 R.S. Horne 1994–1998 and Avisoft saslab pro version R. Specht 1990–1995, Berlin). Only calls from genetically identified males were used. We analysed calls for eight acoustic parameters: duration of acoustic emissions (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) and number of pulses (n). To avoid possible auto-correlation, acoustic parameters were averaged for each individual.

### 2.3. Morphometry

Measurements were performed on anaesthetized animals with a calliper (0.1 mm accuracy) Five variables were measured (body length (BL), tibia length (TL), first toe length (FTL), metatarsal tubercle length (MTL) and metatarsal tubercle height (MTH). These measurements were used to calculate the morphometrical ratios (FTL/MTL, TL/MTL, TL/MTH, BL/TL, BL/MTL, FTL/MTH) that are known to discriminate the three forms of the *esculenta* synklepton [23].

### 2.4. Statistical analysis and graphics

Both call records and morphometrical ratio variation were assessed by an ANOVA in order to specify whether differences were significant or not among taxa. A principal component analysis (PCA) was performed using ADE software [29]. Bivariate templets were also drawn.

## 3. Results

### 3.1. Genetic identification

The alleles found in the data set and their specificity corresponded to previous literature [28, 30]. In regard to specific markers at four to six loci, each individual exhibited a multilocus genotype that corresponded to a specific taxon. Both LDH-B, MPI, PGM showed clear discrimination between *lessonae* and *ridibunda* genomes while clear discrimination between *perezi* and *ridibunda* genomes was revealed by CK-A, AHH and α-GDH (*table I*; see an example in *figure 2*). Thus, 100 % of the individuals were correctly and unambiguously identified.

### 3.2. Variation within and among taxa

ANOVA demonstrated that morphometric ratios and call characteristics significantly differed among species (*tables II* and *III*).

The two first eigenvalues of the PCA performed on call variables accounted for 81.25 % of variation (PC1 = 61.78 %; PC2 = 19.47 %). The PCA performed on morphometrical ratios accounted for 80.74 % of variation for the two former components (PC1 = 61.15 %; PC2 = 19.59 %).

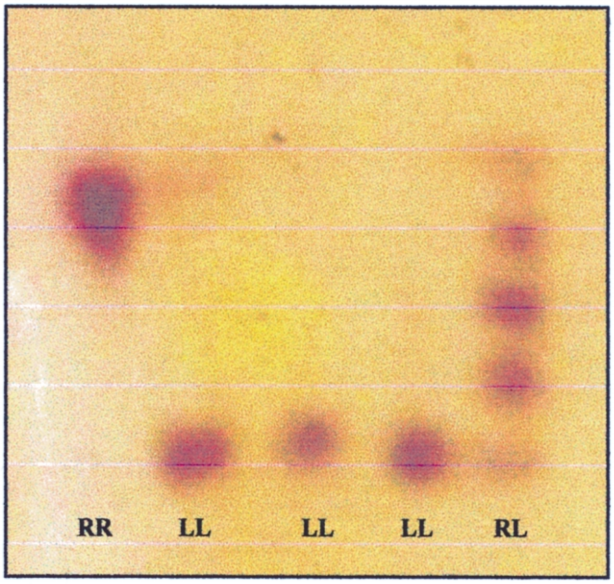

**Figure 2**. Examples of water frog specific markers revealed by analysis of protein polymorphism.
At the LDH-B locus (tetrametric in Ranidae), the faster allele is specific for the *ridibunda* genome, while the slower proved to be a *lessonae* genome. Homozygotes represent species and heterozygotes hybrids. RR = *R. ridibunda*; LL = *R. Lessonae*; RL = *R. kl. esculenta.*

PCA factorial maps (*figure 3.a, b*) illustrated mean intertaxonomic differences that were significant according to ANOVA. However, such maps also showed that intrataxon variation was great and differences sometimes low at the individual level as seen in overlapping zones.

For call features (*figure 3.a*), PC1 mainly supported variation in the duration of the calls, number of pulses and frequency at the beginning while the second component mainly differentiated individuals according to the amplitude. *R. perezi* and *R.kl. grafi* exhibited shorter songs with low amplitude and higher frequency at the beginning, *R. lessonae* and *R. ridibunda*, longer ones with a higher amplitude and a lower frequency at the beginning. *R. kl. esculenta* exhibited intermediate characteristics.

Considering morphometrical features (*figure 3.a*), PC2 strongly supported variation in BL/MTL while the first component mainly differentiated individuals according to TL/MTL (but also with other ratios).

As shown by the overlapping among distinct taxonomic groups on the graphics, several individuals bear more resemblance to individuals of different taxa than with individuals of their own group. This remark is true both for bivariate templets (*figure 4*) and multivariate analyses (*figure 3*). Such overlapping makes taxon identification based on morphometrical ratios or call characteristics difficult. The great inter-specific variation showed that the specific mate recognition system is still open.

**Table II**. Variation in values for morphometric ratios among water frog taxa.

| Species | | BL/TL | TL/MTH | FTL/MTL | TL/MTL | BL/MTL | FTL/MTH |
|---|---|---|---|---|---|---|---|
| *R. lessonae* | mean | 1.94 | 15.95 | 2.05 | 7.32 | 14.17 | 4.46 |
| *n* = 20 | standard error | 0.08 | 3.36 | 0.23 | 0.60 | 1.16 | 0.96 |
| *R. perezi* | mean | 1.79 | 28.31 | 2.59 | 9.80 | 17.51 | 7.71 |
| *n* = 18 | standard error | 0.09 | 6.47 | 0.28 | 0.95 | 1.52 | 1.92 |
| *R. kl. grafi* | mean | 1.79 | 26.31 | 2.50 | 9.45 | 16.92 | 6.93 |
| *n* = 11 | standard error | 0.04 | 4.43 | 0.11 | 0.62 | 0.95 | 0.92 |
| *R. ridibunda* | mean | 1.91 | 38.07 | 2.52 | 10.38 | 19.80 | 9.23 |
| *n* = 54 | standard error | 0.10 | 9.82 | 0.37 | 1.27 | 2.55 | 2.51 |
| *R. kl. esculenta* | mean | 1.87 | 22.92 | 2.28 | 8.47 | 15.79 | 6.11 |
| *n* = 38 | standard error | 0.12 | 5.39 | 0.29 | 0.92 | 1.58 | 1.37 |
| ANOVA, F value | | 8.97 | 41.96 | 12.36 | 42.13 | 42.51 | 28.52 |
| *P* value | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

## 4. Discussion

### 4.1. Variation among water frogs

Males of genetically distinct species of water frogs and their hybrids exhibited, as expected, a large variability in allozymes, call patterns and morphometric traits. Such a variability has significant taxonomic and evolutionary implications regarding the heuristic value of the specific mate recognition system concept in the speciation process. Although Fisher's theorem [25] predicted that diversity decreases with natural selection especially in fitness-related characteristics, intra-taxon variation is high enough to reduce inter-taxonomic differences. Thus, several individuals bear more resemblance to individuals of different taxa than with individuals of their own group.

### 4.2. Taxonomic implications

Call patterns did not basically differ in most of the studied acoustic parameters. Only the courtship call of *R. lessonae*, the most northern species, is characterized by a faster rhythm of sonorous pulses. The distinct species and their hybrids could not be positively field-identified considering most of the acoustic parameters. Indeed, numerous characteristics of the different courtship calls overlapped and it could be hazardous to base a specific determination on the call patterns.

A great overlap was also found in most of morphological traits in males of all taxa as demonstrated by multivariate and bivariate analyses. Berger [22] and Wijnands and Van Gelder [23] proposed to discriminate *R. ridibunda, R. lessonae* and *R. kl. esculenta* by drawing a bivariate templet based on morphometric ratios such as FTL/MTL and TL/MTL. However, taxa overlapped considerably, preventing any reliable determination based on such a ratio (*figure 4.a*). These results are congruent with preliminary data [31]. In addition, the present study contributes to the documentation of morphometric variation of south-western taxa (*R. perezi* and *R. kl. grafi*) and allows them to be compared with *R. ridibunda, R. lessonae* and *R. kl. esculenta*. The morphological differences between *R. perezi* and *R. kl. grafi* are slight and these taxa can often be confounded with *R. kl. esculenta* making field identification very difficult in sympatric areas of southern and western France. Thus, it will be deceptive to base taxon determination on raw body measurements or on call analyses.

### 4.3. Evolutionary implications

Water frogs were chiefly characterized by large variations of most of their characteristics. Such variations

**Table III**. Variation in values for call variables among water frog taxa.

| | | Duration | Beginning frequencies | End frequencies | Maximum frequencies | Amplitude | Number of pulses |
|---|---|---|---|---|---|---|---|
| *R. lessonae* | mean | 1 121.29 | 1 601.65 | 1 723.87 | 1 943.87 | 342.22 | 33 |
| *n* = 24 | standard error | 253.08 | 191.22 | 170.08 | 100.17 | 176.46 | 7.32 |
| *R. kl. esculenta* | mean | 880.18 | 1 792.5 | 1 926.97 | 2 129.26 | 329.07 | 15.94 |
| *n* = 34 | standard error | 183.57 | 89.02 | 88.05 | 109.58 | 85.16 | 2.96 |
| *R. kl. grafi* | mean | 533.55 | 2 081 | 2 021.27 | 2 379.36 | 298.36 | 8.82 |
| *n* = 11 | standard error | 125.92 | 221.47 | 358.83 | 298.74 | 120.3 | 3.56 |
| *R. perezi* | mean | 429.95 | 2 085.05 | 1 941.35 | 2 315.75 | 230.7 | 6.55 |
| *n* = 20 | standard error | 81.36 | 217.40 | 146.06 | 184.40 | 65.94 | 1.10 |
| *R. ridibunda* | mean | 1 162.5 | 1 712 | 1 906 | 2509 | 797 | 8 |
| *n* = 4 | standard error | 14.85 | 304.05 | 60.81 | 57.98 | 246.1 | 0 |
| ANOVA, F value | | 51.48 | 26.58 | 8 | 26.79 | 17.47 | 124 |
| *P* value | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

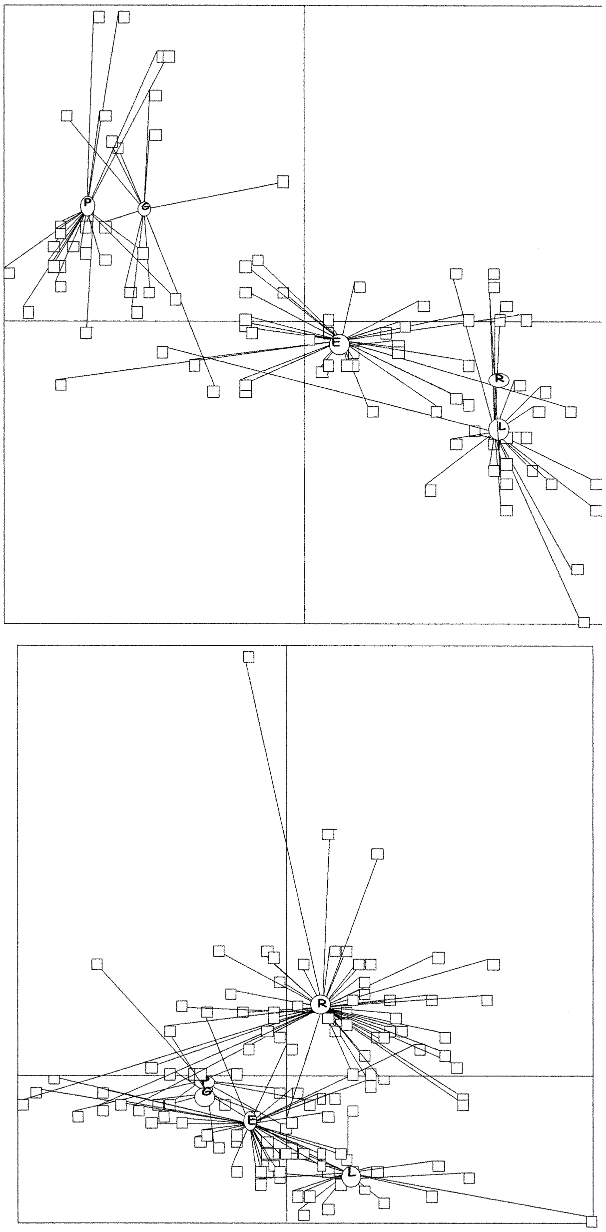

**Figure 3**. PCA factorial maps.
**a)** Call patterns. **b)** Morphometrical ratios.
Each taxa is represented by its barycentre on the factorial map and each individual of the group is linked to this barycentre.
L = *R. lessonae*; P = *R. perezi*; G = *R. kl. grafi*; R = *R. ridibunda*; E = *R. kl. esculenta*.

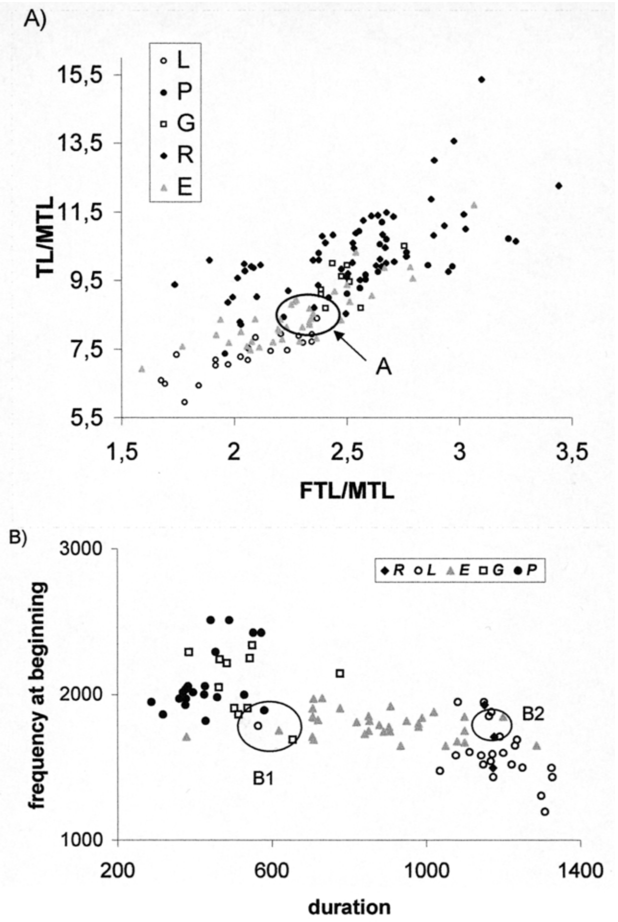

**Figure 4**. Bivariate templets based on **A**) usual morphometrical ratios (Berger, 1996), **B**) call characteristics.
L = *R. lessonae*; P = *R. Perezi*; G. = *R. kl. grafi*; R = *R. ridibunda*; E = *R. kl. esculenta*. Zones A, B1 and B2 highlight zones of the graph (A and B, respectively) where males of different taxa exhibited very similar features, either morphologically (A) or for call (B1 and B2).

increasing resemblance among taxa may favour hybridization events and especially the hybridogenetic process. The close similarity in call pattern between most species attested that the courtship call occurs rather as an attractive sexual signal than as a prezygotic isolation mechanism in water frogs. Furthermore, hybrids showed intermediate characteristics that poorly differ from the mate recognition system of parental species. It may favour such hybrid-species matings that are required to maintain hybrid lineages in the context of hybridogenetic process. The fact that *R. esculenta* females are easily attracted by calls produced by *R. lessonae* males [32] supports such expectations.

They are two hypothesis on the origin of *R. kl. grafi* stemming from a primary hybridization either 1) between *R. ridibunda* and *R. perezi*; or 2) between *R. kl. esculenta* and *R. perezi*. The difference between *R. kl. esculenta* and *R. perezi* is lower than the difference between *R. ridibunda* and *R. perezi* both for morphological and call features. Our results suggest that *R. kl. grafi* originated rather from a *R. kl. esculenta*–*R. perezi* primary hybridization than a *R. ridibunda*–*R. perezi* primary hybridization.

Although the five taxa lived in sympatry in southern and western France [26, 27], no evidence for a character reinforcement differentiating mate recognition was given. When focusing on particular zones of bivariate templets (see zone A and B of *figure 4*), it appears that a female has the choice among several males of distinct taxa with very similar morphometrical or call features. Such graphics suggest that specific mate recognition system is not strictly closed by isolating mechanisms, providing opportunities for various combinations of inter-taxonomic matings.

Emphasizing the concept of specific mate recognition system, Paterson [1] presumed that prezygotic isolation mechanisms predominate in the speciation process as a causal mechanism, while postzygotic mechanisms only resulted from the former divergence as incidental side-effect of evolutionary changes. Our results, however, require a moderation of this design. In fact, regarding both morphometric values and call patterns, the similarity among water frog species did not prevent an incomplete genome compatibility. Indeed, both experimental crosses between different water frog species and the genome exclusion in hybridogenetic process revealed such a relative incompatibility [33, 34]. These postzygotic mechanisms, reducing the possible speciation by hybridization, occur despite few changes in the specific mate recognition system. Consequently, our results also raise the issue of the potential significance of postzygotic divergences in the speciation process.

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