DNA content and genome composition of diploid and triploid water frogs belonging to the *Rana esculenta* complex (Amphibia, Anura)

Maria Ogielska, Piotr Kierzkowski, and Mariusz Rybacki

**Abstract:** The Central European water frog *Rana esculenta* L., 1758 is a natural hybrid between *Rana lessonae* Camerano, 1882 (LL) and *Rana ridibunda* Pallas, 1771 (RR). Hybrids are usually diploid (RL) or triploid (LLR or RRL). Distinguishing LL from RL, RR from RL, and LLR from RRL according to external morphology is ambiguous. In this study we checked whether the DNA content in erythrocyte nuclei measured by image cytometry is useful in determination of the taxonomic status of diploids (LL, RR, and RL) and the genome composition of triploids (LLR and RRL). For exact and direct identification of parental species, as well as for determination of genome composition in hybrids, we applied actinomycin D – 4′,6-diamidino-2-phenylindole chromosome staining to metaphase plates. We analyzed 43 LL, 12 RR, and 32 RL diploids, and 37 LLR and 19 RRL triploids. All diploid hybrids had 2n = 26 chromosomes, and all triploid hybrids had 3n = 39 chromosomes. Neither aneuploid nor mosaic hybrids were detected. The expected numbers of 13 *R. lessonae* (L) and 13 *R. ridibunda* (R) chromosomes in RL hybrids were recorded in about 31% of individuals. In the rest of the sample the composition was variable, ranging from 9 to 14 R chromosomes and the corresponding number of L chromosomes. The expected composition of 26 L and 13 R chromosomes was detected in about 32% of LLR triploids, whereas in the rest of the sample the composition of chromosomes ranged from 8 to 15 R chromosomes and the corresponding number of L chromosomes. The expected numbers of 26 R and 13 L chromosomes were detected in about 26% of RRL triploids, whereas in the rest of the sample the composition of chromosomes ranged from 19 to 28 R chromosomes and the corresponding number of L chromosomes. The DNA content densitometry showed that RR and RL diploids had 9.5% and 3.8% more DNA, respectively, than LL diploids. These differences, although statistically significant, were not sufficient to unequivocally discriminate LL from RL and RR from RL. Triploids had about 50% more DNA than LL diploids (49% in LLR and 51% in RRL), but these differences were too small for unequivocal determination of their genome composition.

**Résumé :** La grenouille verte *Rana esculenta* L., 1758 d’Europe centrale est un hybride naturel de *Rana lessonae* Camerano, 1882 (LL) et de *Rana ridibunda* Pallas, 1771 (RR). Les hybrides sont généralement diploïdes (RL) ou tri-ploïdes (LLR ou RRL). La distinction des LL des RL, des RR des RL et des LLR des RRL d’après les caractères morphologiques externes donne des résultats ambigus. Nous avons vérifié si le contenu de l’ADN des noyaux des érythrocytes, mesuré par cytométrie par analyse d’images, permettait de déterminer le statut taxinomique des diploïdes (LL, RR et RL) et la composition du génome des triploïdes (LLR et RRL). Afin d’identifier directement et avec précision les espèces parentales et de déterminer la composition du génome des hybrides, nous avons coloré les chromosomes des plaques en métaphase à l’actinomycine D et au 4′,6-diamidino-2-phénylindole. Nous avons analysé 43 diploïdes LL, 12 RR et 32 RL, ainsi que 37 triploïdes LLR et 19 RRL. Tous les hybrides diploïdes possédaient 2n = 26 chromosomes et tous les hybrides triploïdes 3n = 39 chromosomes. Nous n’avons observé ni hybride aneuploïde, ni hybride en mosaïque. Les nombres attendus de 13 chromosomes de *R. lessonae* (L) et de 13 de *R. ridibunda* (R) ont été notés chez environ 31 % des hybrides RL; dans le reste de l’échantillon, la composition était variable, allant de 9 à 14 chromosomes R avec un nombre complémentaire de chromosomes L. La composition attendue de 26 chromosomes L et de 13 R a été retrouvée chez environ 32 % des triploïdes LLR; dans le reste de l’échantillon, la composition chromosomique variait de 8 à 15 chromosomes R avec un nombre complémentaire de chromosomes L. La composition attendue de 26 chromosomes R et de 13 L a été retrouvée chez environ 26 % des triploïdes RRL; dans le reste de l’échantillon, la composition chromosomique variait de 19 à 28 chromosomes R avec un nombre complémentaire de chromosomes L. La mesure du contenu en ADN par densitométrie indique que les diploïdes RR et RL avaient respectivement 9,5 % et 3,8 % plus d’ADN que les diploïdes LL. Bien que statistiquement significatives, ces différences ne suffisent pas pour


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Introduction

In Central Europe, water frogs are represented by three taxa: the pool frog (*Rana lessonae* Camerano, 1882) (genotype LL), the lake frog (*Rana ridibunda* Pallas, 1771) (genotype RR), and their natural hybrid, the edible frog (*Rana esculenta* L., 1758) (genotype RL). The diploid hybrid genotype is composed of two genomes, one from each of the parental species (for review, see Graf and Polls-Pelaz 1989). Hybrids reproduce by hybridogenesis (Tunner 1970, 1974); the genome of one of the parental species is rejected premeiotically from the germ line and the remaining one is duplicated and undergoes meiosis (for review, see Graf and Polls-Pelaz 1989). Besides haploid gametes, hybrids can produce diploid eggs and sperm, giving rise to triploid progeny (Uzzell et al. 1975; Berger and Roguski 1978; Günther et al. 1979; Berger et al. 1986; Graf and Polls-Pelaz 1989; Brytcha and Tunner 1994; Rybacki 1994). It is generally accepted that triploid hybrids are of two types: (1) those with two *R. lessonae* (L) and one *R. ridibunda* (R) genome (genotype LRL), and (2) those with two R and one L genome (genotype RRL) (Uzzell et al. 1975, 1977; Hammer 1977; Berger 1979; Günther et al. 1979; Berger et al. 1986; Rybacki and Berger 2001).

With a few exceptions (Günther 1977; Berger et al. 1978; Tunner 1979, 1980; Polls-Pelaz and Graf 1988; Plötner and Klinkhardt 1992), discrimination between diploid and triploid water frogs is straightforward because there are distinct differences in erythrocyte size (Günther 1977, 1990; Berger and Roguski 1978; Berger et al. 1978, 1986; Polls-Pelaz and Graf 1988; Plötner and Klinkhardt 1992; Mikulíček and Kotlik 2001) and DNA content in erythrocyte nuclei (Ogielska-Nowak 1978; Vinogradov et al. 1990). Taxonomic identification of diploids (RR, LL, and RL) and determination of presumed genome composition of triploids (LLR and RRL) in most studies have been based on external morphology and morphometry (Berger 1966, 1988; Günther 1975, 1990; Uzzell et al. 1975; Berger and Truszkowski 1980; Berger and Günther 1988; Rybacki and Berger 2001). In most cases, morphological features of RR individuals allow their discrimination from RL individuals (Berger 1966, 1970, 2000; Günther 1973; Juszczyk 1987; Kotlik and Šúlova 1994); however, some difficulties have also been reported (Pagano and Joly 1998; Lodé and Pagano 2000). Morphological differences between LL and RL individuals, however, are often too small for correct taxonomic identification (Günther 1973; Ebendal 1979; Wijnands 1979; Kotlik and Šúlova 1994; Gubányi 1995; Lodé and Pagano 2000). The same doubts arise when LLR and RRL triploids are considered (Günther 1975; Uzzell et al. 1975; Berger and Roguski 1978; Günther et al. 1979; Tunner 1979, 1980, 2000).

As reported previously, RR diploids have more DNA than LL diploids (Mazin and Borkin 1979; Kierzkowski et al. 2002). For this reason, some authors used measurements of DNA content in erythrocyte nuclei to distinguish between LL, RR, and RL diploids (Vinogradov et al. 1990; Borisovskiy and Rosanov 2003; Borkin et al. 2003) and between LLR and RRL triploids (Vinogradov et al. 1990). These results, however, were never verified by electrophoresis of marker enzymes, nor by chromosome analysis.

It should be emphasised that taxonomic identification of water frogs based on morphological or cytological features, such as erythrocyte size and DNA content in erythrocyte nuclei, is still valuable. These methods can be applied during fieldwork and allow one to examine great numbers of animals without killing them. This is particularly important now, when amphibian populations are declining and prognostic classification of population composition is required. The aim of this study was to determine whether measurements of relative DNA content in erythrocyte nuclei by image cytometry might be an unequivocal method of taxonomic identification of diploids (LL, RR, and RL) and determination of genome composition of triploids (LLR and RRL). For direct identification of L and R genomes, we applied a fluorescence double-staining technique, which enables discrimination between R and L chromosomes by the differential fluorescence of pericentromeric heterochromatin regions (Heppich et al. 1982; Bucci et al. 1990; Tunner and Heppich-Tunner 1991).

Materials and methods

Altogether, 143 adult water frogs from eight localities on the southern shore of the Baltic Sea (northern Poland) were analysed: 88 hybrids (RL, RRL, and LLR) (26 females and 62 males), 43 LL diploids (6 females and 37 males), and 12 RR diploids (11 females and 1 male). Taxonomic status of each frog was determined by chromosome analysis (see below). All specimens were captured according to the Polish legal regulations concerning wild species protection. Frogs were collected under the following permits: OP 4072/218/96, OP 4072/218/98/4501, and OP 4201/144/99 for studies on protected species (Polish Ministry of the Environment), and 13/02 (Local Ethics Commission for Experiments on Animals).

For each animal, blood was taken from cut finger tips and smeared on a microscope slide. The smears were air-dried for 1 h, fixed in freshly made Carnoy’s fixative (3 parts ethanol and 1 part acetic acid) for 5 min, and stored in darkness at room temperature. The smears were then hydrolysed in HCl (5 mol/L) at room temperature for 1 h and stained with Schiff reagent for 30 min. To ensure the accuracy of measurements, all smears were processed under the same standardized conditions (Böcking et al. 1995).

DNA content measurements were done with a Kontron image analysis system (KS400, v. 3.0; Kontron Elektronik GmbH, Munich, Germany) connected to a Carl Zeiss Axioskop 20 microscope with an integrated stabilized power supply.
supply. From each smear about 400 undamaged and properly stained nuclei were automatically selected. Area (S) and mean gray level (I₀) were measured for each nucleus. Mean background gray level was measured in the 20 pixel × 20 pixel square in the center of each image (Hardie et al. 2002). Optical density (D) of each nucleus was calculated according to the formula \( D = \log(I₀/I₁) \). Integrated optical density (IOD) for each nucleus was then calculated according to the formula \( IOD = S \times D \). For each specimen, mean IOD values were calculated. For each series, control smears of LL blood were added as a reference for further standardization of the series. For each animal, the mean IOD value was divided by the mean IOD value of the corresponding control. Therefore, the individual IOD values were standardized (IOD_ST) and could be compared between series.

Statistical analysis was done with STATISTICA® 5 (StatSoft Inc. 1995). Normality of distributions was tested with the Shapiro–Wilk test, and variance homogeneity with Levene’s test. Comparisons between groups were done by means of variance analysis (ANOVA).

After blood smears had been taken, each animal was injected peritoneally with 1 mL of 0.3% colchicine (Sigma) 24 h before preparation. Immediately before tissue preparation the frogs were anesthetized with a 0.25% solution of 3-aminobenzoic acid ethyl ester (Sigma) in water. The intestine was dissected, hypotonized in distilled water for 20 min, and fixed in freshly made Carnoy’s fixative. The tissues were stored in fixative at −20 °C.

Chromosome counting was done on 10–20 complete metaphase plates. To prepare the plates, inner epithelial cells were stained for 5 min with 2% orceine in 70% acetic acid and then squashed under the cover slip.

Genome composition was determined on 10–20 metaphase plates. Intestinal epithelial cells were put on a slide in a drop of 70% acetic acid and squashed under a cover slip. The slides were put on dry ice until frozen, and the cover slips were removed. Chromosomes were stained with acridine orange (AO) and Schiff’s reagent (Schweitzer 1976) and Heppich et al. (1982). Slides were examined using a Carl Zeiss microscope equipped with a fluorescence lamp with appropriate filters.

Results

According to chromosome analysis after orceine staining, 87 diploids with 2n = 26 chromosomes and 56 triploids with 3n = 39 chromosomes were recognized. No aneuploid animals were detected. The diploid chromosome set consisted of five large and eight small pairs of metacentric and submetacentric chromosomes.

After identification of diploids and triploids, we examined their genome composition following acridine orange (AO) and Schiff’s reagent (Schweitzer 1976) and Heppich et al. (1982). Slides were examined using a Carl Zeiss microscope equipped with a fluorescence lamp with appropriate filters.

According to the hybridogenetic rule, the RL genotype should consist of 13 L and 13 R chromosomes (13 + 13 pattern). The triploid genotype should consist of 26 L and 13 R chromosomes (26 + 13 pattern). However, the hybridogenetic rules were defined mainly on the basis of analysis of the phenotype of progeny resulting from various crosses of hybrids and their parental species, and electrophoresis of selected enzymes (reviewed...
hybrids can differ from the expected 13 + 13 and 26 + 13 and the results presented in this paper suggest that the chromosome composition of the hybrids differs from the expected patterns. For about 68% of diploid RL hybrids, we detected 19–28 R chromosomes, instead of 26. Some doubts concerning the number of R chromosomes in diploid RL hybrids were mentioned by Heppich (1978) and Heppich and Tunner (1979; Tunner 1980, 2000). The results presented in this paper suggest that the chromosome composition of the hybrids can differ from the expected 13 + 13 and 26 + 13 patterns. For about 69% of diploid RL hybrids, we detected 9–14, instead of 13, R chromosomes. For about 68% of triploids classified as LLR, we detected 8–15 R chromosomes, instead of 13. For about 73% of triploids classified as RRL, we detected 19–28 R chromosomes, instead of 26. Some doubts concerning the number of R chromosomes in diploid RL hybrids were mentioned by Heppich (1978) and Heppich and Tunner (1979), who made karyotype analyses after the C-banding method. There are also papers describing the lack of a genome dosage effect on electrophoretic bands of serum albumins or several enzymes from triploid hybrids (Günther and Lübcke 1979), Mezhzherin and Morozov-Leonov (1997), and Pagano and Schmeller (1998), and are also a result of substitution of single R chromosomes by L chromosomes (or vice versa). Uzzell et al. (1975) suggested that irregularities and deviations from hybridogenetic rules during oogenesis in RL diploids can lead to patterns other than 13 + 13 or 26 + 13 in the offspring. Some kinds of recombined gametes might also be produced by triploid females, as was described by Alves et al. (1998) (reviewed by Alves et al. 2001) for fishes of the Leuciscus albunoides Steinacher, 1866 complex. Such conclusions must be tentative until more detailed studies, necessary to confirm the existence of hybrids with genome composition patterns other than 13 + 13 or 26 + 13, are undertaken. New results may contribute to an understanding of complicated and often unclear models of inheritance in some water frog populations.

The present study is the first in which DNA content in erythrocyte nuclei has been studied in a large sample of water frogs with known genotypes. Three aspects should be considered here: (1) differences in DNA content between diploids and triploids; (2) differences between diploid taxa (LL, RR, and RL); and (3) differences between LLR and RRL triploids. For aspects 2 and 3, great accuracy of measurements was required because small differences were expected. The stability of the light source during measurements is crucial for DNA image cytometry (Jonker et al. 1997; Puech and Giroud 1999; Ogielska et al. 2001). We applied our original software procedure for light intensity stabilization, which will be published elsewhere (P. Kierzkowski et al., unpublished data).

DNA content measurement is a precise method for identification of diploid and triploid water frogs. In our sample, triploids had 43.2% more DNA than diploids, and ranges of IOD_ST values did not overlap, which is consistent with results provided by other authors (Ogielska-Nowak 1978; Vinogradov et al. 1990). Determination of ploidy level by DNA densitometry was also successful in other diploid-polyploid populations, such as salamanders of the Ambystoma jeffersonianum (Green, 1827) complex (Uzzell 1964), salmonid fishes (Johnstone and Lincoln 1986), and toads of the Bufo viridis Laurenti, 1768 complex (Borkin et al. 1986; Stöck 1997; Stöck and Große 1997; Stöck et al. 2002).

In our sample of triploids, RR had the highest, LL had the lowest, and RL had intermediate DNA content. Similar results have been obtained by other authors (Mazin and Borkin 1979; Vinogradov et al. 1990; Kierzkowski et al. 2002; Borisovskiy and Rosanov 2003; Borkin et al. 2003). However, we cannot agree with Lada et al. (1995), Borisovskiy and Rosanov (2003), and Borkin et al. (2003), who used DNA content measurements for verification of the taxonomic status of RR, LL, and RL diploids identified on the basis of morphological characters.

Fig. 6. Medians, quartiles, and range of mean values of DNA content in erythrocyte nuclei for water frogs with different genome compositions.

Table 1. DNA content (standardised integrated optical density (IOD_ST); see text) in erythrocyte nuclei of parental species Rana ridibunda (RR) and Rana lessonae (LL) and of diploid (RL) and triploid (LLR, RRL) hybrids (Rana esculenta).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Number of individuals</th>
<th>DNA content (IOD_ST)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>R. lessonae</td>
<td>43</td>
<td>1.05</td>
</tr>
<tr>
<td>R. ridibunda</td>
<td>12</td>
<td>1.15</td>
</tr>
<tr>
<td>R. esculenta (RL)</td>
<td>32</td>
<td>1.09</td>
</tr>
<tr>
<td>R. esculenta (LLR)</td>
<td>37</td>
<td>1.56</td>
</tr>
<tr>
<td>R. esculenta (RRL)</td>
<td>19</td>
<td>1.58</td>
</tr>
<tr>
<td>R. esculenta</td>
<td></td>
<td>1.58</td>
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<tr>
<td>R. lessonae</td>
<td>43</td>
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<td>R. ridibunda</td>
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<td>32</td>
<td>1.09</td>
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<td>R. esculenta (LLR)</td>
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basis of external morphology. As shown in the present study, the verification of genome composition of diploids on the basis of DNA measurements is not reliable, owing to small differences in DNA content between the taxa and variability of DNA content within each taxon (see Table 1). The same problem was discussed by Collares-Pereira and Moreira da Costa (1999) and Próspero and Collares-Pereira (2000), who reported that differences in DNA content between diploid hybrids of the fishes of the *L. alburnoides* complex and their parental species are too small to identify their taxonomic status (reviewed by Alves et al. 2001).

The same doubts are relevant to the study of Vinogradov et al. (1990), in which the authors assumed that triploid frogs with lower DNA content should have an LLR genome composition and frogs with higher DNA content should have an RRL genome composition, without verification of the genome composition by any other method. Our results show that such an assumption is misleading. On average, RRL triploids had more DNA than LLR triploids, but the ranges of IOD_ST values overlapped to a large extent. Because the DNA content of hybrids depends strictly on the DNA content of the parental species, variation in DNA content within LL and RR diploids (Mazin and Borkin 1979; Kierzkowski et al. 2002; this study) can lead to differences in DNA content between triploids with the same genome composition, or to the same DNA content in triploids with different genome compositions. For these reasons, identification of the genome composition of water frog hybrids on the basis of DNA content is at least equivocal.

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complex (Teleostei, Cyprinidae) assessed by flow cytometry. 
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