Ann. Zool. Fennici 48: 56–66 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 31 March 2011

Genome dosage effect and hybrid morphology — the case of the hybridogenetic water frogs of the *Pelophylax esculentus* complex

Piotr Kierzkowski[1,*], Łukasz Paśko[1], Mariusz Rybacki[2], Małgorzata Socha[1] & Maria Ogielska[1]

[1)] *Laboratory of Biology and Conservation of Vertebrates, Institute of Zoology, University of Wrocław, ul. Sienkiewicza 21, PL-50-335 Wrocław, Poland (corresponding author's e-mail: piotrk@biol.uni.wroc.pl)*
[2)] *Research Center for Agricultural and Forest Environment, Polish Academy of Sciences, ul. Bukowska 19, PL-60-809 Poznań, Poland.*

Received 16 Mar. 2010, revised version received 9 Dec. 2010, accepted 17 Jan. 2011

Kierzkowski, P., Paśko, Ł., Rybacki, M., Socha, M. & Ogielska, M. 2011: Genome dosage effect and hybrid morphology — the case of the hybridogenetic water frogs of the *Pelophylax esculentus* complex. — *Ann. Zool. Fennici* 48: 56–66.

Western Palearctic water frogs offer a unique possibility to study the genome dosage effect (GDE). There are two morphologically distinct species, *Pelophylax ridibundus* RR and *Pelophylax lessonae* LL, and their hybridogenetic hybrid *Pelophylax esculentus* (RL, LLR or RRL). It is supposed that RL have intermediate morphological features, LLR are more similar to *P. lessonae*, and RRL more similar to *P. ridibundus*. We tested if the morphology of the water frogs reflects the GDE, and whether it can be used in the field for determination of the genome composition. Mean values of the indices DP/CI, T/CI and F/T followed the order LL–LLR–RL–RRL–RR. After applying discriminant and canonical analyses 89% RR, 95% LL, 91% RL, 84% LLR and 52% RRL were correctly classified. Surprisingly, the L haplotype had bigger influence on morphology than the R haplotype — all hybrid genotypes were morphologically closer to *P. lessonae* than to *P. ridibundus*.

Introduction

Morphological aspect of genetic variability may be studied in two ways: (1) as individual morphological changes caused by expression/silencing of particular genes and/or their variants, and/or (2) as quantitative correlations of genetic and morphological differences between taxons. The latter approach, thanks to the development of genetic and statistical methods, allows studying relationships between morphology and the genome composition with the use of a huge amount of various data and with regards of various levels of genome organization. This approach encompasses also studies on the correlation between large-scale genome changes, such as hybridization and polyploidization, and their influence on morphological variability. Manifestation of this correlation is a Genome Dosage Effect (GDE), known mainly in plants (for review *see* Chen & Ni 2006), which assumes more or less linear changes in the hybrid and

allopolyploid morphology towards either of the parental species, depending on its genetic contribution into hybrid genomes.

Hybrid organisms carry chromosome admixture from at least two species in their gene pool, as a consequence of interspecific hybridization. Depending on a nature of interacting species, hybridization may result in clonal or bisexual reproduction (Allendorf *et al*. 2001). An incompatibility between chromosomes deriving from different parental species leads to disturbances in meiosis and causes hybrid sterility. In some cases, however, hybrids are able to produce fertile gametes and take part in breeding. Usually once disturbances in reproduction are overcome and F1 fertile hybrids are established, repeated backcrossings to one of the parental species alters a share of genes contributed from progenitors and effects of hybridization will gradually disappear in next generations (Mallet 2005). However, a fraction of hybrid animals retains a permanent F1 hybrid state from generation to generation owing to altered meiotic mechanisms and reproduces clonally (parthenogenesis, gynogenesis) or hemiclonally (hybridogenesis); (reviewed by Suomalainen *et al*. 1987, Dawley 1989, Ogielska 2009). Due to disturbances of meiosis such hybrids also produce diploid unreduced gametes, which result in allopolyploid individuals with various shares of parental genomes. This variation may be reflected as GDE in hybrid morphology.

Western Palearctic water frogs form a complex that offers a unique possibility to study GDE. The complex comprises two species, *Pelophylax ridibundus* and *Pelophylax lessonae*, and their hybridogenetic hybrid *Pelophylax esculentus*. The species are diploid (genotype RR in *P. ridibundus* and LL in *P. lessonae*, 2n = 26), whereas *P. esculentus* hybrids are diploid (RL, 2n = 26) or triploid (Uzzell & Berger 1975, Uzzell *et al*. 1975, Ogielska *et al*. 2004). Hybrids are fertile and produce both haploid and diploid gametes. The latter give rise to male and female triploids with various proportions of parental genomes (LLR or RRL, 3n = 39). Because of the clear morphological differences between *P. ridibundus* and *P. lessonae* (Berger 1966, Plötner 2005, 2010), effect of hybridization should be reflected by an intermediate morphology of the diploid hybrids, whereas the effect of polyploidization as GDE in the allotriploids. It is supposed that LLR triploids are more similar to *P. lessonae*, and RRL triploids more similar to *P. ridibundus* (Uzzell *et al*. 1975, Hemmer 1977, Uzzell *et al*. 1977, Berger 2000). Those assumptions, however, were never verified by data based on a large sample of water frogs with known genotypes. Data about morphology of triploid water frogs with the genome composition verified in any way is scarce — Hemmer (1977) and Ebendal and Uzzell (1982) describe only a few individuals (verified by protein electrophoresis) and Tunner (2000) reported only on frogs with genotype LLR (verified by chromosome analysis) and provided data for only one, not widely used morphological index LT/CI (body length/*callus internus* length).

The current paper concentrates on two aspects: one more general related to morphological effects of large-scale genome changes, and the other more specialized and related to herpetological issues, because morphology is a simple tool used in the field for identification of taxons and estimation of the genome composition of the hybrids (Berger 2000, Plötner 2010). Therefore, the aim of this study was to determine (1) whether the genome composition of the water frogs of the *P. esculentus* complex is reflected by their morphology and — if yes — whether it displays GDE, and (2) if the morphological features of a given water frog individual can be used in the field for determination of its genome composition.

Material and methods

Adult water frogs of both sexes (174 in total) were analysed: 39 *P. ridibundus* RR (7 females, 32 males), 39 *P. lessonae* LL (4 females, 35 males), 32 *P. esculentus* RL (11 females, 21 males), 43 *P. esculentus* LLR (5 females, 38 males), and 21 *P. esculentus* RRL (13 females, 8 males). Frogs were collected from 10 sites on the southern shore of the Baltic Sea: Baczysław (B), Bielawskie Błota (BB), Dębki (D) lake Dołgie Wielkie (DW), lake Gardno (G), Kukułowo (K), Rozewie (R), Wysoka Kamieńska (WK), Wysoka Żwirownia (WZ), Żurawiowa Łąka

(ZL) and from two sites in the Barycz river valley: Ruda Milicka pond Polny (RMP) and Ruda Milicka pond Dwojak (RMD). Frogs were sorted to 2n and 3n on the basis of the size of the erythrocytes, which is a well known and in most cases a reliable method (Günther 1977, Plötner & Klinkhardt 1992, Polls Pelaz & Graf 1988). From each animal, an air dried blood smear from a cut toe tip was made on a microscopic slide. Measurements were carried out using a PC computer equipped with a KS400 image analysis software (Kontron Elektronik), connected to a Carl Zeiss Axioskop 20 microscope. From each specimen, long axes of 40 randomly chosen erythrocytes were measured. After sorting the frogs into 2n and 3n we carried out a chromosome analysis to verify the ploidy level. The genotype of the frogs was determined on the basis of the differential chromosome staining with AMD/DAPI, with special attention being paid to chromosomes of the 10th pair (Heppich *et al*. 1982), which are easily distinguished by their secondary constriction. Details on the chromosome analysis of the studied frogs are presented in Ogielska *et al*. (2004).

For each specimen *femur* length (F), *tibia* length (T), *digitus primus* length (DP), and *callus internus* length (CI) were measured with an electronic calliper (accuracy 0.01 mm) and morphological indices DP/CI, T/CI and F/T were calculated. We describe the ranges of the morphological index values for each genotype in our sample and then apply discriminant and canonical analyses to all three indices separately for diploids and triploids. This approach allowed for reducing the character space for each analysis. Statistics were calculated using Statistica 8.0 (StatSoft Inc. 2007). For each genotype group (LL, RR, RL, LLR, RRL), the following conditions were tested: normality of distributions of DP/CI, T/CI and F/T indices by a Shapiro-Wilk test, and homogeneity of variances by Levene's test. Significance of differences in DP/CI, T/CI and F/T between genotype groups was tested by ANOVA when distributions were normal and variances homogeneous, otherwise using a non-parametric Kruskal-Wallis test. A discriminant analysis was performed on the log-transformed index values (to remove the non-normal distributions in some cases), separately for 2n and 3n groups. For the diploid groups, a canonical analysis was also carried out (there are only two groups of triploids, and canonical analysis cannot be done with such data). Within each ploidy group, genotype was taken as a grouping variable (LL, RR, RL in case of 2n, and LLR and RRL in case of 3n) and indices DP/CI, T/CI and F/T as dependent variables. To verify the existence of a genome dosage effect, and to estimate the influence of the genome composition/site affinity on the individual's morphology (Plötner *et al*. 1994), groups of water frogs defined by both genotype and site as a grouping variable were analysed together. Log-transformed values of DP/CI, T/CI and F/T were dependent variables. Only 11 groups with six or more individuals were considered in the analysis. Discriminant and canonical analyses were carried out, and an MST (Minimum Spanning Tree) was graphically combined with the distribution of group means along the first two canonical vectors (Root 1, Root 2) using NTSYSpc 2.20u (Applied Biostatistics Inc. 1986–2008). For better visualisation of the GDE, relationship between the proportion of the haplotypes L and R in the genome and canonical Root 1 values (averaged for groups with the same genotype, coming from different sites) was drawn.

Results

Basic statistical description

Sorting the frogs into diploid (LL, RR, RL) and triploid (LLR, RRL) groups on the basis of the erythrocytes is unambiguous: ranges of the values do not overlap (*see* Fig. 1 and Table 1). Significant differences were noted between all pairs of diploids and between all diploid–triploid pairs (ANOVA: $F = 352.70$, $p < 0.05$; all Tukey tests showed $p < 0.05$). No differences in the erythrocyte long axes between LLR and RRL were noted.

The DP/CI index values were significantly different between all pairs of genotypes (ANOVA: $F = 188.19$, $p < 0.05$; Tukey test: $p < 0.05$ between all RL, LLR and RRL pairs, and $p < 0.01$ between the rest of the pairs). For diploids, the values of DP/CI did not overlap and

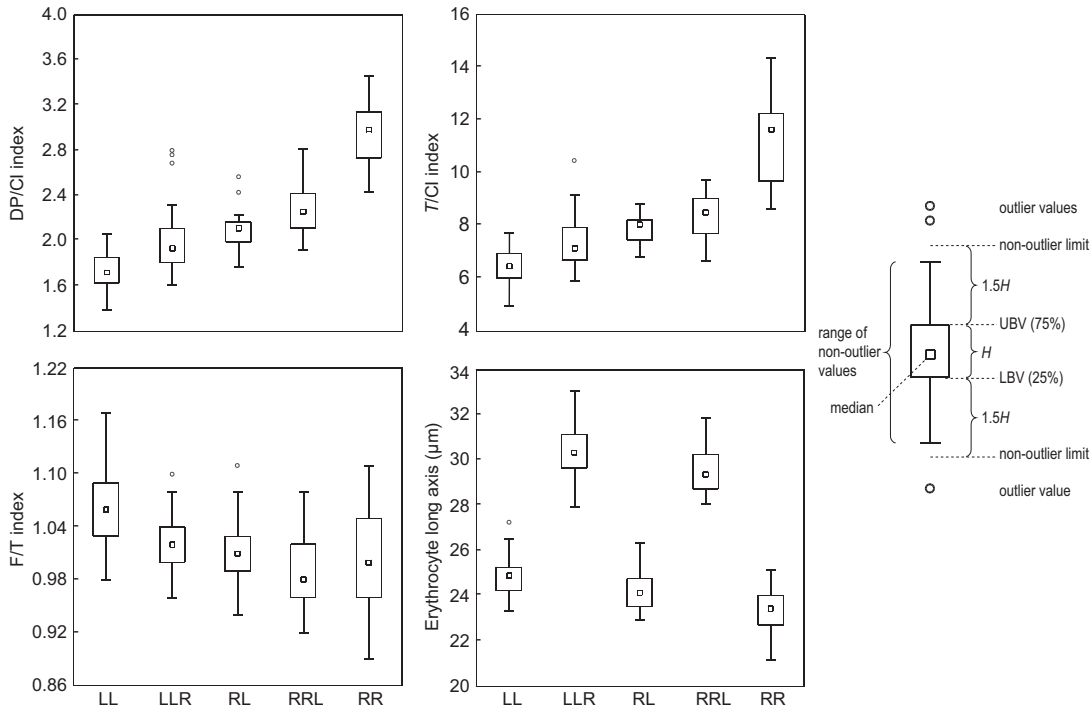

Fig. 1. Statistics for DP/CI, T/CI, F/T and an erythrocyte long axis for water frogs with known genotype. UBV = upper box value, 75th percentile; LBV = lower box value, 25th percentile; *H* = height of the box; non-outlier values are those located between UBV + 1.5*H* and LBV + 1.5*H* (after StatSoft Inc. 2007, modified).

Table 1. Statistics for DP/CI, T/CI, F/T and an erythrocyte long axis of the water frogs with known genotype. Min and max are the outliers (values beyond the non-outlier limit in Fig. 1; StatSoft Inc. 2007). Means were calculated for features with normal distributions, otherwise only medians are shown.

Feature	Genotype	Statistics							
		Min	Min non-outlier	Mean	SD	SE	Median	Max non-outlier	Max
DP/CI	RR	–	2.44	2.93	0.28	0.04	2.98	3.46	–
	LL	–	1.39	1.75	0.15	0.02	1.72	2.06	–
	RL	–	1.77	2.09	0.17	0.03	2.11	2.23	2.57
	LLR	–	1.61	–	–	–	1.91	2.32	2.80
	RRL	–	1.92	2.27	0.23	0.04	2.26	2.82	–
T/CI	RR	–	8.63	11.29	1.41	0.22	11.63	14.35	–
	LL	–	4.96	6.43	0.64	0.10	6.46	7.72	–
	RL	–	6.82	7.89	0.55	0.10	8.02	8.80	–
	LLR	–	5.89	–	–	–	7.13	9.15	10.47
	RRL	–	6.64	8.29	0.95	0.21	8.49	9.72	–
F/T	RR	–	0.89	1.00	0.05	0.01	1.00	1.11	–
	LL	–	0.98	1.06	0.04	0.01	1.06	1.17	–
	RL	–	0.94	–	–	–	1.01	1.08	1.11
	LLR	–	0.96	1.02	0.03	0.01	1.02	1.08	1.10
	RRL	–	0.92	0.99	0.04	0.01	0.98	1.08	–
Erythrocyte long axis (µm)	RR	–	21.2	23.37	0.98	0.16	23.41	25.18	–
	LL	–	23.36	24.9	0.90	0.14	24.9	26.53	27.26
	RL	–	22.93	24.2	0.85	0.15	24.13	26.35	–
	LLR	–	27.96	30.33	1.24	0.19	30.32	26.35	–
	RRL	–	28.09	29.5	1.04	0.23	29.37	31.86	–

allowed for a correct classification of all parental species as either LL or RR, and of 59.4% (n = 19) as RL hybrids. For triploids, the values did not overlap for 51.2% (n = 22) LLR and 4.8% (n = 1) RRL.

Differences between the T/CI index values were significant for all group pairs (Kruskal-Wallis test: $H = 126.38$, $p < 0.01$; Mann-Whitney U-test: $p < 0.01$), except for RL and RRL. For diploids, values of the T/CI index for LL and RR individuals did not overlap, and for 56.2% (n = 18) RL hybrids. For triploids the values did not overlap for 15.6% (n = 10) LLR.

Differences between the F/T index values were significant for all group pairs (Kruskal-Wallis test: $H = 43.25$, $p < 0.01$; Mann-Whitney U-test: $p < 0.05$), except for RL and RR, RL and LLR, and RR and RRL. Ranges of the values of F/T indices did not overlap for 48.7% (n = 19) RR and 7.7% (n = 3) LL. Ranges of the F/T indices between RR and RL did not overlap for 3.13% (n = 1) RL, and between LL and RL for 9.38% (n = 3) LL and 7.7% (n = 3) RL. For triploids, ranges of F/T did not overlap for 2.33% (n = 1) LLR and 19% (n = 4) RRL.

Discriminant and canonical analyses of diploids LL, RR, and RL

In diploids, the variance of DP/CI was homogeneous, and distributions of DP/CI, T/CI, and F/T were normal for all genotypes. Therefore, most of the conditions for the multivariate analyses were met. The discrimination between LL, RR, and RL was good (Wilk's $\lambda = 0.10$, $p < 0.01$). Discrimination power was the highest for DP/CI, and lower for T/CI and F/T (partial λ, *see* Table 2). For all indices, discrimination was statistically significant. F/T had the lowest redundancy ($1 - R^2 = 3\%$), whereas DP/CI and T/CI indices had similar redundancies (26% and 27%, respectively) (Table 2). Discrimination between the species was clear, and no LL was classified as RR, or vice versa. About 10% of LL individuals and 5% of RR individuals were, however, classified as RL (Table 3).

Two canonical vectors — Root 1 and Root 2 — were extracted from the data. Root 1 explained 99% (eigenvalue = 8.13), and Root 2 explained 1% (eigenvalue = 0.05) of the variability. Root 1 was statistically significant (χ^2 test: $p < 0.01$) and

Table 2. Summary of discriminant function analyses for indices of 2n and 3n water frogs.

Ploidy level	Dependent variables	Wilk's λ	Partial λ	p	R^2	$1 - R^2$
2n	log DP/CI	0.15	0.71	0.01	0.74	0.26
	log T/CI	0.12	0.86	0.01	0.73	0.27
	log F/T	0.11	0.93	0.03	0.96	0.03
3n	log DP/CI	0.74	0.96	0.11	0.49	0.51
	log T/CI	0.71	0.99	0.48	0.48	0.52
	log F/T	0.75	0.94	0.05	0.89	0.11

Table 3. Number of 2n and 3n water frog specimens classified by discriminant analyses to genotype groups.

Actual genotype	Properly classified (%)	Predicted genotype				
		RR	RL	LL	LLR	RRL
P. ridibundus RR	89.7%	35	4	0	–	–
P. esculentus RL	90.6%	3	29	0	–	–
P. lessonae LL	94.9%	0	2	37	–	–
P. esculentus LLR	83.7%	–	–		36	7
P. esculentus RRL	52.4%	–	–		10	11
Total	91.8% (2n), 73.4% (3n)	38	37	35	46	18

was responsible for separation of the genotypes, mostly on the basis of the DP/CI and T/CI indices (Table 4). Root 2 reflects the influence of the F/T index, which is minor as compared with that of DP/CI and T/CI. Diploid individuals are discriminated mostly along Root 1, for which DP/CI and T/CI indices are important (Fig. 2 and Table 4). The greater the DP and T values, and the smaller the CI value, the greater the value of Root 1, and the closer a particular individual is to the RR group. LL and RR are situated in two separate groups, and RL hybrids are situated between them, but closer to the LL group (Fig. 2). This may suggest that the L haplotype has a stronger influence on the water frog morphology than the R haplotype.

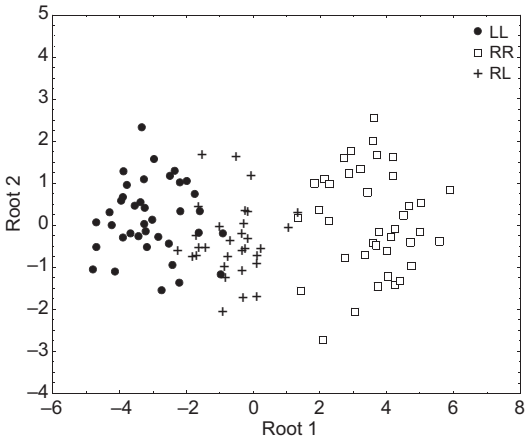

Fig. 2. Values of Root 1 and Root 2 for diploid water frogs *Pelophylax ridibundus* RR, *Pelophylax lessonae* LL, *Pelophylax esculentus* RL.

Discriminant analysis of triploids LLR and RRL

In triploids, variances of the DP/CI, T/CI and F/T indices were homogeneous, and only the distribution of DP/CI for LLR was not normal. Therefore, most of the conditions for the multivariate analyses were met. The discrimination between LLR and RRL was weak, but statistically significant (Wilk's $\lambda = 0.71$, $p < 0.01$). Discrimination power (partial λ) for all indices was similar, but only the F/T index was statistically significant (Table 2). The F/T index had also the lowest redundancy ($1 - R^2 = 11\%$), whereas DP/CI and T/CI had about 50% repeated information. About 84% of LLR and 52% of RRL were correctly classified (Table 3).

Discriminant and canonical analyses for diploids and triploids with site affinity

For the 11 samples with at least six individuals, an analysis was performed with the indices DP/CI, T/CI, and F/T as dependent variables, and genotype and affinity to the sites as grouping variables. All distributions were normal (Shapiro-Wilk's test), but variances for all indices were not homogenous (Levene's test). In case when some conditions are not met, multivariate analyses should not be done, if there is a correlation between mean and standard deviation (Dytham 2003). However, only for T/CI the correlation was statistically significant ($r = 0.86$, $p < 0.01$), and for DP/CI and F/T it was small and not significant, so multivariate analyses were carried out.

The discrimination between groups was good (Wilk's $\lambda = 0.11$, $p < 0.01$). Discrimination power was high and statistically significant for all indices (partial λ, Table 5). F/T had the lowest redundancy ($1 - R^2 = 0.05\%$) , whereas DP/CI and T/CI had similar redundancies (27% and 30%, respectively) (Table 5). Three vectors were extracted from the data, Root 1 (eigenvalue = 6.3, 96.72% of the variability), Root 2 (eigenvalue = 0.18, 2.85% of the variability) and Root 3 (eigenvalue = 0.03, 0.43% of the variability). Roots 1 and 2 explained 99.57% of the total variability. Root 1 was statistically significant (χ^2 test: $p < 0.01$) and was responsible for separation of the genotypes on the basis of DP/CI and T/CI indices (Table 6). Root 2 reflected the influence of the F/T index, similarly as shown in Table 4. Mean values of Root 1 and Root 2 for

Table 4. Factor structure matrix for a canonical analysis of 2n water frogs.

Dependent variables	Root 1	Root 2
log DP/CI	0.89	0.25
log T/CI	0.82	–0.03
log F/T	–0.19	0.98

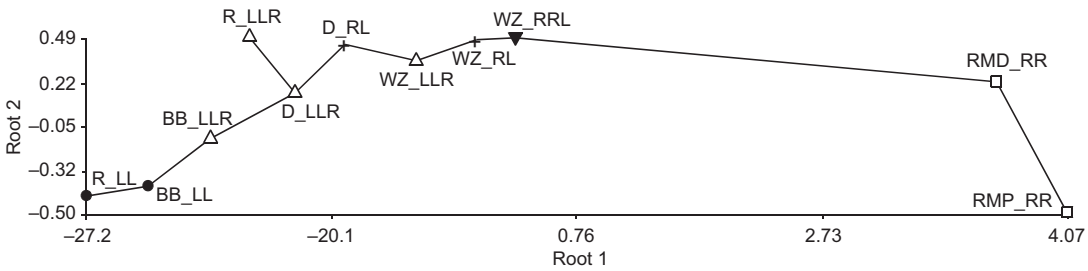

Fig. 3. Mean values of Root 1 and Root 2 for water frogs grouped by genotype/site affinity, combined with MST (Minimum Spanning Tree). Sites: R = Rozewie, BB = Bielawskie Błota, D = Dębki, WZ = Wysoka Żwirownia, RMP = Ruda Milicka pond Polny, RMD = Ruda Milicka pond Dwojak; LL, RL, RR, LLR, RRL = genotypes of water frogs.

groups of individuals were combined with the MST tree (Fig. 3). The distribution along Root 1 reflected the genome composition with a clear GDE, although one of the four LLR samples fell between the two RL samples. The L haplotype seems to have a greater influence on the morphological variability as compared with that of the R haplotype, as all hybrid genotypes with at least one L haplotype were situated closer to *P. lessonae* in their morphology (on the left half of the Root 1 axis in Fig. 3). The distribution along Root 2 reflected the influence of site affinity on the morphological variability. Among all possible pairs of populations having the same genotype, differences in Root 1 were the largest in case of the RR individuals originating from the Barycz river valley (sites RMD and RMP). Site affinity seems to have no influence, either on LL individuals (groups from the sites R and BB had similar values of Root 2), or on RL individuals (groups from sites D and WZ had similar values of Root 2), and moderate influence on LLR individuals. Because Root 1 explained about 97%, and Root 2 only about 3% of the total variability, the genome composition in every case seems to be more important than site affinity in its influence on morphology of the frogs.

Genome dosage and the morphology

The relationship between the proportion of haplotypes L and R in the genome and canonical Root 1 values averaged for each genotype (on the basis of canonical analysis for diploids and triploids with site affinity) is plotted (Fig. 4), GDE can be clearly observed, as the correlation is approximately linear ($r = –0.96$, $p < 0.01$). The percentage increase of Root 1 values (as a measure of morphology) is nearly proportional to percentage increase of L in the genotype for the genotype sequence RR–RRL–RL–LLR–LL. The most conspicuous shift of hybrid morphology towards *lessonae* is observed when we compare RR with RRL. In this case, an increase of the L haplotype input is about 33%, whereas the shift towards LL morphology is about 56%. It results in indices of RRL placed in half way between RR and LL and those of RL shifted towards LL (Fig. 4). A dosage of L haplotype in the rest of the sequence (RL–LLR–LL) is less conspicuous. The relationship between genetic contribution of particular haplotypes and morphology displays more or less linear changes in the hybrid and allopolyploid morphology towards either of the parental species, depending on the part of its genetic contribution, and thereby clearly shows GDE in the hybrids.

Table 5. Summary of discriminant function analyses for indices for 2n and 3n frogs with site affinity.

Dependent variables	Wilks' λ	Partial λ	p	R^2	$1 – R^2$
log DP/CI	0.15	0.73	0.01	0.73	0.27
log T/CI	0.14	0.82	0.01	0.70	0.30
log F/T	0.13	0.84	0.01	0.95	0.05

Table 6. Factor structure matrix for a canonical analysis for 2n and 3n frogs with site affinity.

Dependent variables	Root 1	Root 2
log DP/CI	0.89	–0.15
log T/CI	0.84	0.05
log F/T	–0.19	–0.99

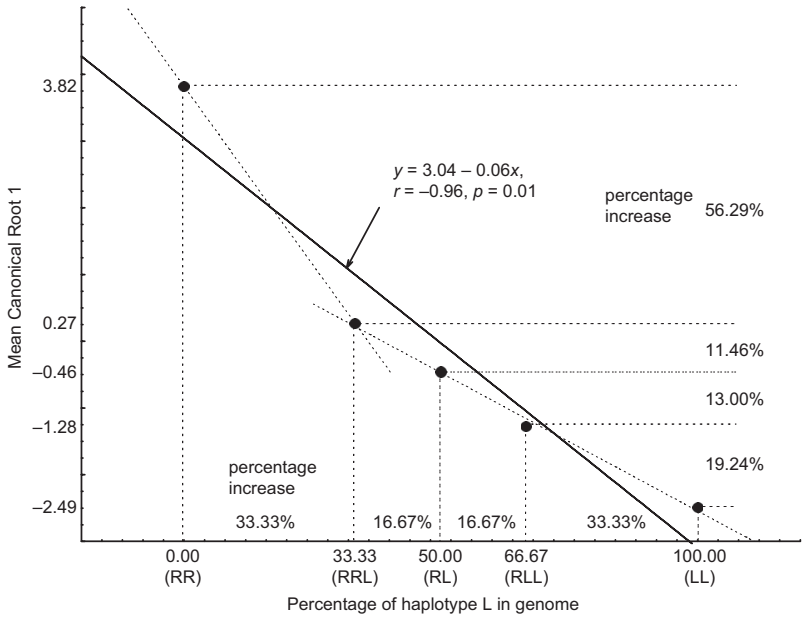

Fig. 4. Mean values of Root 1 for each genotype, calculated on the basis of the canonical analysis for groups genotype/site affinity, plotted against the percentage increase of the L haplotype in the water frogs genome.

Discussion

Hybridization and polyploidization (allopolyploidization) are best known in plants (Otto & Whitton 2000), but were reported also from reptiles, amphibians and teleost fishes (Bogart 1980, Schultz 1980, Vrijenhoek 1989, Alves *et al*. 2001, Kearney *et al*. 2009). GDE has been noted e.g. in north American fish *Poeciliopsis monacha–P. lucida* (Schultz 1969, Cimino 1972) and *Phoxinus eos–P. neogaeus* complexes (Goddard & Schultz 1993). In *Cobitis*, hybrid biotypes with various ploidy levels were encountered, e.g. in the *Cobitis sinensis–Iksookimia longicorpus* complex, where GDE is expressed in intermediate body proportions and coloration of hybrids (Kim & Lee 1990, 1995, 2000). In amphibians, clearly visible GDE was reported in hybrids of European fire-bellied toads *Bombina bombina* and *B. variegata* (Vörös *et al*. 2007, Yanchukov *et al*. 2006).

The present study is the first that describes GDE in morphology of a large sample of five forms of water frogs from the *P. esculentus* complex (LL, RR, RL, LLR, RRL), with the genome composition determined by cytogenetics. Although the use of as many morphological parameters as possible was suggested e.g. by Føg (1994) and recently by Krizmanić (2008), we focused on three main morphological indices (DP/CI, T/CI, and F/T) because they are most often used for taxonomical identification of central European water frogs (Juszczyk 1987, Berger 2000, Plötner 2005) and measurements needed for their calculation can be easily taken in the field.

Despite data obtained from the chromosome analysis of *P. esculentus* and presumed deviations from the expected 13 + 13 (diploid) or 26 + 13 (triploid) chromosome pattern (Ogielska *et al*. 2004), for the scope of this study we assumed that only "pure" RL, LLR and RRL exist. For determination of the genome composition we paid attention mostly to the chromosomes of the 10th pair, which are very well distinguishable because they carry the secondary constrictions.

It has been reported that morphological features of *P. esculentus* hybrids with the same genotype composition may differ among populations (Plötner *et al*. 1994, Tunner 2000). In the present study, however, the influence of site affinity on morphology was an order of magnitude lower than the influence of the genotype (Fig. 3). Owing to this, we pooled together frogs with the same genotype, but originating from different sites.

Successful identification of diploid and triploid hybrids in the field is possible on the basis

of the erythrocyte measurements. In our sample, all diploids and triploids were correctly identified (Fig. 1 and Table 1), which was confirmed by the chromosome analysis. This is also supported by the data of other authors (e.g. Günther 1977, Berger *et al*. 1978, Berger & Roguski 1978, Berger *et al*. 1986, Polls Pelaz & Graf 1988, Plötner & Klinkhardt 1992, Ogielska *et al*. 2004).

Within diploid and triploid groups, the extent of GDE in morphology was not always sufficient for successful differentiation between the genotypes. *Pelophylax ridibundus* formed a clearly separate sub-group with no overlapping of DP/CI and T/CI indices with *P. lessonae*, and with only slight overlapping with *P. esculentus* RL. More overlapping was observed between *P. lessonae* and *P. esculentus* RLs (Fig. 1 and Table 1). This supports the results of other authors, who suggested a possibility of mistaking at least some of *P. lessonae* for *P. esculentus* RL (Ebendal 1979, Wijnands 1979, Kotlik & Šůlova 1994, Plötner *et al*. 1994, Gubányi 1995, Pagano & Joly 1998). A multivariate analysis carried out by us allowed for a correct classification of 91.8% of the diploid frogs (Table 3). Those results show that it is possible to discriminate between diploid forms of water frogs using the DP/CI and T/CI indices (F/T is of minor importance).

Mean values of the morphological indices of the water frogs from the *P. esculentus* complex follow the order LL–LLR–RL–RRL–RR (Fig. 1 and Table 1). Moreover, GDE was observed also in triploid erythrocytes that were larger in LLR than in RRL (Fig. 1) (although those differences were not statistically significant), which might reflect the differences between the parental species (LL have larger erythrocytes than RR). We did not test the body size of the frogs, but Christiansen *et al*. (2010) recently reported that this parameter, too, is subject to GDE. In our study morphological differences in studied indices among triploids were not sufficient for clear discrimination between LLR and RRL; respectively, 84% and 52% individuals were correctly classified (Table 3).

The influence of L and R haplotypes on the external morphology of triploid hybrids is not equal (Fig. 4). Only 16% of LLR were classified as RRL (hence, they were closer to RRL in morphology), but as much as 48% of RRL were classified as LLR (Table 3). Therefore, the presence of L is more stronger reflected in LLR than R in RRL. Similar results were obtained for diploid RL hybrids, which are situated between LL and RR groups, but closer to LL (Fig. 2). This suggest that the L haplotype has stronger influence on the morphology (indices) of the hybrids than the R haplotype. This relationship between the genome and morphology can also be seen in Figs. 3 and 4: one haplotype L added to RR places the resultant RRL close to the mid-values of Root 1. All genotypes with higher proportion of L (1/2, 2/3) are placed closer to "pure" LL. In contrast, morphological similarity of *P. esculentus* hybrids to *P. ridibundus* was recently reported by Krizmanić (2008), but the author did not specify whether the hybrids were diploid or triploid, nor did he verify the genome composition of the analysed individuals.

In the literature, morphological variability of triploid *P. esculentus* with verified genome composition is documented for 49 adults (Hemmer 1977, Ebendal & Uzzell 1982, Tunner 2000). The values of DP/CI and T/CI indices, however, are presented only for 5 LLR and 3 RRL adult individuals (verified by the protein electrophoresis) (Hemmer 1977, Ebendal & Uzzell 1982), so here the sample size is too small to compare the influence of the L and R haplotypes on the morphology. For the remaining 41 individuals, all with LLR genotype verified by the chromosome analysis, only the rarely used LT/CI index is presented (Tunner 2000). Nevertheless, specimens described by Tunner (2000) showed a somewhat "mosaic" pattern of the gene expression — their body size, colouration and hibernation behaviour were more similar to those of RR, whereas their *callus internus* was of the size and shape more typical of LL. Discrepancies in GDE expression can be also observed in e.g. the fish *Cobitis taenia–C. elongatoides* complex. Populations that live in the Moscov River display more distinct GDE (Vasil'ev *et al*. 1989) than populations from central Europe (Kotusz 2008). Such results indicate that the phenotype of hybrids may not always be a linear expression of the ratio of the parental genomes.

Shift in the values of the indices towards the values of *P. lessonae*, observed in hybrids

in the present study may be caused by the fact that most of the investigated individuals originated from regions where mixed *P. lessonae–P. esculentus* populations predominate. We may assume that it can be beneficial for the hybrids to resemble morphologically individuals of the species with which they co-occurr, as was suggested by Tunner (2000), but whether this shift results from population genetics (e.g. presence of hemiclone lineages resulting in *lessonae*-like phenotypes), environmental pressure (e.g. elimination of *ridibundus*-like tadpoles or froglets) or mating choice is not known at the moment.

Another interesting phenomenon was recently described for hybrids of firebellied toads *Bombina bombina* and *Bombina variegata*. Here, GDE is expressed not only in morphology (Vörös *et al*. 2007), but also in habitat preferences (Yanchukov *et al*. 2006). Because *P. lessonae* differs from *P. ridibundus* in the preferred habitat, it would be interesting to see if those preferences in hybrids would also be subject to GDE, as is the morphology. This would constitute additional factor influencing distribution of the central European water frogs. Such broad-scale comparative studies has yet to be carried out, providing a unique possibility to study the genetic and physiological factors that determine the behaviour.

Acknowledgements

Frogs were collected under the following permits: Polish Ministry of Environment Protection and Forestry for performing studies on protected species OP 4072/218 /96; OP 4072/218/98/4501; OP 4201/144/99, and II Local Commission for Ethics in Experiments on Animals 13/02. Study was financed by the State Committee for Scientific Research (Poland), grant no. 6P04C 061 19.

References

Allendorf, F. W., Leary, R. F., Spruell, P. & Wenburg, J. K. 2001: The problems with hybrids: setting conservation guidelines. — *Trends in Ecology & Evolution* 16: 613–622.

Alves, M. J., Coelho, M. M. & Collares-Pereira, M. J. 2001: Evolution in action through hybridization and polyploidy in an Iberian freshwater fish: a genetic review. — Genetics 111: 375–385.

Applied Biostatistics Inc. 1986–2008: NTSYSpc 2.20u software.

Berger, L. 1966: Biometrical studies on the population of green frogs from the environs of Poznań. — *Annales Zoologici* (Warszawa) 23: 303–324.

Berger, L. 2000: *Płazy i gady Polski. Klucz do oznaczania*. — Wydawnictwo Naukowe PWN, Warszawa–Poznań.

Berger, L., Hotz, H. & Roguski, H. 1986: Diploid eggs of *Rana esculenta* with two *Rana ridibunda* genomes. — *Proceedings of The Academy of Natural Sciences of Philadelphia* 138:1–13.

Berger, L., Roguski, H. & Uzzell, T. 1978: Triploid F2 progeny of water frogs (*Rana esculenta* complex). — *Folia Biologica* 26: 135–152.

Berger, L. & Roguski, H. 1978. Ploidy of progeny from different egg size classes of *Rana esculenta* L. — *Folia Biologica* 26: 231–248, Kraków.

Bogart, J. P. 1980: Evolutionary implication of polyploidy in amphibians and reptiles. — In: Lewis, W. H. (ed.), *Polyploidy. Biological relevance*: 341–378. Plenum Press New York and London.

Chen, Z. J. & Ni, Z. 2006: Mechanizms of genomic rearrangements and gene expression changes in plant polyploids. — *BioEssays* 28: 240–252.

Christiansen, D., Jakob, C., Arioli, M., Roethlisberger, S. & Reyer, H.-U. 2010: Coexistence of diploid and triploid hybrid water frogs: population differences persist in the apparent absence of differential survival. — *BMC Ecology* 10: 14.

Cimino, M. C. 1972: Egg-production, polyploidization and evolution in a diploid all-female fish of the genus *Poeciliopsis*. — *Evolution* 26: 294–306.

Dawley, R. M. 1989: An introduction to unisexual vertebrates. — In: Dawley, R. M. & Bogart, J. P. (eds.), *Evolution and ecology of unisexual vertebrates*: 1–18. New York State Museum, Albany New York, USA.

Dytham, C. 2003: *Choosing and using statistics: a biologist's guide*. — Blackwell Publishing, Malden, MA.

Ebendal, T. & Uzzell, T. 1982: Ploidy and immunological distance in Swedish water frogs (*Rana esculenta* complex). — *Amphibia-Reptilia* 3: 125–133.

Ebendal, T. 1979: Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). — *Mitteilungen aus dem Zoologischen Museum in Berlin* 55: 143–152.

Føg, K. 1994: Waterfrogs in Denmark: population types and biology. — *Zoologica Poloniae* 39: 305–330.

Goddard, K. A. & Schultz, R. J. 1993: Aclonal reproduction by polyploid members of the clonal hybrid species *Phoxinus eos-neogaeus* (Cyprinidae). — *Copeia* 3: 650–660.

Gubányi, A. 1995: Biometrical investinagtion of water frogs in the Szigetköz Landscape Protection Area. — *Miscellanea Zoologica Hungarica* 10: 117–126.

Günther, R. 1977: Die Erythrocytengröße als Kriterium zur Unterscheidung diploider und triploider Teichfrösche, *Rana „esculenta"* L. (Anura). — *Biologische Zeitblätter* 96: 457–466.

Hemmer, H. 1977: Studien an einer nordwetdeutschen Grünfroschpopulation als Beitrag zur Bestimmungsproblematik und zur Rolle der Selektion im *Rana esculenta*-Komplex. — *Salamandra* 13: 166–173.

Heppich, S., Tunner, H. G. & Greilhuber, J. 1982: Premeiotic chromosome doubling after genome elimination during spermatogenesis of the species hybrid *Rana esculenta*. — *Theoretical and Applied Genetetics* 61: 101–104.

Juszczyk, W. 1987: *Płazy i gady krajowe*. — Państwowe Wydawnictwo Naukowe, Warszawa.

Kearney, M., Fujita, M. K. & Ridenour, J. 2009: Lost sex in the reptiles: constraints and correlations. — In: Schön, I., Martens, K. & van Dijk, P. (eds.), *Lost sex the evolutionary biology of parthenogenesis*: 447–474. Springer, Dordrecht–Heidelberg–London–New York.

Kim, I.-S. & Lee, J.-H. 1990: Diploid-triploid Hybrid Complex of the Spined Loach *Cobitis sinensis* and *C. longicorpus* (Pisces, Cobitidae). — *Korean Journal of Ichthyology* 2: 203–210.

Kim, I.-S. & Lee, J.-H. 1995: Interspecific hybridization between triploid hybrid Fish, *Cobitis sinensis-longicorpus* and two diploid species from Korea. — *Korean Journal of Ichthyology* 7: 71–78.

Kim, I.-S. & Lee, J.-H. 2000: Hybridization experiment of diploid-triploid cobitoid fishes, *Cobitis sinensis-longicorpus* complex (Pisces, Cobitidae). — *Folia Zoologica*, 49: 17–22.

Kotlik, P. & Šůlova, K. 1994: Syntopic occurence of three taxa of water frogs in Czech Republic. — *Zoologica Poloniae* 38: 417–424.

Kotusz, J. 2008: Morphological relationships between polyploid hybrid spined loaches of the genus *Cobitis* (Teleostei, Cobitidae) and their parental species. — *Annales Zoologici* (Warszawa) 58: 891–905.

Krizmanić, I. I. 2008: Water frogs (*Rana esculenta* complex) in Serbia — morphological data. — *Archives of Biological Sciences*, Belgrade 60: 449–457.

Mallet, J. 2005: Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* 20: 229–237.

Ogielska, M. (ed.) 2009: *Reproduction in amphibians*. — Science Publishers, U.S.

Ogielska, M., Kierzkowski, P. & Rybacki, M. 2004: DNA content and genome composition of diploid and triploid water frogs belonging to the *Rana esculenta* complex (Amphibia, Anura). — *Canadian Journal of Zoology* 82: 1894–1901.

Otto, S. P. & Whitton, J. 2000: Polyploid incidence and evolution. — *Annual Review of Genetics* 34: 401–437.

Pagano, A. & Joly, P. 1998: Limits of the morphometric method for field identification of water frogs. — *Alytes* 16: 15–23.

Plötner, J. & Klinkhardt, M. 1992: Investigations on the genetic structure and the morphmetry of a pure hybrid population of *Rana* kl. *esculenta* (Anura, Ranidae) in North Germany. — *Zoologischer Anzeiger* 229: 163–184.

Plötner, J. 2005: Die Westpaläarktischen Wasserfrösche. — *Beiheft Zeitschrift für Feldherpetologie* 9: 1–160.

Plötner, J. 2010: Möglichkeiten und Grenzen morphologisher Methoden zur Artbestimmung bei europäischen Wasserfröschen (*Pelophylax esculentus*-Komplex). — *Zeitschrift für Feldherpetologie* 17: 129–146.

Plötner, J., Becker, C. & Plötner, K. 1994: Morphometric and DNA investigations into European water frogs (*Rana* kl. *esculenta* Synklepton (Anura, Ranidae)) from different population systems. — *Journal of Zoological Systematics and Evolutionary Research* 32: 193–210.

Polls Pelaz, M. & Graf, J. D. 1988: Erythrocyte size as an indicator of ploidy level in *Rana* kl. *esculenta* before and after metamorphosis. — *Alytes* 7: 53–61.

Schultz, R. J. 1969: Hybridization, unisexuality and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. — *American Naturalist* 103: 605–619.

Schultz, R. J. 1980: Role of polyploidy in the evolution of fishes. — In: Lewis, W. H. (ed.), *Polyploidy. Biological relevance*: 313–340. Plenum Press New York and London.

StatSoft Inc. 2007: *STATISTICA (data analysis software system)*, ver. 8.0. — www.statsoft.com.

Suomalainen, E., Saura, A. & Lokki, J. 1987: — *Cytology and Evolution in Parthenogenesis*. — CRC, Boca Raton.

Tunner, H. G. 2000: Evidence for genomic imprinting in unisexual triploid hybrid frogs. — *Amphibia–Reptilia* 21: 135–141.

Uzzell, T. & Berger, L. 1975: Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae* and their hybridogenetic associate, *Rana esculenta*. — *Proceedings of The Academy of Natural Sciences of Philadelphia* 127: 13–24.

Uzzell, T., Berger, L. & Günther, R. 1975: Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia, Salientia). — *Proceedings of The Academy of Natural Sciences of Philadelphia* 127: 81–91.

Uzzell, T., Günther, R. & Berger, L. 1977: *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia, Salientia). — *Proceedings of the Academy of Natural Sciences of Philadelphia* 128: 147–171.

Vasil'ev, V. P., Vasil'eva, E. D. & Osinov, A. G. 1989: Evolution of a diploid-triploid-tetraploid complex in fishes of the genus *Cobitis* (Pisces, Cobitidae). — In: Dawley, R. M. & Bogart, J. P. (eds.), *Evolution and ecology of unisexual vertebrates*: 153–169. New York State Museum, Alabany, New York.

Vörös, J., Szalay, F. & Barabás, L. 2007: A new method for quantitative pattern analysis applied to two European *Bombina* species. — *Herpetological Journal* 17: 97–103.

Vrijenhoek, R. C. 1989: Genetic and ecological constraints on the origins and establishment of uni- sexual vertebrates. — In: Dawley, R., Bogart, J. (eds.), *Evolution and ecology of unisexual vertebrates*: 24–31. New York State Museum, Albany, New York.

Wijnands, H. E. J. 1979: Partial ecological isolation of *Rana lessonae* and *Rana esculenta* as a mechanism for maintenance of the hybrid form, *Rana esculenta* (Anura, Ranidae). — *Mitteilungen aus dem Zoologischen Museum in Berlin* 55: 131–142.

Yanchukov, A., Hofman, S., Szymura, J. M., Mezhzherin, S. V., Morozov-Leonov, S. Y., Barton N. H. & Nürnberger, B. 2006: Hybridization of *Bombina bombina* and *B. variegata* (Anura, Discoglossidae) at a sharp ecotone in western Ukraine: comparisons across transects and over time. — *Evolution* 60: 583–600.