The Ploidy and Genetic Structure of Hybrid Populations of Water Frogs *Pelophylax esculentus* Complex (Amphibia, Ranidae) of Ukraine Fauna

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Abstract—The complex study, including allozyme variability and cytometry of hybrid populations of green frogs *Pelophylax esculentus* (L., 1758) complex has confirmed that the only region of Ukraine where allodiploid are encountered frequently is the Severski Donets basin (9% of all hybrids). In other areas, only two polyploidy hybrids (0.9%) and one probably autopolyploid individual of each parental species have been registered. According to allozyme specters, all three polyploidy hybrids from the Severski Donets basin were males and belonged to biotype *P. esculentus* (=*lessonae*) − 2 ridibundus, and their population in this region has halved during the past decade.

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INTRODUCTION

The complex of western palaearctic water frogs *Pelophylax esculentus* (L., 1758) s.lato where no less than eight new species have been described [1] is interesting not only because of its hidden genetic variety but also due to peculiarities of interspecies relations, including the exchange of genetic information [2−5]. The hybridization of the two most dominant and widespread species of lake *P. ridibundus* (Pallas, 1811) and pond *P. esculentus* (L., 1758) (=*lessonae* (Camerano, 1882) frogs is at the base of these processes that result in the creation of allodiploid hybrids *P. ridibundus-esculentus*, which are usually considered as the specific taxon, clade *P.* kl. *esculentus* [6, 7]. These hybrids are fertile but reproduce only by crossbreeding because F_2 hybrids are not vital [8−10]. Their gametogenesis occurs in two ways: with the creation of haploid or sometimes diploid gametes.

The first type of gematogenesis in green frogs occurring in most cases is usually called hybridogenesis when the elimination of one of the parental genomes in a hybrid occurs during gametogenesis [11−13]. As a result, the diploid generation appears at backcrossing: either this is characteristic of one of the parental species in whose genome there is an introgression of the genetic material of the second parental species [2−5], or hybrids-allodiploids with the genetic structure of a F_1 hybrid or hybrids with a recombination of genetic material. Since descendants obtain one nonrecombinant genome from one of the parental species, the method of sexual reproduction was defined as polyclonal reproduction.

The ameiosis reproduction of hybrids *P. ridibundus-esculentus* is unique and can be found in single geographical populations whose hybrids produce haploid gametes. Parthenogenesis is absent but allotriploid hybrids appear as the result of the fusion of diploid hybrid gamete and haploid parental species [14−16]. It should be mentioned that the character of gametogenesis can be indeterminate and therefore hybrids from the same reservoir can produce only haploid gametes and others only diploid gametes [17] or both diploid and triploid gametes. There are cases described when the females produce only haploid gametes and males divide into two groups, producers of haploid and diploid gametes, respectively [18].

The geographical distribution of triploid hybrids has a mosaic character. Mostly they can be found in the reservoirs of Western, Northern, and Central Europe: in Germany [12−14], Poland [15, 16, 18], Denmark [17], France [19], Sweden [20], Hungary [21], Slovakia [22] and the Netherlands [23]. In these areas, the frequency of polyploids vary within a wide range (from 4 to 100%) and in case the polyploids are dominant, it is thought that hybrids [17] are able to carry out autonomous reproduction without the involvement of parental species. There are few examples of all-hybrid populations and their stability over time has not been studied. In the greater part of Eastern Europe (Belarus, Russia, Ukraine, Latvia, and

Estonia), polyploids are not found [11−14]. The single exception is Severski Donets [24, 25], which is the southeastern border of the combined habitation of parental species. The polyploids within the Ukrainian part of the basin of this river were on average 20% of the studied individuals [24] that allowed the conclusion on the occurrence of mass polyploidy.

Because of the disjunctive location of polyploid populations of green frogs, the question on the presence of triploid hybrids within Ukraine, whose populations are the link connecting polyploid settlements of northwestern Europe and Eastern Ukraine, is still not clear. At the same time, one cannot say that studies of the genetic structure of hybrids on the territory of Ukraine were not undertaken. However, most of these were carried out without specific analysis of the ploidy and the conclusions on diploid state of the hybrids in these works were made based on the character of electrophoresis specters, which, as is well-known [26], has a clear effect on the dose gene in triploid frogs. This method of the identification of polyploids is reliable in case of a series of polyploids, while single cases can be skipped. Other studies [27] aimed to determine ploidy but were undertaken without gene marking and without consideration of the genetic structure of hybrids. Moreover, conclusions based only on cytometry studies are not reliable because their resolution for the separation of triploid biotypes is not high [28]. Thus, there is a demand for an integrated study of the genetic structure of the green frog of Ukrainian fauna, including both gene marking as well as the analysis of the ploidy of the genome.

MATERIAL AND METHODS

The series of frogs collected during 2007−2008 in reservoirs of Ukraine and covering almost the whole territory where there is hybridization of pond and lake frogs served as the subject of the study. In total, they include 34 samples (table) including 1070 individuals.

Analysis of the allozyme variability of some enzymes encoded by proper loci was carried out in all species and included: aspartate aminotransferase (*Aat*-1, *Aat*-2), analyzed in muscles; lactate dehydrogenase (*Ldh*-B) in muscles or buds; and nonspecific esterases (*Es*-1) and albumin (*Alb*), identified in tissues of buds and liver. It is certain that the loci *Ldh*-B, *Alb*, and *Alb*-2 were species-specific. The latter locus was rarely used compared to the former two because of often its specters combined with specters of polymorphic locus *Aat*-1. Loci *Aat*-1 and *Es*-1 were polymorphous and diagnostically valuable only for populations from western regions. Electrophoresis was carried out in 7.5% polyacrylamide gel in a tris-EDTA-borate system of buffers [29].

Ploidy was determined by measurement of the erythrocyte size according to the method tested by Schemeller et al. [19] and used for the mass analysis of
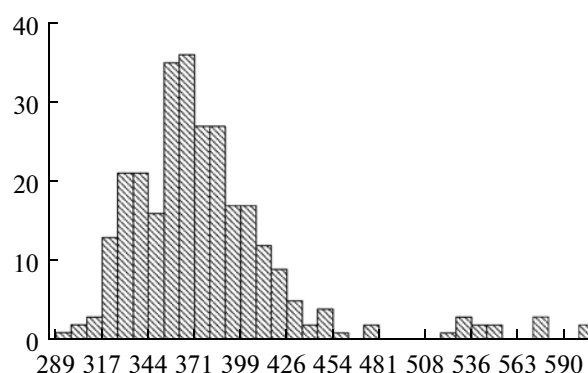
Distribution of green frog species (hybrids and parental species) according to the average area of erythrocytes: the horizontal axis is the average square of the erythrocytes, μm^2, and the vertical axis is the number of species.

materials in population studies [15]. The size of erythrocytes in single individuals was determined by the calculation of the average obtained after the measurement of 20 randomly selected morphologically normal cells.

RESULTS

The distribution of frogs according to average individual sizes of erythrocytes is shown in the figure. Obviously, in this case, there are two different distributions according to volume. The first one includes over 98% of the studied samples and includes frogs with the erythrocytes covering an area from 264 to 458 μm, with an average area of 359 ± 1.3 μm. This group includes, as individuals, two parental types as hybrids. The second distribution includes only 17 individuals with the erythrocytes' size in a diapason ranging from 500 to 609 μm, with an average value of 553 ± 9.4 μm and includes mostly hybrids and single individuals of the parental species. The average values of these two distributions is expressed as a ratio 1 : 1.5, which complies with an increase of the area of the erythrocytes in triploid individuals in comparison with diploid individuals, which is usually not less than 33% [19]. The single species with an average value of 472 μm according to its specter and genotype Ldh-$B^{64/77\text{-}100}$ is the hybrid *P. esculentus−2 ridibundus*. It was probably aneuploid.

As a result of the biochemical gene marking, 16 biotypes of green frogs were identified, including parental species, and various hybrid and differentploid forms (table). The lake frog *P. ridibundus*, a species that has recently been actively widening its area had the largest mass. This area includes about 56% of all species. The share of the second parental species *P. esculentus* was lower and amounted to about 10%. Hybrids *P. esculentus* × *P. ridibundus* made up 34% of frogs. This value is greater for the *P. esculentus* species and shows primarily the possibility of autonomous

The composition of studied selections of green frogs in Ukraine

Selection	Coordinates Lat/Long	P. ridibundus			P. esculentus–ridibundus								P. esculentus–2 ridibundus			P. esculentus	
		2n R	2n Ri	3n	F1 ♂	F1 ♀	F1 h	F1 ?	B ♂	B ♀	B ?	Rec ♀	♂	♀	?	2n	3n
Merla river	50.0/35.0	9	1														
Gaidari village	49.6/36.3	51			81	1			1				12		1		
Luganka river	48.0/37.7	12			12								1				
Sukhoi Torets river	48.8/37.6	31							1								
Stanichno–Luganskoe	48.6/39.4	40			46												
Gomol'sha	49.6/36.3															54	
Krasnokutsk town	50.0/35.1	19						8									1
Vorskla river	49.6/34.5	26	1					1									
Niznii Dnepr	49.6/34.5	10	1														
Erchiki village	46.6/32.7		1		7	4		2								4	
Feofaniya	50.0/29.6	16															
Teterev river	50.6/31.0	17			1		1										
Samara river	50.2/28.6	17															
Novobelichi	48.77/35.31	27	3													4	
Romen river	50.4/30.3	12	2													1	
Sivorotka river	50.7/33.4	1	1													5	
Bogdanovka village	50.8/34.9	29			1												
Irsha river	48.5/36.1							3								6	
Pushcha-Voditsa	50.5/28.4				3				10							2	
Sluch river	50.6/30.4	18		1	1												
Seim river	51.3/32.9	40			10	2		10	8	1	7					11	
Shevchenko village (canal)	51.1/33.2	1			1						1					12	
Oster river	51.0/31.9				5	1			16								
Berezani town	49.4/24.9	8															
Zhukov village	49.5/24.9	18			1												
Podol'e	48.9/25.5	56			22	4		5									
Kamenka river	48.7/30.2	26						9								4	
Nizhnii Dunai	45.4/29.6	26			11											1	
Manevichi village	51.3/25.5				8	20											
Shatskie lakes	51.4/23.9	19										1		1			
Gorin' river	50.6/26.7	12	3		20												
Total		541	13	1	230	32	1	38	36	1	8	1	13	1	1	104	1

Note: R – Pelophylax ridibundus; Ri – Pelophylax ridibundus with introgressions; F1 – hybrids of first generation; B – backcrosses; Rec – hybrids-recombinants; h – hermaphrodite; ? – sex is not determined due to young age.

reproduction for hybrids and, second, that hybridization with the lake frog can be a reason of its gradual disappearance within the south area. Hybrid species appeared as the result of reverse breeding with species of parental species (lake frogs with introgressions of genetic material from pond frog and allodiploids with recombinations of genetic material), and in total made up 5% of the number of studied species (table).

Fifteen of seventeen triploid frogs were hybrids and two were parental species. The species identified as the *P. ridibundus* was characterized with an average size of erythrocytes 502 μm and did not have any introgressions of *P. ridibundus* genes. Another triploid species with average sizes of erythrocytes of 501 μm in allozymes was determined as *P. esculentus*. Thus, the share of polyploids among the studied frogs was 1.6%, and their share among hybrids was 4% and among parental species their share was 0.3%. Most of the polyploid hybrids (13 species) were identified in Severskii Donets, one in Nizhnii Dnepr, and one in the lakes of Volin. The peculiarities of specters in a particular locus, *Ldh*-B, which provides us with reliable and reproducible results, and also *Aat*-1 confirm that all frogs belong to biotype *P. esculentus—2 ridibundus*, or, as they are usually interpreted by researchers of green frogs [16], are hybrids of the RRL-type. The hybrid species are characterized either by triheterozygous specters of *Ldh*-B$^{64/77-100}$, where the products of two alleles inherent to *P. ridibundus* were represented, or by genotypes with double products of one of the genes often belonging to this species, the *Ldh*-B$^{64/77-100}$, and less often, the *Ldh*-B$^{64/77-77}$. The hybrid triploid from Volin whose genotype carries alleles *Ldh*-B^{88} and *Ldh*-B^{100} and the dose gene in its specter cannot be determined due to the close mobility of alleles' products when its biotypical diagnostic was carried out in locus *Aat*-1, whose specter had a clear prevalence of dose genes specific for *P. ridibundus*.

All triploid hybrids were found in populations inhabited together with hybrids, which were mostly males, only by the lake frog (table). It indirectly shows that triploid hybrids are created by the backcrossing of allodiploids, producing diploid sperm, with females *P. ridibundus*. Triploid species of parental species, which occur infrequently, are probably of autoplyploid origin.

DISCUSSION

The study of the genetic structure of hybrid populations of frogs, including the analysis of allozymes and cytometry, confirms the results obtained previously. First, the rarity and scarcity of the hybrid polyploid in populations of green frogs of Eastern Europe and, second, the relatively high number of hybrid triploids of frogs in the Severski Donets basin. The populations of the Dnieper, Dniester, Zapadnii Bug, and Danube included 0.9% polyploids from the total amount of analyzed hybrids, while in the Severski

Donets basin their share was about 9%, although this value is half the value that was noted previously for this region [24]. This should draw attention to the absence in our material of individuals of biotypes *P. 2 esculentus—idibundus* whose frequency, as has been previously established by the same researchers [24] was not less than in biotypes *P. esculentus—2 ridibundus*. It should be mentioned that the equal representation of two alternative biotypes in this region is doubtful because the second parental species *P. esculentus* in the floodplain of Severski Donets was always small and therefore the backcrossing of hybrid males with females of this species was a rare event and there should only be a few of these hybrids. The reverse breeding of hybrid males with females of *P. ridibundus* are normal and the only way of reproduction for hybrids in populations of Severki Donets, therefore in case of the production of a small number of diploid sperm by the hybrids, the triploid *P. esculentus—2 ridibundus* can be produced.

The estimations obtained in this experiment on the distribution of polyploidy and the correlation of triploid biotypes in Servski Donets can differ from the results of previous studies [24] due to two reasons. The first reason is the insufficient sensitivity of the previously used method of flow cytometry that does not allow us to divide two alternative triploid biotypes according to genome size, which results in an artificially increased number of hybrids of *P. 2 esculentus-ridibundus* in this river system. This aspect has already been discussed by other researchers [24]. The second reason, explaining the decrease in the number of polyploid species is related to the drastic alteration of floodplain stations, drying wetlands, and salinity of lakes that has occurred in Severskii Donetsk over the past decade. As a result, the pond frog, which is rare in the floodplain of this river and vulnerable at the southern border area, has disappeared and thus hybridization has terminated. Besides, hybrids, which are more suited to life on land than lake frogs, having found themselves in drought conditions, have also disappeared. As a result, the very rare hybridization, caused by the drastic reduction in the number of pond frogs and hybrids, could lead to hybridization cases, which resulted in the appearance of triploids, occurring very rarely.

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