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The study of three hemiclonal population systems of *Pelophylax esculentus* complex from the Seversko-Donetskiy center of green frogs' diversity

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The structure of hemiclonal population systems (HPS) of *Pelophylax esculentus* complex from the Iskov pond (Gaydary village, Zmiyiv District), the Lower Dobritskiy pond (the region of Gomolsha river floodplain, Dobritskiy yar, the territory of the National Park "Gomolshanski lisy") and from the pond near the village Zhovtneve (Vovchanskiy District, Kharkiv Region) was studied. All studied HPS were defined as REt-type. We observed the triploid hybrids number increasing in the HPS from the Iskov pond. The large part of triploid hybrids was found in the HPS of the Lower Dobritskiy pond. It was observed that all triploid hybrids from the pond near the village Zhovtneve were females. There was significant bond between the diploid erythrocytes size and sex of the frogs. It seems that triploids in this HPS are originating in other way compared to other HPS. There were two groups of females differ in body size which possibly corresponds to different ontogenetic strategies of green frog females. The males have a normal size distribution.

Key words: *hemiclonal population systems, Seversko-Donetskiy center of green frogs diversity, Pelophylax esculentus complex, species composition, triploid hybrids, size distribution.*

Дослідження трьох популяційних систем *Pelophylax esculentus* complex Сіверсько-Донецького центру різноманіття зелених жаб

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Вивчена структура геміклональних популяційних систем (ГПС) *Pelophylax esculentus* complex, що мешкають в Іськовому ставку (с. Гайдари, Зміївський район), Нижньому Добрицькому ставку (район заплави р. Гомольша, Добрицький яр, територія НПП «Гомільшанські ліси» у тому ж районі) та у ставку сел. Жовтневе (Вовчанський район Харківської області). Всі вивчені ГПС належать до REt-типу. В ГПС Іськового ставу в 2013 р. зареєстровано зростання частки триплоїдів порівняно з 2012 р. В ГПС Нижнього Добрицького ставку знайдена велика кількість триплоїдних гібридів. В ГПС сел. Жовтневе триплоїди представлені тільки самками. Серед диплоїдів цього ставка зареєстрований значущий зв'язок розмірів еритроцитів із статтю жаб. Ймовірно, механізм відтворення триплоїдів в ГПС сел. Жовтневе відрізняється від такого двох інших систем. Розмірний розподіл демонструє наявність двох груп самок, яка, вірогідно, відповідає двом онтогенетичним стратегіям. Розподіл самців має нормальний характер.

Ключові слова: *геміклональні популяційні системи, Сіверсько-Донецький центр різноманіття, Pelophylax esculentus complex, видовий склад, триплоїдні гібриди, розмірний розподіл.*

Исследование трех популяционных систем *Pelophylax esculentus* complex Северско-Донецкого центра разнообразия зеленых лягушек

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Изучена структура гемиклональных популяционных систем (ГПС) *Pelophylax esculentus* complex, обитающих в Иськовом пруду (с. Гайдары, Змиевской район), Нижнем Добрицком пруду (район поймы р. Гомольша, Добрицкий яр, территория Национального природного заповедника «Гомольшанские леса») и в пруду пос. Жовтневое (Волчанский р-н Харьковской области). Все исследованные ГПС относятся к REt-типу. В ГПС Иськова пруда зарегистрировано увеличение количества триплоидных гибридов в 2013 г. по сравнению с 2012 г. В ГПС Нижнего Добрицкого пруда обнаружено высокое содержание триплоидных гибридов. В ГПС пруда пос. Жовтневое триплоиды представлены только самками. Для диплоидов в этом пруду показана значимая связь размеров эритроцитов с полом лягушек. Видимо, возникновение триплоидов в данной ГПС связано с иным механизмом, чем в двух других изученных ГПС. В размерном распределении всех лягушек показано наличие двух групп самок, которое, возможно, соответствует различным онтогенетическим стратегиям. Распределение самцов носит нормальный характер.

Ключевые слова: *гемиклональные популяционные системы, Северско-Донецкий центр разнообразия, Pelophylax esculentus complex, видовой состав, триплоидные гибриды, размерное распределение.*

Introduction

There are three main forms of European green frogs (*Pelophylax esculentus* complex): the pool frog, *Pelophylax lessonae* (Camerano, 1882), the lake frog, *Pelophylax ridibundus* (Pallas, 1771), and the edible frog, *Pelophylax esculentus* (Linnaeus, 1758). In 1964, L. Berger discovered the green frogs' phenomenon of interspecific hybridization (Berger, 1964) and has found that *P. esculentus* is an interspecies hybrid. The main distinctive feature of green frogs is a hemiclonal inheritance comprising the clonal transfer of one of the parental genomes to gametes, i.e. without recombination. In the case of crossing between hybrid frogs producing gametes of the same parental species, offspring is usually unviable. This phenomenon is called hybridolysis (Plötner, 2005).

Apart from that, different forms of the green frogs from *Pelophylax esculentus* complex are able to live together and breed in the same area despite of their ecological specificity. This property determines the existence of various types of hemiclonal population systems (HPS), which differ by the presence and dominance of the one or another form, as well as the nature of the gametes produced by hybrid forms (Shabanov et al., 2009; Shabanov, Litvinchuk, 2010).

There are several basic types of HPS (Uzzell, Berger, 1975):

L-type – ordinary populations of *P. lessonae*, the pool frog;

R-type – ordinary populations of *P. ridibundus*, the lake frog;

E-type – so called «pure» populations of hybrids, *P. esculentus*;

LR-type – population systems of *P. lessonae* and *P. ridibundus*; breeding produces no hybrids;

LE-type – population systems of *P. lessonae* and *P. esculentus*;

RE-type – population systems of *P. ridibundus* and *P. esculentus*;

LER-type – mixed systems, comprising both parental species and their hybrids.

It is common to indicate the presence of triploids in HPS by adding the letter "t" to mark the population system. For example, REt-type systems compose of *P. ridibundus*, as well as di- and triploid *P. esculentus*; Et-type systems consist of diploid and triploid hybrids only.

The features of Seversko-Donetskiy center of green frogs' diversity. The region of the HPS' high diversity is associated with the Seversky Donets river basin within Kharkiv and Donetsk Regions. Moreover, polyploid hybrids are often found in this region (Borkin et al., 2004), called Seversko-Donetskiy center of green frogs' diversity (Shabanov et al., 2009). The population systems of R, RE, REt and Et-types with *P. esculentus* of both sexes have been found in the Seversky Donets river valley. Coexisting di- and triploid hybrids is typical for Seversky Donets floodplain and adjacent habitats (Korshunov, 2010).

Seversko-Donetskiy center of green frogs' diversity is defined by the following: (i) *P. ridibundus* and various forms of *P. esculentus* inhabit the center; mature *P. lessonae* are absent; individual representatives of the parental species have been recorded only at the stage of fingerlings (froglets before the first hibernation). Subsequently all genomes of the *P. lessonae* are transmitted via diploid and triploid hybrids. (ii) Diploids of the both sexes are numerous among *P. esculentus* in the diversity center, as well as a significant number of triploids of the both sexes: LRR (more numerous) and LLR, and also single immature tetraploids LLRR were found. (iii) Both sexes' specimens, producing either *P. lessonae* gametes or *P. ridibundus* gametes, or both *P. lessonae* and *P. ridibundus* gametes were observed among diploid *P. esculentus*. (iv) Genetic diversity of *P. lessonae* genomes transmitted via hybrid frogs in this center is not lower than that of F1 hybrids of those regions inhabited by both parental species (Mezhzherin et al., 2010). (v) The recombinant specimens have been registered among green frogs from this center i.e. *P. ridibundus* genome has fragments of the *P. lessonae* genome (Mezhzherin et al., 2005).

The history of previous researches. The most long lasting studies of the HPS structure were undertaken at the Iskov pond (hereinafter – IpHPS), situated near the biological station of V.N.Karazin Kharkiv National University (Gaidary village, Zmiyiv District, Kharkiv Region). Initially the E-type system was described there (Lada, 1995). This HPS is represented by almost exclusively diploid specimens of the *P. esculentus* of both sexes. However, because the pond was hauled down, significant change of the HPS structure occurs. After the pond restoring, spawning areas almost without females have been observed for a long time. Tadpoles haven't been recorded in the pond since 2004 till 2011, when tadpoles and then fingerlings were observed in large quantities. Further observations have led us to conclusion that the frog reproduction in the pond is restoring. Suspected mechanisms of the HPS reproduction, considering females coming from other spawning habitats as a source of new clonal genomes, have been described (Meleshko et al., 2012). This allows us to assume that the dynamics of the IpHPS structure is of considerable interest to study.

HPS of Lower Dobritskiy Pond (hereinafter – DpHPS, near the Gomolsha river valley, the territory of National Park "Gomolshanski lisy") was a subject of primary interest. The single specimen of the hybrid tetraploid frog was recorded close to the habitat of this HPS (Borkin et al., 2004). In the top of that, high proportion of triploid hybrids was recorded for this HPS.

The HPS of the Zhovtneve (hereinafter – ZhpHPS, Vovchanskiy District, Kharkiv Region) is located far away from the two latter. The high proportion of triploid hybrids is typical for the green frogs from this HPS.

In all HPS which were in focus of our studies, according to the data of Yu.Rozanov and S.Litvinchuk, RL genotypes were recorded. In addition to that, RRL triploid form was recorded from the IpHPS, and RL – in the DpHPS and ZhpHPS. Their numbers have not been estimated earlier, because separate specimens of particular interest were collected instead of the representative samples. The genotypes and ploidy level of specimens were determined by DNA flow cytometry. The details of the technique have been published previously (Borkin et al., 2001).

The study of these three HPS can provide valuable information for understanding the reproduction processes within *Pelophylax esculentus* complex in the Seversko-Donetskiy center of green frogs' diversity.

Materials and methods

The research was undertaken in June-July, 2012–2013. The mature green frog individuals were collected from Iskov Pond (Gaydary village, Zmiyiv District), Lower Dobritskiy Pond (near the Gomolsha river valley, Dobritskiy tract, territory of the National Park "Gomolshanski lisy") and from the pond in the Zhovtneve village (Vovchanskiy District, Kharkiv Region). Collection sites and the information about the samples are composed in Table 1.

Table 1.

The studied samples of green frogs

Locality	Geographic coordinates	2012, number	2013, number	Total number	
Iskov pond	N 49˚ 37′ 35″ E 36˚ 17′ 07″	161	93	254	433
Lower Dobritskiy Pond	N 49˚ 33′ 22″ E 36˚ 18′ 39″	25	76	101	
pond in the village Zhovtneve	N 50˚ 08′ 04″ E 36˚ 46′ 01″	43	35	78	

Field work was carried out using standard techniques. Animals were caught at night with a light tube or in the daytime manually or with a hand net. Captured frogs were placed in wet canvas or mesh bags or in plastic boxes. In some cases, during the temporary handling of the animals in the laboratory, they were placed in cans or plastic bottles, which lay on its side without water, once a day frogs were washed with running water.

Determination of the species and sex was carried out visually based on complex of the morphological characters (Korshunov, 2010; Lada, 1995). It is known that visual determination of green frogs based on morphological features does not allow making a precise assumption on their species status. In particular, the triploid hybrid individuals are harder to distinguish visually from individuals of the parental species due to the effect of gene dosage. However, determination of ploidy level by measuring the average length of erythrocytes, the results of which are regularly confirmed by karyological research, significantly reduces the probability errors in the determination.

All specimens from the ZhpHPS aliquot, 99 individuals from the DpHPS aliquot and 183 individuals from the IpHPS aliquot were analysed cytologically, i.e. erythrocytes size was determined. The air dried blood smears of each specimen were made according to the standard technique: a drop of blood obtained from a frog finger was coated on a slide glass and smeared with a thin layer of the second slide edge (Bondarieva et al., 2012). Smears were dried up and photographed under high magnification (13 of a light microscope using a USB-eyepieces camera. The object-micrometer was photographed under the same conditions. Major axis length measurement of 15–20 erythrocytes was performed on photos using the PDF XChange Viewer software. All measurements are given in μ. Sex composition of samples is composed in table 3.

Results and discussion

The composition of the samples studied is represented in Tables 2, 3. Only data for specimens with cytologically determined ploidy level are given in Table 2; Table 3 includes all sample data. The diploid specimens of the *P. esculentus* dominated in number of green frogs taxocenes of the IpHPS (Table 2). It should be noted that we have registered a significant ($p<0.02$) increasing in the proportion of triploid *P. esculentus* in 2013 in comparison with 2012.

Table 2.

Determination of ploidy of individuals from studied HPS

Sample	Year	*P. ridibundus* 2n, number (%)	*P. esculentus*, 2n, number (%)	*P. esculentus*, 3n, number (%)	Total number
Iskov pond	2012	3 (3%)	96 (96%)	1 (1%)	100
	2013	8 (10%)	68 (82%)	7 (8%)	83
Lower Dobritskiy Pond	2012	1 (4%)	19 (83%)	3 (13%)	23
	2013	9 (12%)	47 (62%)	20 (26%)	76
Pond in the village Zhovtneve	2012	3 (7%)	28 (65%)	12 (28%)	43
	2013	7 (20%)	18 (51%)	10 (29%)	35
Total number (%)		31 (8.5%)	276 (76.5%)	53 (15%)	360

Previously it was assumed that the IpHPS crisis was associated with the loss of all clonal genomes except the male *P. lessonae* genome (Shabanov et al., 2006). The resumption of frog reproduction in the pond during the past years could potentially indicate that the HPS got new clonal genomes. We can only assume what kind of genomes was brought in the pond and by which specimens. It is most likely that the observed change of triploid hybrids proportion in 2013 is also a consequence of the ongoing transformations caused by penetrating of new clonal genomes into HPS. We assume that HPS state is premature in this case, and now it is going to the populational equilibrium.

Table 3.

Sex composition of the studied HPS

Sample	Year	Females, number (%)	Males, number (%)	Total, number
Iskov pond	2012	34 (21)	127 (79)	161
	2013	13 (14)	80 (86)	93
Lower Dobritskiy Pond	2012	-	25 (100)	25
	2013	30 (40)	46 (60)	76
Pond in the village Zhovtneve	2012	18 (42)	25 (58)	43
	2013	17 (49)	18 (51)	35
Total number (%)		112 (26)	321 (74)	433

DpHPS comprised mainly by the diploids of the *P. esculentus* (table 2). A lot of triploid hybrids were recorded there as well as in the previous studies (Borkin et al., 2004). The difference in the proportion of triploids in the samples of 2012 compared to samples of 2013 was not significant. Notably in 2012 exclusively males occur in the sample from this HPS. In 2013, the proportion of females was about 40% (table 3). Apparently, this difference is not associated with a change in the composition of HPS and with distinction in time of the samples collecting. In 2012, the frogs have been caught during the spawn by capturing the males in the spawning areas, but in 2013 the collections were done after spawning.

The results of the ZhpHPS study are of particular interest. Diploids of the edible frogs also dominate there. *P. ridibundus* makes up to 7% of the frogs total number in the sample in 2012 and 20% in the sample in 2013 (table 2), but such difference in the sample composition is statistically insignificant. Triploids of *P. esculentus* accounted for about 30% of the studied samples and were represented exclusively by females (group I, fig. 1). At the same time, triploid hybrids were represented by both sexes in the two previously

described HPS. This distinction indicates that a mechanism of triploids' reproduction in the ZhpHPS is different.

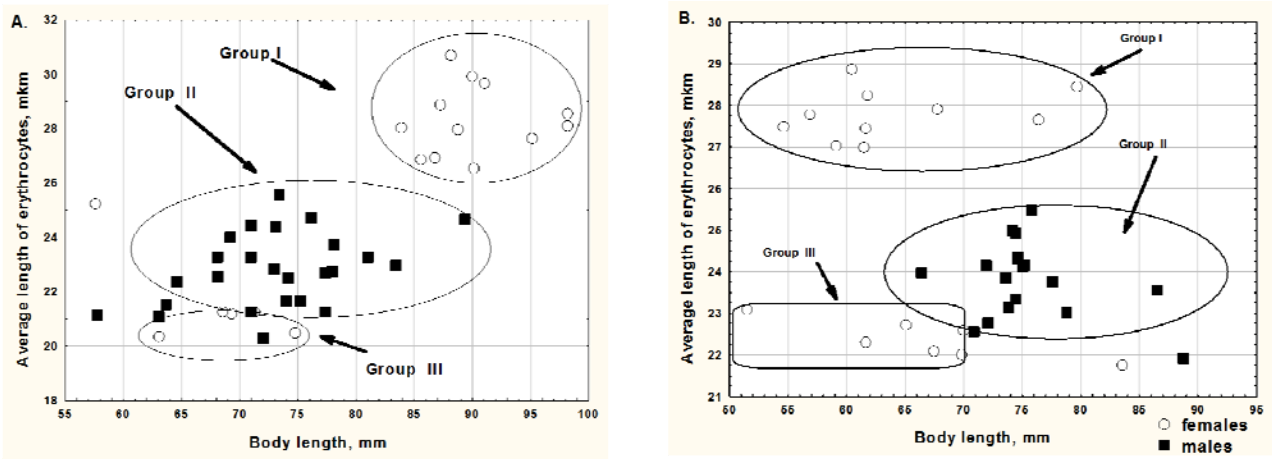

Fig. 1. The length of erythrocytes dependence on body length for green frogs from the pond in the village Zhovtneve. A – sample 2012; B – sample 2013

The second interesting feature of the ZhpHPS was a significant difference in the size of red blood cells of diploid males (group II, fig. 1) and diploid females (group III, fig. 1): size of diploid males' erythrocytes was significantly higher. Once it was assumed that there were both di- and triploid individuals among males. This assumption was tested using karyoanalysis and proved to be wrong (O.Mikhailova, personal communication). We should state that samples from 2013 consist of females with a much smaller body size than in 2012. This is probably due to the fact that in 2012 collection of the material was done during spawning, and in 2013 – after it.

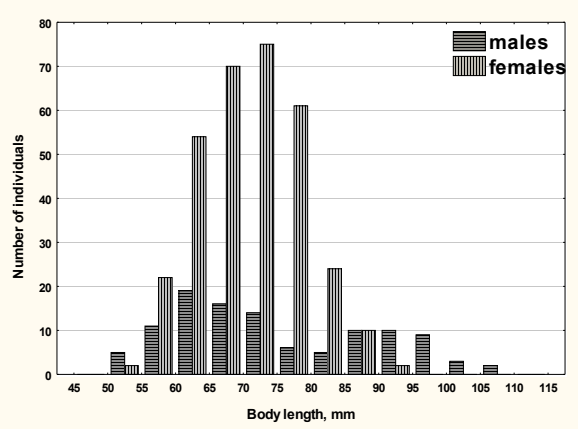

Fig. 2. Distribution of body size of green frogs from the samplings of 2012–2013 years

In addition, we examined the size distribution in the all frogs HPS. The pattern of distribution was found to be similar for all the studied HPS, so all studied samples were considered in fig. 2 together. Distribution of the female size has two peaks corresponding to the relatively small and larger individuals. This result can be considered as an indirect confirmation of the assumption (Usova, 2010) about two growth strategies specific to female of the green frogs: precocity (high fertility with the rapid growth and short life expectancy) and slow-growth (low growth rate and a relatively high life expectancy).

Thus, all studied HPS were REt-type; each of them was characterized by a particular structure. DpHPS was characterized by high percentage of triploid hybrids. Restoration of reproduction was observed in IpHPS, passed through a critical condition. All triploid individuals were females in ZhpHPS. Pattern of the size distribution was similar in all three HPS and characterized by separation of females into relatively small and relatively large individuals.

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