Vestnik zoologii, **48**(6): 511–520, 2014
DOI 10.2478/vzoo-2014-0061

DE GRUYTER OPEN

UDC 597.8:575.853(477)

COMPARATIVE ANALYSIS OF KARYOTYPES OF TWO CRYPTIC SPECIES OF PELOBATID FROGS (AMPHIBIA, ANURA) OF UKRAINE

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Comparative Analysis of Karyotypes of Two Cryptic Species of Pelobatid Frogs (Amphibia, Anura) of Ukraine. Suryadnaya, N. N. — The paper first describes in detail the karyotype of *Pelobates vespertinus* (Pallas, 1771) in comparison with the karyotype of *Pelobates fuscus* (Laurenti, 1768). Comparative morphological analysis of chromosomes has shown that these two cryptic species have a symmetrical karyotype consisting of two-armed chromosomes. It has been established that their chromosome sets consist of 7 pairs of large chromosomes and 6 pairs of small ones. The species differ by the position of the centromere in the chromosomes of the 10th and 11th pairs. The 10th pair of *P. fuscus* is metacentrics, the 11th pair is submetacentric; the 10th pair of *P. vespertinus* is submetacentric, and the 11th one is metacentrics. Secondary constrictions are on the short arms of the 7th chromosome pair. The chromosomal formula for both species is 4 meta-(m) + 7 submeta-(sm) + 2 subtelocentrics (st), 2n = 26, N.F. = 52. Absolute length of all the chromosomes in the karyotype of *P. fuscus* is somewhat larger than that one in the karyotype of *P. vespertinus*. The parameters of relative length are equivalent on average, but they differ in individual chromosomes. On the background of established chromosomal differences between the cryptic species, reliable diagnostic features have not been identified.

K e y w o r d s: karyotype, chromosomes, cryptic species, *Pelobates fuscus*, *Pelobates vespertinus*, Ukraine.

Сравнительный анализ кариотипов двух криптических видов чесночниц рода *Pelobates* (Amphibia, Anura), населяющих Украину. Сурядная Н. Н. — Впервые детально описан кариотип *Pelobates vespertinus* (Pallas, 1771) в сравнении с кариотипом *Pelobates fuscus* (Laurenti, 1768). Сравнительный морфологический анализ хромосом показал, что эти два криптические вида имеют симметричный кариотип, состоящий из двуплечих хромосом. Установлено, что их хромосомные наборы состоят из 7 пар крупных и 6 пар мелких хромосом. Отличаются виды по положению центромеры в хромосомах 10-й и 11-й пар. У *P. fuscus* 10-я пара метацентрик, 11–я — субметацентрик; у *P. vespertinus* 10-я — субметацентрик, 11–я — метацентрик. На коротких плечах 7-й пары хромосом расположены вторичные перетяжки. Хромосомная формула для обоих видов — 4 мета-(m) + 7 субмета-(sm) + 2 субтелоцентрика (st), 2n = 26, N.F. = 52. Абсолютная длина всех хромосом в кариотипе несколько больше у *P. fuscus*, чем у *P. vespertinus*. Параметры относительной длины хромосом в среднем равнозначные, но отличаются по отдельным парам хромосом. На фоне установленных хромосомных отличий между криптическими видами надёжных диагностических признаков не выявлено.

К л ю ч е в ы е с л о в а: кариотип, хромосомы, криптические виды, обыкновенная чесночница, чесночница Палласа, Украина.

Introduction

Issues on the process of species formation (cryptic particularly) in amphibian fauna of Ukraine and neighboring countries have been intensively discussed.

It was established with the use of various biochemical (molecular) methods that the common spadefoot toad is presented by two cryptic species in Ukraine. These species differ significantly in biochemical characteristics and genome size with 6 % differences in the last (Borkin et al., 2001).

The habitat of the common spadefoot toad, *Pelobates fuscus* (Laurenti, 1768), occupies most of the territory of Ukraine (the western part) and its distribution is associated with the basins of the rivers Danube,

Dniester and Dnieper. The Eastern spadefoot toad, *P. vespertinus* (Pallas, 1771), inhabits the eastern part of Ukraine and links up to the Don River drainage, small rivers flowing into the Sea of Azov, as well as the Crimean Peninsula (Litvinchuk et al., 2013). The contact area of these cryptic species (Crottini et al., 2007; Litvinchuk et al., 2013) passes through the territory of Ukraine. Both are found in Kherson and Zaporizhzhya Region. *P. vespertinus* for certain inhabited the Crimea, Lugansk, Donetsk and Kharkiv Region. The common spadefoot toad spreads westward.

Karyological characteristic of species is one of the most important cytological indicators used in the solution of problems in systematics and species formation. It is quite important to establish reliable diagnostic signs at the given stage, what in relation to cryptic species are particularly difficult.

Identification of these signs at the karyotype morphology level is complicated by stable chromosomal conservatism in amphibians when equal number of chromosomes is characteristic not only for the species, but also for the genera and families.

Thus, the aim of our study was to compare the morphological characters and reveal the peculiarities of chromosomes structure in two cryptic species of frog from the territory of Ukraine. It is particularly to be emphasized that the karyotype of *P. vespertinus* was not previously described, and the data on the chromosomes morphology of this species is given in this paper for the first time.

Material and methods

Seventy nine specimens (40 ♀, 39 ♂) (*P. fuscus*— 32 (14 ♀, 18 ♂), *P. vespertinus* — 45 (24 ♀, 21 ♂)) from the territory of Ukraine were used in the study. In total 350 samples (125 — blood / 139 — bone marrow / 86 — testicle): *P. fuscus* — 165 (58/62/45), *P. vespertinus* — 175 (67/77/41) were prepared. All 305 metaphase plates were analyzed: *P. fuscus* — 195, *P. vespertinus* — 110. In the study the samples from the given localities were used: *P. fuscus*: (Dnepropetrovsk Region, Pavlograd District, outskirts of vil. Bulahovka; Zaporizhzhya Region, outskirts of town Energodar; Kherson Region, Tsyurupynsk District, outskirts of vil. Solontsy; Odesa Region, Kiliya District, Vilkovo town); *P. vespertinus* (Donetsk Region, outskirts of Svyatogorsk town; Kharkov Region, Izyum District, outskirts of vil. Snezhkovka; Zaporizhzhya Region, Vasilievsky District, vil. Mayachka; Zaporozhzhya Region, outskirts of Melitopol town; Zaporozhzhya Region, Melitopol District, outskirts of vil. Prilukovka; vil. Terpenie (ibid); Tokmakskij District, outskirts of vil. Snegurovka (ibid); Pologovsky District, outskirts of vil. Semenivka (ibid); Kherson Region, Henichesk District, of vil. Novogrigorevka).

Morphological measurements of chromosomes were performed on 10 plates, 5 for each species (outskirts of Energodar town; outskirts of vil. Bulahovka; Vilkovo town; outskirts of Svyatogorsk town, vil. Prilukovka; vil. Terpenie; outskirts of vil. Snezhkovka). Species belonging of the part of tested animals and all other samples were determined by genome size (Institute of Cytology Russian Academy of Sciences, St. Petersburg).

Standard techniques (Macgregor, Varley, 1986) with some modifications were used for chromosome mounts. 10–15 hours before preparation of the bone marrow and testis mounts the solution of colchicine (concentration 0.8–0.9 %) was intraperitoneally injected. Bone marrow from the femurs was eluted with a solution of KCl 0.75 M. Testis tissue was brought to a homogenous mass (blunt object should be used) and rinsed well with the above solution. Three times (1 per day) intraperitoneal injection of 25 % solution of phytohemaagglutinin (PHA M "Difco") at the rate of 0.2 ml per 20 g of animal body weight preceded the preparing of blood cells mounts. On day 4, and 2 hours (days 5) before the mounts preparation animals were injected with colchicine. Red blood cells (0.2 ml) were poured with 2.0 ml of 0.9 % sodium citrate solution and 0.5 ml of 0.56 % solution of KCl. Tubes with the cell suspension were incubated at 38 °C for 20 min. After centrifugation (5 min) cells were doubly fixed in a solution of glacial acetic acid and methanol (3 : 1 ratio).

Every cell fixation was preceded by a 20-minute room tubes into the cooling chamber. A cell suspension by pipetting was applied to glass slides, followed by air-drying. The resultant mounts were stained with azure-eosin (by Romanovsky).

Karyotype mounts were examined with a binocular 4 microscope objective KONUS # 5306 CAMPUS 1000x, photographed with a digital camera Digital Camera for Microscope DCM300 (5 Mpixels, USB2.0) using ScopePhoto program. Images editing and measurements were carried out with Photoshop and ImageJ respectively. Every four chromosome arms of each homologue were measured. Chromosome arms with secondary constrictions were measured integrally. Selection of homologous chromosomes was carried out by the position of the centromere.

In the analysis all chromosomes parameters were examined. Typology was determined according to the standard classification (Levan et al., 1964; Macgregor, Varley, 1986). For chromosome submetacentric type the expression "more metacentric" chromosome with centromeric index equal to 36–45, and "less metacentric" at centromeric index 26–35 was used (Macgregor, Varley, 1986). Types of chromosomes were determined by average index with a standard statistical error. The significance of differences was tested by the Kolmogorov-Smirnov index using the program Statistica. Mounts are stored in the private collection of the author (Melitopol Institute of Ecology and Social Technologies).

Results

As a result of the morphological analysis it was revealed for *P. vespertinus* and validated for *P. fuscus*, that these cryptic species have symmetrical karyotype, which is represented by two-arm chromosomes. Diploid karyotype of studied species have 26 chromosomes (2n = 26), including 7 pairs of large and small 6 pairs. Chromosomal formula for both species is 4 meta- (m) + 7 submeta- (sm) + 2 subtelocentrics (st), 2n = 26, NF = 52. Sexual dimorphism was not detected.

Primarily, it should be noted that the chromosome dimensions can vary in karyotypes, i. e. with identical typing, homologs can be of different sizes. Despite the fact that the types of chromosomes were determined based on the average index, the variation range (min-max) can be quite wide, up to the presence of different types on different chromosomes in metaphase plates. The morphology of the chromosomes of the two species is somewhat similar, but it can be traced distinctive species specificity.

So the 1st, 2nd, 3rd and 4th pair of the both species are submetacentric what corresponds to the value of centromeric (30–40) and the arm (1.6–1.8) ratios (here and below in fig. 1, 2, table 1). The absolute length of the four pairs of *P. fuscus* in average varies from 17–38 nm, in *P. vespertinus* the range of variation is somewhat smaller (17–26). The relative length of the chromosomes is 9–13. Centromeric index values of the first four pairs showed that the 1st, 2nd, and 3rd pairs are more submetacentric. The 4th pair in both species and 2nd in

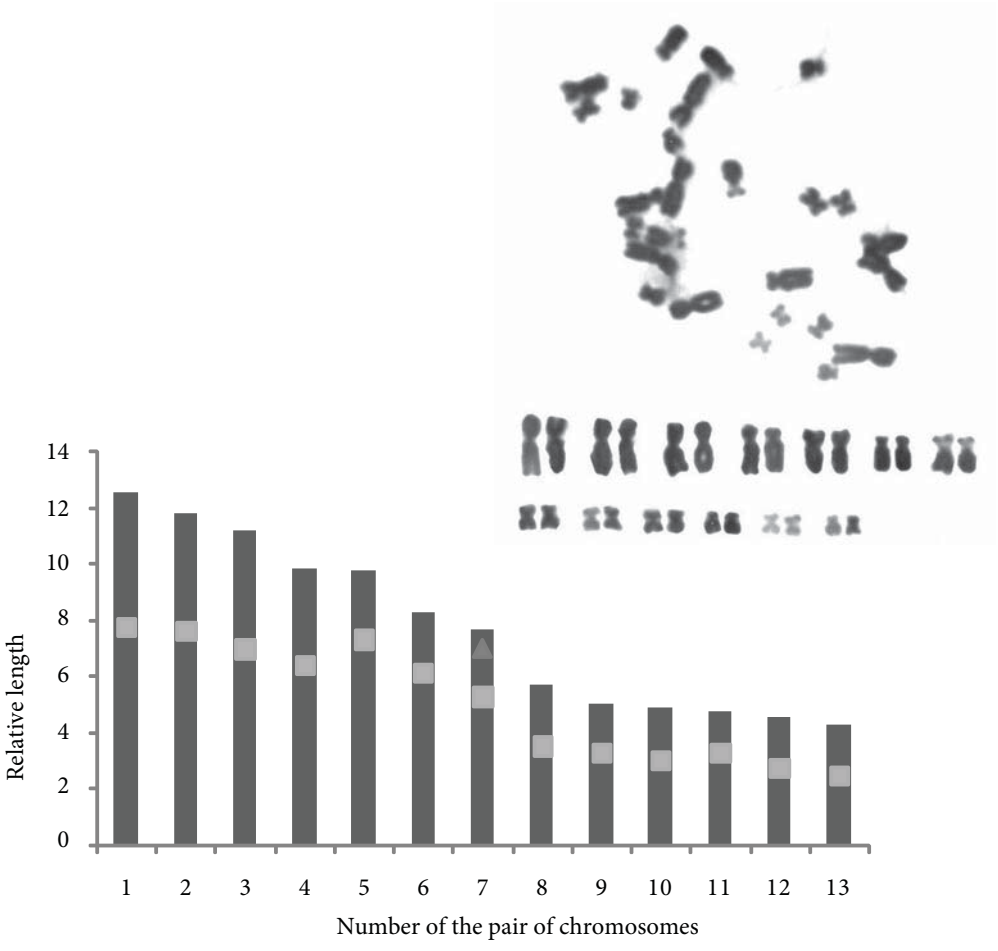

Fig. 1. Karyogram and idiogram of *P. fuscus* of Ukraine.

Рис. 1. Кариограмма и идиограмма *P. fuscus* с территории Украины.

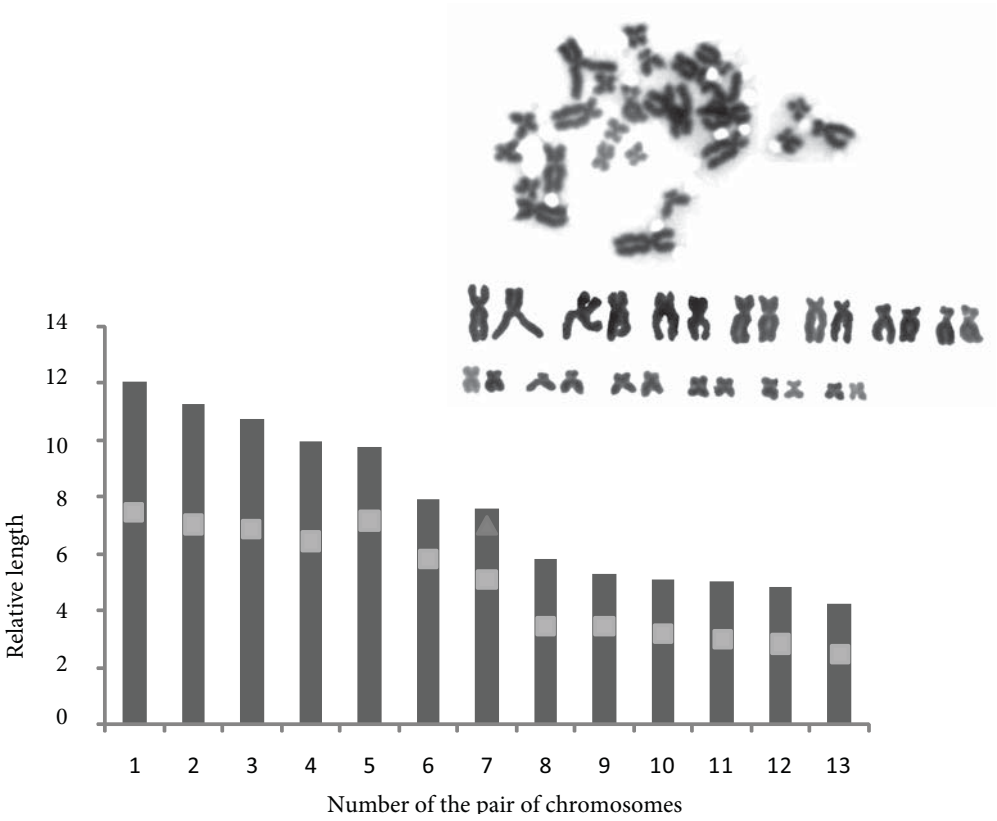

Fig. 2. Karyogram and idiogram of *P. vespertinus* of Ukraine.

Рис. 2. Кариограмма и идиограмма *P. vespertinus* с территории Украины.

P. fuscus are less submetacentric. The 5th and 6th pairs are subtelocentric, with centromere index 22–28 in *P. fuscus*, and 24–28 in *P. vespertinus*. Moreover, the average indicators of this parameter are almost identical. Subtelocentric characteristic is also confirmed by the data of arm ratio. Arms of certain homologues of these pairs may be of different sizes.

The morphology of the 7th pair is visually different, as secondary constrictions were detected on both of its short arms, which can be traced to all analyzed plates (100 %). Values of centromere index (31.04 ± 1.29 in *P. fuscus*, and 32.84 ± 0.85 in *P. vespertinus*) and arm ratio (2.32 ± 0.30, 2.05 ± 0.08, respectively) suggest that on average the pair is submetacentric, although in some plates the short homology arms can be more helical, thereby visually and by measurements, arms with secondary constrictions look relatively short compared to the other investigated chromosomes, which proves subtelocentric character of the given pair of chromosomes. It should be emphasized that in some plates (10 % morphologically processed) 7th pair can be larger than 6th one.

Six pairs of small chromosomes in the karyotype have the following morphology. Thus, 8th pair, according to the centromeric and arm ratios are metacentric. At the same time in *P. fuscus* it can be submetacentrical (34.79–42.19). The absolute length of the 8th pair from *P. fuscus* is larger (12.69 ± 1.37) compared to *P. vespertinus* (11.56 ± 0.56), and relative length parameters are almost identical in both species (5.71 ± 0.22; 5.83 ± 0.12 respectively). In *P. vespertinus* the 9th pair is submetacentric in all investigated plates. Centromeric index varies from 30.19 to 39.51 (table 1), arm ratio value also corresponds to the type. Thus at *P. fuscus* it may have various morphology, despite the fact that on average the steam also relates to submetacentric type (35.58 ± 2.63). As the range of variability displays this pair can

Table 1. Chromosomes parameters *Pelobates fuscus* (*P. f.*) and *Pelobates vespertinus* (*P. v.*) of Ukraine

Таблица 1. Параметры хромосом *Pelobates fuscus* (*P. f.*) и *Pelobates vespertinus* (*P. v.*), населяющих Украину

Pairs of chromosomes	Species	Long arm, nm M ± m (min–max)	Short arm, nm M ± m (min–max)	Absolute length, nm M ± m (min–max)	Relative length (R.L.) M ± m (min–max)	Arm ratio (A.R.) M ± m (min–max)	Centromere index (Ci.) M ± m (min–max)	Type of chromosomes
1	*P. f.*	17.23 ± 1.97 (13.11–23.59)	10.78 ± 1.32 (97.34–14.77)	28.01 ± 3.28 (20.45–38.36)	12.52 ± 0.32 (11.59–13.53)	1.61 ± 0.05 (1.50–1.79)	38.39 ± 0.67 (35.89–39.97)	SM
	P. v.	14.79 ± 0.52 (13.58–16.31)	9.13 ± 0.34 (8.18–10.16)	23.92 ± 0.82 (22.04–26.47)	12.08 ± 0.21 (11.53–12.83)	1.62 ± 0.04 (1.48–1.69)	38.17 ± 0.58 (37.11–40.31)	
2	*P. f.*	17.02 ± 2.14 (12.99–22.87)	9.29 ± 0.73 (7–32–11.60)	26.30 ± 2.83 (20.31–34.47)	11.79 ± 0.15 (11.36–12.16)	1.81 ± 0.11 (1.50–2.13)	35.78 ± 1.40 (31.94–39.94)	SM
	P. v.	14.04 ± 0.97 (10.82–16.10)	8.51 ± 0.55 (7.33–10.43)	22.55 ± 1.41 (18.15–26.44)	11.33 ± 0.27 (10.57–12.02)	1.66 ± 0.09 (1.48–1.95)	37.81 ± 1.18 (33.94–40.39)	
3	*P. f.*	15.52 ± 1.80 (11.52–20.82)	9.53 ± 1.10 (7.22–13.00)	25.05 ± 2.88 (18.74–33.82)	11.20 ± 0.24 (10.62–11.93)	1.63 ± 0.06 (1.46–1.81)	38.04 ± 0.84 (35.56–40.61)	SM
	P. v.	13.63 ± 0.56 (12.56–15.73)	7.80 ± 0.56 (5.63–8.73)	21.44 ± 0.98 (18.19–24.22)	10.80 ± 0.08 (10.59–11.01)	1.78 ± 0.12 (1.54–2.23)	36.24 ± 1.51 (30.95–39.29)	
4	*P. f.*	14.29 ± 1.70 (11.24–18.96)	7.61 ± 0.61 (6.34–9.56)	21.90 ± 2.31 (17.58–28.52)	9.84 ± 0.25 (9.36–10.70)	1.86 ± 0.08 (1.66–2.10)	35.12 ± 0.97 (32.29–37.58)	SM
	P. v.	12.85 ± 0.60 (11.11–14.64)	6.97 ± 0.36 (4.86–9.13)	19.82 ± 0.80 (17.10–21.63)	10.00 ± 0.09 (9.77–10.29)	1.86 ± 0.10 (1.65–2.25)	35.18 ± 1.18 (30.81–37.73)	
5	*P. f.*	16.26 ± 1.77 (13.21–20.95)	5.44 ± 0.53 (4.23–7.22)	21.70 ± 2.28 (17.44–28.17)	9.75 ± 0.23 (9.30–10.57)	2.99 ± 0.12 (2.68–3.35)	25.18 ± 0.72 (22.97–27.11)	ST
	P. v.	14.27 ± 0.59 (10.06–15.94)	5.18 ± 0.18 (4.66–5.88)	19.46 ± 0.72 (17.05–21.32)	9.82 ± 0.05 (9.70–10.00)	2.76 ± 0.09 (2.51–3.04)	26.69 ± 0.65 (24.76–28.50)	
6	*P. f.*	13.49 ± 1.24 (10.85–16.71)	4.81 ± 0.43 (3.75–5.90)	18.30 ± 1.64 (14.73–22.61)	8.27 ± 0.24 (7.76–9.17)	2.81 ± 0.10 (2.46–3.00)	26.32 ± 0.69 (24.98–28.91)	ST
	P. v.	11.57 ± 0.46 (10.62–13.19)	4.19 ± 0.18 (3.52–4.58)	15.76 ± 0.56 (14.32–17.54)	7.97 ± 0.23 (7.09–8.31)	2.77 ± 0.12 (2.48–3.07)	26.60 ± 0.85 (24.58–28.75)	
7	*P. f.*	11.61 ± 1.14 (8.80–15.07)	5.17 ± 0.49 (3.86–6.38)	16.78 ± 1.36 (13.03–21.45)	7.62 ± 0.34 (6.48–8.37)	2.32 ± 0.30 (1.75–3.47)	31.04 ± 1.29 (22.39–36.40)	SM
	P. v.	10.14 ± 0.56 (9.06–11.54)	4.95 ± 0.28 (4.44–6.02)	15.10 ± 0.78 (13.78–17.47)	7.61 ± 0.22 (7.07–8.02)	2.05 ± 0.08 (1.90–2.34)	32.84 ± 0.85 (29.98–34.46)	
8	*P. f.*	7.79 ± 0.98 (5.96–11.08)	4.91 ± 0.42 (3.76–5.91)	12.69 ± 1.37 (9.96–16.99)	5.71 ± 0.22 (5.22–6.39)	1.58 ± 0.09 (1.37–1.87)	39.01 ± 1.29 (34.79–42.19)	M
	P. v.	6.86 ± 0.23 (6.05–7.40)	4.70 ± 0.37 (3.77–5.91)	11.56 ± 0.56 (9.82–13.00)	5.83 ± 0.12 (5.57–6.25)	1.48 ± 0.08 (1.20–1.61)	40.44 ± 1.32 (38.25–45.46)	
9	*P. f.*	7.20 ± 0.74 (5.49–9.74)	4.02 ± 0.56 (2.58–5.91)	11.23 ± 1.15 (9.02–14.25)	5.04 ± 0.07 (4.80–5.18)	1.87 ± 0.21 (1.33–2.50)	35.58 ± 2.63 (28.60–42.89)	SM
	P. v.	6.91 ± 0.38 (6.04–8.21)	3.68 ± 0.15 (3.19–4.07)	10.60 ± 0.41 (9.23–11.76)	5.35 ± 0.12 (4.93–5.65)	1.89 ± 0.12 (1.53–2.31)	34.87 ± 1.48 (30.19–39.51)	
10	*P. f.*	6.68 ± 0.83 (5.31–9.27)	4.22 ± 0.31 (3.68–5.06)	10.89 ± 11.12 (9.01–14.14)	4.90 ± 0.07 (4.70–5.11)	1.56 ± 0.89 (1.41–1.90)	39.16 ± 1.27 (34.44–41.43)	M
	P. v.	6.39 ± 0.35 (5.82–7.64)	3.74 ± 0.26 (3.20–4.55)	10.13 ± 0.31 (9.08–10.84)	5.12 ± 0.06 (4.93–5.29)	1.75 ± 0.19 (1.33–2.39)	36.94 ± 2.37 (29.52–42.97)	SM
11	*P. f.*	7.16 ± 0.4 (6.18–8.36)	3.30 ± 0.56 (2.38–5.17)	10.46 ± 0.98 (8.56–13.28)	4.72 ± 0.10 (4.48–5.10)	2.32 ± 0.22 (1.57–2.68)	30.74 ± 2.26 (27.19–38.93)	SM
	P. v.	6.02 ± 0.24 (5.31–6.63)	4.00 ± 0.18 (3.56–4.58)	10.02 ± 0.32 (8.87–10.62)	5.06 ± 0.06 (4.83–5.19)	1.52 ± 0.08 (1.24–1.70)	39.92 ± 1.33 (37.04–44.73)	M
12	*P. f.*	5.93 ± 0.53 (5.01–7.27)	4.02 ± 0.21 (3.54–4.77)	9.95 ± 0.72 (8.55–12.04)	4.53 ± 0.15 (4.02–4.97)	1.43 ± 0.07 (1.34–1.72)	40.63 ± 1.13 (36.72–42.81)	M
	P. v.	5.70 ± 0.33 (4.55–6.54)	3.92 ± 0.19 (3.27–4.30)	9.62 ± 0.51 (7.82–10.84)	4.85 ± 0.22 (4.46–5.54)	1.45 ± 0.03 (1.39–1.52)	40.81 ± 0.42 (39.67–41.82)	
13	*P. f.*	5.33 ± 0.25 (4.71–6.10)	3.92 ± 0.28 (3.33–4.85)	9.24 ± 0.52 (8.04–10.95)	4.23 ± 0.21 (3.64–4.75)	1.37 ± 0.04 (1.26–1.51)	42.27 ± 0.78 (39.85–44.29)	M
	P. v.	4.99 ± 0.21 (4.34–5.57)	3.49 ± 0.13 (3.10–3.81)	8.48 ± 0.29 (7.44–9.12)	4.28 ± 0.07 (4.02–4.39)	1.43 ± 0.06 (1.25–1.57)	41.20 ± 1.06 (38.82–44.46)	
Total length	*P. f.*			222.51 ± 21.72 (176.41–282.15)				
	P. v.			198.45 ± 7.68 (172.89–218.54)				

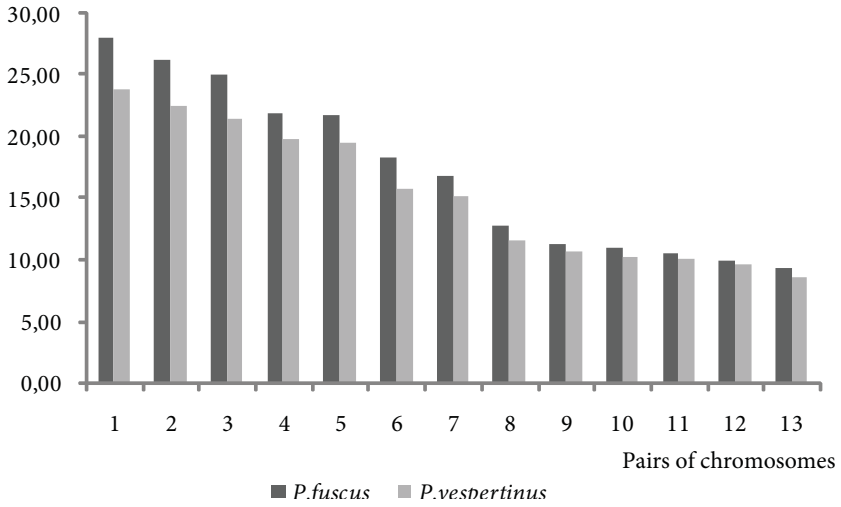

Fig. 3. Absolute length of chromosomes in spadefoots (*Pelobates*) of Ukraine. nm.

Рис. 3. Абсолютная длина хромосом у чесночниц (*Pelobates*) Украины, нм.

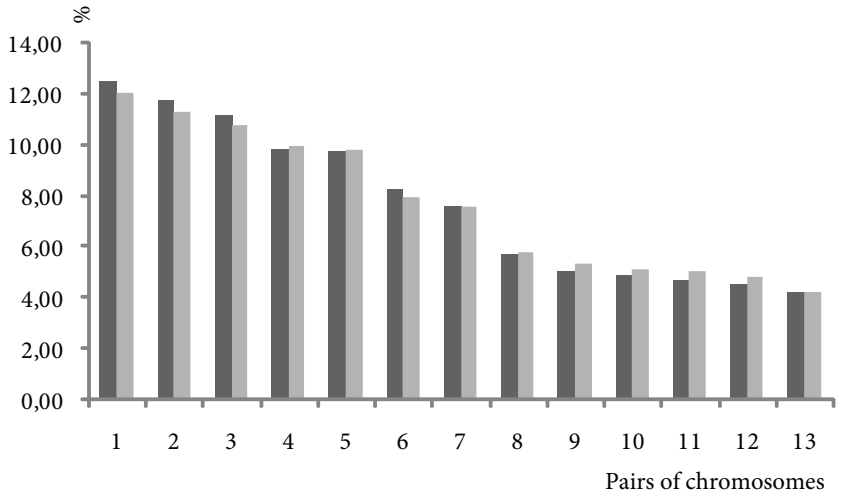

Fig. 4. Relative length of chromosomes in spadefoots (*Pelobates*) of Ukraine.

Рис. 4. Относительная длина хромосом у чесночниц (*Pelobates*) Украины.

be subtelocentric and metacentric (28.60–42.89) on different metaphase plates. In absolute and relative length of these chromosomes are virtually identical.

The 10th pair, on the contrary, is more variable in *P. vespertinus* (29.52–42.97), with a mean of centromere index (36.94 ± 2.37) and arm ratio (1.75 ± 0.19), and indicates the submetacentric character of the pair in this species. The 10th pair in *P. fuscus* is metacentric. Value of the center-dimensional and arm ratios is 39.16 ± 1.27 and 1.56 ± 0.89, respectively. Also the difference by type of chromosomes is established for the 11th pair. It is of metacentric type in *P. vespertinus*, and submetacentric in *P. fuscus*. To be more precise, in the latter species the pair is less submetacentric and quite variable, centromere index varies from 27.19 to 38.93.

The 12th and 13th pairs are metacentrics. By the 12th pair *P. fuscus* is more variable (centromere index within 36.72–42.81), but the value of arm ratio in both species is practically the same, which confirms the determined type of chromosomes. The 13th pair is the smallest metacentric in the karyotype.

By parameters of the absolute length of each pair and all the chromosomes in the karyotype in general are larger in *P. fuscus* in comparison with *P. vespertinus* (fig. 3) (the average total length of the karyotype, 222.51 ± 21.72 nm and 198.45 ± 7.68 nm, respectively). A discernible pattern is not observed when comparing the parameters of the relative length of each chromosome separately. So, the 1st, 2nd, 3rd and 6th pairs are larger in *P. fuscus*, the 7th pair has the same value, all the remaining pairs, on the contrary, are larger in *P. vespertinus* (fig. 4). Statistical comparison of the differences of all studied parameters of the chromosomes has showed that differences between the studied cryptic species are not significant (p-level > 0.10).

Discussion

In this paper the karyotype of *P. vespertinus* is described in detais for the first time. At present, the literature contains information about the karyotype of *P. fuscus* only. Therefore, in the discussion the comparative analysis was carried out using published data only on this species. Thus, data on the number of chromosomes is given in the works of Morescalchi et al. (1977). Types of chromosomes were described in detailes by B. Mészáros (1972) from Hungary. In the works of German researchers (Schmidt, 1980; Schmidt, Guttenbach, 1988) analysis of *P. fuscus* chromosomes using the methods of differential staining has been presented. On the territory of Ukraine (Manilo, Radchenko, 2004) the karyotype of *P. fuscus* was analyzed for Kyiv, Chernigov and Transcarpathian Regions.

It was confirmed that the total number of chromosomes in the karyotype of *P. fuscus* is 13 pairs (2n = 26), which was confirmed for *P. vespertinus* too. Our result concerning 7 large and 6 pairs of small chromosomes in two studied species (7 + 6) differs from the published data, except for the work of Ukrainian colleagues (Manilo, Radchenko, 2004), where the separated groups coincide with their data. Distribution of large and small chromosomes is extremely important in speciation of amphibians (Morescalchi et al., 1977). It should be emphasized that these size groups are clearly seen on the presented karyogram (fig. 1, fig. 2) and are typical for all studied metaphases.

In the first papers on *P. fuscus* chromosomes (Mészáros, 1972; Morescalchi et al., 1977), and on the basis of these results, in the recent works (Nöllert et al., 2012), the given groups have been defined as 6 large and 7 small chromosomes (6 + 7). Perhaps this difference is explained by the fact that the seventh pair of chromosomes with secondary constrictions on its short arms can vary greatly in size (see below).

Results regarding the different sizes of homologous chromosomes at an identical morphology deserve attention, and range of chromosome parameters variation (min–max) tells about the different types of chromosomes on different metaphase plates. There are several reasons for this: a geographical variability and features of the preparation technique / measurement of chromosomes and a certain subjectivity of the researcher. In addition, the cause of these changes can be different degrees of helix. Their separate areas at different stages of cell division are replicated asynchronously. The length of the chromosome varies regularly on different stages of division, when the shortening of longer arms may be faster as compared with short ones (Vogel, Motulsky, 1989).

Premature or asynchronous condensation of certain chromosomal regions between homologous chromosomes can also occur. Centromere regions and areas with secondary constrictions subjected to variability, what leads to heteromorphism of homologues. All this greatly complicates the definition of sizes, shapes, and the individual identification of chromosomes, that should be considered when carrying out such studies, especially for selection of homologous chromosomes.

As for the types of chromosomes, the first four pairs in *P. fuscus* are submetacentric that has been previously shown for the territory of Ukraine (Manilo, Radchenko, 2004) and Hungary (Mészáros, 1972). The same type of the first four pairs of chromosomes is characteristic for other species of this genus, such as *Pelobates syriacus* Boettger 1889 (Uğurtaş

et al., 2001) and *Pelobates cultripes* (Morescalchi, 1967) (assessed visually by karyogram presented in this paper, since the dimensional figures are not available, and the types of chromosomes by themselves have not been identified by authors).

The first pairs are visually quite similar in all investigated plates and this fact deserves consideration. Moreover, the first relatively big pair of chromosomes is characteristic for other types of anurans (Suryadnaya, 2003). This equivalence of the first pairs of chromosomes should be specific for the genus *Pelobates*, and this feature can be clearly traced on the karyograms of different members of the families (Megophryidae, Pelodytidae) close to Pelobatidae, and studied by other researchers (Morescalchi et al., 1977).

The types in other smaller chromosomes up to the pair 9 also coincide. Types in the last three pairs of the smallest chromosomes either varied in different researchers or partly overlap. For example, the type of the 11th pair of chromosome matches with the data of Ukrainian researchers (Manilo, Radchenko, 2004), and this type differs from the Hungarian one (Mészáros, 1972). Type of the 12th pair in this study coincides with the results obtained for the Chernigiv Region (Manilo, Radchenko, 2004). Interpopulation variability is likely to affect on it.

The 7th pair of cromosomes with the secondary constrictions should be also discussed. The fact of their presence in *P. fuscus* has been also described by other authors (Mészáros, 1972; Morescalchi et al., 1977; Schmidt, 1980; Manilo, Radchenko, 2004). This secondary constriction coincides in position with the nucleolar organizer, identified by the method of differential silver staining (Schmidt et al., 1987). These structures are known not to be stained by standard methods, meanwhile the length of chromosome arms, which could potentially contain the secondary constriction varies depending on its expression. In anurans, secondary constrictions are not often stained, or they can be visualized in only one of the homologous chromosomes (Suryadnaya, 2003). For the investigated species absolutely on all the studied plates (100 %) such constriction was detected, and in both of the short arms of each homologs, which is essential in total staining. It can indicate the stability of location of the given part of chromosomes in contrast to many other amphibian species.

The fact that the 7th pair can be larger than the 6th pair is understandable for chromosomes with secondary constrictions, as so-called "sub-spiralization" can occur, what in some cases leads to a lengthening of the arm. This segment in comparison with the rest of the chromosome can be very thin. Incidentally a sign of "sub-spiralization", as well as other individual characteristics of chromosome morphology is found in all somatic cells and in half of germ cells in vertebrates, i. e. inherited as a dominant character (Vogel, Motulsky, 1989).

Since all nuclear DNA in the cell is contained in the chromosomes, the size and number of chromosomes may reflect the size of the genome (Macgregor, Varley, 1986). Therefore, it was assumed that the total length of all chromosomes of a karyotype located at metaphase correlates with the amount of DNA contained in the chromosomes (Mészáros, 1972). Number of chromosomes is equal in both studied species, and by the absolute length of chromosomes was larger in *P. fuscus*. It should be noted that genome size, i. e. the amount of nuclear DNA determined by flow DNA cytometric, conversely, is smaller in *P. fuscus* (average 8.83 pg) in comparison with *P. vespertinus* (average 9.35 pg). These values do not overlap, but the differences are stable (Borkin et al., 2001; Khalturin et al., 2003). Thus, the size of the chromosomes is bigger in *P. fuscus,* although the size of the genome is bigger in *P. vespertinus*. Perhaps discrepancy is due to the less accurate methods of routine kariology (measurements by photographs) in comparison with the measurement of genome size. To clarify this discrepancy, more research is needed.

But yet identified chromosomal differences between these two species in the position of the centromere in the chromosomes of the 10th and 11th pairs can not be recognized as distinct diagnostic characters for *P. fuscus* and *P. vespertinus* from Ukraine. Statistical

differences between these two species according to the pairs of chromosomes were not reliable. This can be explained by a relatively small amount of processed plates, and of course, the data should be checked on a large amount of material.

However, it should be emphasized that in cryptic speciation and chromosomal conservatism of amphibians the probability to reveal the reliable diagnostic features at the chromosomes level can not exist.

Studied features (morphology of specimens, the environment and other aspects of the biology of the species) do not allow identifying reliable diagnostic signs in spadefoot toads (Lada et al., 2005). Currently, many factors have not been sufficiently studied concerning so complex form of speciation. There is an urgent need for further revealing of the weightiest characters to distinguish cryptic species.

Conclusions

In the study for the first time the karyotype of *P. vespertinus* was described in details. It was defined that cryptic species *P. fuscus* and *P. vespertinus* have symmetrical karyotype consisting of 26 double-armed chromosomes. On individual metaphase plates the same types of chromosomes, or their homologs vary in length and variability of chromosomal parameters indicate different typing of chromosomes, in contrast to the general mean value.

Differences were defined by the types of 10th pairs and 11th pairs. In *P. fuscus* 10th pair is metacentric, 11th pair is submetacentric; in *P. vespertinus* 10th pair is submetacentric, 11th pair is metacentric. The secondary constriction was identified on the short arm of the 7 th pair. Chromosomal formula for both species is 4 meta- (m) + 7 submeta- (sm) + 2 subtelocentrics (st), 2n = 26, NF = 52. There are 7 pairs of large and small 6 pairs of chromosomes in the karyotype.

Absolute length of all chromosomes in the set is more in *P. fuscus*. The values of this index for all treated metaphase plate (n = 10) overlap. Parameters of chromosome length ratio in average are the same for both species and for individual chromosomes are greater in *P. vespertinus*. It is possible that this index refers to the proportion of each chromosome in the whole genome. Statistically significant differences between two cryptic species have not been identified, respectively chromosomal features can not be used for species identification of frog on the territory of Ukraine. Nevertheless, the obtained materials are of certain scientific value, as described in details karyotypes open the possibility of a targeted search for new reliable signs in frog at the chromosomal level, and the karyological study of cryptic species is one of the most important aspects of the problem of the species in whole.

It is my pleasant duty to thank my colleagues — Yu. V. Karmyshev, S. Boyko, I. Antonenko, A. Belikov, Ju. Zvyagintseva, and the Scientific Director Yevgen Maksimovic Pysanets. I am particularly grateful to G. Mikitinets and O. Manuilova. This paper was prepared thanks to comprehensive care of S. N. Litvinchuk. I would like to offer my special thanks to Yu. M. Rozanov who carried out measuring the size of the genome in common spadefoot toads. I express my deep gratitude to the reviewers — L. J. Borkin and D. V. Skorinov. I wish to acknowledge V. Lysenko, S. Pavlenko, K. Rentyuk, the undisputed mastermind M. Jo.Stroeva for their full support.

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Received 15 August 2014
Accepted 26 November 2014