The physiological and genetic differences between flycatchers (*Ficedula albicollis* vs. *Ficedula hypoleuca*)

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Abstract

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The paper studies interspecies physiological and genetic differences between the white-collared flycatcher and the pied flycatcher of the genus *Ficedula*. The fact that the flycatchers are capable of interspecies hybridization is one more reason for particular interest to these species. Using our own-developed method of taking venous blood from the eye's sinus, we took blood samples from birds in the protected area of Homilshanski Forests, Kharkiv, Ukraine, to study their leukogram patterns. The bird feathers were also collected for genetic analysis -- to identify interspecies differences with application of the ISSR technique. It was revealed that the percentage of heterophiles in the nestlings of the pied flycatcher was lower than in the nestlings of the white-collared flycatcher. The micronucleus test did not reveal any significant difference in the interspecies groups. The spectra of amplification products obtained with the primer $(AGC)_6 G$ showed that the white-collared flycatchers had a more heterogeneous structure. The study of the leukogram, micronucleus test, and the ISSR analysis can be especially effective in the study of intra-species genetic differentiation.

Keywords

flycatchers, ISSR inter-microsatellite analysis, leukogram, micronuclei

Introduction

The domestic chicken karyotype is a model karyotype for the class Aves (Derjusheva, 2014; Rodionov, 1996). Currently, most of the nucleotide and protein sequences entered into the GenBank belong to the domestic chicken and turkey (Animal Genome, 2017; Genome Size, 2017). The discovery of B-cells and tumour viruses are among examples of using birds as model objects in fundamental biology and medicine (Brown et al., 2003; Romanov et al., 2004), and bird embryos are ideal for studying the development of vertebrate animals (Stern, 2004).

Though the structure of the chicken genome is in many respects comparable to that of warm-blooded animals, it still remains unique. A certain similarity is observed when studying and comparing particular regions of individual chromosomes in chickens and mammals. This suggests that the maps of mammalian nucleotide sequences may be used in positional cloning. The avian karyotype is characterized by the multiplicity and heterogeneity of chromosomes. It includes several macrochromosomes (3–8 μm) and numerous microchromosomes (Schmid et al., 2000). The birds, unlike the mammals, have also an additional family of "superheavy" isochors – H4 (Caccio

et al., 1997), which portion in the avian genome is quite significant and ranges from 9 to 13%. It can partly be explained by the presence of the GC (guanine-cytosine)- enriched bird satellite, making up about 5% of the genome (KADI et al., 1993). The avian genome also includes so-called microchromosomes, in whose structure the "light" isochores of the families L1 and L2 are always absent (SAZANOV et al., 2003) – thus making them similar to telomeric regions of macrochromosomes. The bird genome is almost three times smaller than the mammal's average. Investigating the size of the avian genome and cells of the immune system, scientists have found a positive correlation between the size of the bird genome and that of erythrocytes and nuclei (ROMANOV et al., 2004). Presumably, the value of the cell mass is connected with the need to accelerate metabolism during flight (KADI et al., 1993). This genomic feature in birds allows us to talk about the presence not only of the structural but also functional difference of the avian genomes.

Despite similarities in the functioning of systems of birds and mammals, demonstrating for example by the leukocytic-neutrophilic physiological cross-section (BESSARABOV, 2009), there are also a number of characteristic features in the structure and functioning of the immune system (YEZDAKOVA et al., 2008). Thus, the thymus and bursa of Fabricius belong to the central lymphoid organs of birds but the lymphocytes forming in the bursa of Fabricius are different from those forming in the spleen and red bone marrow. Large bursa lymphocytes are characterized by a well-developed Golgi apparatus and endoplasmic reticulum (YEZDAKOVA et al., 2008). Therefore, at the cytological and cytogenetic levels, the study of the avian genome will provide qualitatively new opportunities to investigate patterns of functioning of the eukaryotic cell nucleus and organism as a whole – in the light of physiological changes and ecological relationships among genome elements in representatives of various taxonomic groups.

A resource base has already been created for the family Cathartidae and the order Ciconiiformes (KAMEDA and GOODRIDGE, 1991; ROMANOV and DODGSON, 2006). Genomic libraries have been designed for the Japanese quail, pigeon, goose, emu, two species of passerines – the red-winged blackbird and the brown-headed cowbird (EDWARDS et al., 1998; LONGMIRE et al., 1999; ROOTS and BAKER, 2002; SHIINA et al., 1999; TAKAHASHI et al., 2003), and for the domestic duck (MOON and MAGOR, 2004).

In practice, various BAC clones are used for understanding and mapping genomes in various mammals (primates, cats, dogs, cows, pigs) (KELLNER et al., 2005; THOMAS et al., 2002). A certain number of molecular markers and genomic databases have been created for the duck and goose (HUANG et al, 2006; MAAK et al., 2003), dove (TRAXLER et al., 2000), budgerigar (KAMARA et al., 2007), pheasant (BARATTI et al., 2001), peacock (HALE et al., 2004, HANOTTE et al., 1991), Japanese quail (KAYANG et al., 2002; PANG et al., 1999), ostrich (TANG et al., 2003), emu (TAYLOR et al., 1999) and a limited number of other

bird species. These tools should facilitate the construction of recombination genetic maps that have been recently successfully realized for such object as a duck (HUANG et al., 2005). These maps were constructed by a method of segregation analysis of microsatellite markers. A comparative map of microsatellite markers of passerines has also been initiated (HANSSON et al., 2005). In 2002, Schutz and colleagues have conducted a microsatellite scanning to find the QTL related to the feeding behaviour and social motivation of birds (SCHÜTZ et al., 2002).

Ecological changes underpin the transformation of internal structures of individual species, being a driving force for the rearrangement of immune systems of organisms. An assessment of the ecological situation in a particular area can be greatly simplified if the damaging agents affecting organisms are revealed. For this purpose, the micronucleus test and leukogram evaluation are often used. At the same time, the organism adaptation to the environment depends on the contribution of certain allelic variants to different genes. To assess a possibility of this approach, we performed an analysis of the DNA sections (microsatellite loci) homological to the ERV1-2C-LTR bovine retrovirus (leukemia virus), and also a cytological blood test and micronucleus test of the pied flycatcher and white-collared flycatcher in the protected area of "Homilshanski Forests" (Kharkiv, Ukraine).

Materials and methods

Haematological method of research

We studied the birds *Ficedula*, including 52 of *Ficedula albicollis* (14 adults and 38 nestlings 10–12 day-old) and 34 of *Ficedula hypoleuca* (12 adults and 22 nestlings 10–12 day-old), nested in the city of Kharkiv, over the period 2015–2017 (n = 86). Adult birds were captured with mist nets.

More often, an axillary vein is used for taking haematological material (IMANGULOV et al., 2004), but if the bird is small, let alone the nestling, the head decapitation is applied (DONNIK et al., 2015), or blood is taken from the leg vein (VISSER et al., 1991) or the second finger of one of the legs after cutting off half or less of a claw phalanx (VALKYUNAS et al., 2001).

We developed our own method of taking venous blood from birds. Thus, to take a blood sample, the venous sinus of the eye was punctured with a haematocrite capillary. Blood then flowed into the capillary which, for this purpose, was selected ahead for each bird (DRAHULIAN et al., 2018a).

To obtain a smear, we introduced a capillary with a light screw-like motion under the eyeball, closer to the corner of the eye. The blood coming through the capillary was placed on a properly marked watchglass (DRAHULIAN et al., 2018b).

The blood was cleaned by a cotton swab and the arrest of bleeding was controlled (PAULKE and HAASE, 1978;

IMANGULOV et al., 2004). After that, the smears were placed on slide plates with the aid of a narrower bevelled-edge spreader slide. The blood smears were air-dried until the wet shine disappeared. After that, they were fixed in Mai-Grunwald stain for 30 seconds, then rinsed with water and finally dyed in Romanowsky stain from 25 to 40 minutes (ALMAZOV and RYABOV, 1963.).

The calculation of leukocytes for the identification of the leukogram pattern was carried out in the bird blood smears, using a microscope with an immersion lens of 200 cells (50 in each corner) (VARTANYAN and KARAPETYAN, 1959.). To calculate the leukogram, a special keyboard calculator was used, with each key marked with the initial letter of the leukocyte name (IRISOV et al., 1980). The calculation of erythrocytes with micronuclei was performed for 1,000 cells (CEA et al., 1983).

Molecular and genetic method of research

The method of molecular and genetic control by DNA marking (ROOTS and BAKER, 2002; SHIINA et al., 1999; TAKAHASHI et al., 2003) is promising for the identification of the bird species and the results of species crossing (hybrids), and as such it will soon apparently become an advanced technology. This technique has proven itself as a reliable method for assessing the genetic and population statuses of some bird ecotopes, as a criterion for determining the influence of paratypical and selection factors on the structure of the studied samples, as well as a tool for the genetic certification of flycatcher species.

A genetic system of the ISSR intermicrosatellite analysis has been proposed by Eva Zinkevich as a method for fingerprint analysis in various biological objects (ZIETKIEWICZE et al., 1994).

The application of the ISSR technique for the investigation of bird genomes allowed identifying certain DNA fragments specific for the white-collared flycatchers. Thereby, these fragments can be used to identify a flycatcher species.

The research was carried out in the Genetics Department of the Institute of Animal Breeding and Genetics of the National Academy of Sciences of Ukraine. The genetic analysis was performed using the sampled bird feathers, with the application of the ISSR technique (ZIETKIEWICZE et al., 1994).

Preparation of the samples included a thorough grinding of feathers in a glass homogenizer, with gradu-

ally adding 200 μl of lysis buffer; 150 μl of the resulting homogenate was used to extract the DNA. The DNA extraction was carried out using the standard commercial package "Sorb-B" of the "Amplisens" company (Moscow, Russia) by the guanidine-isothiocyanate method according to the manufacturer 's instructions.

A polymerase chain reaction was carried out according to the following procedure (Table 1).

The structure of the used primer: S1: 5'–$(AGC)_6$G–3' (ISSR) (KURILENKO and SUPRUN, 2015). For the amplification, a standard set of the "mplisens" company (Moscow, Russia) was used, the reaction mixture for the PCR (polymerase chain reaction) with the ISSR primers contained 2.5 μL of the reaction buffer (16.6 mmol/L of $(NH4)_2SO_4$; 67.0 mmol/L of Tpic-HCl (pH 8.8), 0.01% of Tween-20; 2.0 mmol/L of $MgCl_2$; 2 mmol/L of dNTP); 100 pM of the primer (0.7 μL); 4 activity units of Tag-polymerase (0.2 μL); 2 ng of DNA-sample (2 μl); the deionized water to get a total volume of the mixture of 10 μL.

The amplification products were separated by horizontal electrophoresis in IXTBE buffer using 1.5% agarose gel and stained in ethidium bromide solution. Gel documentation was used by photographing the electrophoregrams placed on a transilluminator in UV radiation with a wavelength of 340 nm using an orange light filter. The size of the received amplicons was controlled using a standard M15 molecular weight marker. To determine the allele frequencies on the basis of the processed profiles of the sample, the matrix of the output data was created with the presence (1) or absence (0) of the band in a certain position of the profile according to the results of the three performed amplifications for each DNA sample. The matrix of the output data was entered in a corresponding file of the standard computer program (Microsoft Exel) intended for the data processing of polylocus typing. Probability of the received patterns was evaluated according to recommendations of PLOKHINSKY, 1969.

Results

We studied 86 representatives of the genus Ficedula, including 52 of *Ficedula albicollis* (14 adults and 38 nestlings 10–12 day-old) and 34 of *Ficedula hypoleuca* (12 adults and 22 nestlings 10–12 day-old). All results of the research are presented in Table 2.

Table 1. Amplification parameters of ISSR using the primer $(AGC)_6$G. For amplificators with active regulation of temperature liquid volume in a tune – 10 μL

Amplification phases	Temperature	Time	Number of cycles
Initial denaturation of DNA	94 ºC	4 min	1
	94 ºC	1 min	
Annealing of primers	57 ºC	2 min	29
	72 ºC	4 min	
Final synthesis	72 ºC	7 min	1
	4 ºC	Storage	

Table 2. Blood indices of the nestlings (above the line) and adults (below the line) of the white-collared flycatcher and pied flycatcher

Indices	White-collared flycatcher	Pied flycatcher
Number of erythrocytes with micronucleus abnormalities, ‰	4.28 ± 0.52 ------------------- 3.73 ± 0.28	4.66 ± 0.21 ----------------------- 4.00 ± 0.19
Leukogram, %: heterophiles	15.00 ± 1.06** ------------------------- 12.68 ± 0.47•••	16.33 ± 0.21*** ----------------------- 9.45 ± 0.57
Lymphocytes	72.85 ± 1.96*** ------------------------- 82.89 ± 0.52••	76.33 ± 1.47*** ----------------------- 85.54 ± 0.87
Eosinophils	2.71 ± 0.28 ----------------------- 3.36 ± 0.25	3.33 ± 0.21 ----------------------- 3.09 ± 0.25
Monocytes	3.71 ± 0.28*** ------------------------- 0.86 ± 0.20••	5.33 ± 0.91*** ----------------------- 1.81 ± 0.51
Basophils	5.71 ± 1.79*** ---------------------- 0.42 ± 0.19	4.33 ± 0.42*** --------------------- 1.00 ± 0.30

** $p < 0.01$, *** $p < 0.001$ between nestlings and adults of the same species, •• $p < 0.01$, ••• $p < 0.001$ between nestlings of the white-collared flycatcher and the pied flycatcher.

Table 2 shows that the mean indices of erythrocytes with micronuclei in the white-collared and pied flycatchers were within the norm for mammals and birds (CEA et al., 1983, KURSA et al., 2005), not differing significantly between the species and age bird groups. We did not find any significant difference in the leukogram between the adults of two species. However, the difference in the leukogram distribution was found between the nestlings of the white-collared and pied flycatchers. The heterophile (granulocyte) indices were higher in the white-collared flycatcher nestlings by 3.23% (12.68 ± 0.47) ($p < 0.001$), and those of lymphocytes and monocytes were higher in the pied flycatcher nestlings by 2.65% (85.54 ± 0.87) ($p < 0.01$) lymphocytes and by 0.95% (1.81 ± 0.51) ($p < 0.01$) monocytes. This may indicate some difference in the immune system development, although, as mentioned above, there was no particular leukogram difference between the adults of the two species. It seems that with age, the immune systems of two species are levelled and become similar.

It should be noted that we found an intra-species difference between the age groups. Thus, the lymphocyte percentage in the white-collared flycatcher decreased with age by 10.04% (72.85 ± 1.96) ($p < 0.001$), and in the pied flycatcher – by 9.21% (76.33 ± 1.47) ($p < 0.001$), but monocytes instead increased by 2.85% (3.71 ± 0.28) ($p < 0.001$) in the white-collared flycatcher, and by 3.52% (5.33 ± 0.91) ($p < 0.001$) in the pied flycatcher. Also, in the pied flycatcher, the heterophile percentage statistically significantly increased with age by 6.88% (16.33 ± 0.21) ($p < 0.001$) and that of basophils increased by 3.33% (4.33 ± 0.42) ($p < 0.001$). The basophils indices in the white-collared flycatcher were higher in the adult birds by 5.29% (5.71 ± 1.79) ($p < 0.001$). That is, in adults of the two species of the studied birds, the percentage of cells of the myeloid series increased and the percentage of those of lymphoid series decreased. This corresponds to the physiological cross-section, observed in broiler chickens (BESSARABOV, 2009).

Further, we carried out an ISSR microsatellite analysis with the primer (AGC) 6G for the collected material (feathers). The results of amplification products are shown in Fig. 1.

Using the primer (AGC)6 G with the DNA of the pied and white-collared flycatchers revealed a quite high level of genetic polymorphism. Thus, the total sum of amplicons formed in the PCR with the selected primer ranged from 9 in the pied flycatcher to 14 in the white- collared flycatcher. The length of amplicons ranged between 270–1,400 bp. A majority of PCR-loci (83.33% of the total number of loci) received with this primer were of average size. Four PCR loci (16.67%) were less than 500 bp long. Each fragment of DNA was considered as an individual genome locus, absence of the fragment in the spectrum of the primer was recognised as a homozygote for the recessive allele, and the presence – as the presence of a homozygote for the dominant allele or a heterozygote.
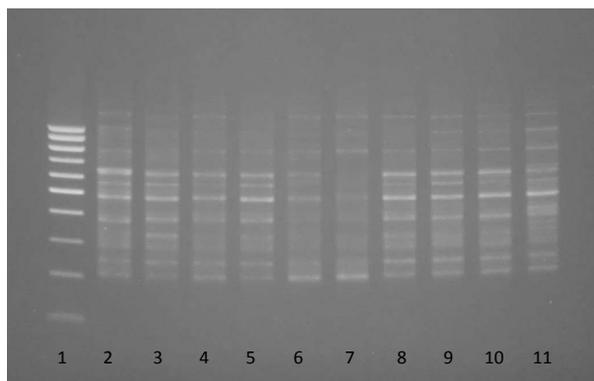
Fig.1. Screening ISSR with the primer (AGC)$_6$G: 1-marker, 2, 3, 4, 5, 8, 9, 10, 11 – *Ficedula albicollis*; 6, 7 – *Ficedula hypoleuca*. Electrophoresis exposure time – 40 minutes of exposure, 250 W.

Discussion

Relationships between the bursa, immune system and digestive system

The structure and functioning of the bird immune system has a number of peculiarities (Turitsyna et al., 2013). The central lymphoid bird organs are the thymus and bursa of Fabricius; the latter, in fact, is a projection of a primary gut (Yezdakova et al., 2008). We suppose that this peculiarity brings some differences in the regulation of the karyotype stability and immunity in birds. Thus, to understand interspecies difference of the family Muscicapidae, the diet of the pied and white-collared flycatchers should be studied.

Feeding and differences in the leukogram between nestlings of the pied and white-collared flycatchers

Observations reported from the Voronezh region of Russia, based on ration of nestlings of pied flycatchers and white-collared flycatchers (Ivanov, 2004), provide an opportunity to talk about differences in their diets. These observations have shown that the nestling diet of the white-collared flycatchers was dominated by larvae (76.4%), including 60.6% of caterpillars, and 14.2% of Homoptera larvae. The nestling diet of pied flycatchers was dominated by imagoes (75.0%), including Hymenoptera – 27.8%, spiders – 14.4%, Diptera – 12.5%, and beetles – 8.6%.

The percentage of spiders eaten by pied flycatchers was rather high, making up for about 94%. This shows that the percentage of spiders eaten by white- collared flycatchers made up for about 6% (Polchaninova and Prishada, 1994). This research coincides with Chaplygina et al. (2011), reporting that the percentage of spiders in diet of white-collared flycatchers was 5%. The main part of the diet of flycatchers is made up of beetles, dipterans, butterflies (Chaplygina et al., 2015).

In a situation where the nest-holes are occupied by other species, such as robin, the food chains of flycatchers change, and this leads to a change in the physiological parametres (Chaplygina et al., 2016). Changes in the diet, often depend on weather conditions, time of day and season, which indicates a certain dietary plasticity. However, we make a conclusion that a major part of the diet of the white-collared flycatchers consists of caterpillars and larvae, while that of the pied flycatchers mostly includes imagoes.

The concentration of carotene in white-collared flycatchers was also studied. The studies have shown that the carotene concentration is positively correlated with the presence of caterpillars in the feed, which also provides antioxidant protection and regulation of immune processes. Török et al. (2007), comparing the diet of nestlings, noted that pied flycatchers prefer imagos, and white collared flycatchers prefer caterpillars. It can be assumed that this preference increases the level of carotene in the latter species. Ivanov (2005) has also noted that β-carotene positively correlates with testosterone concentration in the egg yolk. This may indicate an increased tolerance to the oxidative stress and to pathogenic microorganisms (Török et al., 2007).

Leukogram

The leukocyte percentages received in our experiment did not differ from those presented in the literature sources (Cichoń et al., 2001; Lugaskova et al., 2005). Our intraspecies research showed that both species were characterized by a system of physiological cross-sections. The bird haemogram undergoes changes in the postembryonic development period. With age, the number of leukocytes increases almost twice. This essential growth occurs due to the increase in lymphocytes and decrease in granulocytes (Bessarabov, 2009). However, the importance of our leukogram studies for the two species of flycatchers lies in the revealed difference in the leukogram percentages of chicks that we associate with their diet composition. Thus, the heterophyle (granulocyte) indices were higher in the nestlings of the white-collared flycatcher, while lymphocyte indices were higher in the nestlings of the pied flycatcher. In many bird studies, the decline of heterophiles in the leukogram has indicated their decreasing bactericidal and antitoxic blood functions (Miklyaeva et al., 2017). Though, there was no age-related difference in the leukogram between the adults of the two species (Table 2). It is assumed that with age, the immune species of two species is levelled and becomes similar.

Micronuclei

The birds are a quite numerous and widespread class, characterized by an evolutionary stability of their karyotype. Therefore, they are always selected as a model object in genetics, phylogenetics, ecology. For a cytogenetic research, the analysis of mature erythrocytes can be used. But despite the bird is a fairly good model for studying biological patterns, there is a lack of data characterizing the peculiarities of genome instability in individual species. The assessment of genome instability in the field also en-

tails some difficulties. Consequently, the studies on avian cytogenetic are scanty, and every work is valuable. Thus, to determine the cytogenetic parameters of the genome instability in the pied and white-collared flycatchers, we had to compare the obtained data with other birds species (Zúñiga-Gonzales et al, 2001).

The first thing to be mentioned in the micronucleus analysis of bird erythrocytes is that they slightly differ from those in mammals. Most often, the micronucleus analysis for mammals is carried out on leukocytes (Cea et al., 1983), while for birds – on erythrocytes.

This is not only because of the differences between the chromosomes of mammals and birds (in birds, micro-chromosomes have been found, in which structure light isochores of the families L1 and L2 are always absent (Sazanov et al., 2003)), but also due to the spatial organization of actin filaments of erythrocytes and leukocytes. During movements, the cytoskeleton microfilaments of erythrocytes are rearranged in analogy to leukocytes. And this ability of the cytoskeleton activity enables the migration and phagocytosis responses of bird red blood cells (Chernyavsky et al., 2012).

When counting micronuclei in birds, all possible forms can be found. First of all, this is a common micronucleus, round in shape, smaller than the main nucleus, and located separately from it (Fig. 2, I). A kidney-shaped nucleus (Fig. 2, II), emarginated nucleus (Fig. 2, III), and caudate nucleus (Fig. 2, IV) are also distinguished.

There is an opinion that the kidney-shaped nucleus arises as a result of amplification of a certain part of the genome (Kursa et al., 2005). Caudate nuclei indicate the presence of dicentric chromosomes in the metaphase. The cauda may form when the chromatin bridges break between two cells (Kursa et al., 2005). The causes of the origin of erythrocytes with emarginated nuclei have not yet been fully studied.

In our studies, conducted under favourable environmental conditions, only classical micronuclei were detected in erythrocytes (Fig. 2, I). Their number in the sexually mature individuals of the white-collared flycatcher was 4.2 ± 0.58‰, in the nestlings 3.8 ± 0.32‰. In the sexually ma-

ture individuals of the pied flycatcher, the number of erythrocytes with micronuclei constituted 4.6 ± 0.33‰, and in the nestlings 4.14 ± 0.26‰. The indices of a micronucleus test were within normal limits that had been established for few other bird species of birds (Kursa et al., 2005). Age-related and interspecific differences in the number of micronuclei were not revealed.

We believe that the micronucleus test on the family Muscicapidae will be subject to further analysis and approbation. It is necessary to establish the maximum allowable limits for the micronucleus test for this family for further understanding and application of the songbird genetics.

Results with microsatellites

The ISSR-PCR, widely used for the taxonomic and phylogenetic comparison and as a means of mapping of many organisms, has allowed receiving genomic fingerprints of a number of animals and fish. The primer $(AGC)_6 G$, homological with the section of the bovine leukosis, was found to be informative regarding the microsatellite loci of genomic DNA for mapping of the channel catfish (Khmeleva et al., 2009). This primer is also quite effectively applied for the analysis of DNA polymorphism in horses (Kurilenko and Suprun, 2015) and cattle (Glazko and Glazko, 2015).

The peculiarity of microsatellite loci is in their distribution in conservative, weakly varying regions of DNA and in their ability to demonstrate high levels of intra-species polymorphism.

A comparative analysis of distribution density of the nucleotide sequences, potentially predisposed to the formation of non-canonical DNA structures (G4 quadruplexes) along the length of env and pol genes of the bovine leukemia virus, indicates a possible relationship between the increased density of non-canonical structures of nucleic acids in the genes, with the products interacting with a receptor system of the cells – targets of retroviral infection, and their genetic heterogeneity, supported in a "host-pathogen" system (Glazko and Kosovsky, 2013).
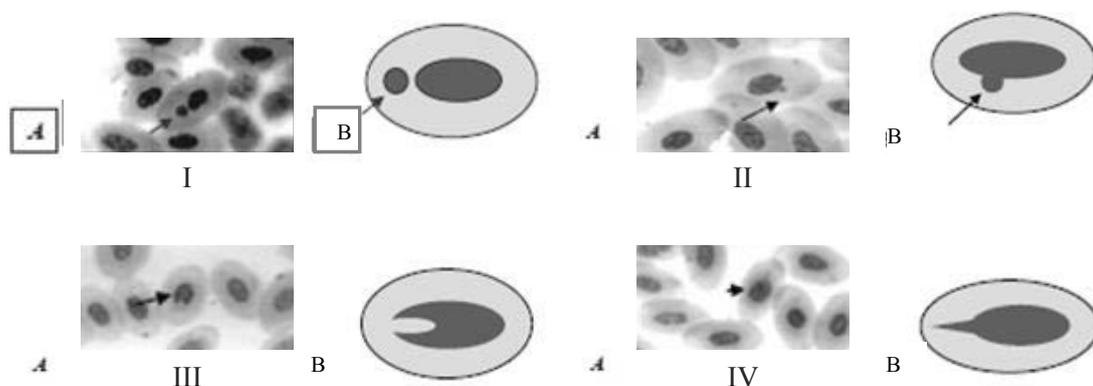

Fig. 2. A mature erythrocyte (A – photo, B – scheme): I – with a micronucleus; II – with a kidney-shaped nucleus; III – emarginated nucleus; IV – caudate nucleus (Kursa et al., 2005).

Our studies have revealed that the white-collared fly-catchers demonstrate more heterogeneous structure (Table 1). It can be expected that the known genetic heterogeneity of the retroviruses genes, with products directly interacting with the receptor system of the host cells, derives, to a certain extent, from the increased density of localization of non-canonical structures in those genomic regions of retroviruses which play a key role in the retrovirus infectivity. It allows us to consider new approaches for detection. It can also be expected that the increased variability of such sequences reflects the phylogeny of the two species. An intriguing fact is that the PCR analysis of the pied flycatchers, not infected with the bovine leukemia virus, found fragments of relatively low density.

Conclusions

1. Literary analysis of the studied relationships between the diets of the studied nestlings of the family Muscicapidae showed that the prey items eaten by the nestlings of the pied flycatchers and white-collared flycatchers were different. The pied flycatchers prefer imagoes, while white collared flycatchers more frequently choose caterpillars. We assume that consumption of caterpillars raises the carotene content in the white collared flycatchers. In its turn, the level of carotene could have an impact on the formation on heterophiles. We have found that the heterophyle percentage in the nestlings of the white-collared flycatcher exceeded by 3.23% ($p < 0.001$) that in the nestlings of the pied flycatcher. Supposedly, the reduction of heterophiles indicates the decrease in bactericidal and antitoxic blood functions.
2. Studying the karyotype stability of two flycatcher species we did not find any significant interspecies or age-related difference. However, we think that it is necessary to establish the maximum permissible limits of the micronucleus test for this family − for further understanding and application of the songbird genetics.
3. Using the primer $(AGC)_6 G$ for the genomic scanning of flycatchers allows obtaining polylocus spectra of the amplification products the polymorphism of which is closely related to retrotranspositions and products of recombination between retrotransposons. There is a definite trend to an increasing frequency of the sections identical to retrotransposons, or to the products of their combination in the white-collared flycatchers. Apparently, the spectra of the amplification products obtained with the primer (AGC)6 G may be especially effective for the study of intra-species genetic differentiation.

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