UDC 575
DOI: 10.2298/GENSR1501311R
Original scientific paper

SCREENING OF B CHROMOSOMES FOR PRESENCE OF TWO GENES IN YELLOW-NECKED MICE, *Apodemus flavicollis* (Mammalia, Rodentia)

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Rajičić M., T. Adnađević, G. Stamenković, J. Blagojević and M. Vujošević (2015*): Screening of B chromosomes for presence of two genes in yellow-necked mice, Apodemus flavicollis (Mammalia, Rodentia)* - Genetika, Vol 47, No. 1, 311-321.

B chromosomes (Bs) are a very heterogeneous group of extra chromosomes. In various species Bs occur with different nucleotide sequences ranging from repetitive to protein coding. In yellow-necked field mice, *Apodemus flavicollis* Bs are small euchromatic chromosomes and untill now, only few molecular analyses have been conducted. In this study we examined *A. flavicollis* individuals with different number of Bs for presence of two genes, C-KIT and 18S rRNA. The C-KIT proto-oncogene was found on Bs in three Canidae species and one Cervidae species. This gene is a coding receptor critical for proliferation and cell differentiation of hematopoietic, melanoblast and primordial germ cells, and is highly conserved within mammals. While using semiquantitative PCR, we did not notice any difference in the C-KIT band intensity among animals with different number of Bs (0-3). The presence of only one copy of C-KIT gene was confirmed using real time-PCR on genomic DNA of *A. flavicollis* specimens with different number of Bs. rRNA genes in eukaryotes' genome are organized like units of tandem repeated sequences. The units form distinct clusters on one to several chromosome pairs. rRNA genes were found on Bs in different species including two species of genus *Apodemus*. One particular sample with 2 Bs showed the number of 18S rRNA gene about three times that of the calibrator 0 B sample. This result can indicate the presence of 18S rRNA gene on Bs, but its confirmation requires the implementation of other methods. Still, we can neither confirm nor deny the existence of pseudogen of tested target genes, or lose of exon 1 of C-KIT protooncogen in Bs of *A. flavicollis*. Our findings are further discussed.

Key words: Apodemus flavicollis, B chromosomes, C-KIT, 18S rRNA, semiquantitative PCR

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INTRODUCTION

B chromosomes (Bs) are supernumerary chromosomes in a standard karyotype. They are found in all main groups of plants, animals and fungi reaching an average of nearly 15%. B chromosomes are defined as dispensable supernumerary chromosomes which do not recombine with members of the basic A chromosome complement and do not behave according to rules of the Mendelian segregation law. Different aspects of B chromosomes biology were carefully reviewed, among others by JONES and REES (1982), CAMACHO *et al*. (2000), BURT and TRIVERS (2006) and HOUBEN *et al*. (2013). It was generally believed that most of B chromosomes do not harbour genes (JONES and REES, 1982; CAMACHO *et al.,* 2000), but recent findings revealed that Bs of some species are rich in gene-derived sequences. Clusters of rRNA genes are found on Bs in many different species (STITOU *et al.,* 2000; TRIFONOV *et al.,* 2002; MATSUBARA *et al.,* 2004). In ascomycete fungus, *Nectria haematococca*, several functional genes bringing resistance to an antimicrobial compound produced by its host garden pea, *Pisum sativum*, were mapped on their B chromosome (HAN *et al.,* 2001; RODRIGUES-CARRES *et al.,* 2008; COLEMAN *et al.,* 2009). In cichlid fish, *Lithochromis rubripinnis* and Hochstetter's frog, *Leiopelma hochstetteri*, Bs play role in sex determination (YOSHIDA *et al.,* 2011). Recent sequence characterization of the rye, *Secale cereale,* showed that Bs are rich in gene-derived sequences (MARTIS *et al.*, 2012). Transcription of few protein encoding genes in this species has been associated with Bs (HOUBEN *et al.,* 2013). Furthermore, large autosomal segment was discovered in all B chromosomes and B-derived transcripts of the Siberian roe deer (TRIFONOV *et al.,* 2013). The proto-oncogene C-KIT is the first unique autosomal gene found on the mammalian Bs. Copies of this gene with intron-exon boundaries has been first found on all Bs in two Canidae species, red fox (*Vulpes vulpes*) and racoon dog (*Nyctereutes procynoides*), that diverge from common ancestor more than 12.5 million years ago, which indicated common origin of Bs in these species (GRAPHODATSKY *et al.,* 2005; YUDKIN *et al.,* 2007). GRAPHODATSKY *et al.* (2005) proposed that Bs could be beneficial for their carrier due to absence of accumulation of multiple mutations in coding region of C-KIT of canid B-chromosomes. The presence of this gene was later detected on Bs of one more Canidae species, Japanese racoon dog (MAKUNIN *et al.,* 2014) and the most recently on one cervid species, brown brocket deer, *Mazama gouazoubira* (MAKUNIN *et al.,* in preparation). In three Canidae species nine out of ten examined chromosomal regions on Bs were found to be species-specific except the C-KIT containing region (MAKUNIN *et al.,* 2014). The authors proposed that some sequences are re-used for B chromosome in various lineages independently as an alternative explanation for the presence of the same gene on B chromosomes in different species. That raises a question are C-KIT genes present on Bs of other mammalian species.

The C-KIT proto-oncogene represents the cellular homologue of V-KIT, the oncogene of HZA feline sarcoma virus (BESMER *et al.,* 1986). The gene encodes the transmembrane receptor KIT, which is a type-III tyrosine kinase receptor, a protein consisting of an extracellular ligand-binding region (5 immunoglobulin-like domains), a single transmembrane spanning region (hydrophobic domain), and a cytoplasmic region. There has been reported a high conservation in sequence for the C-KIT gene within mammals (MA *et al.,* 1999). The function of C-KIT receptor is critical for proliferation and cell differentiation of hematopoietic, melanoblast and primordial germ cells (ASHMAN, 1999). Most mastocytomas and intestinal stromal tumours attested in human, mouse, dog, and rat are caused by C-KIT mutations (HEINRICH *et al.,* 2002; BOISSAN *et al.,* 2000). Pigmentation disorders in mouse, pig, goat, and human are also associated with different C-KIT

mutations (CHABOT *et al.,* 1988; FLEISHMAN *et al.,* 1991), as well as sterility in adult mouse (FENG *et al.,* 1997).

In eukaryotes, each unit of tandemly repeated sequences of ribosomal genes (rRNA) is composed of tree genes coding for 5S, 18S and 28S ribosomal RNA, separated by two intergenic transcribed spacers (ITS) and an intergenic spacer (IGS). These tandemly repeated sequence units form distinct clusters at the nucleolus organizer regions (NORs) of one to several chromosome pairs. In genus *Apodemus*, the distribution pattern of the 18S-28S rRNA genes is widely different among species, from conserved predominantly located on chromosomes 7 and 8 in species from Asia, to clusters spread on numerous chromosomes in species from Palearctic region (BOESKOROV *et al.,* 1995; MATSUBARA *et al.,* 2004). In *A. flavicollis* karyotype, depending on number and location of active rRNA clusters, there are two cytotypes present. The first cytotype has NORs localized on 6-8 chromosomes in telomeric region and on 1-2 in pericentromeric region. In second cytotype NORs are localized only on telomere region on 4-8 chromosomes (KARTAVSEVA *et al.,* 2002). Many studies have confirmed the presence of rRNA genes on Bs in different mammalian species: *Rattus rattus* (STITOU *et al.,* 2000), *Apodemus peninsulae* (TRIFONOV *et al.,* 2002; MATSUBARA *et al.,* 2004), *Akodon montensis* and *Oryzomys angouya* (SILVA and YONENAGA-YASSUDA, 2004). The 5S genes were also found on B chromosome in *A. agrarius* (MATSUBARA *et al.,* 2004).

The presence of Bs in mammals is below 2% (VUJOŠEVIĆ and BLAGOJEVIĆ, 2004). In the light of this fact genus *Apodemus* is an exceptional one with almost one third of the species having Bs. In yellow necked field mouse, *Apodemus flavicollis*, Bs are found at different frequencies in almost all populations (VUJOŠEVIĆ *et al.,* 1991; WÓJCIK *et al.*, 2004; KARTAVTSEVA, 2002; VUJOŠEVIĆ *et al.*, 2007). Cytological findings showed that Bs, in this species, exhibit a euchromatic nature and show homology in distribution of G- and C-bands with certain small A chromosomes (VUJOŠEVIĆ and ŽIVKOVIĆ, 1987). Prior studies showed that three cDNA fragments were differentially expressed due to the presence of B chromosomes in *A. flavicollis* (TANIĆ *et al.,* 2005). Therefore, the aim of this study was to evaluate C-KIT and 18S rRNA genes copy number in specimens of *A. flavicollis* with different number of B chromosomes using semiquantitative PCR and real time- PCR (RT-PCR) analyses.

MATERIALS AND METHODS

The study was performed on DNA samples of ten specimens of *A. flavicolis,* five males and five females, collected at three different localities in Serbia (Mt. Tara, Mt. Avala and Mt. Cer) with different number of Bs. Four of the specimens used in the study had no Bs, two of them had 1B, three animals had 2Bs, and two had 3Bs. The animals were live-trapped using Longworth traps provided with hay and food, and treated according to the legal and ethical guidelines current in the countries where they were sampled. Chromosomes were prepared directly from bone marrow cells using the standard technique (HSU and PATTON, 1969). The presence and number of Bs were determined from 30 analyzed metaphase figures. All animals with more than 48 chromosomes (standard complement) were considered to have Bs. The genomic DNA (gDNA) was extracted from liver tissues using DNeasy Blood and Tissue Kit (Qiagen, Germany).

Semiquantitative PCR analyses

In order to estimate copy number of C-KIT genes in DNA samples of individuals with and without B chromosomes, we performed miltiplex PCR. In order to quantify the efficiency of

C-KIT amplification, the shadow protein gene (*Sprn*), which is a single copy gene, was used as an internal control. Genomic DNA was amplified in the same PCR reaction with two primer pairs selected by softwer OligoAnalizer as a convenient: CK12 forward primer 5'-CCTGGTCTTAGAGGGCACAG-3' and CK2 reverse primer 5'-AAAGCATCACCAAACTCGCC-3'. Estimated PCR product was 589 bp length segment of C-KIT gene exon 1. And the control sequence of the shadow protein gene (*Sprn*) amplification was obtained with MusSPRd forward primer 5'-GATGGAGTTTAGCCTGGTCT-3', and MusSPRu reverse primer 5'- CAATTCTGCCCAGTAGGATG-3'. Estimated PCR product was 478 bp length. PCR was performed in 1x PCR Dream Taq buffer, 10mM dNTPs, 20μM of each primer, 1U of Dream Taq polymerase (Thermo Scientific Inc.) and 200ng gDNA. PCR products were obtained by program: 95°C for 2 min, 34 cycles of 95°C (30s), 60°C (30s) and 72°C (1min); and final extension at 72°C for 5 min, and analyzed on 1,2% agarose gel. Standard molecular weight marker (GeneRuler 100 bp DNA Ladder) was used in electrophoretic run. The intensity of the C-KIT band and the internal control (*Sprn* gene) within one sample and between different samples was visually scored and compared.

Real time-PCR

In order to avoid subjectivity and get more precise results, the same primer pairs for C-KIT and *Sprn* sequence together with the same PCR program were used for RT-PCR. It was performed on gDNA from eight individuals, with 0B, 1B, 2B and 3B chromosomes (including three individuals used in multiplex PCR as control). RT-PCR was obtained in separate tubes for target and control gene, in duplicates, including no template control.

RT-PCR was also used to estimate copy number of 18S rRNA gene in DNA samples of individuals with and without B chromosomes. 18S rRNA gene target sequence was obtained by the forward (5'-AGTTCCAGCACATTTTGCGAG-3') and reverse (5'-TCATCCTCCGTGAGTTCTCCA-3') primers. The control sequence for this reaction was a 216bp long fragment of exon 2 of the MHC class II DRB gene which is a single copy gene, obtained by the forward JS1 (5'-GAGTGTCATTTCTACAACGGGACG-3') and reverse JS2 primer (5'-GATCCCGTAGTTGTGTCTGCA-3').

Relative quantification was used to assess the potential existence of different copy number of two tested target genes depending on Bs number within the analysed DNA samples. The results were normalized to the endogenous control for each target gene, and reported as a fold change relative to a calibrator sample. The DNA sample from 0B individual was used as the calibrator. The comparative Ct method was used for relative quantification, which was calculated from the threshold cycle (Ct) values generated during PCR. The following equation was used:

$$\text{Relative Quantity} = 2^{-\Delta\Delta Ct}$$

The ΔCt is calculated by normalizing the Ct of the target sequence with the Ct of the endogenous control (Ct target – Ct endogenous control). The ΔΔCt is then calculated by subtracting the average ΔCt for the calibrator sample from the corresponding average ΔCt for the target sample. The relative levels of the target gene amplification are expressed as a fold change relative to the calibrator sample. A relative quantity of one gene copy indicates no changes in amplification levels. In case of the 18S rRNA gene, we simplified it by treating the sample with 0B, as calibrator with one gene copy. The ratio of C-KIT or 18S rRNA to each reference gene to the calibrator >2 indicates the presence of target gene in more than one copy (cut-off 2).

RESULTS AND DISSCUSION

In this study we evaluated relative copy number of two genes, C-KIT and 18S rRNA, in *A. flavicollis* individuals with different number of B chromosomes, using semiquantitative PCR and real time- PCR (RT-PCR) analyses.

To estimate the relationship between number of Bs and C-KIT copy number, we firstly performed the semiquantitative PCR on DNA from five yellow-necked mice without Bs, and with 1-3 Bs (Fig. 1). Comparing the intensity of C-KIT bands and *Sprn* bands within as well as between the lanes, we did not notice any difference of C-KIT band intensity between individuals with and without Bs.

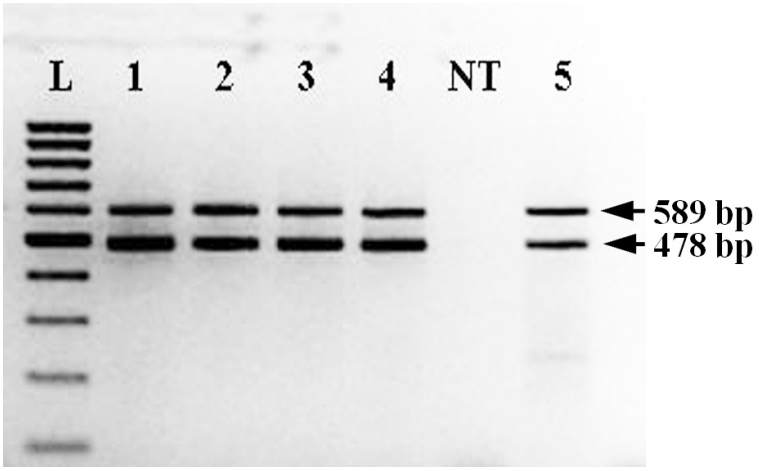
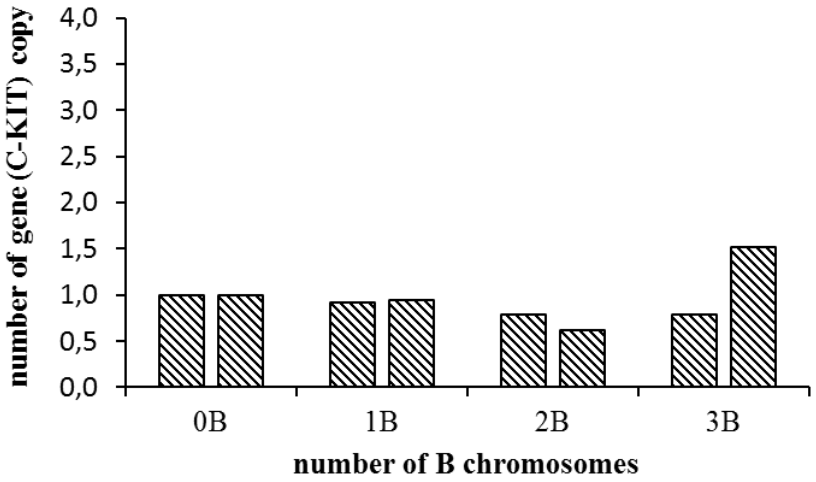


Fig. 1. PCR amplification of C-KIT and *Sprn* (control) genes from *A. flavicollis* genomic DNA; Lanes 1 and 2 represent PCR product amplified on gDNA from specimens with 0B, lane 3 represent PCR product amplified on gDNA from specimens with 2Bs, lanes 4 and 5 represent PCR product amplified on gDNA from specimens with 3Bs. L – DNA ladder 100-1000bp. The 589–bp (upper) band corresponds to C-KIT product and 478-bp (lower) band corresponds to *Sprn* product. NT - no template control.

Having performed RT-PCR on gDNA of *A. flavicollis* specimens with different number of Bs, we did not record existence of more than one copy of C-KIT gene in any of 8 tested samples (Fig. 2a).

The analysis of the 18S rRNA gene showed that only one sample with 2Bs had about 3 times increased the number of 18S rRNA gene copy compared to the 0B calibrator sample (Fig. 2b). The other analysed samples, both with and without B chromosomes, did not show any difference in copy number of 18S rRNA gene when compared to the calibrator sample.

a)



b)

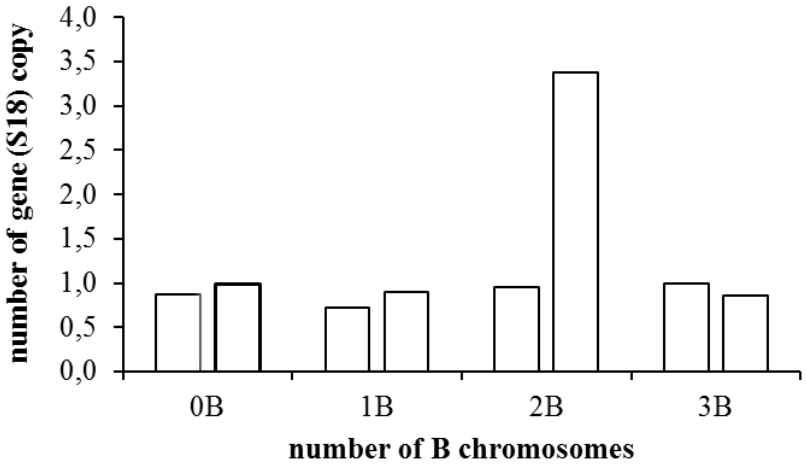


Fig. 2.Relative quantification of a) C-KIT b) 18S rRNA gene copy number from genomic DNA of *A. flavicollis*

Considering the presence, number and structure, B chromosomes are highly heterogeneous in different species. In various organisms, Bs can contain repetitive elements, telomeric sequences (WURSTER-HILL *et al.,* 1988), ribosomal DNA clusters (MATSUBARA *et al.,*

2004), and histone genes (TERUEL *et al.,* 2010). Various protein-coding sequences were detected on Bs of fungi (HAN *et al.,* 2001, RODRIGUES-CARRES *et al.,* 2008, COLEMAN *et al.,* 2009), cichlid fish (YOSHIDA *et al.,* 2011), fox and raccoon dog (GRAPHODATSKY *et al.,* 2005; YUDKIN *et al.,* 2007), and roe deer (TRIFONOV *et al.,* 2013).

The occurrence of Bs has been reported in six *Apodemus* species: *A. flavicollis* (SOLDATOVIĆ *et al.,* 1972), *A. peninsulae* (HAYATA, 1973), *A. sylvaticus* (VUJOŠEVIĆ and ŽIVKOVIĆ, 1987), *A. mystacinus* (BELCHEVA *et al.,* 1988) *A. agrarius* (KARTAVTSEVA, 1994), and *A. argenteus* (OBARA and SASAKI, 1997). There are wide differences in the number of Bs and frequency of individuals with Bs among *Apodemus* species (VUJOŠEVIĆ *et al.* 2007). The origin and molecular structure of Bs were mainly studied in *A. peninsualae* (KARAMISHEVA *et al.,* 2002; TRIFONOV *et al.,* 2002; RUBTSOV *et al.,* 2004; MATSUBARA *et al.,* 2004, 2008). In this species, Bs are meta-, submeta-, acrocentric, and dotlike derivates of autosomes. There were three families of repetitive sequences derived from autosomes that were independently amplified on Bs. The 18S-28S rRNA genes appeared to be localized to meta- or submetacentric Bs (MATSUBARA *et al.,* 2004). It is suggested that Bs appeared in *A. peninsulae* independently of other *Apodemus* species (MATSUBARA *et al.,* 2008), and that different types of Bs within this species have multiple origins (TRIFONOV *et al.,* 2002; MATSUBARA *et al.,* 2004). The fact that Bs of *A. flavicollis* are euchromatic (VUJOŠEVIĆ and ŽIVKOVIĆ, 1987) suggests the possible existence of active genes.

Proto-oncogene C-KIT have important role for proliferation and cell differentiation of hematopoetic, melanoblast and primordial germ cells (ASHMAN, 1999), and its sequence is highly conserved within mammals (MA *et al.,* 1999). The presence of such gene, with complete exon-intron boundaries, on all B chromosomes in two Canidae species, suggests common origin of B chromosomes. They also showed that this gene has preserved transcription activity. A more recent research, on the contrary, showed that C-KIT copy on Bs of the red fox is not translated or was not completely functional (MAKUNIN *et al.,* 2014). In the same paper it was revealed that Bs of three canidae species contained ten autosomal regions, with at least four different proto-oncogenes or tumor suppressor genes.

It is estimated that a common ancestor of *Murinae* and *Canidae* families lived about 83.3 and 91.8 Mya (MEREDITH *et al.,* 2011). Considering such a long divergence time, as well as the fact that both *Muridae* and *Canidae* genomes have been extensively reorganized during evolution (GRAPHODATSKY, 2007), the possibility that Bs in these two families have the same origin is unexpected. Furthermore, the comparative study of structure and molecular organization of raccoon dog and Asian wood mice Bs suggests independent origin of B chromosomes in these two mammalian species (TRIFONOV *et al.,* 2002). Considering this fact potential presence of C-KIT gene on Bs of *A. flavicollis* would not indicate common origin of *A. flavicollis* and Canidae Bs. Considering the studies performed until now, the origin of Bs differ between individuals as well as between species within genus *Apodemus* (MATSUBARA *et al.,* 2004*)*. Four groups of *Apodemus* species have diverged from the same ancestor more recently (8-10 Mya) (SERIZAWA *et al*., 2000) and cytogenetic studies indicate that their Bs originated independently. We did not detect more than one copy of C-KIT exon 1, in all tested DNA samples of *A. flavicollis* containing from 0-3 B chromosomes. However, we were not able to either exclude the presence of pseudogene, or the loss of exon 1 of C-KIT proto-oncogene on Bs of *A. flavicollis.*

MATSUBARA *et al.* (2004) showed that the distribution patterns of the 18S-28S rRNA genes in seven *Apodemus* species were well correlated with the phylogenetic relationships determined using mitochondrial *cyt* b and nuclear IRBP genes by Serizawa *et al.* (2000). Genes for

rRNA are organized in clusters and spread widely in genome of *A. flavicollis* (BOESKOROV *et al.,* 1995). Depending of the number and localization of active rRNA genes (NORs) there were two cytotypes defined (KARTAVTSEVA *et al.,* 2000). Although rRNA genes have been found on Bs in different species (GREEN 2004), they have never been found on the Bs of the studied species. We recorded an increased number of 18S rRNA gene copies in only one sample, an individual with 2Bs. This can indicate a possible presence of this gene on Bs of the mentioned individual. The used method was not sufficient to confirm whether this is an active gene or a psudogene. Furthermore, it is possible to presume that all studied Bs are not the same, or that they originated from different chromosomes of A set, so some of them could carry rRNA genes while the other could not. Fluorescent *in situ* hybridization should resolve questions regarding presence and origin of 18S rRNA gene copies on Bs in the species.

ACKNOLEDGEMENTS

Support provided by the Ministry of Science and Technological Development of the Republic of Serbia (Grant 173003). We are very grateful to Prof. Jelena Milašin and her team for expert advices and technical support for the part of the research.

Received December 25th, 2014
Accepted February 25th, 2015

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PROVERA PRISUSTVA DVA GENA NA B HROMOZOMIMA KOD ŽUTOGRLOG MIŠA, *Apodemus flavicollis* (Mammalia, Rodentia)

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Izvod

B hromozomi su veoma heterogena grupa dodatnih hromozoma. Kod razlčitih vrsta, B hromozomi poseduju različite nukleotidne sekvence od repetitivnih do onih koji kodiraju proteine. Kod žutogrlog miša, *Apodemus flavicollis,* B hromozomi su mali euhromatični hromozomi i do sada je na njima urađeno nekoliko molekularnh analiza. U ovom radu ispitivali smo prisustvo dva gena, C-KIT i 18S rRNA, kod jedinki vrste *A. flavicollis* sa različitim brojem B hromozoma. C-KIT protoonkogen je pronađen na B hromozomima kod tri vrste iz familije Canidae i jedne vrste iz familije Cervidae. Ovaj gen, visoko konzerviran kod sisara, kodira receptor značajan za proliferaciju i diferencijaciju hematopoetičnih ćelija, ćelija melanoblasta i primordijalnih germinativnih ćelija. Upotrebom semikvantitativnog PCR-a, nisu uočene razliku u intenzitetu traka karakterističnih za C-KIT gen između jedinki sa različitim brojem (0-3) B hromzoma. Prisustvo samo jedne kopije C-KIT gena potvrđeno je upotrebom RT-PCR na genomskoj DNK jedinki *A. flavicollis* sa različitim brojem B hromozoma. rRNK geni u genomu eukariota su organizovani u jedinicama sastavljenim od tandemski ponovljenih sekvenci. Ove jedinice formiraju različite klastere na jednom ili više hromzomskih parova. rRNK geni su nađeni na B hromozomima različitih vrsta uključujući i dve vrste roda *Apodemus*. Samo jedan uzorak sa 2B hromzoma je pokazao oko tri puta povećan broj kopija rRNK gena u poređenju sa 0B kalibrator uzorkom. Ovakav rezutat može da ukaže na prisustvo 18s rRNK gena na B hromozomima, ali da bismo to potvrdili neophodna je upotreba drugih metoda. Na osnovu ovog istraživanja nije moguće potvrditi niti opovrgnuti prisustvo pseudogena, kao ni gubitak egzona 1 C-KIT protoonkogena na B hromozomima *A. flavicollis*.

Primljeno 25. XII 2014.
Odobreno 25. II. 2015.