



# Cytogenetic Characteristic of *Apodemus epimelas* (Nehring, 1901) (Rodentia: Muridae) from Bulgaria: Constitutive Heterochromatin and Nucleolar Organiser Region Distribution

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**Abstract:** The taxonomic status of the western broad-toothed mouse, *Apodemus epimelas* (Nehring, 1901), was established in 2005 based on variations in morphological and molecular genetic data. Previously, it was considered part of the eastern broad-toothed mouse, *Apodemus mystacinus* (Danford & Alston, 1877). This species is endemic to the western and southern Balkans, including Bulgarian populations along the Struma River. The distribution of the constitutive heterochromatin and the nucleolar organiser regions was examined using C- and NOR banding techniques. The results of this study show that the species has a diploid chromosome number of  $2n=48$ , with  $FN=52$ , consisting mainly of acrocentric chromosomes and only two small metacentric autosomal pairs. The distribution and extent of C-bands at pericentromeric regions varied among chromosome pairs and primarily appeared at centromeric positions of the autosomes. The X chromosome was consistently the second largest acrocentric chromosome with distinct intercalated C-bands, while the Y chromosome was present as a small, entirely heterochromatic acrocentric chromosome. Here, we present the distribution of nucleolar organiser regions (NORs) in six pairs of autosomes for the first time. The centromere heterochromatin and NORs mixed (centromeric and telomeric) locations characterised the reported species' cytotype, providing valuable insights into the cytogenetic characteristics of this species.

**Key words:** *Apodemus epimelas*, karyotype, C-banding, NOR's

## Introduction

The genus *Apodemus* Kaup, 1829 has been the subject of a long-standing scientific debate concerning the characterisation and number of its species, as well as their phylogeography and evolutionary history. Currently, the genus includes over 20 field and wood mice species (MUSSEr & CARLETON 1993), widely distributed throughout

the Palaearctic region. These species are typically categorised into 3 subgenera. In the past, the genus was conventionally segmented into three subgenera: *Apodemus*, found from central Europe to eastern Asia, *Sylvaemus* occurring in the western Palaearctic, and eastern Palaearctic *Alsomys* (ZIMMERMANN 1962). MUSSEr et al. (1996) later categorised wood mice as *Sylvaemus*, *Apodemus* and *Argenteus*. Recent taxonomic revisions (SERIZAWA

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et al. 2000, SUZUKI et al. 2003) have identified two major subgenera: *Apodemus* and *Sylvaemus*. The subgenus *Apodemus* encompasses most species with an East Asian distribution and *Apodemus agrarius*, which has a discontinuous Eurasian range. The subgenus *Sylvaemus* Ognev, 1924 consists of most species with distributions in Europe and the Near East. In Europe, the subgenus *Sylvaemus* is represented by five species: *Apodemus flavicollis* (Melchior, 1834), *Apodemus sylvaticus* (Linnaeus, 1758), *Apodemus alpicola* (Heinrich, 1952), *Apodemus microps* (Kratohvil & Rosickiy, 1952), and *Apodemus mystacinus* (Danford & Alston, 1877).

*A. mystacinus*, occurring on the Balkan Peninsula and the Middle East, is morphologically distinguishable from other *Sylvaemus* species. There are ongoing debates regarding its classification. Some authors (RIETSCHEL & STORCH 1974, STORCH 1975) have classified it as a member of the separate subgenus *Karstomys* Martino, 1939, while others have placed it in *Sylvaemus*, based on morphological, chromosomal, and genetic data (MUSSEY et al. 1996, MARTIN et al. 2000). According to several studies, the validity of *Karstomys* remains disputed (CORBET 1978, MUSSEY et al. 1996, NIETHAMMER 1978). Nevertheless, it is widely accepted that *A. mystacinus* is not closely related to other *Sylvaemus* species. It remains uncertain if the allocation of a distinct subgenus is warranted. MICHAUX et al. (2002) suggest that more studies need to provide conclusive evidence regarding this matter. FELTEN et al. (1973) propose the recognition of two subspecies for the species: *A. mystacinus epimelas*, distributed on the Balkan Peninsula, and nominal *A. mystacinus mystacinus* of the Middle East. MITCHELL-JONES et al. (1999) indicated that the Balkan *A. epimelas* and Anatolian *A. mystacinus* were different species. The significant differences between the two were documented earlier by STORCH (1977), who provided the primary discriminatory data. STORCH observed distinct occlusal patterns between the two species regarding molars  $M_1$  and  $M_2$ . In particular, the posterior cingulum is free in most *A. epimelas*, while it fuses with the cusp tuberculum t8 in *A. mystacinus*.

The splitting hypothesis of *A. mystacinus* into two separate subspecies, *A. mystacinus epimelas* and *A. mystacinus* s.str. has been supported in recent years by morphological and molecular data (FILIPPUCCI et al. 2002, MICHAUX et al. 2002, 2005, VOHRALÍK et al. 2002). Based on the variation of morphological and biochemical genetic data, MEZHHERIN (1997) considered subspecies *A. mys-*



**Fig.1.** Topographic locations of the sampling sites. The red dots denote the sampling sites of this study, and the blue dot represents the only previous research on *A. mystacinus* karyotype in Bulgaria (BELCHEVA et al. 1988).

*tacinus epimelas* to be a separate species from *A. mystacinus mystacinus*, a position supported by phylogenetic analyses of protein electrophoresis of 28 to 38 loci (FILIPPUCCI et al. 2002), mtDNA cytochrome b and 12S rRNA sequences (MICHAUX et al. 2002), sequences of the nuclear IRBP gene and two mitochondrial regions, the cytochrome b gene, and the D-loop region (MICHAUX et al. 2005).

These studies have given rise to the splitting of *A. mystacinus* into two separate species: *A. mystacinus* and *A. epimelas* (WILSON & REEDE 2005). The Western broad-toothed field mouse (*A. epimelas*) is endemic to the western and southern Balkans, where it is found in Croatia south of Bosnia and Herzegovina, southern Serbia, Kosovo, Montenegro, Albania, Macedonia, southwestern Bulgaria, and Greece (KRYŠTUFEK & VOHRALÍK 2016).

Chromosomal studies have not confirmed the distinct genetic divergence between populations of *A. mystacinus* and *A. epimelas* distributed on either side of the Bosphorus. In recent years, the scrutiny of the karyotype of *A. epimelas* has witnessed a decline in attention, believed to be due to its conservatism and the absence of recorded chromosomal variations. Other than the C-banding analysis conducted on populations from Greece, cytogenetic characterisation of this species has yet to be accomplished. This study aims to cytogenetically characterise the Bulgarian populations of *A. epimelas* by analysing the localisation of constitutive heterochromatin and Nucleolus Organiser Regions (NORs). This would improve the description of the species' karyotype and facilitate in future comparative analyses.

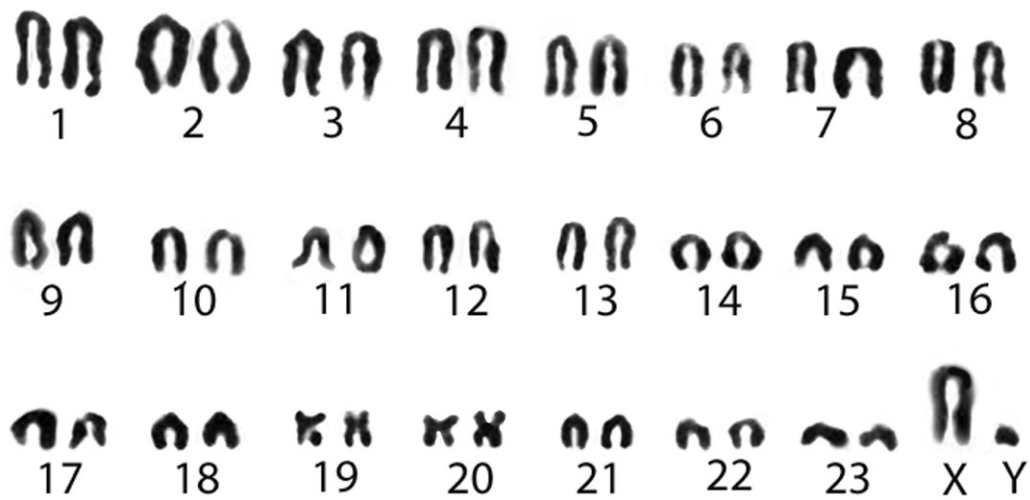


Fig.2. Male karyotype of the Western broad-toothed field mouse (*A. epimelas*) from southwestern Bulgaria (Rupite).

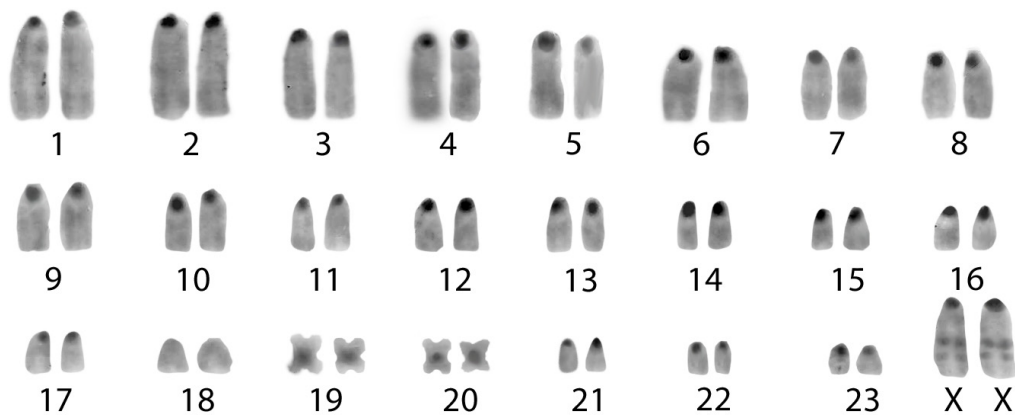


Fig.3. Female C-banded karyotype of the Western broad-toothed field mouse (*A. epimelas*) from southwestern Bulgaria (Koprivlen).

## Materials and Methods

### Specimen collection

Five specimens of *A. epimelas*, three males and two females, were live-trapped from two populations in Southwest Bulgaria: Rupite village (N 41.44367°, E 23.24308°) and Koprivlen village (N 41.52197°, E 23.79312°) (Fig. 1). The coloration pattern and body dimensions were the main distinguishing features of *A. epimelas* from other *Apodemus* species.

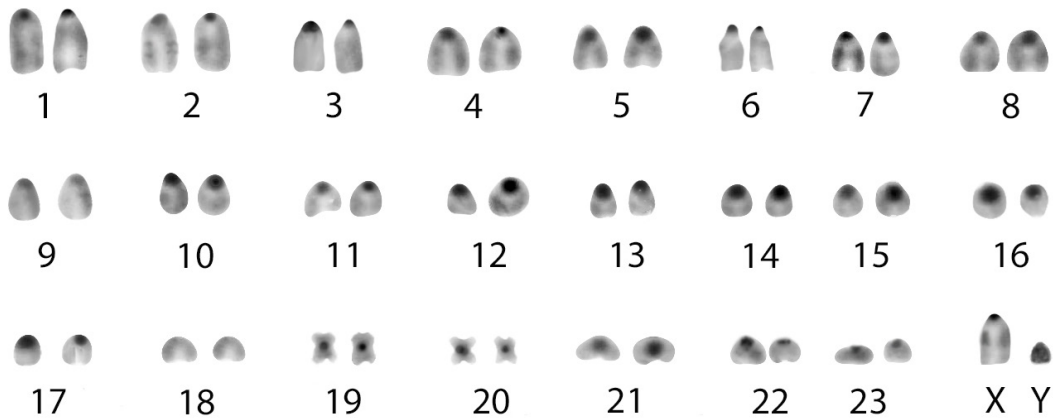
### Cytogenetic analysis

Mitotic chromosome preparations were obtained from bone marrow using standard cytogenetic techniques (ROTHFELS & SIMINOVITCH 1958). The chromosomes were subjected to hypotonic treatment with 0.56% KCl in water for 20 minutes and subsequently fixed with methanol/acetic acid (3:1) before being air-dried. The conventional stained

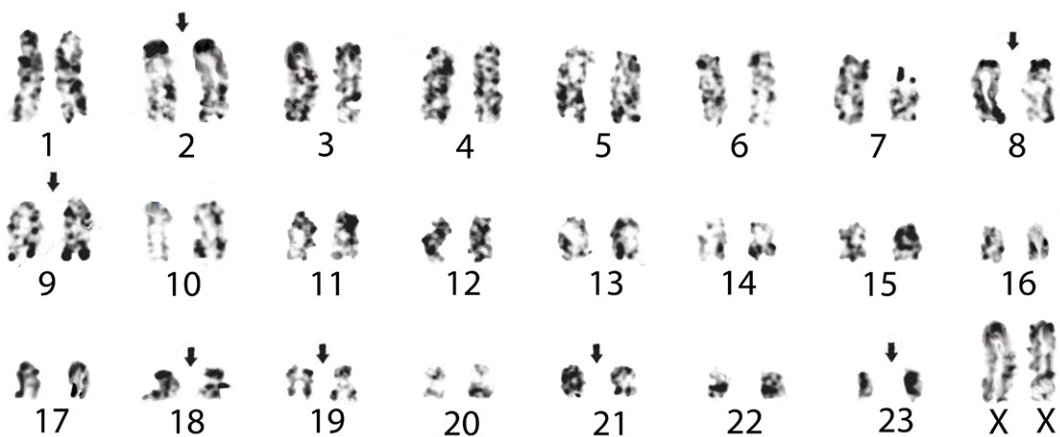
karyotypes arranged the chromosomes by size in descending order. To determine the distribution of constitutive heterochromatin and NORs, we conducted differential C- and NOR-banding through standard procedures (SEABRIGHT 1971, SUMNER 1972, GOODPASTURE & BLOOM 1975, PELLICCIARDI et al. 1990). We analysed twenty metaphase plates per individual.

## Results

In Bulgaria, the diploid chromosome number of *A. epimelas* was established at  $2n=48$  (FN=52). The autosomes consisted of 21 acrocentric pairs that decreased in size continuously. Additionally, there were two pairs of small bi-armed chromosomes (No. 19 and No. 20) that were barely distinguishable from each other by centromere position and size (Fig. 2). The sex chromosomes were acrocentric,



**Fig.4.** Male C-banded karyotype of the Western broad-toothed field mouse (*A. epimelas*) from southwestern Bulgaria (Rupite).



**Fig.5.** Female Ag-banded karyotype of the Western broad-toothed field mouse (*A. epimelas*) from southwestern Bulgaria (Rupite).

and the Y chromosome was the smallest acrocentric chromosome in the set.

The heterochromatin was located in the centromeric regions of the chromosomes, except for the 18th autosome pair, as revealed by C-banding (Fig. 3). This observation was consistent in both sexes and across all metaphase plates of each individual analysed (Fig. 4). Heterochromatin blocks of varying sizes, as well as visible, distinct intercalated bands on X-chromosomes, were identified (Fig. 3). Additionally, a faint C-staining was observed in the centromeric region of the metacentric chromosomes. The Y chromosome was entirely heterochromatic (Fig.4).

The study established the existence of NORs in six chromosomal pairs for the first time. The localisation of NORs was found to be mixed, with four pairs (№ 2, № 8, № 18, and № 23) showing pericentromeric NORs and the other two (№ 9 and №21) displaying telomeric NORs (Fig. 5). NORs were identified in the telomeric regions of one of

the biarmed autosome pairs. Notably, the location of NORs at the telomeric position was less frequent than at the centromeric position.

## Discussion

The karyotypes of the wood mice of the subgenus *Sylvaemus* have relatively uniform basic characteristics. The karyotypes of the studied *Apodemus* species are also rather homogenous, concerning the predominant diploid number of 48 largely acrocentric chromosomes. This uniformity extends to the karyotype of a related allopatric species, *A. epimelas* and *A. mystacinus* ( $2n=48$ ,  $NFa=50$ ,  $NF=52$ ;  $X=A$ ,  $Y=A$ ). The autosomal complement comprises 21 acrocentric and two metacentric pairs. Both sex chromosomes in both species are acrocentric (ZIMA & KRÁL 1984). The only recorded differences between the two species are related to a population with only one biarmed autosomal pair described from Greece (GIAGIA et al. 1985) and Bulgaria (BELCHEVA et al.

1988). Additionally, an individual from the Pernik region in Bulgaria was found to have a B-chromosome (BELCHEVA et al. 1988).

The karyotype data established for European populations of *A. mystacinus* can now be exploited for the species *A. epimelas*. BELCHEVA et al. (1988) presented the first karyotype characterisation of the Bulgarian populations previously known as *A. mystacinus* but now classified as *A. epimelas*. They studied two females from the Pernik region and identified two distinct karyotypes: one with two pairs of metacentric chromosomes (FN=52) and the other with only one pair of biarmed chromosomes (FN=50). Furthermore, the authors reported an individual with a supernumerary B-chromosome. It is worth noting that the first karyotype variant reported in this study is consistent with the first report of the karyotype of the species by SOLDATOVIC et al. (1969). ZIMA et al. (1997) reported differing karyotype characteristics. They described the smaller autosomal pairs as biarmed. In contrast, GIAGIA et al. (1985) reported only one metacentric chromosomal pair in the chromosomal complement. Previous studies (SOLDATOVIC et al. 1969, 1975, BELCHEVA et al. 1988, ZIMA et al. 1997, ROVATSOS et al. 2008) have indicated a higher frequency of karyotypes with two small metacentric chromosome pairs in *A. epimelas*, which is consistent with our current findings. Our research did not detect any supernumerary B-chromosomes, in contrast to the report from BELCHEVA et al. (1988). No other karyotype studies have been reported for the B-chromosomes in *A. mystacinus* or *A. epimelas*. This study presents the karyotypes of individuals inhabiting the species' habitats in southwestern Bulgaria. The karyotype description of the species is based primarily on the northern population, as shown in Figure 1. This may be clarifying the lack of reciprocation in the outcomes obtained.

This study presents the first distribution analysis of constitutive heterochromatin in *A. epimelas* from Bulgaria. The research shows that the 18th autosomal pair lacks centromeric blocks of heterochromatin, in contrast to the only previous study of heterochromatin distribution in *A. epimelas* populations in Greece (ROVATSOS et al. 2008). Therefore, this is the first report to present the absence of heterochromatin blocks in certain chromosomal centromeric regions in this species. Additionally, our study provides the first analysis of the C-banding pattern on the Y-chromosome.

This study details the first description of the distribution of NORs in the karyotype of *A. epimelas*. The presence of NORs in six pairs of chromosomes is consistent with the findings of OBARA et

al. (2007) study, which indicates that the number of NOR pairs in *Apodemus* species ranges between one and eleven. This range demonstrates a geographical correlation. Species from the Far East, such as Japan and Taiwan, display one to three pairs, while those from West Europe and Central Asia have five to eleven pairs. This suggests that the *Apodemus* genus may have diversified in two distinct geographic directions, with the number of NOR pairs either decreasing or increasing.

Mammals inhabiting the Balkan Peninsula may share biogeographical and phylogenetic links with faunal complexes in Asia Minor and the Near East (ZIMA 2004). The chromosomal data obtained in this study on *A. epimelas* provides definite evidence for the existing relationships between the related allopatric species, *A. epimelas* and *A. mystacinus*. No differences in the standard karyotype between the two species were found. The standard karyotype of *A. mystacinus* has been documented in Turkey by DOĞRAMACI & KEFELIOĞLU (1991), ÇOLAK et al. (2004), and ARSLAN & ZIMA (2014), as well as in neighbouring regions such as Israel and Jordan by ZIMA & MACHOLÁN (1995) and SÖZEN et al. (2008).

These results are consistent with the hypothesis proposed by HOSEY (1982) that *A. epimelas* may have originated from a population of *A. mystacinus* that migrated across a Bosphorus land bridge linking the Balkan Peninsula, Turkish Thrace and northwestern Anatolian Turkey some 10,000-20,000 years ago during the Pleistocene. Following the increase in sea levels during the post-Pleistocene period, the Black and Marmara Seas reconnected through the Bosphorus channel, leading to the genetic isolation of the species. MICHAUX et al. (2002) suggest that the isolation of the Balkan and Near East groups of *A. mystacinus* and the subsequent differentiation of *A. epimelas* may be due to cooling intervals that occurred in the late Pliocene or early Pleistocene, resulting in low population densities.

The molecular-genetic differences between the two species do not reflect the chromosomal conservatism observed in both. The absence of cytogenetic research on *A. mystacinus* also precludes potential comparative analysis of cytotypes between the two species. Notably, cytogenetic studies have often failed to establish distinct taxonomic status among well-differentiated species. The reliability of chromosomal variation caused by changes in heterochromatin distribution and amount as an indicator of taxonomic separation remains unclear (ZIMA 2004). Therefore, additional cytogenetic analysis of *A. mystacinus* is necessary to conclude potential chromosomal divergence among the species.

By examining the cytogenetic makeup of Bulgarian populations of *A. epimelas*, the research shed light on the unique chromosome-specific characteristics and variations of constitutive heterochromatin and nucleolar organiser regions. The study contributes to our understanding of the species' cytogenetic diversity and opens up new avenues for further investigation into the evolutionary processes shaping Bulgarian populations. The findings of this study have significant implications for both cytogenetics and the conservation of biodiversity in Bulgaria.

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