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## Karyological differences between two *Apodemus* species in Bulgaria

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### Abstract

Described on the basis of a karyological study two cytotypes of Bulgarian wood mice (genus *Apodemus*, subgenus *Sylvaemus*) which differ completely in the distribution of their constitutive heterochromatin, the position of NOR as well as the pattern of G-banding in several autosomes and the X-chromosome. Their identification based on known European and Transcaucasian karyotypes was carried out. In addition to three previously described cytotypes in Transcaucasian mice, two cytotypes are distinguished in Bulgarian specimens. One of them apparently corresponds to *A. flavicollis* now absent in Transcaucasia. The other cytotype appears to belong to “*sylvaticus*” genomes by virtue of the large amounts of distal heterochromatic areas. Compared to some previously reported European examples of *A. sylvaticus*, here called “*sylvaticus*-E1”, it was classified as “*sylvaticus*-E2” similar to an Austrian sample. The data obtained clearly contradict the generally accepted karyological conservatism of species of the genus *Apodemus*.

### Introduction

Contrary to a widely spread opinion of karyological conservatism of 48-chromosome karyotypes in species of *Apodemus* (subgenus *Sylvaemus*), variability does exist but is revealed only after differential staining of chromosomes. Firstly, these differences concern heterochromatin distribution (ENGEL et al. 1973; BEKASOVA et al. 1980; GAMPERL et al. 1982; HIRNING et al. 1989) demonstrated mainly in European mice. Another important characteristic seems to be the localization of nucleolus organizer regions (NORs) on acrocentric chromosomes (HIRNING et al. 1989; BULATOVA et al. 1991).

Using these markers at least three cytotypes were recently found in Transcaucasus. All cytotypes or their combinations demonstrate sympatric distribution without any introgression in Azerbaijan (NADJAFOVA 1989). At the same time, absence of true *A. flavicollis* in this region was confirmed based on karyological evidence (KOZLOVSKY et al. 1990; BULATOVA et al. 1991).

Data obtained from karyological and parallel biochemical analyses (VORONTSOV et al. 1989; MEZHHERIN 1990) have shed doubt on the widely held opinion of LARINA (1958) concerning hybridization of Transcaucasian wood (*A. sylvaticus*) and yellow-necked (*A. flavicollis*) mice in nature.

Due to overlapping of diagnostic morphological characters new biochemical criteria have been suggested which can be used for unambiguous determination of these species in southern Europe (BRITTON-DAVIDIAN et al. 1991).

To date, improved karyological techniques for revealing intrachromosomal differentiation have not been applied for the analysis of these populations. For the first time we obtained data from differential staining of chromosomes from individuals of *Apodemus* from Bulgaria and compared these with Transcaucasian samples previously identified.

## Material and methods

Karyological analysis was carried out on samples of mice not yet identified to the species level which had been caught at three sites in Bulgaria, in the autumn of 1989 and in spring of 1990. Six individuals (5 ♂♂, 1 ♀) were caught in the mountain region of Stara Planina (near Karlukovo village, 100 km north-east from Sofia), 11 individuals (3 ♂♂, 8 ♀♀) on Vitosha mountain (village Lozen, vicinity of Sofia) and 4 individuals (2 ♂♂, 2 ♀♀) in the Thrace lowland (village Goleminovo, vicinity of Pazardjik).

During 1990–1991 all animals were karyotyped. The colour peculiarities of the pelage, presence and shape of the chest spot and main body measurements were registered. The skulls of specimens are deposited in the laboratory of microevolution and domestication of mammals (head Prof. V. N. ORLOV), Severtzov Institute of Evolutionary Morphology and Ecology of Animals.

Comparative karyological analysis of metaphase plates was performed using conventional methods. The technique used for NORs localization and identification of NOR-bearing chromosomes was described earlier (BULATOVA et al. 1991).

## Results

All studied animals except two from Lozen with additional chromosomes had the same diploid number common to all representatives of the genus *Apodemus*, i.e.  $2n = 48$ . All chromosomes were acrocentric. Mice from Lozen and two individuals from Karlukovo had exclusively a centromeric localization of heterochromatin which was considered to be a specific feature of the cytotype "*flavicollis*" (ENGEL et al. 1973). All studied mice from Goleminovo and 4 ♂♂ from Karlukovo belong to the quite different cytotype, close to "*sylvaticus*" because of the almost exclusively telomeric localization of the heterochromatin (ENGEL et al. 1973; GAMPERL et al. 1982). This cytotype was denoted as "*sylvaticus*-E2" for reasons discussed below.

### The cytotype "*flavicollis*"

Among 13 examined animals 11 (3 ♂♂, 8 ♀♀) had a diploid chromosome number of 48 (Fig. 1a). In two individuals (♂ and ♀) from Lozen the diploid chromosome number varied in different cells from 48 to 51, obviously due to the presence of 1–3 additional chromosomes. These are small acrocentrics revealed among constant elements of the set by C- and G-patterns.

The X-chromosome is identified according to the G-banding as one of the largest chromosomes. Additional chromosomes are stained rather dark and without distinct G-bands (Fig. 1d).

Structural heterochromatin revealed when using C-staining is distributed almost uniformly throughout centromeric regions of all chromosomes of the basic set. Telomeric blocks were not found on any of the chromosomes (Fig. 1b). The Y-chromosome is rather large, with a length not less than half that of the X-chromosome, and completely heterochromatic (Fig. 1c). Generally, additional chromosomes are of smaller size and have a weaker intensity of C-staining compared with the Y-chromosome (Fig. 1b).

In the silver-stained metaphase preparations NORs are located exclusively in telomeric areas of autosomes (Fig. 1f). The maximum number of NOR-bearing chromosomes was 9 pairs. However, the majority of cells on a slide revealed fewer numbers of NORs which confirms the simultaneous functional activity of only part of the available nucleolus organizers. Moreover, NORs may be expressed on only one of two homologues of the same pair.

All individuals of this population had similar ochre colouring of back pelage. Most mice had a "collar"-shaped chest spot, one male being without a chest spot at all. Length of the body varied from 91.0 to 115.0 mm, most mice had damaged tails, so they could not be used for analysis. Foot length varied between 22.0–24.5 mm, and ear length between

15.4–19.0 mm. Both individuals with additional chromosomes had a round chest spot, and a rather large body as well as foot length.

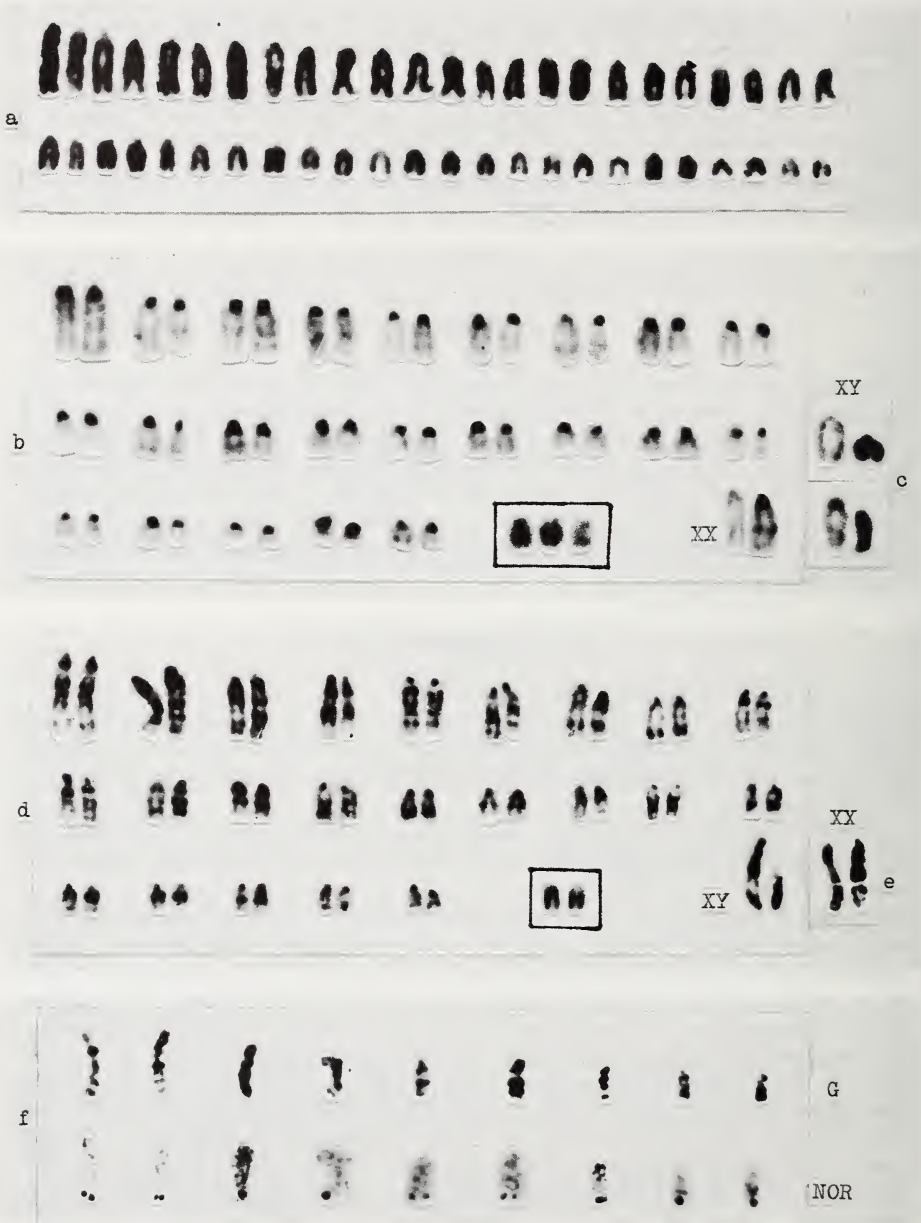


Fig. 1. Karyograms of *A. flavicollis* (Lozen): a: conventional staining by Giemsa (♀); b: C-banding (♀); c: Y-chromosome variants due to different level of spiralization; d: G-banding (♂); e: comparison of a G-banded X-chromosome of *A. sylvaticus* (right) and *A. flavicollis* (left); f: G-restaining of NOR-carrying autosomes from different karyograms. In outset = additional chromosomes





Fig. 2. Karyograms of *A. sylvaticus*: a: conventional staining by Giemsa (♀) (Goleminovo); b: C-banding (♀) (Goleminovo); c: C-banded gonosomes of a male (Goleminovo); d: G-banding (♂) (Karlukovo); e: G-banded X-chromosomes of a female (Goleminovo); f: G-restaining of NOR-carrying chromosomes from different karyograms

### The cytotype “*sylvaticus*-E2”

The diploid number of chromosomes was found to be stable in the karyotypes of all studied animals (6 ♂♂, 2 ♀♀),  $2n = 48$ , additional chromosomes were not found in any individuals (Fig. 2a).

Using G-staining all autosomes and X-chromosomes were identified (Fig. 2d). The pair of sex X-chromosomes of both females was heteromorphous in size and peculiarities of differential staining. They have the same pattern of G-bands length-wise except for the pericentromeric region where the lengthy homologue has an additional dark G-band (Fig. 2e). It is worth noting that in the karyotypes of all examined males only the lesser homologue was found.

Compared with the above-described cytotype “*flavicollis*”, distribution of heterochromatin in this cytotype is quite different. Heterochromatin is concentrated only in telomeric regions of small and medium-sized autosomes. Their number can attain 12 pairs. In the remainder of the autosomes heterochromatin was not found at all. On only some plates a small centromeric block and/or intercalary band of heterochromatin was observed below the centromere in the largest autosome No. 1. Another peculiarity of this cytotype seems to be the presence of a pronounced pericentromeric block of heterochromatin in the pair of large chromosomes of females, which corresponds to the X-chromosome identified by G-staining. Both homologues differ significantly by the amount of heterochromatic material (Fig. 2b). The Y-chromosome is medium-sized acrocentric and completely heterochromatic; the intensity of its staining corresponds to the staining of heterochromatic blocks of autosomes and the X-chromosome (Fig. 2c).

NORs were found to be located both in telomeric as well as centromeric areas of several chromosomes (Fig. 2f). It should be noted that the centromeric location occurs more rarely than the telomeric. On some plates centromeric NORs were not observed at all. Analysis of a large number of metaphases retained using different schemes (NOR-Giemsa, NOR-C, NOR-G) allowed us to conclude that NOR-bearing chromosomes belong to three different autosomal pairs. There were no more than 8 pairs of small and medium-sized chromosomes with a telomeric location of NORs. Similar to “*flavicollis*” studied here, NORs can be revealed either in both homologues of a pair or only in one.

## Discussion

Traditionally in the fauna of Bulgaria two species of *Apodemus* – *A. sylvaticus* and *A. flavicollis* – can be recognized, taking into account combinations of some morphological and ecological peculiarities. Reliability of assignment of each species depends on the sample size. Unambiguous individual identification of wood mice to the species level is ensured only when using genetic markers. Both sympatric and allopatric populations of Bulgarian *A. sylvaticus* and *A. flavicollis* can be differentiated clearly when using electrophoretic (BRITTON-DAVIDIAN et al. 1991) and karyological (our data) analyses.

So far, two cytotypes were described based on this material. One of these is undoubtedly characteristic for the true yellow-necked mouse, *A. flavicollis*. One peculiarity of this cytotype is the centromeric location of the heterochromatin in all autosomes and the X-chromosome and another is the exclusively telomeric distribution of NOR (ENGEL et al. 1973; GAMPERL et al. 1982; HIRNING et al. 1989). Variations in the diploid chromosome number due to several additional, or B-chromosomes are possible in “*flavicollis*” cytotype (WOLF et al. 1972; SOLDATOVIČ et al. 1975; KRÁL et al. 1979; ZIMA 1984; SABLINA et al. 1985; BRITTON-DAVIDIAN et al. 1991).

C-stained karyotypes of *A. flavicollis* from Germany (ENGEL et al. 1973; HIRNING et al. 1989), Austria (GAMPERL et al. 1982) and the Leningrad region of Russia (SABLINA et al.

1985) have been described. The localization of NORs has been studied in one German population near Ulm (HIRNING et al. 1989), and in the Ukraine, Estonia and Lithuania (BOESCOROV et al. 1990). According to these authors, the Y-chromosome was reported to be a medium-sized acrocentric, its size not being less than a half of the X-chromosome. Only in one Austrian population (Achenwald) was the Y-chromosome identified at the level of the smallest autosomes using conventional staining (KRÁL et al. 1979).

The second cytotype found in Bulgarian mice is characterized by a completely different distribution of heterochromatin and mixed (centromeric as well as telomeric) localization of NORs, as well as varying pattern of G-banding in several autosomes and the X-chromosome in comparison to *A. flavicollis* (Figs. 1d, e; 2d). In this type of chromosome set variability in the size of X-chromosomes at the expense of an increase or decrease in pericentromeric heterochromatin is observed. It should be noted that in two males of geographically rather distant populations (Goleminovo and Karlukovo) only one variant of a heteromorphous X-chromosome with relatively smaller content of heterochromatin was discovered.

The presence of telomeric heterochromatin is characteristic for the mice identified as *A. sylvaticus* by biochemistry and morphology. For the first time karyological peculiarities of *A. sylvaticus* compared to *A. flavicollis* were described by ENGEL et al. (1973) according to their study of mice from Southern Germany (Freiburg).

Comparison of the data in the literature and our results confirms the existence of two cytotypes within European "*sylvaticus*", which we denoted as "*sylvaticus*-E1" and "*sylvaticus*-E2".

The "*sylvaticus*-E1" cytotype refers to mice earlier studied from Freiburg and the geographically close population of Ulm (Southern Germany). In the karyotype of these mice not more than 5 autosomal pairs were reported to carry telomeric blocks of heterochromatin. In addition, almost all chromosomes had centromeric heterochromatin. In the X-chromosome no centromeric heterochromatin has been revealed (ENGEL et al. 1973; HIRNING et al. 1989).

*A. sylvaticus* from Austria (GAMPERL et al. 1982) and from Bulgaria (our data) seem to correspond to another cytotype "*sylvaticus*-E2". Karyotypes of these populations can be distinguished by the presence of centromeric heterochromatin in the only large pair of autosomes with varying heterochromatic block and by exclusive distribution of the remainder of heterochromatin in distal areas of several small and medium-sized chromosomes.

It is worth noting that the cytotype "*sylvaticus*-E2" does not seem to match any of the Transcaucasian karyotypic forms known to us (KOZLOVSKY et al. 1990; BULATOVA et al. 1991). At the same time some similarities in the heterochromatin and distribution of NORs could be seen in European "*sylvaticus*-E1" and the Azerbaijanian "t-form" (KOZLOVSKY et al. 1990).

We also failed to find the "*flavicollis*" cytotype in individuals of so-called "yellow-necked" mice from the Eastern Tanscaucasus although up to now the fauna of wood mice in this region has been considered to be represented by two species – *A. sylvaticus* and *A. flavicollis* – and by a number of mixed populations, presumably of hybrid origin (LARINA 1958).

The conclusion can be drawn that the species composition of mice in the regions of Transcaucasus and Balkans (particularly in Bulgaria) appears to have little in common.

In this connection we would like to emphasize that marked genetical (here cytogenetical) differences within the subgenus *Sylvaemus* are not unexpected. Indeed, in Europe the species *A. sylvaticus* is known to be highly differentiated both at the level of classical systematics and biochemical genetics (BERRY 1970; GEMMEKE 1980). At all, about two dozens subspecies were identified on European islands and in some regions of the continent, some of them superseding the species rank. It seems quite opportune to



reinvestigate their status on the basis of newly available approaches, among which the chromosome analysis seems to play an important role.

Unambiguous identification of *A. flavicollis* and complex *A. sylvaticus* cytotypes could be useful for clarifying species borderlines between their genomes. For example, additional chromosomes were reported by ZIMA (1984) for *A. sylvaticus* individuals. The critical examination of such cases is of both theoretical and practical interest.

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### Zusammenfassung

#### *Chromosomenunterschiede zweier Apodemus-Arten in Bulgarien*

Waldmäuse der Gattung *Apodemus* (Subgenus *Sylvaemus*) aus Bulgarien wurden karyologisch untersucht. Es wurden zwei Karyotypen gefunden, die sich grundsätzlich in der Menge des konstitutiven Heterochromatins, in der Lage der NOR-Regionen und im G-Bandenmuster verschiedener Autosomen und des X-Chromosoms unterscheiden. Beide wurden mit bereits bekannten Karyotypen aus Europa und Transkaukasien verglichen. Einer der bulgarischen Karyotypen scheint *A. flavicollis* zu repräsentieren, eine Art, die in Transkaukasien offenbar nicht vertreten ist. Der andere Karyotyp wird wegen seines großen Gehaltes an Heterochromatin *A. sylvaticus* zugeordnet. Diese Art ist karyologisch heteromorph und wird vorläufig in zwei Gruppen aufgeteilt: „*sylvaticus*-E1“ (Süddeutschland) und „*sylvaticus*-E2“ (Österreich, Bulgarien). Die Befunde zeigen, daß die Arten der Gattung *Apodemus* genetisch variabler sind als bisher angenommen.

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