KARYOLOGICAL RELATIONSHIPS AND BIODIVERSITY OF THE PINE VOLEs OF AZERBAIJAN: DIFFERENTIATION OF SPECIES FROM THE GREATER AND LESSER CAUCASUS MOUNTAINS

Gias N. Kuliev and John W. Bickham

ABSTRACT

We conducted a karyological investigation of the pine voles Microtus (Terricola) and Microtus (Hyrcanicola) inhabiting Azerbaijan. Differentially and non-differentially stained karyotypes of three species (M. (H.) schelkovnikovi, M. (T.) majori, and M. (T.) daghestanicus) revealed a constant diploid number (2n = 54) and variation in fundamental number (FN = 58-62). Differential staining revealed pericentric inversions accounted for variation in FN and also revealed variation in NOR position and number as well as in C-band heterochromatin amount and distribution. Two subspecies of M. majori from the Greater and Lesser Caucasus, respectively, differed in heterochromatin and NOR distribution and possibly represent distinct species. The non-differentially stained karyotype of a fourth species, M. nasarovi (2n = 38, FN = 58) from the Lesser Caucuses, was found to be highly distinct from the previously considered conspecific M. daghestanicus (2n = 54, FN = 58) from the Greater Caucuses. We conclude that there are at least four species, and possibly five, of pine voles in Azerbaijan and further systematic studies should be conducted to determine the taxonomic status of the recognized subspecies of M. majori. The Greater and Lesser Caucasian Mountains appear to have served as refugia providing for differentiation among species pairs of pine voles.

Key words: Azerbaijan, karyotypes, Microtus (Hyrcanicola), Microtus (Terricola), pine voles

INTRODUCTION

The pine voles Microtus are an intriguing group of 14 species of terrestrial rodents in which, despite more than 170 years of investigation (McMurtrie 1831; Miller 1912; Ognev 1950; Ellerman and Morrison-Scott 1951; Alekperov 1959; Shidlovskij 1976; Niethammer and Krapp 1982; Chaline et al. 1988; Gromov and Erbaeva 1995; Macholan et al. 2001; Nadachowski 2007) many unresolved issues still remain concerning their taxonomy, phylogenetic relations, and features of their karyotypic evolution. Three species of pine voles are generally considered to inhabit the Caucasus and Transcaucasia and are the focus of this study (M. schelkovnikovi, M. majori, and M. daghestanicus).
Until the 1970s some researchers referred the Caucasian pine voles to subspecies of *M. subterraneus* (Ellerman and Morrison-Scott 1951; Kuzyakin 1963; Bobrinsky et al. 1965), and morphological features of the skull and body have not fully resolved their status and position. However, the estimated numbers of Caucasian pine vole species according to most investigators has varied from two to three and subspecies from three to five (Schidlovsky 1919, 1941, 1976; Ognev 1950; Alekperov 1959; Gromov and Polyakov 1977). Schidlovsky (1919, 1941, 1976) referred the pine voles to the genus *Pitymys* (*Arbusticola*) with two species, *P. majori* and *P. daghestanicus*. *P. schelkovnikovi*, which is endemic to the Lenkaran area (Talysh Mountains) in southern Azerbaijan, was referred to by him as *P. majori schelkovnikovi*. Yet, later he recognized *P. schelkovnikovi* but relegated *P. daghestanicus* to a subspecies of *P. majori*. Recently, Chaline et al. (1988) erected the subgenus *Terricola* to include the Palaearctic species of pine voles, and subsequently Nadachowski (2007) relegated *P. schelkovnikovi* to a new subgenus (*Hyrcanicola*). Currently most researchers recognize in the Caucasus and Transcaucasia three separate species of pine voles: *M. (H.) schelkovnikovi*, *M. (T.) majori*, and *M. (T.) daghestanicus*.

Using non-differential staining, Ivanov and Tembotov (1972) have described the karyotypes of pine voles of the Caucasus and concluded that they belong to four karyotypic groups including: 2n = 54, FN = 58; 2n = 52, FN = 58; 2n = 54, FN = 62; and 2n = 54, FN = 60. Subsequent studies employing the use of modern high resolution karyological techniques have resulted in the recognition of 13 karyotypically distinct forms within *Hyrcanicola* and *Terricola*; 11 of them are found within *M. daghestanicus* (Akhverdyan et al. 1992). Kuliev (1979) described the standard and G-band karyotypes of pine voles from Azerbaijan including *M. (T.) majori* and *M. (H.) schelkovnikovi*, and Kuliev and Kasumova (1996) described the standard and G-band karyotypes of those two species plus *M. (T.) daghestanicus*. Those two studies have shown that *M. schelkovnikovi* is the most distinct of the three species and differs by several rearrangements. The objective of this paper is to build upon the study of Kuliev (1979) and Kuliev and Kasumova (1996) by describing in more detail and with additional staining methods the karyotypes and chromosomal banding patterns of the three species of pine voles *M. (H.) schelkovnikovi* Satunin 1907, *M. (T.) majori* Thomas 1906, and *M. (T.) daghestanicus* Schidlovsky 1919, which are generally recognized to occur within the territory of Azerbaijan, and to investigate the phylogenetic relationships and the taxonomic status of these species.

**Materials and Methods**

Specimens were collected in the field over a 30-year period (1973-2003) on trips arranged by the Institute of Zoology of the National Academy of Sciences of Azerbaijan. The animals were caught within the territory of the Azerbaijan Republic both in the Greater (Gussar district, Zakatal district) and Lesser Caucasus (Gedabek district, Lenkaran natural area, Lerik). Karyotypic analyses were performed on 90 specimens (41♀, 49♂) representing the three traditionally recognized species of pine voles inhabiting the country (*M. schelkovnikovi*, *M. majori*, and *M. daghestanicus*). Mitotic chromosome preparations were prepared from bone marrow cells and stained by non-differential staining (Giemsa) as well as differential techniques (G- and C-banding, and AgNOR staining). Table 1 presents the localities, sample sizes (N), diploid numbers (2n), and fundamental numbers (FN; the number of arms of the autosomes and the X chromosome) for the animals used in this study.
Table 1. Species, collecting localities, sample sizes (N), diploid numbers (2n), and fundamental numbers (FN) of the pine voles examined in this study. Vouchers including dry specimens, fluid preserved specimens, and/or microscope slides or film negatives are deposited in the mammal collection at the Institute of Zoology, Azerbaijan National Academy of Sciences, Baku.

<table>
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<th>Species</th>
<th>Locality</th>
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<th>2n</th>
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<td>*Microtus (Terricola)</td>
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<td>schelkovichki</td>
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<td>54</td>
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<td>*Microtus (T.) majori</td>
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<td>a) Hanaba village</td>
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<td>b) Galajic village</td>
<td>6</td>
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<td>b) Muhag village</td>
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<td>b) Novoivanovka village</td>
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<td>Dashkesan district,</td>
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<td>a) Gabagtapa village</td>
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<td>*Microtus (T.) daghets-</td>
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<td>tanicus</td>
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Results

*Microtus schelkovnikovi* Satunin 1907 has 2n = 54 and FN = 62. Four pairs of autosomes are biarmed and the other 22 pairs are acrocentric. The X chromosome is a large-sized acrocentric chromosome, and the Y chromosome is small and acrocentric (Fig. 1). G-bands (Fig. 2) allow for the identification of all chromosomal pairs and revealed no polymorphisms.

*Microtus majori* Thomas 1906 has 2n = 54 and FN = 60. The first and second pairs of autosomes are submetacentric and the remaining 24 pairs are acrocentric and grade in size from large to small. The X chromosome is a metacentric of approximately the same size as the first pair of autosomes, and the Y chromosome is a medium-sized acrocentric (Fig. 3). G-bands (Fig. 4) allow for the identification of all chromosomal pairs.

Differences in position and amount of the structural heterochromatin were found between individuals caught in the Lesser and Greater Caucasus Mountains. In animals from the Zakatal district (the Greater Caucasus), distinct heterochromatic blocks are present near the centromeres of four chromosome pairs (20, 23, 25 and 26). In the shorter arm of the X chromosome a small heterochromatin block is present but no heterochromatin was observed at the centromere or in the short arm. The Y chromosome is heterochromatic (Fig. 5).

In karyotypes of *M. majori* from the Lesser Caucasus (vicinity of Gedabek) heterochromatic blocks were revealed near the centromeres of two chromosome pairs (tentatively identified as pairs 13 and 25). In the ostensible long arm of the metacentric X chromosome, a small but distinct interstitial heterochromatin block is present but no heterochromatin was observed at the centromere or in the short arm. The Y chromosome was heterochromatic (Fig. 6).

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Differences between populations were also observed in the number and locations of the nucleolus organizer regions (NORs), although in all cases, NORs were found at the centromeric ends of acrocentric autosomes. In animals from the Greater Caucasus, very distinct NORs were observed on six pairs (tentatively identified as pairs 6, 8, 9, 10, 13, and 20) of autosomes. In pairs 18 and 24, NORs were present but were less distinct (Fig. 7). Ag-NOR staining of chromosomes in animals of the Lesser Caucasus revealed distinct NORs in five pairs of chromosomes (tentatively identified as 13, 14, 15, 16, 22) and less distinct NORs in two pairs (17 and 23) (Fig. 8).

*Microtus daghestanicus* Schidlovsky 1919 has 2n = 54 and FN = 58. Except for the smallest pair, all autosomes are acrocentric. The first pair is significantly bigger than the rest and the smallest pair (26) is metacentric. After the first pair, the autosomes form a gradually decreasing gradient in size. The X chromosome is large and metacentric and the Y is small and acrocentric (Fig. 9). G-bands (Fig. 10) allow for the identification of all autosomal pairs and the sex chromosomes.

C-band heterochromatin is distributed as small, faintly discernible blocks in the centromere sites of all of the acrocentric autosomes and the X chromosome. No interstitial heterochromatin was observed on the X chromosome, but the smallest (metacentric) autosomal pair has a distinctly heterochromatic short arm, centromere and pericentromeric region (Fig. 11).

Comparisons of the general features of the karyotypes of the three species show that their karyotypes have diverged from one another by means of pericentric inversions and changes in the distribution of heterochromatin and NORs. Such rearrangements do not alter the diploid number but are reflected in variation in the FN which ranges from 58 to 62. Differences were also found in the structure of the Y chromosomes of the three species. *Microtus daghestanicus* possessed a Y that was reduced in size relative to the other two species. C-bands showed the increase in size is due to an increase in the amount of heterochromatin.

Comparisons of G-bands among the three species confirmed the conclusions based upon examination of non-differentially stained karyotypes. Figure 12 shows G-bands for autosomal pairs 1, 2, 4, 7, 8 and the X chromosome. There is evidence for a pericentric
inversion in pair 1 in which *M. daghestanicus* and *M. schelkovnikovi* have acrocentrics whereas *M. majori* has a submetacentric. Similarly a pericentric inversion is indicated for pair 2 which is acrocentric in *M. daghestanicus* and submetacentric in *M. schelkovnikovi* and *M. majori*. Likewise, pairs 4, 7, and 8 are biarmed in *M. schelkovnikovi* and acrocentric in the two other species and most likely all of these chromosomal pairs have evolved by inversions in *M. schelkovnikovi*. The X chromosome is acrocentric in *M. schelkovnikovi* and metacentric in the two other species. In this case both an inversion and addition of interstitial heterochromatin is indicated.

Schidlovsky (1938) described *M. (T.) nasarovi*; however, this form has been relegated to a subspecies of *M. daghestanicus* by most authors. Although we do not have differential staining data for this species, the non-differentially stained karyotype is quite distinct in possessing 2n = 38 and FN = 58. The karyotype consists of eight pairs of large meta- and submetacentric chromosomes, nine pairs of small acrocentrics which are very close in size; the X chromosome is metacentric and the Y chromosome is one of the smallest chromosomes of the complement (Fig. 13). Thus, the karyotype differs from all other of the *M. daghestanicus* complex both in number (2n = 38 compared to 2n = 54 in *M. daghestanicus*) and morphology of chromosomes. Specimens of this species can only be found within the Lesser Caucasus in the areas of Murovdag and Garabah ridges, in Gek-gel National Park, and also in Gedabek district (Novoivanovka village).

Figure 1. Non-differentially stained karyotype of *M. shelkovnikovi*.

Figure 2. G-band karyotype of *M. schelkovnikovi*.
Figure 3. Non-differentially stained karyotype of *Microtus majori*.

Figure 4. G-band karyotype of *Microtus majori*.
Figure 5. C-band karyotype of *M. majori* of a specimen from the Greater Caucuses Mountains (Zagatal, Gabzdara).

Figure 6. C-band karyotype of *M. majori* from the vicinity of Gedabek in the Lesser Caucuses.
Figure 7. Locations of NORs in *Microtus majori* from the Greater Caucuses Mountains (Zakatal Region, Gabizdara).

Figure 8. Locations of NORs in *Microtus majori* from the Lesser Caucuses Mountains, vicinity of Gedabek.
Figure 9. Non-differentially stained karyotype of *M. daghestanicus*.

Figure 10. G-band karyotype of *Microtus daghestanicus*. 
Figure 11. C-band karyotype of *Microtus daghestanicus*.

Figure 12. Comparisons of G-bands and non-differential staining of autosomal pairs 1, 2, 4, 7, and 8 and the X chromosome for three species of pine voles.
The comparative analysis of differentially and non-differentially stained chromosomes of the pine voles of Azerbaijan detected considerable variation. All of the species studied differ by diploid number and/or FN and additional minor alterations in heterochromatin and NORs were observed that are not reflected in 2n or FN.

Previous studies have shown the presence of several forms within *M. daghestanicus* which differ in chromosome number while FN is constant. Ivanov and Tembotov (1972) and Hatuhov (1982) described three karyotypically distinct populations: 2n = 54, FN = 58; 2n = 52, FN = 58; 2n = 38, FN = 58. Akhverdyan et al. (1992) recognized 11 karyotypically distinct forms including three with odd diploid numbers (2n = 43, FN = 58; 2n = 45, FN = 58; and 2n = 53, FN = 58). In this study we failed to detect any specimens or populations with odd diploid numbers despite the fact that our study was conducted in all regions of Azerbaijan except for the Nakhichevan Autonomous Republic. We can confirm only the two distinct forms representing the species *M. daghestanicus* and *M. nasarovi* with 2n = 54, FN = 58 and 2n = 38, FN = 58, respectively. The status of all the other previously reported karyotypically distinct forms is, in our opinion, dubious but additional studies are needed.

Gromov and Erbayeva (1995) recognized two subspecies of *M. majori* in Azerbaijan: *M. majori ciscaucasicus* Ognev 1924 occupies the forest zone of the Greater Caucasus, and *M. majori suramensis* Heptner 1948 occurs in the forest zone of the Lesser Caucasus. This study shows differences between the subspecies in amount and distribution of C-band heterochromatin and NORs. Specifically, *M. m. suramensis* possesses a virtual absence of centromeric heterochromatin in the autosomes whereas *M. m. ciscaucasicus* has distinct centromere blocks of heterochromatin present in the autosomes. The X chromosome of *M. m. ciscaucasicus* has a narrow intercalary band of heterochromatin in the middle of the shorter arm and a large block of distinct heterochromatin is present near the centromere in the long arm. In the specimens of *M. m. suramensis* the metacentric X chromosome has no intercalary heterochromatin in the shorter arm but it does possess a
band in the ostensible long arm, although not as large as seen in *M. m. ciscaucasicus*. The Y chromosomes are acrocentric in both subspecies and entirely consist of heterochromatin. However, in *M. m. suramensis* the Y chromosome is somewhat bigger than in *M. m. ciscaucasicus*.

Differences were also detected between the two subspecies for NORs. Distinct, brightly stained nucleolus organizer regions were detected in six chromosome pairs (6, 8, 9, 10, 13 and 20) and less distinctly stained NORs were detected in pairs 18 and 24 of *M. m. ciscaucasicus* from the Greater Caucasus. Whereas distinct nucleolus organizer regions were detected in eight chromosome pairs (13, 14, 15, 16, and 22) and less distinct NORs on pairs 17 and 23 of *M. m. suramensis* from the Lesser Caucasus.

In this study we used differential chromosome staining patterns to detect homologous chromosomal regions and map rearrangements in the pine voles occupying Azerbaijan. Although it is outside the scope of this paper, these data will be useful in determining the evolutionary relationships, natural history, and taxonomic status of species and populations from both the Greater and Lesser Caucasus Mountains. Chromosomal banding data have been used as genetic markers in phylogenetic and systematic studies of mammals for decades (Baker and Bickham 1980; Baker et al. 1985).

Recent molecular studies have confirmed that often chromosomal rearrangements track speciation events and thus might be good indicators of biodiversity patterns (Baird et al. 2009).

Based on the results obtained in our study we conclude that there are at least four species of pine voles that occupy the Caucasuses and Transcaucasia: *M. daghestanicus*, *M. schelkovnikovi*, *M. majori*, and *M. nasarovi*. Evidence that *M. nasarovi* is a valid species is based on the highly distinct nature of its karyotype compared to its presumed conspecific *M. daghestanicus*, and its disjunct distribution. The karyotypic differences observed between the two subspecies of *M. majori* indicate that these are likely distinct species. We studied specimens from three and four localities of *M. m. ciscaucasicus* and *M. m. suramensis*, respectively (Table 1). Because the karyotypic differences were consistent between the two subspecies at multiple sites, it is likely that the Greater and Lesser Caucasus represent different phylogeographic regions. Molecular-genetic analyses are required to determine the degree of genetic differentiation among these forms. Nonetheless, the clear karyotypic differentiation among the subspecies of *M. majori* and between *M. daghestanicus* and *M. nasarovi* are strong indications that the Greater and Lesser Caucasus Mountain ranges have served as refugia for the diversification of species of pine voles.

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