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# Molecular phylogenies of the genus *Marmota* (Rodentia Sciuridae): comparative analysis

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Phylogenetic reconstructions of the genus *Marmota* were compared using data based on mitochondrial genes *cit* b (KRUCKENHAUSER et al. 1999, STEPPAN et al. 1999, HERRON et al. 2004), *NADH-dehydrogenase subunit 4* (KRUCKENHAUSER et al. 1999) and *D-loop* sequences and inter-SINEs nuclear DNA pattern (our data) as molecular markers. These studies present the evidence of a recent origin and North American descent of marmots. Most of the data reveal Nearctic and Palearctic groups of species, but the composition of the groups differ. *M. monax* and *M. broweri* are grouped with Nearctic or Palearctic marmots on different trees. Existence on mitochondrial and nuclear DNA trees of closely-related species groups, such as *bobak*- and *camtschatica*-groups, reflects a recent Pleistocene evolution. Low mitochondrial and high nuclear genetic distances in the pair *M. menzbieri-M. caudata* probably indicate their remote hybridization. A genetic affinity of *M. vancouverensis* and *M. caligata* demonstrates insufficient time since their separate evolution for the accumulation of species-specific molecular differences. Causes of variance in different molecular phylogenetic trees of *Marmota* are discussed. A consensus phylogenetic tree of the genus *Marmota* is proposed.

KEY WORDS: *Marmota*, phylogeny, molecular markers, consensus phylogenetic tree.

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## INTRODUCTION

Phylogenetic research is important for studying the history and evolution of living organisms. Moreover, phylogenetic data are important for the construction of natural systems and taxonomy. With the development of molecular genetic techniques used in phylogenetic studies, phylogenetic reconstructions, based on classical morphology, are tested with the help of molecular markers. Molecular genetic approaches, in some cases, resolve issues related to relationships. Methods of constructing phylogenetic trees based on differences in the nucleotide sequences have been widely applied. For this purpose different molecular markers are used to assess the variability of some parts of mitochondrial and nuclear genomes or the general variability of the genome (Bannikova 2004, Noor & Feder 2006). Phylogenetic trees derived from the use of different markers may differ in general topology and in the position of individual branches within a cluster. These differences may be explained by the influence of peculiarities of marker inheritance, different rates of base substitutions in different parts of genomes that are selected and the composition of an investigated sample.

Phylogenetic relationships in the genus *Marmota* have been investigated by the analysis of fossil and morphological traits (Gromov et al 1965, Hoffmann et al. 1979, Hafner 1984, Cardini & O'Higgins 2004). The dominant idea of the North American origin of this genus was based on these studies. However, there is a view that marmot ancestors formed in Eurasia (Bibikov 1967, Erbaeva 2003). Contentious issues about the ancestral form of the Palearctic species and the number and order of marmot migrations through the Beringian Bridge remained obscure. Phylogenetic relationships among Palearctic species were not entirely clear due to the lack of paleontological data and undefined morphological differences between species which are traditionally grouped in the *bobak*-group (*M. bobak, M. baibacina, M. sibirica, M. himalayana* and sometimes *M. camtschatica*).

The problems of marmot phylogeny have been examined additionally using other approaches, such as karyological (Hoffmann & Nadler 1968, Vorontsov & Lyapunova 1970, Brandler et al. 2008), biochemical (Mezhzherin et al. 1999), immunogenetical (Zholnerovskaya 2002) and bioacoustical (Nikol'skii 1984). However, many questions have not been resolved because of the limitations of these methods. For example, karyological studies differentiated Nearctic species well, but revealed the lack of differences in chromosome numbers of most Palearctic species having similar diploid numbers (2n = 38). Only 2 of 9 Palearctic species are differentiated on this basis: *M. camtschatica* (2n = 40) and *M. kastschenkoi* (2n = 36) (Brandler et al. 2008). In summary, the above mentioned studies reveal: (1) there are 15 species in the genus *Marmota*; (2) North American species are more distinct than Eurasian; (3) *M. marmota* is the oldest Palearctic species; (4) amphiberingian species *M. broweri* and *M. camtschatica* may be or not be sister species; (5) relationships of members of the *bobak*-group are not clear.

The first molecular-genetic studies of squirrels were initiated by N.N. Vorontsov in Russia and R. Hoffmann in the United States in 1991 (Lyapunova et al. 1995). Sequencing of *cytochrom b* (*cyt* b) was used in this study,

and the first phylogeny of the genus *Marmota* was constructed (STEPPAN et al. 1999). Later M.D. Herron and others used these data obtained from GenBank for reconstructing a phylogeny of the entire family Sciuridae (HERRON et al. 2004). Simultaneously with Steppan's group Austrian researchers sequenced *cyt* b (KRUCKENHAUSER et al. 1999). Their sample included a smaller number of species and some samples were used by both research groups. In addition, Kruckenhauser and others used *NADH-dehydrogenase subunit 4* (*ND4*) gene as another mitochondrial marker. Another study of phylogeny of the genus *Marmota* used the DNA-DNA hybridization method (GIBOULET et al. 2002), but they examined only four species of marmots. Later we tested marmot phylogeny using other mitochondrial (mtDNA) and nuclear (nDNA) genome markers (our unpublished data). The phylogenetic trees based on results of different markers differ both in the general topology and in the position of individual branches inside clusters. In consideration of the use of molecular data in modern phylogenetic reconstructions there is strong need to weigh accuracy of analysis and interpretation of such data. For this purpose we have tried to compare and analyze phylogenetic trees available in the literature and those produced by us. The main goals of this study are: (1) to find similarity and difference in these phylogenetic trees; (2) to determine the cause of the differences; (3) to define those relationships among *Marmota* species that are well-supported by the available data and those relationships that are still ambiguous.

## MATERIALS AND METHODS

We used published and our unpublished phylogenetic trees consisting of all or the majority of marmots species for comparison and analysis. Some mtDNA (*cyt* b, *ND4* and *D-loop*) and nDNA (MIR and B1-dID) markers were used in these reconstructions (Table 1). Mitochondrial genome markers such as *cyt* b, *ND4* and *D-loop* or Control region are widely used in modern phylogenetic studies. MIR (mammalian interspersed repeat) and B1-dID (KRAMEROV et al. 1999) are SINEs (short interspersed nuclear elements) which are dispersed throughout the genome in the hundreds of thousands.

Table 1.

Descriptions of analyzed phylogenetic trees.

| Molecular marker | Number of *Marmota* species | Number of specimens | Molecular method | Reference |
|---|---|---|---|---|
| *cyt* b | 15 | 25 | Sequencing | STEPPAN et al. 1999 |
| *cyt* b | 10 | 10 | Sequencing | KRUCKENHAUSER et al. 1999 |
| *cyt* b | 15 | 22 | Sequencing | HERRON et al. 2004 |
| ND4 | 6 | 6 | Sequencing | KRUCKENHAUSER et al. 1999 |
| D-loop | 13 | 22 | Sequencing | Our unpublished data |
| MIR and B1-dID | 13 | 51 | inter-SINE-PCR | BRANDLER et al. in press |

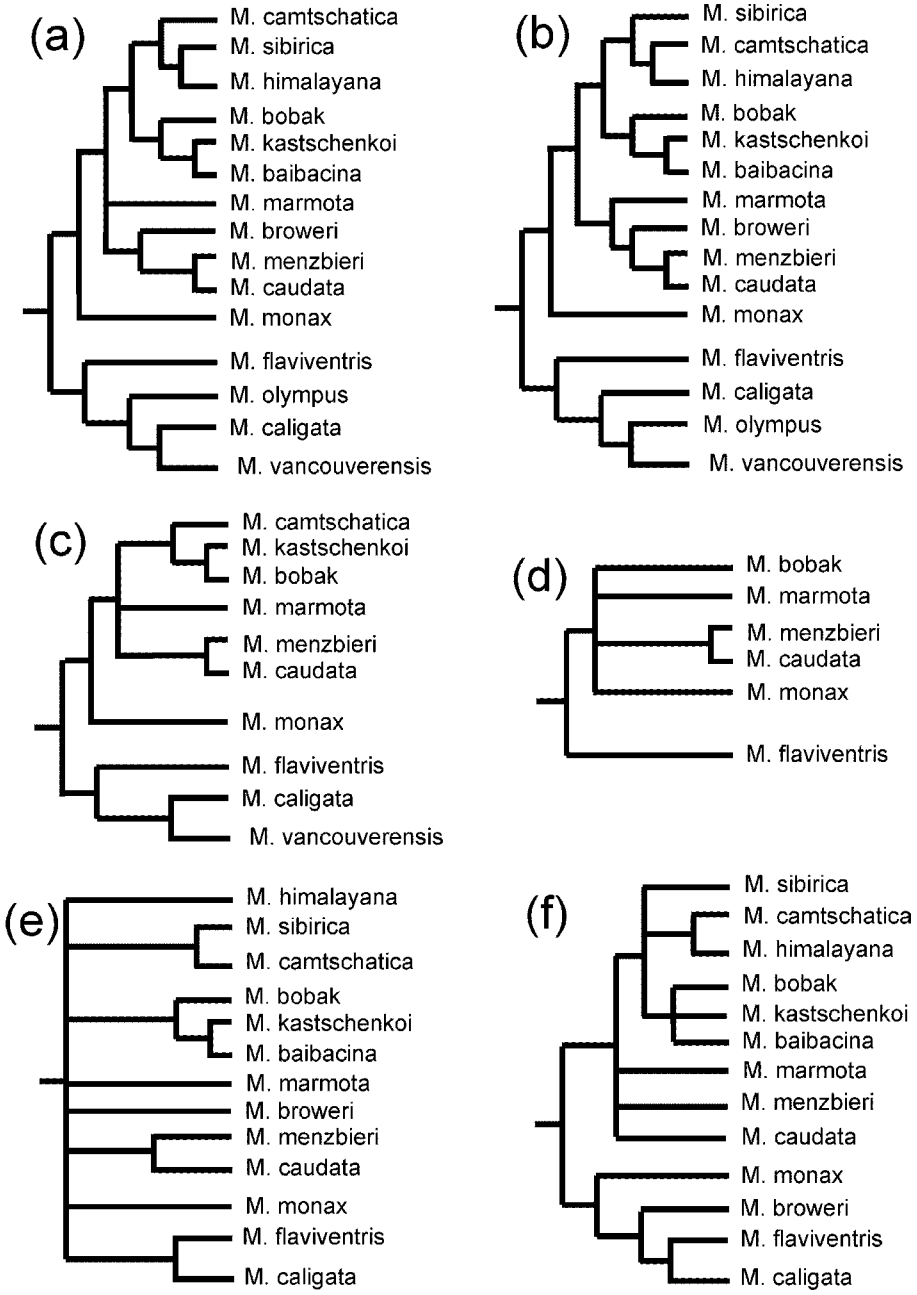


Fig. 1. — Phylogenetic schemes based on data of: sequencing of *cyt* b – (a) STEPPAN et al. 1999, (b) HERRON et al. 2004, (c) KRUCKENHAUSER et al. 1999; *ND4* – (d) KRUCKENHAUSER et al. 1999; (e) *D-loop* (our unpublished data); (f) MIR and B1-dID IS-PCR (BRANDLER et al. in press).

These markers are used in a method of inter-SINE-PCR that is based on amplification and detection of variability of the SINE-flanked DNA-fragments and indicate general variability of the whole nuclear genome (JURKA et al. 1995).

Only the *Marmota* cluster was taken from the general ground squirrel tree of HERRON et al. (2004) for the analysis. D-loop tree was made on the basis of sequencing of 875 bp by the neighbor joining (NJ) method. Combined data on MIR and B1-dID fingerprints were used in inter-SINE-PCR analysis by maximum parsimony (MP) and NJ methods.

Original phylogenetic trees differ in topology and the composition of the samples. For the purpose of simplifying the comparisons, all clusters, including more than one sample per species, are reduced to one branch and respective trees are presented in the form of the species clustering schemes. Bootstrap value was used for the estimation of dichotomy support. When bootstrap support was below 50%, we considered clusters to be unresolved (Fig. 1). We accept as the taxonomic background the *Marmota* taxonomy described in WILSON & REEDER (2005), with the exception that *M. kastschenkoi* should be elevated from a subspecies of *M. baibacina* to species status on the basis of karyological data (BRANDLER 2003). When previous work used *M. kastschenkoi* as a subspecies of *M. baibacina,* we present these samples as a separate species in our schemes.

Direct gene-trees topology comparisons approach (modified from NOOR & FEDER 2006) was used for finding common and distinct features of the different phylogenetic trees. We assume well-supported evolutionary events were on all or most trees and events that are distinct on different trees we consider as ambiguous. A consensus tree of the genus *Marmota* was constructed from all available data.

## RESULTS AND DISCUSSION

All mtDNA markers either support the union of the North American species *M. flaviventris*, *M. caligata*, *M. olympus* and *M. vancouverensis* into one group and place *M. monax* and *M. broweri* into the cluster of Eurasian marmots (Fig. 1a-d) or does not identify their positions because of low bootstrap value (Fig. 1e). As opposed to mtDNA data, nDNA markers divide North American and Eurasian species into different clusters (Fig. 1f).

In most trees the group *M. sibirica*, *M. himalayana*, *M. camtschatica* stood out with variable branching. Possibly these species originated by divergence of a single ancestral form during a short period of time. The same is true for the species group *M. bobak*, *M. baibacina*, *M. kastschenkoi,* which cluster on all trees. The last two species join into a separate cluster, and *M. kastschenkoi* is the youngest of all Palearctic species as demonstrated by a karyological study (BRANDLER et al. 2008). *M. marmota* is situated at the root of the Palearctic group in all trees. This supports the concept that it is closely related with the ancestral form of the Palearctic species (BIBIKOV 1967). North American *M. flaviventris* and *M. caligata* group with good support; *M. flaviventris* shows more ancestral traits. *M. caligata*, *M. olympus* and *M. vancouverensis* are closely-related species. Among them *M. vancouverensis* is the youngest, others are older and are variously placed on the trees when all three species are present (Fig. 1a-b). It should be noted that samples of *M. olympus* DNA were not good because old collected material was used for its preparation. Therefore, these data could not be considered exact. *M. monax* demonstrates more ancestral traits than the other species. Despite mitochondrial markers that join

it with the Palearctic species (Fig. 1a-d), and nuclear ones, with Nearctic species (Fig. 1f), this species is always situated close to the root of the tree. All mitochondrial markers indicate a close relationship of *M. menzbieri* and *M. caudata* (Fig. 1a-e). On the contrary, nDNA data place it at different branches with genetic distances equal to middle values for species (Fig. 1f).

Thus, there are general regularities of distribution and clustering of species of the genus *Marmota* supported by all the molecular markers. At the same time, there are essential differences in topology of the phylogenetic trees constructed on the basis of the same data. For example, an order of branching in clusters "sibirica, himalayana, camtschatica" and "flaviventris, caligata, olympus, vancouverensis" is different in the trees based on the same sequences of *cyt* b (Fig. 1a-b).

Distinctions among *Marmota* phylogenetic trees may be explained by diverse factors. First, there may be different rates for the accumulation of substitutions inside the studied parts of the genome. Changes in the mitochondrial genome are 5-10 times faster in comparison with the nuclear genome (Brown et al. 1979). In turn, different parts of the mitochondrial genome change at different rates. Gene *cyt* b codes a functionally important protein; therefore, most of the allowed substitutions are restricted to synonymic ones by selection pressure. On the contrary, *D-loop* is a noncoding sequence of mtDNA, and all nucleotides might be changed with equal probability, and this leads to high variability of this sequence. That causes a diverse resolution of the respective trees. *D-loop* tree is badly resolved for old species and groups (Fig. 1f). One possible explanation of this phenomenon is a loss of synapomorphies because of high variability of corresponding sequences of this part of mtDNA. At the same time clusters of younger species are well supported. It is necessary to mention that the *D-loop* marker is more suitable for studying closely-related species and intra-species groups (Bannikova 2004).

The possible source for disparity of the trees obtained by studying parts of mitochondrial and nuclear genomes might be maternal and non-Mendel inheritance of mtDNA (Chikuni et al. 1995). Mitochondrial DNA is inherited only from the mother to the offspring in the cytoplasm of the egg and stays unchanged for many generations. The hybridization results in introgression of mtDNA from one species to another, while the nuclear genome is inherited in a different way and stays species-specific. This may be the cause of different locations of *M. menzbieri* and *M. caudata* on mitochondrial (Fig. 1a-e) and nuclear (Fig. 1f) trees. This discordance may be explained by the hypothesis of the remote hybridization of these species (Lyapunova et al. 2003). Small differences between mitotypes of these two species are due to the accumulation of different mutations in the species genomes after their isolation.

A combination of the sample for analysis and an out-group is essential to form the topology of trees. That may be the reason for differences in the trees proposed on the basis of the same sequence data of *cyt* b by various research groups (Steppan et al. 1999) (Fig. 1a), (Herron et al. 2004) (Fig. 1b). In the latter study more species were used (114 species in 21 genera of Sciuridae). Taking into account the similarities of other conditions of both studies (sequence data and statistical methods of analysis) we assume that the cause of differences in topologies of these trees was due to the increased number of species in the second study.

We have tried to combine the results based on using different molecular markers. We have identified nodes that are supported by the majority of the data, and those that are still unresolved or disputed (Fig. 2). First of all, there are two major groups. The first, undoubtedly, includes the North American species of *M. flaviventris*, *M. caligata*, *M. olympus* and *M. vancouverensis*. The second consists of all Palearctic marmots. *M. flaviventris* is the oldest and *M. vancouverensis,* the youngest in this American group according to all the data. The position of *M. olympus* is vague because it is placed differently on each trees (Fig. 1a-b) and its DNA sample was of bad quality. The high genetic affinity of *M. vancouverensis* and *M. caligata* shows the short time since their separate evolution for the accumulation of species specific molecular differences. *M. marmota* apparently is the oldest Palearctic marmot. In the group consisting of *M. sibirica*, *M. himalayana* and *M. camtschatica* different molecular trees show various orders of branching. Apparently, *M. sibirica* and *M. himalayana* are derivatives of approximately similar age from one ancestral form. Adding karyological data for this group analysis (BRANDLER et al. 2008) leads to the assumption that *M. himalayana* may have more ancestral features and *M. camtschatica* is the youngest in this group. The last assumption is also supported by paleontological data (ERBAEVA 2003). *M. bobak*, *M. baibacina* and *M. kastschenkoi* are

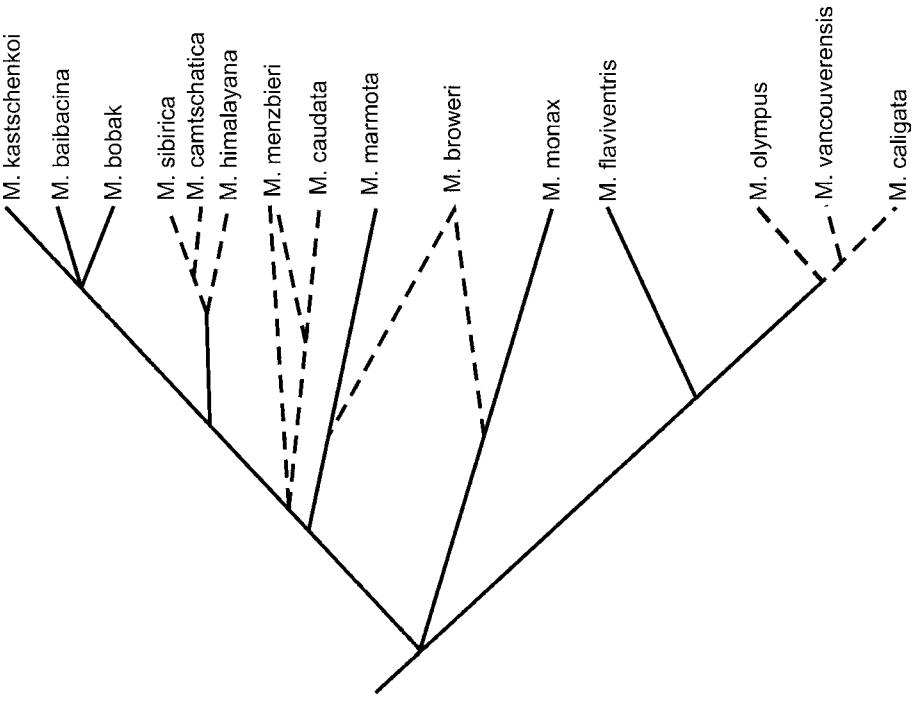


Fig. 2. — Consensus tree of *Marmota* phylogeny based on summarized molecular-genetic data. Solid lines mark well-supported species branches, broken lines mark weakly-supported or disputable branches.

the youngest Palearctic species, thus *M. kastschenkoi* is a recent derivative of *M. baibacina*. Generally, the separation of closely-related species groups, such as *bobak*- and *camtschatica*-groups, both on mitochondrial and on nuclear trees reflects a recent Pleistocene evolution of Palearctic marmots.

*M. monax* clusters with the American and Eurasian marmots in compliance with different constructions. However, it is undoubtedly the most primitive of living marmots. A position of *M. broweri* is the most disputable. It can associate both with *M. marmota* and with *M. monax*. It is unequivocally possible to say that *M. broweri* is not a sister species of *M. camtschatica* as was presumed earlier on the basis of chromosomal structure (VORONTSOV & LYAPUNOVA 1976, LYAPUNOVA et al. 1992).

The positions of *M. menzbieri* and *M. caudata* are not clear. According to the mitochondrial DNA data they divided more recently. In this case it is necessary to assume rapid morphological evolution. On the other hand, according to the nuclear DNA data they are older and primitive representatives of Palearctic marmots. Rapid morphological evolution for an isolated form in *Marmota* was demonstrated for *M. vancouverensis* (CARDINI et al. 2007). But *M. vancouverensis* differentiated under strong isolation on an island in contrast to *M. menzbieri* and *M. caudata*. Unfortunately, a comparison of these examples is not quite complete because there are no data of nuclear genome variability in the *caligata*-group. In the case of *M. menzbieri* and *M. caudata* the hypothesis of remote hybridization of these species in which traces remained in the mitochondrial genome (LYAPUNOVA et al. 2003), explains better the disharmony in mtDNA and nDNA results then their rapid morphological evolution.

In summary, despite some ambiguity in the molecular results, the general topology of the phylogenetic tree of *Marmota* is well resolved. However, there are some gaps in our knowledge of marmot phylogeny as mentioned above: (1) relationships within the *camtschatica*-group and *caligata*-group; (2) the position of *M. broweri*; (3) the background of differences in mitochondrial and nuclear genomes of *M. menzbieri* and *M. caudata*. To resolve these questions it is important: (1) to use samples from all marmot species in each investigation; (2) to increase the number of samples and their distribution per each species; (3) to use new material for *M. olympus*; (4) to use more different molecular markers for different specific purposes; e.g., application of more variable markers, such as *D-loop*, may establish relations of young, closely-related species, and use of nDNA markers will permit the comparison of the evolution of *menzbieri-caudata* and *caligata-vancouverensis-olympus*.

Addition of molecular data with other (morphological, paleontological, karyological, etc.) data will make the picture more complete. Construction of a consensus phylogeny is possible only on the basis of the complex use of markers of different levels of the organic structure and estimation of their weight.

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