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Molecular phylogeny and evolution of the Asian lineage of vole genus *Microtus* (Rodentia: Arvicolinae) inferred from mitochondrial cytochrome *b* sequence

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To examine phylogenetic relationships within the Asian lineage of voles (*Microtus*) belonging to subgenus *Alexandromys*, the mitochondrial cytochrome *b* gene (*cytb*) was sequenced for its representatives, and the results were compared with the cytogenetic, morphological, and paleontological data. In all the trees inferred from maximum likelihood, parsimony, and Bayesian phylogenetic analyses, the Asian clade is subdivided into highly supported *Alexandromys s.s.* and moderately supported *Pallasiinus* lineages. Four subclades are recovered within *Alexandromys*: (1) *Microtus maximowiczii* and *Microtus sachalinensis*; (2) *Microtus miiddendorffii s.l.*, *Microtus mongolicus* and *Microtus gromovi*; (3) *Microtus fortis*; and (4) *Microtus limnophilus.* Thus, *M. limnophilus* demonstrates clear affinities to *Alexandromys s.s.* but not to *Microtus oeconomus* (subgenus *Pallasiinus*), which was always regarded as its sibling species. The results obtained indicate *M. mongolicus* as a member of *Alexandromys* but not of the *Microtus arvalis* group, thus being concordant with the cytogenetic data. The mitochondrial data support the species status of *M. gromovi*; moreover, its placement as a part of a trichotomy with *M. miiddendorffii s.l.* and *M. mongolicus* contradicts the traditional affiliation of *M. gromovi* with *M. maximowiczii*. The divergence rate of *cytb* third position transversions in *Microtus* is estimated at approximately 8% per Myr, which corresponds to approximately 30% per Myr for all substitution types at all codon positions. The maximum likelihood distance based on complete sequence showed a tendency for a progressive underestimation of divergence and time for older splits. According to our molecular clock analysis employing nonlinear estimation methods, the split between *Alexandromys* and *Pallasiinus* and basal radiation within *Alexandromys* date back to approximately 1.2 Mya and 800 Kya, respectively. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **99**, 595–613.

ADDITIONAL KEYWORDS: Alexandromys – Cricetidae – East Palearctic – molecular clock – molecular taxonomy.

INTRODUCTION

Phylogenetic reconstructions of relationships among species of the diverse genus *Microtus* Schrank, 1798 on the basis of morphological and karyological traits are problematic as a result of mosaicism in their formation. Above the level of species groups (such as the 'arvalis' group), chromosome data might be phylogenetically uninformative as a result of convergent evolution of some traits and conservatism of others (Agadzhanyan & Yatsenko, 1984; Modi, 1996). Although the fossil record of *Microtus* is one of the most detailed among extant rodent genera (Gromov &

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Polyakov, 1977; Rabeder, 1981; Rekovets & Nadachowski, 1995), for many lineages, it is still insufficiently complete for precise phylogenetic reconstructions. The examination of genetic differentiation of common voles demonstrated that some subgenera of *Microtus* are artificial groupings combining unrelated lineages, which is readily explained by abundant morphological parallelisms (Jaarola *et al.*, 2004; Golenishchev & Malikov, 2006). An impressive insight into *Microtus* phylogeny was provided by analyses of variation in mitochondrial DNA (mtDNA) (Conroy & Cook, 2000b; Jaarola *et al.*, 2004). According to the results of the latter study, the initial stage of diversification within the genus resulted in the formation of four to six main branches. One of these clades, designated as the 'Asian' lineage, includes representatives of *Alexandromys* and *Pallasiinus* subgenera (*sensu* Pavlinov, Yakhontov & Agadjanian, 1995). Following this pattern, taxonomic interpretation accepted in MSW3 (Musser & Carleton, 2005) favours lumping these two taxa into a single subgenus *Alexandromys s.l.*. However, most of the species traditionally assigned to these subgenera were absent from the sample examined previously. In the present study, we focus on the subgenus *Alexandromys* Ognev, 1914 *s.s.* (or 'calamorum' species group, *sensu* Ellerman, 1941; Meyer, 1983) that is believed to include six to nine species distributed exclusively in the Eastern Palearctics. The molecular phylogenetic relationships among them have never been examined previously. Although the subgenus was intensively studied previously by means of comparative morphometry, cytogenetics, and experimental hybridization (Meyer *et al.*, 1996), several issues remain unresolved. First, the taxonomic position of *Microtus mongolicus* is unclear. For a long time, this species was considered to be a well differentiated subspecies within polymorphic *Microtus arvalis s.l.*, a species complex that is now demonstrated to be quite distant from *Alexandromys*. Later cytogenetic studies confirmed the full species status of *M. mongolicus* and its affinity to the 'arvalis' group was questioned. Although many studies suggested its close relationship to *Alexandromys* (Orlov, Yatsenko & Malygin, 1983; Agadzhanyan & Yatsenko, 1984; Radjably *et al.*, 1984), the necessity of additional evidence to confirm this hypothesis was always stressed. Moreover, according to an alternative viewpoint, this species might be a sole representative of a separate monotypic lineage (Gromov & Erbajeva, 1995). Second, for some decades, the subgeneric affiliation of *Microtus middendorffii* was also considered controversial (Gromov & Polyakov, 1977). Although molecular data indicate its affinity to *Alexandromys* proper (Conroy & Cook, 2000b), the exact phylogenetic position of *M. middendorffii* within the group remains obscure. In addition, there is still a lack of consensus in the interpretation of the status of *Microtus middendorffii hyperboreus* that is treated either as a subspecies or a distinct species (Litvinov, 2001 versus Meyer *et al.*, 1996). Third, a recent study (Sheremetyeva *et al.*, 2009) inferred the full species status of *Microtus maximowiczii gromovi* (Vorontsov *et al.*, 1988) from cytogenetic and morphometric evidence. However, this conclusion warrants corroboration from a comparative molecular genetic data. The phylogenetic relationships of *M. gromovi* with other species of the group remain to be established. Finally, our sample includes *Microtus limnophilus*, whose status as a sister-species to *Microtus oeconomus* (Malygin, Orlov & Yatsenko, 1990) has never been tested.

Thus, the present study is aimed to elucidate the phylogenetic relationships and to determine the content of the subgenus *Alexandromys*. In our study, we sequenced the mitochondrial cytochrome *b* gene (*cytb*) to incorporate previously published data on *Microtus* species. Both the rate and mode of evolution of this gene make it extremely useful for phylogenetic reconstructions over the expected divergence times corresponding to the level of species and subgenera of voles.

MATERIAL AND METHODS

Specimens examined

Tissue samples were taken from 23 vole specimens. All voucher specimens are deposited in the Zoological Museum of Moscow State University (ZMMU) or in the Zoological Institute RAS (ZIN RAS) in St Petersburg. The list of species, collecting sites, museum catalogue numbers, and GenBank Accession numbers are given in the Appendix (Table A1). Species identifications were based on morphological criteria and performed prior to the molecular analysis. To confirm identification of specimens of *Microtus gromovi*, a partial sequence of *cytb* was obtained for the type specimen (collected in Yakutia-Sakha, Neryungrinskiy District, Bolshoye Toko Lake, July 1986, stored in alcohol, collection of ZMMU, N S-140238).

DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing

Genomic DNA was isolated from ethanol-fixed liver, kidney or muscles by proteinase K digestion, phenol–chloroform deproteinization, and isopropanol precipitation (Sambrook, Fritsch & Maniatis, 1989).

Complete or partial *cytb* was sequenced in 23 voles (Table A1). A gene region that included the whole of the mitochondrial *cytb* gene was amplified by PCR with the forward/reverse primer combination L14728/H15906arvic (Lebedev *et al.*, 2007). DNA of an *M.*

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gromovi type specimen was purified directly using MinElute PCR Purification Kit (Qiagen) after Yang *et al.* (1998) from muscle and bone tissue of the ZMMU museum specimen collected 22 years ago. Most of primers for PCR and sequencing were designed for the present study and are presented in the Appendix (Table A2). Short fragments of the gene were amplified with different combinations of primers: L506MM/H15576MO (220 bp) and L506MM/H669East (185 bp), and two additional primers (L410_East and L555_East) were used for sequencing the 154-bp fragment. To avoid contamination, the extraction and amplification of the DNA of the *M. gromovi* type specimen was performed independently in two different laboratories (Department of Vertebrate Zoology of Lomonosov Moscow University and 'Genom', Engelhardt Institute of Molecular Biology).

Double-stranded PCR usually entailed 30–35 thermal cycles: 30 s of denaturation at 94 °C, 1 min of annealing at 57–62 °C and 1 min of extension at 72 °C. PCR products were visualized on 1% agarose gel and then purified using DEAE Watman or NH_4EtOH. Approximately 10–40 ng of the purified PCR product was used for sequencing with each primer using an autosequencing system ABI 3100-Avant using ABI PRISM®BigDye™ Terminator, version 3.1.

Sequence analysis

To test the significance of compositional heterogeneity, values of disparity index (Kumar & Gadagkar, 2001) for all codon positions combined were calculated in MEGA, version 4 (Tamura *et al.*, 2007). Corresponding *P*-values were estimated with the use of Monte-Carlo tests (5000 replicates). To control for the increased type I error as a result of simultaneous testing of multiple hypotheses, we applied the false discovery rate procedure (Benjamini & Hochberg, 1995) modified for correlated statistics by Benjamini & Yekutieli (2001). The false discovery rate was kept at 0.05.

To reveal saturation for different substitution classes/codon positions, uncorrected pairwise differences (*p*-distances) were plotted against maximum likelihood (ML) distances based on the total data set.

Outgroup choice

We consider *Chionomys* to be the most adequate outgroup for monophyletic *Microtus* s.l., including such taxa as *Lasiopodomys*, *Blanfordimys*, and *Stenocranius*. The phylogenetic position of *Stenocranius* recovered in previous analyses of the *cytb* data was ambiguous (Jaarola *et al.*, 2004; Bužan & Kryštufek, 2008; Robovský, Řičánková & Zrzavý, 2008), suggesting that it might be sister to *Chionomys* or *Chionomys* + *Microtus*. However, nuclear genes (GHR exon 10 and LCAT) provide sufficient evidence for the monophyly of *Microtus s.l.* with respect to *Chionomys* (Abramson *et al.*, 2009; Galewski *et al.*, 2006).

Phylogenetic analysis: general outlines

Phylogenetic analysis was performed in two steps. At the first stage, to reveal taxa phylogenetically close to *Alexandromys* and *Pallasiinus*, we analysed an extended alignment including 215 sequences that represent almost all the known mitochondrial lineages of *Microtus* and *Chionomys* that are divergent by more than 1%. The list of sequences retrieved for this purpose from the GenBank is given in the Supporting information (Table S1). The complete sample includes nine of 11 species that unambiguously belong to subgenera *Alexandromys* and *Pallasiinus* according to contemporary checklists (Musser & Carleton, 2005; Pavlinov, 2006). This dataset was tested for departure from base homogeneity as described above. Phylogenetic relationships were estimated under the ML criterion as implemented in RaxML, version 7.0.4 (Stamatakis, 2006).

At the second stage, we focused on a subset of species that demonstrate affinity to the Asian clade *sensu* Conroy & Cook (2000b), as a consequence of the results of the provisional ML analysis. Because the number of taxa was reduced at this step, a more comprehensive analysis employing several tree reconstruction and molecular dating methods could be accomplished. Here, only seventeen sequences of *Chionomys* and *Microtus* were used as potential outgroups for the Asian clade. The criteria for their choice were two-fold: (1) each of the main lineages of *Microtus*, as revealed in Jaarola *et al.* (2004), should be represented by several species and (2) the nucleotide composition in the selected outgroup taxa should be as similar as possible to that in the ingroup. The latter requirement is justified by the necessity to minimize biases as a result of compositional heterogeneity, of which the effect on phylogenetic inference is hardly predictable a priori (Jermiin *et al.*, 2004).

Tree reconstruction

At the first stage, the ML tree for the complete dataset was inferred in RAxML using rapid hill-climbing algorithm and estimating individual GTR+G models for the three codon positions. Node support values were generated with the implication of rapid bootstrapping option (three independent runs of 300 replicates each).

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To reconstruct the final ML tree, appropriate models of sequence evolution were chosen as implemented in MODELTEST, version 3.7 (Posada & Crandall, 1998) based on Bayesian information criterion. A separate model was determined for each of the three codon positions. The ML tree was reconstructed in TREEFINDER (Jobb, 2008) with simultaneous optimization of tree topology and model parameter values. Rate heterogeneity among sites was modelled assuming a gamma distribution for substitution rates (discrete approximation, four categories) without invoking the proportion of invariant sites. Twenty random trees obtained with the 'Generate Start Trees' procedure were used as the starting point. Bootstrap analysis (1000 pseudoreplicates) was performed with model parameters and partition rates fixed at the values optimal for the ML topology and Neighbour-joining tree tree as the initial topology. Bootstrap support percentages were defined as: 50–69%, low; 70–84%, moderate; and 85–100%, high.

Bayesian phylogenetic analysis (Huelsenbeck *et al.*, 2001) was conducted using the program MrBayes, version 3.12 (Ronquist & Huelsenbeck, 2003). Separate models corresponding to those suggested by MODELTEST were used for the three codon positions with all parameters unlinked. Two separate runs were performed with the set of parameters: four chains, heating parameter = 0.2, five million generations, and sampling frequency = 2000. TRACER, version 1.4 (Rambaut & Drummond, 2007) was employed to check for convergence and to detemine the burn-in fraction.

Phylogenetic maximum parsimony (MP) analyses were performed using PAUP* 4.0b10 (Swofford, 2000). Unweighted MP analysis was performed using a heuristic search starting with stepwise addition trees (random addition sequence, 100 replicates) and employing tree bisection–reconnection branch-swapping. To assess clade stability, 1000 bootstrap pseudoreplicates were analysed using the same options. To check for potential topological bias as a result of saturation of transitions in the third codon positions, additional analysis was conducted with the third positions recoded into purines and pyrimidines.

Molecular clock analysis

Rate heterogeneity among taxa was determined with the use of hierarchical likelihood ratio tests as implemented in PAML, version 4.0 (Yang, 2007) and relative-rate tests as implemented in RRTree (Robinson *et al.*, 1998; Robinson-Rechavi & Huchon, 2000). Taxa for which evidence was found suggesting that they violate clock assumption were excluded from the analysis.

To find the best measure of genetic divergence for our molecular estimates of split times, we examined three sets of node heights obtained from ML ultrametric trees in PAUP*: (1) $D_{1–3}$ as calculated for all codon positions and substitutions types assuming common GTR+I+G model; (2) $D_{1,2,3}$ averaged across values calculated separately for each of the three codon positions applying position-specific models; and (3) D_{tv3} based on third position transversions only (CF+I+G model). Standard errors (and variances) of node heights were estimated using 1000 bootstrap replicates generated in PAUP*.

In our molecular clock analysis, we attempt to accommodate the phenomenon of rate decay (time dependency of rate estimates) that might have serious implications for dating recent (< 1–2 Mya) evolutionary events (Ho *et al.*, 2005, 2008; Henn *et al.*, 2009). Rate decay is manifested as a lack of linear relationship between divergence time and genetic distance, thus introducing bias into split age estimates. A direct analysis of the decay curve could be performed only if multiple fossil-based calibration points were available, which appears to be unfeasible thus far. As a workaround, we considered an additional assumption suggesting that the divergence measure estimated from transversions at the third codon positions (D_{tv3}) experiences only minor to negligible decay. A rigorous test on this assumption would require independent evidence and could not be performed within the limits of the present study. Nevertheless, this supposition does not appear to be unrealistic given that the time dependency of rate in protein-coding genes is just moderate (Henn *et al.*, 2009) and that the mode of evolution for the third position transversions is more clocklike than for other substitution classes (Irwin *et al.*, 1991). At the same time, the hypothesis that D_{tv3} is significantly less affected by rate decay than other measures can be verified directly with the data using a nonlinear regression approach. Finally, provided that D_{tv3} may be used as a reasonable proxy for relative age, we can model the behaviour of the decay curve for other measures, at least within some temporal intervals.

Linearity between the measures was tested using nonlinear estimation techniques as implemented in STATISTICA, version 6 (StatSoft, 2001). In all models, D_{tv3} was treated as the dependent variable. To fit a nonlinear regression, a weighted least-squares loss function was used, with the weights being the inverse of variances of D_{tv3} values. To estimate the standard errors of predicted values, 1000 bootstrap sets of $D_{1–3}$ and D_{tv3} node heights were analysed in an R-environment using a script invoking facilities for nonlinear estimation implemented in the 'stats' package.

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Clock calibration was based on the assumption of Late Pliocene radiation of the basal lineages of *Microtus* (2.2 Mya). By this time the ancestor of *Microtus s.l.* (*Allophaiomys*-like arvicolines) dispersed across the Palearctics (from Europe in the west to China in the east), as well as to Nearctics (Tesakov, Vangengeim & Pevzner, 1999; Zheng & Zhang, 2000; Martin *et al.*, 2008). It is this time slice that marks the onset of divergent evolution of multiple *Microtus* lineages throughout the Northern Hemisphere. Given this consideration, we regard the above-mentioned calibration not as the upper bound, but rather as the expectation for the age of basal radiation in *Microtus*. To account for uncertainty of the calibration date, we used 0.2 Myr as its standard error. Although multifossil calibration might increase the accuracy of age estimates, at this stage, we preferred to base our analysis on a single but credible calibration point. The rational for this approach stems from ambiguity with respect to a phylogenetic interpretation of fossil data and poor resolution of early microtine lineages as a result of both insufficient morphological differentiation and abundant parallelisms (Repenning, 1992; Martin & Tesakov, 1998).

RESULTS

The final alignment contained 1140 bp. Partial sequences longer than 900 bp were included in all analyses. The length of three sequences was much shorter; thus, they were omitted from the data set used for tree reconstruction. Only 154 bp were sequenced for the type specimen of *M. gromovi*. This fragment was identical to the corresponding part of the complete sequence obtained for a vole trapped in the Uda River valley. This allowed us to attribute the latter to *M. gromovi.* The length of the fragment sequenced for the second specimen from the Uda river was 419 bp, and it differed from the first one by two third position transitions only.

The partial sequence obtained for one of the two specimens of *M. limnophilus* was 541 bp long; it showed no change compared to the complete sequence.

Sequence composition and saturation

Base composition conforms to the pattern expected for mammalian mitochondrial protein coding genes (Irwin, Kocher & Wilson, 1991).

The analysis of matrix of *P*-values for disparity index measures (DI) using the false discovery rate controlling procedure showed that base composition in *Microtus* is significantly heterogenous. Despite the conservative nature of the applied method, H_o was rejected in at least 30% of the pairwise tests. Importantly, most of *Alexandromys* and *Pallassiinus* demonstrate a deviant base composition as demonstrated by a higher C/T ratio. The evidence on disparity between members of the Asian clade and other species is summarized in the Supporting information (Table S1). The strongest departure from homogeneity was revealed in pairwise comparisons contrasting *Alexandromys* and *Pallassiinus* against *Chionomys* or *Microtus s.s*. Nevertheless, in all lineages except *Chionomys*, we were able to identify species with a base composition relatively similar to that in the Asian clade (e.g. *Microtus socialis* in *Sumeriomys*, *Microtus duodecimcostatus* in *Terricola*, *Microtus chrotorrhinus* in the New World clade, *Microtus kirgisorum* in the nominotypical subgenus). Within *Alexandromys* and *Pallassiinus*, the observed heterogeneity is relatively weak. The only highly significant test corresponds to disparity between *Microtus kikuchii* (GenBank AF348082) and *M. middendorffii* from Chukotka. However, it should be noted that *Microtus montebelli* and *M. kikuchii* appear to be rather divergent from the rest of the Asian clade whereas, at the same time, they are somewhat closer to other groups of *Microtus*.

Saturation plots (not shown) demonstrate some saturation only for transitions at third codon positions. Nevertheless, the plot for this substitution class still do not reach plateau for ML distance > 0.25, indicating that meaningful phylogenetic signal is retained even when distantly related taxa are compared.

Phylogenetic results: the analysis of the complete data set

The results of the fast ML analysis of the complete data set (Fig. 1) demonstrate that all taxa traditionally attributed to *Alexandromys* proper (*Microtus fortis*, *M. middendorffii*, *M. mongolicus*, *M. maximowiczii*, *Microtus sachalinensis*) constitute a highly supported monophyletic group which, however, also includes *M. limnophilus.* This clade clusters together with unsupported *Pallasiinus* (*M. oeconomus*, *M. montebelli*, *M. kikuchii*), thus reproducing the Asian clade *sensu* Conroy & Cook (2000b). The latter is sister to the lineage containing *Microtus (Phaiomys) leucurus*, *Microtus (?Neodon) irene* and *Microtus (Subgen.?) clarkei* (referred hereafter as the *Phaiomys* clade). In general, the inferred topology of the *Microtus* tree agrees with the previous results (Jaarola *et al.*, 2004; Bužan & Kryštufek, 2008; Bannikova, Lebedev & Golenishchev, 2009) in recovering several well differentiated lineages with varying support (*Microtus s.s.* + *Sumeriomys*, *Terricola*, *Agricola* + *Blanfordimys* and New World clades). *Stenocranius* and *Lasiopodomys* occupy a basal position and are highly divergent from all other taxa.

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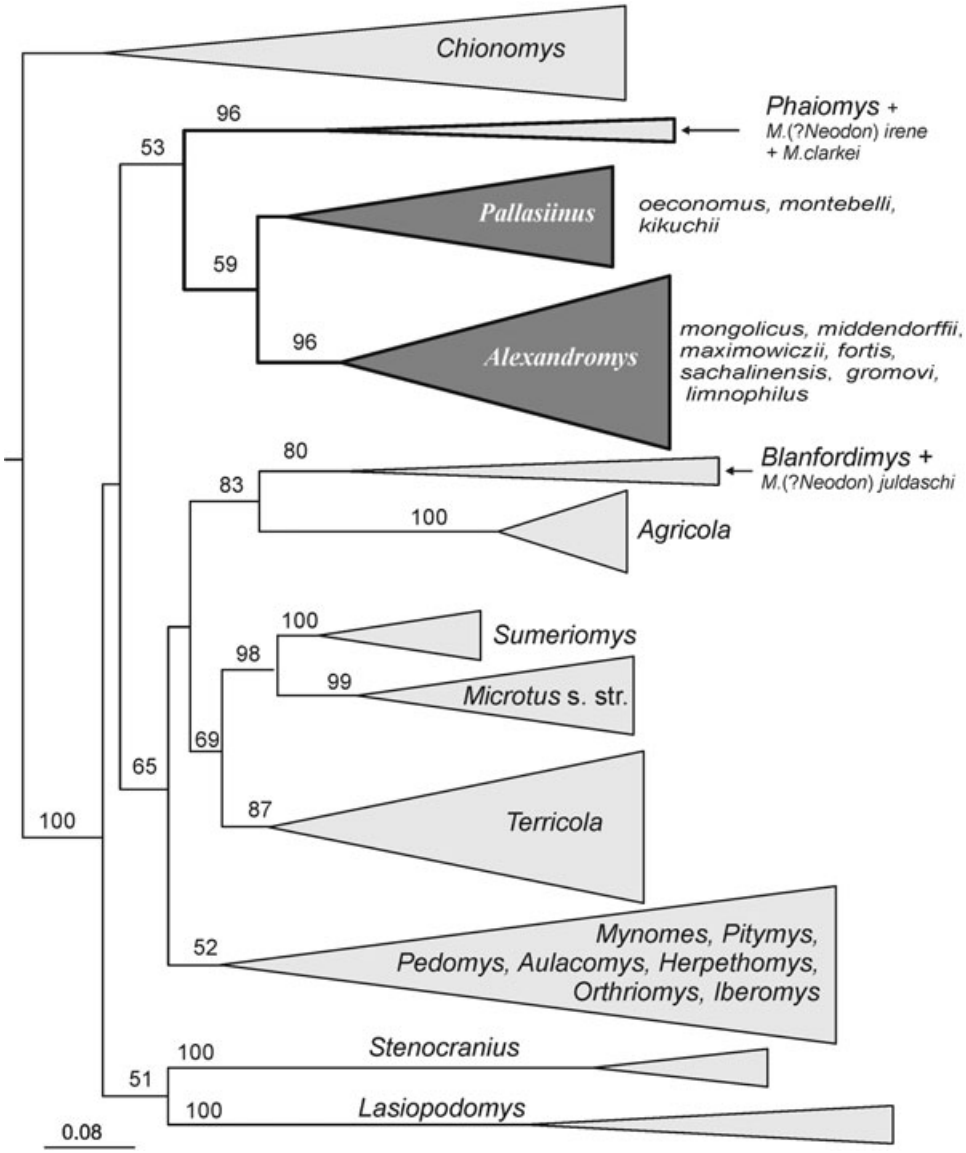

Figure 1. Relationships among major lineages of *Microtus* as inferred by RAxML. Support values estimated with the use of fast bootstrapping (three runs × 300 replicates) are given above branches. *Chionomys* is used as the outgroup for *Microtus s.l.*.

Given this tree pattern, not only *Alexandromys* and *Pallasiinus*, but also members of the *Phaiomys* clade should be included into analysis of relationships within the Asian group at the next stage. However, nucleotide content in both *M. irene* and *M. clarkei* deviates significantly from that in most other Asian voles. Therefore, to control for potential adverse effects of compositional heterogeneity on topological accuracy, all tree searches were performed with *M. irene*, *M. clarkei* and *M. leucurus* either included or excluded. The outgroup selected for the second round of analysis consisted of *Chionomys roberti*, *Chionomys nivalis* and fifteen species of *Microtus s.l.* (see Supporting information, Table S1). Among these, only *M. arvalis* and both *Chionomys* demonstrate highly significant compositional difference from *Alexandromys* and *Pallasiinus*.

Tree reconstructions: Asian clade

The results of Bayesian, ML and MP analyses of the dataset not including the *Phaiomys* lineage are presented in Figure 2A.

In accordance with the recommendations of MODELTEST in the Bayesian analysis, we used a HKY+I+G, HKY+I and GTR+I+G model for the first,

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second, and third codon positions, respectively. The inspection of the Markov chain Monte Carlo samples in Tracer indicates adequate mixing and convergence between the two runs. Stationarity was reached by the 500 000th generation; therefore, the first 10% of the total sample was discarded as the burn-in.

The inferred consensus tree contains strongly supported Asian clade subdivided into highly supported *Alexandromys* and marginally supported *Pallasiinus* lineages. The former subclade includes three groups with high posterior probabilities: (1) *M. maximowiczii* and *M. sachalinensis*; (2) *M. miiddendorffii s.l.*, *M. mongolicus*; and *M. gromovi* (3) *M. fortis* and *M. limnophilus.* The branching order within the second subclade (*M. oeconomus*, + *M. kikuchii* and *M. montebelli*) is supported insufficiently. Bayesian analysis recovers a monophyletic group including most of other major branches of *Microtus* (*Microtus s.s.*, *Terricola*, *Agricola*, *Blanfordimys*, and New World species), and thus places the Asian clade as one of the earliest offshoots within the genus, perhaps, splitting together with the *Lasiopodomys* + *Stenocranius* clade.

The topology of the ML tree is similar to the one reconstructed by MrBayes with the exception of few details (i.e. sister position of *M. miiddendorffii* and *M. mongolicus* relative to *M. gromovi*, basal placement of *Lasiopodomys* + *Stenocranius* clade); however, none of the conflicting groupings receive any bootstrap support. Within the Asian clade, the same two major lineages are recovered; however, the support for *Pallasiinus* group is low (51%). The main groups found within *Alexandromys* in Bayesian analysis retain high to moderate support in the ML tree.

With the MP method, a single most parsimonious tree was retrieved [consistency index (CI) = 0.35; retention index (RI) = 0.58]. Again, the branching order for the Asian clade is the same as in Bayesian analysis, whereas the pattern of intrageneric basal radiation is different but unsupported. Bootstrap support for *M. fortis* + *M. limnophilus* grouping is only 69%. Exclusion of transitions at third codon positions resulted in a decrease of resolution: here, 252 equally parsimonious trees were found (CI = 0.39; RI = 0.65), bootstrap support was somewhat reduced for younger nodes, although the topology of the bootstrap consensus tree is consistent with those of ML, MP, and Bayesian trees. Most likely, the apparent decline of support should be explained by a lack of informative substitutions rather than a presence of conflicting phylogenetic signals.

In general, all three phylogenetic methods produced optimal trees with concordant topologies with respect to the pattern of relationships within the Asian clade. In neither case were highly supported incongruent clades found.

The results of the analyses with the *Phaiomys* group re-included (Fig. 2B) essentially agree with the pattern described above. The monophyly of the clade comprising these three species as well as the grouping of *M. irene* with *M. clarkei* were robustly supported by all three search criteria. In all trees this clade was positioned sister to *Alexandromys* + *Pallasiinus*; however, this placement received high support from Bayesian analysis only. Notably, the inclusion of the *Phaiomys* lineage reduced resolution at the base of the Asian clade, resulting in a substantial decrease in nodal support for both *Pallasiinus* and *Alexandromys* + *Pallasiinus* clades. The support for the monophyly of *Alexandromys*, however, remained unaltered. Moreover, if only *M. leucurus* was excluded from the analysis (results not shown), *M. irene* and *M. clarkei* formed an unsupported association with *M. montebelli* (ML and MP trees), thus compromising the monophyly of the Asian clade *sensu* Conroy & Cook (2000b). *Microtus montebelli* is compositionally closer to *M. irene* and *M. clarkei* (DI = 0.00 and 0.07, respectively; $P = 1.00$ and 0.18) than to *M. oeconomus* (DI = 0.08 and 0.32; $P = 0.004$ and 0.12). Accordingly, we may assume that the observed topological instability can be, at least partly, attributed to the effect of compositional convergence.

The average *p*-distance between haplotypes of *Alexandromys* and *Pallasiinus* is 10%, and the *Phaiomys* group is separated from them by 12%. The difference between the main groups within *Alexandromys*, including distance between *M. fortis* and *M. limnophilus*, is in the range 8.5–9.2%; *M. miiddendorffii s.l.*, *M. mongolicus*, and *M. gromovi* differ by 5.8–6.3%; the average distance between *M. maximowiczii* and *M. sachalinensis* is 5.2%. The most divergent intraspecific haplotype lineages (European and Asian phylogroups of *M. oeconomus*, different lineages of *M. maximowiczii*) differ by up to 4% and 3.5%, accordingly.

Molecular clock

A hierarchical likelihood ratio test was performed in PAML applying a separate GTR+G model for each codon position and using the topology inferred in MrBayes with the *Phaiomys* group excluded. Strict clock assumption was rejected ($\chi^2 = 74.1$, d.f. = 45, $P = 0.004$). After inspecting root-to-tip distances in the ML tree, we identified the *Microtus mandarinus* + *Microtus gregalis* clade as the branch potentially responsible for clock violation. A relative rate test performed in RRTree demonstrates that the rate of synonymous substitutions in these two taxa is significantly higher ($P = 0.01$; however, because this test was conducted a posteriori, its result should be treated with caution). If *M. mandarinus* and *M. gregalis* are omitted, rate constancy holds for the rest of

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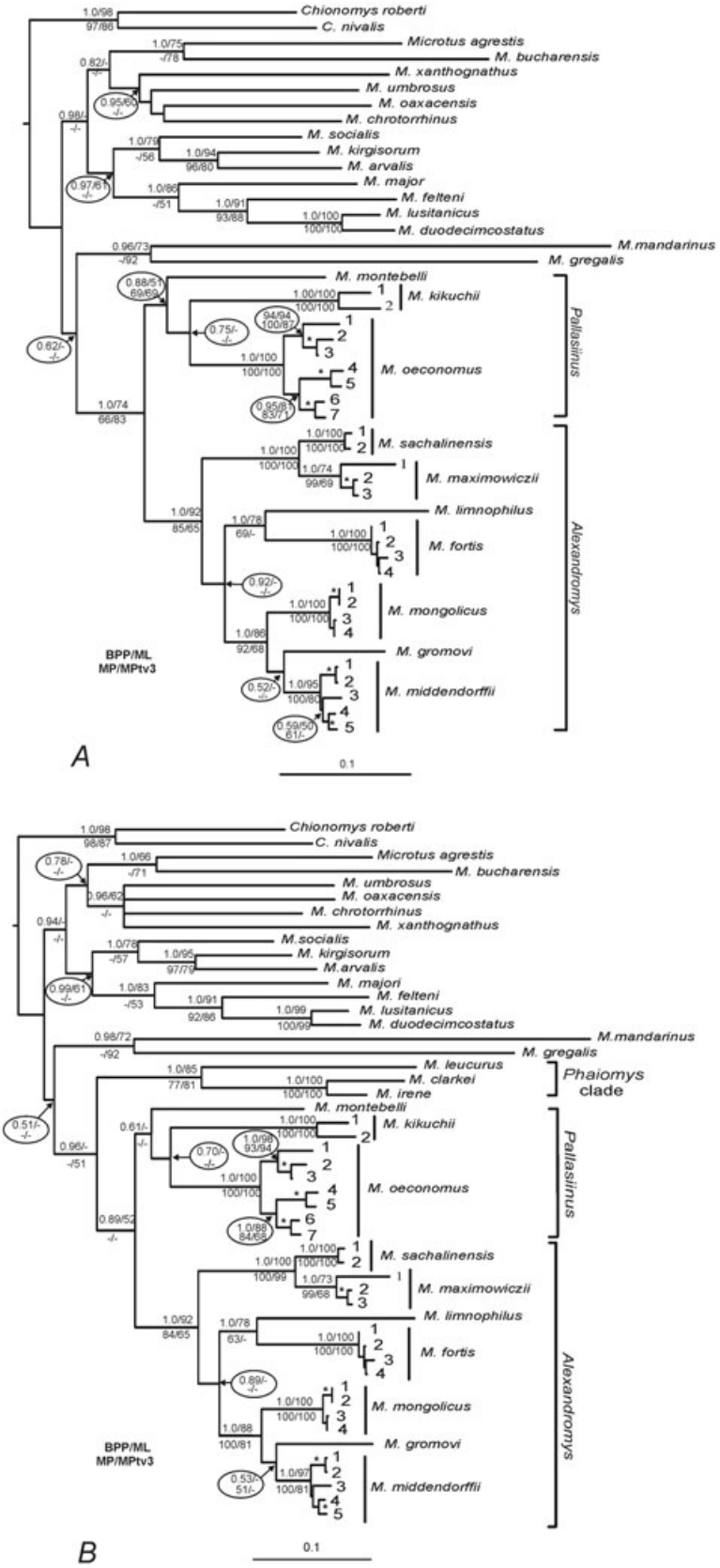

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Figure 2. A, Bayesian *cytb* tree for *Microtus* generated using separate models for the three codon positions. Bayesian posterior probabilities (BPP) and bootstrap values (≥ 50%) for maximum likelihood, maximum parsimony using all substitutions, and maximum parsimony with third position transitions excluded are presented in the following order: $\frac{BPP/ML}{MP/MPtv3}$. Asterisks designate strongly supported intraspecific nodes, bootstrap/BPP values for which are omitted. The *Phaiomys* clade was excluded from the analyses. B, as in (A), but with the *Phaiomys* clade included.

the sample ($\chi^2 = 51.7$, d.f. = 43, $P = 0.17$). Given this result, we decided to exclude the two above-mentioned species from the subsequent molecular clock analysis.

An examination of bivariate plots between measures of sequence divergence based upon third position transversions only (D_{tv3}) and all substitution types at all codon positions ($D_{1–3}$) demonstrates that relationship between them is nonlinear. This conclusion is confirmed by the results of polynomial regression analysis indicating the significance of the quadratic component. Thus, as expected, rate decay proved to be much smaller for the tv3-based distance. If we assume that D_{tv3} accurately reflects time subsequent to clade separation, then $D_{1–3}$ should be biased towards an underestimation of sequence divergence for older splits and, hence, given the position of our calibration point, towards overestimation of dates for all the rest. For example, based on $D_{1–3}$, the time of the *Alexandromys–Pallasiimus* dichotomy is overestimated by approximately 50% (1.6 Mya versus 1.1 Mya for D_{tv3}). It might be assumed that this effect is accounted for by the application of the same evolutionary model simultaneously to all codon positions, thus ignoring variation among them in substitution rate, base frequencies, etc. However, similar, although less pronounced, distortion is observed if D_{tv3} is plotted against node heights averaged across positions and calculated with position-specific models ($D_{1,2,3}$), suggesting that the latter measure is biased as well. Accordingly, it might appear to be logical to base all molecular date estimates on third position transversions exclusively. However, in this case, the number of observed substitutions will be insufficient for an accurate estimation of recent dates. For example, *M. sachalinensis* is separated from *M. maximowiczii* by just seven or eight transversions. To obtain both effective and unbiased estimates, we prefer to use values of $D_{1–3}$ transformed in such a way that they are linearly related to D_{tv3}, and, hence, expectedly to time. Preliminary analysis demonstrated that relationship between $D_{1–3}$ and D_{tv3} can be described with reasonable precision by the equation:

$$D_{tv3} = B/A * [\exp(D_{1–3} * A) - 1]$$

This nonlinear regression (Fig. 3) accounts for 96% of total variation of D_{tv3}, its both coefficients (A and B) are significantly different from zero. Using the above equation, transformed node heights and corresponding estimates of split dates were calculated from $D_{1–3}$-values. The obtained divergence times and their standard errors are given in Table 1. The latter incorporate error as a result of limited sequence length, regression error, and uncertainty of calibration date.

The results obtained suggest that the substitution rate of transversions at third codon position reaches approximately 4% per Myr (SE 0.65%). The rate for all substitution types at all codon positions estimated from transformed values of $D_{1–3}$ is equal to the D_{tv3} rate divided by B, thus being close to 16% per Myr (corresponding to a divergence rate of approximately 32%, SE 7%). Biased divergence rate estimates obtained from the original $D_{1–3}$-values range from more than 30% for most recent splits down to 12–14% per Myr for basal-most nodes.

At this point, it should be noted that our age estimates were inferred from a single calibration point and that our molecular clock analysis relies on the assumption that D_{tv3} increases linearly with time; therefore, the present result can only be considered as preliminary. Given that rate decay is much more rapid for the time period of < 50 Kya (Henn *et al.*, 2009), the estimates for Late Pleistocene splits should be treated with more caution.

DISCUSSION

Phylogenetic relationships within *Alexandromys*

The most obvious result stemming from the present study is the presence of a highly supported lineage including *M. fortis*, *M. maximowiczii*, *M. sachalinensis*, *M. middendorffii* (*s.l.*), *M. mongolicus*, *M. gromovi*, and *M. limnophilus*. The content of this clade corresponds to that of the subgenus *Alexandromys s.s.* (*sensu* Pavlinov *et al.*, 1995), with the exception of *M. limnophilus*. The results obtained confirm a close affinity of *M. mongolicus* to *Alexandromys* but not to the *M. arvalis* group, thus being concordant with the cytogenetic data (Orlov *et al.*, 1983; Meyer *et al.*, 1996).

Four subclades are recovered within *Alexandromys s.s.*, with the relationships among them remaining poorly resolved. The first group comprises *M. maxi-*

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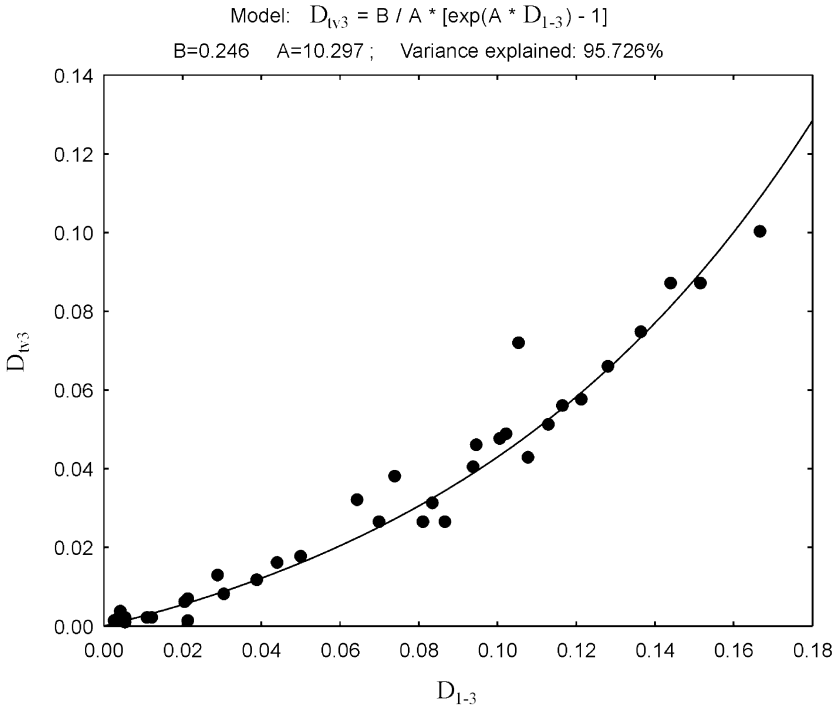

Figure 3. Relationship between node heights of ultrametric maximum likelihood trees calculated from all substitutions at all codon positions of *cytb* gene ($D_{1–3}$) and third codon transversions only (D_{tv3}). The regression line was fit to the data using a nonlinear estimation procedure based on the exponential model.

mowiczii and *M. sachalinensis*. One can expect that *Microtus mujanensis* and *Microtus evoronensis*, which are missing from our sample, also belong to this group because, according to the chromosome data, they are the closest relatives of *M. maximowiczii* (Meyer *et al.*, 1996). Additionally, it was shown that allozyme distances among the three species fall within the range of intraspecific divergence (Frisman *et al.*, 2008).

The second clade corresponds to an unexpected association of *M. middendorffii s.l.*, *M. mongolicus*, and *M. gromovi*, which make up an unresolved trichotomy. A close phylogenetic relationship between *M. mongolicus* and *M. middendorffii* was never demonstrated before, although a similarity between karyotypes of these species was noted by Meyer *et al.* (1996). Our mitochondrial data support the species status of *M. gromovi*. At the same time, the inferred placement of *M. gromovi* as sister to *M. middendorffii* (or *M. mongolicus* + *M. middendorffii*) contradicts its affiliation with *M. maximowiczii* to which it was earlier attributed (Vorontsov *et al.*, 1988). According to previous chromosome data (Meyer *et al.*, 1996; Sheremetyeva *et al.*, 2009), the largest pair of autosomes in both *M. gromovi* and *M. mongolicus* is acrocentric, whereas, in *M. middendorffii*, it is polymorphic, being either acrocentric or subtelocentric. In all other species, chromosomal elements homologous to the first pair of *M. mongolicus* and *M. middendorffii* are either bi-armed (*M. maximowiczii*, *M. sachalinensis*), or rearranged as a result of translocations or fissions (*M. fortis*, *M. mujanensis*, *M. evoronensis*). Therefore, the presence of a large acrocentric could be regarded as a potential synapomorphy for a *middendorffii–mongolicus–gromovi* clade.

The other two groups of *Alexandromys s.s.* are both monotypic, represented by *M. fortis* and *M. limnophilus*, respectively. The mtDNA data in the present study indicate that these two species might be highly divergent sister taxa.

The position of *M. limnophilus* is an unexpected result of the present study. Previously, this species was either synonymized with *M. oeconomus* or considered to be its sibling species. The latter viewpoint is substantiated by the similarity of their karyotypes and hybridization data (Malygin *et al.*, 1990). The Mongolian sample examined in the present study belongs to the subspecies *Microtus limnophilus malygini* (Courant *et al.*, 1999); additional research is necessary to clarify its relationship with the nominal form distributed in Qaidam.

It should be emphasized that, although comparative cytogenetic data on *Microtus* are abundant (Agadzhanyan & Yatsenko, 1984; Meyer *et al.*, 1996), its accurate and comprehensive phylogenetic inter-

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Table 1. The estimates of divergence times and *cytb* divergence

Clade	Divergence time (Mya)	SE (Myr)	Divergence of *cytb* estimated from maximum likelihood ultrametric tree	
			$D_{1–3}$ (GTR+I+Γ model)	D_{tv3} (CF+I+Γ model)
Chionomys/*Microtus*	2.69	0.44	0.333	0.201
Microtus basal radiation	**2.20**	**0.20**	0.302	0.175
Alexandromys/*Pallasiimus*	1.19	0.19	0.215	0.086
Alexandromys basal radiation	0.84	0.14	0.173	0.053
Microtus mongolicus + *Microtus middendorffii*/*Microtus fortis*	0.75	0.13	0.162	0.053
Microtus limnophilus/*Microtus fortis*	0.61	0.12	0.139	0.053
Microtus mongolicus /*M.middendorffii*	0.38	0.08	0.100	0.036
Microtus gromovi/*Microtus middendorffii*	0.32	0.07	0.087	0.033
Microtus middendorffii Yakutia1/Yakutia2	0.009	0.003	0.011	0.005
Microtus middendorffii Yakutia /Chukotka	0.045	0.013	0.021	0.005
Microtus middendorffii Taymyr/Yakutia + Chukotka	0.054	0.015	0.024	0.005
Microtus mongolicus Hentei/Chita	0.008	0.003	0.011	0.003
Microtus fortis basal radiation	0.013	0.005	0.008	0.003
Microtus sachalinensis/*M.maximowiczii*	0.27	0.06	0.077	0.024
Microtus maximowiczii Hentei/Chita	0.12	0.03	0.042	0.014
Microtus kikuchii/*Microtus oeconomus*	0.79	0.16	0.166	0.063
Microtus montebelli/*Microtus oeconomus*	0.95	0.17	0.187	0.081
Microtus oeconomus Beringian clade basal	0.020	0.007	0.014	0.000
Microtus oeconomus Beringian/Siberia	0.11	0.03	0.041	0.013
Microtus oeconomus Siberian clade, basal	0.016	0.006	0.013	0.000
Microtus oeconomus Europe/Siberia + Beringia	0.19	0.05	0.060	0.017
Microtus oeconomus Russia/Sweden	0.012	0.005	0.012	0.000
Microtus oeconomus Russia/Western Europe	0.11	0.03	0.038	0.000
Microtus socialis/*Microtus arvalis*	0.96	0.19	0.189	0.092
Microtus kirgizorum/*Microtus arvalis*	0.54	0.13	0.128	0.065
Terricola basal radiation	1.08	0.20	0.204	0.098
Terricola/*Microtus s.s.*	1.61	0.25	0.256	0.132
Terricola + *Microtus s.s.*/*Agricola*	2.00	0.24	0.287	0.175
Agricola/*Blanfordimys*	1.36	0.24	0.233	0.112

$D_{1–3}$, all substitutions types; D_{tv3}, transversions at third codon positions. Calibration date is given in bold.

pretation has not yet been accomplished. Thus, based on karyotype similarity, two subgroups were recognized within *Alexandromys*, the first of them includes highly polymorphic *M. maximowiczii*, *M. mujanensis*, and *M. evoronensis*, and the second includes *M. mongolicus*, *M. middendorffii*, *M. fortis*, and *M. sachalinensis* (Meyer *et al.*, 1996). The lowest level of differentiation is observed between karyotypes of *M. fortis* and *M. sachalinensis*, which belong to different clades according to the mtDNA data. Nevertheless, given that no chromosomal features were identified as synapomorphies for the second group, we might regard the latter as a paraphyletic assemblage. If so, the apparent conflict between the two patterns should be regarded not as an indication of discordant evolution of chromosomal and mitochondrial characters, but rather as a result of methodological differences.

Intraspecific variation

Our data on intraspecific variation of the species studied are insufficient for comprehensive phylogeographic implications; however, some interesting remarks are worthy of note.

Geographic variation revealed within *M. fortis* is low (sequence divergence < 1%), thus indicating a lack of differentiation between recognized subspecies *Microtus fortis michnoi* (Buryatia) and *Microtus fortis uliginosus* (Korea). Similarly, little interpopulation divergence is found in *M. mongolicus*. By contrast,

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haplotypes of *M. maximowiczii* from the Chita region and those from Hentiyn-Nuruu (Mongolia) are separated by a *p*-distance of more than 3%. To check for correspondence between the distribution of mtDNA lineages and the extensive chromosomal polymorphism reported in this species (Kovalskaya, Khotolkhu & Orlov, 1980), additional research is warranted.

Our sample of *M. middendorffii* s.l. includes specimens of both *hyperboreus* and *middendorffii s.s.* According to Litvinov (2001), voles distributed to the East of the Kolyma river should be attributed to the former taxon, whereas the latter is represented by specimens collected close to terra typica of *middendorffii* (southern Taymyr). However, our data do not reveal any substantial (> 2.0%) variation within this species group and, hence, provide no evidence supporting recognition of *M. hyperboreus* as a distinct species. At the same time, because three groups of haplotypes differing by approximately 1.8% were found within *M. middendorffii s.l.*, it is possible that the pattern of relationships within it may be more complex than the simple *middendorffii s.s./hyperboreus* dichotomy. It is evident that phylogeographic structure of *M. middendorffii s.l.* requires a much more detailed examination before any definite taxonomic conclusions could be made.

Thus, intra-specific geographic variation found in *Alexandromys* falls within the range reported previously for other species of *Microtus* (Brunhoff *et al.*, 2003; Jaarola *et al.*, 2004; Martínková *et al.*, 2007). In neither case did we observe high level of differentiation (> 5%) between populations ascribed to the same species, as demonstrated for *Microtus savii* (Castiglia *et al.*, 2008), *Microtus subterraneus*, *Microtus agrestis* (Jaarola *et al.*, 2004) or *Microtus longicaudus* (Conroy & Cook, 2000a).

Relationships within the Asian clade and its potential sister groups

The results of our phylogenetic analysis are concordant with those of previous mtDNA studies (Conroy & Cook, 2000b; Jaarola *et al.*, 2004) in recovering a monophyletic Asian clade, consisting of *Alexandromys s.s.* and *Pallasiinus* branches. In addition, *cytb* data indicate that the Asian clade might be viewed in a broader sense to include also a third lineage, currently represented by three species endemic to southeast Asia and Tibet (*M. leucurus*, *M. irene* and *M. clarkei*), and placed as the closest sister group to *Alexandromys* + *Pallasiinus.* The same pattern was produced by the combined analysis of genetic and morphological data on Arvicolinae (Robovský *et al.*, 2008). However, the position of the *Phaiomys* clade as part of the Asian lineage was not robustly supported and, moreover, its inclusion resulted in a loss of support for the monophyly of *Alexandromys* + *Pallasiinus*. Therefore, we have to conclude that, based on available mitochondrial evidence, we can neither confidently resolve basal relationships in the Asian clade *s.l.*, nor even confirm its existence. At the same time, it should be stressed that grouping of *M. oeconomus* with *M. kikuchii* and its sister position to *Alexandromys* relative to *Phaiomys*/*Neodon* is supported by the data on GHR exon 10 (Galewski *et al.*, 2006). The monophyly of the Asian clade (in its original definition) gains additional support from Zoo-FISH data (Lemskya, 2008) according to which *Alexandromys* and *Pallasiinus* (represented by *M. maximowiczii* and *M. oeconomus*, respectively) are the closest sister groups with respect to members of *Lasiopodomys*, *Stenocranius*, *Terricola*, *Blanfordimys*, *Agricola*, *Sumeriomys*, *Microtus s.s.*, and, finally, *M. (Subgen.?) clarkei*.

In all our analyses, the support for the *Palassiinus* branch is either low or missing, critically depending on the presence/absence of the *Phaiomys* clade. From a cytogenetic standpoint, the three species with $2n = 30$ (*M. oeconomus*, *M. kikuchii*, *M. montebelli*) are extremely similar because they share the same homologous banding patterns of almost all chromosomes and demonstrate specific synaptic behaviour of the sex chromosomes at meiotic prophase (Mekada *et al.*, 2001). Nevertheless, the validity of this and other groupings is worthy of being tested with a larger data set, including less known Asian taxa such as *Volemys*, *Neodon s.s.* and *Proedromys*, of which the relationships with the major microtine lineages remain to be established.

Based on allozyme data, the close relationship among East Palearctic *Pallasiinus*, *Alexandromys*, *Lasiopodomys*, and *Stenocranius* was suggested (Mezhzherin, Zykov & Morozov-Leonov, 1993). In our analysis *Lasiopodomys* and *Stenocranius*, albeit highly divergent between themselves, form a separate clade, well differentiated from other main branches of *Microtus*. This finding is consistent with the pattern recovered from the data on two nuclear genes (Abramson *et al.*, 2009). However, concerning the putative association of this lineage with the Asian clade, the *cytb* data are insufficient to reject or confirm it.

The rate of molecular evolution

Based on the *cytb* data for *Microtus*, we have demonstrated that relationship between ML distances for the complete sequence and tv3 sequence divergence is nonlinear. It means that if the latter is supposed to increase linearly with time, the former would systematically underestimate the divergence between deeper nodes even if the best fit model (such as suggested

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by MODELTEST) is applied. This pattern could be directly related to the apparent acceleration of molecular evolution for recent events, as discussed by Ho *et al.* (2005), or regarded as a manifestation of some more general phenomenon (Wayne, van Valkenburgh & O'Brien, 1991). It may be suggested that the observed decay of substitution rates could be accounted for, to a large extent, by the presence of mutation hot spots in mammalian mtDNA and a high rate of evolution of site-specific mutation rates (Galtier *et al.*, 2006), which makes any adequate correction for multiple hits problematic. The results obtained in the present study highlight the necessity for a proper choice of methods for molecular dating and indicate the potential inadequacy of a straightforward approach based on simplistic models.

The divergence rate of third position transversions in *Microtus* is estimated at 8% per Myr, which corresponds to an instantaneous rate of up to 32% per Myr for all substitutions types at all codon positions. This value is much higher than the previous estimate of 13% per Myr obtained by Conroy & Cook (2000b) when applying a molecular clock approach essentially similar to the present one. Given that the same single calibration point was used in both studies, this discrepancy should be attributed to the effect of rate decay, which was ignored previously. Taking into account the accumulating bulk of data elucidating high variability of mitochondrial substitution rates in mammals (Nabholz *et al.*, 2008) and its apparent acceleration in *Microtus* (Triant & deWoody, 2006), we regard our rate estimate as plausible.

Paleontological evidence and evolution of the Asian lineage

The fossil record in the modern geographic range of *Alexandromys* and *Pallasiinus* voles is relatively scarce. At the beginning of the Early Pleistocene (approximately 1.8–1.5 Mya), most *Microtus* show primitive *Allophaiomys* dental morphology (Gromov & Polyakov, 1977; Rabeder, 1981; Rabeder, 1986; Erbajeva, 1998), and no lineages can be recognized yet. By the late Early Pleistocene, however, a number of faunas document the origin of *oeconomus*-type variability of m1. Mid Early Pleistocene faunas in West Siberia show a distinct percentage of *oeconomus*-like morphotypes among the dominant *Allophaiomys* samples (Zazhigin, 1980; Smirnov, Bolshakov & Borodin, 1986). Some of these early voles were described from north-eastern Siberia as *Microtus reservatus* (Zazhigin, 1998) and from Hebei (China) as *Microtus minoeconomus* (Zheng & Cai, 1991). In Eastern Europe, an abrupt (i.e. most likely migrational) appearance of *oeconomus*-like forms (*Microtus protoeconomus*; Rekovets, 1994) occured slightly later, between 1.0 and 0.8 Mya (Rekovets, 1994).

The genetic unity of diverse fossil *Microtus* in the Middle-Late Pleistocene of northern Asia is possibly emphasized by widespread *oeconomus*-like (ratticepoid) morphotypes of m1; for example, Middle Pleistocene *Microtus epiratticeps* from Choukoutien localities 1 and 3 in eastern China (Young, 1934; for other cases, see Agadjanian & Erbaeva, 1983; Frolova, 1985; Smirnov *et al.*, 1986; Zazhigin, 1998; Kuznetsova & Tesakov, 2004). A morphological transition from a primitive *oeconomus*-like morphotype to an advanced one was described for the endemic Japanese lineage comprising fossil Middle-Late Pleistocene *Microtus epiratticepoides* and recent *M. montebelli* (Kawamura, 1988).

Most recent 'eastern' *Microtus* species have advanced five-triangled m1. Only *M. oeconomus* and *M. limnophilus* retain four-triangle structure of this molar. *Microtus middendorffii*, *M. mongolicus*, *M. sachalinensis* and most other 'eastern' *Microtus* all occasionally show *oeconomus*-like morphotypes as the indication of the ancestral condition. Thus, there are sound reasons to assume affinities of all these voles marked by a specific dental complication. Another peculiar character shared by many extant species in the group is the relatively thick posterior enamel bands (trailing edges) on lower molars retaining a primitive layer of tangential enamel that is normally reduced in most arvaloid 'western' *Microtus* (von Koenigswald, 1980). Furthermore, *M. middendorffii*, *M. gromovi*, *M. sachalinensis*, and occasionally some other 'eastern' species have a very complex M3 with a peculiar occlusal field formed by fused T5 and T6 triangles (Fig. 4).

A close look at the genetic and morphological characters shows a conspicuous picture of mosaic and parallel evolution of dental characters. A striking mismatch between the mitochondrial phylogeny and the distribution of dental characters is evident. The most dentally simple *M. oeconomus* and *M. limnophilus* appear to retain almost identical plesiomorphic condition, whereas they also show a high level of genetic differentiation. Plesiomorphic thick enamel can be present in some closely-related species (*M. middendorffii* and *M. gromovi*), whereas the other species of the same group (*M. mongolicus*) shows well differentiated enamel. By contrast, two former species have the most advanced complex M3 and the latter species is distinct in the relatively simple structure of this molar, etc. The acquisition of a very complex M3 with frequently fused T5–T6 occurred independently at least in two lineages (*M. middendorffii* (+ *M. gromovi*) and *M. sachalinensis* + *M. maximowiczii*).

The palaeontological data do not contradict the suggested timing of most of the cladogenetic events

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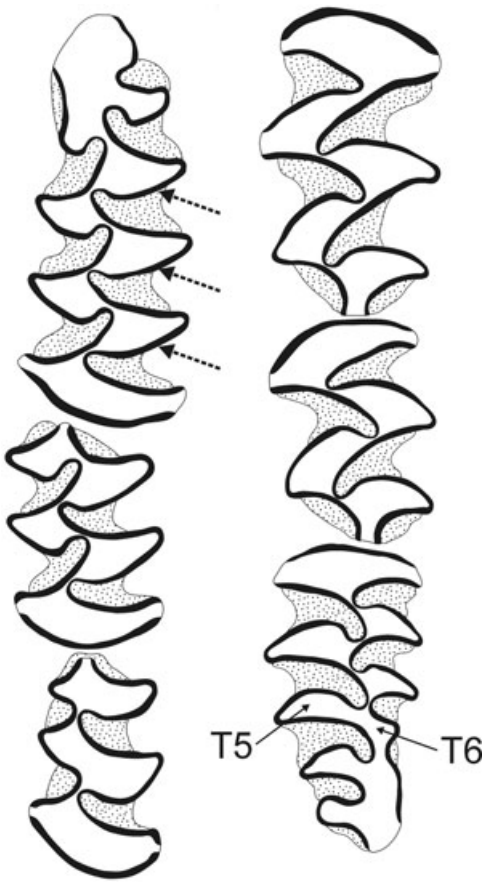

Figure 4. Upper (right) and lower (left) molar rows of *Microtus gromovi* (type specimen, ZMMU S-140238). Solid arrows indicate the position of T5 and T6 triangular prisms forming common occlusal field in M3. Dashed arrows indicate the thick enamel layer on the trailing edge of m1.

(Table 1). First, as indicated in the present study, the known fossil record does fit the hypothesis of splitting into different groups of eastern *Microtus* no later than the late Early Pleistocene, supporting our estimate of the split between *Alexandromys* and *Pallasiinus* at approximately 1.2 Mya. A possible last common ancestor of the two lineages could be *M. reservatus*. The presence of multiple lineages of eastern *Microtus* in the Middle Pleistocene supports the main species group differentiation in *Alexandromys* during this interval.

The divergence time between *M. miiddendorffii*, *M. mongolicus*, and *M. gromovi*, as well as between *M. maximowiczii* and *M. sachalinensis*, dates back to the late Middle Pleistocene. Hence, a widespread large form of *Microtus* from early Middle Pleistocene faunas of Eastern Europe (approximately 0.8–0.5 Mya), which is sometimes interpreted as a member of the *M. middendorffii* group (Agajanian, 1981), can hardly be regarded as such. In many of the reported cases, however, well differentiated enamel and occlusal elements do not match the 'eastern' *Microtus* and indicate a different unrelated lineage.

With respect to other lineages of *Microtus*, it is worth noting that our age estimates are consistent with the fossil record indicating the first appearance of *Terricola* and *Microtus s.s.–Sumeriomys* approximately 1 Mya (Agusti, Oms & Pares, 1999; Cuenca Bescos, Canudo & Laplana, 2001; Maul & Markova, 2007).

In conclusion, we should emphasize that the examined group of *Microtus* is characterized by intensive chromosomal evolution that presumably promotes the rapid formation of reproductive barriers. Therefore, it might be expected that introgression of alien mitotypes is unlikely and, consequently, our mitochondrial tree should not deviate considerably from the true species phylogeny.

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APPENDICES

Table A1. List of specimens analysed (*Pallasiinus* and *Alexandromys* subgenera)

Species	Collecting locality	Museum catalogue number, tissue or field code	Tree (Figs 2, 3) code	*Cytb* sequences	
				Length, bp	GenBank Accession number
Microtus (Alexandromys) middendorffii	Russia, Krasnoyarsk Territory, Dolgano-Nenets Autonomous District, Kureyka River, 68.166°N, 92.760°E	S-181782 f.c.218	1	1140	**FJ986314**
Microtus (Alexandromys) middendorffii	Russia, Krasnoyarsk Territory, Dolgano-Nenets Autonomous District, Kureyka River, 68.166°N, 92.760°E	S-181793 f.c.219	1	1140	**FJ986315**
Microtus (Alexandromys) middendorffii	Russia, Republic of Yakutia-Sakha, Ust′-Mayskiy District, mouth of Chabda river, tributary of Maya, 59.777°N, 134.814°E	S-183385 f.c.731	5	1140	**FJ986316**
Microtus (Alexandromys) middendorffii	Chukotka	f.c.midd ZIN	3	991	**FJ986313**
Microtus (Alexandromys) middendorffii	Russia, Republic of Yakutia-Sakha, Nizhnekolymsky District, Cherskiy, Little Kol' Kovaya River, 69.240°N, 158.300°E	f.c.39309 Burke museum	4	1143	AF163898
Microtus (Alexandromys) mongolicus	Mongolia, Selenge Aymak, upper Ero river, 49.083°N , 107.283°E	S-182195 f.c.5	1	1140	**FJ986304**
Microtus (Alexandromys) mongolicus	Mongolia, Selenge Aymak, upper Ero river, 49.083°N , 107.283°E	f.c.7	2	1134	**FJ986305**
Microtus (Alexandromys) mongolicus	Russia, Chita Region, Krasnokamenskiy District, 6 km S of Kuytun, 50.120°N, 118.670°E	S-180448 f.c.141	3	1140	**FJ986309**
Microtus (Alexandromys) mongolicus	Russia, Chita Region, Krasnokamenskiy District, 6 km S of Kuytun, 50.120°N, 118.670°E	S-180449 f.c.148	4	1140	**FJ986310**
Microtus (Alexandromys) maximowiczii	Mongolia, Central Aymak, Hentiyn-Nuruu, 47.992°N, 107. 375°E	S-179480 f.c.3	1	1140	**FJ986303**
Microtus (Alexandromys) maximowiczii	Russia, Chita Region, Kalganskiy District, Kozlovo, 51.190°N, 118.930°E	S-178597 f.c.90	2	1140	**FJ986311**
Microtus (Alexandromys) maximowiczii	Russia, Chita Region, Sretenskiy District, Chachakan River, right bank of Shilka R., 52.320°N, 118.210°E	S-178602 f.c.92	3	1140	**FJ986312**
Microtus (Alexandromys) sachalinensis	Sakhalin Region, Noglikskiy District, Chayvo Bay, 52.530°N, 143.060°E	f.c.26	1	1134	**FJ986317**
Microtus (Alexandromys) sachalinensis	Sakhalin Region, Noglikskiy District, Chayvo Bay, 52.530°N, 143.060°E	f.c.19	2	1133	**FJ986318**
Microtus (Alexandromys) fortis	Mongolia, Selenge Aymak, upper Ero river, 49.083°N , 107.283°E	S-182280	2	1140	**FJ986308**
Microtus (Alexandromys) fortis	Russia, Chita Region, Aleksandrovo-Zavodskiy District, upper Kher-Khira River, 50.410°N, 118.110°E	S-178587 f.c.96	1	1140	**FJ986307**
Microtus (Alexandromys) fortis	Russia, Buryatia, Dzhidinskiy District, Dodo-Ichetuy, 50.583°N. 105.500°E.	S-179172 f.c.186	4	1140	**FJ986306**
Microtus (Alexandromys) fortis	Korea, Kyonggi Prov., Dong Du Chun		3		AF163894
Microtus (Alexandromys) gromovi	Russia, Khabarovsk Region, Uda River, 25 km above Chumickan	S-176552 f.c.335		1140	**FJ986319**
Microtus (Alexandromys) gromovi	Russia, Khabarovsk Region, Uda River, 25 km above Chumickan	S-176537 f.c. 320		419	**FJ986320**
Microtus (Alexandromys) gromovi	Russia, Republic of Yakutia-Sakha, Neryungrinskiy District, Bolshoye Toko Lake, 56.050°N, 130.810°E	S-140238		154	**FJ986321**
Microtus (Alexandromys) limnophilus	Mongolia, Kobdo Aymak, vicinities of Chadman, 47.692°N, 92.769°E	S-181025 f.c.175		1140	**FJ986323**
Microtus (Alexandromys) limnophilus	Mongolia, Kobdo Aymak, vicinities of Chadman, 47.692°N, 92.769°E	S-181017 f.c.165		543	**FJ986324**
Microtus (Pallasiinus) oeconomus	Russia, Orenburg Region, Kuvandykskiy District, source of Pismenka River, 4 km S of Pervomaisk, 51.350°N, 57.450°E	S-178553 f.c.86	3	1140	**FJ986325**
Microtus (Pallasiinus) oeconomus	Russia, Irkutsk Region, Koty	f.c.189	7	1140	**FJ986326**
Microtus (Pallasiinus) oeconomus	Netherlands		1	1143	AY220006
Microtus (Pallasiinus) oeconomus	Sweden		2	1143	AY219994
Microtus (Pallasiinus) oeconomus	Alaska		4	1143	AY220045
Microtus (Pallasiinus) oeconomus	Russia, Kuril islands		5	1143	AF163902
Microtus (Pallasiinus) oeconomus	Russia, Magadan Region		6	1143	AY305206
Microtus (Pallasiinus) kikuchii	Taiwan		1	1143	AF163896
Microtus (Pallasiinus) kikuchii	Taiwan		2	1143	AF348082
Microtus (Pallasiinus) montebelli	Japan, Honshu			1143	AF163900

Accession numbers for the specimens sequenced in the present study are given in bold.

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Table A2. Primers used for polymerase chain reaction amplification and sequencing of the *cytb* gene in the genus *Microtus*

Primer	Sequence (5′ to 3′)	Reference
L14728	GACATGAAAAATCATCGTTGTTATT	Lebedev *et al.* (2007)
H15906arvic	ACTGGTTTACAAGACCAGTGTAAT	Lebedev *et al.* (2007)
H15576MO	GATCGTAGGATGGCGTAGG	Brunhoff *et al.* (2003)
L555_East	GCCCTTTATCATCACCGCCCTA	Present study
L410_East	AAATATCATTCTGAGGAGCCACAGTAATC	Present study
H669_East	CACCTAGGAAGTCTTTGATTGTGTAG	Present study
L506MM	AGACAAAGCCACCCTAACACGATT	Present study

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Species examined in the study, base composition in *Microtus* and *Chionomys* and the results of pairwise tests for compositional homogeneity between *Alexandromys–Pallasiinus* and other microtine lineages (sequences presented in Fig. 2 are marked by asterisks).

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