Mammalian Biology 76 (2011) 258–267

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.de/mambio

Original Investigation

Phylogeny of Palearctic vole species (genus *Microtus*, Rodentia) based on mitochondrial sequences

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ARTICLE INFO

Article history:
Received 4 February 2010
Accepted 22 April 2010

Keywords:
Microtus fortis species-group
Microtus oeconomus
Mitochondrial control region
Phylogeography

ABSTRACT

Within the species-rich rodent genus *Microtus*, the *Microtus fortis* species-group is not well studied so far. We investigated DNA sequences of the mitochondrial control region in taxa of this group to assess the inter- and intraspecific variation and differentiation of populations, and to establish a molecular phylogeny. For comparison, samples of *Microtus oeconomus* covering the species distribution range were analyzed. Within the *M. fortis* group five distinct highly supported lineages were found. Four of them represent single species: *Microtus fortis*, *Microtus sachalinensis*, *Microtus hyperboreus*, and *Microtus gromovi*. The fifth clade comprises *Microtus mujanensis*, *Microtus evoronensis* and *Microtus maximowiczii*. Genetic distances between these five lineages range from 5.4% to 9.2%. The distinct position of *M. gromovi* confirms the proposed species status suggested by earlier chromosome and cranial-morphological investigations. The trees also indicate that *M. hyperboreus* belongs to the *M. fortis* group and is the sister group of *M. gromovi*. Genetic diversity is rather high within the East Asian *M. fortis* species-group, which is also characterized by high chromosomal variation as determined in previous studies. The phylogeographic relationships found in *M. oeconomus* are in accordance with previous findings based on the mitochondrial *cytochrome b* gene. There are tree main haplogroups (Europe, Siberia, Beringia) found in this Holarctic species. The genetic distances between these groups in the mitochondrial control region range from 3.4% to 4.1%.

In general, genetic diversity and species richness of voles in the Eastern Palearctic implies that this region might have provided ideal conditions for the radiation of this species group.

Introduction

The species rich genus *Microtus* comprises more than 60 extant species distributed throughout the Palearctic and Holarctic (Chaline et al. 1999). In general, the subdivision of the genus *Microtus* into subgenera and species groups is very complicated and controversial (Ellerman 1941; Ellerman and Morrison-Scott 1951; Gromov and Polyakov 1977; Zagorodnyuk 1990; Meyer et al. 1996), and has been revised several times in the past. This is in particular true for composition and designation of the East Palearctic species groups. For example, the species of the East Palearctic *Microtus fortis* group (formerly *Microtus calamorum*; see below) were in the past assigned to two (*M. fortis* and *M. maximowiczii*; Gromov and Polyakov 1977) or three (*M. fortis*, *Microtus middendorfii*, and *M. maximowiczii*; Zagorodnyuk 1990) species groups, respectively.

In the past decade, phylogenetic and phylogeographic analyses have been carried out on many representatives of the genus *Microtus* employing molecular methods (e.g., Conroy and Cook 2000; Haring et al. 2000; Brunhoff et al. 2003; Fink et al. 2004; Galbreath and Cook 2004; Jaarola et al. 2004; Hellborg et al. 2005; Martínková et al. 2007; Castiglia et al. 2008; Tougard et al. 2008; Tryfonopoulos et al. 2008; Bannikova et al. 2010). However, most of these analyses focused on West Palearctic species. In this paper we analyze the systematics and phylogeography of the East Palearctic *M. fortis* species-group which belongs to the subgenus *Alexandromys* (Zagorodnyuk 1990). This group was formerly named *M. calamorum* group (Ellerman 1941) including a set of East-Asian species: *M. calamorum* Thomas, 1902, *Microtus clarkei* Hinton, 1923, *Microtus ungurensis* Kastschenko, 1913, *M. fortis* Buchner, 1889, and *Microtus michnoi* Kastsch., 1910. Because *M. calamorum* is currently not valid any more (Allen 1940; Ognev 1950) we will use the name "*M. fortis*" species-group henceforth. According to Meyer et al. (1996) this group comprises the following species, which share similar habitat preferences: (1) *M. fortis*, (2) *M. maximowiczii* Schrenck, 1858, (3) *Microtus mujanensis* Orlov and Kovalskaya, 1978, (4) *Microtus evoronensis* Kovalskaya and Sokolov, 1980, and (5) *Microtus sachalinensis*

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doi:10.1016/j.mambio.2010.04.006

Vasin, 1955. The first two are more widely distributed in Eastern Siberia (*M. fortis*, *M. maximowiczii*) as well as in Korea and Eastern China (*M. fortis*). The latter three species occupy rather restricted areas in Eastern Siberia: Buryatia (*M. mujanensis*), Khabarovskyi Krai (*M. evoronensis*) and Sakhalin (*M. sachalinensis*). However, in the same book (Meyer et al. 1996), based on chromosome and morphological analyses, the authors concluded to divide this group into the *M. fortis* group and the *M. maximowiczii* group. The former comprised *M. fortis*, *M. sachalinensis*, *Microtus mongolicus* Radde, 1861, and *M. middendorffi* (Poljakov, 1881). The latter one included *M. maximowiczii*, *M. mujanensis*, and *M. evoronensis*. Moreover, there is no consensus regarding the content of each group. In this paper, we will treat the *M. fortis* group *sensu latu*.

Morphologically, the five species display high intraspecific variability, but there are no clear-cut differences between species. Moreover, several new species such as *M. mujanensis* (Orlov and Kovalskaya 1978), and *M. evoronensis* (Kovalskaya and Sokolov 1980) were described recently owing to chromosomal differences, based on a few samples, without detailed morphological investigations.

Consequently, the taxonomy of these taxa remained unsettled. For *M. maximowiczii*, several authors reported geographic polymorphisms in number and morphology of the chromosomes (Kovalskaya 1977; Kovalskaya et al. 1980; Vorontsov et al. 1988; Meyer et al. 1996; Korobitsyna et al. 2005; Kartavtseva et al. 2008). Stimulated by our previous genetic analyses (Sheremetyeva et al. 2008; Haring et al. 2005) indicating a distinct position of *M. m. gromovi*, the three subspecies *M. m. maximowiczii* Schrenck, 1858, *M. m. ungurensis* Kastchenko, 1913, and *M. m. gromovi* Vorontsov, Boeskorov, Lyapunova and Revin, 1988 were recently analyzed with morphological and karyological methods (Sheremetyeva et al. 2009). It was shown that the individuals of *M. m. gromovi* are differentiated morphologically as well as in chromosome characters. Thus, it was suggested that *M. m. gromovi* should be raised to species status (Sheremetyeva et al. 2009). Allozyme data (Frisman et al. 2009) supported *M. gromovi* as a distinct species. Another confirmation came from a recent study of the subgenus *Alexandromys* based on mitochondrial (mt) *cytochrome b* (*cyt b*)sequences (Bannikova et al. 2010) showing the distinct position of *M. gromovi*. However, that investigation was based on a rather small sample size. In the present study we extended our previous study and analyzed the non-coding mitochondrial control region (CR) using a large sample to elucidate the intra- and interspecific relationships of the taxa comprising the *M. fortis* group. Besides the position of *M. gromovi*, there are several other open questions, e.g., whether the other subspecies, *M. m. ungurensis*, might also represent a cryptic species, or more generally, to which extent it is differentiated from the nominate subspecies. Moreover, two representatives of the *M. fortis* group, the species *M. mujanensis* and *M. evoronensis*, have not been included so far in any molecular analysis. Another question concerns the species *Microtus hyperboreus* Vinogradov, 1933, for which the systematic and taxonomic assignment is unsettled. It might be a synonym of *Microtus middendorfii* (Poljakov, 1881) (=*Arvicola middendorfii* Poljakov, 1881). As the study of Bannikova et al. (2010) revealed a closer relationship of *M. middendorfii* with *M. gromovi*, we included *M. hyperboreus* into our data set, although we obtained only one sample of this species.

To interpret the genetic diversity of the East Palearctic *M. fortis* group with respect to the question of species delimitation, a comparison with intraspecific variation in a related species with a presumably similar evolutionary rate is advisable. Therefore, we analyzed the same genetic marker sequence in the root vole *Microtus oeconomus* Pallas, 1778, a representative of the subgenus *Pallasiinus*, the sister group of the subgenus *Alexandromys*.

The following questions were addressed: (1) What is the degree of genetic differentiation between the representatives of the *M. fortis* group? (2) In particular, to which extent is *M. gromovi* differentiated from its former conspecific *M. maximowiczii*? (3) Are the subspecies of *M. maximowiczii* genetically differentiated? (4) Is there any geographic pattern in the haplotype distribution of the various taxa? (5) How closely is *M. hyperboreus* related to the taxa of the *M. fortis* species group? (6) In general, what are the phylogenetic relationships among these taxa? (7) How can we interpret the genetic variation found within the taxa of the *M. fortis* group, comparing it with the intraspecific variation of *M. oeconomus*?

Material and methods

Samples

Specimens investigated in this study, their geographic origins and types of tissue are listed in Table S1 (Supplementary data). The types of material used were: tissue from study skins (museum collection), liver, muscle or blood preserved in 70% ethanol. Altogether samples of 152 individuals representing eight species and 22 subspecies were analyzed (58 samples representing *M. oeconomus*; 94 samples representing the *M. fortis* group) (Table S1).

DNA from liver, blood and muscle tissue was extracted using the DNAeasy blood and tissue kit (QIAGEN) according to the manufacturer's instructions. DNA extractions from museum material were performed in a 10% Chelex (BioRad) solution containing proteinase K (0.5 mg/ml). After incubation (4 h, 55 °C, with agitation) solutions were heated to 95 °C for 5 min and centrifuged for 1 min. The supernatant was purified using the QIA Quick PCR Purification Kit (QIAGEN) with a final volume of 30–100 μl elution buffer (depending on the expected quantity and quality of DNA). For PCR reactions with DNA from fresh tissue 50–200 ng were used as template DNA. Optimal amounts of template DNA of Chelex extractions were determined empirically (2–10 μl of the DNA solution). If necessary, reamplifications were performed with 1–2 μl template DNA. Negative controls for PCR reactions were performed to screen for contaminated reagents: (i) control extractions (without DNA) instead of template; (ii) reaction with A.d. instead of template. PCR primers for the *tRNA Pro* and *tRNA Phe* genes flanking the mt CR were used to amplify a fragment of approximately 1 kb comprising the complete CR flanked by partial sequences of *tRNA Pro* and *tRNA Phe* genes (Haring et al. 2000): Pro+ (5′-ACCATCAGCACCCAAAGCTG-3′), Phe− (5′-AAGCATTTTCAGTGCTTTGCTT-3′). From samples with low DNA quality smaller fragments were amplified with two other primers in combination with Pro+: mico3− (5′-GTAAAAGAAGCATTAATTAAA-3′) giving rise to a ~420 bp fragment, and mico4− (5′-AGGTAAGAACCAGATGCCT-3′) amplifying a fragment of ~220 bp.

PCR was performed on a Master gradient thermocycler (Eppendorf) in 25 μl with 0.5 units Dynazyme DNA polymerase (Finnzyme OY), 1 μM of each primer and 0.2 mM of each dNTP (Boehringer Mannheim); annealing temperature: 58 °C; 35 reaction cycles. Control reactions of both DNA extraction and PCR amplification were performed. PCR products were extracted from agarose gels using the Qiaquick Gel Extraction Kit (QIAGEN) and either purified and sequenced directly or cloned (TOPO TA Cloning Kit, Invitrogen) prior to sequencing. Sequencing of both strands was performed with universal M13 primers (for cloned PCR products) or with PCR primers (for direct sequencing) by MWG-Biotech (Ebersberg, Germany) and AGOWA (Berlin, Germany).

Phylogenetic analysis

Editing and alignment of sequences were performed using the BioEdit software package version 5.0.9 (Hall 1999). Three different alignments were produced: (1) The alignment of complete CR

sequences (long fragment = Lf) has a length of 981 bp and includes 129 sequences (plus one outgroup sequence). (2) Of 20 additional individuals only the medium length fragment (Mf) was obtained. The resulting alignment of Mf sequences has a length of 384 bp. (3) From three additional samples only the short fragment (Sf) could be amplified, and the resulting alignment of Sf sequences has a length of 180 bp.

Maximum parsimony (MP), and Neighbour-joining (NJ; Saitou and Nei 1987) dendrograms were calculated with the software package PAUP (version 4.0b10; Swofford 2002). MP analyses were based on heuristic searches with the TBR (tree bisection reconnection) branch swapping algorithm and a random taxon addition sequence (1000 replicates) and delayed character transformation (DELTRAN). Gaps were treated as fifth character state. Bootstrap analyses were performed with 1000 replicates for MP (10 random addition replicates) and NJ trees. For NJ trees p distances were used. Bayesian (BI) analyses were performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The model (HKY + I + G) was selected according to the hierarchical Likelihoods Ratio Tests (hLRT) in Modeltest (version 3.7; Posada and Crandall 1998). We applied this model for the trees presented, as it is simpler than that proposed by the Akaike Information Criterion (AIC). However, using various other models did not change the general topology of the tree (data not shown). Two independent runs, starting with random trees, were performed for 3 million generations, each with four Markov chains, and a sampling frequency of every 100th generation. Model parameter values were treated as unknown and estimated separately in each run. The burn-in phase was determined by the time of convergence of likelihood scores. The consensus tree was constructed based on the trees sampled after the burn-in phase from both independent runs.

The number of haplotypes (n_h), number of polymorphic sites (n_p), nucleotide diversity (π), and haplotype diversity (h) were calculated with ARLEQUIN 3.01 (Excoffier et al. 2005). The software PHYLTEST (Kumar 1996) was used to calculate average pairwise p-distances between groups. In PHYLTEST, alignment gap (and missing data) sites are ignored from the distance computation in the Pairwise-Deletion fashion (Kumar et al. 2004). In this case, the evolutionary distance for each pair of sequences is computed by ignoring only those gaps that are involved in the comparison. As an outgroup we aimed to select a representative of the genus *Microtus* which is definitely outside the taxa under investigation. Our ingroup taxa (*M. oeconomus* and the *M. fortis* group) are - according to Jaarola et al. (2004) - combined in a clade comprising the subgenera *Pallasiimus*, *Alexandromys* and *Vloemys*. All other clades of *Microtus* are more or less equally distant from this clade. We chose the previously published sequence of *Microtus subterraneus* (subgenus *Terricola*) as outgroup (AF267271; Haring et al. 2000). The same strategy was used for the partial trees (presenting the relationships of *M. oeconomus* and the *M. fortis* group, respectively): We used in each case a representative of the other group (being rather closely related but clearly outside of the ingroup). The sequences determined in the course of the present study are registered under the GenBank accession numbers listed in Table S1.

Results

Data set of complete sequences

A tree based on a Bayesian analysis of the Lf-sequences is shown in Fig. 1. It has the same main topology as the trees calculated with other tree building algorithms (NJ, MP). This tree gives an overview of the principal divisions and relationships between species, while

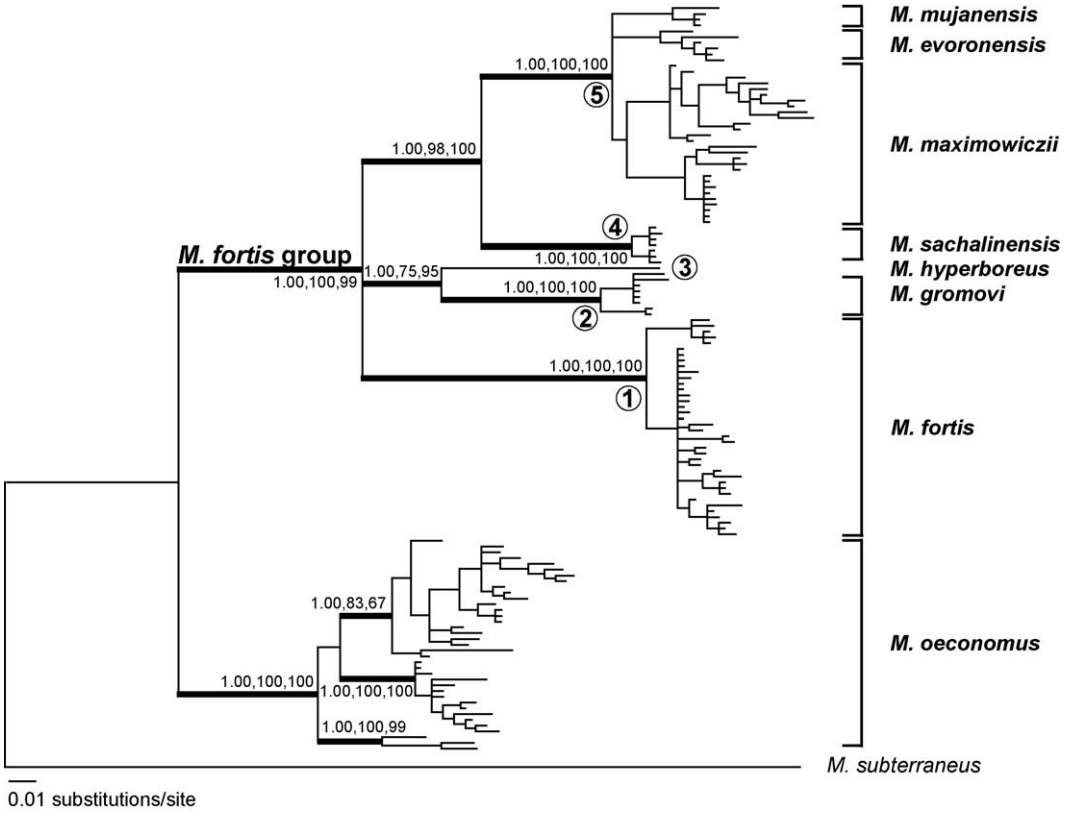

Fig. 1. Bayesian tree based on CR sequences illustrating the major clades of the *M. fortis* group and *Microtus oeconomus*. Support values (BI, NJ, MP) of major nodes are indicated. Branches leading to major clades are depicted with thick lines. Major clades/lineages 1–5 of the *M. fortis* group are indicated on the right. Outgroup: *Microtus subterraneus*.

Table 1
Average pairwise distances and their standard errors between clades of the *M. fortis* group (*p*-distances, in %, calculated from all sequences of the Lf-data set).

	Mmaxmax	Mmaxung	Mmuj	Mevo	Msac	Mgro	Mhyp	Mfor
Mmaxung	2.7 (0.4)							
Mmuj	2.9 (0.5)	2.9 (0.5)						
Mevo	2.7 (0.4)	3.0 (0.5)	2.8 (0.5)					
Msac	5.4 (0.7)	5.4 (0.7)	5.7 (0.7)	5.5 (0.7)				
Mgro	7.4 (0.8)	7.6 (0.8)	7.8 (0.8)	7.4 (0.8)	7.2 (0.8)			
Mhyp	8.2 (0.9)	8.4 (0.9)	9.1 (0.9)	8.4 (0.9)	8.2 (0.9)	6.4 (0.8)		
Mfor	8.8 (0.9)	8.7 (0.9)	9.2 (0.9)	9.1 (0.9)	8.4 (0.9)	8.5 (0.9)	8.3 (0.9)	
Moec	10.9 (0.9)	10.4 (0.9)	10.6 (0.9)	10.4 (0.9)	9.9 (0.9)	9.0 (0.9)	9.8 (0.9)	9.7 (0.9)

Average distances between clades of the *M. fortis* group and *M. oeconomus* are depicted in the last line.
Mfor = *M. fortis*, Mmaxmax = *M. m. maximowiczii*, Mmaxung = *M. m. ungurensis*, Mgro = *M. gromovi*, Mhyp = *M. hyperboreus*, Msac = *M. sachalinensis*, Mmuj = *M. mujanensis*, Mevo = *M. evoronensis*, Moec = *M. oeconomus*.

Table 2
Average pairwise distances and their standard errors between clades of *M. oeconomus* (*p*-distances, in %, calculated from all sequences of the Lf-data set).

	C Europe	N Europe	Siberia	N + C Europe
N Europe	2.1 (0.4)			
Siberia	4.1 (0.6)	3.5 (0.5)		3.9 (0.5)
Beringia	4.1 (0.6)	3.4 (0.6)	3.4 (0.5)	3.9 (0.6)

The European clades are treated separately as well as independently (N + C Europe).

detailed trees (see below) will illustrate the distribution of individual sequences among branches. The main tree is divided into two major clades, one comprising the *M. fortis* group (*M. fortis*, *M. maximowiczii*, *M. sachalinensis*, *M. evoronensis*, *M. gromovi* and *M. mujanensis*) together with *M. hyperboreus*, and the other one *M. oeconomus*. Both clades are supported by high support values (bootstrap values and Bayesian posterior probabilities).

Within the *M. fortis* group there are five main branches that are clearly differentiated with this genetic marker. This part of the tree is in accordance with current taxonomy in some aspects, as several clearly differentiated species form monophyletic groups: *M. fortis* (clade 1), *M. gromovi* (clade 2), and *M. sachalinensis* (clade 4) are highly supported clades. The distinct position of *M. gromovi* with respect to *M. maximowiczii* confirms its proposed species status (Sheremetyeva et al. 2009). *M. maximowiczii* is clearly differentiated, but its relationship to *M. evoronensis* and *M. mujanensis*, which are found within the same clade (clade 5), is unrelsoved. *M. hyperboreus* (one single sequence, lineage 3 in Fig. 1) appears as the sister group of *M. gromovi*. The grouping of clades 5 and 4 (*M. maximowiczii*, *M. evoronensis*, *M. mujanensis* plus *M. sachalinensis*) as sister clades is highly supported.

The second major clade representing *M. oeconomus* is divided into three clades, each of them highly supported. However, the relationships among them are not resolved.

Sequence diversity

Comparisons of average *p*-distances and haplotype diversities between and within clades of the *M. fortis* group and *M. oeconomus* are given in Tables 1–3. As clade 5 in Fig. 1 comprises two subspecies of *M. maximowiczii* and the two species *M. everonensis* and *M. mujavensis* we present average distances within the whole clade as well as for the subclades separately. Similarly, for the European samples of *M. oeconomus* distance values were calculated for the whole group as well as for the Central European samples separately. However, it has to be mentioned that some of the clades contain only a few sequences and thus the values have to be compared with caution.

Distances within clades (Table 3) range between 0.7% and 1.9% with the highest values in *M. m. maximowiczii* as well as between the Siberian and the European clades of *M. oeconomus*. Haplotype diversity is high in all clades (close to 1.0), irrespective of the number of sequences and the number of variable sites.

Partial trees and geographic distribution of samples

Trees based on partial sequences (Mf sections) were calculated with Bayesian, NJ and MP analyses. We present NJ trees as they reflect distances between sequences directly. In those cases where

Table 3
Genetic diversity within clades of the *M. fortis* group and of *M. oeconomus*.

	D (r)	π	h	$n/l/n_h/n_p$
M. fortis group				
Clade 1—*M. fortis*	0.7 (0–2.1)	0.0089 (0.0047)	1.0 (0.0075)	33/959/33/61
Clade 2—*M. gromovi*	0.3 (0–1.6)	0.0090 (0.0053)	1.0 (0.0625)	8/957/8/23
Clade 3—*M. sachalinensis*	0.4 (0–0.6)	0.0075 (0.0045)	1.0 (0.0764)	7/961/7/15
Clade 4	2.3 (0–3.9)	0.0276 (0.0137)	1.0 (0.0058)	39/963/39/121
Subclades of clade 4				
M. m. maximowiczii	1.9 (0.2–2.2)	0.0160 (0.0085)	1.0 (0.0270)	14/961/14/57
M. m. ungurensis	1.0 (0–1.6)	0.0098 (0.0054)	1.0 (0.0270)	14/960/14/38
M. mujanensis	0.8 (0–1.2)	0.0084 (0.0057)	1.0 (0.1768)	4/958/4/15
M. evoronensis	0.7 (0–1.7)	0.0110 (0.0064)	1.0 (0.0962)	6/959/6/27
M. oeconomus				
C Europe	1.1 (1.1)	–	–	–
N Europe	–	–	–	–
C Asia	1.4 (0–3.0)	0.0165 (0.0085)	1.0 (0.0147)	21/954/21/69
Beringia	0.7 (0–1.7)	0.0122 (0.0066)	1.0 (0.0302)	13/981/13/58
N + C Europe	1.8 (1.1–2.1)	–	–	–

D = Average pairwise *p*-distances (*p*-distances, in %, calculated from all sequences of the Lf-data set) within clades, r = range of distances; π = nucleotide diversity (standard deviation, SD); h = haplotype diversity (SD); n = number of sequences; l = length of sequences; n_h = number of haplotypes; n_p = number of polymorphic sites. The European clades are treated separately as well as independently (N + C Europe). Nucleotide and haplotype diversities were not calculated for clades with less than four sequences.

the algorithms resulted in topological changes (concerning clades) they are mentioned in the text. Support values of all analyses are included in the figures.

Fig. 2 shows the relationships within the *M. fortis* group. The geographic origins of the samples and distribution of subspecies are depicted in Figs. S1 and S2 in Supplementary data. In general, the tree reflects current taxonomy. The only discrepancy with respect to subspecies assignment is found in *M. maximowiczii*. One of the clades contains solely representatives of *M. m. ungurensis*, whereas the other clade comprises all individuals of *M. m. maximowiczii* but also four individuals, which would be ascribed – according to their geographic origin (Chitinskaya Oblast) – to *M. m. ungurensis*. For the two specimens, from which only Mf fragments were obtained, the assignment is unambiguous: Mevo-1 is identical to three other specimens of *M. evoronensis* (Mevo-7, Mevo-8, Mevo-9), and a *M. m. ungurensis* sample from Buryatia (Mmaxung-1) clusters with Mmaxung-21 from Chita Region.

The distribution ranges and the origin of samples of *M. fortis* are shown in Fig. S2. Within the *M. fortis* clade two groups become apparent (Fig. 2). The larger group comprises individuals from all regions covering the northern part of its distribution range including various islands in the Sea of Japan, while some individuals from Amur Region and Matveyev Island cluster in the second group. Thus, no clear clustering according to geography is observed and also no substructuring according to subspecific division (*M. f. pelliceus*, *M. f. michnoi*). Several sequences from distant regions

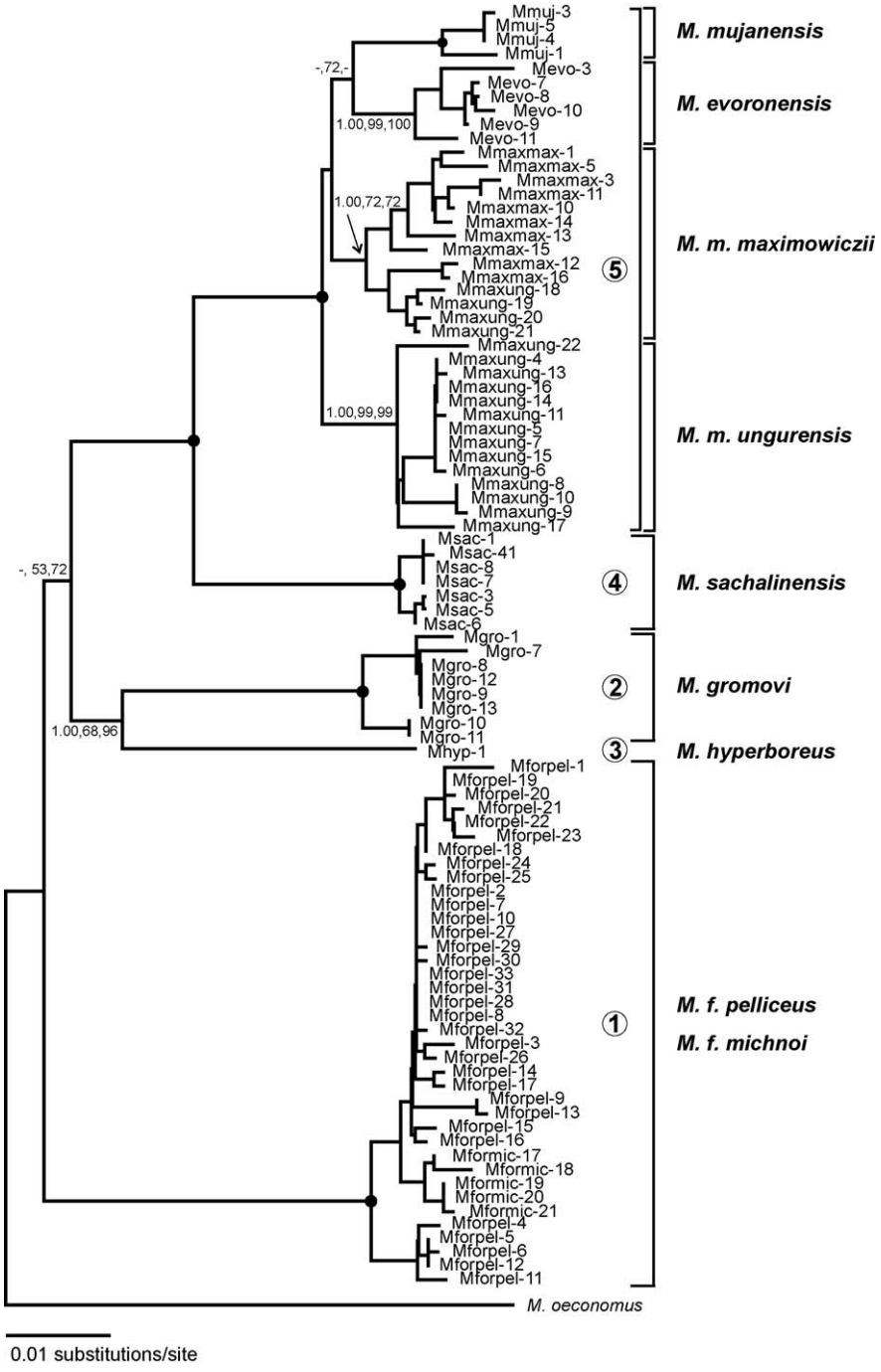

Fig. 2. Phylogenetic relationships of the *M. fortis* group (NJ tree). Major clades/lineages 1–5 and support values (BI, NJ, MP) of major nodes are indicated. Black circles indicate nodes with maximal support in all analyses. Outgroup: *Microtus oeconomus* (Moecrat-8).

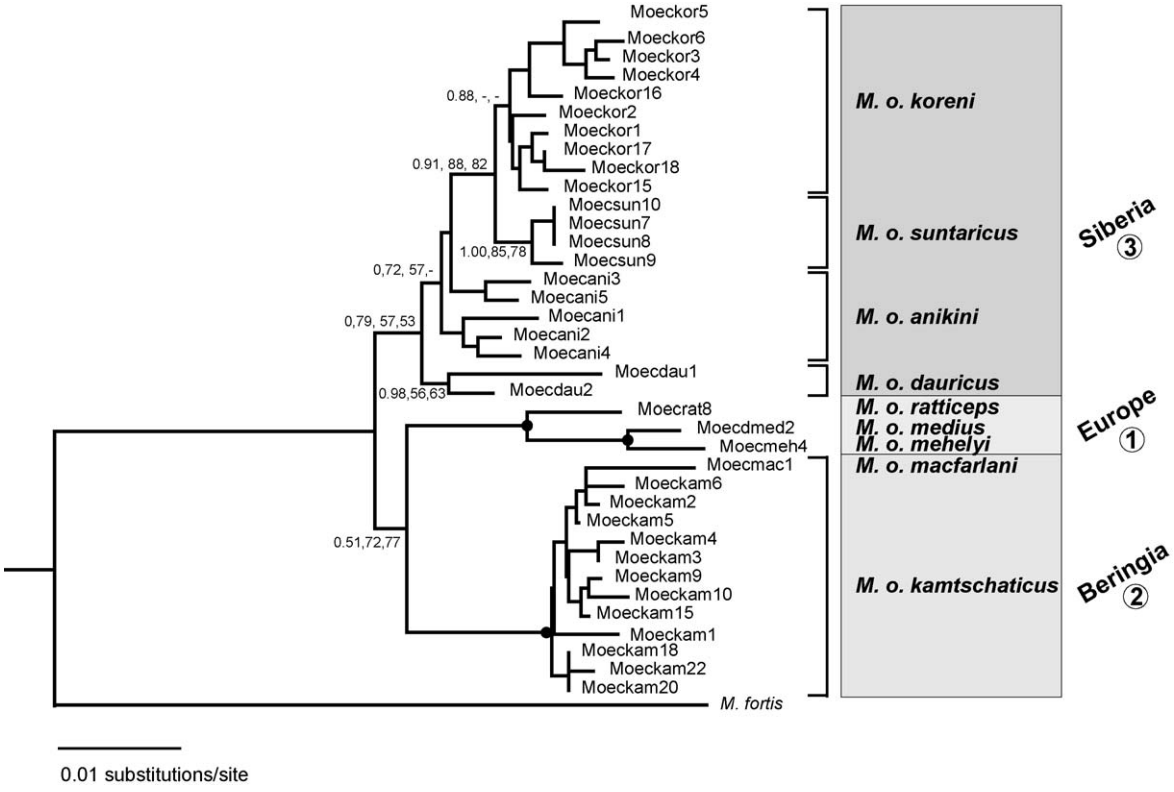

Fig. 3. NJ tree of *Microtus oeconomus*. Major clades 1–3 (Europe, Siberia, Beringia) are highlighted with grey shaded boxes. Support values (BI, NJ, MP) of major nodes are indicated. Black circles indicate nodes with maximal support in all analyses. Outgroup: *Microtus fortis* (Mforpel-1).

are identical or almost identical, while on the other hand, some sequences from the same locality are rather distinct (e.g., Mforpel-14 and Mforpel-18 from Blagoveshensk). Samples from Matveyev Island (Mforpel-5, Mforpel-6, Mforpel-12) cluster together in a separated clade, but in this clade there are also two sequences that represent other distinct localities.

The relationships within *M. oeconomus* based on the long fragments are depicted in Fig. 3 and the geographic origins of the samples are shown in Fig. S3. Distances within and between the three groups are listed in Tables 2 and 3 and haplotype diversities in Table 3. The three clades comprise different groups of subspecies: (1) the European clade with *M. o. mehelyi*, *M. o. ratticeps*, *M. o. medius*, (2) the Beringian clade with *M. o. kamtschaticus* and *M. o. macfarlani*, and (3) the Siberian clade with *M. o. koreni*, *M. o. anikini*, *M. o. suntaricus*, *M. o. dauricus*. In this tree the Siberian clade splits off from the basal node, which is not consistent with the Bayesian tree in Fig. 1. But the grouping of the remaining two clades, the Beringian clade and the European clade is only poorly supported, and thus the relationships of the three main clades remain unresolved. In the Mf-tree (not shown) several additional samples of *M. o. koreni*, *M. o. kamtschaticus*, *M. o. ratticeps*, *M. o. suntaricus*, and *M. o. medius* as well as three additional subspecies (*M. o. kjusjurensis*, *M. o. hahlovi*, *M. o. altaicus*) are included. They cluster as expected in the Siberian clade, e.g., *M. o. hahlovi* with *M. o. kjusjurensis* and *M. o. altaicus* with *M. o. anikini*, but in general the relationships are not clearly resolved. While in the Lf-tree (Fig. 3) at least the subspecies of the Siberian clade *M. o. koreni*, *M. o. suntaricus* and *M. o. dauricus* represent monophyletic groups, this is not the case in the Mf-tree. The only sequence obtained from an individual of the nominate subspecies *M. o. oeconomus* is an Sf-sequence (Moecoec-2 from Kazakhstan). It falls within the variation of the Siberian clade (3), but is not closely related to any of these sequences. This exemplifies that the phylogenetic information of the shorter sequences is rather limited. The European subspecies, however, form monophyletic groups in all trees, even in that from Sf-sequences. Concerning the short sequences (tree not shown), the assignment of two more individuals of *M. o. mehelyi* (Moecmhe-2, Moecmeh-3) is clear: they are identical with Moecmeh-4 (Lf), which is not surprising since they come from adjacent localities in Burgenland/Austria near Lake Neusiedl. Furthermore, the sequence of Moecmeh-4 is very similar (0.75% *p*-distance) to a sequence published in GenBank (accession number AJ616853), which is of Hungarian origin and thus belongs to *M. o. mehelyi* too.

Discussion

We established a molecular phylogeny of the taxa comprising the *M. fortis* group of the genus *Microtus* based on the sequence of the mt CR. For comparison, samples of *M. oeconomus* covering the species distribution range were analyzed with the same marker sequence. Shorter sequences isolated from additional specimens were used to assign those individuals to the various clades obtained with the complete CR sequence.

Genetic divergences and taxonomic considerations

The trees based on the complete CR sequences revealed five highly supported lineages within the *M. fortis* group, which are clearly separated by average distances ranging from 6.2% to 9.2% (i.e., *M. mujanensis* + *M. evoronensis* + *M. maxomowiczii*, *M. sachalinensis*, *M. hyperboreus*, *M. gromovi*, and *M. fortis*). Although the relationships among these lineages are not completely resolved, as one node is only poorly supported, several questions could be clarified: We could show that *M. hyperboreus* belongs to the *M. fortis* group, being the sister group of *M. gromovi*. Moreover, *M. maximowiczii*, is more closely related to *M. sachalinensis* than to *M. gromovi*, its former conspecific. The average genetic distances between *M. gromovi* and the two clades of *M. maximowiczii* (7.4% and 7.6%)

are higher than between *M. maximowiczii* and, e.g., *M. sachalinensis* (5.4%), and almost as high as between *M. maximowiczii* and *M. fortis* (8.8%). The high genetic distance and the fact that *M. gromovi* and *M. maximowiczii* are not sister groups confirm our earlier genetic results (Sheremetyeva et al. 2008; Haring et al. 2005) as well as the proposed species status of *M. gromovi* based on recent chromosome and cranial-morphological investigations (Sheremetyeva et al. 2009). The results are in accordance with those obtained recently by Bannikova et al. (2010), who investigated the mt *cyt b* gene in the subgenus *Alexandromys*, including also one complete *cyt b* sequence of *M. gromovi*. Unfortunately, that study and ours are not congruent with respect to marker sequence and taxon sampling. Bannikova et al. (2010) did not include *M. mujanensis*, *M. evoronensis*, and *M. hyperboreus*, but analyzed three further species that were not included in our study (*M. middendorfii*, *M. mongolicus*, and *M. limnophilus*). Moreover, only a few sequences of *M. maximowiczii* are included in the study of Bannikova et al. (2010). However, both analyses show that concerning the definition of species groups, most proposals published so far do not correspond to the genetic results.

Are the haplogroups in accordance with current subspecific division? The subspecies of *M. maximowiczii* (*maximowiczii* and *ungurensis*) are not reciprocally monophyletic. Four *M. m. ungurensis* individuals from Chita Region cluster with *M. m. maximowiczii*. This might be explained by wrong assumptions regarding the distribution ranges of the two subspecies. A problem is that the distribution ranges of any subspecies of *M. maximowiczii*, as in East Palearctic species of the genus *Microtus* in general, are not defined clearly. Therefore, subspecific assignment is usually not straightforward and often accomplished only tentatively based on the geographic information. Postulating that the two mt clades represent *M. m. maximowiczii* and *M. m. ungurensis* respectively, the distribution range of *M. m. maximowiczii* (as deduced from the sample localities) would extend much further to the west than presumed, and the four individuals from the northern Chita Region would represent in fact *M. m. maximowiczii*. Certainly, future population analyses of chromosomal variation in combination with data of mt haplotypes should be the basis for subspecies delineations (see below).

Both clades of *M. maximowiczii* are combined in one clade which also includes *M. evoronensis* and *M. mujanensis*. The latter are monophyletic groups. These four taxa are more or less equally distant from each other (2.9–3.0%). For comparison, these distances fall within the intraspecific variation of the related species *M. oeconomus* being even lower than the values differentiating the three main haplogroups of *M. oeconomus* (3.4–4.1%). Thus, genetic distances provide no arguments that would corroborate species status of *M. evoronensis* or *M. mujanensis*. Recent allozyme analyses (Frisman et al. 2009) also indicated that the interspecific allozyme differentiation of the chromosomally polymorphic species *M. maximowiczii*, *M. evoronensis*, and *M. mujanensis* does not exceed the intraspecific differences found in *M. oeconomus*, *M. fortis*, and *M. maximowiczii*. However, chromosome analyses (Meyer et al. 1996) show that *M. evoronensis* and *M. mujavensis* are characterized by peculiar chromosome morphology and banding patterns compared to the variants reported for *M. maximowiczii*.

For *M. maximowiczii* considerable polymorphism of chromosome number and morphology has been reported (Kovalskaya 1977; Kovalskaya et al. 1980; Golenishchev and Radjabli 1981; Meyer et al. 1996; Kartavtseva et al. 2008), with chromosome numbers ranging from $2n = 36–44$ (NF = 52–62). Moreover, various rearrangements of chromosomes (Robertsonian fusions/fissions, inversions as well as tandem fusions of middle sized metacentrics with formation of large metacentrics) have been observed in *M. maximowiczii* and there is a geographic pattern in the occurrence and frequencies of these chromosomal variants (Kovalskaya et al. 1980; Kartavtseva et al. 2008). According to those investigations, at least four groups of chromosomal variants are found in *M. maximowiczii*, one in *M. m. maximowiczii* and three in *M. m. ungurensis*. As mentioned above, it would be important to perform comprehensive investigations over the whole distribution ranges to reveal whether specific chromosomal forms correlate with mt haplogroups.

In *M. fortis* there is no clear geographic pattern in the distribution of haplotypes. Compared to *M. maximowiczii*, intraspecific genetic variation is lower (average 0.7%) and the two subspecies investigated (*pelliceus* and *michnoi*) are not clearly differentiated. The structure of the *M. fortis* clade is rather shallow with a polytomy of several small subclades. One of these subclades is composed by the five individuals of *M. f. michnoi* (Mformic-17, Mformic-18, Mformic-19, Mformic-20, Mformic-21). Subspecies in *M. fortis* were described based on morphology, and later Kovalskaya et al. (1991) discovered differences among subspecies in number and chromosomal locations of heterochromatine blocks. The morphological differentiation among subspecies of *M. fortis* might be caused by drift or local adaptation which sometimes occurs rather fast and therefore need not necessarily be reflected in mt patterns. Unfortunately, the picture remains incomplete, as three subspecies of *M. fortis* could not be analyzed so far, because they occur in China and North Korea only and were not available for our analysis.

In *M. oeconomus* the three main clades correspond partly to the four groups distinguished by Brunhoff et al. (2003) based on *cyt b* sequences. Our clades 2 and 3 represent the Central Asian and Beringian groups, while clade 1 combines the North and Central European groups of Brunhoff et al. (2003). In our opinion the term "Central Asian clade" used by Brunhoff et al. (2003) is not very accurate, because this clade has a Siberian distribution extending to the Russian Far East. We designated this clade as Siberian clade. In the present study we covered several additional areas of the distribution range of *M. oeconomus*, thus complementing the picture of the phylogeographic structure of this species. We did not concentrate on European samples, which were studied in detail by Brunhoff et al. (2003), but instead included more Asian ones.

The mt data may provide additional information for a taxonomic revision of *M. oeconomus*. The subspecies of *M. oeconomus* have been described based on several morphological characters, e.g., coloration, body and tail length, ear size (Ognev 1950; Vinogradov and Gromov 1952; Bobrinsky et al. 1965; Vorontsov et al. 1986; Pavlinov and Rossolimo 1987; Kostenko and Allenova 1989; Kostenko 2000; Abramson and Tikhonova 2005). However, some researchers regard several subspecies as invalid considering their morphological differentiation as insufficient (e.g., Pyastolova 1971). The chromosome number in *M. oeconomus* is rather uniform ($2n = 30$) (Makino 1950; Matthey 1954; Raush and Raush 1968; Kral 1972; Kozlovskii and Khvorostyanskaya 1978; Belyanin et al. 1986; Vorontsov et al. 1986; Frisman et al. 2003). The only exceptions are voles from two isolated regions in Central Sweden (*M. o. ratticeps*), with $2n = 30–32$ (Fredga and Bergstrom 1970; Fredga et al. 1980). Despite the scarceness of morphological data corroborating the subspecific division, the genetic differentiation provides clear hints. In our tree the European subspecies comprise distinct mt clades and the same is true for some of the Siberian subspecies (e.g., *M. o. suntaricus*, *M. o. koreni*, *M. o. dauricus*), but not for others (*M. o. anikini*, *M. o. macfarlani*). These genetic indications should stimulate comprehensive morphometric analyses covering the whole distribution ranges to further test the differentiation of the various subspecies.

Molecular dating of splits

It would be interesting to date the splits in the molecular phylogeny resulting from our data. However, such efforts are hampered by the problem of determining the divergence rate. Despite the fact

that 2% per million years is repeatedly reported and cited as "the standard mt rate", such a standard rate does not seem to exist, neither for vertebrates in general nor for mammals. There is a wide variation of mutation rates in mammals and, although for some rodents an elevated rate has been reported, this is still a matter of debate (e.g., Martin and Palumbi 1993; Kumar and Subramanian 2002; Kumar 2005; Triant and Dewoody 2006; Bininda-Emonds 2007). For the genus *Microtus*, Brunhoff et al. (2003) estimated divergence times on the assumption that the evolutionary rate of mt DNA (as that of rodents in general) might be three to five times higher than the "standard" mammalian rate. They arrived at estimates ranging from 6% to 10% per million years for their *cyt b* sequences resulting in estimated divergence times for the various groups between 0.29 and 0.49 Mya. Bannikova et al. (2010), using a single calibration point (radiation of the basal lineages of *Microtus* at 2.2 Mya), estimated the divergence rates of "more than 30% for most recent splits down to 12–14% per Myr for basal-most nodes". Unfortunately, there are not enough reasonable calibration points and therefore, at the moment any assumption for a substitution rate appears arbitrary and we did not attempt to establish such datings for our marker sequence.

Phylogeographic considerations

The occurrence of distinct mt lineages is often explained by glacial climatic changes and differentiation within isolated refugia. In fact, concerning *M. oeconomus* this appears plausible to a certain extent. The phylogeography of *M. oeconomus* has been discussed in detail previously (Brunhoff et al. 2003) and our results are generally in accordance with those analyses which were based on a different mt marker (*cyt b*). The unresolved trichotomy of the three main mt lineages Europe, Siberia, and Beringia in our tree suggests that they arose by a more or less simultaneous radiation. The division of the European lineage into a northern (*M. o. ratticeps*) and a central group (*M. o. mehelyi*, *M. o. medius*) observed by Brunhoff et al. (2003) is in accordance with our results, but the differentiation is not so pronounced in our CR data set. With respect to the three main groups the Ural mountains and the Verkhoyansk Range could have acted as barriers, especially in glacial periods. Galbreath and Cook (2004), who investigated the phylogenetic structure of the Beringian clade of *M. oeconomus* using the mt *cyt b* gene and a short section of the CR as well as the nuclear ALDH1 intron, found evidence for population isolation and differentiation caused by glacial advances.

Considering the species and interspecies diversity in the *M. fortis* group one might ask whether the differentiation of these lineages is also a consequence of isolation in glacial refugia in East Siberia. In contrast to *M. oeconomus*, where two distinct prominent mountain ranges may have acted as geographic barriers, the distribution of the *M. fortis* group, ranging from Baikal region to Sikhote Alin in the Russian Far East, may have imposed completely different constraints. This region comprises highly structured areas with many high mountain massifs crossed by various river systems. The early separation of the lineage leading to *M. gromovi* and *M. hyperboreus* as implied by our trees can be explained by their geographic distribution in the northern parts of the Russian Far East, which is bounded by high mountain ranges in the southeast (Stanowoj Mountains) resulting in long term isolation of these taxa. For *M. sachalinensis*, isolation and subsequent divergence on Sakhalin Island appears likely. However, the Tatarsky Strait, which separates Sakhalin from the Siberian mainland, is only 7 km wide and 5 m deep at its narrowest part. Therefore, several times throughout the Pleistocene, when sea levels were considerably lower than today, the Mayima land bridge emerged repeatedly connecting Sakhalin Island to the mainland (Dobson 1994; Millien-Parra and Jaeger 1999; Bogatov et al. 2006). It seems possible that during those periods *M. sachalinensis* was able to extend its range to the mainland but so far no traces of such colonization have been found. Maybe this was impeded because the appropriate habitats at the mainland were occupied by related species. The first split in the radiation of the *M. fortis* group is that of *M. fortis*. It probably dates back to the Pliocene. From all the taxa it has the most southern/southeastern distribution. Thus, one could assume that the first split separated a southern ancestral *M. fortis* lineage from a more northerly distributed ancestor of the remaining taxa.

Concerning *M. hyperboreus* we could show its affiliation with the *M. fortis* group. Unfortunately we could not include samples of *M. middendorfii* in our study, thus, there are still many open questions concerning its relationships with *M. hyperboreus*. *M. middendorfii* is distributed from Yamal Peninsula in the west to low Kolyma River in the east. *M. hyperboreus* occurs from low Yenisey River in the west (a possible contact zone with *M. middendorfii*) to upper Indigirka and Kolyma Rivers in the east. Thus, the range of *M. hyperboreus* is further in the south compared to that of *M. middendorfii*, whose distribution range in East Siberia is restricted to the tundra and forest-tundra zones. In Northeast Yakutia, the ranges of both forms are separated by the zone of coniferous taiga of the Kolymo-Indigirka lowland (Meyer et al. 1996). To which extent *M. middendorfii* and *M. hyperboreus* are genetically differentiated remains to be investigated. As the presumed distribution ranges of both species are divided by the Verkhoyansk Range, which in the case of *M. oeconomus* is supposed to have acted as a barrier between the Siberian and Beringian group, it would be interesting to investigate if this mountain range similarily caused a genetic differentiation between *M. middendorfii* and *M. hyperboreus*.

Comparisons between M. oeconomus and the M. fortis group

The taxa of the *M. fortis* group as well as *M. oeconomus* occur in similar habitats and have more or less the same ecological requirements. Although our data have to be considered with caution, as for some clades only a few individuals have been sampled so far, from the genetic diversities (average within group distances and their ranges, Table 3) observed in all these taxa, there is no indication for recent genetic bottlenecks during the last glacial maximum. Interestingly, the diversity values within the two clades of *M. maximowiczii* are rather similar or even higher than within the *M. oeconomus* clades, despite the fact that the distribution range of *M. maximowiczii* is considerably smaller compared to the clades of *M. oeconomus*. Moreover, each of the mt main clades of the *M. fortis* group (Fig. 2), of which some are considered as distinct species, occupies a much smaller distribution range than the phylogeographic groups of *M. oeconomus*. Species richness of voles in the Eastern Palearctic implies that southeastern Siberia (and maybe also Mongolia and Eastern China) might have provided ideal conditions for diversification and speciation. This is comparable with the radiation of the subgenus *Terricola* (gen. *Microtus*) in southern Europe (Alpine, Dinaric, and Balkan regions), e.g., the diversification of the *Microtus savii* species group (Chaline 1987) in the Pyrenees and the Apennine Peninsula, the evolution of the *M. savii* complex in Italy (Galleni et al. 1998; Castiglia et al. 2008), or the *Microtus multiplex* complex in the Alpine-Dinaric region (Haring et al. 2000; Tvrtković et al. in press). In these regions the highly structured habitats (mountains, peninsulas) in connection with glacial advances may have been the main factor for species diversification.

Another factor that has to be considered is the tendency to produce chromosomal rearrangements which is obviously characteristic for the taxa of the *M. fortis* group, but not for, e.g., *M. oeconomus*. The genus *Microtus* is one of the most karyotypically variable rodent groups (Maruyama and Imai 1981), although the various species seem to be quite different concerning their disposition to produce rearrangements. Also the tendency of het-

erochromatin accumulation (e.g., in the sex chromosomes) is different among species (Mitsainas et al. 2008). The emergence of chromosomal rearrangements might be due to some kind of chromosomal instability, but their fixation in chromosomal races might be just a consequence of the fragmented population structure characteristic for the *M. fortis* group species, which would allow new variants to increase rapidly in frequency just by genetic drift and/or inbreeding in smaller, isolated subpopulations. The high probability of speciation induced by chromosomal variation (so-called chromosome speciation) within this group was supposed by Frisman et al. (2009). In any case, however, the combination of biogeographic peculiarities in East Siberia in combination with high chromosomal variation might be the reason of the species richness of the *M. fortis* group.

Acknowledgements

This work was supported by the ÖAD (Österreichischer Austauschdienst, Project no. I.9/2001), the Russian Foundation for Basic Research, Projects no. 01-04-02006 and no. 06-04-48969, and the Far East Branch of the Russian Acad. Sci., Project no. 09-I-ОБН-03. We are grateful to I. Kartavtseva, M. Tiunov, V. Kostenko, V. Nesterenko, V. Korablev, M. Pavlenko, K. Korobytsina, L. Frisman (Vladivostok, RU), L. Voyta (Chita, RU), E. Zholnerovskaja (Novosibirsk, RU), G. Bojeskorov (Yakutsk, RU), N. Dokuchaev, M. Zasypkin, F. Chernyavsky (Magadan, RU), E. Lyapunova, N. Bulatova (Moscow, RU), B. Herzig (Vienna, A), M. Jaarola (Bialowieza, Poland), and M. Pavlicev (St. Luis, MO, USA), for providing vole tissue samples. We are greatly indebted to Wilhelm Pinsker for fruitful discussions and for critically reading the manuscript and to Barbara Däubl for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mambio.2010.04.006.

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