Original Investigation

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

Elisabeth Haringa,*, Irina N. Sheremetyeva, Alexey P. Kryukov

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; Martinková 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 Microtus fortis species-group which belongs to the subgenus Alexandromys (Zagorodnyuk 1990). This group was formerly named M. calamarum group (Ellerman 1941) including a set of East-Asian species: M. calamarum Thomas, 1902, Microtus clarkei Hinton, 1923, Microtus ungurensis Kastschenko, 1913, M. fortis Buchner, 1880, and Microtus michnovi Kastsch., 1910. Because M. calamarum 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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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. m. majaensis), Khabarovsky Krai (M. e. evoronensis) and Sakhalin (M. s. s. chalinskensis). 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. s. chalinskensis, Microtus mongolicus Radde, 1861, and M. middendorffi (Poljakov, 1881). The latter one included M. maximowiczii, M. m. majaensis, and M. e. 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. m. majaensis (Orlov and Kovalskaya 1978), and M. e. evoronensis (Kovalskaya and Sokolov 1980) were described recently owing to chromosomal differences, based on a few samples, without detailed morphological investiga-

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. 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 ana-

The supernatant was purified using the QIA Quick PCR Purifica-

Materials 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, muscle and blood tissue was extracted using the DNAeasy blood and tissue kit (Qiagen) according to the manu-

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 contaminating DNA. PCR primers for the rRNA Pro and rRNA 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 M. majaensis 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 middendorffi (Poljakov, 1881) (=Arvicolam middendorffi Poljakov, 1881). As the study of Bannikova et al. (2010) revealed a closer relationship of M. middendorffi 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. for-

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 Likelihood 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 \( (\pi) \), 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 Pallasimius, 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
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. sachalensis*, *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. sachalensis* (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 unresolved. *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. sachalensis*) 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. evoronensis* and *M. mujanensis* 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. 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>
<thead>
<tr>
<th>Table 1</th>
<th>Average pairwise distances and their standard errors between clades of the <em>M. fortis</em> group (p-distances, in %, calculated from all sequences of the Lf-data set).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmaxung</td>
<td>Mmaxung</td>
</tr>
<tr>
<td>2.7 (0.4)</td>
<td>2.9 (0.5)</td>
</tr>
</tbody>
</table>

Average distances between clades of the *M. fortis* group and *M. oeconomus* are depicted in the last line.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Average pairwise distances and their standard errors between clades of <em>M. oeconomus</em> (p-distances, in %, calculated from all sequences of the Lf-data set).</th>
</tr>
</thead>
<tbody>
<tr>
<td>C Europe</td>
<td>N Europe</td>
</tr>
<tr>
<td>N Europe</td>
<td>2.1 (0.4)</td>
</tr>
<tr>
<td>Siberia</td>
<td>4.1 (0.6)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Genetic diversity within clades of the <em>M. fortis</em> group and of <em>M. oeconomus</em>.</th>
</tr>
</thead>
</table>
| D (r) | π | h | n/lh
| 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. sachalensis* | 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. m. maximowiczii* | 0.8 (0–1.2) | 0.0084 (0.0057) | 1.0 (0.1768) | 4/958/4/15 |
| *M. hyperboreus* | 0.7 (0–1.7) | 0.0110 (0.0064) | 1.0 (0.0962) | 6/957/6/27 |
| *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; nH = number of haplotypes; np = 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...
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 Blagoveshesnk). 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. 3. 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. raticeps*, *M. o. medius*, (2) the Beringian clade with *M. o. koreni*, *M. o. suntaricus*, and (3) the Siberian clade with *M. o. altaicus*, *M. o. sutchiensis*. 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. kamschaticus*, *M. o. raticeps*, *M. o. suntaricus*, and *M. o. medius* (as well as three additional subspecies (*M. o. kiusjuresis*, *M. o. hahlovi*, *M. o. altaicus*) are included. They cluster as expected in the Siberian clade, e.g., *M. o. hahlovi* with *M. o. kiusjuresis* and *M. o. altaicus* with *M. o. anikini*, but in general the relationships are not clearly resolved. While in the MF-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 (Moecmhe-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* (Moecmehe-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. sacha-linensis*, *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. maxomowiczii*, 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. maxomowiczii* (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. evoronenisci, and M. hyperboreus, but analyzed three further species that were not included in our study (M. middendorffii, 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. evoronenisci 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. evoronenisci 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. evoronenisci, 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. evoronenisci and M. mujanensis 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; Golenishev 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 metacenters with formation of large metacenters) 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.

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. rattiiceps) 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 Verkhoyans 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 located 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 southeastern/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 similarly 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 (Gallieni et al. 1998; Castiglia et al. 2008), or the Microtus multiplex complex in the Alpine-Dinaric region (Haring et al. 2009; Tverkovic 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 ([Mitsaina 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 chromosomal instability, but their fixation in chromosomal races might be just a consequence of the fragmented population structure charac-

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Appendix A. Supplementary data


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