RESEARCH ARTICLE

Phylogenetic relationship and time of divergence of *Mus terricolor* with reference to other *Mus* species

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Abstract

Mitochondrial DNA control region of *Mus terricolor*, three aboriginal species *M. spretus*, *M. macedonicus*, *M. spicilegus*; the Asian lineage *M. caroli*, *M. cervicolor*, *M. cookii*; and the two house mice, *M. musculus domesticus* and *M. m. castaneus* were analysed to estimate the substitution rate, phylogenetic relationship and the probable time of divergence. Results showed that *M. spretus*, *M. caroli* and *M. terricolor* are highly diverged from each other (*caroli*/*terricolor* = 0.146, *caroli*/*spretus* = 0.147 and *terricolor*/*spretus* = 0.122), whereas *M. spretus* showed less divergence with two house mice species (0.070 and 0.071). Sequence divergence between *M. terricolor* and the Palearctic group were found to be ranging from 0.121 to 0.134. Phylogenetic analysis by minimum evolution, neighbour-joining, unweighed pair group method with arithmetic mean and maximum parsimony showed almost similar topology. Two major clusters were found, one included the Asian lineage, *M. caroli*, *M. cookii* and *M. cervicolor* and the other included the house mice *M. m. domesticus*, *M. m. castaneus* and the aboriginal mice *M. macedonicus* and *M. spicilegus* along with *M. spretus*, forming the Palearctic clade. *M. terricolor* was positioned between the Palearctic and Asian clades. Results showed that Palearctic-*terricolor* and the Asian lineages diverged 5.47 million years ago (Mya), while *M. terricolor* had split around 4.63 Mya from their ancestor. *M. cervicolor*, *M. cookii* and *M. caroli* diverged between 4.70 and 3.36 Mya, which indicates that *M. terricolor* and the Asian lineages evolved simultaneously. *M. spretus* is expected to have diverged nearly 2.9 Mya from their most recent common ancestor.

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Introduction

The house mouse is an Eurasian polytypic species with five currently recognized subspecies *Mus musculus domesticus*, *M. m. musculus*, *M. m. castaneus*, *M. m. molossinus* and *M. m. gentilulus* included in the Palearctic group along with three aboriginal species *M. macedonicus*, *M. spicilegus* and *M. spretus* (Boursot *et al*. 1993; Sage *et al*. 1993; Prager *et al*. 1998; Guénet and Bonhomme 2003; Geraldes *et al*. 2008; Bonhomme *et al*. 2011; Yonekawa *et al*. 2012). It is also the most studied species in various fields of biology, especially from the stand point of evolutionary biology. Among these, *M. m. castaneus* (Waterhouse 1842; Philippines) has been considered as more polymorphic with high genetic variability (allozymic, nuclear and mtDNA) having retained more ancestral polymorphism than the other *M. musculus* subspecies and *Mus* species of the Indian subcontinent (Boursot *et al.* 1996; Din *et al.* 1996; Phifer-Rixey *et al.* 2012). In spite of extensive studies, the relationships of *M. musculus* with other species, namely, *M. terricolor*, *M. caroli* and *M. spretus* are not yet well established and the time of divergence of these species from most recent common ancestors is still a controversy (Tucker 2007). *M. caroli* found in Asian continent (Musser and Carleton 2005) is less studied than house mice. The exact relationship of *M. spretus* Lataste, 1883 to the house mouse is unclear, however, it may represent the earliest evolutionary offshoot of the Palearctic group (Lundrigan *et al.* 2002) as substantial genetic diversity exists within the species (Mahler *et al.* 2008). Two other well-defined aboriginal species have nonoverlapping ranges in eastern Europe. *M. spicilegus* is restricted to the steppe grassland regions lying in the north and west of the Black Sea spreading over Bulgaria, Romania and Ukraine and *M. macedonicus* is restricted to the eastern Mediterranean region across Greece and Turkey (Bonhomme *et al.* 1978, 1983; Sage 1981).

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M. terricolor Blyth, 1851 is the indigenous common pygmy field mouse of India and was known as *M. dunni* until Musser and Carleton (1993) synonymised the two. *M. terricolor* coexists with its sibling species *M booduga*. Both species have $2n = 40$ chromosomes and constitute the lineage of Indian pygmy field mice. *M. terricolor* is characterized by a large submetacentric X and a large acrocentric Y chromosome (Matthey and Petter 1968; Markvong *et al*. 1975; Sharma and Garg 1975; Manjunatha and Aswathanarayana 1979; Sen and Sharma 1983; Sharma *et al*. 1986). Three divergent karyotypes are found in *M. terricolor* due to presence of variable number of heterochromatic short arms in homozygous conditions (Sen and Sharma 1983; Sharma *et al.* 1986, 1990) and are designated as chromosome types I, II and III. *M. terricolor* chromosomal type I with all acrocentric autosomes having heterochromatic minute arms is widely distributed throughout the subcontinent except in southern peninsular region, while chromosome type II found in Mysore and Erode, and type III is distributed in Chennai, Tirupati and Madurai which has heterochromatic short arms on autosome pairs 1 and 3 and 1, 3 and 6, respectively. The short arm of X and the entire Y are heterochromatic (Sharma *et al.* 1986, 1990). The three chromosomal variants of the *M. terricolor* complex are sufficiently diverged to merit consideration as incipient species (Chatterjee *et al.* 1994). Based on mitochondrial DNA restriction fragment length polymorphism (RFLP), Chatterjee *et al.* (1994) have also commented that *M. booduga – M. terricolor* lineage might have evolved simultaneously with other lineages but not before the *cervicolor*, *caroli* and *cookii* lineage, however, the diversification of the *M. terricolor* complex appears to be a recent evolutionary event. Multidisciplinary studies also indicate that *M. terricolor* complex is in the active stage of evolutionary differentiation (Sharma *et al.* 1990; Sharma 1996). But compared to other *Mus* species, the information on the phylogenetic relationship of *M. terricolor* lineage with other *Mus* species is scanty.

The mitochondrial DNA (mtDNA) is a good genetic marker to evaluate the phylogenetic relationships and to estimate the probable time of divergence of species from their most recent common ancestor (MRCA) (Spradling *et al.* 2001; Galewski *et al.* 2006). The mutation rate of mtDNA is several orders of magnitude higher than that of nuclear genes (Ingman *et al.* 2000). The mutation rate in the two hyper variable regions (HVR I and II) of the noncoding control region of mtDNA has been shown to be $0.075–0.165 \times 10^6$ substitution per site per year (Stoneking *et al.* 1992; Tamura and Nei 1993; Hasegawa *et al.* 1998). Related studies have indicated that rodent mtDNAs evolve much faster than those of large mammals (Rice 1971; Benveniste *et al.* 1977; Brownell 1983; Wu and Li 1985). Britten (1986) reviewed the differences of rates in different taxonomic groups and concluded that the rodent mitochondrial DNA has evolved approximately five times as fast as that of hominoids and birds. Due to the different rate of mutation of mitochondrial DNA in different species, the accurate estimation of the time of divergence is difficult. However, palaeontological data together with the molecular data have improved the accuracy of estimation of the rate of DNA evolution and hence the accuracy of the molecular clock.

To have an insight into the enigmatic relationship of the Eurasian species, the present study embodies the analysis of sequence divergence and the divergence time using the sequences of mtDNA control region of *M. terricolor*, *M. caroli*, *M. spretus* together with other *Mus* sequences retrieved from GenBank.

Materials and methods

Animal and DNA collection

A total of 25–50 individuals of *M. terricolor* were captured from the rice fields of the Dooars region (26°31′21″N–27°54′00″N/80°30′05″E–89°49′30″E) located in the northern part of West Bengal, India, and cytogenetically analysed. Seven populations having sample size ranging between 2 and 25 individuals were used in molecular studies. Out of these, one individual was used in the present study, while the rest are to be analysed and published as a population study separately. Identity of the species was confirmed by chromosomal analysis following air drying method as described by Lee and Elder (1980) and modified by Baker *et al.* (1982). Total DNA from wild-derived strains of *M. spretus* (SPRET/Eij, stock number 001146) and *M. caroli* (Caroli/EiJ, stock number 000926) were obtained from the Jackson Laboratory (Bar Harbor, USA).

Mitochondrial DNA preparation

M. terricolor mitochondrial DNA was isolated using the method of Chatterjee and Rao (1984). Tissues were chopped and washed in cold PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), about 1 g tissue/mL of cold homogenization buffer (0.3 M sucrose, 0.002 M EDTA pH 8.0, 0.025 M Tris pH 8.0) was homogenized. The homogenate was centrifuged at $1000 \times g$ at 4°C for 15 min and the mitochondrial pellet was lysed in lysis buffer (0.01 M Tris, 1 mM EDTA pH 8.0, 0.1 M NaCl) with SDS to the final concentration of 1%, incubated at 37°C for 5 min and left at room temperature for 10 min. Five M NaCl was added to the lysate to a final concentration of 1 M, mixed slowly and kept in ice for 1–2 h for complete lysis. After separation of the proteins and high molecular weight DNA (centrifugation at $20,000 \times g$) the supernatant was extracted thrice with phenol/chloroform. DNA was ethanol precipitated, dried and resuspended in Tris-EDTA (pH 8.0).

Polymerase chain reaction (PCR) amplification and sequencing

The D-loop region of the mtDNA from *M. terricolor* (wild), *M. caroli* (Caroli/EiJ) and *M. spretus* (SPRET/EiJ) was amplified either from total DNA or purified mtDNA using three pairs of primers (table 1). DNA was amplified in

Table 1. Primer pairs used for amplification of D-loop region of mtDNA.

Forward primer		Reverse primer	
15093	5′-TTCCGTCCAATCACCCAAATC-3′	15956	5′-GACTATGTGCTGTCCTTTCAAGCC-3′
16003	5′-CATTAGTCCGCAAAACCCAATC-3′	00226	5′-TTATCACTGCTGAATCCCGTGG-3′
15704	5′-AAACCAACAACCCGCCCACCATTG-3′	00227	5′-TTTATCACTGCTGAATCCCGTG-3′

35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and in the final cycle for 3 min. Taq DNA polymerase, 10× Taq buffer and primers were used as 1.25 U, 1× and 1 μM, respectively, in the final reaction mixture. Other components used were as per the manufacturer's specification. The amplified PCR products were checked by separating on 2% agarose gel in 1× TAE buffer (0.04 M Tris - acetate, 0.001 M EDTA) containing 0.5 μg/mL ethidium bromide with 100-bp DNA ladder as size markers (figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). The amplified DNA was purified through the QIAquick PCR purification kit. Sequencing reactions were performed for both strands using forward and reverse primers using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystem, Foster City, USA). The reaction products were then purified by Performa® DTR Gel Filtration Cartridges (Edge Biosystem, Gaithersburg, USA) from unused BigDye and primers and analysed on ABI3130 DNA Sequencer (Applied Biosystem).

Sequence alignment and phylogeny

For phylogenetic analysis of *M. terricolor* (JF487791), *M. spretus* and *M. caroli*, 23 sequences of the mtDNA control region (CR) with flanking regions were retrieved from GenBank (accession number in parenthesis) and added to the analysis. These comprised one sequence each of *M. terricolor* (NC010650), *M. caroli* (AF355601) and *M. spretus* (U47539), two sequences of *M. cookii* (KJ530561 and KJ 530562), three sequences each of *M. m. castaneus* (U47534, JN416655 and JN416656), *M. m. domesticus* (AY172335, AJ843863 and AJ843835), *M. spicilegus* (U47538, U47536 and HG421219), *M. cervicolor* (KJ530559, KJ530560 and NC025269), four sequences of *M. macedonicus* (AJ286326, AJ286328, AJ286329 and U47535) and one sequence each of *Rattus rattus* (X04735) and *Nannospalax ehrenbergi* (AJ440450). *R. rattus* and *N. ehrenbergi* were included as out groups. Sequences obtained from the control region and the flanking tRNA genes of mtDNA were aligned (figure 2 in electronic supplementay material) using multiple alignment algorithms in CLUSTAL W (Thompson *et al.* 1994). The maximum parsimony analysis was performed on each partitioned and on the combined matrix. Phylogenetically informative characters were unordered and equally weighed, gaps were treated as missing characters. Clade stability was assessed by bootstrap analyses (Felsenstein 1985). All bootstrap analyses included 1000 replicates. A maximum likelihood analysis was carried out on each data partition to calculate neighbour-joining (NJ) tree. The tree was drawn to scale, with branch lengths in the same unit as those of the evolutionary distances used to infer the phylogenetic tree (Tamura *et al.* 2004, 2007). The evolutionary history was inferred using NJ (Saitou and Nei 1987), maximum evolution (ME) methods (Rzhetsky and Nei 1992) and unweighed pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973). The evolutionary distances were computed using the p-distance (Nei and Kumar 2000) and maximum composite likelihood model (Tamura *et al.* 2004) which are in the units of the number of base differences per site. MEGA 5.1 (Tamura *et al.* 2011) has been used for all phylogenetic analyses. The maximum parsimony (MP) (Nei and Kumar 2000) tree was obtained using the min-mini heuristic algorithm.

Relative rate test

Tajima's relative rate test (Tajima 1993) was also conducted using three sets of comparison. These sets were with outgroup *Nannospalax* to ingroups *Rattus* and *M. m. domesticus*; *Rattus* to *Nannospalax* and *M. m. domesticus* and *M. m. domesticus* to *Rattus* and *Nannospalax*. Tests showed equality of evolutionary rate between sequences of outgroups and each of the two ingroups.

Similar tests were also carried out for each subspecies and species of *Mus* considering any one of them as outgroup. No significant substitution rate heterogeneity was detected between the subspecies under comparisons.

Estimation of divergence times

To obtain tentative divergence times of different species using molecular clock, the outgroups, preferably with similar evolutionary rate were considered to calibrate the clock. Hence, *R. rattus* and *N. ehrenbergi* were included as outgroups in the present study. The divergence time between *Mus* and *Rattus* is commonly used as a calibration point. Estimated time of divergence of *Rattus* and *Mus* by different workers varied from 10 million years ago (Mya) (Smith and Patton 1999) to 41.9 Mya (Huchon *et al.* 2000), whereas the fossil data indicate a date no later than 14 Mya (Jacobs and Pilbeam 1980). The murid fossils from Pakistan and a reexamination of the oldest known murids indicated that the *Mus–Rattus* split occurred about 8–11 Mya (Jacobs 1978; Flynn *et al.* 1985). Benton and Donoghue (2007) suggested that *Mus* and *Rattus* split have a lower bound of 12.3 Mya and an upper bound of 11 Mya for minimum and maximum age constraints of the mouse–rat divergence. In the present study, based on the paleontological data, the time of *Rattus–Mus* split has been considered as 12 Mya (Jacobs and Downs 1994), which was preferentially used as a calibration point.

Results

All the individuals of *M. terricolor* studied were cytogenetically found to be chromosome type I ($2n = 40$) having all acrocentric autosomes with large submetacentric X and large acrocentric Y chromosomes in the complement as characteristic. The short arm of X and the large Y were heterochromatic (Rudra and Bahadur 2013).

Sequence divergence

Twenty-four sequences of the control region of *Mus* were aligned and compared. Sequences of *Nannospalax* and *Rattus* were taken as outgroups. Base differences at each site were taken into account with a uniform rate as the mtDNA control region is noncoding, therefore, there is no preference for the third base position of codon to mutate highly. Patterns among lineages were treated as homogenous. Result of sequence divergence considering one sequence from each taxa using p-distance method is documented in table 2. *Nannospalax* and *Rattus* were found to be highly diverged from other *Mus* species as expected. The genetic divergence of *M. terricolor* with *M. cervicolor*, *M. cookii* and *M. caroli* were 0.159, 0.139 and 0.146, respectively, while among *M. cervicolor*, *M. cookii* and *M. caroli* sequence divergence ranged between 0.091 to 0.133, i.e. moderately low with least sequence divergence within the group between *M. cervicolor* and *M. cookii* (0.091). *M. terricolor* showed a sequence divergence of 0.122 with *M. spretus* while 0.128, and 0.121 were observed with *M. spicilegus* and *M. macedonicus*, respectively. Sequence divergence between *M. terricolor* and Palearctic group was found to range from 0.121 to 0.134 which is lower than *cervicolor – cookii – caroli* lineage. *M. spretus* mtDNA seems to be highly diverged from *cervicolor – cookii – caroli* lineage (0.130–0.153) than *macedonicus–spicilegus* (0.070 and 0.063) and *domesticus–castaneus* lineage (0.071 and 0.070). The sequence divergence between *domesticus–castaneus* was found to be 0.032 which was lower than the sequence divergence between *macedonicus* and *spicilegus* (0.047).

Phylogenetic analysis

The possible evolutionary history of *Mus* and the first split among the lineages was evaluated and delineated. The evolutionary history was inferred using the NJ, ME, UPGMA and MP methods. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Phylogenetic trees constructed by p-distance and maximum composite likelihood methods revealed similar topology of species, thus only trees obtained by p-distance for NJ, UPGMA and ME are shown in figures 1, 2 and 3. Transition and transversion were given equal weight.

Phylogenetic trees constructed using NJ (figure 1), ME (figure 2) and UPGMA (figure 3) revealed almost similar branching patterns regarding the relationship of house mice and different *Mus* species. Analysis showed two distinct groups of clusters consisting of the Palearctic group of *Mus* species in one cluster and the Asian species *caroli – cookii – cervicolor* in other group (figures 1, 2 and 3). *M. terricolor*, an Asian species with relatively high sequence divergence (table 2) is distinct and rather being incorporated in a clade positioned between the Palearctic clade and the clade formed by Asian groups of *Mus* species (figures 1, 2 and 3) and also supported by maximum parsimony tree (figure 4). Radiation tree (figure 5) provides a clear picture about the branch point of *M. terricolor* implying common ancestry with Palearctic group.

M. macedonicus and *M. spicilegus* with low sequence divergence (table 2) are phylogenetically placed closely in a clade, while two commensal species of house mice, *M. m. castaneus* and *M. m. domesticus* are clearly grouped in another clade close to the aboriginal species *M. macedonicus* and *M. spicilegus*. Both the lineages are included in the Palearctic group that diverged from their MRCA with high bootstrap support. *M. spretus* is separately joined to the Palearctic clade by a deep node with low bootstrap support (figures 1, 2 and 3) which is also supported by the radiation tree by UPGMA showing the ancestry (figure 5) and clearly indicating the early divergence of *M. spretus* than the other Palearctic group of *Mus* species.

Table 2. Estimates of evolutionary divergence in pairwise comparisons considering one sequence from each species and subspecies of *Mus* including outgroups, *R. rattus* and *N. ehrenbergi*.

	1	2	3	4	5	6	7	8	9	10	11
1. *N. ehrenbergi*	****										
2. *R. rattus*	0.314	****									
3. *M. caroli*	0.287	0.248	****								
4. *M. terricolor*	0.286	0.248	0.146	****							
5. *M. m. castaneus*	0.270	0.242	0.143	0.128	****						
6. *M. m. domesticus*	0.273	0.243	0.145	0.134	0.032	****					
7. *M. spretus*	0.287	0.255	0.147	0.122	0.070	0.071	****				
8. *M. cookii*	0.296	0.252	0.126	0.139	0.130	0.139	0.130	****			
9. *M. spicilegus*	0.275	0.246	0.135	0.128	0.052	0.070	0.063	0.126	****		
10. *M. macedonicus*	0.270	0.237	0.120	0.121	0.064	0.068	0.070	0.124	0.047	****	
11. *M. cervicolor*	0.283	0.258	0.133	0.159	0.150	0.153	0.153	0.091	0.146	0.141	****

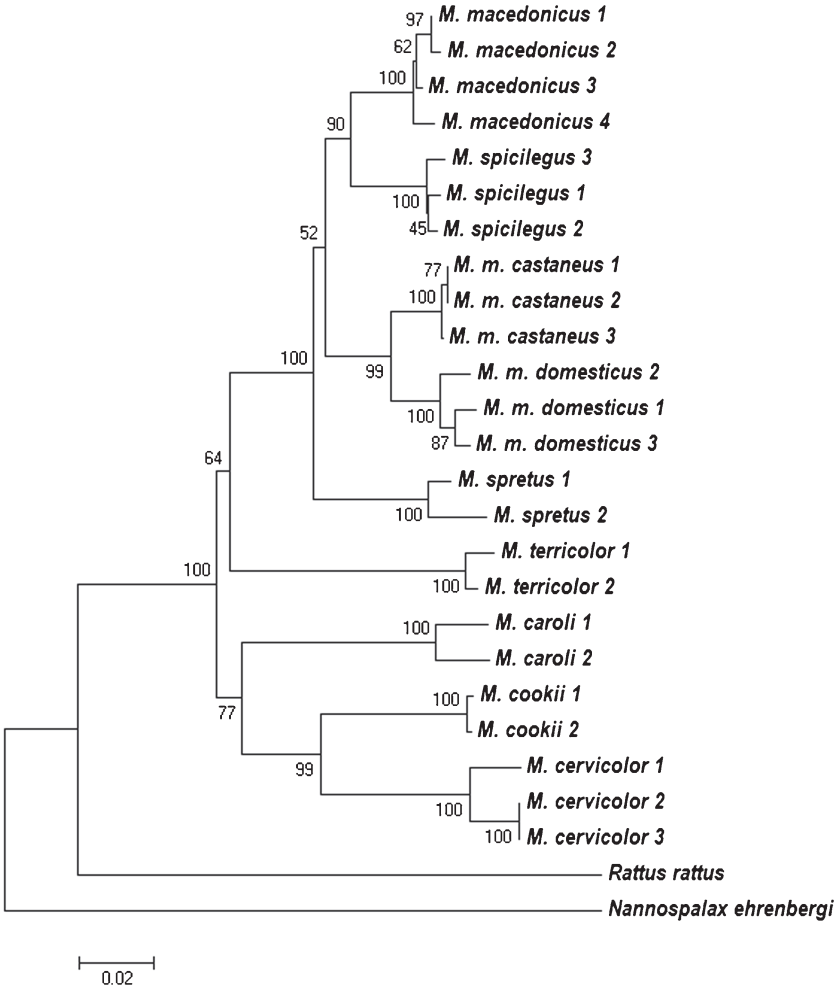

Figure 1. Phylogenetic tree based on mtDNA control region for nine species and subspecies of *Mus* inferred using NJ methods by p-distance model. Bootstrap values (percentage occurrence in 1000 replicates) for internal nodes are given at each node (below or above the branches). *R. rattus* and *N. ehrenbergi* were used as outgroups. Scale bar = 0.02 unit.

In all phylogenies by different methods, *M. caroli*, *M. cookii* and *M. cervicolor* showed similar branching pattern where *cookii* and *cervicolor* were grouped closely as sister taxa and *caroli* being shown as the earlier diverged taxon from common ancestor of *caroli – cookii – cervicolor* lineage. However, the deepest node was found for the branch of *M. caroli* and *M. terricolor* after divergence of *Rattus* and the common ancestor of *Mus* indicating that maximum number of mutational events had occurred in *M. caroli* and *M. terricolor*, while the node of house mice is less deep, indicating a low number of mutational events.

In maximum parsimony (figure 4) analysis (a single parsimonious tree was presented out of two most parsimonious trees (length = 714) with the consistency index of 0.619893, the retention index of 0.796771, and the composite index of 0.563517 (0.493913) for all sites and parsimony-informative sites in parenthesis), *M. spretus* with high bootstrap values embedded within the clade made the position of *M. spretus* debatable. The *M. spicilegus* and *M. macedonicus* separately branched off from the Palearctic lineage though with low bootstrap value.

Nannospalax ehrenbergi, a near relative of *Mus* and *Rattus*, was taken as outgroup to count the number of events occurred along the branches and in each lineage of *Mus* species and *Rattus*. Comparing one sequence from each lineage, the total number of nucleotide substitutions (excluding insertion/deletion) were estimated. A total of 212 mutational events were estimated that occurred after the separation of *Mus* lineage from *Rattus* lineage leading to *M. m. domesticus*, while 105 events were estimated before the divergence of different *Mus* lineages and 98 events in *Rattus* after divergence from *Mus* lineage.

Time of divergence

In this study, the rate of change of murid mtDNA was calculated taking 12 Mya (*Rattus* and *Mus* divergence time) as a calibration point. Tajima's relative rate test was also

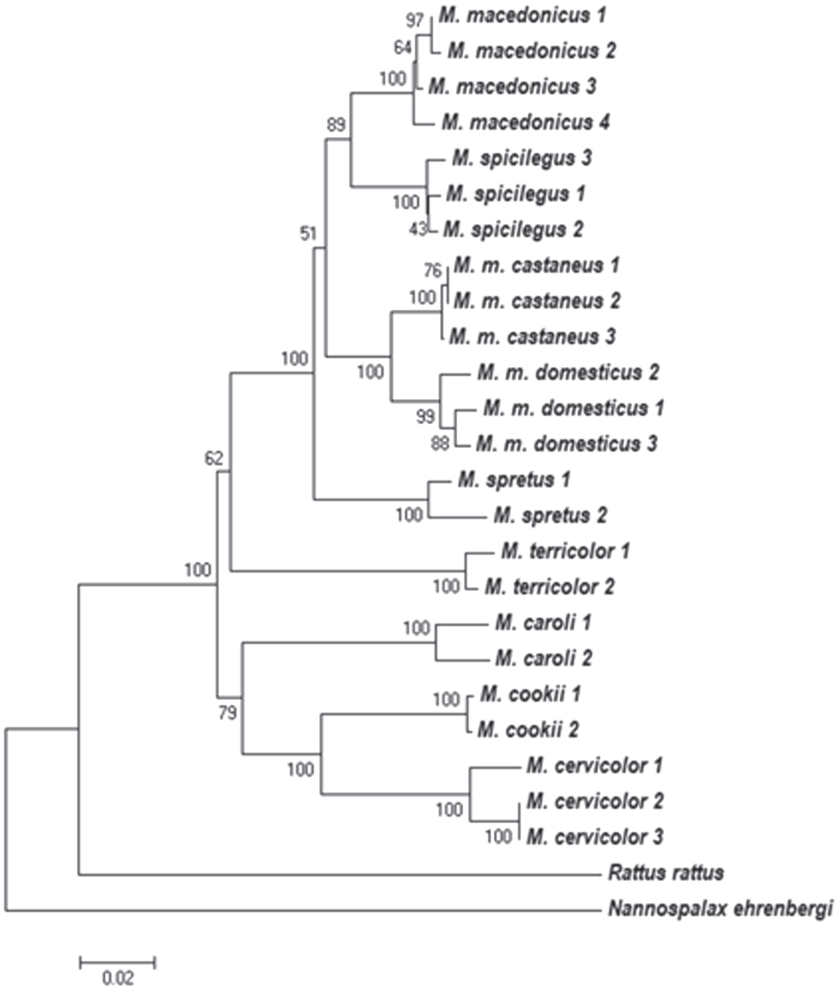

Figure 2. Phylogenetic tree inferred using ME methods by p-distance model based on mtDNA control region for nine species and subspecies of *Mus*. Occurrence of bootstrap values and outgroups are similar as in figure 1.

conducted to verify the rate of evolution of the sequences. Three sets of tests were conducted using *Nannospalax*, *Rattus* and *M. m. domesticus* as outgroups and χ^2 values obtained were 0.01, 0.15 and 1.14, respectively, at 95% confidence level and 1 degree of freedom which accepts the null hypothesis. Therefore, it is apparent that the rate of evolution is equal between lineages.

Using *Rattus–Mus* divergence date and nucleotide substitution data, a substitution rate of 0.0136 per base per Mya was calculated giving equal weightage to transitions and transversions in *Rattus–Mus* lineage. Substituting the rate 0.0136 per site per Mya, the time of divergence for different *Mus* species were estimated (table 3). According to our estimation, *Rattus* and the ancestor of different *Mus* species diverged to some extent, 8.61 Mya (node A, figure 5), while the ancestor of *Mus* (node B) diverged into two major lineages, ∼5.47 Mya. One of them gave rise to the *cervicolor – cookii – caroli* lineage. *M. caroli* diverged earlier from *M. cookii* and *M. cervicolor* nearly 4.70 Mya, while *M. cookii* and *M. cervicolor* diverged later from their ancestor, ∼3.36 Mya. The earliest offshoot of the other lineage is *M. terricolor* which branched off from its ancestor around 4.63 Mya. *M. spretus* diverged early around 2.90 Mya, whereas *M. spicilegus – M. macedonicus* and *M. m. castaneus–M. m. domesticus* lineages split nearly 1.75 Mya and 1.40 Mya, respectively.

Discussion

Different species of house mice and field mice from various parts of the world have been extensively studied to infer their phylogenetic relationship (Chevret *et al.* 2005; Macholan *et al.* 2012), but the position of the Indian pygmy field mouse *M. terricolor* is uncertain (Tucker 2007). Our results of sequencing of mtDNA control region of *M. terricolor*, *M. spretus* and *M. caroli* and the comparison of these sequences with two house mice species *M. m. domesticus*, *M. m. castaneus* and other *Mus* species shed some light on their phylogenetic relationships.

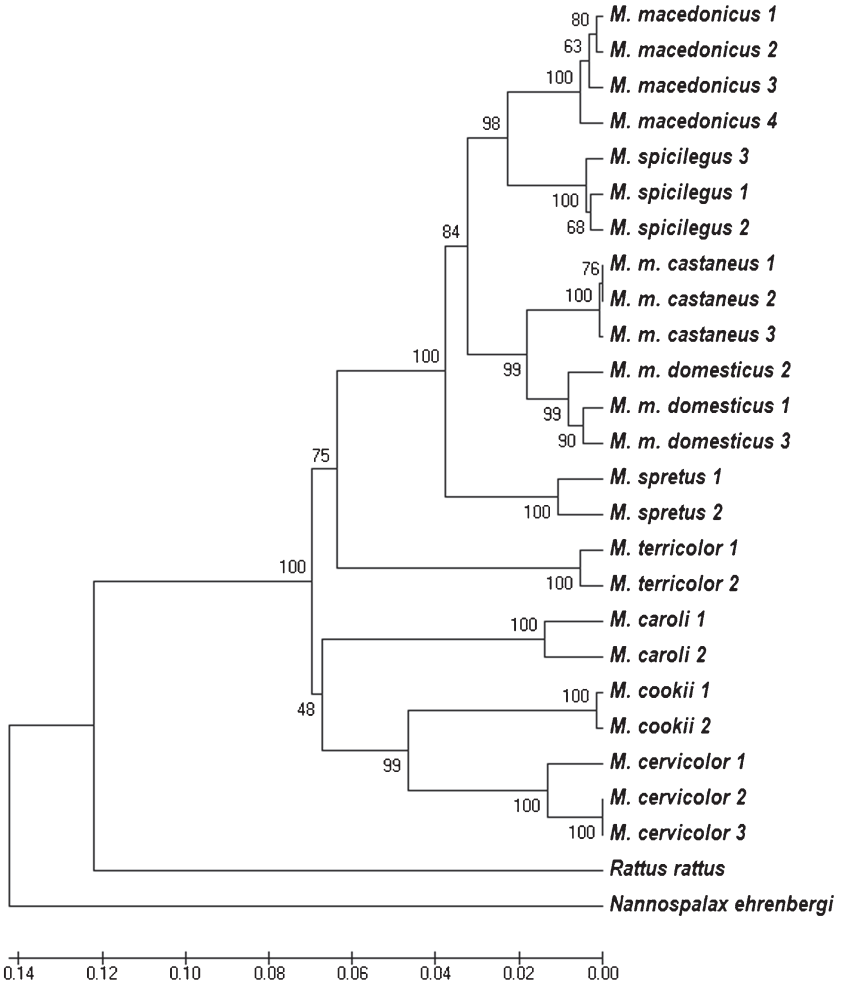

Figure 3. Phylogenetic tree based on mtDNA control region for nine species and subspecies of *Mus* inferred using UPGMA methods by p-distance model. Occurrence of bootstrap values and outgroups are similar as in figure 1.

Phylogenetic relationships

The Indian pygmy field mice, *M. terricolor* (= *M. dunni*) has not been studied much with respect to its phylogenetic relationships with other *Mus* species. However, the branching of *M. dunni* shown by Guénet and Bonhomme (2003) or *M. terricolor – M. booduga* (Tucker 2007) remain uncertain. In the present study, relatively high sequence divergence and phylogenetic analyses by NJ (Saitou and Nei 1987), ME (Rzhetsky and Nei 1992), UPGMA (Sneath and Sokal 1973), MP (Nei and Kumar 2000) and radiation tree by UPGMA clearly indicated that *M. terricolor* lineage diverged earlier from the other lineages leading to the Palearctic group of *Mus* species shortly after the divergence of Palearctic and Asian lineages. This is also supported by maximum parsimony analysis with high bootstrap support (figure 4).

M. caroli, *M. cervicolor* and *M. cookii* lineage established as a monophyletic group by She *et al.* (1990), Lundrigan *et al*. (2002), Chevret *et al.* (2005) is further confirmed by our phylogenetic analysis. However, there is a strong evidence that *M. cervicolor* and *M. cookii* have a closer phylogenetic relationship as sister taxon than that with *M. caroli*, which is distantly related as basal taxa (Chevret *et al.* 2005; Tucker *et al.* 2005). Similar branching topology has been obtained in the present study with high bootstrap support in all phylogenetic analysis except UPGMA where the relationship of *M. caroli* as basal taxa is weakly supported (48%).

M. spretus has been consistently placed in the Palaeartic clade either occupying a basal position (NJ, ME, UPGMA) thereby supporting the results obtained by Prager *et al.* (1996) and Tucker *et al.* (2005) or embedded within the clade (MP) as proposed by Lundrigan *et al.* (2002), and Guénet and Bonhomme (2003). Therefore, the position of *M. spretus* remains enigmatic. The high sequence divergence of *M. spretus* from other members of the Palearctic clade and its outgroup appearance within the clade supports the view that the lineage leading to *M. spretus* diverged earlier before split of the lineage leading to other species of the Palearctic clade (Prager *et al.* 1996 and references there in). The instability in position of *M. spretus* within the clade may be due to the rapid speciation of this Palaeartic lineage (Lundrigan *et al.* 2002) or may be due to

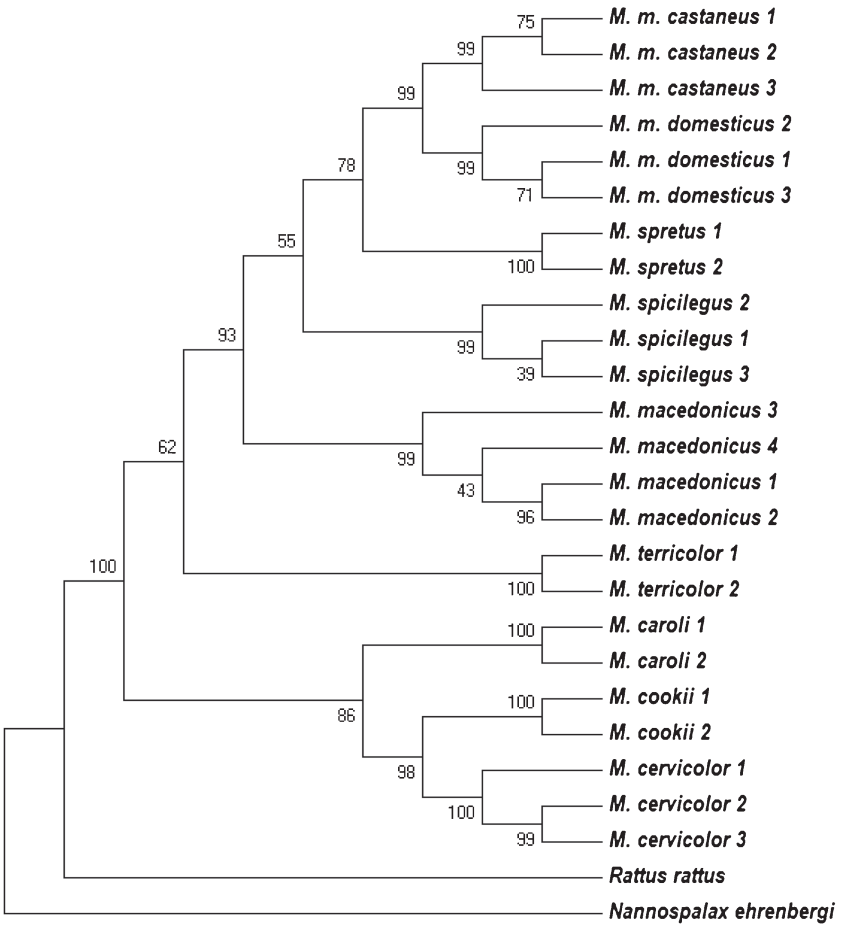

Figure 4. Maximum parsimony tree based on mtDNA control region for nine species and subspecies of *Mus*. Bootstrap values for internal nodes are given at each node (below or above the branches). *R. rattus* and *N. ehrenbergi* were used as outgroups.

differences in selection pressure between *M. musculus* and *M. spretus* (Mahler *et al.* 2008) after the diversification of the lineage.

M. spicilegus and *M. macedonicus* appeared as close relative within the Palearctic clade with lower sequence divergence (0.049) between them, which corroborates the results of Prager *et al.* (1996), Lundrigan *et al.* (2002), Auffray *et al*. (2003) and Tucker *et al.* (2005). However, MP analysis showed to some extant conflicting result which does not group the two taxa together which is consistent with the result of Fort *et al.* (1985) and Tucker *et al.* (1989). MtDNA of two commensal species of house mice *M. m. domesticus* and *M. m. castaneus* are closely similar with very low sequence divergence (0.037) and are always grouped together in our phylogenetic analysis.

Time of divergence

Different workers suggested the separation of *M. terricolor* lineage from *M. musculus* ancestor ~3 to 4 million years ago (Guénet and Bonhomme 2003; Suzuki *et al.* 2004). Our data estimate the divergence date for *M. terricolor* lineage (4.63 Mya) to be marginally earlier.

The estimated time of divergence of the Palearctic-*terricolor* and Asian (*caroli – cookii – cervicolor*) lineage is 5.47, which is in accordance with the estimation of Chevret *et al.* (2005) for the Palearctic–Asian lineages is 5 Mya. The subsequent divergences of the extant taxa within the lineages have been shown to be occurring from 4.4 to 4.2 Mya (Chevret *et al.* 2005). Our estimation of divergence date of *caroli* from *cookii–cervicolor* (4.7 Mya) and for *M. terricolor* (4.63 Mya) is slightly more than the estimate proposed by Chevret *et al.* (2005), which may be due to their considering different regions of mtDNA in analysis or using different *Rattus*/*Mus* divergence time as calibration point. Time of divergence of *M. terricolor* and *caroli – cookii – cervicolor* lineage reflect a parallel evolution of the two lineages which also supports the view of Chatterjee *et al.* (1994).

The estimated time of divergence of *M. spretus* from *M. musculus* has been shown to be 1 – 3 Mya (She *et al.* 1990; Suzuki *et al.* 2004, 2013; Chevret *et al.* 2005). Our estimated divergence time of *M. spretus* from the lineage of other species of house mice and lineage of *M. macedonicus* and *M. spicilegus* is ~2.90 Mya, which is almost similar to the above findings. In the present study, the divergence date of *M. macedonicus* and *M. spicilegus* is 1.75 Mya, which is

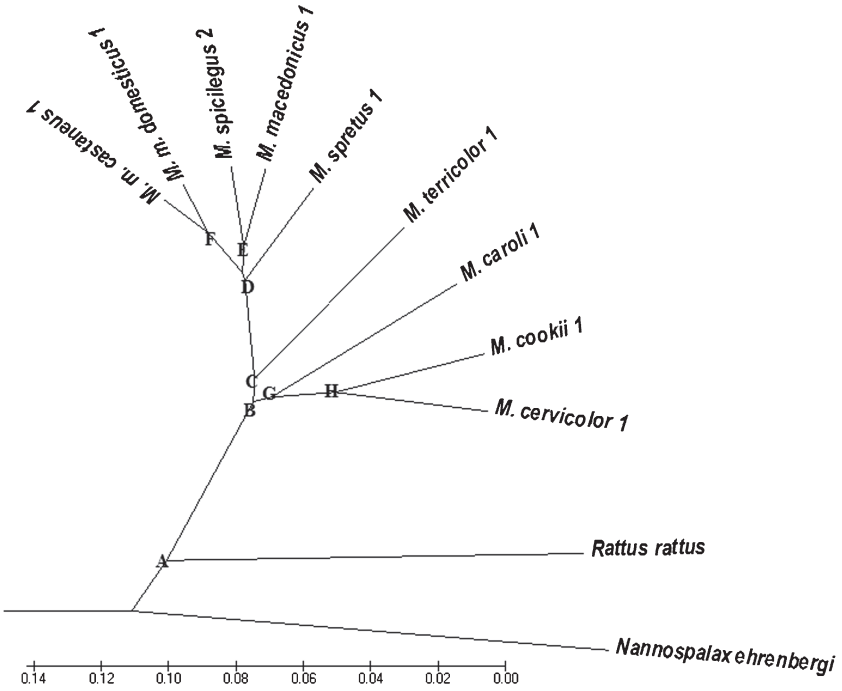

Figure 5. UPGMA tree showing the split and radiation of the species and subspecies of *Mus*. Letters indicate the split of the lineages at the node (see also table 3).

more than the estimation done earlier based on scnDNA, i.e. 0.17 – 0.29 Mya (She *et al.* 1990) and mitochondrial DNA 1147 Kya (Macholan *et al.* 2012).

Suzuki and workers in their earlier study estimated the time of divergence of *M. musculus* subspecies as 0.1–1.5 Mya (Suzuki *et al.* 2004), but their later studies showed the time of divergence between 0.37 and 0.47 Mya (Suzuki *et al.* 2013). Our estimation of *M. domesticus–M. castaneus* lineage (1.40 Mya) is within the range shown by Suzuki *et al.* (2004). However, the estimation by Suzuki *et al.* (2004) was based on mitochondrial and nuclear genes, whereas our estimation is based on mtDNA control region, which is highly polymorphic than protein coding genes. The time of divergence based on mtDNA RFLP of two Asian subspecies of house mice, *M. m. castaneus* and *M. m. bactrianus* with *M. m. domesticus* was 2.1–2.5 Mya (Yonekawa *et al.* 1981), which is higher than our estimation. According to Geraldes *et al.* (2008), *M. musculus* species began to diverge about 0.5 Mya with all three subspecies (*M. m. musculus*, *M. m. domesticus* and *M. m. castaneus*) diverging within a short time interval. Their estimation is based on the mutation rate of 0.041×10^{-6} in the control region which is also higher than our result.

The present study allows us to infer that *M. terricolor* lineage that evolved parallely with *caroli – cookii – cervicolor* lineage, is distinct and has diverged earlier than other species of the subgenus *Mus*. However, more molecular data need to be generated based on genes both conserved as

Table 3. Estimation of time of divergence of different species and subspecies of *Mus* based on mtDNA control region. Nodes indicate the split of lineages from their MRCA showed in figure 3.

Node	MRCA estimated	Estimated time of divergence in Mya using the formula, substitution per base per Mya/2 substitution rate and $t = D/2r$*
A	*Rattus* and *Mus*	8.61
B	Palearctic-*terricolor* lineage and Asian lineage	5.47
C	Palearctic lineage and *M. terricolor* lineage	4.63
D	House mice – *macedonicus* group and *M. spretus*	2.90
E	*M. macedonicus* and *M. spicilegus*	1.75
F	*M. m. domesticus* and *M. m. castaneus*	1.40
G	*M. caroli* and *M. cookii –M. cervicolor*	4.70
H	*M. cookii* and *M. cervicolor*	3.36

*t = D/2r, where t is the time in Mya, D is the proportion of base pair difference between two sequences and r is the rate of divergence per base pair per Mya.

well as nonconserved to ascertain the phylogenetic position accurately.

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