

## Accelerated molecular evolution in *Microtus* (Rodentia) as assessed via complete mitochondrial genome sequences<sup>★</sup>

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**Abbreviations:** MtDNA – mitochondrial DNA; *Numt* – nuclear copies of mitochondrial fragments

### Abstract

*Microtus* is one of the most taxonomically diverse mammalian genera, including over 60 extant species. These rodents have evolved rapidly, as the genus originated less than 2 million years ago. If these numbers are taken at face value, then an average of 30 microtine speciation events have occurred every million years. One explanation for the rapid rate of cladogenesis in *Microtus* could be the karyotypic differentiation exhibited across the genus: diploid numbers range from 17 to 64. Despite the striking chromosomal variability within *Microtus*, phenotypic variation is unremarkable. To determine whether nucleotide substitution rates are also elevated in voles, we sequenced the entire mitochondrial DNA (mtDNA) genome of the Eurasian sibling vole (*Microtus rossiaemeridionalis*). We compared this genome to another previously sequenced vole mtDNA genome (*Microtus kikuchii*) and performed pairwise sequence comparisons with the mtDNA genomes of ten additional mammalian genera. We found that microtine mtDNA genomes are evolving more rapidly than any other mammalian lineage we sampled, as gauged by the rate of nucleotide substitution across the entire mtDNA genome as well as at each individual protein-coding gene. Additionally, we compared substitution rates within the cytochrome *b* gene to seven other rodent genera and found that *Microtus* mtDNA is evolving fastest. The root cause of accelerated evolution in *Microtus* remains uncertain, but merits further investigation.

### Introduction

Rodents account for over 40% of the ~4500 extant mammalian species, with most of the taxonomic variation found within Muridae (Musser & Carleton, 1993). One of the most diverse murid

lineages is the subfamily Arvicolinae (lemmings, muskrats, and voles). Of roughly 120 arvicoline species distributed across 21 genera, more than 60 are found within the vole genus *Microtus*, which is thought to have originated 0.5–2 million years ago (MYA) (Repenning, 1980; Chaline, et al., 1999). If 2 MYA is correct, and assuming a strictly bifurcating genealogical history, then an average of 30 microtine speciation events occur every million years. Given that most vertebrates average about

<sup>★</sup>The mitochondrial genome sequence from this article has been deposited with the GenBank database under accession number DQ015676.

2–3 million years per speciation event (Avice, Walker & Johns, 1998), this would suggest that speciation rates are  $\sim 60\text{--}100 \times$  higher in *Microtus* than in other vertebrates. Taken at face value, this means a new *Microtus* species evolves every  $\sim 30,000$  years.

There are at least three explanations for this apparently rapid rate of speciation. First, the genus could be much older than thought, but this is unlikely (see citations above). However, even if we assume that the genus actually arose 5 MYA, then the mean rate of speciation would be 60 species/5 million years or a speciation event every  $\sim 80,000$  years. This rate of cladogenesis would still be  $>20$  times faster than in an average mammalian lineage. Second, the taxonomy could be invalid. In other words, if microtine biologists were notorious splitters, one might expect to see an apparent increase in the speciation rate. This is unlikely for several reasons, including the uncommon karyotypic differentiation across the genus (discussed below) and because many species were discovered inadvertently (and others probably remain undiscovered). For example, *M. arvalis* and *M. rossiaemeridionalis* occur in sympatry and are morphologically indistinguishable. Nevertheless, they have distinct karyotypes, allozyme profiles, mitochondrial DNA (mtDNA) sequences, and nuclear DNA sequences (Baker et al., 1996; DeWoody, 1999). Thus, there is little reason to believe the taxonomy is grossly wrong. If the chronology and the taxonomy are correct even to a first approximation, one must consider a third possibility: microtine voles are evolving at a rapid rate.

Several lines of genetic evidence support the latter explanation. For example, species diversity within *Microtus* has been attributed to the flexible nature of the karyotype and its rapid rate of evolution, among the fastest reported for mammals (Maruyama & Imai, 1981). The diploid number for the most recent common ancestor of *Microtus* is thought to have been 56, but contemporary species range from  $2n = 17$  to  $2n = 64$  (Modi, 1987). This karyotypic variability suggests that chromosomal rearrangements have played a significant role in microtine speciation.

In addition to their rapidly evolving karyotype *per se*, microtines are an evolutionary enigma with respect to their sex chromosomes. For example, the X chromosome harbors  $\sim 20\%$  of

the entire *M. agrestis* genome (Nanda et al., 1988). Additional chromosomal oddities include females that bear Y chromosome-specific sex-determining regions (Fernández et al., 2002), conspecific males and females possessing different chromosome numbers (Charlesworth & Dempsey, 2001), and nonrandom inactivation of X chromosomes in hybrid crosses (Nesterova et al., 2001).

Despite extraordinary chromosome variability, the genus *Microtus* is not morphologically diverse. In fact, there is very little morphological differentiation among most microtines and cryptic species are common (Baker et al., 1996; Fink, Excoffier & Heckel, 2004; Jaarola & Searle, 2004). Many such cryptic species can be distinguished with either mitochondrial or nuclear gene sequences (DeWoody, 1999; Belfiore et al., 2003), but the shallow genealogy has made phylogenetic reconstruction of *Microtus* difficult. As a result, some systematic relationships within the genus remain unresolved (Conroy & Cook, 2000; Jaarola et al., 2004). There have been a number of studies describing chromosomal evolution among congeners (Burgos, Jiménez & Díaz de la Guardia, 1989; Mazurok et al., 2001; Mekada et al., 2001), but they have not reconciled microtine evolutionary history or defined a causal role for chromosomal rearrangements in the speciation process. Presumably, chromosomal rearrangements do not dramatically alter gene expression and/or function or we might see concordant morphological variability. Nucleotide substitution rates may or may not co-vary with structural changes in the genome, but mitochondrial cytochrome *b* sequences suggest that molecular evolution is rapid in *Microtus* (Conroy & Cook, 2000).

Single-gene surveys can be valuable, but rates of molecular evolution vary widely across genes and the idiosyncrasies of a lone gene may not accurately reflect patterns of genome-wide evolution. To this end, the mitochondrial genome can be a valuable tool for the study of evolutionary rates, particularly with regard to comparisons among genes or among taxonomic lineages. The eutherian mammalian mtDNA molecule contains 13 protein-coding genes, 22 tRNA genes, 2 rRNA subunits and the control region (displacement loop). We sequenced the entire mtDNA genome of *M. rossiaemeridionalis* to determine the relative rate of molecular evolution in *Microtus*. We compare this

genomic sequence to that of the conspecific Taiwan vole, *M. kikuchi* (Lin, Waddell & Penny, 2002), then contrast the *Microtus* mtDNA genomes with dyads of other conspecific mammals to examine 1) rates of molecular evolution across mtDNA genes and 2) rates of molecular evolution across mammalian genera. If evolution in *Microtus* is rapid relative to other mammals, we might expect to see evidence of such within its mtDNA.

## Materials and methods

### PCR amplification and sequencing

DNA extract from a single individual of *M. rossiaemeridionalis* was obtained from The Museum of Texas Tech University (TK 57718). We downloaded from GenBank the DNA sequences of complete rodent mtDNA genomes (vole: *Microtus kikuchii* NC\_003041; mouse: *Mus musculus* NC\_005089; rat: *Rattus norvegicus* NC\_001665) and complete or partial rodent mitochondrial genes (mice: *M. m castaneus* AB183255, *Peromyscus maniculatus* AF374576; lemmings: *Dicrostonyx groenlandicus* AY261992, *Lemmus lemmus* AY261993; voles: *Clethrionomys glareolus* AY332678, *Arvicola terrestris* AY332681, *M. nivalis* AY332687, *M. subterraneus* AY332685, *M. agrestis* AY332684, *M. arvais* AY332683, *C. gapperi* U83808, *M. pennsylvanicus* U83806; muskrat: *Ondatra zibethicus* U83809). We aligned these sequences using Sequencher 4.1 (GeneCodes) and identified conserved areas along the mtDNA genome from which to design PCR primer pairs (Table 1). To avoid

amplifying *numts*, long-distance PCR was used to amplify five overlapping fragments that were at least 2.8 kb in length (Table 1) [the mean length of mammalian *numts* is 194 bp (Richly & Leister, 2004)].

PCRs were performed in a final volume of 25  $\mu$ l and included 1X ThermoPol Buffer (New England BioLabs), 0.2 mM dNTPs, 0.2  $\mu$ M each primer, 2 U *Taq* DNA polymerase (New England BioLabs) and 0.02 U *Pfu* DNA polymerase (Stratagene). The thermal profile consisted of an initial denaturation at 94° for 2 min followed by 32 cycles of 94° for 1 min, 55 ° for 30 s and 72 ° for 4 min. The 32 cycles were followed by a single elongation step at 72 ° for 8 min. PCR products were cleaned with Qiaquick purification kits (Qiagen) then sequenced in both directions with the amplification as well as internal primers using BigDye v.3.1 (Applied Biosystems) following manufacturer's protocol modified to one-eighth reactions. When we were unable to obtain clean sequences directly from PCR products, shorter products were amplified and cloned into a pGEM vector (Promega). We manually aligned amplicons with the mtDNA genome of *M. kikuchii* and considered them mitochondrial in origin if protein-coding genes possessed open reading frames using the mammalian mtDNA genetic code.

### Comparative sequence alignments

In addition to the mtDNA genome sequence alignment for *Microtus*, we downloaded from GenBank all available mtDNA genome sequences for mammalian congeneric pairs ( $n = 10$  genera as

Table 1. Primer pairs used to amplify *M. rossiaemeridionalis* mitochondrial genome and sizes of their expected products

Primer name	Primer pairs (5' → 3')	Expected product size (bp)
Mr_Cytb/Leu	CCTATTATACAGTCAAAGACTTCT	4224
Mr_Leu/Cytb	TTAGGGAGAGGATTTGAACC	
Mr_CO1-2	CATCAATATGAAACCACCAG	3602
Mr_Gly	GAWCTAACTGATTGGAAGTCAG	
Mr_CO3	CTCSGAAGTATTYTTCTTTG	2842
Mr_Leu	GTTCCCTAAGACCAAYGGATTAC	
Mr_Ser	GCAAGARCTGCTAAYTCATG	3413
Mr_Cytb/ND4	TAAAATTAGTGCTAGTACGCCA	
Mr_Leu	CAGAGTCAGGAAATTGCGTA	3265
Mr_CO1	GTATTGTRATYCTGCGGCTAG	

Table 2. Mammalian mitochondrial genomes used in this study and their associated GenBank accession numbers

Species	Common name	Accession number
<i>Balaenoptera musculus</i>	blue whale	NC_001601
<i>Balaenoptera physalus</i>	finback whale	NC_001321
<i>Bos grunniens</i>	domestic yak	NC_006380
<i>Bos taurus</i>	cow	NC_006853
<i>Equus asinus</i>	ass	NC_001788
<i>Equus caballus</i>	horse	NC_001640
<i>Macaca mulatta</i>	rhesus monkey	NC_005943
<i>Macaca sylvanus</i>	Barbary ape	NC_002764
<i>Microtus kikuchii</i>	Taiwan vole	NC_003041
<i>Microtus rossiaemeridionalis</i>	sibling vole	DQ_015676
<i>Muntiacus muntjak</i>	Indian muntjac	NC_004563
<i>Muntiacus reevesi</i>	Chinese muntjac	NC_004069
<i>Ochotona collaris</i>	collared pika	NC_003033
<i>Ochotona princeps</i>	American pika	NC_005358
<i>Pan paniscus</i>	pygmy chimpanzee	NC_001644
<i>Pan troglodytes</i>	chimpanzee	NC_001643
<i>Pteropus dasymallus</i>	Ryukyu flying fox	NC_002612
<i>Pteropus scapulatus</i>	little red flying fox	NC_002619
<i>Rhinolophus monaceros</i>	Formosan lesser horseshoe bat	NC_005433
<i>Rhinolophus pumilus</i>	Okinawa least horseshoe bat	NC_005434
<i>Ursus arctos</i>	brown bear	NC_003427
<i>Ursus maritimus</i>	polar bear	NC_003428

of March 2005) and assembled comparative sequence alignments for each pair (Table 2). Because the rapidly evolving control region (d-loop) could disproportionately inflate the nucleotide divergence between members of a congeneric pair, we assembled a second set of sequence alignments that consisted of the entire mtDNA genomes minus the control regions. Finally, we assembled separate sequence alignments for all mtDNA protein-coding genes and the control region.

#### Data analysis

Various values of nucleotide diversity were estimated using DnaSP (Rozas et al., 2003), MEGA version 2.1 (Kumar et al., 2001) and PAUP\* (Swofford, 2002). We calculated the average number of nucleotide differences per site between two sequences for all mtDNA gene and genome

sequence pairs. We tested whether there was a difference between the number of nucleotide substitutions in mtDNA genome datasets with and without the control region using a chi-square goodness of fit test. Because there is a pronounced transition bias in animal mtDNA (Brown et al., 1982; Irwin, Kocher & Wilson, 1991), we calculated the number of transversions per site at all sites and at four-fold degenerate sites within protein-coding genes for genomic dyads. Additionally, we calculated the number of synonymous and nonsynonymous substitutions per site within protein-coding genes for genomic dyads. We then performed an analysis of variance on each genomic dyad, blocking by gene, followed by Dunnett's pairwise comparison procedure to detect any differences in the distribution of nucleotide substitutions between *Microtus* and the remaining genera. This includes the number of nucleotide substitutions, the number of transversions at all sites and at four-fold degenerate sites within protein-coding genes, and the number of synonymous and nonsynonymous substitutions per site within protein-coding genes. Our single gene comparisons consisted of point estimates rather than distributions and thus statistical tests were not warranted. However, some interesting patterns emerge from these data (see Results).

Taxonomic designations usually do not reflect absolute dates of nodes in evolutionary trees (Johns & Avise, 1998). Thus, our genome comparisons among lineages could be biased if some genera are significantly older than others. We directly compared the genetic divergence within *Microtus* to that within other genera irrespective of their date of origin. We also considered chronology by correcting all nucleotide substitution values using published dates of evolutionary divergence for each conspecific species pair. We only used dates derived from non-mtDNA data (i.e., paleontological, chromosomal, nuclear or allozyme) to avoid the circularity of using divergence times originally estimated from mtDNA gene trees (Table 3).

#### Rodent comparisons

*Microtus* was the only rodent genus with two complete mtDNA sequences available from GenBank for congeneric species. However, we thought it important to gauge evolution in *Microtus* against other rodent genera and did so using cytochrome *b*

Table 3. Provisional divergence estimates for mammalian genera used in this study and their associated sources

Genus	Divergence Time (MYA)	Sources (MRCA)
<i>Balaenoptera</i>	5	Barnes, 1976; Barnes Domning and Ray, 1985
<i>Bos</i>	1.2	Kurtén & Anderson, 1980; Olsen, 1990; Ritz et al., 2000
<i>Equus</i>	4	Simpson, 1951; Lindsay, Opdykem and Johnson, 1980
<i>Macaca</i>	2	Delson, 1980; Rook, Mottura and Gentili, 2001; Abegg and Thierry, 2002
<i>Microtus</i>	1.5	Repenning, 1980, 1990; Chaline et al., 1999
<i>Mutiacus</i>	1	Ma et al., 1985; Groves and Grubb, 1990; Dong, 1993
<i>Ochotona</i>	1.8	Kurtén and Anderson, 1980; Mead, 1987; Hafner and Sullivan, 1995
<i>Pan</i>	1.8	Stone et al., 2002; Yu et al., 2003
<i>Pteropus</i>	5.3	Colgan and Flannery, 1995; Kirsh et al., 1995
<i>Rhinolophus</i>	1.8	Maree and Grant, 1997; Servent, Francis and Ricklefs, 2003
<i>Ursus</i>	1	Kurtén, 1964; Ray, 1971

MRCA = most recent common ancestor.

sequences from GenBank. Comparative cytochrome *b* alignments were assembled for seven additional genera from a variety of major rodent lineages: (*Chaetodipus*: *C. arenarius* AY926400, *C. spinatus* AY926398; *Ctenomys*: *C. coyhaiquensis* AF071753, *C. rionegrensis* AF538376; *Geomys*: *G. bursarius* AY393941, *G. texensis* AY395301; *Microtus*: *M. kikuchii* (NC003041), *M. rossiaemeridionalis* (DQ015676); *Mus*: *M. musculus* NC005089, *M. spretus* AY224678; *Rattus*: *R. norvegicus* NC001665, *R. rattus* AB033702; *Sigmodon*: *S. fulviventer* AF293400, *S. hispidus* AF425214; *Tamias*: *T. striatus* AF147650, *T. minimus* AY292715). Values of nucleotide diversity were calculated as for whole genomes and all values were

Table 4. Provisional divergence estimates for rodent genera used in this study and their associated sources

Genus	Divergence Time (MYA)	Sources (MRCA)
<i>Chaetodipus</i>	5.3	Wahlert, 1993; Korth, 1994
<i>Ctenomys</i>	1.8	Reig, 1989; Lacey, Patton and Cameron, 2000
<i>Geomys</i>	4.0	Kurtén and Anderson, 1980; Spradling et al., 2004
<i>Mus</i>	3.0	Bonhomme et al., 1984; Thaler, 1986
<i>Rattus</i>	4.0	Baverstock et al., 1983; Odom, Robichaux and Deininger, 2004
<i>Sigmodon</i>	2.5	Baskin, 1986; Peláez-Campomanes et al., 2005
<i>Tamias</i>	1.8	Nadler, 1977; Levensen et al., 1985

MRCA = most recent common ancestor.

corrected for times of divergence (Table 4). Again, because these single gene comparisons consisted of point estimates rather than distributions, statistical tests were unwarranted.

## Results

### *Microtus mitochondrial genome*

We amplified the entire mtDNA genome of *M. rossiaemeridionalis* in five overlapping fragments ranging in size from 2.8–4.2 kb (Table 1). We collected bidirectional sequence data for >90% of the entire genome and did not encounter any contamination by nuclear copies of mtDNA sequences (*numts*). All protein-coding regions possessed open reading frames, and we identified incomplete stop codons that are presumably completed during polyadenylation (Ojala, Montoya & Attardi, 1981). The total length of the mitochondrial sequence was 16,283 bp and its gene order was standard for eutherian mammals. The entire genome sequence has been deposited in the GenBank database (accession #DQ015676).

Base compositions of the two microtine mtDNA genomes were highly consistent (*M. rossiaemerdionalis*/*M. kikuchii*: adenosine 0.327/0.330, cytosine 0.277/0.262, guanine 0.136/0.136, thymine 0.260/0.273). The two *Microtus* genomes differed at 2034 nucleotide sites (12.5%) and the transition/transversion ratio was 2.57. The vast majority of the nucleotide substitutions were singletons. There was one 37 bp indel in the control region, but there were no repetitive features that confounded sequencing or genomic alignments.

#### Congeneric species alignments

The difference between the proportion of nucleotide substitutions in mtDNA genome datasets with and without the control region was not significantly different ( $df = 10$ ;  $p = 0.83$ ;  $\chi^2 = 5.8$ ). Thus, results reported herein were derived from sequence alignments that included the control region.

If one qualitatively ranks the pairwise sequence divergence between congeneric mtDNA genomes, *Microtus* is the highest (fastest) among surveyed

mammalian genera (Figure 1a). Quantitatively, the divergence within *Microtus* is greater than all congeneric pairs (Dunnett's pairwise  $p < 0.05$ ) except *Pteropus*. After correcting for divergence times, *Microtus* is significantly different from all genera (Dunnett's pairwise  $p < 0.05$ ) including *Pteropus* (Figure 1b). Likewise, values for corrected estimates of the number of transversions per site were also highest for *Microtus* at all sites and at four-fold degenerate sites (Dunnett's pairwise  $p < 0.05$ ; Figure 2) as were corrected estimates for the number of synonymous and nonsynonymous substitutions (Dunnett's pairwise  $p < 0.05$ ; Figure 3).

In theory, the whole genome results could be biased by one or two rapidly evolving genes (Pesole et al., 1999). When we break the analysis down gene-by-gene, we see that every protein-coding gene as well as the control region is evolving rapidly in *Microtus* (Figure 4). Dyads of complete mitochondrial genome sequences were unavailable for other congeneric rodents; therefore, we compared entire cytochrome *b* sequences from GenBank and determined that *Microtus* is

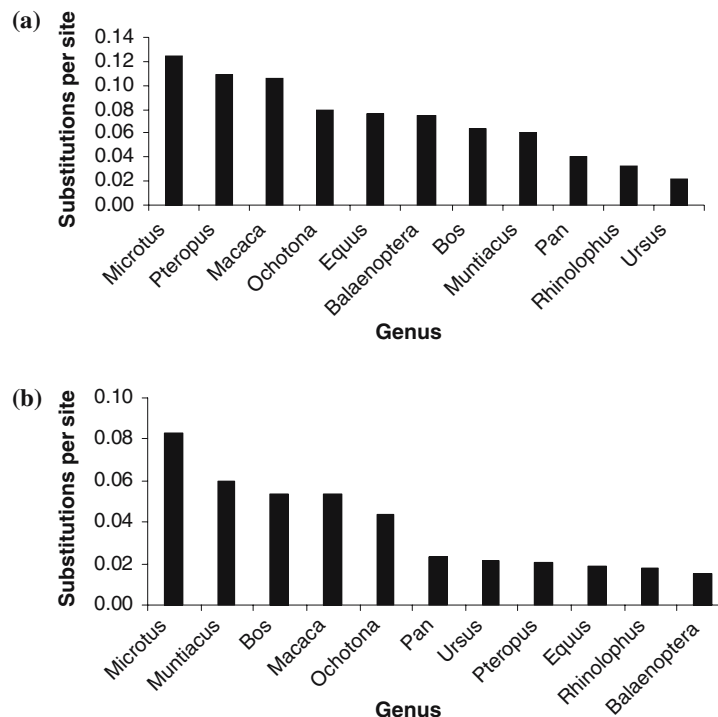


Figure 1. Number of nucleotide substitutions per site between two sequences for the mitochondrial genomes of congeneric mammalian species pairs. (a) Uncorrected for times of divergence. (b) Corrected for times of divergence. *Microtus* is significantly different from all other taxa in the corrected analyses and all except *Pteropus* in the uncorrected analyses.

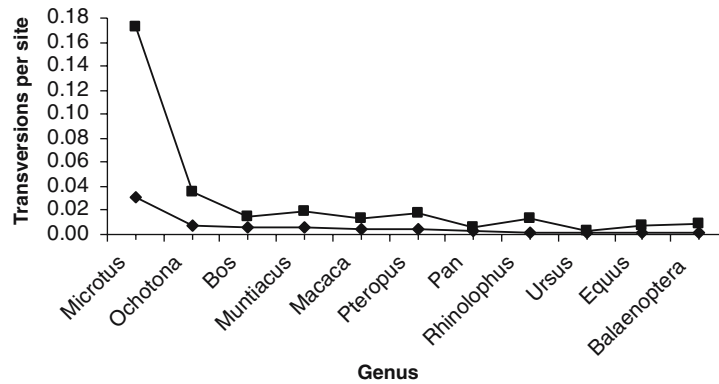


Figure 2. Number of transversions per site at all sites ♦ and at four-fold degenerate sites ■ for all protein-coding genes. Data have been corrected for times of divergence. *Microtus* is significantly different from all other taxa at all sites as well as at four-fold degenerate sites.

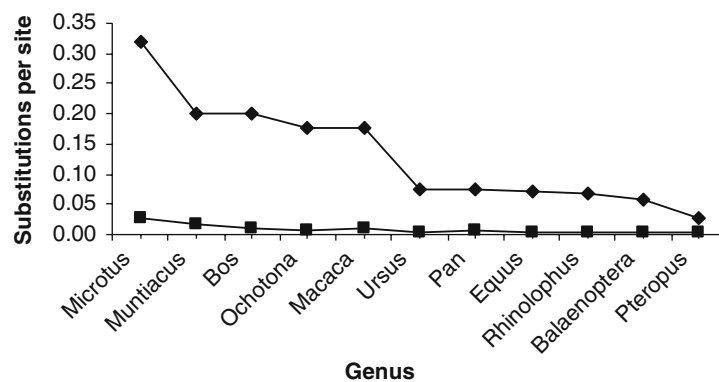


Figure 3. Number of synonymous substitutions per site ♦ and nonsynonymous substitutions per site ■ for all protein-coding genes. Data have been corrected for times of divergence. *Microtus* is significantly different from all other taxa for both synonymous and nonsynonymous substitutions.

also evolving rapidly when compared to other rodents (Figure 5).

## Discussion

Our data indicate that the *Microtus* mtDNA genome is evolving much more rapidly than any other sampled vertebrate. Coupled with the chromosome data cited herein, it seems apparent that microtines are evolutionarily quite exceptional. Our results indicate that the number of nucleotide substitutions in *Microtus* mtDNA is almost twice that of other mammalian genera (Figure 1b), transversions in protein-coding genes are 3–6 times more frequent in *Microtus* (Figure 2) and synonymous substitutions are up to four times as

frequent (Figure 3). In the separate gene analyses, *Microtus* is evolving rapidly at all protein-coding genes as well as the non-coding control region (Figure 4).

Rodent genomes are known to evolve more rapidly than other vertebrates (Wu & Li, 1985; Cooper et al., 2003; Mouse Genome Sequencing Consortium 2002), and mtDNA evolution may be negatively correlated with body size/generation time and positively correlated with metabolic rate (Martin & Palumbi, 1993). Like voles, pikas (*Ochotona*) and horseshoe bats (*Rhinolophus*) have small bodies, rapid metabolic rates and short generation times and thus might be expected to evolve rapidly. However, our comparisons reveal that their rates of evolution pale in comparison to those of voles (Figures 1–4).

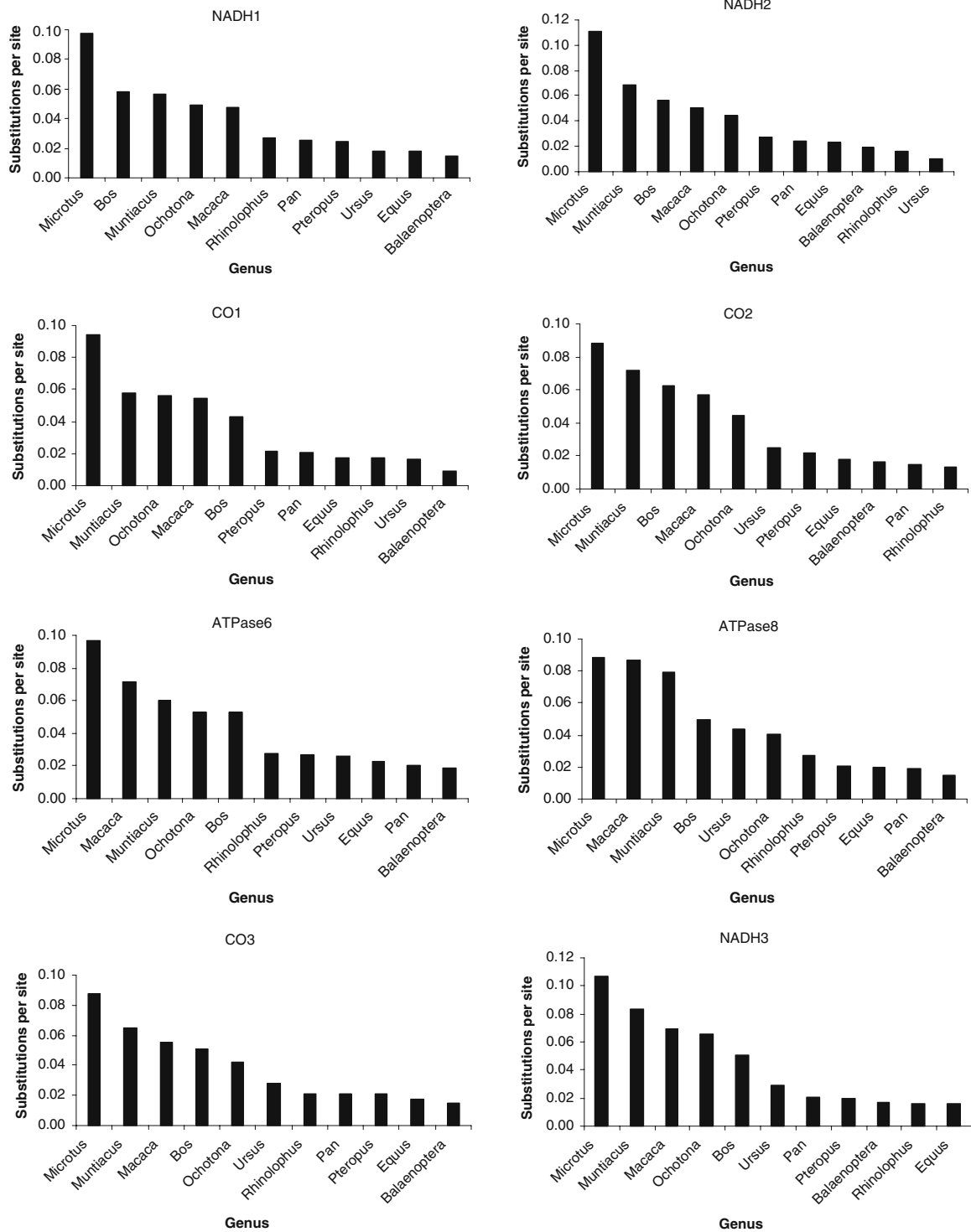


Figure 4. Number of nucleotide substitutions per site between two sequences for the mitochondrial protein-coding genes and control region of congeneric mammalian species pairs. Data have been corrected for times of divergence.

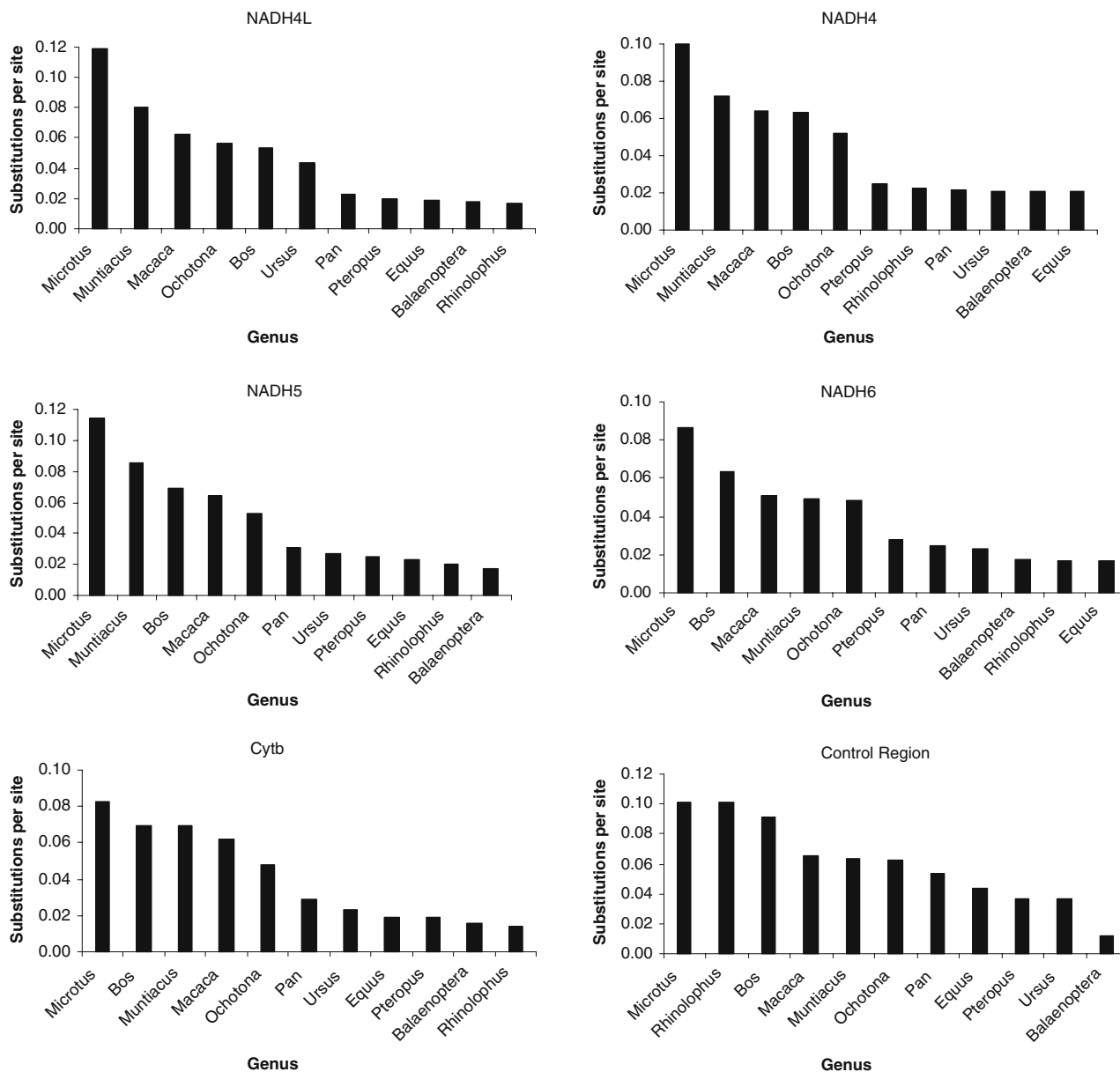


Figure 4. Continued.

In the cytochrome *b* comparisons, we find that *Microtus* is evolving faster than all other rodent genera surveyed and over twice the rate of *Mus* or *Rattus* (Figure 5). Likewise, rates of nucleotide substitution in tuco-tucos (*Ctenomys*) are considerably slower than *Microtus* (Figure 5) even though tuco-tucos have a rate of karyotypic evolution comparable to *Microtus*. Diploid numbers range from  $2n = 10$  to  $2n = 70$  in *Ctenomys* and, although the genus is over 3 million years old,

they are thought to have experienced a rapid diversification less than 2 million years ago (Castillo, Cortinas & Lessa, 2005). If chromosome rearrangements are indeed affecting nucleotide substitution rates in *Microtus*, they do not seem to be doing so at a comparable rate in tuco-tucos.

Cytogenetic studies of rodents have revealed a karyotypically diverse group with high levels of chromosomal variability (Patton & Sherwood, 1983), but most clades are dominated by a

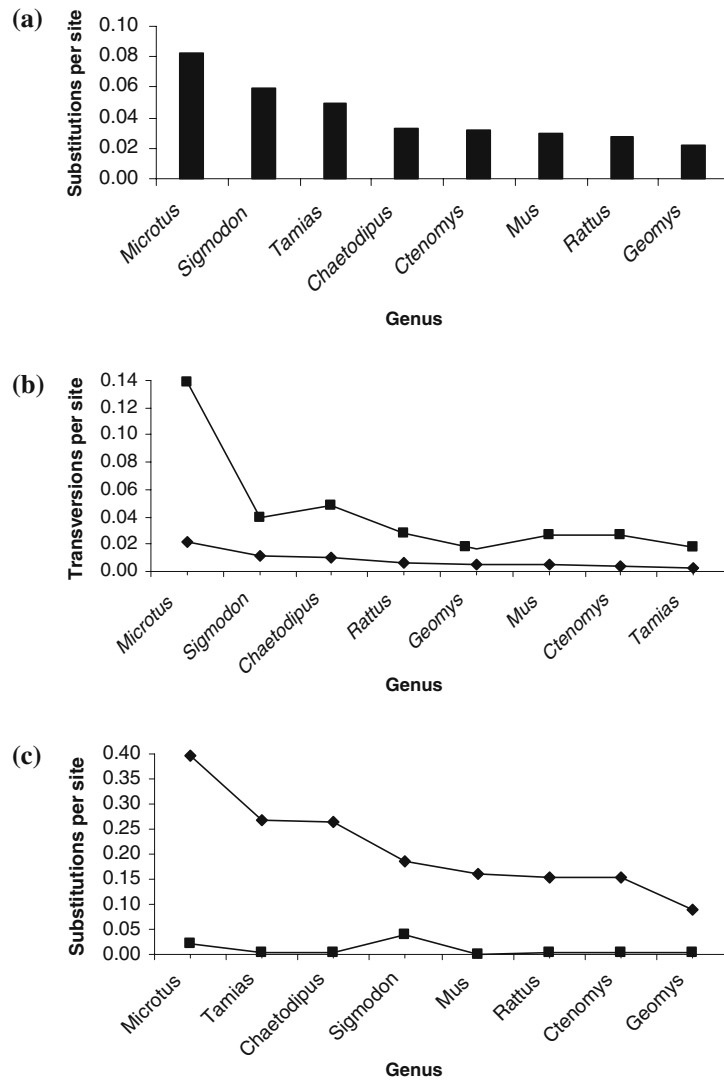


Figure 5. Rodent cytochrome *b* comparisons. (a) Number of nucleotide substitutions per site between two sequences. (b) Number of transversions per site at all sites ◆ and at four-fold degenerate sites ■. (c) Number of synonymous substitutions per site ◆ and nonsynonymous substitutions per site ■. Data in all panels have been corrected for times of divergence.

karyotypic pattern in which only one or few rearrangements account for most of the variability. In sharp contrast, ten different types of chromosomal rearrangements have been reported within arvicoline rodents (Modi, 1987). Many such chromosomal arrangements are presumably the cause (or result) of speciation (Mazurok et al., 2001). Whether karyotypic differentiation actually caused speciation within *Microtus* may be debatable, but it seems clear there is at least a correlation between chromosomal rearrangements and speciation. Many of the deep arvicoline lineages, including other genera of voles and muskrats, have conserved

karyotypes ( $2n = 52-54$ ) that do not depart widely from the primitive arvicoline karyotype of  $2n = 56$ . Only after the explosive radiation of *Microtus* does the arvicoline karyotype begin to differentiate.

Rodents are not the only mammals with flexible karyotypes that we considered. Muntjak deer (*Muntiacus*) also displays drastic karyotypic variation ( $2n = 6-46$ ), and one species within the genus has the lowest diploid chromosome number known among mammals,  $2n = 6$  (Wurster & Benirschke, 1970). *Muntiacus* is thought to have arisen ~2 MYA (like *Microtus*) and there is little

phenotypic diversity within the genus (again like *Microtus*). However, with regard to extant species, the microtines are more than  $6 \times$  as diverse:  $>60$  *Microtus* species versus ten *Muntiacus* species (Musser & Carleton, 1993; Wang & Lan, 2000). Furthermore, rates of mtDNA nucleotide substitution are much more rapid in *Microtus* than in *Muntiacus* (Figures 1–4).

The provisional divergence dates used to correct the substitution rates in this study were largely based on the paleontological record (Tables 3 and 4). Our intentions were to avoid the circularity of using divergence dates based on mtDNA gene trees for estimating genome-wide mtDNA substitution rates. The estimation of divergence times from molecular data and the issue of rate constancy among taxa have been controversial since their inception (Avice, 2004). If one were to use the standard measure of 2% divergence per million years inferred for animal mtDNA (Brown, George & Wilson, 1979), the estimated time of *Microtus* mtDNA genome divergence would have been  $\sim 6$  MYA. This date sharply conflicts with the fossil record (Repenning, 1980, 1990; Chaline et al., 1999), but could be explained by the coalescence of the mtDNA gene trees prior to speciation (Avice, 2000). Even if one ignores the fossil record and chooses to believe the molecular clock (i.e., *Microtus* is 6 million years old), then we must reconcile how  $>60$  species have arisen in only 6 million years. This rate (100,000 years per speciation event) must be compared to the average mammalian rate of 2.2 million years per speciation event, a 22-fold difference.

Our data clearly indicate that *Microtus* is evolving rapidly at the nucleotide level, but shed no light on how mitochondrial variation is linked to karyotypic diversity. As in most species, hybridization is uncommon in microtines (Baker et al., 1996). Therefore, chromosomal rearrangements could lead to reproductive isolation, which could then lead to speciation. However, it is unclear how such chromosomal rearrangements might influence rates of nucleotide substitution in the cytoplasmic mtDNA genome. An alternative explanation is that the elevated rate of nucleotide substitution somehow leads to an increased incidence of chromosomal rearrangement.

The question remains as to whether a rapid rate of molecular evolution in the mtDNA genome might be coupled with a rapid rate of molecular

evolution within the nuclear genome. It is well-established that the human mtDNA genome evolves more rapidly ( $\sim 10 \times$ ) than the nuclear genome (Brown et al., 1979), but it is unclear whether this ratio is constant across mammalian lineages. Because the mtDNA genome contains a minimal amount of genetic information, many proteins critical to the function of the mitochondria must be imported from the nucleus where they are encoded (Stojanovski et al., 2003). While the mtDNA and nuclear genomes are mechanistically linked, it is not known if their relative rates of nucleotide substitution are linked as well. For example, if rates of nucleotide substitution are largely controlled by generation time or metabolic rate (Martin & Palumbi, 1993; Li 1997), one might expect to see concordant relative rates of substitution in both cytoplasmic and nuclear genomes. Alternatively, one can hypothesize that a mutation in a nuclear gene critical to mtDNA replication (e.g., mtDNA polymerase) could result in an elevated rate of mtDNA evolution but have no influence on substitution rates in the nuclear genome. Thus, rates of molecular evolution across genomes may or may not be correlated within any particular lineage.

The genetic diversity exhibited by *Microtus* contrasts sharply with its phenotypic homogeneity. Thus, genes responsible for morphological diversity do not seem to be influenced by either the rapid rate of mtDNA nucleotide substitution or by chromosomal rearrangements. It would be advantageous to understand how these two facets became uncoupled during the course of *Microtus* evolution. Perhaps the speciation has been so rapid that the phenotypic characters have not yet had time to diversify, or perhaps most of the genetic variation is functionally neutral (i.e., purifying selection is strong). Because *Microtus*' evolutionary history has proven difficult to reconstruct due to its rapid diversification, many molecular studies are concerned with reconstructing phylogenetic relationships. Future molecular studies on *Microtus* might uncover novel patterns of speciation or reveal evidence of concordance between evolutionary rates of nuclear and mtDNA genomes.

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