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Molecular phylogeny and biogeography of Oriental voles: genus *Eothenomys* (Muridae, Mammalia)

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Abstract

Oriental voles of the genus *Eothenomys* are predominantly distributed along the Southeastern shoulder of the Qinghai-Tibetan Plateau. Based on phylogenetic analyses of the mitochondrial cytochrome *b* gene (1143 bp) obtained from 23 specimens (eight species) of Oriental voles collected from this area, together with nucleotide sequences from six specimens (two species) of Japanese red-backed voles (*Eothenomys andersoni* and *Eothenomys smithii*) and five species of the closely related genus *Clethrionomys*, we revised the systematic status of *Eothenomys*. We also tested if vicariance could explain the observed high species diversity in this area by correlating estimated divergence times to species distribution patterns and corresponding paleo-geographic events. Our results suggest that: (1) the eight species of Oriental voles form a monophyletic group with two distinct clades, and that these two clades should be considered as valid subgenera—*Eothenomys* and *Anteliomys*; (2) *Eothenomys eleusis* and *Eothenomys miletus* are not independent species; (3) Japanese red-backed voles are more closely related to the genus *Clethrionomys* than to continental Asian *Eothenomys* taxa; and (4) the genus *Clethrionomys*, as presently defined, is paraphyletic. In addition, the process of speciation of Oriental voles appears to be related to the Trans-Himalayan formation via three recent uplift events of the Qinghai-Tibetan Plateau within the last 3.6 million years, as well as to the effects of the mid-Quaternary ice age.

Keywords: Phylogeny; Cytochrome *b*; Oriental voles; *Eothenomys*; Clethrionomyini; Biogeography; Trans-Himalayan Ranges; Speciation

1. Introduction

Oriental voles are traditionally included in the genus *Eothenomys* (Muridae: Clethrionomyini), and inhabit the Trans-Himalayan Ranges of Southwest China, small parts of Northeast Burma and the Assam province in India (Fig. 1). According to the fossil record, this group is of recent origin, and most likely diversified during the late Pliocene (Zheng, 1993). It is assumed that speciation events within this group are linked to historical changes in the geography of their main distribution habitat, the Trans-Himalayan Ranges, which have been severely affected by several uplift events along the Qinghai-Tibetan Plateau. These geological processes have been considered to play a fundamental vicariant role in species

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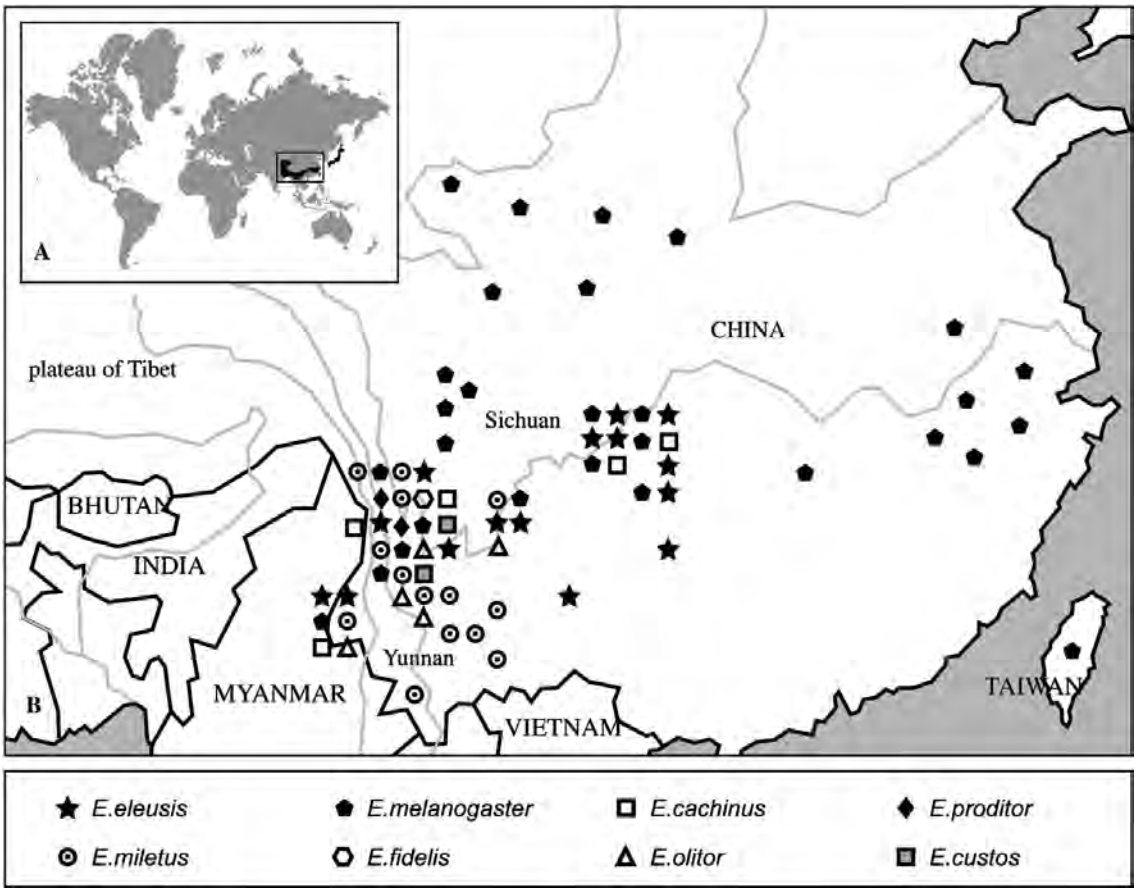

Fig. 1. (A) Map showing the distribution of Oriental voles (Continental mainland) and Japanese red-backed voles (Japan). (B) Distribution of the eight species of Oriental voles and the locations of sites where the specimens of each species have been reported (after Wang and Li, 2000).

divergence of many other vertebrates endemic to this region (Chen et al., 1998; Pang et al., 2003; Yu et al., 2000). Thus, we wanted to test whether the uplift of the Qinghai-Tibetan Plateau also facilitated speciation and adaptation processes of Oriental voles.

The taxonomy of the genus *Eothenomys* is under considerable debate, primarily due to the inherent morphological plasticity among members of this group and to subjectivity regarding the descriptions of some species. This is reflected by the contrasting definitions of the subgenera and genera ascribed to the group (Table 1). Indeed, 7–9 nominal species have been assigned to the genus *Eothenomys* under the subtribe Clethrionomyini based on morpho-anatomical characters or cytological data (Allen, 1940; Corbet, 1978; Ellerman and Morrison-Scott, 1951; Hinton, 1923, 1926; Musser and Carleton, 1993; Wang and Li, 2000; Yang et al., 1998; see Table 1 for summary). Allen (1940) further classified the genus *Eothenomys* into three subgenera: *Eothenomys*, *Anteliomys*, and *Caryomys*. Under this classification scheme, the subgenus *Eothenomys* contains species with the first upper molars displaying three outer and four inner salient angles, and the last upper molars exhibiting three or four outer salient angles. The subgenus *Anteliomys* is comprised of species with the first upper molars possessing three outer and three inner salient angles. The subgenus *Caryomys* includes only two species, both of which have inter-bedded molar triangles in the first and second lower molars. Ma and Jiang (1996) revised the taxonomic status of the subgenus *Caryomys* and elevated it to genus rank based on its karyotype ($2n = 54$) compared to the karyotypes of other species in *Eothenomys* ($2n = 56$) (Chen et al., 1994; Yang et al., 1998). They left only two subgenera in *Eothenomys*, *Eothenomys* and *Antelionomys*, as was also suggested by Wang and Li (2000) (Table 1).

Early classification schemes generally subdivided the subtribe Clethrionomyini into two groups based on the morphology of the molars: *Clethrionomys* (where species have rooted molars) and the *Eothenomys*/*Caryomys* complex (where species have rootless molars). However, following this scheme, the position of Japanese red-backed voles was ambiguous since these species possess rooted molars that appear quite late in adult life. Consequently, Japanese red-backed voles, which were traditionally included by most authorities in *Eothenomys*, are now sometimes reassigned to their own genus, *Phaulomys*, Thomas (1905) (Musser and Carleton, 1993; Kawamura, 1988;

Table 1
Different opinions on the taxonomy of the genus *Eothenomys*

Wang and Li (2000)	Allen (1940)	Hinton (1923, 1926)	Musser and Carleton (1993)
Genus *Eothenomys*	Genus *Eothenomys*	Genus *Eothenomys*	Genus *Eothenomys*
Subgenus *Eothenomys*	Subgenus *Eothenomys*	—	—
E. melangoster	*E. melangoster*	*E. melangoster*	*E. melangoster*
E. me. melanogaster	*E. me. melanogaster*	*E. me. melanogaster*	—
E. me. mucronatus	*E. me. melanogaster*	*E. me. mucronatus*	—
E. me. colurnus	*E. me. colurnus*	*E. me. colurnus*	—
E. me. libonotus	—	*E. me. libonotus*	—
E. cachinus	—	*E. me. cachinus*	—
E. eleusis	*E. eleusis*	*E. me. eleusis*	—
E. eleusis aurora	*E. miletus aurora*	*E. me. aurora*	—
E. miletus confinii	*E. eleusis*	*E. me. confinii*	—
E. miletus miletus	*E. miletus miletus*	*E. me. miletus*	—
E. miletus miletus	*E. miletus miletus*	*E. fidelis*	—
Subgenus *Antelionomys*	Subgenus *Antelionomys*	—	—
E. olitor	*E. olitor*	*E. olitor*	*E. olitor*
E. proditor	*E. proditor*	*E. proditor*	*E. proditor*
—	—	Genus *Antelionomys*	—
E. chinensis	*E. chinensis*	*A. chinensis*	*E. chinensis*
E. c. chinensis	*E. c. chinensis*	*A. c. chinensis*	—
E. c. tarquinius	*E. c. tarquinius*	*A. c. tarquinius*	—
E. wardi	*E. c. wardi*	*A. wardi*	—
E. custos	*E. custos*	*A. custos*	*E. custos*
E. c. custos	*E. c. custos*	*A. c. custos*	—
E. c. rubellus	*E. c. rubellus*	*A. c. rubelius*	—
E. c. hintoni	*E. c. hintoni*	—	—
Genus *Caryomys*	Subgenus *Caryomys*	Genus *Evotomys*	—
Ca. inez	*E. inez*	*Ev. rufocanus shanseius*	*E. inez*
Ca. eva	*E. eva*	*Ev. r. shanseius*	*E. eva*
—	—	*Ev. r. regulus*	*E. regulus*
Genus *Clethrionomys*	Genus *Clethrionomys*	—	—
C. rufocanus shanseius	*C. rufocanus shanseius*	*Ev. rufocanus shanseius*	*E. shanseius*
Genus *Phaulomys*	—	—	Genus *Phaulomys*
P. andersoni	—	*Ev. r. andersoni*	*P. andersoni*
P. smithii	—	*Ev. r. smithii*	*P. smithii*

"—" Indicates that the taxon is not recognized by this author.

Suzuki et al., 1999). Wang and Li (2000) accepted this designation and hypothesized that the subtribe Clethrionomyini includes four valid genera: *Clethrionomys*, *Eothenomys*, *Caryomys*, and *Phaulomys*. Yang et al. (1998) summarized all available karyotype data and discussed the putative evolutionary relationships among the main lineages of the Clethrionomyini. These species are diploids and generally possess chromosome numbers between 54 and 56 with a fundamental arm number between 54 and 60. However, cytological data sometimes provides discordant results. For example, Yang et al. (1998) reported that the karyotype of the Yulong vole (*Eothenomys proditor*) (distributed in Lijiang region, Northwest Yunnan of China) exhibit a dramatically different diploid chromosome number ($2n = 32$). In addition, these authors suggested that karyotype data do not provide enough convincing evidence to elucidate the phylogenetic relationships within this group. A comprehensive phylogeny based on unambiguous characters and appropriate phylogenetic reconstruction methods is still required to shed light on the classification and evolutionary history of this group. In this context, Cook et al. (2004) recently examined the molecular systematics of red-backed voles, and suggested that the genus *Clethrionomys* is paraphyletic with respect to both *Eothenomys* and *Alticola*. However, important taxa from the genus *Eothenomys* were not intensively sampled for this study, with only one Oriental vole species included. Thus, it is imperative to include additional species from the genus *Eothenomys* to better investigate the phylogenetic relationships among the subtribe Clethrionomyini.

The levels of genetic divergence typically found between sister species and their congeners are usually in the range in which the mitochondrial cytochrome *b* (cyt *b*) gene is phylogenetically informative. The cyt *b* gene is usually not affected by severe saturation effects involving superimposed nucleotide substitutions (Johns and Avise, 1998; Meyer, 1993; Moritz et al., 1987). Hence, it has often been used to reconstruct phylogenetic relationships within and among numerous vertebrate groups (Andrews et al., 1998; Irwin et al., 1991; Johns and Avise, 1998), including arvicolid rodents (Cook et al., 2004; Iwasa and Suzuki, 2002; Suzuki et al., 1999). To explore the molecular phylogenetic relation-

ships of Oriental voles and their taxonomic affiliation with other members of the subtribe Clethrionomyini, we thus sequenced their mitochrondrial DNA cyt *b* gene. Drawing on this data, the goals of this study were: (1) to elucidate the phylogeny of *Eothenomys* from the Southeast border default region of the Qinghai-Tibetan Plateau; (2) to revise the taxonomic status of Oriental voles as well as other species in the subtribe Clethrionomyini with reference to the molecular phylogeny constructed; e.g., we wanted to test whether the rank of genus or subgenus assigned to groups such as *Eothenomys*, *Anteliomys*, and *Phaulomys* are valid; (3) to investigate if the divergence events within the group are correlated with recent uplift events of the Qinghai-Tibetan Plateau. To achieve this final goal, we compared divergence times inferred from cyt *b* data with the orogenic events and corresponding biogeographic distribution patterns of voles from this particular area.

2. Materials and methods

2.1. Data collection

Voles were collected along the Southwestern shoulder of the Trans-Himalayan Ranges (Fig. 1). The voucher numbers and localities of the collected samples are listed in Table 2. Except for *Eothenomys fidelis*, specimens

Table 2
Taxonomic sampling, accession numbers, and geographic area of origin

Species	Sample number	Haplotype	Sample locality	Accession No.
Eothenomys eleusis	*E. eleusis* 003	*E. eleusis* 003	Mount Wuliang, Jingdong, YN	AY426678
	E. eleusis 009	*E. eleusis* 009	Mount Wuliang, Jingdong, YN	AY426679
E. miletus	*E. miletus* 014	*E. miletus* 014	Mount Yulong, Lijiang, YN	AY426683
	E. miletus 029	*E. miletus* 029	Mount Wuliang, Jingdong, YN	AY426684
	E. miletus 030	Same as *E. miletus* 014	Mount Wuliang, Jingdong, YN	
	E. miletus 044	Same as *E. miletus* 029	Mount Wuliang, Jingdong, YN	
	E. miletus 98823	*E. miletus* 98823	Mount Ailao, YN	AY426685
	E. miletus 98830	*E. miletus* 98830	Mount Ailao, YN	AY426686
E. cachinus	*E. cachinus* 088	*E. cachinus* 088	Zhaotong, YN	AY426675
E. fidelis	*E. fidelis* 084	*E. fidelis* 084	Lijiang, YN	AY426680
	E. fidelis 97599	Same as *E. fidelis* 84	Lijiang, YN	
E. melanogaster	*E. melanogaster* 201039	*E. melanogaster* 201039	Mount Wawu, SC	AY426681
	E. melanogaster 201040	*E. melanogaster* 201040	Mount Wawu, SC	AY426682
E. custos	*E. custos* 98810	*E. custos* 98810	Lijiang, YN	AY426676
	E. custos 98812	*E. custos* 98812	Lijiang, YN	AY426677
	E. custos 98814	Same as *E. custos* 98810	Lijiang, YN	
	E. custos 98820	Same as *E. custos* 98810	Lijiang, YN	
E. proditor	*E. proditor* 97585#	*E. proditor* 97585	Mount Yulong, Lijiang, YN	AY426691
	E. proditor 97592#	Same as *E. proditor* 97585	Mount Yulong, Lijiang, YN	
E. olitor	*E. olitor* 105	*E. olitor* 105	Zhaotong, YN	AY426687
	E. olitor 106	*E. olitor* 106	Zhaotong, YN	AY426688
	E. olitor 98448	*E. olitor* 98448	Zhaotong, YN	AY426689
	E. olitor 98449	*E. olitor* 98449	Zhaotong, YN	AY426690
E. andersoni	*E. andersoni* CH	*E. andersoni* CH	Central Honshu, JP	AB037290
	E. andersoni NH	*E. andersoni* NH	Northern Honshu, JP	AB037281
	E. andersoni WH	*E. andersoni* WH	Western Hunshu, JP	AB037296
E. smithii	*E. smithii* NH	*E. smithii* NH	Northeastern Honshu, JP	AB037305
	E. smithii SHI	*E. smithii* SHI	Shikoku, JP	AB037313
	E. smithii	*E. smithii**	Honshu, JP	AB104508
Clethrionomys glareolus	*C. glareolus*	*C. glareolus**	Unknown	AF119272
C.rutilus	*C. rutilus*	*C. rutilus**	Unknown	AF119274
C. rex	*C. rex*	*C. rex**	Unknown	AB031582
C. rufocanus	*C. rufocanus*	*C. rufocanus**	Unknown	AB031580
C. gapperi	*C. gapperi*	*C. gapperi**	Unknown	AF272639
Microtus clarkei	*C. clarkei*	*C. clarkei* 103	Zhongdian, YN	AY641526
Arvicola terrestris		*Arvicola terrestris**	Unknown	AF119269
Microtus gregalis		*Microtus gregalis**	Unknown	AF163895
Ellobius fuscocapillus		*Ellobius fuscocapillus**	Unknown	AF126430
Myopus schisticolor		*Myopus schisticolor**	Unknown	AF119263
Phenacomys intermedius		*Phenacomys intermedius**	Unknown	AF119260
Ondatra zibethicus		*Ondatra zibethicus**	Unknown	AF119277
Volemys kikuchii		*Volemys kikuchii**	Unknown	AF348082
Synaptomys borealis		*Synaptomys borealis**	Unknown	AF119259

Note. # Denotes formalin-fixed tissues; * denotes sequences from GenBank; YN, Yunnan; SC, Sichuan; and JP, Japan,

were identified based on external characteristics and skull morphology following the system of Wang and Li (2000) (see Table 1). *E. fidelis* was defined according to its unique cytological pattern (Yang et al., unpublished data). Twenty-three specimens comprising seven Oriental vole species plus *E. fidelis* were included in the current study. Where subspecies exist, we used nominal subspecies nomenclature. Despite several collection expeditions, we failed to obtain the Oriental vole species, *Eothenomys wardi* and *Eothenomys chinensis*. Clarke's vole *Microtus clarkei* (this study), together with eight species of Arvicolinae (sequences retrieved from GenBank) were chosen as outgroup taxa (Table 2). The strategy of multiple outgroup sampling was used to avoid inappropriate selection of outgroups, which might result in misleading conclusions about the phylogeny of the ingroup (Adachi and Hasegawa, 1995; Dalevi et al., 2001; Garcia-Moreno et al., 2001; Hillis, 1996).

Genomic DNA was extracted from 21 freshly frozen voles following Luo et al. (1999). Two formalin-fixed specimens were extracted according to Xiao et al. (1997). Two universal cyt *b* primers: L14724 5′-CG AAGCTTGATATGAAAAACCATCGTTG-3′ (Pääbo and Wilson, 1988) and H15915R 5′-GGAATTCATCT CTCCGGTTTACAAGAC-3′ (Irwin et al., 1991) were initially used to amplify and sequence the cyt *b* gene. PCRs were conducted in a total volume of 50 μl PCR cocktail that included 1× buffer with 0.15 mmol $MgCl_2$ (Sina-American), 0.25 mM dNTPs (Amersco), 1 U *Taq* DNA polymerase (Sina-American) and 25–50 ng genomic DNA. Following a 2-min denaturing period at 95 °C, PCR was conducted for 40 cycles at 95 °C for 60 s, 50 °C for 60 s, and 72 °C for 80 s, followed by a final extension at 72 °C for 5 min. Based on partial cyt *b* sequences obtained, two internal primers (CYTBL320 5′-GCAG TTTACTACGGCTCCTAC-3′ and CYTBH370 5′-GC CCATAAATGCTGTTGCTAT-3′) were designed for subsequent reactions. The PCR condition with L14724 and CYTBH370 was: 2 min at 95 °C followed by 35 cycles of 95 °C for 50 s, 56 °C for 45 s, and 72 °C for 50 s; and the PCR condition with CYTBL320 and H15915R was: 2 min at 95 °C, and 40 cycles of 95 °C for 50 s, 50 °C for 50 s, and 72 °C for 60 s. Both reactions concluded with a posterior extension of 5 min.

PCR products were purified with a gel extraction kit (Watson BioMedical). Double-stranded PCR products were directly sequenced from both directions with an ABI 377 automatic sequencer (Perkin–Elmer) using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA polymerase FS, Applied Biosystems). The inadvertent amplification and possible inclusion of nuclear pseudo-gene sequences was checked by observing if the obtained sequences translated properly, that is, whether they possessed conventionally positioned start and stop codons, and no false stop codons, insertions or deletions. One pseudo-gene sequence was detected for *Eothenomys olitor*. This sequence was discarded from the analyses.

2.2. Data analyses

All sequences were aligned using the DNASTAR software package 5.0 (DNASTAR) and confirmed by eye. The program DAMBE 4.1.19 (Xia and Xie, 2001) was used to identify haplotypes and to analyze saturation plots. Other parameters (variable sites, parsimony informative sites, and base composition biases) were obtained from PAUP 4.0b10 (Swofford, 2002).

We performed a wide array of phylogenetic analyses using different methods to gauge the robustness of our resulting hypotheses. These methods were maximum parsimony (MP), neighbor-joining with maximum likelihood distance (NJ), maximum likelihood (ML) as implemented in PAUP* Version 4.0b10 (Swofford, 2002), and a Bayesian approach as implemented in MrBayes ver.2.01 (Huelsenbeck and Ronquist, 2001). Likelihood ratio tests (Goldman, 1993a,b; Huelsenbeck and Crandall, 1997), as implemented in MODELTEST 3.06 (Posada and Crandall, 1998), were employed to choose models for model-based methods (NJ, ML, and Bayesian analyses). The HKY + G + I model (Hasegawa et al., 1985) was selected by MODELTEST. All model parameters were estimated via the maximum likelihood procedure as implemented in PAUP* through an iterative process (Swofford et al., 1996, p. 445). The Shimodaira–Hasegawa test, as implemented in PAUP*, was used to test alternative phylogenetic hypotheses (Shimodaira and Hasegawa, 1999). Four independent MCMC chains were simultaneously run for 1,000,000 replicates by sampling one tree per 100 replicates with the Bayesian procedure. We discarded the first 100 trees as part of a burn-in procedure, and used the remaining 9900 sampling trees (whose log likelihoods converged to stable values) to construct a 50% majority rule consensus tree. In addition to Bayesian posterior probabilities, node supports were assessed using ML, MP, and NJ bootstraps (Felsenstein, 1985) with 120, 1000, and 1000 replicates, respectively.

To estimate divergence times, we first tested for consistency of molecular evolution rate of the cyt *b* gene sequences in different lineages using PHYLTEST2.0 (Kumar, 1996) and following the method of Takezaki et al. (1995). Owing to the inconsistency of the evolutionary rate in *Eothenomys custos*, divergence times and rates among lineages were estimated by r8s version 1.5 (Sanderson, 2003), since this program enables estimations of divergence time regardless of evolutionary rate inconsistencies. The earliest fossils of *Eothenomys* from the Trans-Himalayan area are recorded from the early Pleistocene, but no direct ancestor has yet been detected in Chinese fossil layers (Zheng, 1993). In Japan, however, the fossil record is

relatively complete and suggests that the divergence between the genus *Clethrionomys* and the ancestor of Japanese red-backed voles lived in the late Pliocene or early Pleistocene (Kawamura, 1988). For this study, we took the early Pleistocene divergence of Japanese red-backed voles and the genus *Clethrionomys* (1.80 million years ago; Mya) (Kawamura, 1988) as a calibration point to infer divergence times for the different lineages of Oriental voles.

3. Results

3.1. Sequence variations and phylogenetic information

The entire coding region of the cyt *b* gene was sequenced from 23 Oriental voles (Table 2), and deposited in GenBank (Accession Nos. AY426678–AY426690). Including the start and stop codons, all sequences were 1143 bp—the same as other related mammalian groups (Irwin et al., 1991; Iwasa and Suzuki, 2002). A total of 252 nucleotide sites were variable, 53 of which were parsimony-informative. Seventeen haplotypes were identified from the 23 sequences. The following taxa shared the same haplotype: *Eothenomys miletus* 030 and *E. miletus* 014; *E. miletus* 044 and *E. miletus* 029; *E. fidelis* 97599 and *E. fidelis* 084; *E. custos* 98814, *E. custos* 98820, and *E. custos* 98810; and *E. proditor* 97592 and *E. proditor* 97585. There were no shared haplotypes between different species, implying that no gene flow occurred.

The final dataset for phylogenetic analyses included 17 unique haplotype sequences from the 23 Oriental vole specimens, together with six sequences from 2 Japanese red-backed vole species (*Eothenomys andersoni* and *E. smithii*), five *Clethrionomys* sequences and nine sequences from eight genera of outgroup taxa. Base composition bias across taxa was not detected (p value = 1). The relative saturation test was performed on transitions and transversions (Fig. 2). The plots appeared to become

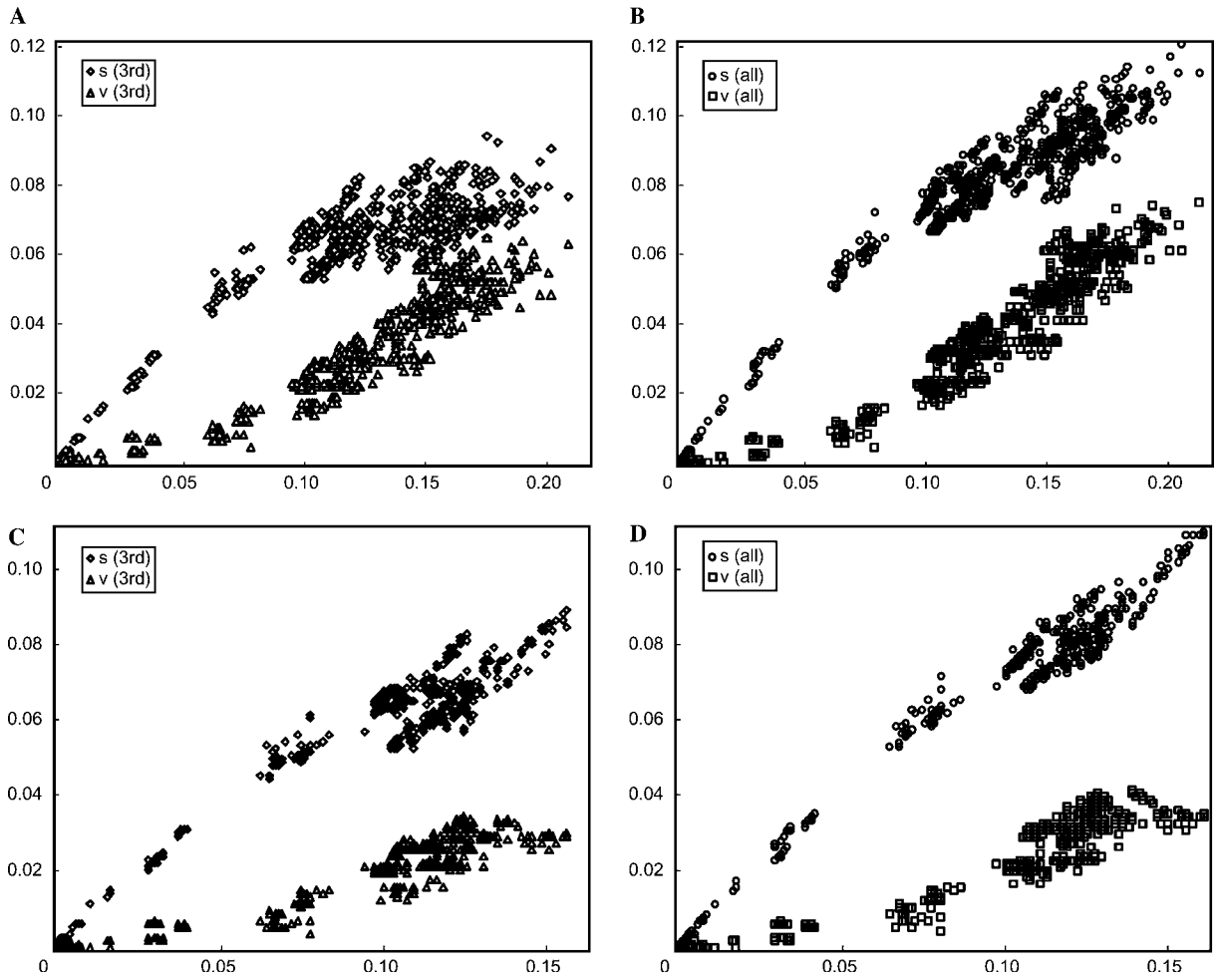

Fig. 2. The relative saturation test (Jukes and Cantor (1969) distances versus uncorrected pairwise distance) performed on transitions (s) and transversions (v) by considering all positions and the third codon position, respectively. Analysis involving 27 sequences from the subtribe Clethrionomyini and 9 outgroup taxa are shown in (A,B); analysis involving ingroup taxa sequences only are shown in (C,D).

saturated when outgroup taxa were included (Figs. 2A and B), notably in the case of substitution type on transitions at the third codon position (Fig. 2A). Nevertheless, neither substitution type exhibited a clear saturation plateau in Figs. 2C and D, suggesting a low frequency of multiple substitutions in our dataset among the ingroup taxa. The average pairwise distance between taxa was 26.42%; the maximum pairwise distance (64.1%) was recorded between *Myopus schisticolor* and *Arvicola terrestris*, and the minimum distance (0.084%) was found between *E. miletus* 14 and *Eothenomys eleusis* 9 (see Appendix A for details).

3.2. Phylogenetic analyses

Fig. 3 shows the ML tree constructed from a set of 37 cyt *b* sequences and confirming the monophyly of the subtribe Clethrionomyini. The other methods produced very similar topologies (data not shown). The primary differences concerned the interrelationships among *Eothenomys cachinus*, *E. fidelis*, and the complex of *E. miletus* and *E. eleusis*. In these cases, the internal branches were extremely short and the related statistical support below 50%. Three major clades within the subtribe Clethrionomyini were identified (Fig. 3). Clade A contained all the nominal species in the subgenus *Eothenomys* (Wang and Li, 2000) (Table 1). Clade B contained the three species ascribed to the subgenus *Anteliomys* (Wang and Li, 2000). Clades A and B appeared to be sister-groups and included all eight species of the genus *Eothenomys* from the Southwestern shoulder of the Trans-Himalayan Range. The monophyly of both clades A and B were highly supported by posterior probability (100%) and ML bootstrap analysis (81 and 89%, respectively), but received mediocre bootstrap support from the MP and NJ analyses (51–83%; Fig. 3). When using only closely related outgoup taxa of clades A and B in the analyses, such as Japanese red-backed voles or *Clethrionomys*, bootstrap support for the monophyly of clades A and B increased dramatically (in the MP tree, support for this grouping increased from 51 to 100%, whereas in the NJ tree it increased from 70 to 97%). This finding is in agreement with the results of the saturation test described above (Fig. 2).

Clade C contained both Japanese red-backed vole species plus the five *Clethrionomys* species. These results were consistent regardless of the tree building method used. Support for the monophyly of Japanese red-backed voles plus *Clethrionomys* was strong, with node support values of 100, 94, 90, and 96% from posterior probability, ML, MP, and NJ bootstrap analyses, respectively.

Within clade A, cyt *b* sequences of *E. miletus* and *E. eleusis* exhibited a notably high degree of similarity (Appendix A). The most widely distributed species, *Eothenomys melanogaster*, was placed at the basal position of this clade (Fig. 3). However, the interrelationships between *E. cachinus*, *E. fidelis*, and the species complex of *E. miletus* and *E. eleusis* were unresolved. The interrelationships among the three species of clade B (*E. custos*, *E. proditor*, and *E. olitor*) were fully resolved in terms of bootstrap support and posterior probability (Fig. 3). *E. custos* diverged first, with *E. olitor* and *E. proditor* appearing to be more derived sister-taxa. Within clade C, the Japanese red-backed voles *E. andersoni* and *E. smithii* comprised a monophyletic group nested together with two species of the genus *Clethrionomys* (Fig. 3). In fact, *Clethrionomys rex* and *Clethrionomys rufocanus* appeared to be more closely related to Japanese red-backed voles than to the other three *Clethrionomys* sampled in this study, *Clethrionomys glareolus*, *Clethrionomys rutilus*, and *Clethrionomys gapperi*.

3.3. Divergence time estimations

Based on the relative rate test, all vole lineages exhibited a constant rate except for evolutionary heterogeneity between *E. custos* and the other Oriental voles or the species of *Clethrionomys*. Divergence times were estimated using the split between *Clethrionomys* and Japanese red-backed voles (1.80 Mya; Kawamura, 1988) as a calibration point (Fig. 4). Molecular-clock estimates for the divergence of *Eothenomys* and *Clethrionomys* was 2.70 Mya (mean rate = 6.208% per site per million years, SD = 0.228% for all estimates), falling within the time frame of the first severe uplift of the Qinghai-Tibetan Plateau (3.6–2.6 Mya; An et al., 2001; Zheng et al., 2000). The divergence between the subgenera *Eothenomys* and *Anteliomys* (clades A and B of Fig. 4) was calculated to be 2.08 Mya. Interestingly, our estimate of the split between Japanese red-backed voles and the clade leading to the *Clethrionomys rex*/*C. rufocanus* complex (0.90 Mya) is nearly identical to that calculated for the radiation of the other three *Clethrionomys* species (0.90–1.02 Mya).

4. Discussion

4.1. Systematics of the subtribe Clethrionomysi: are Japanese red-backed voles more closely related to Oriental voles than to other species?

Miller (1896) first proposed the subgenus *Eothenomys* (which included Oriental and Japanese red-backed voles) and Hinton (1923, 1926) subsequently designated it as a valid genus. Contrary to this suggestion, and regardless of the tree reconstruction methods employed, our phylogenetic analyses consistently grouped all Oriental vole species from the genus *Eothenomys* into a monophyletic clade separate from Japanese red-backed voles (Fig. 3). In fact, Japanese red-backed voles (*E.*

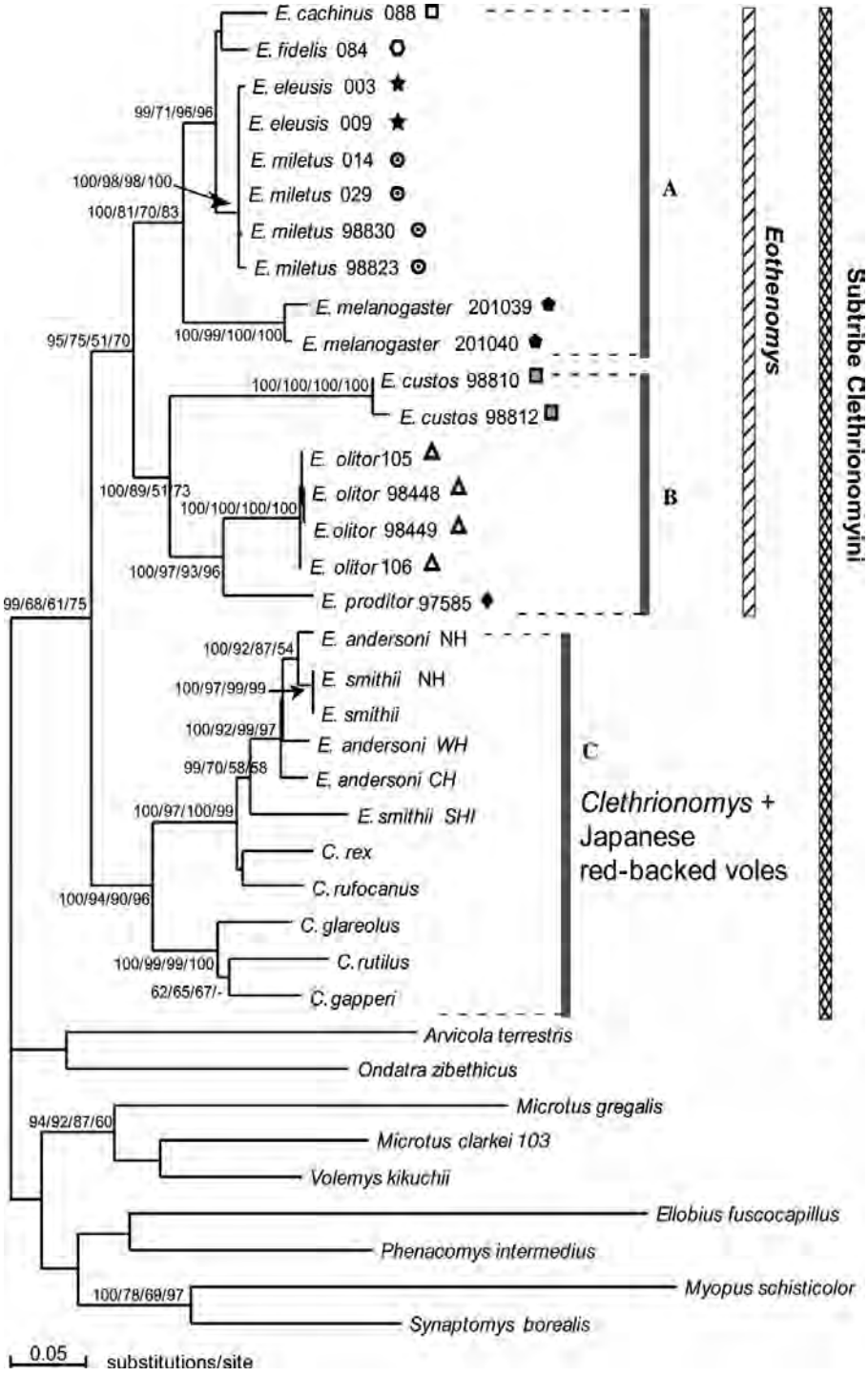

Fig. 3. Maximum-likelihood tree using the HKY+G+I model depicting the relationship of Oriental voles, Japanese voles, *Clethrionomys*, and associated outgroup taxa. ML score is 8904.73819. Numbers represent node supports inferred from Bayesian posterior probability, ML bootstrap, MP bootstrap, and NJ bootstrap analyses, respectively. The symbols of the species are the same as in Fig. 1B.

andersoni and *E. smithii*) appear to be more closely related to the genus *Clethrionomys*, especially *C. rex* (endemic to Japan) and *C. rufocanus* (Gray red-backed vole, a widely distributed species in Siberia) than to continental Asian *Eothenomys* species (Oriental voles). Thomas (1905) established the genus *Phaulomys* for Japanese red-backed voles based on their differentiated external characters. Fossils ascribed to *Clethrionomys* are recorded from the Early Pleistocene of Moldavia (the early Khaprovsk fauna; Gromov and Polyakov, 1977) and *Clethrionomys* are a predominant element of the arvicolid fauna of the Japanese Middle Pleistocene. Some fossils

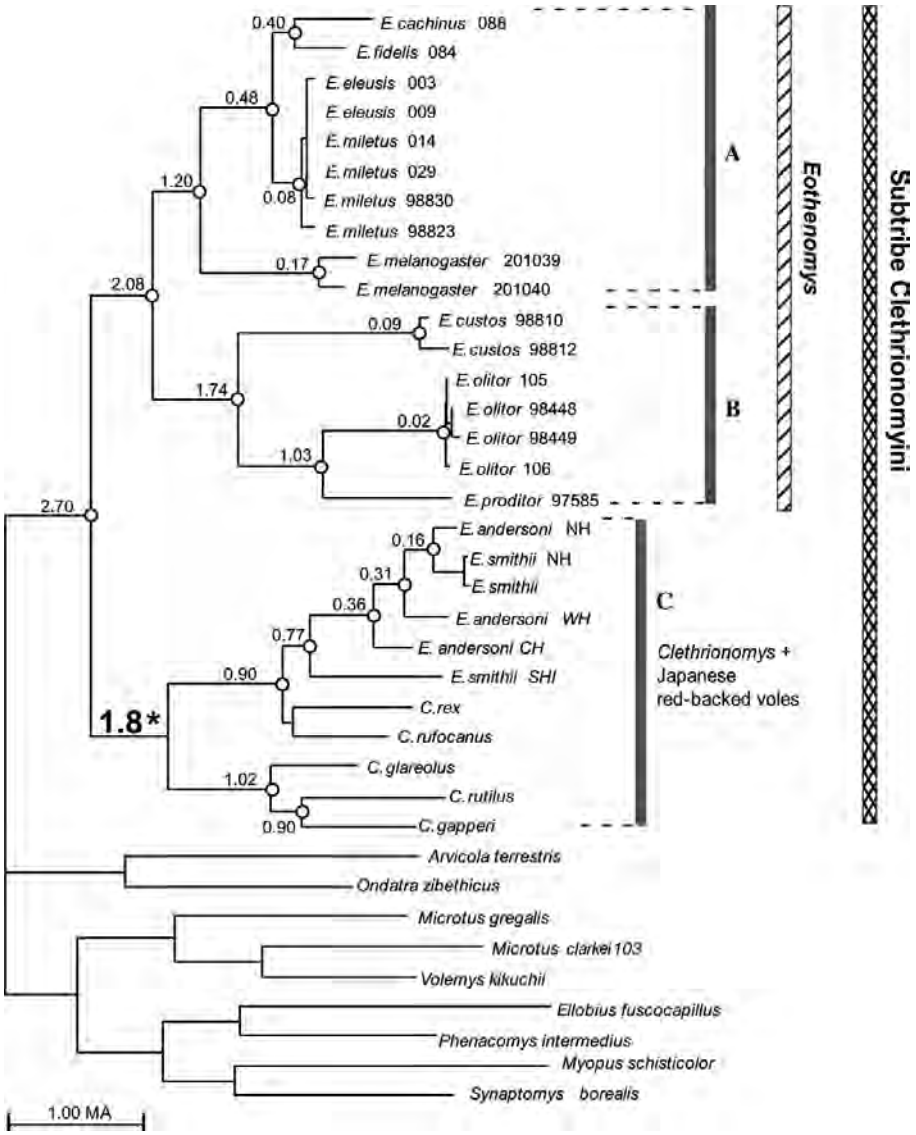

Fig. 4. Phylogenetic relationships and divergence time of Oriental voles. The early Pleistocene divergence between Japanese red-backed voles and the genus *Clethrionomys* (1.80 Mya) (Kawamura, 1988) was taken as a calibration point (node marked with an asterisk) to infer divergence times of the different lineages. Estimated divergence dates, in millions of years, are shown on individual nodes.

of a transitional form of *Clethrionomys*—"*Phaulomys*" have been reported from Japan (Kawamura, 1988). In the late Pleistocene, the transitional form diverged into the two Japanese red-backed vole species: *E. andersoni* and *E. smithii*. According to Kawamura (1988), the extant species of *Clethrionomys* and *Phaulomys* may have shared a common ancestor. This evidence, together with the biogeographical distribution patterns of the extant species indicates that Oriental and Japanese voles of the genus *Eothenomys* have separate, distinct evolutionary histories. Our phylogeny supports this hypothesis and, mirroring the results of Cook et al. (2004), suggest that the genus *Eothenomys* is paraphyletic (Fig. 3). Based on this evidence, we recommend that Japanese red-backed voles should be considered as a separate genus, *Phaulomys*, as initially defined by Thomas (1905). In addition, the closely related taxa, *C. rex* and *C. rufocanus*, should also be included in this genus. Comparable to the molecular topology of Cook et al. (2004), our results also provide support for the paraphyly of the genus *Clethrionomys*. Since both Cook et al. (2004) and the present study used the same molecular marker, cytochrome *b*, the congruent results are not surprising. Additionally, the hypothesis of monophyly for the genus *Clethrionomys* as well as monophyly for the genus *Eothenomys* were both statistically rejected when we compared the ML scores between the optimal ML tree and constrained monophyletic trees using the Shimodaira–Hasegawa test as implemented in PAUP* ($p = 0.00097$). Thus, based on the combined evidence from these two studies, we suggest that *C. glareolus*, *C. rutilus*, and *C. gapperi* should be retained in their

original genus. Interestingly, Cook et al. (2004) suggested *Alticola macrotis* formed a monophyletic clade with this latter grouping. Though this species was not included in our final analysis, a subsequent (post-submission) analysis using our dataset suggested *Alticola* formed robust clade with *C. glareolus*, *C. rutilus*, and *C. gapperi*, congruent with the topology presented by Cook et al. (2004). However, because there is cyt *b* sequence data available for only one species in this genus, additional taxon sampling is required to confirm this relationship.

Finally, our molecular topology suggests *Clethrionomys* and *Phaulomys* form a clade separate from that of Oriental voles (*Eothenomys*) plus perhaps the genus *Caryomys* (unfortunately, specimens were not obtained during the course of this study). Similarly, Cook et al. (2004) noted that *E. melanogaster* (the only Oriental vole included in their study) formed a distinct subclade within the subtńbe Clethrionomyini. It is noteworthy that all *Eothenomys* species within clades A and B (see Fig. 3), together with *Caryomys*, have rootless molars (which can be considered as a synapomorphy for this group), while those of clade C have rooted molars. Moreover, the genus *Caryomys* might be the sister-group to *Eothenomys* since species in *Caryomys* complex present some unique molar characteristics (e.g., opposite molar triangles of the first lower molar alternating and separate), and a karyotype ($2n = 54$) that differs from that generally found in *Eothenomys* ($2n = 56$). Based on these distinctions, we recommend that the generic ranking of *Eothenomys* should be maintained. This designation is in contrast with the suggestion that *Alticola*, *Eothenomys*, *Phaulomys*, and *Clethrionomys* should be amalgamated into a single genus: *Clethrionomys* (Cook et al., 2004).

4.2. Molecular systematics of the genus Eothenomys

Different hypotheses have been forwarded on the subgenus classification of *Eothenomys* (Ellerman and Morrison-Scott, 1951; Hinton, 1923; Ma and Jiang, 1996; Musser and Carleton, 1993). Wang and Li (2000) suggested to keep only two subgenera within *Eothenomys* (Table 1). Our phylogenetic analyses provided strong support for the monophyly of Oriental voles (*Eothenomys*) and support the classification of two valid subgenera, *Eothenomys* and *Anteliomys* (clades A and B of Fig. 3). A morphological differentiation between these two subgenera is the number of inner salient angles on the last upper molar (see Section 1). Interestingly, these two clades can also be distinguished from each other by their distribution patterns. Species in clade A have widespread distribution patterns, while species in clade B are restricted more or less to the Trans-Himalayan Ranges (Figs. 1A and B).

At the species level, all species in clades A and B except for *E. custos* were initially considered as subspecies of *E. melanogaster* (Allen, 1924; Ellerman and Morrison-Scott, 1951; Hinton, 1923, 1926; Osgood, 1932). However, Thomas (1921) proposed *E. cachinus* as a valid species. When *E. miletus* was proposed as a valid species distinct from *E. melanogaster*, Allen (1940) and Wang and Li (2000) used *E. fidelis* as a synonym of *E. miletus*. Recently, Wang and Li (2000) summarized a suite of morphological data and suggested four valid species occurred in the subgenus *Eothenomys* (clade A) and five valid species in the subgenus *Anteliomys* (clade B) (Table 1). Our phylogenetic results are in line with those of Wang and Li (2000). There are three distinct lineages in clade B (*E. custos*, *E. olitor*, and *E. proditor*) and three to four lineages in clade A (Fig. 3).

According to our phylogenic topology *E. cachinus*, *E. fidelis*, and *E. miletus*/*E. eleusis* appear to be more closely related to each other than to *E. melanogaster* (Fig. 3). Moreover, based upon our divergence time estimates (Fig. 4), the initial speciation event leading to the present day Oriental vole species occurred approximately 2.1 Mya whereas the split between *E. melanogaster* and the other members of the subgenus *Eothenomys* occurred about 1.2 Mya. Thus, our results reject the hypotheses of Hinton (1923, 1926) and Ellerman and Morrison-Scott (1951), which considered all species in clade A to be subspecies of *E. melanogaster*. In fact, *E. cachinus* and *E. fidelis* appear to be neither subspecies of *E. melanogaster* nor a synonym of *E. miletus* as previously suggested (Hinton, 1923; Wang and Li, 2000). However, it should be noted that the interrelationships among *E. cachinus*, *E. fidelis*, and *E. miletus*/*E. eleusis* were unresolved in our analyses. To clarify their relationships and designation as valid species, a broader sampling strategy and more information, such as interbreeding and behavior and ecological data, are still required. Significantly, however, our results suggest that separate species designations for *E. eleusis* and *E. miletus* may not be warranted. Although *E. eleusis* and *E. miletus* were proposed as separate subspecies or species (Allen, 1940; Hinton, 1923; Musser and Carleton, 1993; Thomas, 1912a,b; Wang and Li, 2000), the cyt *b* sequences from these two taxa were nearly identical. Indeed, pairwise distances between these two taxa (0.08–0.86%) were consistently smaller than pairwise distances between the intraspecific haplotypes of other species, e.g., the pairwise distance between the two *E. melanogaster* sequences was 1.78%, while that between conspecifics of *E. andersoni* ranged from 1.69 to 3.60% (Appendix A). Moreover, *E. eleusis* and *E. miletus* share the same karyotype: $2n = 54A + XY$ (A, A) (A: acrocentric chromosome) (Yang et al., unpublished data). This combined evidence suggests that *E. eleusis* and *E. miletus* should not be considered as a separate species at the genetic level. It should be cautioned, however, that because phylogenetic relationships inferred from single gene studies might be biased due to gene-tree effects,

evidence from additional molecular markers (i.e., nuclear genes) are required to independently assess this finding (Chen et al., 2003), and address the potential problem of hybridization events between these "species" (Sang and Zhong, 2000).

4.3. The evolutionary history of Eothenomys and its biogeography

In comparison to the biogeographical distributions of many other vole species in the subtribe Clethrionomyini, Oriental voles are generally found at lower latitudes, mainly in the Southwestern region of China. Only *E. melanogaster* is found in central and eastern China and Taiwan (Fig. 1). Conversely, most other species have overlapping ranges in the Trans-Himalayan region (Wang and Li, 2000). The evolutionary history of how and when ancestral Oriental vole species spread into these particular lower latitude areas is unknown.

The present Trans-Himalayan Range includes various north–south extending ranges and adjacent mountainous areas on the east skirts of the Qinghai-Tibetan Plateau. The geological configuration of this area is complicated, as it is composed of several non-uniform landform assemblages. However, three main areas may be defined, the western high-mountain and gorge area, the northeastern piedmont plain-gorge area and the southeastern plateau-lake basin area. The first two areas belong to the Qinghai-Xizang (Tibetan) plateau while the third is a part of the Yunnan-Guizhou Plateau (Li and Wang, 1986). Geological studies have indicated that the uplift events of the Tibetan plateau occurred most intensely and frequently between 2.6 and 3.6 Mya (An et al., 2001; Zheng et al., 2000). These large-scale uplifts caused strong orogenic movement, including the formation of the Trans-Himalayan Range. This occurrence heightened climate change in East Asia, especially that related to the severity of summer and winter monsoons (An et al., 2001). This period was also characterized by large-scale glaciations in the Northern Hemisphere. Based on the divergence times estimated from our molecular data, the Oriental vole clade (clade A + B in Fig. 4) arose about 2.70 Mya. This event is within the latter time frame of the paleo-geographic and paleo-climate change episode mentioned above, implying that the early speciation of Oriental voles is likely related to this major orogenic uplifting. Additionally, mapping the patterns of biogeography onto our phylogeny suggests that the lower latitude Oriental voles are derived taxa. On balance, these results imply that the ancestor of all Oriental voles evolved in the northern part of Asia and underwent a large-scale expansion to the south during the period 2.70–2.08 Mya.

As noted earlier, the radiation of Oriental voles is probably recent, and most likely began about 2 Mya according to the fossil record (Zheng and Li, 1990; Zheng, 1993). Interestingly, two successive orogenic movements occurred near the edge of Qinghai-Tibetan plateau about 2.5 and 1.6 Mya, respectively (Liu et al., 1986; Yu et al., 2000 and references therein), followed closely by the mid-Quaternary Ice age. Notably, the inferred divergence times for the early radiation of the subgenera *Eothenomys* and *Anteliomys* (1.20 and 1.74 Mya, respectively; Fig. 4) correspond to these geographic occurrences. Thus, these recent geological and glacial events likely acted to isolate vole populations, and probably account for the high species diversity of voles found in this area today. However, more detailed information about the distribution of these endemic species and the paleo-geography from this area, together with additional taxon sampling, are still required to develop a clearer picture of the evolutionary history of Oriental voles in Southeast Asia.

Acknowledgments

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Appendix A

ML pairwise distances by HYK variant model

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1 *E. eleusis 003*																																					
2 *E. eleusis 9*	0.25																																				
3 *E. fidelis 84*	3.49	3.17																																			
4 *E. miletus 14*	0.42	0.17	3.38																																		
5 *E. miletus 29*	0.34	0.08	3.27	0.25																																	
6 *E. miletus 98823*	0.86	0.60	3.61	0.78	0.69																																
7 *E. miletus 98830*	0.43	0.17	3.40	0.34	0.25	0.78																															
8 *E. cachinus 88*	4.58	4.24	4.23	4.46	4.35	4.35	4.49																														
9 *E. melanogaster 201039*	10.16	9.71	10.97	10.00	9.86	9.86	10.07	11.36																													
10 *E. melanogaster 201040*	9.90	9.45	10.04	9.74	9.60	9.61	9.81	10.75	1.78																												
11 *E. custos 98810*	17.99	17.40	17.47	17.39	17.40	17.19	17.93	18.08	22.97	21.18																											
12 *E. custos 98812*	19.38	18.78	19.07	18.77	18.78	18.57	19.34	18.64	23.39	21.59	1.11																										
13 *E. olitor 105*	14.96	14.41	14.27	14.41	14.24	14.76	14.89	14.70	19.00	17.13	18.78	19.55																									
14 *E. olitor 106*	15.07	14.52	14.38	14.51	14.34	14.87	15.00	15.17	19.16	17.27	18.93	19.71	0.17																								
15 *E. olitor 98448*	15.33	14.78	14.64	14.77	14.60	15.12	15.26	15.07	19.00	17.13	19.18	19.96	0.17	0.34																							
16 *E. olitor 98449*	15.44	14.88	14.74	14.88	14.71	15.24	15.37	15.18	19.16	17.68	18.84	19.37	0.34	0.51	0.17																						
17 *E. proditor 97585*	16.33	15.76	15.99	15.75	15.57	15.74	15.85	13.84	19.49	18.69	19.16	19.93	9.81	9.87	10.11	10.20																					
18 *E. andersoni NH*	17.27	16.86	18.37	16.85	16.86	17.25	17.21	18.68	22.15	20.84	26.25	27.92	21.99	22.19	22.46	22.50	21.15																				
19 *E. andersoni CH*	19.53	19.11	19.90	19.10	19.11	19.99	19.49	20.11	22.70	21.35	28.09	28.78	22.88	23.09	23.36	23.39	22.42	3.08																			
20 *E. andersoni WH*	18.45	18.03	19.53	18.02	18.03	18.91	18.40	18.18	23.02	21.20	28.24	29.42	23.23	23.46	23.73	23.76	21.89	3.08	3.60																		
21 *E. smithii NH*	17.22	16.81	18.32	17.21	16.81	17.66	17.16	17.94	21.68	20.83	27.73	28.90	22.54	22.75	23.02	23.06	21.26	1.69	3.59	3.39																	
22 *E. smithii SHI*	21.81	21.63	21.73	22.07	21.63	21.61	22.05	21.52	24.76	23.42	24.24	25.91	26.44	26.70	26.94	26.94	24.36	8.11	8.64	8.99	8.11																
23 *E. smithii AB104508*	17.42	17.01	18.52	17.41	17.01	17.87	17.36	18.15	21.45	21.05	27.97	29.14	22.77	22.99	23.26	23.29	21.47	1.79	3.48	3.29	0.25	8.24															
24 *C. rex*	17.00	16.39	16.70	16.38	16.59	16.77	16.72	17.03	21.54	19.79	25.80	26.92	21.55	21.51	22.02	22.06	19.44	8.15	8.38	8.16	8.56	9.60	8.70														
25 *C. rufocanus*	18.92	18.29	17.72	18.69	18.50	18.69	18.65	18.60	20.11	19.21	25.76	26.96	23.60	23.82	24.08	23.63	20.40	8.31	7.60	8.35	8.31	9.21	8.17	7.72													
26 *C. glareolus*	19.60	18.94	19.18	19.15	19.16	18.50	19.55	20.88	22.46	21.04	26.33	27.08	20.37	20.55	20.83	20.88	21.42	14.67	15.77	16.21	15.37	17.64	15.55	16.39	14.09												
27 *C. rutilus*	20.64	19.96	20.17	20.17	20.19	19.49	20.37	18.23	23.57	24.15	27.09	28.08	26.19	26.45	26.70	26.19	22.57	15.33	17.23	15.41	15.70	18.14	15.51	15.57	16.03	9.84											
28 *C. gapperi*	20.29	20.06	18.84	20.28	20.29	20.09	19.68	20.30	22.43	20.61	26.78	28.01	21.79	22.00	22.27	22.31	21.38	15.08	16.12	15.11	15.04	18.21	15.21	15.32	15.52	8.40	9.64										
29 *Arvicola terrestris*	32.60	32.95	33.98	32.65	33.24	32.92	33.63	34.77	35.68	34.06	41.18	43.56	38.99	39.44	38.92	38.78	45.54	38.30	39.42	37.53	40.33	40.97	40.62	37.78	38.59	39.34	38.27	36.19									
30 *Microtus gregalis*	40.93	39.91	38.71	40.23	39.91	41.31	41.12	41.23	39.52	37.77	52.98	55.59	48.08	48.71	48.75	48.47	48.43	43.60	43.72	42.28	44.90	47.62	44.56	44.94	46.46	42.14	44.19	44.98	56.08								
31 *Microtus clarkei 103*	32.80	31.95	34.25	31.94	31.95	31.10	32.88	32.76	33.61	31.71	41.98	41.63	34.69	35.08	34.69	34.60	34.52	31.21	31.15	29.34	31.52	30.81	31.79	33.27	31.47	32.45	36.70	34.83	44.21	36.29							
32 *Ellobius fuscocapill*	39.56	38.91	40.99	38.57	38.91	39.22	38.44	43.03	41.71	38.66	43.00	42.90	39.38	40.49	39.70	40.80	40.20	40.44	44.10	43.44	40.25	44.86	41.19	41.14	46.50	43.05	45.45	42.65	53.22	61.60	54.88						
33 *Myopus schisticolor*	49.47	49.88	54.41	50.19	49.88	48.87	50.86	52.42	52.02	50.08	57.32	58.48	58.72	59.17	58.72	57.87	62.99	48.54	51.11	47.57	49.55	48.15	50.52	49.73	51.96	54.29	59.38	58.15	64.07	63.89	56.27	69.72					
34 *Phenacomys intermedi*	30.52	30.87	28.71	31.16	30.87	30.27	31.55	32.00	29.17	29.22	39.79	42.52	33.46	33.85	33.46	34.44	37.71	32.72	35.83	34.42	34.08	33.67	34.93	31.38	34.11	34.95	35.98	38.11	42.35	41.31	41.64	46.20	54.96				
35 *Ondatra zibethicus*	26.84	27.67	28.41	27.40	27.67	27.13	28.50	28.36	34.58	32.70	32.58	34.46	33.31	33.67	33.32	32.71	33.30	32.29	34.18	32.01	34.46	34.55	34.17	33.97	32.76	32.14	33.06	33.19	34.67	42.92	38.54	50.22	50.20	37.33			
36 *Volemys kikuchii*	27.81	27.01	29.87	27.26	27.01	26.71	27.83	31.20	29.25	27.72	36.63	39.17	33.47	33.85	34.06	33.97	31.63	27.93	27.82	28.23	27.33	26.65	28.10	28.21	29.65	28.61	30.80	30.02	37.00	30.17	18.74	47.39	50.61	35.03	33.15		
37 *Synaptomys borealis*	43.53	43.97	42.28	43.60	43.97	42.87	44.59	42.78	44.47	43.13	45.34	46.88	49.69	50.37	50.38	49.39	42.34	39.07	39.17	40.21	39.58	38.71	40.52	37.48	40.61	40.53	42.45	39.92	47.08	55.48	43.62	55.62	40.22	35.47	35.61	36.54	

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