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TAXONOMIC RELATIONSHIPS AMONG *PHENACOMYS* VOLES AS INFERRED BY CYTOCHROME *b*

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Taxonomic relationships among red tree voles (*Phenacomys longicaudus longicaudus*, *P. l. silvicola*), the Sonoma tree vole (*P. pomo*), the white-footed vole (*P. albipes*), and the heather vole (*P. intermedius*) were examined using 664 base pairs of the mitochondrial cytochrome *b* gene. Results indicate specific differences among red tree voles, Sonoma tree voles, white-footed voles, and heather voles, but no clear difference between the 2 Oregon subspecies of red tree voles (*P. l. longicaudus* and *P. l. silvicola*). Our data further indicated a close relationship between tree voles and *albipes*, validating inclusion of *albipes* in the subgenus *Arborimus*. These 3 congeners shared a closer relationship to *P. intermedius* than to other arvicolids. A moderate association between *pomo* and *albipes* was indicated by maximum parsimony and neighbor-joining phylogenetic analyses. Molecular clock estimates suggest a Pleistocene radiation of the *Arborimus* clade, which is concordant with pulses of diversification observed in other murid rodents. The generic rank of *Arborimus* is subject to interpretation of data.

Key words: *Arborimus*, cytochrome *b*, mitochondrial DNA, molecular systematics, *Phenacomys*, voles, taxonomy

Tree voles (*Phenacomys longicaudus*, *P. pomo*) are small arboreal mammals that live in the coniferous forests of western Oregon and coastal northwestern California (Fig. 1). They dwell in nests in the forest canopy, and subsist on a diet of conifer needles. There is considerable interest in the ecology and conservation of tree voles because they are endemic to a relatively small region, have unusual life history characteristics, are potentially threatened by loss of forest habitat, and are 1 of the primary food sources for the northern spotted owl (*Strix occidentalis*—Forsman et al. 1984, 2004; United States Forest Service and United States Bureau of Land Management 1993, 2000; Verts and Carraway 1998). Of particular interest is the possibility that human manipulation of forests might lead to subpopulations of tree voles that are genetically or demographically isolated. This concern is difficult to evaluate because little is known about tree vole population structure, population trends, range, taxonomy, and general ecology (Hayes 1996; Johnson and George 1991; Murray 1995; Verts and Carraway 1998).

Currently, scientists and managers need a clearer understanding of taxonomic relationships within the tree vole complex and their close relatives in order to develop management recommendations. There are 2 subspecies and 3 congeners with overlapping ranges and taxonomic uncertainty relative to the red tree vole (Fig. 1). They include the dusky tree vole (*P. l. silvicola*), which inhabits the west slope of the Coast Ranges in Tillamook and Lincoln counties, Oregon, and the Oregon red tree vole (*P. l. longicaudus*) to the south and east in the remainder of the range; the Sonoma tree vole (*P. pomo*) in northwestern California; the white-footed vole (*P. albipes*) in western Oregon and northwestern California; and the heather vole (*P. intermedius*), which occurs over most of boreal North America, including high elevations in the Oregon Cascades and mountains of eastern Oregon (Verts and Carraway 1998). *P. l. silvicola* was initially described as *P. silvicola* (Howell 1921) and later relegated to subspecific status based on blood protein similarities (Johnson 1968). Johnson and George (1991) first recognized *P. pomo* as a sister species to *P. longicaudus* based on karyotype, reproductive barriers, and body size. The geographic zone delineating species limits between *longicaudus* and *pomo* is uncertain, but occurs somewhere along the Oregon–California border or the Klamath river in California (Johnson and George 1991; Murray 1995).

Since discovery of the first tree vole in 1890 (True 1890), taxonomists have debated whether *longicaudus*, *pomo*, and *albipes* should be included in the genus *Phenacomys* with the heather vole (*P. intermedius*) or in a separate genus, *Arborimus* (Howell 1926; Johnson 1973; Johnson and Maser 1982; Taylor 1915). Taylor (1915) initially erected the subgenus *Arborimus* to separate *longicaudus* (and presumably *pomo*) from *intermedius*,
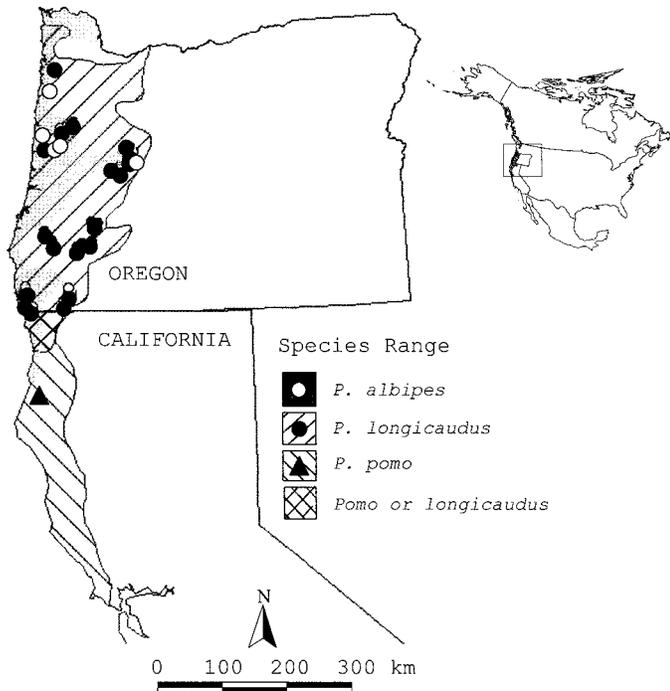
FIG. 1.—Species distribution and sample collection locations of red tree voles (*P. longicaudus*, closed circles), Sonoma tree voles (*P. pomo*, triangles), and white-footed voles (*P. albipes*, open circles), used to assess taxonomic relationships within genus *Phenacomys*.

TABLE 1.—Collection locations of samples used to assess taxonomic relationships among red tree voles (*P. l. longicaudus*), dusky tree voles (*P. l. silvicola*), white-footed voles (*P. albipes*), and heather voles (*P. intermedius*) in Oregon, and Sonoma tree voles (*P. pomo*) in California. Physiographic provinces were assigned according to Franklin and Dyrness (1973). Sample size (*n*) is divided according to DNA source (t = soft tissue and museum skin; b = bone).

Taxa	Physiographic province	County	Samples
P. l. silvicola	Coast Ranges	Tillamook	8t
P. l. longicaudus	Coast Ranges	Benton, Coos	4t, 4b; 1t
		Lane	2t
	Western Cascades	Lane, Linn, Douglas	6b, 3b, 3t
	Klamath Mtns	Curry, Josephine	5b, 5b
		Douglas	6t
P. pomo	Klamath Mtns	Humboldt (California)	5t
P. albipes	Coast Ranges	Yamhill, Lincoln	1t, 1t
		Benton, Lane	2t, 1t
	Western Cascades	Lane	1b
P. intermedius	Western Cascades	Lane	1b
	High Cascades	Jefferson	1b

tentatively including *albipes* in this subgeneric ranking. Johnson (1973) proposed elevation of subgenus *Arborimus* to full generic rank, a designation that remains controversial (Hayes 1996; Musser and Carleton 1993; Verts and Carraway 1998). Johnson and Maser (1982) recommended inclusion of *albipes* in the genus *Arborimus* based on examination of skeletal elements and preserved soft parts. Biochemical analyses further supported a close relationship between *albipes* and tree voles (Murray 1995). However, those results also suggested that *intermedius* was more closely related to *Microtus* than to *Arborimus*. In a review of paleontological records of extinct and extant *Phenacomys*, Repenning and Grady (1988) erected a new subgenus for *albipes* (*Paraphenacomys*) and kept *longicaudus* and *intermedius* in their own subgenera (*Arborimus* and *Phenacomys*, respectively). Furthermore, they speculated that *longicaudus* and *intermedius* shared a more recent common ancestor than *albipes*. *Longicaudus*, *pomo*, and *albipes* differ from other microtine rodents by bacular morphology (Hamilton 1946; Johnson 1973; Johnson and Maser 1982), and all 3 congeners plus *intermedius* differ from other Microtinae based on cranial and morphological traits (Hamilton 1946; Hinton 1926; Howell 1926).

Although there is general consensus that *P. longicaudus*, *pomo*, and *albipes* are related to each other, their relative relationship to *P. intermedius* remains untested (Murray 1995; Musser and Carleton 1993). In this study, we used sequence data from a region of the mitochondrial cytochrome *b* gene (*Cytb*) to explore phylogenetic relationships among *Phenacomys* voles in the Pacific Northwest. *Cytb* has been shown to be useful in

elucidating relationships of closely related taxa (e.g., Edwards and Bradley 2002; Sullivan et al. 1997). Our objectives were to examine taxonomic placement of *longicaudus*, *pomo*, and *albipes* relative to *intermedius* and other arvicolid congeners; to explore species-level differences between *longicaudus* and *pomo;* and to examine phylogenetic relationships between subspecies *P. l. longicaudus* and *P. l. silvicola*.

MATERIALS AND METHODS

Samples.—Samples of *P. longicaudus* were collected throughout its range (Table 1; Fig. 1). Because of the difficult nature of collecting samples of red tree voles in the wild (Howell 1921; Huff et al. 1992), we used several sampling techniques: DNA was isolated from tissue samples of live specimens, carcasses, museum specimens, and bones recovered from regurgitated pellets of spotted owls. Use of skeletal remains recovered from owl pellets as a source of DNA from small mammals has been shown to be free of cross-contamination from other prey consumed by owls (Taberlet and Fumagalli 1996). Tissue samples from 11 live specimens were obtained by hand capturing *P. l. longicaudus* at their arboreal nests and snipping approximately 5 mm from the tip of the tail prior to their release. Tissue samples were preserved in 1 ml of tissue storage buffer (100 mM Tris-HCl pH 8, 100 mM EDTA, 10 mM NaCl, and 0.5% SDS) stored at −80°C until DNA extraction. We obtained tail tissue from 5 carcasses recovered from pitfall traps in Benton (*n* = 3) and Lane (*n* = 2) Counties, Oregon (K. Martin, Oregon State University) and 8 tissue samples of *P. l. silvicola* collected in Tillamook County, Oregon by M. Johnson (Burke Museum of Natural History, University of Washington; specimen voucher numbers 34486–88, 34491–94, 34496). The remaining 23 *P. l. longicaudus* samples were obtained from skulls and mandibles of tree voles recovered from spotted owl pellets collected in Benton, Curry, Josephine, eastern Lane, and eastern Linn counties (Oregon). Identification of vole skulls and jaws was based on osteological skull and dental characteristics as described by Maser and Storm (1970).

Tissue samples from *P. pomo* were obtained (following guidelines from Oregon State University animal care and use committee) from 5 individuals captured and released after collecting fresh tail snips (Table 1; Fig. 1). We also used stomach wall tissue from 5 *P. albipes*

specimens collected in the Coast Ranges of western Oregon (Martin and McComb 2002), and 1 skull extracted from a spotted owl pellet collected in the Western Cascades (Table 1; Fig. 1). *P. intermedius* samples were obtained from bones of 2 specimens recovered from spotted owl pellets (Table 1). One *P. intermedius Cytb* sequence obtained from GenBank (AF 119260—Conroy and Cook 1999) was included in phylogenetic reconstruction.

DNA extraction from bones.—We used a modification of the procedure described by Lambert et al. (2002) to isolate DNA from bones. Briefly, 30–50 mg of bone was separated from a vole skull or lower jaw (about one-half the total jaw). Each sample was cleaned by scraping with a sterile razor blade and was then ground to powder by smashing with a hammer. Bone powder was transferred to a tube containing 750 µl of 0.5 M EDTA and placed at room temperature for 24 h for decalcification. Bone samples were pelleted in a microcentrifuge at $14000 \times g$ for 5 min and the EDTA was drawn off. The bone pellet was then resuspended in 500 µl bone lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM NaCl, 0.9% SDS and 0.5 M EDTA pH 8.0) with dithiothreitol (DTT) and Proteinase K added for a final concentration of 1 µg/µl and 0.45 µg/µl, respectively. This solution was incubated at 37°C in an agitating shaker for 48–96 h until most or all of the bone was dissolved. Twenty-five µl of 20 mg/ml Proteinase K was added every 24 h until extraction. Following digestion, DNA was extracted twice using equal volumes of phenol (saturated with 10 mM Tris pH 8.0) and chloroform : isoamyl alcohol (24:1). The aqueous solution was desalted and concentrated using a Micron 30,000 MW cutoff filter (Amicon Bioseparations) and purified using a Qiagen DNeasy kit. The DNA was eluted from the column in 200 µl buffer AE. One µl of elutant was used in each polymerase chain reaction (PCR).

DNA extraction from tissue.—We used the Cooper (1994) method for DNA isolation from museum tissues. Briefly, a 1 mm^2 tissue sample was chopped with a sterile razor blade, placed in 600 µl of tissue lysis buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 mM NaCl, 500 µg/ml Proteinase K, 10 mg/ml DTT, and 1% SDS) and incubated for 72 h, with 25 µl of 20 mg/ml additional Proteinase K added every 24 h. DNA was then extracted, desalted, and purified following the same procedure as for bones. We isolated DNA from tail snips and stomach tissue using a standard lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM NaCl, and 2% SDS with 600 µg/ml Proteinase K), incubating overnight at 37°C, and extracting twice with equal volumes of Tris-saturated phenol and once with chloroform : isoamyl alcohol (24:1). The aqueous solution was desalted and concentrated using a Micron 30,000 MW cutoff filter. DNA samples were quantified using a Hoefer TKO 100 fluorometer and diluted to 100 ng/µl for use in PCR amplification.

PCR amplification and sequencing.—A 664 base pair portion of the *Cytb* gene was amplified using PCR under the following conditions: 93°C denaturing phase for 12 min followed by 40 cycles of denaturing at 93°C (30 s), annealing at 50°C (45 s), an extension cycle at 72°C (1 min), followed by a final extension cycle at 72°C for 10 min. Amplification reactions were performed in 40 µl volumes under the following concentrations: 50 mM Tris-HCl pH 8.3, 2.5 mM MgCl$_2$, 1.5 pmol each forward and reverse primer, 500 µg/ml bovine serum albumin (BSA), 10 units of Taq Gold (Promega), and 0.2–100 ng DNA. It was necessary to use extremely low concentrations of DNA when amplifying DNA isolated from bone and museum tissue due to the presence of PCR inhibitors (Hagelberg 1994; Lambert et al. 2002). Negative controls were run with all reactions and extractions. All sequences were archived in GenBank (accession numbers AY338766–33825).

Universal primers designed by Irwin et al. (1991: L14724/H15915) were used to redesign primers specific to *Phenacomys* (numbers corresponding to *Mus* 14348–15202, Bibb et al. 1981): RTV-F: 5′–CGAGACGTAAAYTATGGCTGA–3′; RTV-R 5′–GAGGCT-ACTTGTCCAATTATGA–3′. PCR amplification was checked by visualization with ethidium bromide on 1% agarose gels. Successful reactions were cleaned and concentrated by centrifuge dialysis using Micron 30,000 MW cutoff filters (Amicon Bioseparations). Bidirectional sequencing reactions were performed with ABI Big Dye chemistry using an ABI 3100 capillary system, located in the Central Services Laboratory at Oregon State University.

Nuclear copies of *Cytb* have been detected across several rodent families (Patton and Smith 1994; Smith et al. 1992). The absence of stop codons, frameshifts, ambiguous bases, and the distribution of substitutions per position suggest that these sequences are a portion of the *Cytb* coding gene and not a nuclear pseudogene. Sequences were compared to a GenBank archived *Cytb* sequence of *P. intermedius* (AF119260—Conroy and Cook 1999).

Sequence analysis.—Sequences were manually aligned and edited using BioEdit version 5.0.6 (Hall 2001). Arlequin ver. 2.000 (Schneider et al. 2000) was used to identify redundant haplotypes and to determine numbers of transitions and transversions per nucleotide site. Basic sequence statistics were calculated using MEGA version 2.1 (Kumar et al. 2001). Genetic distances (d) among species were calculated with the Tamura-Nei model (Tamura and Nei 1993).

Cytb sequence data have recently received much attention within the family Muridae. Data have been examined at several taxonomic levels, from variation within populations to diversification of the entire family (Conroy and Cook 1999; Martin et al. 2000; Smith and Patton 1991). However, data are lacking that summarize the degree of genetic divergence at the generic level within the subfamily Arvicolinae. Therefore, we aligned all available published GenBank sequences (Appendix I) to our 664 bases of *Cytb* for comparison of uncorrected sequence divergence and TrN genetic distance among 12 of the 17 genera within subfamily Arvicolinae (Carleton and Musser 1984).

Phylogenetic analysis and testing of molecular clock.—We used phylogenetic reconstruction to evaluate whether *P. longicaudus*, *P. pomo*, and *P. albipes* would cluster with *P. intermedius* or if they would form a separate clade within Arvicolinae by using single haplotypes of each *Phenacomys* species, 3 *Microtus* species, and 3 *Clethrionomys* species. *Mus musculus* and *Peromyscus* species were used as an outgroup. Next, we evaluated taxonomic relationships at the subspecies and species level within *Phenacomys* using all *Phenacomys* haplotypes and *Microtus* species as an outgroup. Previously published sequences of outgroup taxa and 1 *P. intermedius* reference sequence (AF 119260—Conroy and Cook 1999) were obtained from GenBank (Appendix I). Conroy and Cook (1999) previously identified *Peromyscus* as the closest outgroup to the arvicolines.

Phylogenetic relationships among *Cytb* fragments were reconstructed using PAUP* 4.0*b*10 (Swofford 2002). Presence of a phylogenetic signal was tested using the critical values of skewness (g_1 statistic) of the tree-length distribution. Critical values were computed from a distribution of 10,000 randomly generated trees in PAUP* with significance levels ($P < 0.01$) obtained from Hillis and Huelsenbeck (1992:table 2). We used 3 methods of phylogenetic inference for tree reconstruction: maximum parsimony (MP), neighbor-joining (NJ—Saitou and Nei 1987), and maximum likelihood (ML—Felsenstein 1981). Maximum parsimony trees were generated by excluding invariable sites, using heuristic search with 10 random addition replicates, TBR, and MulTrees options, and equally weighted and unordered character states. We used hierarchical likelihood ratio tests (hLRTs) implemented in program Modeltest v. 3.06 (Posada and Crandall 1998) to examine the fit of 56 models of

TABLE 2.—Tamura-Nei genetic distances and molecular clock divergence estimates with standard error (see text for explanation) among the red tree vole (*Phenacomys longicaudus*), the Sonoma tree vole (*P. pomo*), the white-footed vole (*P. albipes*), and the heather vole (*P. intermedius*) using 664 bases from the cytochrome-*b* gene of mtDNA.

Taxa	1	2	3	4
1. *P. longicaudus*	—	4.22 ± 0.006	4.15 ± 0.006	9.48 ± 0.010
2. *P. pomo*	5.7	—	2.51 ± 0.005	8.44 ± 0.010
3. *P. albipes*	5.6	3.4	—	9.04 ± 0.011
4. *P. intermedius*	12.6	11.1	12.2	—

TABLE 3.—Tamura-Nei (1993) genetic distance among 12 genera in the subfamily Arvicolinae using the same 664 bases of *cyt-b* as were used for *Phenacomys longicaudus* (see text). Each genus is represented by one species previously published in GenBank (Appendix I) except for *Phenacomys* (this study).

	Tamura-Nei Genetic Distance (%)										
Taxa	1	2	3	4	5	6	7	8	9	10	11
1. *Alticola*	—										
2. *Arvicola*	15.1	—									
3. *Clethrionomys*	11.8	17.6	—								
4. *Dicrostonyx*	16.1	17.1	15.3	—							
5. *Ellobius*	16.9	20.1	21.1	19.6	—						
6. *Eothenomys*	10.3	16.0	8.5	14.9	17.3	—					
7. *Lagurus*	14.7	16.9	17.1	16.4	19.6	16.1	—				
8. *Lemmus*	17.7	17.8	19.8	14.3	19.5	17.1	18.0	—			
9. *Microtus*	15.7	15.2	17.4	15.8	16.6	14.3	15.9	17.6	—		
10. *Ondatra*	16.3	17.2	17.8	14.1	18.3	16.1	17.5	17.1	17.1	—	
11. *Phenacomys*	19.2	18.0	21.2	16.8	19.8	18.1	19.2	20.8	19.5	18.8	—
12. *Synaptomys*	17.1	18.2	17.9	14.7	20.2	17.3	17.7	15.1	19.1	15.6	17.8

nucleotide substitution to the sequence data, and to estimate the initial transition/tranversion ratios, the gamma distribution shape parameter alpha (α), and proportion of invariable sites (*I*) for application to ML and NJ analysis. We used empirically determined nucleotide frequencies from Modeltest for all analysis. After a ML search, parameters were re-estimated using parsimony based estimates of α and the substitution rate matrix and a new search was performed. We continued this process until the topology and parameter estimates stabilized, using the Yang-Kumar model of α (Swofford et al. 1996). Modeltest selected the Tamura-Nei (TrN) model of sequence evolution for the 2 phylogenetic reconstructions detailed above (TrN+G, α = 0.1849, α = 0.2678, respectively). Robustness of phylogenies was assessed using 1,000 (MP, NJ) or 100 (ML) bootstrap (bs) replications (Felsenstein 1985). We tested if sequences were evolving in a clock-like fashion by generating trees using the same ML methodology described (e.g., Modeltest to generate model of sequence evolution and successive approximation to refine these parameters) and a maximum likelihood ratio test to test for violation of molecular clock (TrN+G, α = 0.4166—Felsenstein 1981; Huelsenbeck and Crandall 1997). We then calibrated our molecular clock using the *Mus–Rattus* split of 14 million years ago (mya) to estimate time since coalescence within *Phenacomys* (Jacobs and Pilbeam 1980). *Cytb* sequences of *Mus musculus* and *Rattus norvegicus* used for molecular clock were obtained from Genbank (NC001569—Bibb et al. 1981; NC00165—Gadaleta et al. 1989, respectively). Due to computational time constraints using TrN+G+clock, the *longicaudus* portion of the *Phenacomys* data set was pruned to include only 5 of the most common haplotypes.

RESULTS

Basic sequence statistics.—Characteristics of *Phenacomys* species *Cytb* sequences were similar to other mammalian taxa (Irwin et al. 1991), including some arvicolid genera (Conroy and Cook 1999). Analysis of a 664 base pair (bp) fragment from the *Cytb* gene revealed 118 (17.8%) sites with variable nucleotides, of which 108 (16.3%) were parsimony informative. The majority of substitutions were in the 3rd codon positions (n = 98), followed by 1st (n = 17), and 2nd (n = 3). This 664 bp fragment translated to 221 codons. Within these 221 codons, 120 contained substitutions, of which 17 were nonsynonymous. Of nonsynonymous substitutions, 7 changes were species specific (*intermedius* n = 3, *pomo* n = 2, n = 1 each *albipes* and *longicaudus*), 1 was shared by *albipes* and *longicaudus*, and 9 were phylogenetically uninformative. Transitions outnumbered transversions by 98 to 32. Average

nucleotide frequencies were: adenine 31.1%, cytosine 26.5%, guanine 12.3%, and thymine 30.1%. Twenty-nine haplotypes were identified among 47 *longicaudus Cytb* sequence fragments. There were no redundant haplotypes among *pomo*, *albipes*, or *intermedius*.

Average Tamura-Nei intraspecific genetic distances (*d*) among *Phenacomys* voles were *longicaudus*, 1.1% (n = 47; range 0–2.1%); *pomo*, 0.4% (n = 5; range = 0.2–0.8); *albipes*, 0.2% (n = 6; range = 0.1–0.5); and *intermedius*, 1.3% (n = 2). Larger sample size and wider geographic sampling of *longicaudus* relative to *pomo* and *albipes* most likely affected differences among intraspecific *d*, and thus is probably not indicative of greater or lesser genetic diversity within taxa. Interspecific *d* among *longicaudus*, *pomo*, and *albipes* ranged from 3.4–5.7% (Table 2). *P. longicaudus*, *pomo*, and *albipes* differed from *intermedius* by 11.1–12.6%. Tamura-Nei genetic distance among genera ranged between 8.5% (*Eothenomys–Clethrionomys*) and 21.1% (*Ellobius–Clethrionomys*), with an overall average of 17.0% (Table 3). Uncorrected sequence divergence ranged from 7.9% (*Eothenomys–Clethrionomys*) to 17.9% (*Ellobius–Clethrionomys*), with an overall average of 14.9%.

Phylogenetic reconstruction.—Critical values of skewness were significant for the entire arvicolid data set (g_1 = −0.80, P < 0.01—Hillis and Huelsenbeck 1992:table 2) and combinations of genera therein, indicating that our data contain true phylogenetic signal and not random noise. All 3 phylogenetic methods were largely concordant and indicated a relationship between *P. intermedius* and what we henceforth refer to as the *Arborimus* clade, consisting of *albipes*, *pomo*, and *longicaudus*. Therefore, only a NJ phylogram is shown (Fig. 2). All genera clustered together as would be expected from current taxonomic organization of genera. Similar to Conroy and Cook (1999), we found that the relationship among *Microtus*, *Clethrionomys*, and the *Phenacomys–Arborimus* clade was unstable. Tree reconstruction with MP (1 most parsimonious tree, length = 368; consistency index, CI = 0.551; rescaled consistency index,
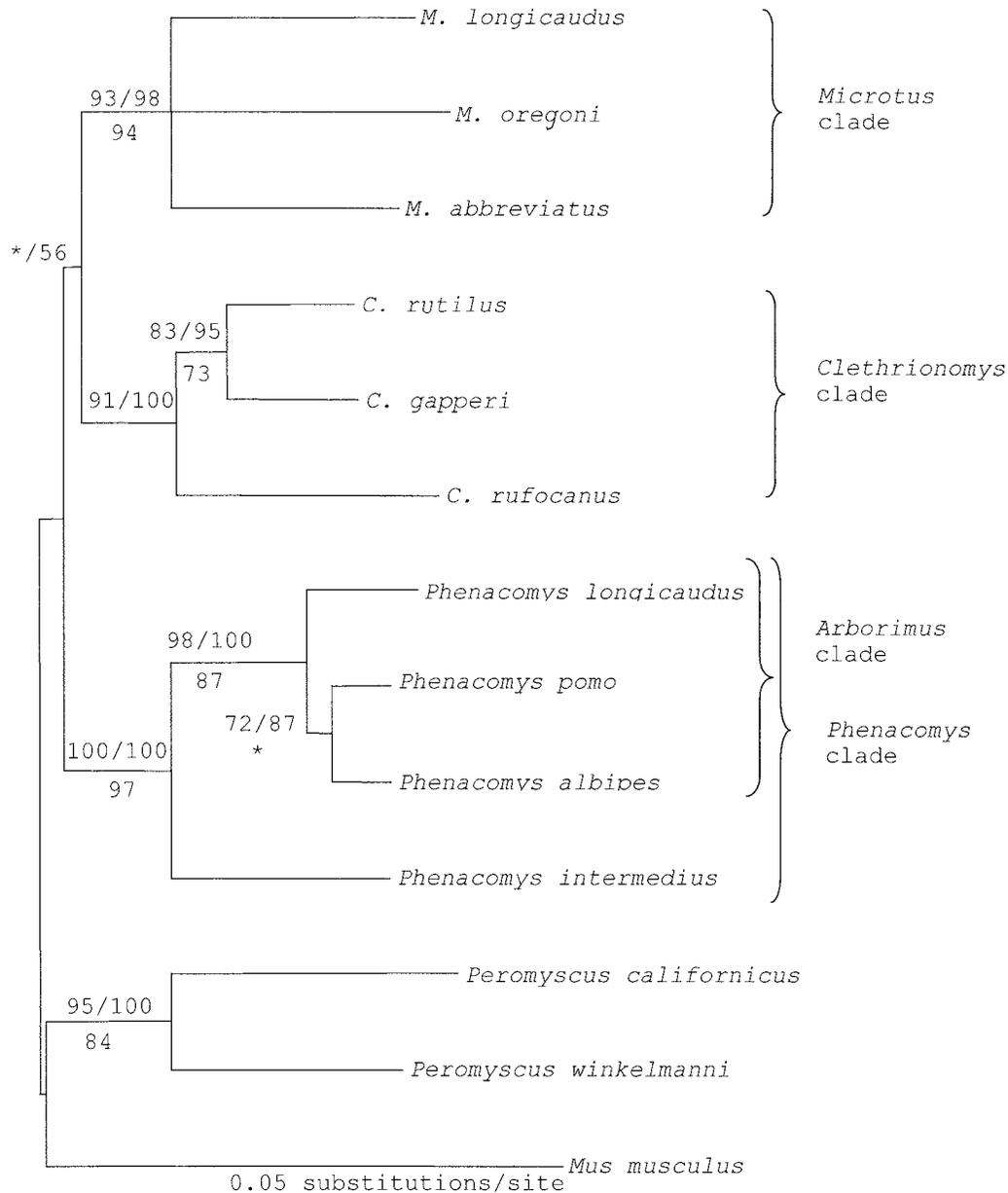
Fig. 2.—Neighbor joining phylogram (calculated with the Tamura-Nei [TrN+G] model of genetic distance, $\alpha = 0.1849$) depicting phylogenetic reconstruction of *Phenacomys*, *Microtus*, and *Clethrionomys* using 664 bases from the cytochrome *b* gene. *Mus musculus* and 2 *Peromyscus* species are used as the outgroup. Numbers above lines indicate maximum parsimony followed by neighbor-joining bootstrap support (1,000 replications); numbers below indicate maximum likelihood (100 replications, TrN+G, $\alpha = 0.1849$).

RI = 0.479) indicated a polytomy at the node joining these three genera, whereas NJ showed weak support for a sister relationship between *Clethrionomys* and *Microtus* (bs = 56). In contrast, ML ($-\ln = 3547.19$) indicated weak support for a sister relationship between *Microtus* and the *Phenacomys–Arborimus* clade (bs = 56). The 2nd major difference among trees was that the node separating *pomo* and *albipes* from *longicaudus* in MP and NJ (MP bs = 72, NJ bs = 87) trees was reduced to a polytomy using ML. We used topological constraints to force monophyly of *P. longicaudus* and *P. pomo* to see if it significantly improved ML scores (tree with constraint $-\ln = 3547.19$, tree without constraint 3547.58). Felsenstein (1988) suggests that if tree topology differs by one branch, as it

does in this case, a likelihood ratio test with 1 degree of freedom can be used to determine significance of results. Trees with and without constraints of tree vole monophyly were not significantly different ($P = 0.37$, $d.f. = 1$).

Critical values of skewness were also significant for the entire *Phenacomys* data set and all combinations of species therein ($g_1 = -0.72$, $P < 0.01$—Hillis and Huelsenbeck 1992:table 2). Tree reconstruction using all *Phenacomys* haplotypes produced 1 most parsimonious tree (length = 368, CI = 0.674, RI = 0.848) that was topologically identical to NJ and ML ($-\ln = 2660.39$) analyses, therefore only a NJ phylogram is presented (Fig. 3). Again, all methods provided strong support for the division between *P. intermedius* and the

FIG. 3.—Neighbor joining phylogram (calculated with the Tamura-Nei [TrN+G] model of genetic distance, α = 0.2678) showing phylogenetic reconstruction using 664 base pairs of the mitochondrial cytochrome *b* gene of Oregon red tree voles (*Phenacomys longicaudus longicaudus*, PLL), dusky tree voles (*P. l. silvicola*, PLS), Sonoma tree voles (*P. pomo*, PP), white-footed voles (*P. albipes*, PA), and heather voles (*P. intermedius*, PI), using 3 *Microtus* species as an outgroup. Numbers above lines indicate maximum parsimony (MP) and NJ bootstrap support (MP / NJ, 1000 replications), numbers below maximum likelihood (TrN+G, α = 0.2678, 100 replications). Physiographic provinces are as follows: CR = Coast Ranges, WC = Western Cascades, and KM = Klamath Mountains.

Arborimus clade (bs ≥ 99). All phylogenetic reconstructions produced weak structuring within the "*longicaudus*" clade, but without a clear geographic-haplotypic association. The subspecies *P. l. longicaudus* and *P. l. silvicola* did not separate within the "*longicaudus*" clade. Within the *Arborimus* clade, all methodologies indicated strong support (bs = 86–100) for the separation of *longicaudus* from a sister node containing *pomo* and *albipes*. Support for the sister relationship between *pomo* and *albipes* was moderate (MP, bs = 81) to low (NJ, bs = 70, ML = 53). ML analyses differed from MP and NJ by overall lower bootstrap support at major nodes.

The null hypothesis of a molecular clock for *Phenacomys* species plus *Mus* and *Rattus* was not rejected, suggesting that this portion of *Cytb* has evolved in a clocklike fashion (Trn+G+clock, −ln = 2410.26, Trn+G −ln = 2403.06, lrt = 14.39, *P* = 0.70, *d.f.* = 18). *Mus* and *Rattus* percentage genetic distance (TrN) was 18.9%, which equates to around 1.35% sequence divergence per million years. Applying this to our molecular clock places the divergence of the *Arborimus* clade from *intermedius* around 8.4–9.4 mya (Table 2). Divergences

within the *Arborimus* clade would have occurred 2.51–4.22 mya (Table 2).

DISCUSSION

Although the systematic position of *Phenacomys* within Arvicolinae is presently unresolved by molecular methods (Conroy and Cook 1999; Modi 1996; Murray 1995), this study is the 1st that provides definitive evidence for the close relationship between *Phenacomys* and *Arborimus*. Moreover, our results substantiate the inclusion of *albipes* within *Arborimus*. Our findings are in agreement with Johnson and Maser (1982) and Murray (1995) who suggested *albipes* was more closely related to *longicaudus* and *pomo* than to *intermedius*. In contrast to Murray (1995), we did not find that *intermedius* clustered with *Microtus*.

Repenning et al. (1987) proposed a Beringian ancestry of *Phenacomys*, with the divergence of *Phenacomys* from a progressive branch of *Mimomys*, called *Cromeromys*, occurring 3.7–4.2 mya. Two separate lineages within *Phenacomys* were recognizable as early as 2.1 mya (Repenning et al. 1987;

Repenning and Grady 1988, and references therein). The 1st lineage, subgenus *Paraphenacomys* Repenning, contained 3 species, 1 of which was thought to have persisted as the extant lineage *albipes*. The 2nd lineage was proposed to have diverged into *longicaudus* and *intermedius* as recently as 400,000 years ago (Repenning and Grady 1988). This proposed sequence of speciation events conflicts with our Cy*tb* data, Murray's (1995) biochemical data, and Johnson and Maser's (1982) morphological data.

Recently, much attention has been placed on applying a molecular clock to estimate ancient species divergence. However, results from clock data have been controversial, particularly for rodents. In general, molecular estimates of the *Mus–Rattus* divergence has been considerably earlier than what is found in the fossil record. Using 658 nuclear genes, Kumar and Hedges (1998) estimated this divergence to have occurred 40 mya, whereas Adkins et al. (2001) used 2 nuclear genes and 1 mtDNA gene and estimated this divergence occurred 23 mya. Calibrating our clock with the *Mus–Rattus* split of 14 mya places the separation of the *Arborimus* clade from *P. intermedius* occurring around 8.4–9.4 mya. In a review of paleontological evidence, Chaline and Graf (1988) cite the recent origin of arvicolids at about 5 mya. Our data for the split of *Arborimus* from *Phenacomys* obviously predates the estimated origin of the subfamily. Ignoring this, using our molecular clock data, *longicaudus* would have shared a most recent common ancestor with *albipes* and *pomo* 4.14–4.22 mya and *Pomo* and *albipes* would have shared an ancestor as recently as 2.51 mya ago. It is difficult to make meaningful comparisons of molecular estimates of species diversification dates given that a variety of calculation techniques have been used to generate these data (e.g., Conroy and Cook 1999; Kumar and Hedges 1998).

Elevation of subgenus *Arborimus* to full genus has been controversial (Hayes 1996). Taylor (1915), who 1st proposed the placement of tree voles in the subgenus *Arborimus*, was concerned that elevation of *Arborimus* to full genus carried the risk of obscuring their close taxonomic relationship with *Phenacomys*. Conversely, Johnson (1973) and Johnson and Maser (1982) summarized character differences between the 2 groups and concluded that the diversity of characters between them equaled or exceeded what is currently accepted as valid criteria for generic rank in other microtine rodents. Our data clearly demonstrate the close relationship between the *Arborimus* clade and *P. intermedius* relative to other arvicolids. Regardless of the taxonomic rank of the *Arborimus* clade, its inclusion with *Phenacomys* at a generic or supergeneric level is appropriate.

There are increasing reports of ranges of genetic distance within genera overlapping differences between genera (Martin et al. 2000; this study). For example, Smith and Patton (1991) reported sequence divergences of 3–21% among species in tribe Akodontini, subfamily Sigmondontidae, whereas Conroy and Cook (2000a) observed genetic distances from 1.5–18% within the single genus *Microtus*. Genetic distances between *Phenacomys intermedius* and members of the *Arborimus* clade were similar to those observed among other arvicolid genera.

However, overall genetic distances within the *Arborimus* clade were lower than most distances observed within *Microtus* (Conroy and Cook 2000a).

The polyphyly of tree voles and relationship of *P. albipes* to *P. pomo* was surprising. Several possibilities provide explanations for these results. First, Cy*tb* is a single, maternally-inherited gene, and thus any tree reconstruction gives rise to a matriarchal gene tree that might not represent the true species tree (Nei 1987). Tree discordance can occur due to evolutionary rate heterogeneity, stochastic lineage sorting (Neigel and Avise 1986; Tajima 1983), or introgressive hybridization (e.g., Ferris et al. 1983). We can eliminate evolutionary rate heterogeneity as an issue because our data conform to a molecular clock. The remaining 2 possibilities, lineage sorting and introgressive hybridization, should be further explored using biparentally or paternally-inherited nuclear markers. Despite alternative explanations for our findings, the possibility exists that *albipes* did share a more recent common ancestor with *pomo* than did *longicaudus*; cryptic speciation has been documented in paraphyletic lineages of white-throated wood-rats (*Neotoma albigula*—Edwards et al. 2001) and ravens (*Corvus corax*—Omland et al. 2000) in North America.

Subspecies designations in *P. longicaudus* deserve further attention. The original description of *P. l. silvicola* (Howell 1921) was based on differences in pelage color and cranial characteristics, some of which have not been consistently present in subsequent analyses (Johnson and George 1991). The absence of detectable genetic differences between *P. l. longicaudus* and *P. l. silvicola* (Johnson 1968; this study) and lack of consistently verifiable morphological differences suggest that subspecific status might not be warranted.

Our data contribute to growing support (Murray 1995) for Johnson and George's (1991) description of *pomo* and *longicaudus* as distinct monophyletic species. Splitting tree vole species somewhere near the Oregon–California border is consistent with numerous phylogeographic breaks observed in plants, reptiles, amphibians, birds, and mammals in the Klamath Mountains of southern Oregon and northern California (e.g., Demboski and Cook 2001; Haig et al. in press; Janzen et al. 2002; Soltis et al. 1997; Wagner 2001).

Resolution of tree vole taxonomy is important because it might influence forest management decisions for tree voles and other forest mammals, particularly if listing under the Endangered Species Act becomes an issue. Huff et al. (1992) suggested that *P. longicaudus* might be the most vulnerable arboreal rodent to local extinctions resulting from the loss or fragmentation of old-growth forests. *P. albipes* is a forest dwelling, riparian-associated species that appears to be rare (Verts and Carraway 1998), and thus might inherently have disjunct populations and be particularly susceptible to genetic divergence resulting from habitat fragmentation. At present, the most pressing issues are to better define the zone of demarcation between *P. longicaudus* and *P. pomo* and to develop a better understanding of local and regional genetic variation within *Arborimus* species. We are currently investigating regional and local genetic variation in *longicaudus* and hope that our work will stimulate additional studies in

California. As forests in the Pacific Northwest become increasingly fragmented, the effects of deforestation on local populations of *Arborimus* voles must be evaluated in order to assure conservation of these very unique mammals.

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APPENDIX I

GenBank accession codes for species used in comparison of Tamura-Nei (1993) genetic distances among Arvicolinae and/or for phylogenetic reconstruction using 664 base pairs of cytochrome-*b*. Species used only in phylogenetic reconstruction are indicated by an asterisk.

Taxa	Accession code	Reference
Alticola macrotis	AAD43891	Conroy and Cook 1999
Arvicola terrestris	AF159400	Martin et al. 2000
*Clethrionomys gapperi**	AF272639	Cook et al. 2001
*Clethrionomys rutilus**	AB072222	Iwasa et al. 2002
Clethrionomys rufocanus	AF429814	A. Dekonenko, in litt.
Dicrostonyx torquatus	AJ238425	Fedorov and Goropashnaya 1999
Ellobius fuscocapillus	AF126430	Conroy and Cook 1999
Eothenomys smithii	BAC57598	M. Iwasa and H. Suzuki, in litt.
Lagurus lagurus	AAL27604	A. Dekonenko, in litt.
Lemmus trimucronatus	AAD43894	Conroy and Cook 1999
Microtus abbreviatus	AF163890	Conroy and Cook 2000a
*Microtus longicaudus**	AF187230	Conroy and Cook 2000b
*Microtus oregoni**	AF163903	Conroy and Cook 2000a
*Mus musculus**	NC001569	Bibb et al. 1981
Ondatra zibethicus	AAD43895	Conroy and Cook 1999
Phenacomys longicaudus	AY338789	This study
*Peromyscus californicus**	AF155393	Tiemann-Boege et al. 2000
*Peromyscus winkelmanni**	AF131930	Bradley et al. 2000
Synaptomys borealis	AAD43877	Conroy and Cook 1999