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Phylogenetic Relationships of Cottontails (*Sylvilagus,* Lagomorpha): Congruence of 12S rDNA and Cytogenetic Data

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The genus *Sylvilagus,* which comprises the New World cottontail rabbits, contains several commercially important as well as endangered (or threatened) species. Understanding the evolution of this group is pertinent to their management and conservation. The purpose of this study was to examine the evolutionary history of the cottontails using sequence data from the mitochondrial 12S rRNA gene. The 12S data provide a robust phylogeny which was supported under a variety of phylogenetic approaches and transition/transversion (Ti/Tv) weighting schemes. Stem and loop regions of the gene were analyzed separately and two different methods of estimating Ti/Tv ratios were employed. The phylogeny obtained was consistent with available cytogenetic information. The 12S data indicate that separate generic status for the pygmy rabbit, *Brachylagus idahoensis,* is warranted based on its phylogenetic position and sequence divergence values. Additionally, the taxa which are geographically adjacent are also phylogenetically closely related; for example, the marsh rabbit, *S. palustris,* and the swamp rabbit, *S. aquaticus,* are sister taxa, as are the mountain cottontail, *S. nuttallii,* and desert cottontail, *S. audubonii.* This finding suggests that recent vicariance events might explain the diversification of several cottontail lineages.

INTRODUCTION

Cottontails, members of the genus *Sylvilagus* (Leporidae, Lagomorpha), are an important part of many New World ecosystems and are regarded as major game species in certain areas. Additionally, several species are classified as rare and endangered by the IUCN (Chapman and Flux, 1990). As such, understanding the evolutionary relationships of the taxa within *Sylvilagus* is a valuable tool in the ecological and commercial management of these species. Compared to *Lepus* (the hares and jack-rabbits), cottontails are less cursorial and have relatively shorter limbs, and their young are mostly altricial at birth.

The genus, which comprises 13 species (Hoffman, 1993), is distributed throughout North and Central America and extends into the northern half of South America. Although many of the cottontails are widely distributed (e.g., *S. audubonii, S. floridanus, S. bachmani*), some of the Mexican species are extremely restricted; for example, *S. graysoni* and *S. mansuetus* occur only on a few islands in the Gulf of California (Angermann *et al.,* 1990; Chapman and Ceballos, 1990). The Omilteme cottontail, *S. insonus,* is, perhaps, the least-known species (<10 specimens examined) and is limited to high elevations in Guerrero, Mexico (Chapman and Ceballos, 1990).

The current estimates of species within *Sylvilagus* may also be incorrect; in part, this is due to the difficulties in delimiting the taxa on morphological grounds and is compounded by the likely presence of cryptic forms among some of the more widely distributed species. For example, the New England cottontail was thought to occur from Maine to Alabama, with its distribution bound closely to the Appalachian mountain range in the eastern United States. However, recent cytogenetic and morphometric studies revealed the presence of two distinct species in what was conventionally regarded a single taxon, *S. transitionalis.* As currently recognized, *S. transitionalis* is limited to boreal habitat, while *S. obscurus* is a high alpine species restricted to the Appalachian mountain chain and associated mountain balds (Chapman *et al.,* 1992). Substantial morphological differences have also been noted in the North American and South American populations of the eastern cottontail, *S. floridanus* (Diersing, 1981). Thus, the current estimates of population size and distribution may be flawed for some of the more widely ranging species.

The phylogenetic position of the pygmy rabbit, *Brachylagus idahoensis,* relative to *Sylvilagus* has been the subject of taxonomic debate. Although described as a distinct genus, it was later included as a

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member of *Sylvilagus* (see Green and Flinders, 1980). In contrast, others (Dawson, 1981; Chapman and Flux, 1990) considered this leporid to be an early offshoot outside the main leporid radiation. Recent phylogenetic analyses of the 12S rRNA and cytochrome *b* mitochondrial genes suggest that *B. idahoensis* is closely related to *Sylvilagus* (Halanych and Robinson, submitted). However, the species representation in that study was insufficient to determine unequivocally if *B. idahoensis* fell within the *Sylvilagus* clade. Consequently, we have included the monotypic *Brachylagus* together with a *Lepus* representative and six *Sylvilagus* species in order to more accurately assess the phylogenetic position of this enigmatic species.

Previous estimates of *Sylvilagus* phylogeny are based largely on cytogenetic data (Robinson *et al.,* 1983a, 1984; see also Chapman and Ceballos, 1990). In these studies, Robertsonian fusions (or centric fusions) of chromosomes provide the main source of phylogenetic information and are responsible for the range in diploid numbers from $2n = 38$ to $2n = 48$. The only cottontail species which shows some variation in this mode of chromosomal evolution is *S. transitionalis,* which has $2n = 52$, and is thought to differ from the presumed ancestral karyotype ($2n = 48$; Robinson *et al.,* 1981) through the presence of two centric fission events (Ruedas *et al.,* 1989).

To gain a better understanding of the evolutionary relationships among cottontails, we employed sequence data (728 bp) from the mitochondrial 12S ribosomal subunit gene in parsimony, neighbor-joining, and maximum-likelihood analyses. Although this study lacks some species of cottontails, including taxa with very limited distributions (e.g., *S. graysoni, S. insonus, S. mansueteus*), it provides the most rigorous phylogenetic analysis of the cottontails to date. As such, it provides an important framework upon which future comparative studies can be based.

Our analyses show that the 12S rRNA gene can be a powerful molecular marker for determining interspecific relationships and relatively recent evolutionary events in these mammals. Moreover, in an attempt to identify which region of the 12S gene provides phylogenetic signal, we analyzed stem and loop positions separately. Empirical estimations of the transition (Ti) to transversion (Tv) ratio were made by two different methods and confirm earlier reports (e.g., Springer and Douzery, 1996) that the stem regions appear less prone to mutational saturation by transitions than loop regions.

MATERIALS AND METHODS

Data Collection

Table 1 lists the species analyzed and the GenBank accession number for the 12S rDNA data used in this study. The aligned data set has been submitted to TREEBASE (http://phylogeny.harvard.edu/treebase) or can be obtained from the authors on request.

TABLE 1

Taxa Used in the Phylogenetic Analysis and Their GenBank Accession Numbers

Specific name	Common name	GenBank accession number
Brachylagus idahoensis	Pygmy rabbit	U58921[a]
Lepus americanus	Snowshoe hare	U58923[a]
Sylvilagus aquaticus	Swamp rabbit	U58927
Sylvilagus audubonii	Desert or Audubon's cottontail	U58928
Sylvilagus floridanus	Eastern cottontail	U58929
Sylvilagus nuttallii	Mountain or Nuttall's cottontail	U63886
Sylvilagus obscurus	Appalachian cottontail	U63887
Sylvilagus palustris	Marsh rabbit	U63885
Pronolagus crassicaudatus	Greater red rock rabbit	U31044[b]

[a] Halanych and Robinson (submitted).
[b] Whiteford (1995).

Total genomic DNA was extracted from fibroblast cells established from ear clippings of the various species (for collection localities see Robinson *et al.,* 1983a,b, 1984; Chapman *et al.,* 1992, for the taxonomic treatment of *S. transitionalis–S. obscurus*) following Maniatis *et al.* (1982). The oligonucleotide primers, A and D, developed by Allard and Honeycutt (1992) were used to amplify an approximately 820-bp region of the 12S ribosomal gene corresponding to the region from the 3′ end of helix 6 to the 3′ end of helix 33 (as numbered in van de Peer *et al.,* 1994). Standard PCR techniques were employed (see Halanych and Robinson, submitted, for cycling parameters), and amplified fragments were purified by agarose gel electrophoresis using the Cleanmix system from Talent, Inc. The use of biotinylated primers during PCR allowed the generation of single-stranded templates for sequencing. Complete sequences for both strands were obtained using the Sequenase v2.0 kit from U.S. Biochemical in conjunction with the PCR oligonucleotide primers and the internal oligonucleotide primers B and C (Allard and Honeycutt, 1992). Fragments were visualized using 6% acrylamide gel electrophoresis and standard audioradiographic techniques (Hillis *et al.,* 1990).

Phylogenetic Analyses

The alignment was produced with the multiple alignment program Clustal W (Thompson *et al.,* 1994) and corrected by hand for obvious alignment errors. One region, 6 bp in length, that could not be unambiguously aligned was excluded from the analyses. The boundaries of the excluded region were trimmed back to the last uninformative character.

The PAUP software package (version 3.1.2d5; Swof-

ford, 1993) was used for parsimony analyses. Additionally, we used PHYLIP version 3.5 (Felsenstein, 1993) for neighbor-joining (DNAdist and Neighbor programs) and maximum-likelihood (DNAml program) estimates, and MacClade version 3.0 (Maddison and Maddison, 1992) to determine various character statistics and tree lengths. All parsimony and neighbor-joining bootstrap values reported here are based on 500 iterations and, in the case of parsimony bootstraps, the general heuristic search algorithm (using the default settings) was employed. Maximum likelihood bootstraps employed only 100 iterations due to computation time. For neighbor-joining and maximum-likelihood bootstrap analyses, the Seqboot and Consense programs of PHYLIP were used. Although the gobal search option and jumble option (seed = 1, 10 iterations) were used for producing single trees under different Ti/Tv weighting schemes for maximum likelihood, they were not employed in the bootstrap analysis due to time. All maximum likelihood searches employed a random sequence addition and had two rate categories (means determined empirically = 0.22 and 1.78) to account for the different rates in stems and loops.

The ratios of 1:1, 2:1, 3:1, and 10:1 were used to weight transversions over transitions in the analyses. Although these ratios were arbitrarily chosen, the empirical ratio for stem regions, loop regions, and the entire 12S data set was determined by two different methods. First, the "state changes and stasis" option of MacClade was used to count the average number of Ti and Tv events on 100 equiprobable random trees (Halanych, 1996). Although this method is easily biased by saturation effects, they did not pose a problem for this data set (because of the limited mutational saturation, see below).

The second method involved a maximum-likelihood estimation of Ti/Tv by determining the Ti/Tv value which produces the best log-likelihood score (Felsenstein, personal communication). This was done with the DNAml program in PHYLIP employing the user-defined tree option in order to reduce computation time. In this case, the topology in Fig. 1a was inputted as a predefined user-tree upon which the log-likelihood score for several different Ti/Tv values could be analyzed. The tree topology was extremely robust and was repeatedly recovered under a variety of weighting schemes (see below).

Pronolagus crassicaudatus was designated as the outgroup to polarize the characters and root the resultant trees. This species is clearly distinct from the other leporid lineages and occupies a basal position in leporid phylogeny (Halanych and Robinson, submitted). The *L. americanus* sequence was included to help assess the relationship of *B. idahoensis* relative to the *Sylvilagus* clade.

Considerable attention has been given to the mode and tempo of molecular evolution in the stem versus loop regions of the ribosomal subunit genes (e.g.,

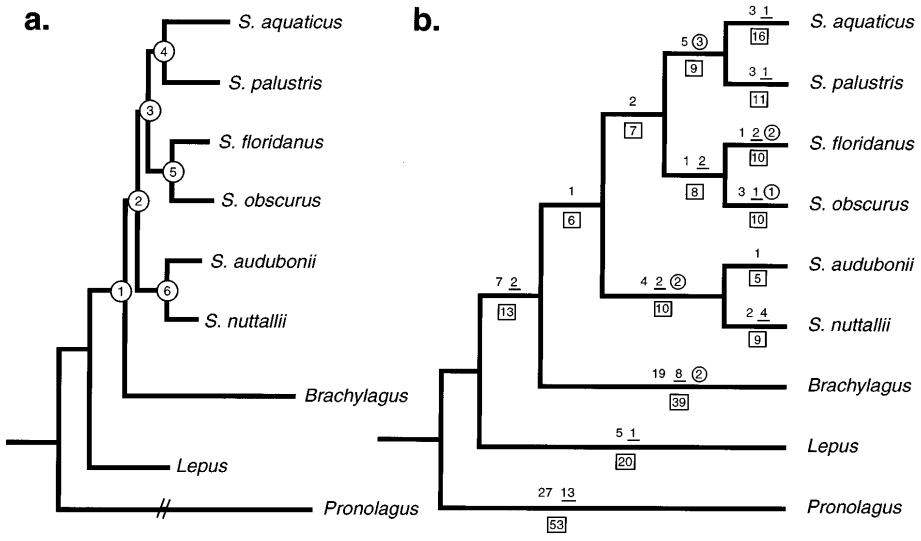

FIG. 1. (a) The topology obtained for all parsimony, neighbor-joining, and maximum likelihood analyses conducted on the entire 12 S rDNA data set. The only exception to this outcome was with the parsimony reconstruction when transversions were weighted 10 times transitions (see text for details). The internal nodes are numbered to correspond with the bootstrap results given in Table 3. The branch length is proportional to the amount of change along the branch produced by neighbor joining with a Jin and Nei (1991) model with an empirically derived correction for among-site rate variation ($\alpha = 0.42$) when the empirical Ti/Tv value is 3:1. This tree also served as the input tree for the maximum likelihood calculation of the empirical Ti/Tv ratios. (b) The topology produced by the combined 12S rDNA and cytogenetic parsimony analysis using the branch and bound algorithm. All characters were weighted equally and the tree was 229 steps with a CI of 0.786 (CI minus uninformative characters was 0.629). The number and types of character changes are mapped along the branches, but only for unambiguous characters (i.e., characters with a CI = 1 on this topology): plain text = transition events, underlined text = transversion events, and circled text = cytogenetic characters (i.e., number of centric fusions). The boxed text shows branch length (i.e., total number of changes along a branch) when all characters are included and when accelerated character transformation (ACCTRAN option) is assumed.

Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993; Springer and Douzery, 1996). Therefore, using the secondary structure model of Springer and Douzery (1996) for a generalized mammalian 12S secondary structure, our 12S rDNA data were divided into stem and loop regions. Loops are defined here as all unpaired nucleotides, while stems are those nucleotides that exhibit base pairing (Dixon and Hillis, 1993). The 6-bp region which could not be unambiguously aligned occurred in the loop region between stems 17 and 18 as determined by Springer and Douzery (1996). The leporid sequence fits the generalized mammalian model well. There was one nucleotide pair downstream of stem 11 which is considered unpaired in the published model but in leporids, this pair contained a C and a G and was therefore counted as a stem position.

Cytogenetic Data

By comparing the *Sylvilagus* karyotypes to that of *Lepus*, which is thought to have retained the ancestral leporid condition, Robinson *et al.* (1984) were able to identify the chromosomes that had been involved in the evolution of karyotypes of the extant taxa. We used their data to determine whether there was any concordance between the cytogenetic results and those obtained from 12S rDNA. The information from Robinson *et al.* (1984, Fig. 8a) was coded as binary characters for incorporation into a combined phylogenetic analysis (their Fig. 8b was not considered here because it hypothesized an unobserved intermediate chromosomal state, the fusion of chromosomes 17/21). The character state descriptions and data matrix are shown in the appendix. All of the characters reflect centric fusion events. Because of the flexibility of available software, only a parsimony analysis was conducted on the combined 12S rDNA and cytogenetic data.

RESULTS

Table 2 list the numbers of positions analyzed, variable positions, and informative positions for the entire 12S data set, the stem regions, and the loop regions. The g_1 statistic, which is a measure of phylogenetic signal, is also shown and indicates that the data contain more signal than expected at random (Hillis and Huelsenbeck, 1992). The calculated g_1 values are relatively robust even when various combinations of taxa, including the outgroup ($g_1 = -0.69$) or taxa in the well-supported tips, are excluded.

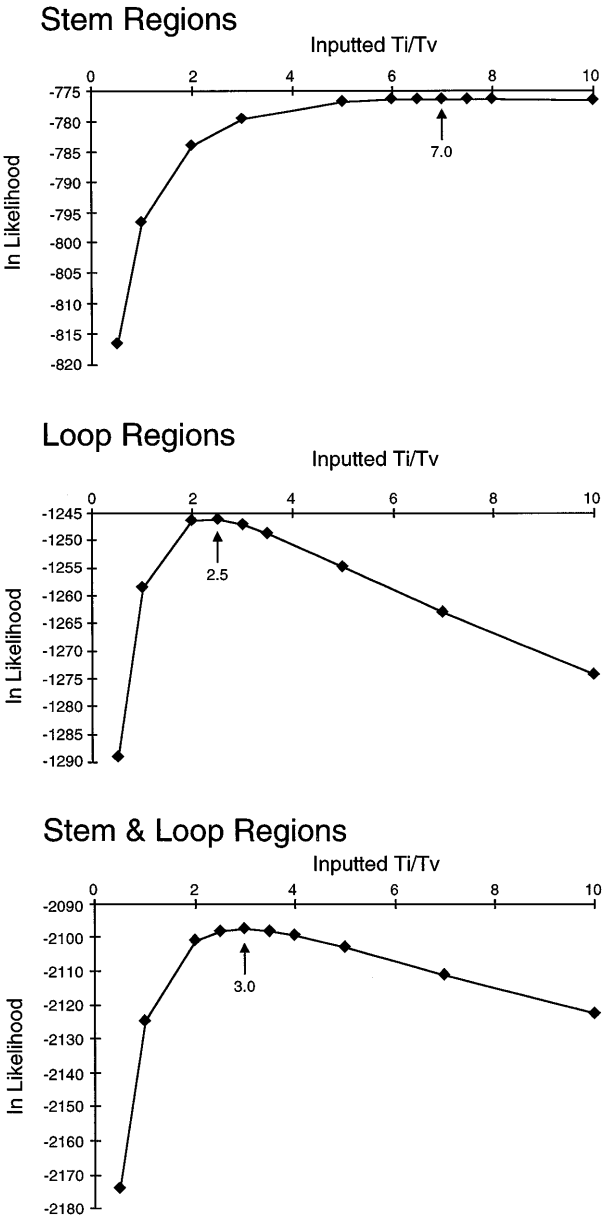

FIG. 2. The empirical Ti/Tv estimates using the DNAml program of PHYLIP and the user-defined tree shown in Fig. 1a. The values along the abscissa are the inputted Ti/Tv ratios, and the values on the ordinate are the maximum likelihood scores obtained. The results for stems only, loops only, and both regions combined are shown. The arrows indicate the value that had the highest likelihood score.

TABLE 2

Phylogenetic Information Present in the Three 12S rDNA Data Sets Examined

	Stems	Loops	Stems and loops
Positions analyzed	334	388	722
Variable characters	53	92	145
Percentage variable characters	15.9%	23.7%	20.1%
Informative characters	12	47	59
Percentage informative characters	3.6%	12.11%	8.2%
g_1 statistic	−1.159	−0.687	−0.812

Ti/Tv Estimates

Both methods of calculating the empirical Ti/Tv value for the data sets gave similar results. The maximum likelihood estimates are depicted in graphical form in Fig. 2. The values for the Ti/Tv ratios derived

from MacClade are 2.74 = minimum, 3.18 = average, and 3.50 = maximum for the entire 12S sequence; 6.31 = minimum, 7.18 = average, and 7.40 = maximum for stems only; and 2.29 = minimum, 2.65 = average, and 2.99 = maximum for loop regions only. To reduce computation time in subsequent analyses, the empirical values were rounded off to 7:1, 2.5:1, and 3:1 and used respectively for the stem regions, the loop regions, and the stem and loop regions combined.

Phylogeny Reconstruction

When transversions were weighted three times transitions, the branch and bound algorithm of PAUP produced a single most parsimonious tree of 329 steps (Fig. 1a). The same single tree is also recovered under an equal (215 steps, CI = 0.781, CI excluding uninformative characters = 0.618) or a 2:1 weighting scheme (272 steps). Under the extreme transversion weighting of 10:1, the topology of the single most parsimonious tree (715 steps) differed in that *B. idahoensis* clustered with *S. palustris* within the *Sylvilagus* clade, and *S. aquaticus* clustered with *S. nuttallii* and *S. audubonii.* These groupings were not supported by bootstrap analysis. The bootstrap values obtained under all weighting schemes are shown in Table 3.

The topology in Fig. 1a was also recovered in all the maximum-likelihood and neighbor-joining reconstructions of the entire 12S data set. For both methods, weighting schemes that considered transversions equal, twice, 3 times, and 10 times less frequent than transitions were employed. The neighbor-joining analysis was based on a Jin and Nei (1991) correction model with $\alpha = 0.42$ to account for among-site rate variation; this value was calculated from the data using the approach described in Sullivan *et al.* (1995). Figure 1a shows the neighbor-joining tree with proportional branch lengths, and the bootstrap support for both the neighbor-joining and the maximum-likelihood analyses are shown in Table 3. The distance estimates used to reconstruct the branch lengths in Fig. 1a are presented in Table 4. For comparative purposes, the absolute pairwise distances between taxa are also shown.

TABLE 3

Bootstrap Results for Internal Nodes Labeled in Fig. 1a under Various Reconstruction Methods and Weightings

			Nodes[a]					
Dataset	Method	Ti/Tv	1	2	3	4	5	6
Stems and Loops	Parsimony	1:1	92	52	—	53	89	95
		2:1	89	—	—	—	86	93
		3:1	83	—	—	—	77	88
		10:1	58	—	—	—	52	68
	Neighbor joining	3:1	92	74	65	65	84	95
	Maximum likelihood	3:1	96	59	53	59	84	96
12S and Cytogenetic	Parsimony	1:1	92	51	—	87	84	99
Loop only	Parsimony	1:1	72	—	—	—	89	96
		2:1	76	—	—	—	83	95
		2.5:1	76	—	—	—	78	91
		3:1	74	—	—	—	74	88
		10:1	54	—	—	—	—	67

[a] Values of <50% are not shown and all values represent bootstrap percentages.

TABLE 4

Distances Based on the Total 12S rDNA Sequence Examined: Above Diagonal—Distances Calculated with the Jin and Nei (1991) Correction ($\alpha = 0.42$), Below Diagonal—Absolute Distances[a]

	Bid	*Lam*	*Saq*	*Spa*	*Sfl*	*Snu*	*Sau*	*Sob*	*Pcr*
Bid	—	0.0944	0.0906	0.0838	0.0848	0.0823	0.0793	0.0814	0.1483
Lam	62	—	0.0726	0.0665	0.0601	0.0619	0.0601	0.0634	0.1098
Saq	60	49	—	0.0388	0.0492	0.0472	0.0432	0.0495	0.1265
Spa	56	45	27	—	0.0339	0.0520	0.0493	0.0465	0.1267
Sfl	55	40	33	23	—	0.0476	0.0461	0.0249	0.1214
Snu	52	40	31	34	31	—	0.0210	0.0412	0.1124
Sau	53	41	30	34	31	14	—	0.0435	0.1182
Sob	54	43	34	32	17	27	30	—	0.1254
Pcr	93	71	81	81	76	69	76	80	—

[a] Taxa are designated by the first letter of their generic name and the first two letters of their species name.

Stems versus Loops

Although the phylogeny obtained is well supported and is robust irrespective of the reconstruction method or Ti/Tv weighting scheme employed, it is desirable to understand how different regions of the gene evolve since this information can be applied to future phylogenetic studies. To this end, stem regions and loop regions were analyzed separately to determine differences in their evolutionary patterns. There was a high percentage of adenine (mean across taxa = 48.0%) and a low percentage of guanine (mean across taxa = 10.0%) in the leporid loop regions (Table 5), confirming trends in other species (Vawter and Brown, 1993; Springer *et al.*, 1995; Springer and Douzery, 1996). The preponderance of adenosines in loop regions may promote hydrophobic interactions with proteins (Gutell *et al.*, 1985) and may therefore be maintained by selective forces.

As indicated above, there was a marked difference in the occurrence of transition events relative to transversion events in stem versus loop regions (Fig. 2). Ti/Tv ratios close to one suggest that mutational saturation may have occurred (alternatively, it can be argued that too brief a period of time has passed to allow for the accumulation of sufficient numbers of mutations). This phenomenon can be illustrated by plotting the nucleotide substitutions (absolute or percent) against divergence time (see Fig. 7 in Janczewski *et al.*, 1995; Fig. 4 in Springer and Douzery, 1996). Because the Ti/Tv values of the cottontail data are much greater than one, however, excessive mutational saturation has appar-

TABLE 5

Percentage Nucleotide Composition of Different Regions of the 12S Ribosomal Subunit Gene[a]

	Stems				Loops				Stems and loops			
Taxon	A	C	G	T	A	C	G	T	A	C	G	T
Bid	23.7	22.8	26.9	26.6	47.0	21.7	10.9	20.4	36.2	22.2	18.3	23.3
Lam	23.4	22.8	26.6	27.2	48.7	23.3	9.3	18.7	36.9	23.1	17.4	22.6
Saq	22.5	24.3	27.3	25.8	48.2	24.1	9.8	17.9	36.3	24.2	17.9	21.6
Sau	23.4	23.1	26.6	26.9	48.8	21.6	10.1	19.7	36.9	22.3	17.8	23.1
Sfl	24.2	23.3	25.5	27.0	49.6	21.2	9.1	20.1	37.7	22.2	16.8	23.3
Snu	23.8	23.5	26.3	26.3	48.8	22.4	9.7	19.1	37.0	23.0	17.5	22.5
Sob	23.1	23.1	26.6	27.2	47.5	22.5	9.9	20.1	36.1	22.7	17.7	23.4
Spa	23.4	23.4	26.6	26.6	47.9	23.8	9.6	18.7	36.5	23.6	17.5	22.4
Pcr	21.6	24.6	27.6	26.1	46.2	24.4	11.4	17.9	34.8	24.5	18.9	21.7
Mean	23.2	23.4	26.7	26.7	48.0	22.8	10.0	19.2	36.5	23.1	17.8	22.7
Bias[b]		0.045				0.307				0.153		

[a] Taxa are designated by the first letter of their generic name and the first two letters of their species name.

[b] Calculated as in Irwin *et al.* (1991).

ently not occurred, and both stems and loops should yield phylogenetic information.

By conducting separate phylogenetic analyses of the stem and loop regions, we were able to determine how the signal in these regions differed temporally. Under all of the weighting schemes employed (1:1, 2:1, 3:1, 10:1, and the empirical value of 7:1), the stem regions failed to resolve any of the relationships within the *Sylvilagus–Brachylagus* clade when a branch and bound parsimony search was employed. All weighting schemes produced the same 13 trees, a strict consensus of which had only one resolved node (that uniting *Sylvilagus* and *Brachylagus* which had 74% bootstrap support across all weighting schemes).

The loop regions, on the other hand, yielded more resolution within the *Sylvilagus* clade. Weightings of 1:1 and 2:1 produced five most parsimonious trees each, whereas weightings of 3:1 and 10:1 produced a different single most parsimonious tree (all searches were branch and bound). The empirical weighting (2.5:1) recovered all six of these trees as most parsimonious. The 3:1 and 10:1 tree was problematic in that it clustered *B. idahoensis* with *S. palustris*, but this association is due to a single transversion (A to T) in character 278 which is inappropriately overweighed by the 3:1 and 10:1 weighting schemes. (Note: On this tree, the *B. idahoensis–S. palustris* node is also supported by a transversion in character 68, but this character is homoplasic as *S. floridanus* has the same state, T.) Bootstrap results for the loops regions did, however, show strong support for certain associations (Table 3).

Cytogenetic and 12S rDNA Phylogeny

The phylogeny produced by a branch and bound search algorithm when the 12 cytogenetic characters were added (assuming equal weighting for all characters) has the same topology as in Fig. 1a. This tree has a length of 229 and a CI of 0.786 (CI excluding uninformative characters was 0.629). The bootstrap values for the tree are shown in Table 3. Figure 1b shows the distribution of unambiguous (i.e., characters with a CI of one) transversions, transitions, and centric fusions. Under the criteria of Bull *et al.* (1993), the cytogenetic and sequence data are not significantly heterogeneous ($p = 0.47$ for 500 iterations), and therefore can be combined.

DISCUSSION

The 12S rDNA sequence data consistently resolved the same tree topology (Fig. 1a) regardless of the phylogenetic method or the Ti/Tv weighting scheme employed. This indicates that the phylogenetic signal in these data is robust (also suggested by the g_1 statistic) under a wide range of assumptions. Bootstrap analysis, however, revealed some weakly supported nodes and these are discussed in detail below. The only instances where the topology varied involved the use of the more extreme Ti/Tv weighting schemes (e.g., 10:1 for the full data set) in parsimony and, in these cases, the variation resulted from the inappropriate weighting of a single transversion.

Sylvilagus Phylogeny

The phylogeny presented herein, while certainly not complete, does offer several insights into the evolutionary relationships of *Sylvilagus*. Morphological characters that are diagnostic of the *Sylvilagus* clade have been long recognized (see Corbet, 1983; Chapman and Ceballos, 1990) but the proposed inclusion of the pygmy rabbit, *Brachylagus idahoensis*, within the cottontails (Orr, 1940; Hall and Kelson, 1959) has been the subject of considerable taxonomic debate. The arguments of Dawson (1967), Green and Flinders (1980), and Corbet (1983) for the separate generic status are supported by the 12S data. In all of the phylogenetic reconstructions (except some of the parsimony analyses with higher Ti/Tv weighting schemes), *Brachylagus* fell out as the sister taxon to the cottontail clade. However, the branch leading to the *Sylvilagus* clade was very short, as is evidenced by its low bootstrap values. Also, a Kishino-Hagesawa (1989) test found that the maximum likelihood scores for the two equally parsimonious trees in which *Brachylagus* and *Sylvilagus* were not sister taxa were not significantly better than the most parsimonious solution.

Not only does the pygmy rabbit usually fall outside the *Sylvilagus* clade, but it is also quite distinct from the cottontails based on genetic distance. The sequence divergences (based on the 12S data corrected by a Kimura 2-parameter model with a gamma correction; Jin and Nei, 1991) within *Sylvilagus* varied from 2.1 to 5.2%, whereas values between *B. idahoensis* and the various cottontail species ranged from 7.9 to 9.1%

(Table 4). These latter distances are comparable with leporid intergeneric values (see Table 3 in Halanych and Robinson, submitted). Interestingly, the cytogenetic data (Robinson *et al.*, 1984) revealed that, unlike other *Sylvilagus* species, the pygmy rabbit genome was noteworthy for the small amounts of pericentromeric heretochromatin. Thus, while both data sets clearly emphasize its distinctiveness, the mitochondrial sequence data (Halanych and Robinson, submitted) indicate that *Brachylagus* is the sister taxon of *Sylvilagus*.

The swamp rabbit, *S. aquaticus*, and the marsh rabbit, *S. palustris*, are found in water inundated habitats, as their common names imply. This feature provided the original justification for the generic name, *Limnolagus*, proposed by Lyon (1904) and later supported by Corbet (1983). However, based on both the 12S rDNA phylogeny and the cytogenetic data (Robinson *et al.*, 1984), these two species fall well within the *Sylvilagus* clade. The swamp and marsh rabbits have a parapatric distribution in the southeastern United States and are placed as each others closest relative both by the 12S rDNA (with a sequence divergence value of 3.9%) and the cytogenetic data (three unambiguously shared centric fusions), suggesting that the preference for a watery habitat type arose only once in cottontail evolution.

The eastern cottontail, *S. floridanus*, and the Appalachian cottontail, *S. obscurus*, also are closely related (sequence divergence value = 2.5%; Table 4). Greater resolution of their relationships is anticipated since there are other species which are within the clade defined by the last common ancestor of *S. floridanus* and *S. obscurus* which have not been included in this study. Importantly, the New England cottontail, *S. transitionalis*, is reported to share cytogenetic synapomorphies (Ruedas *et al.*, 1989) with an extremely morphologically similar species, *S. obscurus* (referred to as *S. transitionalis* by Robinson *et al.*, 1984; see Chapman *et al.*, 1992, for new species description), to the exclusion of other cottontail species. Additionally, a phylogenetic scheme (Chapman and Ceballos, 1990), based on a variety of data, argues for a recent common ancestry between *S. floridanus* and some of the Mexican cottontails (*S. cunicularis* and *S. graysoni*). These phylogenetic considerations, and the high number of subspecies described for the eastern cottontail (Chapman *et al.*, 1980), suggest that the cottontails within this clade have undergone a relatively recent radiation within a short period.

The desert cottontail, *S. audubonii*, and the mountain cottontail, *S. nuttallii*, are the two most closely related taxa included in our analysis (2.1% sequence divergence; Table 4). They also share three centric fusions (Robinson *et al.*, 1984). The mountain cottontail extends through the western regions of the United States to the north of *S. audubonii*'s range, but there is a broad zone of sympatry between the two (Chapman and Ceballos, 1990). Unfortunately, more information is needed to accurately delimit the distributions of these species before an assessment of the possible biogeographic factors influencing their speciation can be made.

Nonetheless, when the distribution maps (Chapman and Ceballos, 1990) of the species contained in this investigation are considered, there appears to be an association between phylogeny and biogeography. In other words, parapatric species are seemingly each other's closest relatives. This is true for both the *S. nuttallii–S. audubonii* and the *S. aquaticus–S. palustris* clades in Fig. 1a. The situation between *S. floridanus* and *S. obscurus* is more complicated given *S. floridanus*' extensive range and the fact that *S. transitionalis* (the presumed sister taxon of *S. obscurus* based on cytogenetic data; Ruedas *et al.*, 1989) was not included in our study. These data would suggest that vicariance may have played a significant role in *Sylvilagus* evolution.

Congruence with Cytogenetic Data

The 12S rDNA phylogeny shows a remarkable degree of concordance with the cytogenetic phylogeny presented by Robinson *et al.* (Fig. 8, 1984). The only discrepancy between the two studies is the position of *S. obscurus*, which, in the absence of chromosomal synapomorphies, was placed as part of a basal polytomy within the *Sylvilagus* clade. However, the results here indicate that *S. obscurus* is related to *S. floridanus* well within the cottontail clade.

In order to determine if the cytogenetic data could offer any additional support for the topology in Fig. 1a, a combined analysis using 12S and cytogenetic data was performed. The cytogenetic data offer three unambiguous characters (centric fusions) which support the clustering of *S. aquaticus* and *S. palustris*. Based solely on the 12S rDNA data, the bootstrap support for this node was weak, but inclusion of the cytogenetic data resulted in a bootstrap value of 87% (Table 3). The only cytogenetic characters which support deeper branching events contain homoplasy as evidenced by the presence of the same centric fusion in different lineages. For example, the fusion of 12/23 may have occurred twice independently in cottontail evolution (Robinson *et al.*, 1984).

Although we have used cytogenetic data to help resolve the phylogeny of *Sylvilagus*, the reciprocal question can also be addressed. What does phylogeny tell us about chromosomal evolution in this group of mammals? Given that the sister taxa *S. nuttalli*/*S. audubonii* and *S. aquaticus*/*S. palustris* share identical karyotypes, quite obviously, speciation in these taxa has not involved chromosomal rearrangement. Intriguingly, however, the lineages leading to both groups of sister species (as well as to the other cottontails where centric fusion has driven chromosome evolution) are distinguished by a high proportion of monobrachial centric fusions. This type of rearrangement (where one arm of a fusion chromosome is homologous between

populations but the other is nonhomologous) is thought to provide an effective means of disrupting gene flow between populations and of promoting speciation in a wide range of mammals (Baker and Bickham, 1986). Under the conditions of this model, isolated populations become fixed for different chromosomal fusions with each encountering minimal segregational difficulties. However, where hybridization through secondary contact occurs, significant meiotic problems are anticipated and reproductive isolation would ensue; the effectiveness of the reproductive isolation would become greater as the number of monobrachial fusions that distinguish taxa increases. Given the high number of monobrachial centric fusions characterizing the deeper nodes in the cottontail phylogeny, it seems possible that cladogenic events in the lagomorphs were caused, in part, by chromosomal change which was probably mediated by vicariance events.

Conclusions

The 12S rDNA data provide sufficient phylogenetic signal to allow resolution of leporid interspecific relationships. Although the loops and stem regions of the gene are evolving at different modes (Ti/Tv ratios) and rates, the best estimate of phylogeny was made when both regions were included. The robustly supported 12S phylogeny is consistent with previous hypotheses based on cytogenetic data. The pygmy rabbit, *B. idahoensis*, warrants separate generic status based on tree topology and sequence divergence values. *S. aquaticus* and *S. palustris* fall within the cottontail clade, suggesting that they should not be placed in their own genus. The phylogeny also clusters the most geographically close taxa as sister taxa, suggesting that vicariance has played an important role in the recent evolution of cottontails.

APPENDIX: CYTOGENETIC CHARACTERS

The 12 cytogenetic characters used in our analysis are centric fusion products whose identification was established by G-banding (Robinson *et al.*, 1984). The numbering follows that of the *Lepus* standard. The characters were scored as binary: 0 = absent and 1 = present. *Pronolagus crassicaudatus* was scored as "N" for all character indicating that the characters are not applicable.

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	17/19	12/23	14/15	13/15	12/13	14/17	11/22	9/19	17/23	12/21	15/18	8/4
B. idahoensis	0	0	0	0	0	0	0	0	0	0	1	1
L. americanus	0	0	0	0	0	0	0	0	0	0	0	0
S. aquaticus	0	1	0	1	0	1	1	1	0	0	0	0
S. audubonii	1	1	1	0	0	0	0	0	0	0	0	0
S. floridanus	0	0	0	1	0	0	0	0	1	1	0	0
S. nuttallii	1	1	1	0	0	0	0	0	0	0	0	0
S. obscurus	0	0	0	0	1	0	0	0	0	0	0	0
S. palustris	0	1	0	1	0	1	1	1	0	0	0	0
P. crassicaud.	N	N	N	N	N	N	N	N	N	N	N	N

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