Patterns of restriction-endonuclease site and length variation at the nuclear rDNA locus (18S + 28S rRNA gene complex) were examined in rodents. Of the 164 restriction sites mapped for seven species, 22 were conserved (mapping to the 18S, 28S, and 5.8S genes and ITS1) in all three *Onychomys* species as well as in *Mus musculus* and in three closely related peromyscine rodents, *Peromyscus boylii*, *P. eremicus*, and *Reithrodontomys megalotis*. The nontranscribed spacer (NTS) region revealed most of the variation among these taxa, with the patterns of variation grouping into the following categories, (1) intraindividual variation revealing as many as four site-specific repeat types within an individual, (2) intraspecific and interspecific site variation confined to the NTS, and (3) length variation in both the transcribed and NTS regions. Length variation in the 28S rRNA gene was also examined in 17 additional rodent species, and most size differences mapped to the divergent domain, D8, found in sequence comparisons between *Mus* and *Rattus*. The systematic implications of rDNA variation are discussed using the perspective gained from these rodent comparisons.

**Introduction**

The rDNA repeating unit in vertebrates consists of three gene regions (18S, 5.8S, and 28S rRNA genes), two internal transcribed spacers (ITS-1 and ITS-2) which separate each gene, and an external transcribed spacer (ETS) at the 5' end of the 18S rRNA gene (fig. 1). This gene complex is represented in the mammalian genome by multiple copies (50–250) which are located on one to several pairs of chromosomes (Appels and Honeycutt 1986). Each copy or repeating unit is flanked by a nontranscribed spacer (NTS) which has promoter activity and considerable size variation in some vertebrates and invertebrates (Treco et al. 1982).

In recent years the patterns and processes of rDNA evolution have come under close scrutiny, and several observations have been made concerning the evolution of this gene system. First, sequence heterogeneity (as assessed by restriction-site variation) in coding and noncoding regions of rDNA repeats is very low within some species, presumably as the result of concerted evolution (Dover 1982; Arnheim 1983). The process of concerted evolution reduces sequence variation via non-Mendelian mechanisms (unequal exchange, gene conversion, and sequence transposition), resulting in relatively homogeneous sequences within a species. Second, most species examined for rDNA variation reveal both within-individual and between-individual variation, and the distribution and frequency of rDNA variants demonstrate a definite geographic pattern (Suzuki et al. 1986, 1987). This level of within-individual and between-in-
Individual variation presumably reflects a lag time in turnover of variation, resulting from the influx of new variation by mutation and from the fixation of that variation by non-Mendelian processes. Third, patterns of rDNA variation have been used to evaluate the phylogenetic relationships among taxa of several vertebrate groups (Hillis and Davis 1986; Larson and Wilson 1989; Mindell and Honeycutt 1989).

Although a considerable amount of information exists on rDNA structure and evolution, few detailed comparative studies of mammalian rDNA variation have been conducted, with the possible exception of phylogenetic studies on hominoid primates (Wilson et al. 1984; Schmickel et al. 1990). Our objective in the present study is to investigate rDNA variation among rodent species. We have approached this problem by evaluating variation at several taxonomic levels of divergence, including intraindividual, intraspecific, intrageneric, and intergeneric diversity, and in most cases the comparisons have involved restriction-endonuclease-site variation in peromyscine and murine rodents. Existing nucleotide sequence data for *Mus* and *Rattus* (from GenBank; Bilofsky and Burks 1988), together with restriction-site data, have been used to evaluate the types of change, magnitude of change, and overall patterns of divergence seen at the rodent rDNA locus.

Material and Methods
Specimens Examined

Detailed patterns of intraindividual, intraspecific, and interspecific variation were examined in *Onychomys* species collected from the following localities (sample size in parenthesis): *O. leucogaster*-Oregon, Morrow Co. (two); New Mexico, Bernalillo Co. (one), DeBaca Co. (one), Luna Co. (two), and Sandoval Co. (one); and Texas, Lamb Co. (five) and Winkler Co. (three); *O. torridus*-Arizona, Pinal Co. (two); and *O. arenicola*-New Mexico, Eddy Co. (one), Luna Co. (one), Socorro Co. (one), and Valencia Co. (two). In addition to these *Onychomys* genera, intergeneric comparisons included one individual of each of the following species: *Mus musculus*-Harvard Biological laboratory stock; *Rattus norvegicus*-Harvard Biological laboratory stock; *Peromyscus alstoni*-Mexico, Tlaxcala; *P. banderanus*-Mexico, Jalisco; *P. boylii*-Arizona, Pima Co.; *P. eremicus*-Arizona, Pima Co.; *P. maniculatus*-Washington; *P. nudipes*-Costa Rica; *P. thomasi*-Mexico, Oaxaca; *P. pirgensis*-Panama, Darien prov.; *Reithrodontomys megalotis*-New Mexico, Luna Co.; *Reithrodontomys montanus*-Texas, Lubbock Co.; *Ochrotomys nutalli*-Arkansas, Pulaski Co.; *Nectomys sumichrasti*-Mexico, Jalisco; *Nectomys parvipes*-Suriname, Marowijne; *Sigmodon hispidus*-Texas, Lubbock Co.; *Neotoma micropus*-New Mexico, Luna Co.; and *Ototylomys phyllotis*-Costa Rica.

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**FIG. 1.**—Schematic of rRNA gene complex for rodents, including representative regions of N1S, E1S, ITS, and three gene coding regions—18S, 5.8S, and 28S rRNA. p2546 and p119 define the regions covered by the probes used in restriction-site mapping. Conserved restriction-endonuclease sites found in rodent taxa are shown at the top. Restriction-site maps include all geographic variation observed within each species. Restriction sites above/below the maps were conservative/variable sites within that species. All sites were shared between the two *O. torridus* samples examined. Triangles represent presumed insertion/deletion events. Mapped sites affected by the hypothesized insertion/deletion events were aligned with respect to one another. Mapped location of 60 sites used in the analysis of *Onychomys*, *Peromyscus*, and *Reithrodontomys* are shown at the bottom, and these correspond to the 60 sites listed in table 1. Restriction endonucleases mapped are denoted by single letters as follows: A = *Apa* I; B = *Bam* HI; L = *Bcl* I; G = *Bgl* II; T = *Bst* EI; C = *Cla* I; D = *Dra* I; E = *Eco* RI; H = *Hinc* II; N = *Hind* III; K = *Kpn* I; P = *Pst* I; V = *Pvu* II; S = *Sac* I; F = *Sal* I; X = *Xba* I; O = *Xho* I.
Fig. 2.—Insertion/deletion events observed in 28S gene around SacI (S) and PvuII (V) restriction sites. Symbols are as in fig. 1. Numbers indicate the size (in bp) of insertion/deletions. Taxa sharing the Onychomys pattern include Peromyscus maniculatus, P. boylii, P. eremicus, P. banderanus, P. nudipes, P. alstoni, P. pirrensis, Reithrodontomys montanus, R. megalotus, and Neotoma.

Restriction-Endonuclease Mapping

High-molecular-weight DNA was isolated using a modified procedure of Bingham et al. (1981). Approximately 3–5 μg of nuclear DNA was digested with restriction endonucleases according to the manufacturer's specifications (New England Biolabs). The digested DNAs were electrophoresed in a 0.8% agarose gel in 1 × TAE (0.04 M Tris, 0.005 M sodium acetate, 0.001 M disodium ethylenediamine tetraacetate, pH
buffer. The gel was stained with ethidium bromide, and the DNA was transferred to a nylon membrane (GeneScreen Plus; DuPont) by using the Southern (1975) blot procedure, with some modifications for alkaline transfer (Chomczynski and Qasba 1984).

Two rDNA probes were used to map the 18S and 28S rRNA gene complex. These probes were p119, a 4.8-kb insert containing part of the Mus 28S rRNA gene, and p2546, a 1.9-kb fragment containing most of the Mus 18S rRNA gene (fig. 1; Arnheim 1979). All probes were radiolabeled using the random priming method (Feinberg and Vogelstein 1984). After hybridization with a specific probe, the nylon filters were washed (three times at 42°C with 2 × saline sodium citrate, 0.1% sodium dodecyl sulfate; twice at 60°C in 0.1% saline sodium citrate, 0.1% sodium dodecyl sulfate) and were exposed to X-ray film for 18–48 h. Prior to hybridization with a second probe, the old probe was stripped from the filters, and the filters were prehybridized. Both rRNA probes were hybridized separately to all filters examined.

Seventeen restriction endonucleases (for list, see fig. 1) were used to map the 18S and 28S rDNA complex for Onychomys arenicola, O. leucogaster, O. torridus, P. boylii, P. eremicus, Reithrodontomys megalotus, and M. musculus. In addition, Rattus norvegicus, P. alstoni, P. banderanus, P. maniculatus, P. nudipes, P. thomasi, P. pirrensis, Reithrodontomys montanus, Ochrotomys nuttalli, Nyctomys sumichrasti, Neotomys parvipes, S. hispidus, Neotoma micropus, and Ototylomys phyllotis were mapped for length variation in the 28S rRNA gene only. Mapping was accomplished by single and double digests (Nathans and Smith 1975). Of the 136 possible combinations of double digests for the 17 restriction endonucleases used, we performed 52 double digests for each of three species of Onychomys. In addition, Mus controls were run on each gel as an internal comparison vis-à-vis the known sequence of the Mus rDNA repeat. Therefore, equal sized fragments could be confidently oriented to known mapped and sequenced data. Double digests were chosen so as to minimize the distance between a known site within the region of the probe and an unknown site that we wished to map accurately. Geographic variation was assessed mainly by comparison with the known mapped standard for a particular species.

Phylogenetic relationships within the genus Onychomys and among the genera Onychomys, Peromyscus, and Reithrodontomys were determined by parsimony analysis outlined in Swofford’s PAUP (version 3.0, option branch and bound) and Farris’s Hennig 86 (version 1.5, option ie*) computer programs. Each restriction site was treated as an unordered character and was scored as present or absent. The use of heterologous probes confined to the transcribed regions, plus the large size of the mammalian NTS, increases the probability that some restriction sites in the NTS will be undetectable. These sites were coded as “missing data” in the character-state matrix and were used in the parsimony analysis. Mus musculus was used as an outgroup in an effort to determine the polarity of characters in the phylogenetic tree. The criterion for comparing trees derived from parsimony analysis is based on tree length, with the most parsimonious tree being the shortest tree. When two or more trees of equal length were found, a strict consensus tree was constructed for all these trees combined.

Results
Patterns of Variation
Restriction Sites

From the 164 restriction sites mapped in the present study, the rRNA gene coding regions were shown to be highly conservative in all taxa (fig. 1). For instance, of the 21 sites mapped in the gene regions, no variation was observed in the 5.8S or 28S
### Table 1
Coding for Phylogenetically Informative Restriction-Endonuclease Sites for *Onychomys*, *Peromyscus*, *Reithrodontomys*, and *Mus*

| SPECIES          | *BglI* | *XbaI* | *KpnI* | *BglII* | *HindIII* | *PvuII* | *EcoRI* | *Clal* | *SalI* | *KpnI* | *BclI* | *Sacl* | *ApaI* | *PstI* | *PstII* |
|------------------|--------|--------|--------|---------|-----------|---------|---------|--------|--------|--------|--------|--------|--------|--------|
| *O. leucogaster* |        |        |        |         |           |         |         |        |        |        |        |        |        |
| Texas            | 0      | 1      | 0      | 1       | 0         | 1       | 1       | 1      | 1      | 0      |        |        |        |        |
| New Mexico       | 0      | 1      | 0      | 1       | 0         | 1       | 1       | 1      | 1      | 0      |        |        |        |        |
| Oregon           | 0      | 1      | 0      | 1       | 0         | 1       | 1       | 1      | 1      | 0      |        |        |        |        |
| *O. arenicola*, New Mexico |      |        |        |         |           |         |         |        |        |        |        |        |        |
| Arizona          | 0      | 1      | 0      | 1       | 1         | 0       | 1       | 1      | 1      | 0      |        |        |        |        |
| *P. boylii*      | 0      | 0      | 0      | 1       | 0         | 0       | 0       | 0      | 1      | 0      |        |        |        |        |
| *P. eremicus*    | 1      | 0      | 0      | 0       | 1         | 0       | 0       | 0      | 0      | 0      |        |        |        |        |
| *R. megalolis*   | 1      | 0      | 0      | 0       | 0         | 1       | 0       | 0      | 1      | 1      |        |        |        |        |
| *M. musculus*    |        |        |        |         |           |         |         |        |        |        |        |        |        |        |

**Note:** Data are expressed in binary style: 0 = absence; 1 = presence; - = unknown.

*Numbers 1-60 in column heads are the same as those in Fig. 1.*

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... genes, and only one variable site (*SacI*) was found in the 5' end of the 18S rRNA gene. The *SacI* site was absent in *Peromyscus boylii*.

The ITS regions revealed more variation, with nine sites differing among genera. By far the most variable region was the NTS, with more than half of the 132 variable sites being unique to individual species. Most of the NTS variation (intraspecific, interspecific, and intergeneric) involved the regions extending 4.5 kb from the 5' end of the 18S gene and 3.0 kb from the 3' end of the 28S gene (fig. 1, sites 10-26 and 30-48, respectively).

**Length Variation**

Overall repeat length varied from 45.5 kb to 48 kb, with at least three regions of the rDNA repeat contributing to this variation. One of the major sources of variation was the NTS (fig. 1). In this region, species of *Onychomys* and *Mus musculus* revealed size increases of 2 kb and 650 bp, respectively. *Onychomys leucogaster* and *O. arenicola* also revealed a 50-bp addition in the ITS-1. Finally, the size of the 28S rRNA gene varied in length among most rodent genera. A detailed examination of 21 rodent...
species revealed size variation mapping to the 28S gene 3' end, between SacI and PvuII sites (fig. 2).

### Intraindividual Variation

Nine restriction endonucleases revealed multiple repeat types within individuals of *Onychomys*. In all cases except one (SacI site 29 in ITS-2), this variation mapped to the NTS. The greatest number of repeat types within an individual was four, and the average number of repeat types for *O. leucogaster*, *O. torridus*, and *O. arenicola* was 1.64, 1.4, and 1.6, respectively. Some repeat types were unique to a species, but several were shared between species. Four polymorphic sites were shared between *O. arenicola* and *O. leucogaster* (fig. 1, sites 29, 36, 39, and 59).

### Phylogenetic Analysis

A phylogenetic analysis using rDNA site variation was conducted for the three peromyscine rodent genera, *Onychomys*, *Peromyscus*, and *Reithrodontomys* (table 1). Analysis of the 60 phylogenetically informative characters, including the polymorphic sites (all sites were located in the NTS, except for the three sites 27-29 in the ITS), produced 1,500 equally parsimonious trees of length 101 and with a consistency index (Cl) of 0.59. The strict consensus tree (fig. 3) constructed from these data revealed the following: (1) Texas and New Mexico *O. leucogaster* form a clade with respect to Oregon *O. leucogaster*. (2) *Onychomys torridus* is more closely related to *O. leucogaster*...
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than is O. arenicola, with trees depicting either an O. arenicola/O. leucogaster or an O. arenicola/O. torridus relationship being five and six steps longer, respectively. The Onychomys relationships are maintained when a parsimony analysis is conducted without an outgroup, as well as when either Peromyscus or Reithrodontomys is used as an outgroup rather than Mus. (3) The genus Peromyscus is paraphyletic, with P. boylii and P. eremicus grouping closer to Reithrodontomys megalotis.

Patterns of nucleotide sequence change for four regions of the rDNA complex were examined at several levels of evolutionary divergence by using two approaches. First, equation (8) of Nei and Li (1979) was used to calculate δ (nucleotide divergence per nucleotide site) for all mapped sites in Onychomys, Peromyscus, and Reithrodontomys. As can be seen in figure 4, δ increases as taxonomic divergence increases, with the NTS and ITS regions reflecting a faster evolutionary rate as compared with that in the coding region. Second, a divergence profile was constructed using known nucleotide sequence data for Mus and Rattus. A similar trend relative to the rate of divergence depicted by δ values can be seen for regions of the rRNA gene complex.

Discussion

Length Variation

The length variation observed in the rDNA repeat is comparable to that seen in other organisms (Appels and Honeycutt 1986). One major cause of rDNA length
variation in mammals is the deletion/addition of repeats mapped to regions in the NTS, either 3' to the 28S rRNA gene or 5' to the 18S rRNA gene (Erickson and Schmickel 1985; Appels and Honeycutt 1986; Suzuki et al. 1986). In rodents and humans this length variation has been found at several levels of divergence, including intraindividual, intraspecific, and interspecific. Although the exact location of repeat-length variation has not been determined for most species of rodents examined in the present study, interspecific repeat-length differences are apparent and map to the general region of the NTS. The accurate mapping of length variation can be seen in *Onychomys* (fig. 1), in which length differences occur 14 kb 5' to the 18S rRNA gene. This region is somewhat removed from those indicated for *Mus* (Kuehn and Arnheim 1983) and *Homo* (Erickson and Schmickel 1985). As can be seen in figure 1, the major difference in length between *Mus* and the other rodents (a 650-bp addition in *Mus*) does map to the vicinity of the variable region reported by Kuehn and Arnheim (1983). The ITS also demonstrates length variation between species (e.g., *Mus* vs. *Rattus*; Michot et al. 1983), and at least some ITS length variation occurs between species of *Onychomys* (fig. 1), with the remaining rodent taxa being constant in ITS size.

Insertions and deletions within the 28S gene of several vertebrates and other eukaryotes have been reported (Hillis and Davis 1987; Hancock and Dover 1988; Mindell and Honeycutt 1989). These regions of length variation have been termed "expansion segments" or "divergent domains" and represent the major cause of size
difference in the large rRNA gene of eukaryotes (Clark et al. 1984; Hassouna et al. 1984). On the basis of nucleotide sequence comparisons, 12 divergent domains have been located in eukaryotes, with two domains (D2 and D8) revealing most of the size variation between the rodent genera Mus and Rattus (Hassouna et al. 1984). The Mus 28S gene (Hassouna et al. 1984) has two SacI sites bracketing four divergent domains, D7-D10, with a PvuII site mapping within D8. These restriction-endonuclease sites were used to localize deletion/insertion differences among additional rodent 28S genes (fig. 2), and in most cases the size variation maps close to the D8 region found in Mus and Rattus.

The location and size of specific additions/deletions in the 28S gene may provide some phylogenetic information for deriving relationships among rodent genera. Carleton’s (1980) systematic treatment of neotomine-peromyscine rodent genera suggests a closer relationship among the genera Peromyscus, Reithrodontomys, Onychomys, Ochrotomys, and Neotoma, with Ototylomys being more distantly related. As can be seen in figure 2, Ototylomys shares deletions/insertions with Mus and Rattus. Thus, the pattern seen in most peromyscine-neotomine rodents can be considered synapomorphic, with Ochrotomys and P. thomasi possessing uniquely derived types.

Although there seems to be some phylogenetic information relative to 28S deletions/insertions, one must interpret restriction-site maps with considerable caution. While no difference in the size of the 28S rRNA gene could be distinguished between Mus and Rattus when Southern blotting was used, at the nucleotide-sequence level these same regions revealed a 17-31-bp difference between the genera (Hassouna et al. 1984). Therefore, in the absence of direct sequencing, there are limits to one’s ability to assume homology (in an operational sense) between insertions/deletions of similar size.

Site Variation

Patterns of restriction-site variation at the rDNA locus have been used to determine both relationships among species and the patterns of geographic and population variation within species (Suzuki et al. 1987; Mindell and Honeycutt 1989). In most comparative studies the NTS and ITS regions have been shown to be the most variable
Fig. 4.—Nei and Li’s (1979) nucleotide substitutions per nucleotide site, for subregions of rRNA gene complex compared at intraspecific, interspecific, and intergeneric levels by using 86 restriction-endonuclease sites, with no missing data, from peromyscine rodents and Mus. The number of pairwise comparisons were as follows: 116 intraspecific, 115 intrageneric, and 69 intergeneric. Intergeneric distance values are also compared between Mus and Rattus at the nucleotide level from published sequence data (Bilofsky and Burks 1988). For restriction-site values, NTS and ETS were lumped, because of the uncertain location of the site for transcription initiation in the grasshopper mouse, and the ITS regions were grouped to increase sample size.

(Appels and Honeycutt 1986), and, as can be seen both in the restriction-site maps (fig. 1) and in the divergence estimates (fig. 4), these two regions are the most divergent in rodents as well. The utility of these regions for resolving phylogenetic relationships among rodents, is equivocal, however. Multiple repeat types within and between in-
individuals are quite common, and most variation of this type maps to the NTS (La Volpe et al. 1985; Seperack et al. 1988). Similar variation was observed in the present study, and in some cases repeat types defined by a particular restriction site were even shared between species. Within a species, the frequency of particular repeat types does demonstrate patterns of geographic variation similar to those derived from other characters (Suzuki et al. 1986, 1987), and the NTS variation seen in Onychomys is no exception. For instance, the relationships among different geographic samples of O. leucogaster (fig. 3) are congruent with relationships derived from morphological and mitochondrial DNA data (Riddle and Honeycutt 1990), suggesting the utility of the NTS for deriving regional relationships among populations within a species.

Restriction-site variation in the NTS and ITS is less clear with regard to the derivation of phylogenetic relationships among species of peromyscine rodents. First, the close relationship, depicted by the rDNA data, between O. torridus and O. leucogaster relative to O. arenicola is incongruent with the results of most other comparative studies. Allozymes, morphology, and mitochondrial DNA suggest a sister-group relationship between O. leucogaster and O. arenicola (Sullivan et al. 1986; Riddle and Honeycutt 1990). If one were to accept the rDNA phylogeny based on NTS variation, then the patterns of variation seen with these other characters would be more complicated. Chromosomal data, however, generally support the rDNA results, but the chromosomal differences separating the three species of Onychomys present some difficulties with respect to determining character-state polarity (Baker and Barnet 1981). For instance, all chromosomal differences among the three species are the result of additions/deletions of constitutive heterochromatin to/from the short arms of autosomes, and it is impossible to evaluate whether these additions/deletions represent independent events or events resulting from common ancestry. Second, the intergeneric comparisons are also equivocal. The monophyly of Onychomys and the closer relationship between Reithrodontomys and Peromyscus are congruent with the inferences to be drawn from other comparative data sets (Hooper and Musser 1964; Carleton 1980). Nevertheless, the paraphyly demonstrated for the genus Peromyscus is not congruent with other data (Koop et al. 1984). Even in Carleton’s review (Carleton 1980), where Peromyscus is considered paraphyletic, P. boylii and P. eremicus were placed in the same clade.

The comparisons of rodent taxa examined in the present study span a time period of ~4–15 Mya or more (Carleton and Eshelman 1979; Jacobs and Pilbeam 1980; Flynn et al. 1985). The overall results of these comparisons suggest that restriction-site variation at the rDNA locus may be of limited utility in systematic studies of rodents (especially in studies involving intergeneric comparisons), with the evolutionary rates of coding regions being too slow and those of the NTS being too fast. Therefore, we suggest that further studies on rodent rDNA variation be conducted at the nucleotide sequence level and be confined to the more variable regions mapped to the 28S gene or, possibly, to the ITS region.

Acknowledgments

Special thanks to Robert J. Baker, The Museum, Texas Tech University, for frozen tissue from many of the rodent taxa and to Kim Nelson for specimen collection and laboratory assistance. We are also grateful to Ron Adkins, Mike Arnold, Jim Carpenter, Scott Davis, Brian Hanks, David Hillis, Michael Lynch, Masatoshi Nei, David Pilbeam, MaryEllen Ruvolo, Ron Van Den Bussche, Ward Wheeler, Scott Williams, and two anonymous reviewers for comments on earlier drafts of the manuscript. This research was supported by National Science Foundation grant BSR
85084479 to R.L.H. and by grants-in-aid, to M.W.A., from the American Society of Mammalogists and Sigma Xi, The Scientific Research Society. During the course of this research M.W.A. was supported by NIH Genetics Training grant GM07620.

LITERATURE CITED


MASATOSHI NEI, reviewing editor

Received March 29, 1990; revision received July 26, 1990

Accepted July 26, 1990