MITOCHONDRIAL DNA RESTRICTION SITE ANALYSIS OF THE PHYLOGENY OF THE *TRUEI* AND *BOYLII* SPECIES GROUPS OF THE RODENT GENUS *PEROMYSCUS* (CRICETIDAE)

THESIS

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The phylogenetics of eight species of the *Peromyscus truei* and *P. boylii* species groups from 15 populations were analyzed based on mitochondrial DNA sequence differentiation, using 13 hexanucleotide specific restriction enzymes. *P. difficilis*, *P. nasutus*, and *P. attwateri* were found to be members of the same clade. *P. leucopus* was not found to be closely related to any of the species of the *boylii* or *truei* species groups. Phylogenetic interpretations for the remaining species differed based on Wagner and Dollo parsimony analyses. *P. truei* appears to be most closely related to *P. gratus* based on Wagner parsimony and the phenetic analysis, while the relationship of *P. gratus* to other species could not be resolved based on Dollo parsimony.

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CHAPTER I

INTRODUCTION

Background.--In 1966, Hennig called for a holomorphological approach to systematics, advocating the use of the largest number of characters possible in making taxonomic decisions. Since that time, our ability to make systematic recommendations based on different forms of genetic data has steadily improved, providing an increasing number of characters on which to base our conclusions. The rodent genus *Peromyscus* has been readily accessible to systematists and has received much taxonomic attention utilizing a variety of characters. Supraspecific classifications have been made based on pelage color, cranial morphometrics, dental morphology, and more recently, various genetic measures. Taxonomic assignments of species groups have largely been phenetic (based on overall similarity), however, the formation of species groups which reflect common evolutionary decent has always been an underlying goal of systematists.

With the increasing availability of genetic measures of relatedness and cladistic analyses, testing of the monophyly of species groups has received renewed interest (Carleton, 1989). Recently, several systematists have addressed the phylogeny of the members of the *P. boylii* and *P. truei* species groups of *Peromyscus* (Janecek, 1987, 1990; Smith, 1990; Sullivan, et al., in press). The genetic data in these

studies was generated from allozymic and karyotypic approaches. Each of these studies indicated that one or both of these species groups do not represent monophyletic lineages (i.e., groups including one common ancestor and all of its descendants), and many of the phylogenetic conclusions concerning relationships of the species are in direct conflict. Despite the work completed to date and the diversity of approaches, the evolutionary relationships of these species have been particularly difficult to resolve. However, there have been no previous examinations of these species groups utilizing molecular techniques which are currently available. Work utilizing the analysis of DNA sequence divergence should help resolve the phylogenetic relationships of certain species of *Peromyscus* and further our understanding of molecular evolution in this rapidly evolving group of North American rodents.

Systematic history.--The genus *Peromyscus* is an excellent model for the study of speciation. *Peromyscus* is a speciose genus, comprising approximately 53 named species that range over most of North America in a diversity of habitats (Carleton, 1989). Thirteen species groups are recognized in the most recent taxonomic revision of the genus (Carleton, 1989), most of which follow the earlier classification by Hooper (1968) based on morphology and breeding data.

The *truei* species group (Table 1) was originally erected by Osgood (1909) on the basis of morphological similarity and contained P. *truei*, *P. difficilis*, *P. nasutus*, *P. bullatus*, and *P. polius*. In Hooper's (1968) revision, the *truei* species group included *P. truei*, *P. difficilis*, and *P. bullatus*, with *P. nasutus* being listed as a

Table 1. Species group classifications of *Peromyscus*.

P. truei species group (Osgood, 1909)	*P. truei* species group (Hooper, 1968)	*P. truei* species group (Carleton, 1989)
truei	*truei*	*truei* (Shufeldt)
difficilis	*difficilis*	*gratus* Merriam
nasutus	*bullatus*	*bullatus* Osgood
bullatus		*difficilis* (Allen)
polius		*nasutus* (Allen)
P. boylii species group (Osgood, 1909)	*P. truei* species group (Hooper, 1968)	*P. boylii* species group (Carleton, 1989)
boylii	*boylii*	*boylii* (Baird)
pectoralis	*pectoralis*	*levipes* Merriam (?)
oaxacensis	*polius*	*stephani* Townsend
hylocetes	*evides*	*attwateri* Allen
	aztecus	*simulus* Osgood
	hondurensis	*madrensis* Merriam
	oaxacensis	*pectoralis* Osgood
	hylocetes	*polius* Osgood

subspecies of *P. difficilis*. These species are currently considered the only members of this group (Carleton, 1989) with the tentative re-elevation of *P. nasutus* to species level and the additional elevation of *P. gratus* to species level from its original placement as a subspecies of *P. truei* based on karyotypic divergence (Modi and Lee, 1984). *P. truei* occurs in the southwestern United States and in isolated populations in Baja California del Sur, Mexico, while the range of *P. gratus* lies mostly in mainland Mexico extending into southwestern New Mexico and southeastern Arizona. Modi and Lee (1984) reported areas of sympatry in the ranges of *P. truei* and *P. gratus* in southwestern New Mexico with no hybrid individuals being found, supporting their assignment of *P. gratus* to specific status. Allozymic data have further confirmed the distinctness of *P. truei* and *P. gratus* (Janecek, 1990).

The placement of *P. nasutus* as a species distinct from *P. difficilis* remains controversial. *P. nasutus* occurs in the southwestern United States, extending into northern Mexico along the Sierra Madre Oriental. *P. difficilis* occurs in the mountain ranges of Mexico; however, the taxa do not appear to occur in sympatry (Carleton, 1989). Janecek (1990) cited the need for more sampling in northern Mexico to determine if any overlap in ranges exists. The two taxa show slight karyotypic differences (Hsu and Arrighi, 1968; Zimmerman et al., 1975). While both have a diploid chromosome number of 48, *P. nasutus* possesses one more pair of biarmed chromosomes than does *P. difficilis*. Allozymic data have likewise indicated that the two are distinct species, leading to recommendations for the

elevation of *P. nasutus* to its former specific status (Avise et al., 1979b; Zimmerman et al., 1975, 1978). Carleton (1989) recognized *P. nasutus* as one of the species of the *P. truei* species group in his classification, although he cited a lack of breadth in geographic sampling as problematic in determining the relationship of the two taxa. He could not find strong evidence against intergradation of the two taxa. A more recent allozymic study by Janecek (1990), employing a wider range of geographic samples, indicated that *P. difficilis* and *P. nasutus* are not distinguishable allozymically and that *P. nasutus* should be recognized as a subspecies of *P. difficilis*.

Another taxon which has received a great deal of attention is *P. truei comanche*, which occurs as a series of isolated populations in the Texas panhandle along the Llano Estacado. These mice were originally recognized as *P. comanche* by Blair (1943), and then as *P. nasutus comanche* (Hoffmeister, 1951) or *P. difficilis comanche* (Hoffmeister and de la Torre, 1961). This mouse is currently recognized as a subspecies of *P. truei* based on their identical karyotypes (Modi and Lee, 1984). That designation was further supported by morphologic data (Schmidly, 1973a) and allozymic data (Janecek, 1990; Modi and Lee, 1984).

In the last 15 years, the *boylii* group has probably received more attention from mammalian systematists than any other species group of *Peromyscus* (Carleton, 1989). The *boylii* group (Table 1) currently contains eight species (Carleton, 1989). When the species group was defined originally by Osgood (1909), it contained only four species, with *P. attwateri* considered a subspecies of

P. boylii. *P. attwateri* was later elevated to specific status based on karyotypic data (Lee et al., 1972). The distinctness of *P. boylii* and *P. attwateri* has been confirmed by morphologic (Schmidly, 1973b) and allozymic data (Janecek, 1990; Kilpatrick and Zimmerman, 1976; Sullivan et al., in press; Zimmerman et al., 1978).

Evidence has been generated recently which suggests that neither of these species groups represents a monophyletic lineage (Fig. 1). As groups in which one or more of the decendants of the common ancestor may be excluded from the group (paraphyletic) or in which taxa which may not share a recent common ancestor are included (polyphyletic), these non-monophyletic species groups therefore represent artificial taxonomic units. However, it should be possible, given enough synapomorphic characters on which to base a decision, to construct species groups which estimate real evolutionary groupings.

Based on her analysis of allozyme variation, Janecek (1990) reported that *P. attwateri*, *P. difficilis*, and *P. nasutus* may be conspecific rather than belonging to different species groups. Her analysis placed *P. attwateri* in the *truei* group, as she found *P. difficilis* and *P. gratus* to be sister taxa, with *P. boylii* being the most distantly related species. Another analysis of allozyme variation by Sullivan et al. (in press), placed *P. attwateri* and *P. difficilis* as sister taxa, but indicated that they are not conspecific based on several fixed allelic differences. *P. gratus* was found to be more closely related to *P. leucopus* (of the *leucopus* species group) than to *P. difficilis*. Furthermore, Sullivan et al. found *P. difficilis* to be most closely related

to many of the members of the *boylii* group, also in conflict with the analysis by Janecek (1990). Neither of these recent allozymic studies support previous allozymic analyses which showed *P. truei* and *P. gratus* to be sister taxa, closely related to *P. difficilis*, and *P. attwateri* most closely related to *P. boylii* (Avise et al., 1974; Avise et al., 1979b; Zimmerman et al., 1978). Smith (1990) presented karyotypic data which indicated that both species groups are polyphyletic, with each being fragmented into several clades. Smith's data support Janecek's contention that *P. gratus* and *P. difficilis* are sister taxa, being further removed from *P. truei*. Smith (1990) also indicated that *P. truei* belongs to a clade which includes members of both the *boylii* and the *leucopus* groups.

Mitochondrial DNA as a phylogenetic tool.--Sullivan et al. (in press) called for the "application of techniques that will provide a larger number of synapomorphic characters" (i.e., shared, derived characters) or "more sensitive estimates of genetic distance" than are provided by allozyme data to better resolve the phylogeny of these species. There are several reasons why analysis of mitochondrial DNA (mtDNA) may provide a refined approach for dealing with this problem, the most important of which is the rapid rate of evolution of mtDNA compared to that of the nuclear genome. Brown et al. (1982) showed that nucleotide sequence changes in mtDNA may accumulate at a rate five to ten times faster than in nuclear DNA. This tendency is likely to provide a marker which will show greater differentiation over the geological lifetime of a species.

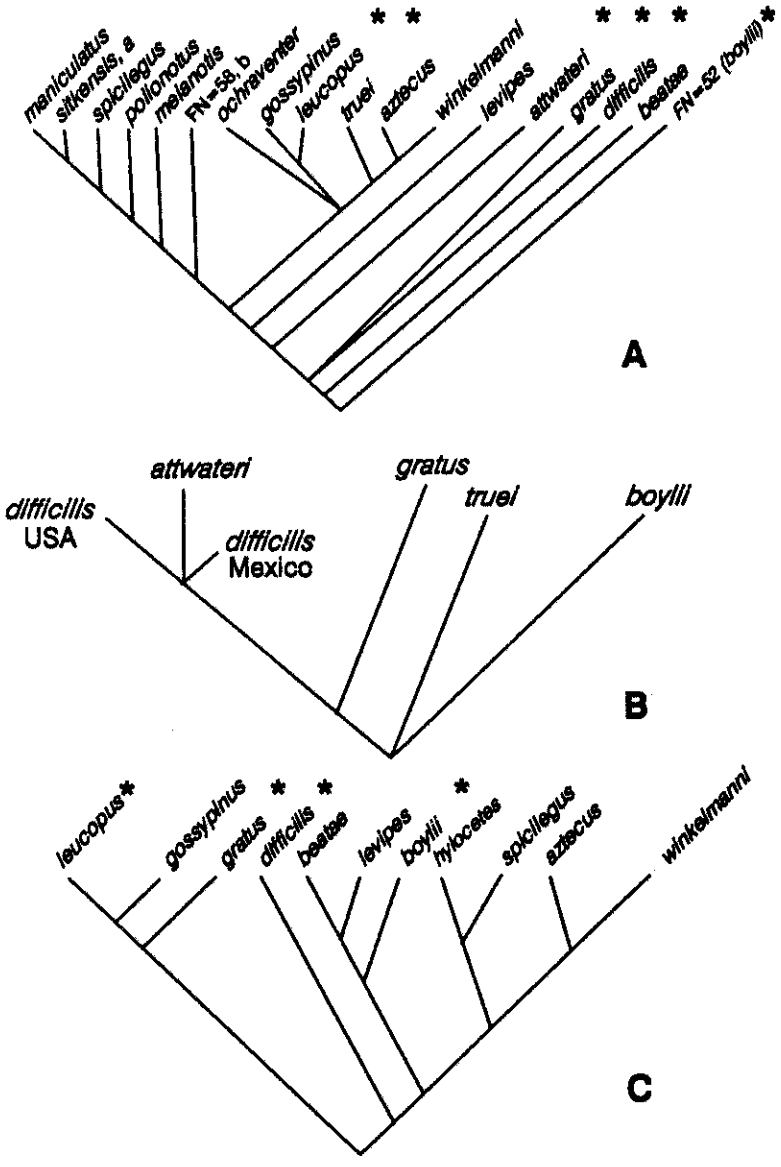

Figure 1. A) Cladogram depicting the relationships of species of the subgenus *Peromyscus* based on karyotypic data (Smith, 1990). B) Unrooted tree depicting the phenetic relationships of species of the *truei* and *boylii* species groups based on allozymic variation (Janecek, 1990). Branch lengths represent relative genetic distance. C) Cladogram of relationships of species of the *boylii*, *truei*, and *leucopus* species groups based on allozyme data (Sullivan et al., in press).

Other important characteristics of mtDNA include its maternal and clonal inheritance. Mitochondrial DNA generally exists in only one form in an individual and does not undergo crossing-over which is experienced by the nuclear genome. All of the mitochondria present in the zygote are contributed by the egg, with the sperm contributing only a haploid set of nuclear chromosomes. Thus the ambiguities created by recombination of nuclear DNA are eliminated.

The small size (approximately 16.5 kilobase pairs) and simplicity of the mitochondrial genome as compared to that of the nucleus is another advantage of mtDNA analysis. This simplicity is due to the fact that the mitochondrial genome is not complicated by redundancy, large intervening sequences, or the presence of heterochromatic regions common to eukaryotic chromosomes (Brown, 1983).

Finally, the protein products of mtDNA are integral to the electron transport chain and aerobic respiration. Therefore, those mutations in the genome that are deleterious are likely to be eliminated from the population by natural selection, leaving only those mutations which are selectively neutral. Indeed, most mutations which have been observed in mtDNA are thought to be selectively neutral, as they are most commonly due to nucleotide substitutions and not additions, deletions, or rearrangements (Aquadro and Greenberg, 1983; Avise and Lansman, 1983; Greenberg et al., 1983), easing the comparison of restriction site maps of different species. A large majority of these substitutions produce no concomitant changes in amino acid sequence due to the relaxed nature of the mtDNA codon recognition system (Anderson et al., 1982; Brown et al., 1982), allowing sequence

variation to accrue at a more rapid rate than may occur in the nuclear genome.

Published studies of mtDNA variation in *Peromyscus* have been limited to interpopulation analyses of three species, *P. maniculatus*, *P. leucopus*, and *P. polionotus* (Ashley and Wills, 1987; Avise et al., 1979a; Avise et al., 1983; Lansman et al., 1983; Nelson et al., 1987). No examinations of species group affinities based on mtDNA data are available to date for these groups. However, mtDNA restriction site analysis has proven informative in determining relationships of other rodent taxa (e.g. Davis, 1986; Riddle and Honeycutt, 1990; Tegelstrom et al., 1988; Yonekawa et al., 1981).

Specific aims.--The purpose of this study was to estimate the phylogenetic relationships of *P. truei*, *P. gratus*, *P. difficilis*, *P. nasutus*, *P. attwateri*, and *P. boylii* based on mtDNA sequence divergence. The relationship of *P. leucopus* (of the *leucopus* group) to these taxa is also of interest due to its karyotypic similarity to *P. truei* (Smith, 1990) and its allozymic similarity to *P. gratus* (Sullivan et al., in press). *P. eremicus* belongs to the *eremicus* species group and is generally placed in the subgenus *Haplomylomys*, while the other species of interest here are members of the subgenus *Peromyscus* (Carleton, 1989). Because *P. eremicus* is not believed to be closely related to the other species being examined, it provides a good outgroup for phylogenetic analyses. The results of restriction endonuclease cleavage site maps of the mtDNA of these species will provide a third independent data set, perhaps with greater resolving power, which can then be compared to the data yielded by allozyme variation and chromosome banding.

Specific hypotheses addressed were: 1) Are *P. difficilis* and *P. attwateri* sister taxa or even conspecific rather than belonging to different species groups? 2) Is the species-level relationship of *P. nasutus* to other *P. difficilis* confirmed? 3) Do *P. attwateri* and *P. difficilis* fall into a clade with *P. boylii* as members of the *boylii* group or are they more closely related to *truei* group members? 4) Are *P. truei* and *P. gratus* most closely related to each other, or is *P. gratus* most closely related to either *P. leucopus* or *P. difficilis* as certain allozymic data suggest? 5) Is *P. truei* more closely related to *P. leucopus* than to other members of the *truei* group as karyotypic data suggest? 6) Is the subspecific relationship of *P. truei comanche* to other *P. truei* confirmed?

CHAPTER II

METHODS

Collection of specimens.--Seven species of *Peromyscus* were collected from 15 naturally occurring populations (Fig. 2, Appendix I) using Sherman™ live traps. Animals were sacrificed in the field according to approved methods (Committee, 1987). Heart, liver, and kidney tissues were removed and placed in liquid nitrogen until they could be returned to the laboratory where they were stored in an ultracold freezer (-80 °C) before DNA isolation.

Standard karyotypes were prepared to assist in species identifications when necessary (Appendix II). Voucher specimens and accessory collections are deposited in the Carnegie Museum of Natural History and the Texas Tech University Museum.

Isolation of mtDNA.--Purified mtDNA was isolated according to the techniques of Lansman et al. (1981) (Appendix III). This procedure involves homogenizing the tissue, removing the nuclei and cell debris through centrifugation, and then pelleting the mitochondria. The mitochondria were then lysed and placed in a CsCl solution containing ethidium bromide. Ultra-centrifugation produced a density gradient which separated the covalently closed, circular mtDNA from nuclear DNA (nDNA), RNA, protein, and glycogen. The

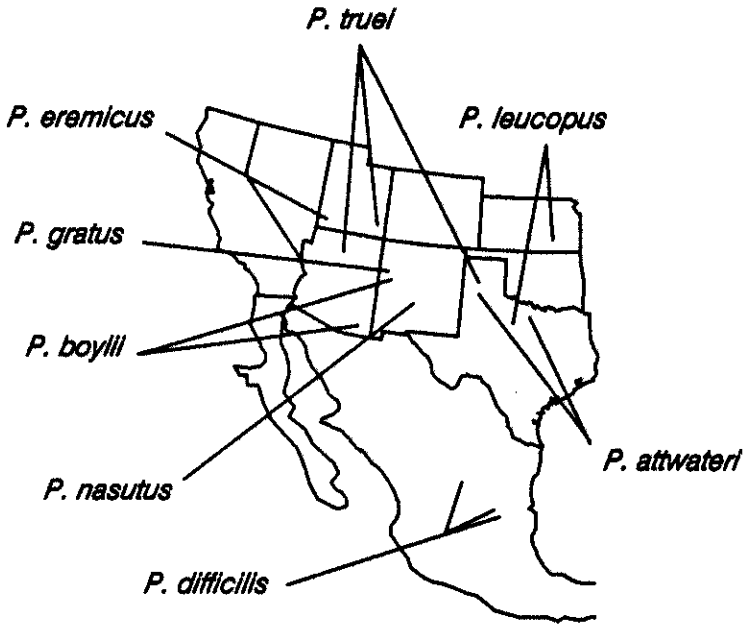

Figure 2. Collection localities for the 15 populations of *Peromyscus* for which mtDNA restriction site maps were generated. Sites correspond with the localities listed in Appendix 1.

DNA was visible under UV illumination as fluorescent bands due to the intercalation of ethidium bromide between the base pairs of the DNA. The bands of mtDNA were removed by puncturing the centrifuge tube with a hypodermic needle and drawing the DNA out with a syringe. Some nDNA was invariably removed with the mtDNA; however, this did not adversely affect subsequent analyses. The mtDNA was further prepared for restriction analysis by removing the ethidium bromide with at least three extractions, using 1 volume of 1-butanol each, and dialyzing for 2 days against TE (0.01 M Tris-HCl, 0.5 mM EDTA, pH 8.0) to remove the CsCl (Maniatis et al., 1982).

Restriction analysis.--Thirteen restriction endonucleases, each of which had six defined nucleotides within their recognition sequences, were used in the analysis. The enzymes *Eco*RV, *Bgl*I, *Bgl*II, *Sal*I, *Bst*EII, *Bsp*106, *Pst*I, *Kpn*I, and *Apa*I were purchased from Stratagene (La Jolla, CA), while *Stu*I, *Bam*HI, *Xho*I, and *Pvu*II were purchased from New England Biolabs (Beverly, MA).

Single and double restriction digestions were carried out on approximately 0.05 μg of DNA in a total volume of 100 μl, according to the manufacturer's recommended temperature and buffer composition. One μl of each enzyme (8 to 12 units, dependent upon stock enzyme concentration) was used per digest.

Following digestion, the mtDNA was precipitated in 2 volumes of ethanol and 25 μl of 3 M sodium acetate, pH 5.2 (Maniatis et al., 1982), and dried under vacuum in a Savant Speed Vac™. The dried DNA was prepared for loading on agarose gels by resuspending in 10 μl of sterile water and 3 μl of carrier dye

(0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll-type 400 in water) (Maniatis et al., 1982).

The mtDNA fragments produced by the restriction enzyme digests were separated by molecular weight using agarose gel electrophoresis. Electrophoresis was carried out on 0.7% or 1.0% agarose gels (Bethesda Research Laboratories, Gaithersburg, MD) overnight at 32 volts (v) or for approximately 6 hours at 65 v in 1 X TBE (0.089 M Tris-Borate, 0.089 M Boric acid, 0.002 M EDTA, pH 8.0). Lambda phage DNA digested with *Hind* III was run on each gel as a molecular weight standard.

Visualization of mtDNA.--MtDNA fragments were visualized through modifications of Southern hybridization (Southern, 1975) recommended by DuPont (Boston, MA) for their GeneScreen*Plus*™ nylon. This process (Appendix IV) involved denaturing the mtDNA in the gel with NaOH, followed by neutralization of the gel. The single-stranded mtDNA was then transferred from the agarose gel onto Magna NT™ nylon (Micron Separations Inc.) using the capillary blot procedure recommended by the manufacturer. The DNA was covalently bound to the nylon using ultraviolet light and hybridized to radioactively-labeled probes.

Evan Hermel of Southwestern Medical University, Dallas, Texas provided the cloned mitochondrial genome of *Mus domesticus* (Strain N2B) which was used as a mtDNA probe. The mtDNA genome is contained in four pUC18 clones, with mtDNA fragment sizes of 7.2, 5.0, 2.7, and 1.0 kilobase (kb) pairs. Purified

recombinant DNA was isolated according to the technique described by Tanaka and Weisblum (1975) (Appendix V).

The four mtDNA probes, along with *Hind*III-cut Lambda phage DNA, were labeled with ^{32}P-dCTP, 3000Ci/mmol (DuPont) in a random primed labeling reaction (Boehringer-Mannheim, Germany). The radioactively-labeled probe DNA was heat denatured just prior to hybridization.

Hybridization was carried out at 65 °C in a shaking waterbath overnight. Excess probe was washed from the nylon, and the nylon was then exposed to x-ray film (X-OMAT AR, Eastman Kodak Company, Rochester, NY) for approximately 24 hours using a Cronex™ Quanta III intensifier (DuPont).

Restriction site mapping.--The mtDNA restriction fragment patterns were visualized on film, permitting the number and size of the fragments produced by each enzyme or pair of enzymes to be determined. Fragments as small as 0.4 kilobase pairs (kb) were observable. The restriction sites produced by each enzyme in an individual's mtDNA were mapped in their positions relative to one another based on the results of the double restriction digests. Restriction site positions were denoted based on a conserved *Bgl*I site which was designated as "0.0". Due to limitations in the accuracy of determining fragment sizes, restriction site positions were determined within an estimated range of 0.3 kb.

Comparisons of the mtDNA's of the taxa were made from restriction site locations. The restriction site maps of the mtDNA's of each population were aligned with one another based on the positions of five conserved sites.

Restriction sites were considered to be in the same position on the different maps if the site was mapped within a 0.3 kb region for both taxa. Side-by-side comparisons of double restriction digests for different species were used when necessary to confirm the homology of map positions.

Phenetic data analysis.--The overall percent sequence divergence between mtDNA haplotypes (δ) was estimated by the method of Nei and Tajima (1983). This method is dependent upon the number of restriction sites possessed by each of the two taxa (M_x and M_y), the number of sites which the two taxa share (M_{xy}), and the number of nucleotides in the recognition sequences of the restriction enzymes used (r). δ was calculated as

$$\delta = (-\ln S)/r$$

where

$$S = 2 M_{xy}/ (M_x + M_y).$$

Percent sequence divergence values were calculated for all possible pair-wise combinations of the taxa. The sequence divergence data were then clustered phenetically based on the unweighted pair-group method using arithmetic averages (UPGMA) (Nei, 1990).

Cladistic data analysis.--Dollo parsimony analysis was performed using PHYLIP (Felsenstein, 1986). The DOLLOP option was employed in order to find the shortest tree which could be generated based on Dollo criteria. Multiple analyses were conducted using varing sequences of species input and global branch swapping in order to assure that all minimum length trees would be found. This

technique does not require outgroup rooting or the construction of a hypothetical ancestor (DeBry and Slade, 1985; Swofford and Olsen, 1990), and no ancestral character polarity was assumed. However, one or more "outgroup" species must be included in the analysis to assist in the decisions of character states for the most recent common ancestor of the species of interest (Swofford and Olsen, 1990). *P. eremicus* served as the outgroup species in these analyses based on its inclusion in a well-defined species group (and possibly to a different subgenus) which is not believed to be closely related to the species of interest (Carleton, 1989).

In order to estimate confidence limits on the predicted phylogeny (Felsenstein, 1985), the DOLBOOT algorithm of PHYLIP was also employed with the global branch swapping option in place. This process involved subsampling the data set, with replacement of various characters to produce a new data set of the same size as the original data set. Different characters were eliminated from each analysis, with other characters being represented more than once. These new data were then analyzed cladistically using Dollo parsimony criteria. Finally, each branch was removed from the resultant cladogram and placed in different positions to determine if a shorter tree was available. After 100 replicates were generated, a tree was produced which showed the number of times that a particular node containing the species at each side of the fork occurred in the subsampled replicates.

The data were also analyzed using Wagner parsimony criteria via the METRO

option of the PHYLIP program. Multiple analyses were conducted using varying random seed numbers to assure that all minimum length trees were found. In each replication, the threshold value was set at 0.01, in order that about 1,000 tree arrangements would be examined in determining the shortest tree. The cladogram was rooted using *P. eremicus* as the outgroup species. Confidence limits of the branch points were estimated from 100 bootstrap replicates using PHYLIP's BOOT algorithm with global branch swapping.

The PAUP program (Swofford, 1985) was also used in analyzing the data based on Wagner parsimony in order to assure that all minimum length trees would be found. The options MULPARS, BANDB (branch and bound), GLOBAL (global branch swapping) were invoked.

CHAPTER III

RESULTS

Phenetic analysis.--Restriction site maps of the 15 populations, representing eight species, consisted of an average of 20.7 sites per map, yielding a total of 66 restriction sites (Appendix VI, Figure 3). Of the 66 sites, 61 were variable, and 45 were phylogenetically informative (characters which were present or absent in two or more taxa, allowing inferences of evolutionary relationships). The five sites which were conserved in all species were distributed throughout the genome (Fig.3). The 1.0, 3.3, and 9.7 kilobase (kb) regions also showed a great deal of conservation. Most regions of the genome which were sampled showed variation, (especially the 2.8, 5.5, 6.7-8.5, and 10.0-10.5 kb regions). These variable and conserved regions are likely to correspond to the variable (e.g., D-loop) and conserved (e.g., cytochrome b) genes of the mtDNA. The uneven distribution of restriction sites throughout the genome could be the result of sampling error.

Sequence divergence values (δ) based on intraspecific and interspecific comparisons of taxa ranged from 0.3% to 16.7% (Table 2). No intrapopulational variation was observed. Interpopulation variation ranged from 0 to 0.8% in comparisons of populations of the same subspecies (i.e., within *P. truei*, *P. boylii*, *P. difficilis*, and *P. attwateri*). Populations of *P. difficilis* from Mexico (representing

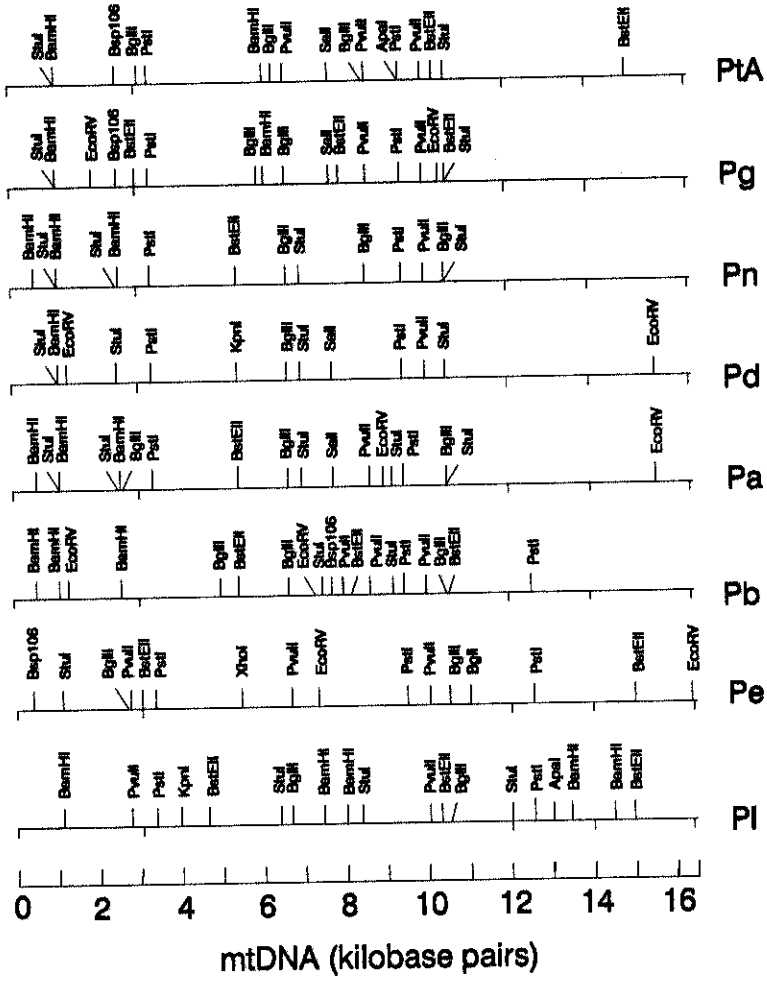

Figure 3. Maps depicting relative positions of cleavage sites of 13 hexanucleotide specific restriction enzymes for representative populations of each of eight species of *Peromyscus*. Conserved sites are represented by marks below the lines (from left to right: *Bgl*I, *Eco*RV, *Apa*I, *Bst*EII, and *Apa*I). Taxon codes are as follows: PtA) *P. truei truei* Arizona and *P. t. comanche*; Pg) *P. gratus*; Pn) *P. nasutus griseus*; Pd) *P. difficilis saxicola, P. d. ssp.,* and *P. d. difficilis*; Pa) *P. attwateri* Briscoe Co.; Pb) *P. boylii* New Mexico; J) *P. eremicus*; Pl) *P. leucopus* Texas.

Table 2. Matrix of percent sequence divergence values (Nei and Tajima, 1983). Taxon codes are as follows: PtA) *P. truei truei* Arizona and *P. t. comanche*; PtU) *P. truei truei* Utah; Pg) *P. gratus*; Pn) *P. nasutus griseus*; Pd) *P. difficilis saxicola, P. d. ssp.,* and *P. d. difficilis*; PaB) *P. attwateri* Briscoe Co.; PaP) *P. attwateri* Palo Pinto Co.; PbN) *P. boylii* New Mexico; PbA) *P. boylii* Arizona; Pe) *P. eremicus*; PlK) *P. leucopus* Kansas; PlT) *P. leucopus* Texas.

	PtA	PtU	Pg	Pn	Pd	PaB	PaP	PbN	PbA	Pe	PlK	PlT
PtA	0	0.8	5.3	8.8	7.7	9.1	7.9	14.2	13.9	10.8	11.2	13.0
PtU		0	6.4	8.0	7.7	9.1	7.9	14.2	13.9	12.4	11.2	13.0
Pg			0	9.3	8.1	9.1	7.9	9.9	9.5	14.1	16.0	16.4
Pn				0	4.3	3.9	3.9	9.1	8.8	11.6	13.5	13.9
Pd					0	5.5	5.1	13.7	12.9	12.9	14.8	15.3
PaB						0	0.7	9.0	8.6	12.7	16.4	16.7
PaP							0	9.0	8.6	14.5	16.4	16.7
PbN								0	0.3	13.5	15.3	15.6
PbA									0	13.1	14.9	15.3
Pe										0	11.9	12.3
PlK											0	2.5
PlT												0

the subspecies *P. d. saxicola* and *P. d. difficilis*) showed no differences in their mtDNA sequences. *P. truei comanche* and *P. truei truei* from Arizona also showed no differences in their mtDNA sequences; each differed from *P. truei truei* from Utah by approximately 0.8% of their mtDNA sequences. The two populations of *P. leucopus* showed higher levels of interpopulation sequence variation, differing by approximately 2.5% of their mtDNA sequences. These two populations represent two subspecies, *P. l. noveborascensis* and *P. l. texanus*, and most likely represent the two described cytotypes of *P. leucopus* which differ by pericentric inversions in three chromosomes (Stangl and Baker, 1984a).

Interspecific sequence differentiation between taxa which are known to be reproductively isolated varied from 5.3% to 16.7%. *P. truei* and *P. gratus* were most similar to each other and differed in 5.3-6.4% of their mtDNA sequences. These two taxa are thought to be closely related and are known to act as biological species. *P. attwateri*, *P. difficilis*, and *P. nasutus*, taxa about which conspecificity has been questioned, exhibited percentage sequence divergences which were intermediate between values found between the cytotypes of *P. leucopus* and between *P. truei* and *P. gratus*. *P. difficilis* and *P. nasutus* differed by approximately 4.3% of their mtDNA sequences, while *P. nasutus* and *P. attwateri* showed sequence divergence of about 3.9%.

UPGMA analysis of the phenetic relationships of the eight species based on percent sequence divergence produced two distinct clusters (Fig. 4). One cluster contained *P. leucopus* and *P. eremicus*, differing from the remaining taxa by an

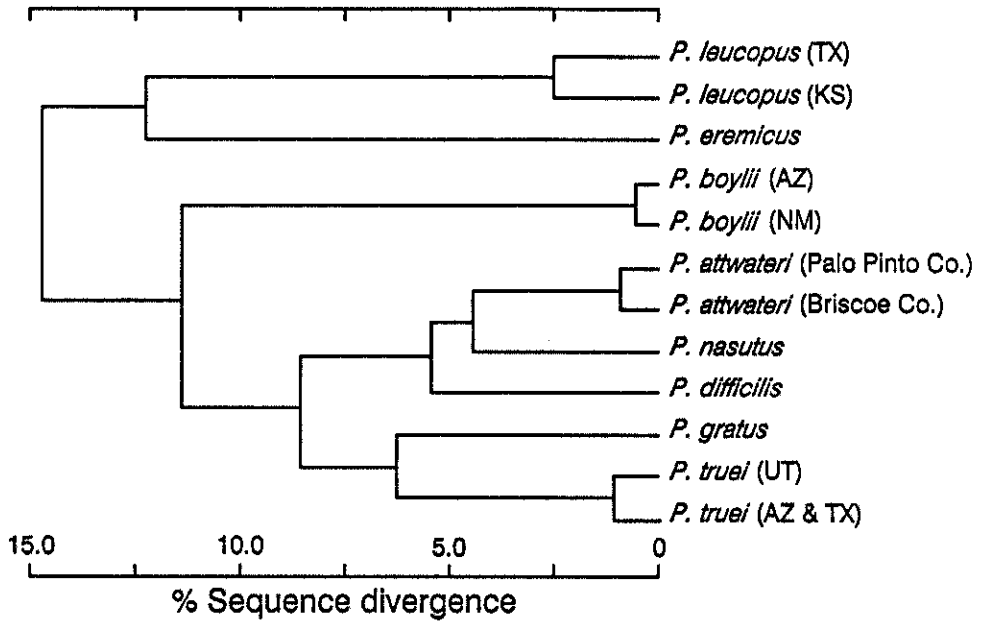

Figure 4. UPGMA analysis of percent sequence divergence values for all possible pair-wise combinations of taxa. Cophenetic correlation coefficient = 0.92.

average of 15% of their sequences. The other cluster consisted of *P. boylii*, *P. attwateri*, *P. difficilis*, *P. nasutus*, *P. gratus*, and *P. truei*. *P. boylii* falls outside the cluster containing *P. truei*, *P. gratus*, *P. difficilis*, *P. nasutus*, and *P. attwateri*, differing from these species by an average sequence divergence of 11.5%.

Cladistic analysis using Wagner parsimony.--Wagner parsimony analysis conducted by PAUP produced three equally parsimonious trees, each 85 steps in length (Fig. 5). The corresponding consistency index for the trees is 0.72 (including autapomorphies) or 0.52 (excluding autapomorphies). Ten replications of the METRO analysis run with PHYLIP found only two of these trees (Fig. 5, A and C). Further replications of the analysis, varying the random seed number, would be required to find the third tree produced by PAUP, however the strict consensus trees produced by both analyses were identical (Fig. 6).

P. truei and *P. gratus* were consistently found to be most closely related to each other based on Wagner parsimony criteria, forming a clade which included none of the other species analyzed. *P. difficilis*, *P. nasutus*, and *P. attwateri* were also placed together consistently, indicating that they are members of one clade. However, the relationships of the three taxa cannot be resolved sufficiently from these data. *P. difficilis* and *P. nasutus* were displayed as sister taxa on two of the trees (Fig. 5, A and B), with *P. attwateri* being placed as their closest relative, while in the third tree (Fig. 5, C), *P. difficilis* and *P. attwateri* were sister taxa, with *P. nasutus* indicated as their closest relative. Therefore, the relationship of these taxa was depicted as an unresolved trichotomy on the consensus tree (Fig. 6).

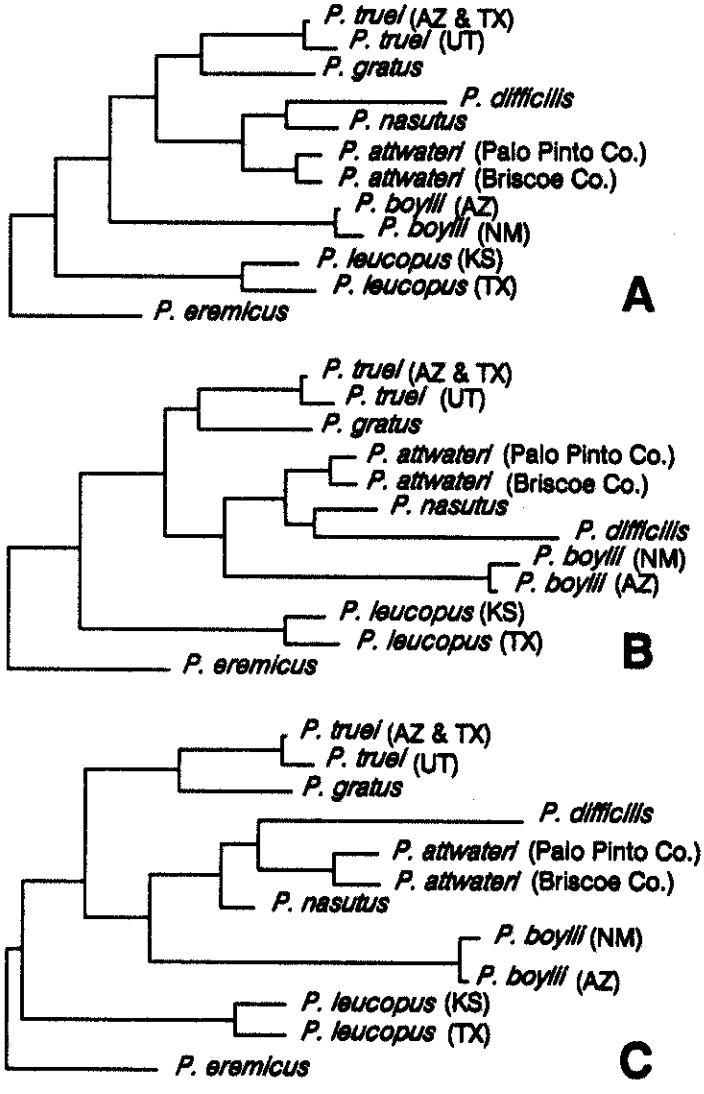

Figure 5. Three equally parsimonious minimum length trees based on Wagner parsimony. All trees have a length of 85 steps and a consistency index of 0.72 (including autapomorphies) or 0.53 (excluding autapomorphies). Branch lengths represent relative genetic divergence.

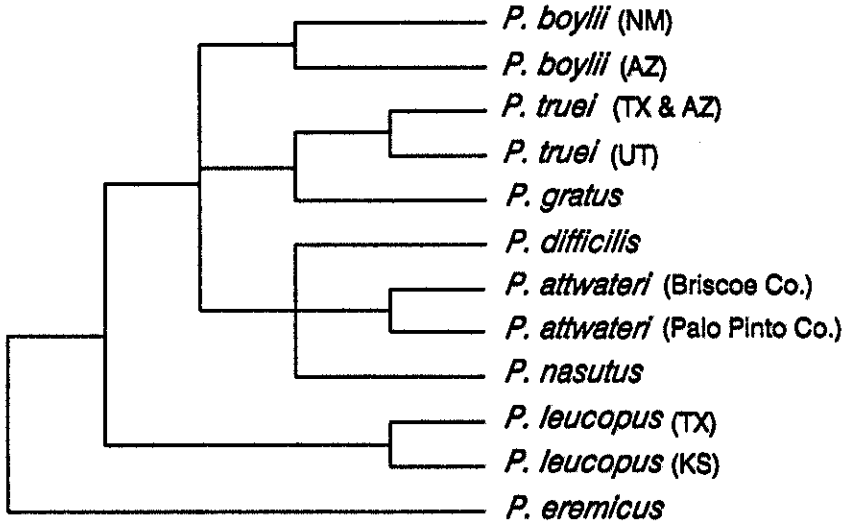

Figure 6. Strict consensus cladogram based on Wagner parsimony.

The relationship of *P. boylii* to the other taxa was also unresolved from these data. *P. boylii* was placed alternately outside the clade containing *P. truei*, *P. gratus*, *P. difficilis*, *P. nasutus*, and *P. attwateri* (Fig. 5, A), and within this large clade as a close relative of *P. difficilis*, *P. attwateri*, and *P. nasutus* (Fig. 5, B and C). This ambiguity was also represented as an unresolved trichotomy on the consensus tree.

P. leucopus was placed consistently outside the clade containing the other ingroup species, indicating that it is not closely related to any of these species. Interestingly, when the Wagner tree was rooted using *P. leucopus* or a combination of *P. leucopus* and *P. eremicus* as the outgroup, there was no change in the topologies of the three most parsimonious trees. This adds further support to the conclusion that *P. leucopus* is not closely related to any of the other ingroup species.

The bootstrap analysis of one of the three trees provided confidence limits which can be placed on the associations of various taxa (Fig. 7). The numbers shown at the nodes indicate the number of times in the 100 replications that the species grouped by that node were associated. Strong associations existed in intraspecific comparisons of *P. truei*, *P. attwateri*, *P. boylii*, and *P. leucopus*, with conspecific populations grouping from 95% to 100% of the time. The association of *P. truei* and *P. gratus* was also strong, with the two taxa being associated 70% of the time. Likewise, the placement of *P. leucopus* outside of the clade containing the other species was supported statistically, as *P. boylii, P. attwateri, P. difficilis, P.*

nasutus, P. truei, and *P. gratus* formed a clade separate from *P. leucopus* 80% of the time. *P. difficilis, P. nasutus,* and *P. attwateri* were associated 65% of the time, however phylogenetic ambiguity within this group was indicated by the low value of 39% associating *P. difficilis* and *P. nasutus*. Apparently, there were many characters which associated *P. difficilis* and *P. attwateri*, and possibly *P. nasutus* and *P. attwateri*, contributing to this ambiguity. The uncertainty in the placement of *P. boylii* in the search for the shortest tree was exhibited by a relatively low number obtained from the bootstrap analysis, as *P. boylii* was placed outside the clade containing *P. attwateri, P. difficilis, P. nasutus, P. truei,* and *P. gratus* only 38% of the time. In many of the bootstrap replicates, *P. boylii* may have been placed within this clade as a close relative of *P. attwateri, P. difficilis*, and *P. nasutus*.

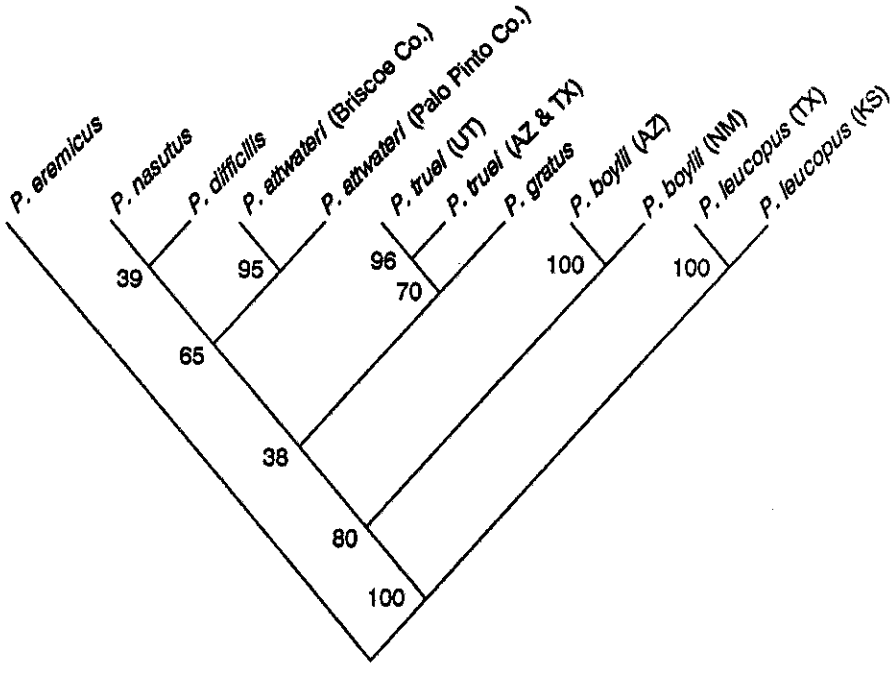

Figure 7. Bootstrap analysis of one of the three most parsimonious cladograms based on Wagner parsimony criteria. Numbers at nodes indicate the number of times the taxa associated by that node were affiliated in 100 subsamples of the data.

Cladistic analysis using Dollo parsimony.--Dollo parsimony analysis of the restriction site data conducted by PHYLIP produced two equally parsimonious trees, each requiring 51 character reversions, in 12 replications of the analysis (Fig. 8). As in the Wagner analysis, *P. attwateri*, *P. difficilis*, and *P. nasutus* were considered to form a clade separately from the other species. The relationships of the three taxa does seem to be resolvable based on Dollo criteria, however. *P. difficilis* and *P. nasutus* appear to be most closely related to each other, with *P. attwateri* as a sister taxon. Like trees A and C of the Wagner analysis (Fig. 5), the tree resulting from Dollo parsimony analysis indicated that *P. boylii* was a close relative of the *attwateri-difficilis-nasutus* clade.

The relationship of *P. truei* and *P. gratus* as predicted by Dollo parsimony was different than that resulting from Wagner parsimony. Based on Dollo parsimony, *P. gratus* appeared to be either closely related to *P. boylii* (Fig. 8, A), or was not closely related to any of the other taxa, but shared a most recent common ancestor with *P. attwateri, P. difficilis, P. nasutus,* and *P. boylii* (Fig. 8, B). This ambiguity was represented by the trichotomy including *P. gratus* on the Dollo parsimony consensus tree (Fig. 9). Unlike the Wagner parsimony analysis, Dollo parsimony did not predict a close relationship between *P. truei* and *P. gratus*.

Dollo parsimony analysis also placed *P. eremicus* and *P. leucopus* as the two most distantly related species of the group. *P. leucopus* was placed more distantly from the other ingroup species than was *P. eremicus*, which has been considered to

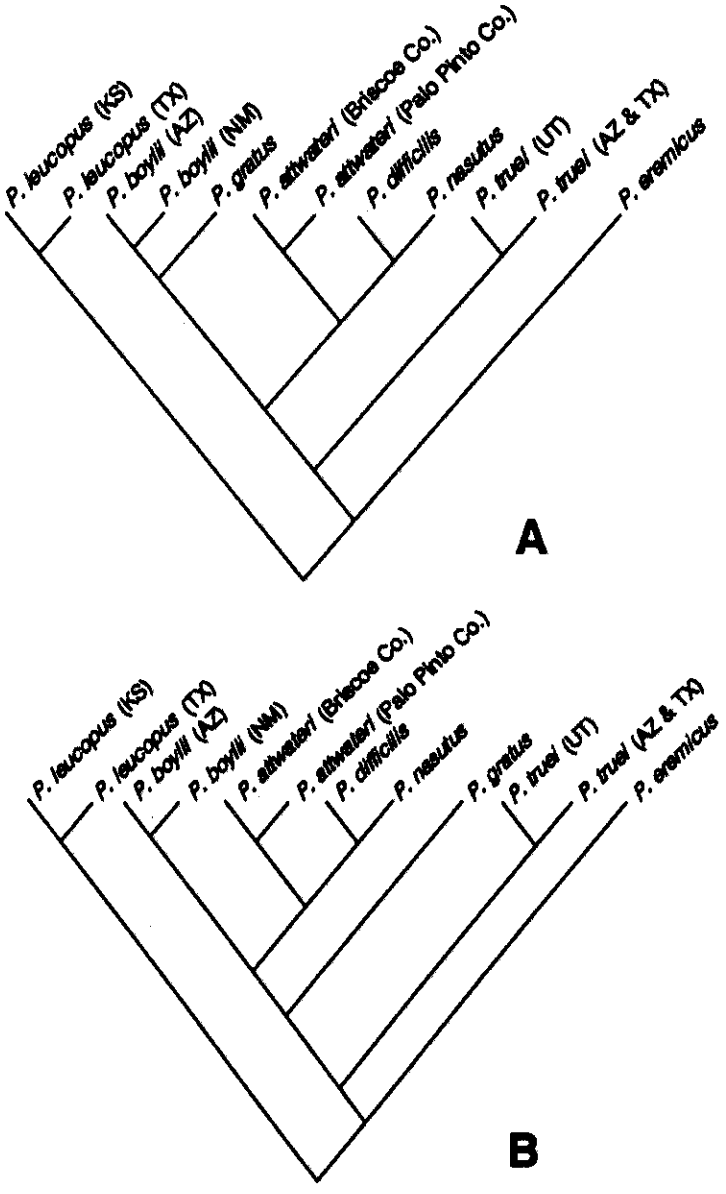

Figure 8. Two equally parsimonious minimum length trees based on Dollo parsimony analysis. Each tree requires 51 character reversions.

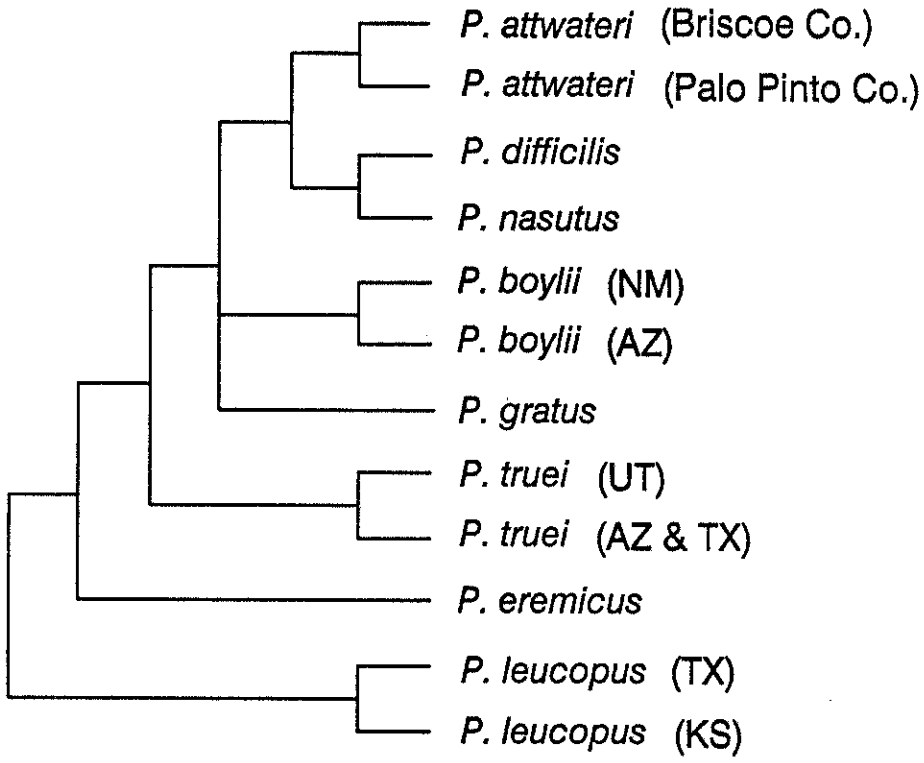

Figure 9. Strict consensus tree based on Dollo parsimony criteria.

belong to a different subgenus (*Haplomylomys*) than do *P. leucopus* and the other species analyzed here (Subgenus *Peromyscus*).

Bootstrap analysis of the Dollo parsimony trees provided an indication of strong and weak branch points of the cladogram derived from the Dollo parsimony analysis (Fig. 10). *P. difficilis* and *P. nasutus* were associated from 63% to 70% of the time, indicating that a relatively high degree of confidence can be placed on their sharing a common ancestor. The relationship of *P. gratus* is ambiguous based on Dollo criteria, indicated by the relatively low bootstrap numbers associating *P. gratus* and *P. boylii* in tree A (41%) and at the node separating *P. boylii, P. difficilis, P. nasutus,* and *P. attwateri* from the other species in tree B (35%). Similarly, the placement of *P. leucopus* outside the clade containing the other species is not supported with a high degree of confidence (54-66%). The existence of a clade containing *P. gratus, P. boylii, P. difficilis, P. nasutus,* and *P. attwateri*, excluding *P. truei* also was not supported strongly by the bootstrap analysis, with these species forming a clade separately from *P. truei* from 51% to 63% of the time.

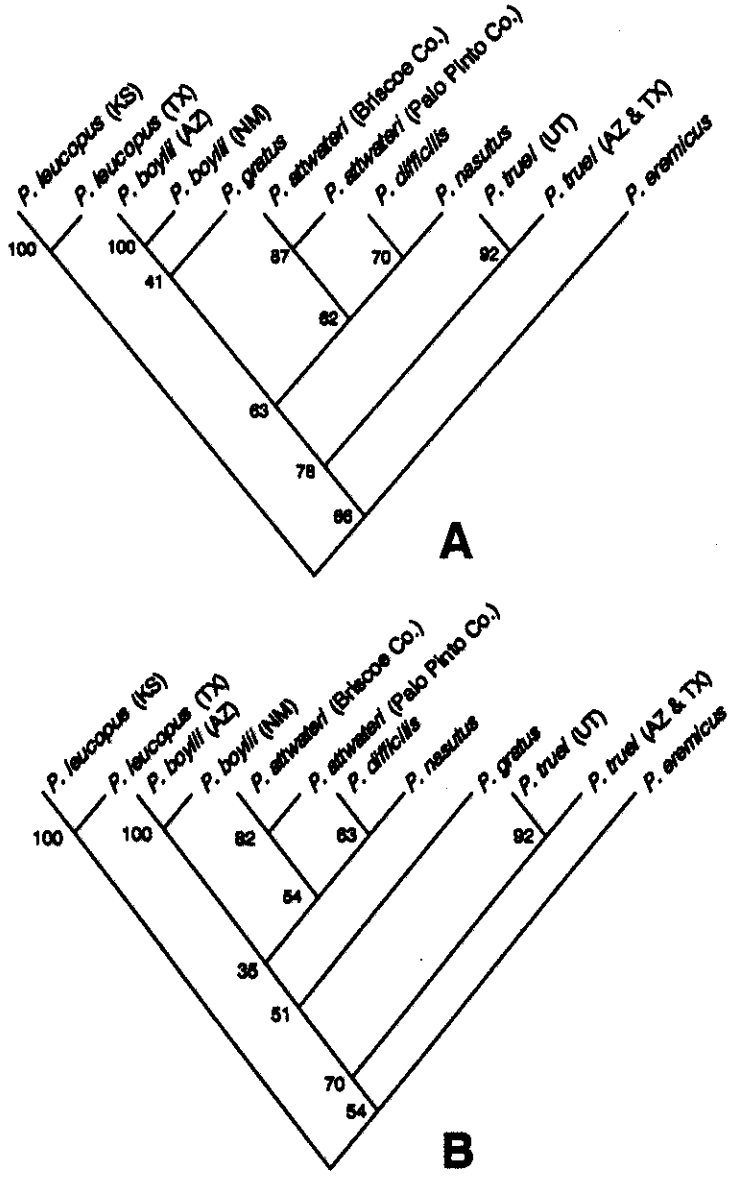

Figure 10. Bootstrap analysis of two minimum length cladograms based on Dollo parsimony. Numbers at nodes indicate the number of times of 100 that the taxa associated by that node were affiliated in 100 replications.

CHAPTER IV

DISCUSSION

Because the systematics of the species of the *boylii* and *truei* species groups has been analyzed using a number of genetic techniques, the groups provide an especially useful model for comparing molecular evolution at varying levels of genomic modification. Karyotypic analyses have provided insight into the major chromosomal changes associated with speciation. Allozymic analyses estimate divergence of nuclear genes, with the limitations of resolving only 33-50% of amino acid substitutions expressed as charge changes on the proteins. MtDNA restriction site analysis provides an estimate of nucleotide divergence in the entire mitochondrial genome. Sequence data for mitochondrial genes, when it becomes available, will provide further information on the evolution of these genes. The information combined from these different components of the genome will provide the best estimates of the phylogeny of these species. More importantly, these different analyses will provide an example of evolution at differing genetic levels, as components of the genome are expected to evolve at different rates throughout time, representing varying components of the "genetic revolution" that Mayr (1963) proposed occurs with the process of speciation.

Systematic conclusions.--One of the objectives of this study was to determine

whether *P. difficilis* and *P. attwateri* are sister taxa or even conspecific rather than belonging to different species groups and also if the species-level relationship of *P. nasutus* to *P. difficilis* is confirmed by mtDNA relationships. The UPGMA analysis indicated that *P. attwateri*, *P. nasutus*, and *P. difficilis* were similar, differing by only 3.9-5.5% of their mtDNA sequences. These levels of sequence divergence are intermediate between variation within species (2.5% between the cytotypes of *P. leucopus*) and between species (5.3-6.4% between *P. truei* and *P. gratus*) observed in this study; although, these values are closer to those found between species. Other studies including within and between species comparisons of mtDNA sequence variation in rodents have shown up to 5.7% sequence divergence within species and as little as 2.9% between reproductively isolated entities (Riddle and Honeycutt, 1990). There do not appear to be consistent levels of mtDNA differentiation associated with distinct gene pools among rodents; therefore, these levels of divergence do not provide strong evidence for either conspecificity or distinctness. Nevertheless, one should not expect different components of the genome to exhibit the same amounts of divergence accompanying the attainment of reproductive isolation. It appears, however, that the three taxa either represent three different species or three genetic and morphologic variants of one species.

Questions of conspecificity cannot be addressed using only one form of data. In addition to the mtDNA data described here, there are karyotypic and allozymic data available for these species. *P. difficilis* and *P. attwateri* have similar

karyotypes, differing by only two chromosomal inversions, one of which occurs on chromosome 6, a chromosome which exhibits a high degree of homoplasy in *Peromyscus* (Smith, 1990). *P. nasutus* and *P. difficilis* differ by only one pericentric inversion. These levels of karyotypic divergence are certainly not high enough to suggest that successful interbreeding between the three would be impossible. *P. leucopus*, for example, exhibits two karyotypic forms which differ by three presumed pericentric inversions, and these cytotypes interbreed freely (Stangl and Baker, 1984a). In the single, wide ranging species, *P. maniculatus*, more karyotypic variation occurs (Sparkes and Arakaki, 1966) than is often found between reproductively isolated entities of *Peromyscus* (Smith, 1990). The proposed mechanism in *Peromyscus* which allows fertility in inversion heterozygotes is heterosynapsis, in which nonhomologous sequences and non-aligned centromeric regions synapse, suppressing the formation of detrimental crossover products within the inverted sequences (Greenbaum and Reed, 1984). Also, more distantly related species of *Peromyscus* (e.g. *P. boylii, P. crinitus,* and *P. hooperi*) show no observable karyotypic differences (Smith, 1990), so relative number of chromosomal inversions are often of little use in assessing conspecificity.

Allozymically, *P. difficilis*, *P. nasutus*, and *P. attwateri* are not well differentiated. No fixed allelic differences were found among the three taxa when a large geographic representation of *P. difficilis* and *P. nasutus* was examined (Janecek, 1990). Sullivan et al. (1991) also found *P. difficilis* and *P. attwateri* to be

similar, yet they reported that it is unlikely that the two are conspecific based on three fixed allelic differences (PGI & TRF, Sullivan et al., 1991) (LDH-3, Zimmerman et al., 1975). However, the sample sizes reported by Sullivan et al. for these two species were limited, including only one population of *P. difficilis* (n=3) and one population of *P. attwateri* (n=2).

All forms of genetic evidence indicate that *P. difficilis, P. nasutus,* and *P. attwateri* are closely related, but they show levels of genomic differentiation intermediate between levels within species and between species. It is likely that the possible conspecificity of these populations cannot be definitively addressed since they are not known to occur in sympatry. Extensive sampling in the Texas panhandle, where the ranges of the two taxa occur in close proximity, suggests that *P. nasutus* and *P. attwateri* are separated by approximately 130 km of unsuitable habitat and therefore do not appear to occur in sympatry (Choate et al., 1991). Sampling in northern Mexico, although not as extensive as sampling in the United States, has failed to discover any sympatric populations of *P. difficilis* and *P. nasutus* (Janecek, 1990). The sympatric occurrence of any of the three taxa is doubtful.

As allopatric taxa which are generally considered to be too morphologically divergent to be named as conspecific, *P. nasutus* and *P. attwateri* conform to Mayr's (1963) description of "superspecies". *P. nasutus* and *P. difficilis* do not show marked morphologic differentiation (Janecek, 1987), but seem to fit these criteria genetically. Mayr states that superspecies occur most commonly when

organisms are closely tied to specific, isolated physiographic features. *P. difficilis* and *P. nasutus* occur in upper-elevation, rocky situations of the mountain ranges of Mexico and the southwestern United States. *P. attwateri* occurs along rocky cliffs which are interspersed throughout the southcentral United States. These restrictive habitat requirements make *P. difficilis, P. nasutus*, and *P. attwateri* typical of groups which exist as superspecies. The absence of sympatry makes it impossible to determine if these closely related taxa are capable of intergrading.

The species group assignment of *P. attwateri*, *P. difficilis*, and *P. nasutus* is also of interest. *P. attwateri* is currently considered a member of the *boylii* group, while *P. difficilis* and *P. nasutus* are assigned to the *truei* group. However, due to the close relationship of these species, it is clear that their traditional placement in different species groups is not warranted, and that the *truei* and *boylii* species groups as they are currently recognized do not represent separate, monophyletic lineages.

Based on the Wagner parsimony analysis, the relationship of *P. attwateri*, *P. nasutus*, and *P. difficilis* to the other taxa cannot be determined. The three taxa either form a clade with *P. truei* and *P. gratus* (Fig.5, A) or with *P. boylii* (Fig. 5, B & C). Based on the Dollo parsimony analysis, these three taxa are more closely related to *P. boylii* and possibly *P. gratus* than they are to *P. truei*. Because there are no well-defined clades consistently containing any of these species as determined from these data, there does not seem to be any warrant for making species group assignments until more species are added to the analysis.

The relationships of *P. gratus* to *P. truei*, *P. difficilis*, and *P. leucopus* is also of interest in light of recent studies which yielded conflicting conclusions (Fig. 1). The relationship of *P. gratus* to the other species based on Wagner and Dollo parsimony analysis of mtDNA restriction maps is not clearcut. Based on Wagner parsimony analysis, *P. gratus* is most closely related to *P. truei*, and the two taxa differ by about 6% of their mtDNA sequence. This finding agrees well with earlier allozymic analyses (Avise et al., 1979; Zimmerman et al., 1978). Although, Janecek (1990) found *P. gratus* to be most similar to *P. difficilis*, and her data did not suggest *P. truei* and *P. gratus* were extremely dissimilar. The Dollo parsimony analysis, however, indicates that the relationship of *P. gratus* is unresolvable. *P. gratus* is either the closest relative of *P. boylii*, or it shares a most recent common ancestor with *P. difficilis*, *P. attwateri*, *P. nasutus*, and *P. boylii*. The possible relationships proposed by the Dollo analysis are not supported by any other published data set. It seems that the Dollo analysis may be too stringent in dealing with these data, as the results of the Wagner analysis are much better supported both by previous studies and by bootstrap analysis.

The relationship of *P. leucopus* to the species of the *boylii* and *truei* species groups is also of interest. Based on mtDNA restriction site data, *P. leucopus* does not appear to be close to any of the species of the *truei* of *boylii* species groups examined. This conclusion is reached based on both Wagner and Dollo parsimony analysis and is supported by the fact that, when the Wagner tree is rerooted using either *P. leucopus* or a combination of *P. leucopus* and *P. eremicus*, no change in

topology of the tree is observed. The conclusion that *P. leucopus* is not closely related to the other species examined in this study agrees well with early allozymic analyses of the relationships of these taxa (Avise et al., 1979; Zimmerman et al., 1978) and with traditional species group assignments based on morphology, and *P. leucopus* has been consistently placed in the *leucopus* species group (Carleton, 1989).

The final question that was addressed in this study concerned the relationship of *P. truei comanche* to other *P. truei*. The taxonomic placement of this mouse has changed a number of times (Chapter I), however its current placement as a subspecies of *P. truei* was substantiated by the mtDNA restriction site data. No differentiation was found between *P. truei comanche* and *P. truei* from Arizona, and only minimal differences (0.8%) were found between *P. truei comanche* and *P. truei* from Utah. This absence of divergence is indicative of a close relationship between these taxa, supporting the conspecificity of the populations. As an isolated population of *P. truei, P. truei comanche* reserves its subspecific designation, despite the lack of observable genetic differentiation in their mtDNA sequences. Interestingly, allozyme data show differences between *P. truei comanche* and other *P. truei*. *P. truei comanche* was the most dissimilar population of *P. truei* analyzed by Janecek (1990).

Comparison of methods of phylogenetic reconstruction based on restriction sites.--Some authors propose that phenetic methods (those based on the overall phenotypic similarity of the organisms) provide sufficiently accurate estimates of

phylogeny that they may be used in the reconstruction of evolutionary lineages (Nei et al, 1985). UPGMA (unweighted pair-group method using arithmetic averages) analysis provides a diagram of clusters based on phenotypic similarity, or in this case, percent nucleotide sequence divergence of mtDNA. However, stronger tests of evolutionary relationships may be made based on changes in individual character states (the presence or absence of restriction sites). Therefore, it is best not to infer evolutionary relationships from the results of the phenetic analysis. However, the UPGMA phenogram provides a useful representation of the similarity of the species being examined and the percent sequence divergence values associated with various combinations of the taxa.

There is still some controversy surrounding the most appropriate cladistic analysis for mtDNA restriction site data (J. Felsenstein, pers. comm.). There are two different approaches which have been employed in phylogeny reconstruction from restriction site data, each differing in its treatment of convergent site losses and convergent site gains.

A restriction site can be lost by a change in any one of the six nucleotides of a recognition sequence. With three alternate nucleotide possibilities which can be substituted for each of the six positions, there are 18 different mutations which would produce a restriction site loss that is only one substitution away from being a recognition site (DeBry and Slade, 1985; Templeton, 1983). Therefore, convergent restriction site losses can be produced rather easily by a number of different mutations.

Convergent restriction site gains are possible; however, the likelihood of their occurrence is considerably lower than that of convergent restriction site losses. If for example, an ancestral species has a mtDNA sequence which is only one nucleotide away from being a recognition sequence for a particular restriction enzyme (e.g., AAATTC rather than GAATTC, which is recognized by *Eco*RI), only one of the eighteen possible single base-pair substitutions will produce a recognition sequence. A mutation in any of the other five positions (15 of the possible 18 point mutations) will produce a sequence which is further from being a recognition sequence (DeBry and Slade, 1985). Therefore, convergent site gains must occur much less frequently than convergent site losses.

"Wagner parsimony" or "maximum parsimony" analysis is a commonly employed cladistic analysis strategy. The primary goal of this method is to produce a cladogram with the fewest number of steps, or character changes, in it. The gain or loss of a restriction site in any branch of the cladogram is considered to be a step; site gains and site losses are treated as being equally likely.

Dollo parsimony analysis is a fundamentally different cladistic approach. In this analysis, convergent site losses are treated as being much more likely than convergent site gains. Therefore, in the construction of the cladogram, a site is allowed to arise only once, but may be lost as many times as necessary to produce the cladogram. While the minimum length tree is still the goal, the cladogram produced by Dollo parsimony will always have more steps than the Wagner parsimony tree due to the more stringent treatment of convergent site gains. This

increased tree length is a result of the fact that a number of site losses will normally be required in order to prevent the assumption of one convergent site gain (J. Felsenstein, pers. comm.).

Dollo parsimony is often considered to be a better estimator of phylogenetic relationships than is Wagner parsimony analysis. The latter may not be an appropriate phylogenetic tool for restriction site data due to its failure to recognize the vast difference in the likelihood of convergent restriction site losses and convergent restriction site gains (DeBry and Slade, 1985; Jansen et al.,1990). However, there are those who would argue that Wagner parsimony analysis is preferable to Dollo parsimony due to the possible "overcorrection" generated by Dollo parsimony (J. Felsenstein, pers. comm.). This issue is far from being resolved, and some propose the use of a "relaxed Dollo" approach, allowing two convergent site gains rather than a specified number of site losses (Swofford and Olsen, 1990). However, computer software is not yet readily available for analyzing data using this approach. Additionally, there are important philosophical problems to be addressed, such as the number of site losses to accept before assuming a convergent site gain, before this approach will be feasible.

The results of the Wagner and Dollo analyses of these mtDNA data differ in several ways. These data seem to provide some insight into the usefulness of the Wagner and Dollo parsimony approaches, based on the confidence limits generated by bootstrap analysis and congruence of the results of each analysis with

the results of allozymic and morphologic analyses.

Both the Wagner and the Dollo parsimony analyses indicate that *P. difficilis, P. attwateri,* and *P. nasutus* are members of a single clade. The inter-relationships among the three taxa cannot be determined based on Wagner parsimony criteria, however, leaving an unresolved trichotomy among the taxa. Dollo parsimony analysis yields only one proposed relationship of the three, with *P. difficilis* and *P. nasutus* being most closely related to each other, as is shown by other forms of data, and *P. attwateri* as their closest relative. Bootstrap analysis indicates that a relatively high degree of confidence can be placed on this arrangement. At this low level of sequence divergence, Dollo parsimony analysis seems to be preferable to Wagner parsimony analysis, since with more closely related taxa, convergent site gains are less likely to be found than in more distantly related taxa.

Wagner and Dollo parsimony analyses also differ in their placement of *P. gratus*. Wagner analysis places *P. gratus* in a clade with *P. truei*; the two taxa also show low levels of sequence divergence in their mtDNA's (5.3-6.4%). This arrangement is supported by earlier allozyme analyses (Avise et al., 1979; Zimmerman et al., 1978) and by previous assumptions that the two were conspecific based on their morphology. Bootstrap analysis indicated a relatively high level of confidence in this relationship (70%). However, the Dollo analysis places *P. gratus* in one of two positions relative to the other taxa: *P. gratus* may be most closely related to *P. boylii* or may be placed on a separate branch, sharing a common ancestor with *P. attwateri, P. difficilis, P. nasutus,* and *P. boylii*. Although

the results of the Dollo analysis may represent real evolutionary relationships, neither of its conclusions concerning the relationships of *P. gratus* to the other taxa have been reached based on any other form of data. In addition, the bootstrap estimates place somewhat lower levels of confidence in these proposed relationships than is placed in the relationship proposed by Wagner parsimony.

Although Dollo parsimony analysis may be intuitively appealing, it is possible that the "overcorrection" it provides in dealing with slightly more distantly related species is problematic occasionally. In cases of taxa which are not extremely closely related, Wagner parsimony analysis may be preferable. Further application of both approaches to phylogenetic reconstruction to real data sets will be needed, especially with regard to species complexes which are understood more fully than is *Peromyscus*, in order to make more informed decisions on appropriate methodologies in dealing with restriction site data.

Congruence of genetic data sets.--Apparently, there is little congruence between the karyotypic evolution of these particular species and mtDNA or nuclear gene evolution as assessed by allozymic analyses. The only point on which the karyotypic data reported by Smith (1990) are moderately supported is by the allozymic data of Janecek (1990) in which *P. gratus* and *P. difficilis* are proposed to be sister taxa. Smith did not find *P. difficilis* and *P. attwateri* to be sister taxa, however, as was indicated by Janecek's allozymic data. Sullivan et al. (1991), using a cladistic analysis of allozymic data, found *P. gratus* and *P. difficilis* to belong to two different, well-defined clades, further contradicting karyotypic data.

The mtDNA data also support the hypothesis of a more distant relationship between these taxa.

An earlier analysis of karyotypic evolution in *Peromyscus* recognized the homoplasious nature of chromosome 6 and eliminated it from cladistic analyses (Rogers et al., 1984). While some authors felt that chromosome 6 provides some important information in resolving certain relationships in *Peromyscus* (Stangl and Baker, 1984b), the elimination of this character provides better resolution of other relationships. Specifically, when chromosome 6 is eliminated from the analysis, *P. attwateri* and *P. difficilis* are found to belong to one clade (Rogers et al., 1984). Although this relationship was not supported by any other data at that time, recent allozymic and mtDNA restriction site data have indicated that this relationship is valid.

On all other points concerning the species analyzed here, the karyotypic data differ from the mtDNA and allozymic data, possibly due to a high degree of homoplasy in karyotypic data. *Peromyscus* is an extremely speciose genus of rodents, in which all species share the same chromosomal number, but a large number of chromosomal inversions are known to exist both between species (Smith, 1990) and within species (Stangl and Baker, 1984a; Sparkes and Arakaki, 1966). The genus is depicted by karyotypic orthoselection, whereby a single mechanism of chromosomal change characterizes karyotypic evolution in the group (Greenbaum et al., 1986). Heterosynapsis is the mechanism which has been proposed to allow these major rearrangements. Fragile sites in chromosomes are

also known to occur, and the possibility that these fragile sites are a mechanism allowing pericentric inversions is being investigated (I. F. Greenbaum, pers. comm.). If there are indeed "hot spots" in the chromosomes of *Peromyscus* which allow inversions to occur, it is likely that these regions allow a great deal of convergent karyotypic evolution in *Peromyscus*. The possibility that major chromosomal rearrangements can occur relatively frequently, creating homoplasy in karyotypic data sets, is supported by observations that identical Robertsonian fusion and fission events in chromosomes occur independently in populations of *Mus domesticus* (Patton and Sherwood, 1983).

It is not yet known what relationship chromosomal mutations have to speciation or what their systematic implications may be. Better understanding of the significance of karyotypic data will come only when more taxa are studied both karyotypically and using more direct measures of relatedness such as actual DNA sequences (Patton and Sherwood, 1983). Comparisons of relationships of the species examined here based on chromosomal data with mtDNA data and allozyme data do not indicate high levels of congruence between the conclusions drawn from the chromosomal data and any other measure of genomic modification.

MtDNA data, while differing from previous allozyme data sets in various ways, show much greater concordance with the proposed relationships based on the nuclear gene products than with karyotypes. For example, the relationship between *P. truei* and *P. gratus* proposed by Wagner parsimony and UPGMA

analyses of mtDNA data are supported by the results of allozymic analyses by Avise et al. (1979) and Zimmerman et al. (1978). Also, the close relationship between *P. difficilis* and *P. attwateri* was previously suggested by allozymic studies by Janecek (1990) and Sullivan et al. (1991). And finally, the placement of *P. leucopus* outside the *truei* and *boylii* species groups is concordant with allozymic studies (Avise et al., 1979; Zimmerman et al., 1978).

It is clear that, although mtDNA restriction site data yield some resolution of the relationships of these species which is supported by other data, homoplasy is a problem in the mtDNA restriction site data. MtDNA provides a useful systematic tool because it shows rapid rates of evolution compared to nuclear DNA sequences. However, this rapid rate of evolution contributes to high degrees of homoplasy when taxa are not closely related. Additionally, restriction site analysis samples the entire mtDNA molecule, including the most rapidly evolving regions, such as the D-loop, allowing the introduction of further homoplasy. Future systematic studies of *Peromyscus* may benefit from nucleotide sequence analysis of more highly conserved mitochondrial genes.

The species group concept in *Peromyscus*.--The concept of the *boylii* and *truei* species groups as monophyletic units is not well supported by any form of genetic data. Although phenetic analysis of the mtDNA data produces two clusters, one of which contains *P. boylii* and the other of which contains the other *truei* and *boylii* species group constituents, cladistic analyses of the data using Wagner and Dollo parsimony do not indicate the existence of two well-defined clades which

can be considered *boylii* and *truei* species groups. It is possible that the species of these groups are all members of one large clade, as these species do appear to be closely related to each other. Distinctions between lineages within this large clade may remain difficult due to the large number of extant species and the possibly larger number of extinct species which are excluded from each phylogenetic analysis. The partial representation of the species groups in all studies utilizing a variety of techniques as well as the variation in the species composition of each study may be one underlying cause for the high degree of incongruence in comparisons of different data sets. Our ability to resolve relationships within the genus *Peromyscus* may improve only when studies include all members of the genus.

APPENDIX I

SPECIMENS EXAMINED

APPENDIX I

SPECIMENS EXAMINED

Collection localities of 15 populations of *Peromyscus* and, in parentheses, the number of specimens examined.

P. truei truei.--ARIZONA: Coconino Co.; 25 mi. NE Flagstaff, elev. appro. 5000 ft. (4); UTAH: Grand Co.; Castle Valley, 12 mi. NE Moab (1).

P. truei comanche.--TEXAS: Randall Co.; Palo Duro Canyon State Park, 2.5 mi. S, 15.6 mi. E Canyon (34° 56' N, 101° 39' W) (2).

P. gratus.--NEW MEXICO: Catron Co.; 1.5 mi. N, 0.75 mi. E Luna, elev. 7200 ft. (33° 51' N, 108° 56' W) (3).

P. difficilis griseus.--NEW MEXICO: Lincoln Co.; Malpais lava flow, 7.0 mi. N, 7.0 mi. W Carrizozo (2).

P. difficilis difficilis.--AGUASCALIENTES: 6 mi. W Rincon de Ramos (1).

P. difficilis saxicola.--HIDALGO: Puerto la Estancia (2); HIDALGO: 5.4 mi. SE, 3.2 mi. S Ixmiquilpan (2).

P. boylii rowleyi.--NEW MEXICO: Catron Co.; Gila National Forest, 0.25 mi. S, 2.5 mi. W Mogollon, elev. 6800 ft. (33° 23' N, 108° 50' W) (1); ARIZONA: Cochise Co.; Coronado National Forest, 2.0 mi. S, 1.5 mi. W Portal, elev. 5800 ft. (31° 53' N, 109° 10' W) (2).

P. attwateri.--TEXAS: Briscoe Co.; 7 mi. N, 3.2 mi. W Quitaque (34° 27' N, 101° 07' W) (4); TEXAS: Palo Pinto Co.; intersection of Highway 4 and Brazos River (2).

P. leucopus noveboracensis.--KANSAS: Butler Co.; 1.0 mi. W of Potwin on Highway 196 (2).

P. leucopus texanus.--TEXAS: Taylor Co.; 3.0 mi. N Merkel (82° 31' N, 100° 01' W) (1).

P. eremicus eremicus.--UTAH: Washington Co.; 13 km N, 6 km W St. George, elev. 4000 ft. (37° 12' N, 113° 38' W) (2).

APPENDIX II

PROCEDURE FOR FIELD KARYOTYPING

APPENDIX II

PROCEDURE FOR FIELD KARYOTYPING

1. Remove femurs immediately after sacrificing and flush marrow with 0.075 M KCl (@ 37 °C) into a centrifuge tube.

2. Agitate to break up marrow and increase volume to approximately 6 ml with the KCl.

3. Add one drop of 0.005% Colchicine and incubate at 37 °C for 32 min.

4. After 31 min. add approximately 1 ml of Carnoy's Fixative (3:1 :: methanol:glacial acetic acid).

5. Centrifuge at 1500 rpm for approximately 1 min. to pellet cells.

6. Decant supernatant and add 1 ml fixative.

7. Agitate to break up pellet and add approximately 3 ml fixative.

8. Centrifuge at 1500 rpm for 1 min.

9. Repeat steps 6-8 three times.

10. After final spin resuspend cells in 2 ml fixative.

11. Prepare three slides for analysis and freeze remaining cell suspension in a cryo vial in Liquid Nitrogen. (Slides may be flamed for test slides, must be air dried for banding.)

12. Stain test slides with Giemsa Stain (two quantities of Giemsa Buffer to one part Giemsa Stain stock soln.) for 2-3 minutes.

13. Rinse slide with a stream of dH_2O and allow to dry on an angle.

Solutions:

0.075 M KCl (KCl aliquoted into 0.56g tubes)

0.005% Colchicine

Carnoy's Fixative: 3:1 :: Methanol: Glacial Acetic Acid

(Made Up Fresh)

Giemsa Stain Stock Soln.

Giemsa Buffer:

0.05 g NaH_2PO_4

0.09 g Na_2HPO_4

100 ml dH_2O

APPENDIX III

MITOCHONDRIAL DNA ISOLATION TECHNIQUE

APPENDIX III

MITOCHONDRIAL DNA ISOLATION TECHNIQUE

1. Mince tissues (up to 10 g) and homogenize in 2 - 3 ml MSB-Ca^{+2} per gram of tissue.

2. Add 0.2 M EDTA to a final concentration of 10 mM (150 μl per 3 ml solution).

3. Centrifuge at 700 x g for 5 min. at 4 °C.

4. Decant supernatant into a fresh 50 ml centrifuge tube making sure not to disturb the debris pellet and repeat spin as in step 3.

5. Decant supernatant into Oakridge Tube and centrifuge at 20K x g for 20 min. at 4 °C to pellet mitochondria.

6. Decant supernatant and resuspend pellet in 10-20 ml of MSB-EDTA. Repeat centrifugation as in step 5.

7. Decant supernatant and resuspend pellet in 3 ml of STE. Add 0.375 ml of 10% SDS. Incubate 3 min. at 37 °C.

8. Weigh out 3.85 g CsCl for each sample and decant in Beckman 0.5" X 2" ultracentrifuge tubes.

9. Add mtDNA suspension and 0.2 ml of ethidium bromide (10 mg/ml in STE). Mix contents well by inversion.

10. Adjust weight to equality using a 1.1 g/ml CsCl solution and cap each tube with a layer of mineral oil to fill the tube.

11. Centrifuge at 36,000 rpm at 20 °C for 30-40 hours in a Beckman 50.1 swinging bucket rotor.

12. Observe tube under ultraviolet light to detect bands of DNA, RNA, glycogen and proteins. Mitochondrial DNA band appears approximately 0.5 cm below the large nuclear DNA band.

13. Remove mtDNA band by puncture with syringe.

14. Remove EtBr with at least 3 extractions with 1 volume of followed by one extraction with 1 volume ether. Heat at 70 °C for about 10 min. after removal of ether to insure that all ether is extracted.

15. Pipet DNA solution into a 1.5 ml centrifuge tube and cover with a single layer of dialysis tubing. Seal the top of the tube with a rubber band and dialyze the inverted tube for 2-3 days in TE buffer, changing buffer twice on the first day and once each following day.

16. DNA can be concentrated by ethanol precipitation and used for further analysis.

Recipes:

MSB (0.21 M Mannitol, 0.07 M Sucrose, 0.05 M Tris-HCl, pH 7.5)

Mannitol	7.65 g
Sucrose	4.79 g
Tris	1.21 g
H_2O	160 ml

adjust pH to 7.5 and bring vol. to 200 ml

MSB-Ca^{+2} (MSB + 3 mM $CaCl_2$)

As above with 0.088 g $CaCl_2$

MSB-EDTA (MSB + 0.01 M EDTA, pH 7.5)

As above with 0.744 g EDTA

STE (0.1 M NaCl, 0.05 M Tris-HCl, 0.01 M EDTA, pH 8.0)

NaCl	1.16 g
Tris	1.21 g
EDTA	0.74 g
H_2O	160 ml

adjust pH to 8.0 and bring vol. to 200 ml

1 X SSC (0.15 M NaCl, 0.015 M Na citrate)

Make as 20 X stock soln.:

175.3 g NaCl

88.2 g Sodium citrate

800 ml H_2O

adjust pH to 7.0 and bring vol. to 1 liter

APPENDIX IV

SOUTHERN HYBRIDIZATION

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SOUTHERN HYBRIDIZATION

1. Cut DNA with restriction enzymes and run on an agarose gel. (65 v = 5.5 to 6 hrs., 32 v = 11 hrs. for 1.0%)

2. Cut the wells off of the gel and clip the top left corner with a razor blade.

3. Place gel in Denaturation soln. (0.4 M NaOH, 0.6 M NaCl) on shaker at room temp. for 30 min.

4. Soak gel in Neutralization soln. (1.5 M NaCl, 0.5 M Tris, pH 7.5) on shaker at room temperature for 30 min.

5. Cut the nylon to the size of the gel and clip the top left corner. Wet nylon in ddH_2O and then soak in 10 X SSC.

6. Layer three sheets of blotter paper which are cut 3 in. longer than the gel on a platform so that the ends form wicks into 10 X SSC. Invert the gel so that it will lay with the DNA side up, and place it in the middle of the blotter paper.

7. Place the nylon on the gel so that the bottom of the nylon lines up with the bottom of the gel. Carefully remove all bubbles by rolling a glass rod over the layers. Lay three to five layers of blotter paper cut to the size of the gel on top of the nylon. Make sure there are no bubbles. Layer six inches or more paper towels on top. Place a plate over the paper towels and top with a weight such as a 500-1000 ml flask of water. Blot overnight.

8. Remove the paper towels and blotter paper. Mark the DNA-side of the nylon with pencil or permanent ink.

9. Bind the DNA to the nylon by exposing to UV (254 nm) light for 3 min. at a distance of 10 cm.

10. Heat prehybridization soln. at 65 °C for 10-15 min. Add 0.58 g NaCl and heat at 65 °C for 10-15 min longer.

12. Place nylon in a Seal-a-Meal bag and seal the top. Then clip a small hole in the corner and pipet the prehybridization mix into the bag. Remove as much of the air as possible and reseal the corner. Incubate at 65 °C in shaking water bath for 15-30 min.

13. Make probe mixture:
To 500 μl of TE, add 100 μl of salmon sperm DNA (100 μg/ml) and the equivalent of 10^6 counts per min. of each probe. Heat at 95 °C for 10 min.

14. Place the probe mix on ice until used.

15. Using a syringe, inject the probe mix into the bag with the nylon, remove all bubbles, and reseal.

16. Incubate at 65 °C in a shaking water bath overnight.

17. Perform three 5-min. washes of the nylon in ca. 500 ml of 2 X SSC at room temperature with mild agitation.

18. Wash the nylon once in ca. 500 ml of 2 X SSC, 1% SDS in a 65 °C shaking water bath for 30 min.

19. Rinse SDS bubbles off of the nylon with 2 X SSC, blot the nylon with a piece of absorbent paper, and then place in a resealable plastic bag. Do not allow the nylon to dry completely if planning to reprobe.

20. Expose film to the nylon for about 24 hrs. to 10 days (depending on the quantity of mtDNA that was used) using an intensifier at -80°C.

** For alternate washing schemes see Micron Separations Inc. information manual

Probe removal

1. Wash nylon in 0.4 N NaOH at 42 °C for 30 min.

2. Wash for 30 min. in probe removal soln. (0.1 X SSC, 0.1% SDS, 0.2 M Tris-HCl, pH 7.5).

3. Check nylon with Geiger counter to determine if any radioactivity remains. If necessary, repeat steps 1 and 2.

4. Prehybridize and rehybridize nylon as before.

Recipes:

DENATURATION SOLN.

35.1 g NaCl

16.0 g NaOH

1 liter water

NEUTRALIZATION SOLN.

1.45 g Tris

0.82 g sodium acetate

0.11 g EDTA

800 ml water

adjust pH to 7.5 and bring vol. to 1 liter

20 X SSC

175.3 g NaCl

88.2 g Sodium citrate

800 ml water

adjust pH to 7.0 and bring vol. to 1 liter

PREHYBRIDIZATION SOLN. (w/o NaCl)

7 ml water

2 ml 50% dextran sulfate

1 ml 10% SDS

(add 0.58 g NaCl just before use)

TE

6.05 g Tris

3.72 g EDTA

0.58 g NaCl

800 ml water

adjust pH to 8.0 and bring vol. to 1 liter

PROBE REMOVAL SOLN.

5 ml 20 X SSC

10 ml 10% SDS

24.22 g Tris

800 ml H_2O

adjust pH to 7.5 and bring vol. to 1 liter

APPENDIX V

RECOMBINANT PLASMID ISOLATION

APPENDIX V

RECOMBINANT PLASMID ISOLATION

1. Inoculate 5 ml of Luria-Bertani (LB) broth with *E. coli* containing the recombinant plasmid. Incubate overnight on shaker at 37 °C (about 250 rpm).

2. Inoculate 1 liter of LB broth with the overnight culture in a 2800-ml flask. Incubate at 37 °C and 250 rpm in gyrorotary shaker incubator.

3. Add 0.17 g chloramphenicol to the broth to cause plasmid amplification.

4. Incubate overnight on shaker at 37 °C.

5. Collect cells in a GS3 rotor, approximately 500 ml per bottle, 6000 rpm for 6 min. at 4 °C.

6. Resuspend cell pellet in approximately 10 ml of 0.15 M NaCl and transfer to a 45 ml Oak Ridge centrifuge tube. (Steps 5 and 6 may be repeated and combined in one tube).

7. Pellet cells in SA600 rotor at 6000 rpm for 6 min. at 4 °C.

8. Resuspend the pellet in 10 ml of 50 mM Tris, 25% sucrose, pH 8.0. Be sure all clumps are broken up.

9. Add 2 ml freshly made lysozyme (5 mg/ml), cap the tube, and mix by inversion. Leave on ice for 5 min.

10. Add 4 ml 0.25 M Na_2EDTA (pH 8.0). Mix by inversion and leave on ice for 5 min.

11. Add 5 ml of 5 M NaCl and mix quickly by inversion. Avoid violent shaking.

12. Add 2 ml 10% SDS. Mix thoroughly and quickly by inversion. Avoid violent shaking.

13. Store on ice in cold box for 2 hrs.

14. Centrifuge at 16,350 rpm for 60 min. at 4 °C in SA600 rotor.

15. Pour supernatant into a graduated cylinder and add 1 volume of isopropanol. Pour into a flask of appropriate size.

16. Place in ultracold for 20 min. or longer.

17. If frozen, allow contents to melt by placing flask in a tray of tap water.

18. Centrifuge in GSA rotor for 20 min. at 8000 rpm.

19. Resuspend pellet in 8 ml of 10 mM Tris, 1mM EDTA, pH 8.0 (TE). Place in a cold box on a stirrer. Stir slowly to avoid foaming. If large pieces remain after 20 min., break up by repeated pipeting. Some DNAase free RNAase may be added to a final concentration of 20 μg/μl.

20. Remove undissolved material by centrifuging at 10,000 rpm for 10 min. in SA600 rotor.

21. To supernatant, add 5.3 g CsCl per 5 ml TE.

22. Add 400 μl of 10 mg/ml ethidium bromide per 8 ml soln. Further work should be done in subdued light to avoid damaging the DNA.

23. Place solution in a Ti1270 (Ultracrimp^{TM}) tube. Balance the tubes with a CsCl/TE solution or with mineral oil. All tubes should be full to avoid their collapsing during centrifugation.

24. Centrifuge at 36,000 rpm for 40 hrs.

25. Observe the tube under UV (366 nm) illumination. The lower DNA band is the plasmid band. Remove this band with a hypodermic needle.

26. Extract the ethidium bromide with saturated butanol.

27. Dialyze against 1 X TE for 3 days (change the buffer at least 3 times).

APPENDIX VI

CHARACTER STATE MATRIX

APPENDIX VI

Character state matrix generated from restriction site analysis of 14 populations of *Peromyscus*. Approximate position of each restriction site is given relative to a conserved *Bgl*I site. Restriction site characters are designated as present (1), absent (0), or undetermined (?). Taxon codes are as follows: A) *P. truei truei* Arizona and *P. t. comanche* B) *P. truei truei* Utah C) *P. gratus* D) *P. nasutus griseus* E) *P. difficilis saxicola* and *P. d. difficilis* F) *P. attwateri* Briscoe Co. G) *P. attwateri* Palo Pinto Co. H) *P. boylii* New Mexico I) *P. boylii* Arizona J) *P. eremicus* K) *P. leucopus* Kansas L) *P. leucopus* Texas.

		TAXON CODE											
ENZYME	POSITION	A	B	C	D	E	F	G	H	I	J	K	L
*Xho*I	5.4	0	0	0	0	0	0	0	0	0	1	0	0
*Kpn*I	4.0	0	0	0	0	0	0	0	0	0	0	1	1
	5.5	0	0	0	0	1	0	0	0	0	0	0	0
	?	0	0	0	0	0	0	0	0	0	0	0	1
	?	0	0	0	0	0	0	0	0	0	0	0	1
*Pst*I	3.3	1	1	1	1	1	1	1	0	0	1	1	1
	9.5-10.0	1	1	1	1	1	1	1	1	1	1	0	0
	12.7	0	0	0	0	0	0	0	1	1	1	1	1
*Sal*I	7.8	1	1	1	0	1	1	1	0	0	0	0	0

		TAXON CODE											
ENZYME	POSITION	A	B	C	D	E	F	G	H	I	J	K	L
*Pvu*II	2.8	0	0	0	0	0	0	0	0	0	1	1	1
	6.7	1	1	0	0	0	0	0	0	0	1	0	0
	8.0	0	0	0	0	0	0	0	1	0	0	0	0
	8.7	1	1	1	0	0	1	1	1	1	0	0	0
	10.0	1	1	1	1	1	0	0	1	1	1	1	1
*Bgl*I	0.0	1	1	1	1	1	1	1	1	1	1	1	1
	3.6	0	0	0	0	0	0	0	0	0	0	1	0
	11.0	0	0	0	0	0	0	0	0	0	1	0	0
*Eco*RV	1.3	0	0	0	0	1	0	0	1	1	0	0	0
	2.0	0	0	1	0	0	0	0	0	0	0	0	0
	3.0	1	1	1	1	1	1	1	1	1	1	1	1
	7.0-7.5	0	0	0	0	0	0	0	1	1	1	0	0
	8.5 or 9.5	0	0	0	0	0	1	1	0	0	0	0	0
	10.3	0	0	1	0	0	0	0	0	0	0	0	0
	15.7	0	0	0	0	1	1	1	0	0	0	0	0
	16.4	0	0	0	0	0	0	0	0	0	1	0	0
*Bsp*106	0.4	0	0	0	0	0	0	0	0	0	1	0	0
	2.8	1	1	1	0	0	0	0	0	0	0	0	0
	7.5	0	0	0	0	0	0	0	1	1	0	0	0

		TAXON CODE											
ENZYME	POSITION	A	B	C	D	E	F	G	H	I	J	K	L
*Stu*I	1.1	1	0	1	1	1	1	1	0	0	1	0	0
	2.5	0	0	0	1	1	1	1	0	0	0	0	0
	6.3	0	0	0	0	0	0	0	0	0	0	1	1
	7.0	0	1	0	1	1	1	1	0	0	0	0	0
	7.7	0	0	0	0	0	0	0	1	1	0	0	0
	8.3	0	0	0	0	0	0	0	0	0	0	1	1
	9.2	0	0	0	0	0	1	1	1	1	0	0	0
	10.5	1	1	1	1	1	1	1	0	0	0	0	0
	12.0	0	0	0	0	0	0	0	0	0	0	1	1
	3.5 or 6.0	0	0	0	0	1	0	0	0	0	0	0	0
*Bst*EII	3.0	0	0	1	0	0	0	0	1	1	0	0	0
	4.7	0	0	0	0	0	0	0	0	0	0	0	1
	5.5	0	0	0	1	0	1	1	1	1	0	0	0
	7.8	0	0	1	0	0	0	0	1	1	0	0	0
	10.0	1	1	0	0	0	0	0	0	0	0	1	1
	10.5	0	0	1	0	0	0	0	1	1	0	0	0
	14.0	1	1	1	1	1	1	1	1	1	1	1	1
	15.0	1	1	0	0	0	0	0	0	0	1	1	1

		TAXON CODE											
ENZYME	POSITION	A	B	C	D	E	F	G	H	I	J	K	L
*Apa*I	9.5	1	1	0	0	0	0	0	0	0	0	0	0
	11.9	1	1	1	1	1	1	1	1	1	1	1	1
	12.7 or 16.0	0	0	0	1	1	0	0	0	0	0	0	0
	13.0	0	0	0	0	0	0	0	0	0	0	1	1
	16.4	1	1	1	1	1	1	1	1	1	1	1	1
*Bgl*II	2.8	0	0	0	0	0	1	0	0	0	1	0	0
	5.0	0	0	0	0	0	0	0	1	1	0	0	0
	6.0	0	0	1	0	0	0	0	0	0	0	0	0
	6.5	1	1	1	1	1	1	1	1	1	0	1	1
	8.6	1	1	0	1	0	0	0	0	0	0	0	0
	10.5	0	0	0	1	0	1	1	1	1	1	1	1
*Bam*HI	0.6	0	0	0	1	?	1	1	1	1	0	0	0
	1.2	1	1	1	1	?	1	1	1	1	0	1	1
	2.6	0	0	0	1	?	1	1	1	1	0	0	0
	3.1	1	1	0	0	?	0	0	0	0	0	1	0
	6.2	1	1	1	0	?	0	1	0	0	0	0	0
	7.5	0	0	0	0	?	0	0	0	0	0	1	1
	8.0	0	0	0	0	?	0	0	0	0	0	1	1
	13.5	0	0	0	0	?	0	0	0	0	0	1	1
	14.6	0	0	0	0	?	0	0	0	0	0	1	1

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