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*Rbp*3 in Geomyid Rodents: Reduced Rate of Molecular Evolution or Evidence for Selection?

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Abstract

DNA sequences from the interphotoreceptor retinoid binding protein gene (*Rbp*3) in pocket gophers (*Geomys*) display an unusually slow rate of molecular evolution relative to other species of rodents. Rates of molecular evolution were examined in pocket gophers and other members of the rodent superfamily Geomyoidea to determine if this phenomenon was restricted to pocket gophers. DNA sequences from the *Rbp*3, mitochondrial 12S ribosomal RNA (12S rRNA), and mitochondrial cytochrome-*b* (*Cyt*b) genes were compared within members of *Geomys*, among members of the Geomyidae, and among members of the Geomyoidea to ascertain rates of molecular evolution for the three genes among the various taxa. A variety of analyses (genetic distance, Tajima's relative rate test, Tajima's neutrality test, coalescence theory, and Hudson, Kreitman, and Aguadé test) indicated that DNA sequences affiliated with *Rbp*3 in species of *Geomys* were evolving at a rate slower than were sequences of members of the Heteromyidae. In addition, there was weak evidence suggesting that the *Rbp*3 gene in other pocket gopher genera (*Cratogeomys*, *Orthogeomys*, *Pappogeomys*, and *Thomomys*) evolved more slowly than in members of the Heteromyidae.

Key words: geomyoid rodents, *Geomys*, interphotoreceptor retinoid binding protein, molecular evolution, pocket gophers, *Rbp*3

Introduction

Pocket gophers of the genus *Geomys* are fossorial rodents distributed throughout the central plains and southeastern regions of the United States and coastal regions of northeastern Mexico (Russell 1968; Hall 1981; Baker et al. 2003; Patton 2005). Distributions of pocket gophers are affected by availability of suitable soil types (Davis 1940; Baker et al. 2003), and as a result, populations generally contain few individuals and are isolated from other conspecific populations. Pocket gophers also are highly territorial leading to a solitary lifestyle with limited vagility (Williams and Baker 1976; Smolen et al. 1980) and non-overlapping home ranges. In addition, past glacial events in the central plains region are thought to have had a major impact on speciation and distributions of members of *Geomys* (Russell 1968; Hart 1978). Studies of genetic

evolution indicate that pocket gophers (probably as a consequence of the above factors) have small effective population sizes and possess low levels of intrapopulational and intraspecific variation; however, variation among populations and species is high and overall levels of heterozygosity is low (Selander et al. 1975; Penny and Zimmerman 1976; Avise et al. 1979; Zimmerman and Gayden 1981; Ruedi et al. 1997).

Recent studies pertaining to systematic relationships among species in *Geomys* have produced DNA sequence data for two mitochondrial genes (12S ribosomal RNA - 12S rRNA, Jolley et al. 2000; cytochrome-*b* - *Cyt*b, Sudman et al. 2006) and one nuclear gene (interphotoreceptor retinoid binding protein - *Rbp*3, Chambers et al. 2009). Although the goals of these studies were to reconstruct phylogenetic relationships among taxa, Chambers et al. (2009) noted unusually low levels of genetic divergence among species for *Rbp*3 relative to the other two genes. Specifically, Chambers et al. (2009) reported an average between species genetic divergence of 0.60% (0.08%-1.5%) for the *Rbp*3 gene, whereas similar comparisons among the same taxa yielded divergence values of 3.67% (0.6%-8.1%) for 12S rRNA and 13.8% (8.1%-21.0%) for *Cyt*b. Although it is well known that nuclear genes evolve at slower rates than do mitochondrial genes, the low level of genetic divergence associated with *Rbp*3 was unexpected given the higher levels of genetic divergence reported for other rodent taxa (Stanhope et al. 1996; Weksler 2003).

The goals of this study were to determine: 1) whether the low rate of molecular evolution in *Rbp*3, as reported by Chambers et al. (2009), is restricted to *Geomys* - or is it typical for other genera of pocket gophers, and 2) if the rate of molecular evolution in *Rbp*3 is a product of the following scenarios: a) population dynamics, b) age of the geomyid lineage, c) reduction of vision as a product of a fossorial lifestyle, or d) selective forces by examining rates of molecular evolution for genes unrelated to *Rbp*3 (12S rRNA and *Cyt*b). To examine these goals, DNA sequences were obtained for *Rbp*3, 12S rRNA, and *Cyt*b in other genera of pocket gophers (*Cratogeomys, Orthogeomys, Pappogeomys*, and *Thomomys*,) and five genera of the rodent family Heteromyidae (*Chaetodipus, Dipodomys, Heteromys, Liomys*, and *Perognathus*). The Heteromyidae (kangaroo rats and pocket mice) is sister to the Geomyidae and together the two families comprise the superfamily Geomyoidea. In general, the Heteromyidae possess larger population sizes and presumably a greater dependence on vision, and therefore offer an opportunity to examine the four scenarios presented above in taxa that have different demographic and natural history traits than the Geomyidae.

METHODS

Taxonomic sampling.—DNA sequences for *Rbp*3, 12S rRNA, and *Cyt*b were either generated in this study or obtained from GenBank for 29 individuals from the Geomyidae: *Geomys* (21 individuals representing 12 species), *Cratogeomys* (2 individuals representing 2 species), *Orthogeomys* (1 individual), *Pappogeomys* (2 individuals from 1 species), and *Thomomys* (3 individuals representing 3 species) and 13 individuals from the Heteromyidae: *Chaetodipus* (3 individuals representing 3 species), *Dipodomys* (4 individuals representing 4 species), *Heteromys* (1 individual), *Liomys* (2 individuals representing 2 species), and *Perognathus* (4 individuals representing 3 species). Three individuals representing *Castor canadensis* were used for outgroup comparisons. GenBank accession numbers and museum voucher numbers are provided in Table 1.

PCR and sequencing methods.—Twenty unreported *Rbp*3 sequences were obtained in this study. Genomic DNA was isolated from approximately 0.1 g of frozen liver or muscle tissue using the Puregene DNA isolation kit (Gentra, Minneapolis, Minnesota). Approximately 1,230 bp near the 5' end of exon 1 of the single-copy *Rbp*3 gene was amplified by the polymerase chain reaction (PCR, Saiki et al. 1988) using primers A, B, D, D2, E2, F, 125F, G, and I (Stanhope et al. 1992; Jansa and Voss 2000; DeBry and Sagel 2001; Weksler 2003; Chambers et al. 2009). Thermal profiles were adapted from those of Jansa and Weksler (2004): initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 25 sec, annealing at 58°C for 20 sec, and extension at 72°C for 60 sec, and a final extension at 72°C for 10 min.

*Table 1. DNA sequences used in this study were either generated herein or were obtained from GenBank (Jolley et al. 2000; Sudman et al. 2006; Chambers et al. 2009). Taxon names and GenBank accession numbers are provided. Abbreviations are as follows: interphotoreceptor retinoid binding protein (*Rbp*3), mitochondrial 12S ribosomal RNA gene (12S rRNA), and mitochondrial cytochrome-*b *gene (*Cyt*b). Abbreviations for identification numbers are as follows: Abilene Christian University Natural History Collection (ACUNHC); Universidad Autónoma de Mexico (CNMA); Instituto Politécnico Nacional Unidad Durango, México (CRD); Rodney L. Honeycutt (H); James L. Patton (JLP); J. Randall Jackson (JRJ); Louisiana Museum of Natural Science (LSUMZ); Las Vegas Tissue Collection (LVT); Michael J. Smolen (MJS); Moore Laboratory of Zoology, Occidental College (MLZ); Mark S. Hafner (MSH); Museum of Vertebrate Zoology, University of California-Berkeley (MVZ); New Mexico Museum of Natural History (NMMNH); Richard M. Pitts (RMP); Scott B. Block (SBB); Scott K. Davis (SKD); Catalog of Mammalian Tissue Collection (T - Catzeflis 1991); Texas Cooperative Wildlife Collection (TCWC); Natural Science Research Laboratory, Museum of Texas Tech University (TTU); and University of Nebraska State Museum (UNSM). Taxa without available sequences and DNA sequences without museum catalog numbers are designated as N/A.*

Taxon		*Rbp*3	12S rRNA	*Cyt*b
Castoridae	*Castor canadensis*	AF297279 (N/A)	AY012111 (N/A)	AF155878 (N/A)
	C. canadensis	AJ427239 (N/A)	AY787828 (N/A)	AF293348 (N/A)
	C. canadensis	GU985155 (TTU 80729)	U67297 (H 2205)	AY793641 (N/A)
Geomyidae	*Cratogeomys castanops*	EU551778 (TTU 69307)	AF048291 (TTU 69280)	L11902 (N/A)
	C. merriami	GU985156 (LSUMZ 7600)	N/A	L11906 (N/A)
	Geomys arenarius	EU551796 (TTU 69208)	AF084292 (TTU 69209)	AY393935 (LSUMZ 31456)
	G. attwateri	EU551794 (TTU 75223)	AF084293 (TTU 69217)	AY393936 (LSUMZ 29596)
	G. breviceps sagittalis	EU551782 (TTU 69299)	AF084294 (TTU 69297)	FJ210793 (TTU 69299)
	G. b. sagittalis	EU551783 (LSUMZ 30723)	EU551799 (LSUMZ 30723)	AY393940 (LSUMZ 30723)
	G. bursarius major	EU551780 (TTU 69304)	AF084296 (TTU 46117)	AY393944 (LSUMZ 29606)
	G. b. majuscules	EU333407 (TTU 76067)	AF084297 (TTU 76065)	AY393945 (LSUMZ 31448)
	G. knoxjonesi	EU551795 (TTU 69233)	AF084295 (TTU 69300)	AY393947 (SBB 8)
	G. jugossicularis halli	EU333414 (TTU 76073)	AF084298 (TTU 76069)	AY393948 (LSUMZ 31464)
	G. j. jugossicularis	EU551781 (LSUMZ 29284)	EU551800 (LSUMZ 29284)	AY393949 (LSUMZ 29284)
	G. lutescens lutescens	EU333411 (UNSM 20858)	AF084299 (TTU 76082)	AY393950 (LSUMZ 31447)
	G. personatus davisi	EU551791 (JRJ 282)	EU551801 (JRJ 282)	AY393951 (JRJ 282)
	G. p. maritimus	EU551788 (SKD 176)	EU551802 (SKD 176)	AY393952 (SKD 176)
	G. p. megapotamus	EU551786 (TTU 69242)	AF084300 (TTU 69239)	AY393958 (MJS 4940)

Table 1. (cont.)

Taxon		*Rbp*3	12S rRNA	*Cyt*b
	G. p. personatus	EU551787 (TTU 104950)	AF084301 (TCWC 54082)	AY393960 (TCWC 54091)
	G. pinetis mobilensis	EU551789 (LSUMZ 29340)	EU551803 (LSUMZ 29340)	AY393961 (LSUMZ 29340)
	G. p. pinetis	EU551790 (TTU 40793)	AF084303 (TCWC 54095)	AY393963 (LSUMZ 29331)
	G. streckeri	EU551792 (TTU 69295)	EU551804 (TTU 69295)	AY393967 (SKD 47)
	G. streckeri	EU551793 (TTU 69251)	AF084302 (TTU 69244)	AY393968 (MJS 4917)
	G. texensis bakeri	EU551785 (TTU 69254)	AF084304 (TTU 69260)	AY393964 (TTU 69260)
	G. t. texensis	EU551784 (TTU 69277)	AF084306 (RMP 2112)	AY393966 (LSUMZ 29605)
	G. tropicalis	EU551797 (TTU 44886)	AF084307 (TTU 44889)	AY393971 (TTU 44866)
	Orthogeomys hispidus	GU985157 (TTU 44899)	N/A	L38470 (N/A)
	Pappogeomys bulleri	EU551779 (TTU 45109)	EU551798 (TTU 45109)	EU880394 (MSH 1697)
	P. bulleri	GU985158 (LSUMZ 8197)	EF156797 (CNMA 41923)	L11900 (N/A)
	Thomomys bottae	AF297277 (MVZ)	AF084289 (TTU 75871)	AF445064 (TTU 109268)
	T. talpoides	AJ427234 (N/A)	N/A	AF215812 (JLP 11726)
	T. umbrinus	GU985159 (TTU 754459)	AF084290 (TTU 75459)	U65290 (MVZ 153745)
Heteromyidae	*Chaetodipus californicus*	AY303217 (MVZ)	EF156784 (MLZ 1843)	AY009242 (LVT 3682)
	C. hispidus	GU985160 (TTU 109682)	EF156787 (LSUMZ 36375)	AY009247 (LVT 1099)
	C. nelsoni	GU985161 (TTU 97994)	N/A	AY009249 (LVT 1075)
	Dipodomys merriami	GU985162 (TTU 97980)	EF156766 (NMMNH 4548)	AY926363 (LVT 1023)
	D. ordii	GU985163 (TTU 109083)	U59173 (N/A)	AF173501 (TTU 48552)
	D. phillipsii	GU985164 (TTU 75585)	AF084288 (TTU 75585)	AF173500 (CRD 1252)
	D. spectabilis	GU985165 (TTU 38443)	EF156772 (NMMNH 14399)	AF173503 (TTU 37019)
	Heteromys gaumeri	FM200057 (N/A)	AJ389547 (T 348)	AJ389536 (T348)
	Liomys pictus	GU985167 (TTU 104906)	EF156781 (CNMA 41912)	DQ168535 (AK 11725)
	L. irroratus	GU985166 (TTU 82301)	EF156780 (LSUMZ 36295)	DQ168501 (TCWC 42048)

Table 1. (cont.)

Taxon		*Rbp*3	12S rRNA	*Cyt*b
	Perognathus amplus	GU985168 (TTU 41754)	N/A	DQ168552 (ACUNHC 22)
	P. flavus	GU985169 (TTU 54627)	EF156791 (LSUMZ 36254)	AY926495 (LVT 702)
	P. flavus	GU985170 (TTU 78913)	U67298 (TCWC 57416)	DQ168551 (TTU 35363)
	P. merriami	GU985171 (TTU 109081)	EF156793 (NMMNM 4728)	AY926409 (LVT 603)

PCR products were purified using the Exosap-II PCR purification kit (USB Corp., Cleveland, Ohio). Amplified gene products were sequenced on an ABI 3100-Avant using ABI Prism Big Dye v3.1 terminator technology (Applied Biosystems, Foster City, California). Primers used to cycle sequence *Rbp*3 included B, D, E2, F, 125F, Geo395R, Geo609F, Geo958R, Geo1405R, and 1000F, (Stanhope et al. 1992; Jansa and Voss 2000; DeBry and Sagel 2001; Weksler 2003; Chambers et al. 2009). Primers beginning with "Geo" were modified from Stanhope et al. (1992) by altering nucleotides so they matched sequences of *Geomys* more specifically. Cycle sequencing reactions were purified using isopropanol cleanup protocols. Sequences were assembled and proofed using Sequencher 4.9 software (Gene Codes, Ann Arbor, Michigan) and chromatograms were examined to verify all base changes and to inspect sequences for heterozygous sites, which were coded following the International Union of Biochemistry (IUB) polymorphic code. MEGA 4.1 software (Kumar et al. 2007) was used to align and inspect sequences for the presence of stop codons and pseudogenes.

Data Analyses.—To examine rates of molecular evolution in the three genes examined in this study (*Rbp*3 - 1,230 bp, 12S rRNA - 870 bp, and *Cyt*b - 1,140 bp), five methods were implemented for data analysis. First, neighbor-joining trees (Saitou and Nei 1987) were generated independently using DNA sequences from each of the three genes so that taxonomic relationships and corresponding branch lengths (indicating rates of molecular evolution) could be compared among genes. The neighbor joining analyses used uncorrected-P genetic distances obtained using the MEGA 4.1 software (Kumar et al. 2007) for each of three respective genes. The uncorrected-P distance was selected to avoid interjecting "rules of molecular evolution" on the DNA sequences as incorporated by the various substitution models commonly used in calculating genetic distances. This choice was crucial so that rates of molecular evolution could be compared as evenly as possible among nuclear and mitochondrial genes. Average uncorrected-P distances were estimated for individuals within each genus and between genera and used to estimate levels of genetic divergence between various taxonomic groups.

Second, Tajima's relative rate test (Tajima 1993) using MEGA 4.1 software (Kumar et al. 2007) was used to ascertain if rates of molecular evolution differed significantly among taxa and among genes. Specifically, this test was implemented to determine if *Rbp*3 sequences in *Geomys*, and geomyids in general, were evolving at rates different (i.e., evidence for rate heterogeneity) than those of heteromyids relative to DNA sequences from 12S rRNA and *Cyt*b. Pairwise comparisons of DNA sequences from each of the three genes were made between species within *Geomys*, between species of *Geomys* and other pocket gophers, and between geomyids and members of the Heteromyidae.

Third, Tajima's neutrality test (D-statistic, Tajima 1989) using MEGA 4.1 software (Kumar et al. 2007) was implemented to determine if DNA sequences were evolving under a neutral model of evolution (Kimura 1983) or under non-random models normally associated with selective forces (directional selection, balancing selection, demographic expansion or contraction, genetic hitchhiking, etc.). Specifically, the neutral model of evolution would be operative, and would remain a viable hypothesis, if rates of molecular evolution at the three loci were not significantly different among *Geomys* and other members of the Geomyoidea.

Fourth, the Hudson, Kreitman, and Aguadé test (HKA test, Hudson et al. 1987) was used to determine if the *Rbp*3 was behaving in a neutral fashion relative to 12S rRNA and *Cyt*b. The HKA test estimates theta (θ) from the following equation, $\theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate. Theta is estimated for each locus based on comparing the intrapopulational genetic variability for one taxon with the interpopulational genetic variability between that taxon and a second. The DnaSP software program (version 5.10.01, Librado and Rozas 2009) was used to estimate theta values at each locus (*Rpb*3,12S rRNA, and *Cyt*b) for six genera of geomyoid rodents (*Chaetodipus, Cratogeomys, Dipodomys, Geomys, Perognathus*, and *Thomomys*) and one outgroup taxon (*Castor*). A chi-square test ($P < 0.05$) was used to identify significant differences among pairwise comparisons of the three loci, with one locus representing observed values and the second locus representing expected values. Significantly different θ values indicated a deviation from neutrality (i.e. selection), with positive selection inferred if μs for each locus were equal and the N_e was unequal and purifying selection inferred if μs for each locus were unequal and the N_e was equal. In other words, under a model of neutrality, (Kimura 1983; Hudson et al. 1987) all loci are expected to possess equal μs if all taxa have the same the N_e; however if taxa have unequal N_{es}, then positive selection acts upon individual loci producing an excess of polymorphisms between species, conversely, if taxa possess unequal μs, then purifying selection generates an excess of polymorphisms within a species.

Fifth, coalescence theory was used to estimate the time of divergence from a hypothetical common ancestor based on DNA sequences from the three genes. If *Rbp*3 sequences coalesce at times similar to those obtained for 12S rRNA and *Cyt*b, then the hypothesis of a slower rate of *Rbp*3 evolution in *Geomys* could be rejected. The software program BEAST v1.5.3 (Drummond and Rambaut 2007) was used to analyze the coalescence process among each gene. All taxa were grouped into all possible taxon sets (e.g., Castorimorpha, Geomyoidea, Geomyidae, Geomyini, *Geomys*, etc.). Two fossil calibrations of ancestral taxa (Castorimorpha - 54.4 MYA, McKenna 1960; Geomyoidea - 45.45 MYA, Walsh 1991) were used as priors on the tree. A normal distribution was used for all point fossil calibrations with standard deviations based on dates from the International Commission on Stratigraphy (Gradstein et al. 2004; Ogg et al. 2008). A relaxed, uncorrelated lognormal clock was used with a GTR + I + G model of substitution based on MrModeltest 2.3 (Nylander 2004) and the Akaike information criterion (Nylander 2004) for each gene. In addition, a Yule species prior was used to date nodes within each gene tree. Each dataset was analyzed twice for 10,000,000 generations (with a 10% burn-in) to obtain an appropriate effective sample size. The log files were combined using LogCombiner v1.5.2 (Drummond and Rambaut 2007) and analyzed for convergence in Tracer v1.4.1 (Rambaut and Drummond 2007). A one-way analysis of variance (ANOVA, $P < 0.05$) was used to compare the mean rates of substitution to determine whether genes were evolving at different rates.

RESULTS

Taxonomic relationships and genetic divergence.—Genetic divergence values, based on uncorrected-P distances, were estimated for individuals within each genus and between genera for the three respective genes (Table 2). Within genera values ranged from 0.66% for individuals within *Geomys* to 5.98% within *Liomys* for *Rbp*3, from 2.38% for individuals within *Perognathus* to 12.02% in *Chaetodipus* for 12S rRNA, and from 11.93% for individuals within *Geomys* to 18.67% in *Cratogeomys* for *Cyt*b. In addition, these values were used to construct a neighbor-joining tree for each of the three genes (Fig. 1). Topologies recovered in the three analyses were similar, although placement of some heteromyid genera differed depending on which gene was analyzed. However, branch lengths, reflecting the number of substitutions per site, were different between genes and between taxa in each tree. For example, in all analyses, branch lengths for individual species of *Geomys* were substantially shorter than for other taxa.

Rate heterogeneity.—Tajima's relative rate test (Tajima 1993) depicted specific taxa that exhibited differential rates of molecular evolution relative to other members of the Geomyoidea based on comparisons within each of the three genes (Table 3). In most intrageneric comparisons, the 12sRNA gene accounted for a greater number of significantly different rates ($P < 0.05$) than the other two genes. However, in comparisons involving members of *Geomys* versus heteromyids and geomyids versus heteromyids, *Rbp*3 depicted a greater number of significantly different rates (Table 3).

Neutral model of molecular evolution.—DNA sequences obtained from the three genes were tested independently for departure from the model of neutrality using Tajima's neutrality test (Tajima 1989) and the HKA test (Hudson et al. 1987). Tajima's neutrality test provided evidence of positive selection or a previous history of having been subjected to a population bottleneck in four instances (Table 4). Two cases

*Table 2. Average genetic distances (uncorrected-P distances) were estimated for each of the three genes examined in this study. Values were estimated by averaging genetic distances for comparisons of selected taxa. Those with a single sequence prohibited the calculation of an average distance and are indicated by N/A. Abbreviations are as follows: interphotoreceptor retinoid binding protein gene (*Rbp*3), mitochondrial 12S ribosomal RNA (12S rRNA), and mitochondrial cytochrome-*b *(*Cyt*b).*

Taxon	*Rbp*3	12S rRNA	*Cyt*b
Within *Geomys*	0.00661	0.03568	0.11926
Within *Cratogeomys*	0.1771	N/A	0.18670
Within *Pappogeomys*	N/A	N/A	N/A
Within *Thomomys*	0.02520	0.07732	0.17970
Within *Chaetodipus*	0.01749	0.12022	0.15510
Within *Dipodomys*	0.01981	0.11107	0.15341
Within *Liomys*	0.05976	0.05472	0.15263
Within *Perognathus*	0.01439	0.02375	0.16910
Within Geomyidae	0.02193	0.06869	0.15867
Within Heteromyidae	0.08326	0.16319	0.21493
Within Geomyoidea	0.07862	0.14693	0.20546

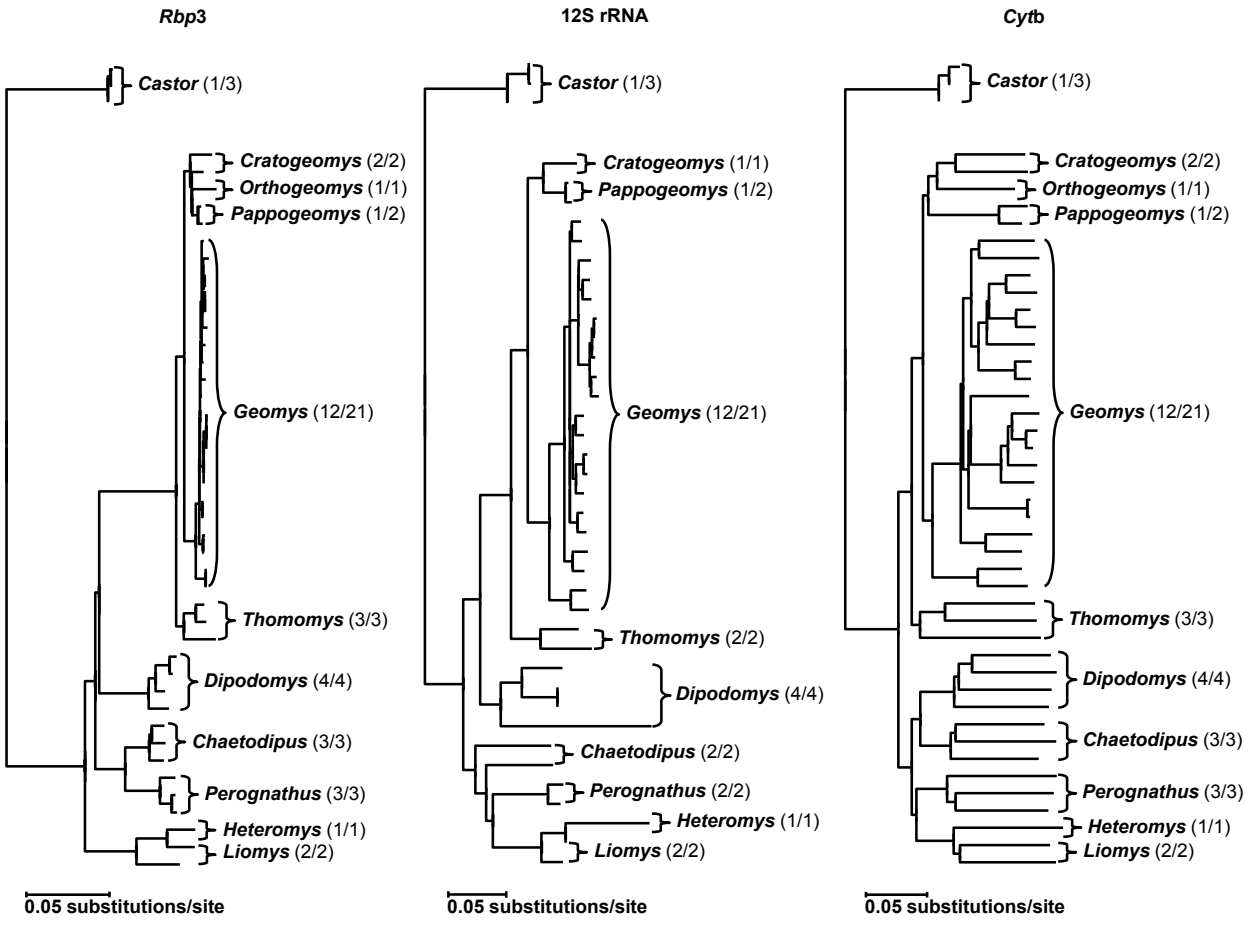

Figure 1. Neighbor joining trees obtained from uncorrected-P genetic distances estimated from DNA sequences obtained from the *Rbp*3, 12S rRNA, and *Cyt*b genes. Only genera are labeled and numbers in parentheses following each genus represent: number of species included per genus (left of slash), and number of DNA sequences included per genus (right of slash).

*Table 3. Number of significant differences (*P < *0.05) in pair-wise comparisons based on Tajima's relative rate test (Tajima 1993). Numbers to left of the slash represent the number of significant comparisons and numbers to right of the slash indicate the number of comparisons attempted. Abbreviations are as follows: interphotoreceptor retinoid binding protein gene (*Rbp*3), mitochondrial 12S ribosomal RNA (12S rRNA), and mitochondrial cytochrome-*b *(*Cyt*b).*

Taxon	*Rbp*3	12S rRNA	*Cyt*b
Within *Geomys*	5/66	9/66	0/66
Within *Cratogeomys*	0/1	0/0	0/1
Within *Pappogeomys*	0/0	0/0	0/0
Within *Thomomys*	0/3	0/1	0/3
Within *Chaetodipus*	0/3	0/1	0/3
Within *Dipodomys*	0/6	3/6	1/6
Within *Liomys*	1/1	0/1	0/1
Within *Perognathus*	0/3	0/1	1/3
Geomys to Other Geomyids	0/84	1/48	3/84
Within Geomyids	4/171	10/120	7/171
Within Heteromyids	3/78	10/55	7/78
Geomys to Heteromyids	81/156	20/132	15/156
Geomyids to Heteromyids	124/247	27/176	25/247

*Table 4. Results from Tajima's neutrality test (Tajima 1993) for each of the three genes examined. The outcome of Tajima's neutrality test is based on Tajima's D statistic. Taxa with sample sizes of ≤ 3 gave inconclusive results and were not included. Abbreviations are as follows: interphotoreceptor retinoid binding protein gene (*Rbp*3), mitochondrial 12S ribosomal RNA (12S rRNA), mitochondrial cytochrome-*b *(*Cyt*b), population bottleneck (PB), positive selection (PS), and balancing selection (BS).*

Taxon	Rbp3	12S rRNA	Cytb
Within *Geomys*	PB or PS (90% CI)	PB or PS (<90% CI)	BS
Within *Dipodomys*	BS	BS	BS
Within Geomyids	PB or PS (<90% CI)	PB or PS (<90% CI)	BS
Within Heteromyids	BS	BS	BS
Within Castorimorphs	BS	BS	BS

involved comparisons of taxa within *Geomys* (*Rbp*3 and 12S rRNA) and two cases involved comparisons of taxa within the Geomyidae (*Rbp*3 and 12S rRNA). Based on this test, heteromyid taxa (generic or family level) and *Cyt*b sequences from all taxa appear to be evolving at neutral rates in all comparisons. In addition, the HKA test (Hudson et al. 1987) indicated that θ values between *Rbp*3 and *Cyt*b were significantly different ($P = 0.0016$). The HKA test did not detect any other significant differences in 24 additional pairwise comparisons of genera and loci, which suggests that purifying selection was responsible for a slower rate of molecular evolution at *Rbp*3 in *Geomys* but that the remaining sequences were evolving at a neutral rate.

Coalescence theory.—The mean rates of evolution (substitutions per site per million years) were 0.0023, 0.0067, and 0.0138 for *Rbp*3, 12S rRNA, and *Cyt*b, respectively. The coefficient of variance for *Rbp*3 and 12S rRNA were high (0.4847, 0.6783) but low for *Cyt*b (0.0872). A one-way ANOVA ($F = 2.1832 \times 1012$, $P \approx 0.0000$) rejected the null hypothesis of equal rates among the three datasets, indicating independent rates of evolution for each gene. In addition, trees obtained from each of the three genes used in the BEAST analysis depicted more recent divergence times for species of *Geomys* based on *Rbp*3 than for the other two genes (Fig. 2).

DISCUSSION

The observation (Chambers et al. 2009) that *Rbp*3 sequences obtained from several species of *Geomys* were evolving at rates slower than sequences obtained from other genes for the same taxa was re-examined using genetic distances (uncorrected-P), relative rate test (Tajima 1993), neutrality tests (Tajima's D statistic, Tajima 1989; HKA test, Hudson et al. 1987), and coalescence theory (BEAST, Drummond and Rambaut 2007). All analyses, whether visual (comparison of genetic distances) or statistically supported (Tajima's relative rate test, Tajima's test of neutrality, HKA test, or coalescence theory) indicated that species of *Geomys* were evolving at a rate slower compared to members of the Heteromyidae. Also, other pocket gopher genera (*Cratogeomys, Orthogeomys, Pappogeomys*, and *Thomomys*) appeared to evolve more slowly than their heteromyid counterparts, although low sample sizes prevented meaningful statistical analyses in some cases.

Although the various analyses performed in this study revealed differences in the molecular evolution of *Rbp*3 in geomyids and heteromyids, with geomyids consistently possessing a slower rate of evolution, it was not clear from a molecular standpoint why geomyids possessed a slower rate. To further investigate this phenomenon, we determined the number of variable sites per codon position (1st, 2nd, and 3rd) for DNA sequences obtained from the two protein-coding genes (*Rbp*3 and *Cyt*b); the 12S rRNA gene was not included as it is not a protein-coding locus. The average number of variable sites (by position) was determined at the generic and familial levels for geomyids and heteromyids (Table 5). A chi-square test was used to detect differences in the observed number of variable sites (represented by the number of changes per position in *Rbp*3) versus the expected number of variable sites (represented by the number of changes per position in *Cyt*b). *Cyt*b was selected as the "expected" value to approximate a neutral rate. Significant differences ($P < 0.05$) were detected among taxa for *Rbp*3 relative to *Cyt*b, with the genera of geomyids possessing a significantly lower number of substitutions, in the 1st and 3rd positions relative to the other taxa (Table 5).

At least four scenarios are possible for explaining the low level of genetic variation in the *Rbp*3 gene in *Geomys* and for pocket gophers in general. First, the product of being fossorial has resulted in pocket gophers being distributed in small isolated populations, susceptible to inbreeding, and generally characterized by low levels of heterozygosity, etc. Also, it is well known that glacial periods had a major impact on the distribution and speciation of *Geomys* (Blair 1954; Russell 1968; Penney and Zimmerman 1976; Heaney and Timm 1983; Mauk et al. 1999) by producing population bottlenecks during glacial maxima. These events may have acted to homogenize or constrain evolution of the *Geomys* genome. However, these arguments seem unlikely given that levels of genetic variation reported

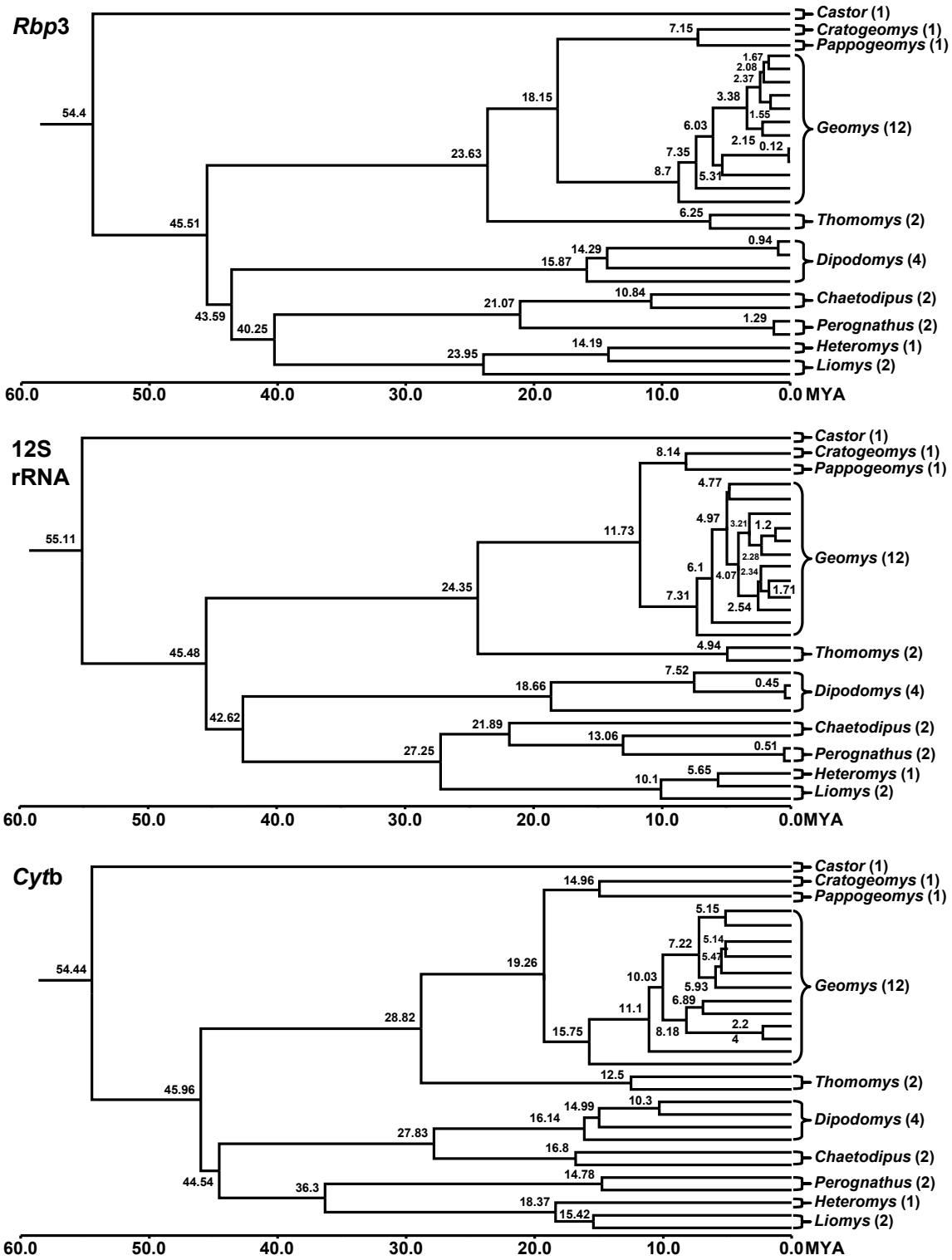

Figure 2. Coalescence trees were generated using the BEAST analysis (Drummond and Rambaut 2007) and DNA sequences obtained from the *Rbp*3 (top), 12S rRNA (middle), and *Cyt*b (bottom) genes. The GTR + I + G model of substitution and two combined runs of 10,000,000 generations (with a 10% burn-in) were used for tree construction. Two fossil calibrations of ancestral taxa (Castorimorpha - 54.4 MYA, McKenna 1960; Geomyoidea - 45.45 MYA, Walsh 1991) were used as priors on the tree. Numbers at nodes reflect approximate coalescence times.

*Table 5. Number of variable nucleotide sites per codon position for the protein-coding genes, interphotoreceptor retinoid binding protein gene (*Rbp*3) and mitochondrial cytochrome-b (*Cyt*b), respectively. For each taxonomic group and associated genes, the following is depicted: average number of bases examined (ANBE), total number of changes per gene, number of changes at the 1st position, number of changes at the 2nd position, number of changes at the 3rd position, and statistical significance (SIGN). A chi-square test (*$P < 0.05$*) was used to determine if the number of nucleotide changes (by position) in the* Rbp*3 gene (treated as observed data) was significantly different relative to changes per position in the* Cyt*b gene (treated as expected data).*

	*Rbp*3					*Cyt*b					
		Changes per Position					Changes per Position				
Taxon	ANBE	Total	1st	2nd	3rd	ANBE	Total	1st	2nd	3rd	SIGN
Within *Geomys*	1,222.6	37	5	5	27	1,124.3	385	68	16	301	Yes
Within *Cratogeomys*	1,208.0	21	4	6	11	1,140.0	133	31	4	98	Yes
Within *Thomomys*	1,222.0	46	4	6	36	1,123.3	288	51	15	222	Yes
Within *Chaetodipus*	1,128.7	30	5	2	23	449.7	101	15	4	82	No
Within *Dipodomys*	1,117.0	40	7	4	29	1,139.8	291	47	8	236	No
Within *Liomys*	1,188.5	71	16	4	51	1,140.0	174	25	3	146	No
Within *Perognathus*	1,140.0	25	2	2	21	1,138.7	271	50	1	220	Yes
Within Geomyidae	1,210.6	134	20	19	95	1,088.6	491	112	32	347	Yes
Within Heteromyidae	1,144.5	273	47	23	203	980.3	504	110	40	354	No
Within Geomyoidea	1,190.4	358	73	41	245	1,044.6	587	151	63	373	Yes

for 12S rRNA and *Cyt*b (Jolley et al. 2000; Sudman et al. 2006; Chambers et al. 2009) are similar to genetic divergences obtained through comparisons with other species of rodents. It is possible that genetic drift "targeted" the *Rbp*3 gene but did not reduce genetic variation in 12S rRNA and *Cyt*b; however, this hypothesis should be further examined as the results of Tajima's neutrality test indicated that population bottleneck and positive selection were both viable explanations for the reduction in molecular evolution of *Rbp*3 in *Geomys* and other geomyids.

Second, contemporary species of *Geomys* may have diverged recently and, consequently, should possess low levels of genetic variation at *Rbp*3. However, several lines of data oppose this hypothesis. For example, fossil evidence (Russell 1968) places the origin of modern species of *Geomys* to be at least 5-7 million years ago (MYA). In addition, Jolley et al. (2000) used rates of molecular divergence estimated from 12S rRNA sequences to hypothesize that extant species of *Geomys* diverged between 2.5 and 5.7 MYA and a similar value (2.5-7 MYA) is obtained if DNA sequences from *Cyt*b (Sudman et al. 2006; Chambers et al. 2009) are used with a molecular divergence rate of approximately 3% per million years. Although coalescence times obtained herein (Fig. 2) for *Cyt*b (15.75 MYA) are greater than those reported by Sudman et al. (2006) and Chambers et al. (2009), coalescence times for *Rbp*3 and 12S rRNA (8.7 MYA and 7.31 MYA, respectively) are comparable to fossil estimates and previous molecular hypotheses. Consequently, a recent divergence time for *Geomys* and concomitant reduction in genetic divergence for *Rbp*3 seems unlikely.

Third, *Rbp*3 encodes a large glycolipoprotein in the interphotoreceptor matrix and is thought to play a role in retinoid transport between retinal photoreceptors and pigment epithelial cells (Borst et al. 1989). It is possible, during evolution of the fossorial lifestyle characteristic of *Geomys* and other species of pocket gophers, that molecular evolution in *Rbp*3 was somehow constrained as a possible consequence of a reduced emphasis on vision as a result of their fossorial lifestyle. Similar observations have been reported in studies of other fossorial genera of mammals, including *Ctenomys* (Borghi et al. 2002) and *Notoryctes* (Springer et al. (1997). However, Feldman and Phillips (1984) concluded that *Geomys* possess a similar retinal pigment epithelium to that observed in other diurnal species (e.g. tree squirrels, ground squirrels, and voles) and actually may have limited visual acuity under low light conditions. We tested this hypothesis by comparing geomyids to heteromyids, and based on data presented herein, we cannot reject a connection between fossoriality and the reduction of molecular evolution in *Rbp*3.

Fourth, selective forces may be acting to reduce or constrain genetic variation at *Rbp*3. Results from Tajima's neutrality test (Tajima 1989), HKA test (Hudson et al. 1987), and BEAST (Drummond and Rambaut 2007) rejected a neutral model of evolution for *Rbp*3 in *Geomys* and geomyids in some analyses. In addition, Tajima's neutrality test and the HKA test also indicated that positive selection was a possible explanation for a slower rate of molecular evolution in *Geomys*, although the mechanisms were not clear.

At this time, there are insufficient data to determine if the reduction of genetic variability in *Rbp*3 in geomyid rodents is a product of fossoriality (small population size, bottlenecks, reduction in development of the geomyid eye, etc.), population dynamics, age of the geomyid lineage, or selective forces (positive selection). Support for positive selection was identified in some analyses, although interpretations of these results were not unambiguous. Further tests of other fossorial mammals (moles, mole rats, ctenomyids, etc.) are needed before broader conclusions can be made.

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