

Genes and Ecology: Accelerated Rates of Replacement Substitutions in the Cytochrome b Gene of Subterranean Rodents

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Abstract: South American tuco-tucos (genus *Ctenomys*) and related coruros (genus *Spalacopus*), North American pocket gophers (family Geomyidae), and African mole rats (family Bathyergidae) are lineages of rodents that colonized the subterranean niche independently. An energetically demanding lifestyle, coupled with the hypoxic atmosphere characteristic of the subterranean environment may change the selective regime of genes that encode proteins involved in cellular respiration. Here, we examined the molecular evolution of the cytochrome b gene, a mitochondrially-encoded gene participating in oxidative phosphorylation, in these lineages and their above-ground relatives. Using maximum-likelihood and Bayesian approaches, we estimated rates of synonymous (dS) and nonsynonymous (dN) substitutions. We found a significantly higher ω ratio (dN/dS) in each of the subterranean groups with respect to their non-subterranean counterparts. Using an alternative procedure that tests for positive selection on quantitative physicochemical amino acid properties, we found that i) subterranean mole rats and tuco-tucos showed more sites whose amino acid properties may be under positive selection in the cytochrome b gene than their non-subterranean relatives, and ii) some of the sites identified to be under selection exclusively in subterranean taxa were shared among all subterranean taxa. The results given by these two approaches are consistent with each other and suggest a link between directional selection at the molecular level and niche shift.

INTRODUCTION

Understanding evolutionary phenomena at the molecular level presents one of the outstanding challenges in biology. The most widely used approach to investigate the potential existence of positive selection at the molecular level among species is to compare the ratio of synonymous (dS) and nonsynonymous substitutions (dN) changes. The ratio dN/dS > 1 (known as ω) indicates positive directional selection [1-8], although this criterium might be too stringent [6, 9, 10], and it probably fails to uncover many events of directional selection [11-14]. Growing interest in this field has led to the development of alternative procedures attempting to overcome the previously described difficulties. One of these evaluates protein-coding nucleotide sequences using well corroborated phylogenetic trees to test for positive selection on quantitative physicochemical amino acid properties [15].

Cytochrome b gene (cyt b) is a key component of bc1, one of the protein complexes involved in oxidative phosphorylation in the mitochondrial membrane whose role in oxidative phosphorylation is well understood [16]. Given that the cellular generation of ATP is a crucial metabolic process, this gene is in general conserved across taxa at the amino acid level. However, it is in principle conceivable that shifts in the ecology of organisms that imply changes in the metabolic demand may be associated with changes in the selection pressure of the proteins that participate in the biochemical pathways of cellular respiration. Supporting that idea, Andrews *et al.* [1] and Adkins and Honeycutt [17]

found that the rate of evolution of the cytochrome b, and cytochrome c oxidase subunit II, respectively, was greater in simians than in non-simian mammals, suggesting an event of concerted evolution. Beside, Nevo *et al.* [18] correlated sequence variation of a portion of the cyt b gene with ecological differences among chromosomal races of blind mole-rats (*Spalax ehrenbergi*), although in that case, the direct link with respiratory function is not clear. More recently, Grossman *et al.* [19] reviewed these and new results and suggested the co-adaptation among nuclear and mitochondrial subunits of the electron transport chain proteins during primate evolution driven by the metabolic demands associated with an expanding cortex. Additionally, McClellan *et al.* [20] found shifts in both the amino acid residue loci and physicochemical properties influenced by positive selection in the cetacean cyt b protein relative to that of artiodactyls. Furthermore, da Fonseca *et al.* [21] found a wide variation in the properties of amino acids at functionally important regions of cytochrome b in species with more specialized metabolic requirements (such as adaptation to unusual oxygen requirements, for example diving in cetaceans, flying in bats, and living at high altitudes in alpacas).

Fossorial rodents constitute an ideal study system to test hypotheses about adaptive evolution driven by important ecological shifts. They live in a subterranean environment characterized by high levels of carbon dioxide, low levels of oxygen and relatively constant temperature and humidity [22]. Given the drastic change in energy requirements [23] and habitat associated with the colonization of the subterranean niche, especially the transition from oxygen-rich to hypoxic atmosphere [24, 25], it is likely that selective regimes of proteins involved in respiration may have changed. Some of these proteins could have experienced positive directional

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Table 1. List of Specimens Used in the Study with their GenBank Accession Numbers. Non-Subterranean Taxa are in Grey

Taxon	Species	Accession Number	Taxon	Species	Accession Number
South American Tuco-tucos, Coruro, and relatives			North American pocket gophers and relatives		
Tuco-tucos (Ctenomyidae)	<i>Ctenomys talarum</i>	AF370699	Pocket gophers (Geomyidae)	<i>Thomomys bottae</i>	U65263
	<i>Ctenomys rionegrenses</i>	AF119114		<i>Thomomys townsendii</i>	U65282
	<i>Ctenomys mendocinus</i>	AF007062		<i>Thomomys umbrinus</i>	U65288
	<i>Ctenomys australis</i>	AF370697		<i>Thomomys bulbivorus</i>	AF155867
	<i>Ctenomys pearsoni</i>	AF119108		<i>Thomomys talpoides</i>	U65291
	<i>Ctenomys maulinus</i>	AF370702		<i>Thomomys monticola</i>	U65292
	<i>Ctenomys tuconax</i>	AF370684		<i>Thomomys mazama</i>	AF215806
	<i>Ctenomys magellanicus</i>	AF370690		<i>Orthogeomys heterodus</i>	U65300
	<i>Ctenomys coyhaiquensis</i>	AF119112		<i>Papogeomys bulleri</i>	L11900
	<i>Ctenomys haigi</i>	AF007063		<i>Cartogeomys tylosinus</i>	AF302183
	<i>Ctenomys tucumanus</i>	AF370691		<i>Cartogeomys gymnurus</i>	AF302179
	<i>Ctenomys argentinus</i>	AF370680		<i>Cartogeomys neglectus</i>	AF302174
	<i>Ctenomys latro</i>	AF370704		<i>Cartogeomys castanops</i>	AF302171
	<i>Ctenomys leucodon</i>	AF007056		<i>Cartogeomys zinseri</i>	AF302170
	<i>Ctenomys sp. "ITA"</i>	AF007047		<i>Cartogeomys fumosus</i>	AF302165
	<i>Ctenomys sp. "MINUT"</i>	AF007053		<i>Cartogeomys goldman</i>	AF302176
	<i>Ctenomys sp. "MONTE"</i>	AF007057		<i>Cartogeomys merriami</i>	AF302160
	<i>Ctenomys steinbachi</i>	AF007044		<i>Geomys pinetis</i>	AF158698
	<i>Ctenomys goodfellowi</i>	AF007051		<i>Geomys bursarius</i>	AF158697
	<i>Ctenomys boliviensis</i>	AF007037		<i>Geomys lutescens</i>	AY393950
<i>Ctenomys lewisi</i>	AF007049	<i>Geomys jugossicularis</i>	AY393949		
<i>Ctenomys frater</i>	AF007046	<i>Geomys texensis</i>	AY393965		
<i>Ctenomys conoveri</i>	AF007055	<i>Geomys knoxjonesi</i>	AY393947		
<i>Ctenomys fulvus</i>	AF370687	<i>Geomys arenarius</i>	AY393935		
<i>Ctenomys opimus</i>	AF007042	<i>Geomys tropicalis</i>	AY393970		
Octodontidae	<i>Spalacopus cyanus</i>	AF007061	<i>Geomys personatus</i>	AY393959	
	<i>Octodon degus</i>	AF007059	<i>Geomys streckeri</i>	AY393968	
	<i>Octodontomys gliroides</i>	AF370706	<i>Geomys atwateri</i>	AY393938	
	<i>Tympanoctomys barrerae</i>	AF007060	<i>Geomys breviceps</i>	AF158689	
Spiny rats (Echimyidae) Outgroup	<i>Makalata didelphoides</i>	L23363	Heteromyidae	<i>Perognathus longuimembris</i>	U65302
	<i>Mesomys hispidus</i>	L23385		<i>Perognathus amplus</i>	U65301
	<i>Proechimys simonsi</i>	U35414		<i>Perognathus apache</i>	AY926412
	<i>Euryzygomatomys spinosus</i>	U34858		<i>Perognathus flavescens</i>	AY926411
<i>Trionomys paratus</i>	U35165	<i>Perognathus alticola</i>		AY926413	
African mole rats and relatives				<i>Perognathus parvus</i>	AY926407
Mole rats (Bathyergidae)	<i>Heterocephalus glaber</i>	AF155870		<i>Dipodomys agilis</i>	U65303
	<i>Bathyergus suillus</i>	AF012242		<i>Dipodomys elephantinus</i>	AY926374
	<i>Bathyergus janetta</i>	AF012241		<i>Dipodomys californicus</i>	AY926368
	<i>Georchus capensis</i>	AF012243		<i>Dipodomys elator</i>	AY926376
	<i>Cryptomys hottentotus</i>	AF012239		<i>Dipodomys margaritae</i>	AY926370
	<i>Cryptomys choma</i>	AF012234		<i>Dipodomys deserti</i>	AY926381
	<i>Cryptomys amatus</i>	AF012233		<i>Microdipodops megacephalus</i>	AF172833
	<i>Cryptomys damarensis</i>	AF012223		<i>Microdipodops pallidus</i>	AY926361
	<i>Cryptomys darlingi</i>	AF012232		<i>Chaetodipus hispidus</i>	AF172832
	<i>Cryptomys mechowii</i>	AF012230		<i>Chaetodipus rudinoris</i>	AY926397
	<i>Cryptomys bocagei</i>	AF012229		<i>Chaetodipus fallax</i>	AY926402
	<i>Heliophobius argenteocinereus</i>	U87527		<i>Chaetodipus spinatus</i>	AY926398
	Outgroup	<i>Thryonomys swinderianus</i>		AJ301644	<i>Chaetodipus pernix</i>
<i>Hystrix africae australis</i>		X70674		<i>Chaetodipus penicillatus</i>	AY926388
			<i>Chaetodipus intermedius</i>	AY926389	
			<i>Heteromys anomalus</i>	DQ168468	
			<i>Heteromys desmarestianus</i>	DQ168467	
			<i>Liomys spectabilis</i>	DQ168550	
			<i>Liomys pictus</i>	DQ168535	
			<i>Liomys irroratus</i>	DQ168501	
			<i>Pedetes capensis</i>	AJ389527	
			<i>Castor fiber</i>	DQ088706	

selection in response to the new habitat requirements. Under this hypothesis, accelerated rates of replacement substitution are expected in subterranean organisms with respect to their non-subterranean sister taxa. On the other hand, effective population size may be smaller in subterranean rodents than in their above-ground relatives [26]. In small populations, however, accelerated rates of replacement substitution also occur if purifying selection against nearly neutral variation was relaxed. A key distinction between the two hypotheses is that we expect to find individual amino acids with ω higher than one in the former but not in the latter.

Here, we characterize the pattern of molecular evolution of the mitochondrial cyt b gene in independent lineages of subterranean rodents, and compare them with their non-subterranean counterparts. Our goal was to test the hypothesis that there is an acceleration of evolutionary rates of amino acid replacement (relative to synonymous changes) of the cyt b gene, in response to a major change in the selection pressure associated with the colonization of the subterranean niche. The four rodent groups of interest are: South American coruro (*Spalacopus cyanus*) and tuco-tucos (genus *Ctenomys*), North American pocket gophers (family Geomy-

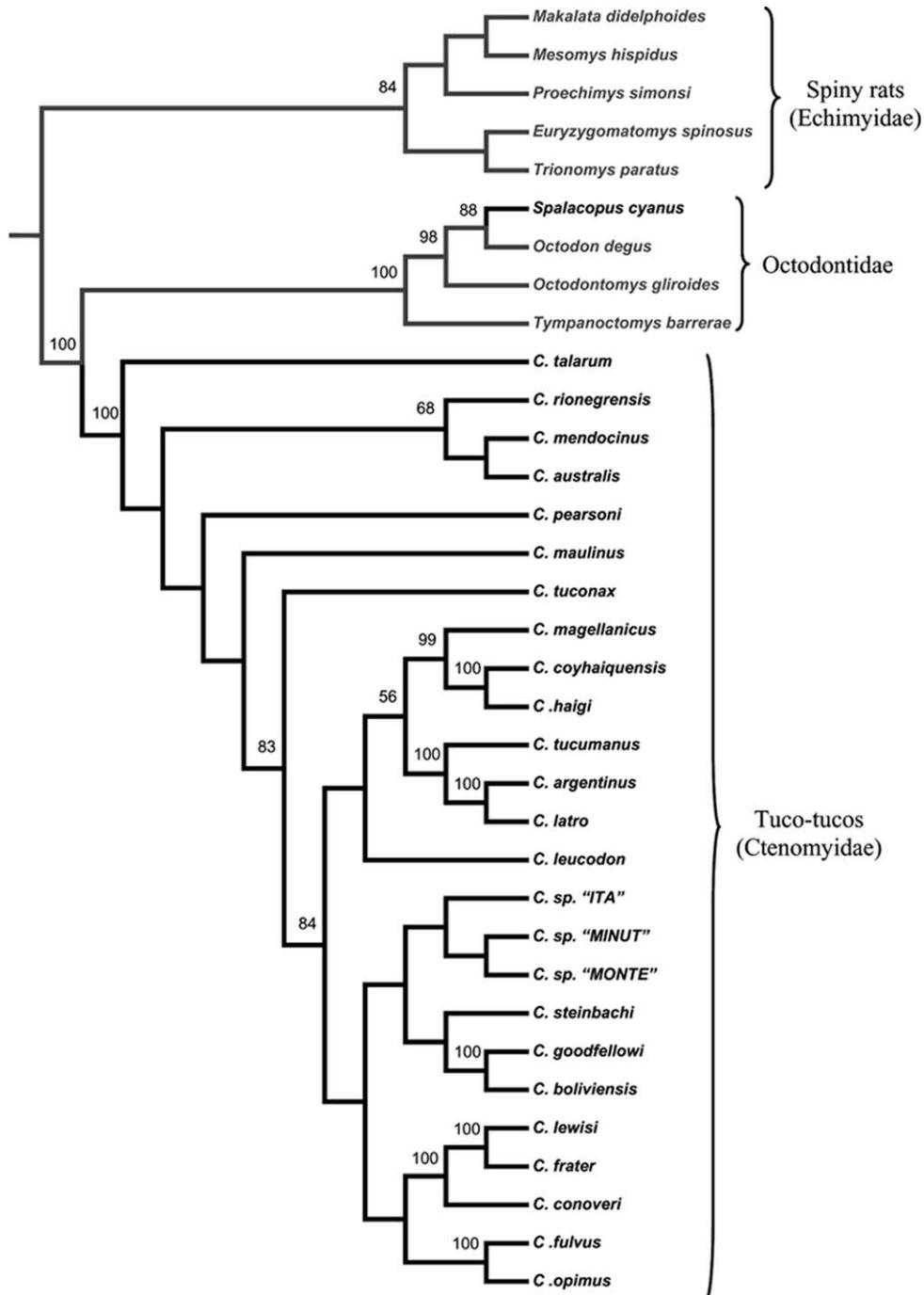


Fig. (1). Topology of the phylogenetic tree of tuco-tucos, coruro and relatives. (*C.* = *Ctenomys*). Non-subterranean taxa are in gray. Spiny rats are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

idae), and African mole rats (family Bathyergidae) (see Table 1 for more details about the species used in this study). *Spalacopus* and *Ctenomys* are closely related genera that colonized the subterranean habitat independently and recently (Plio-Pleistocene) [27, see also 28]. In contrast, pocket gophers are a very distinctive rodent clade that colonized the subterranean niche in the early Oligocene [29]. Members of the family Bathyergidae are known from early Miocene [30]. The substantial differences among these rodent groups in terms of the time span they had to adapt to their subterranean niche may be key to interpreting changes in rates of replacement substitutions in the evolution of the *cyt b* gene.

MATERIALS AND METHODOLOGY

Data and Phylogenies

We restricted our attention to complete *cyt b* gene sequences obtained from GenBank database. Accession numbers are shown in Table 1. We included representatives of three closely related families of South American rodents: a) Ctenomyidae, consisting of species of the subterranean genus *Ctenomys*; b) Octodontidae, which includes a single subterranean species (the coruro, *Spalacopus cyanus*) and non-subterranean species of the genera *Octodon*, *Octodontomys* and *Tympanoctomys*; and c) representatives of the spiny rats (Echimyidae) [31-34]. In pocket gophers, we included se-

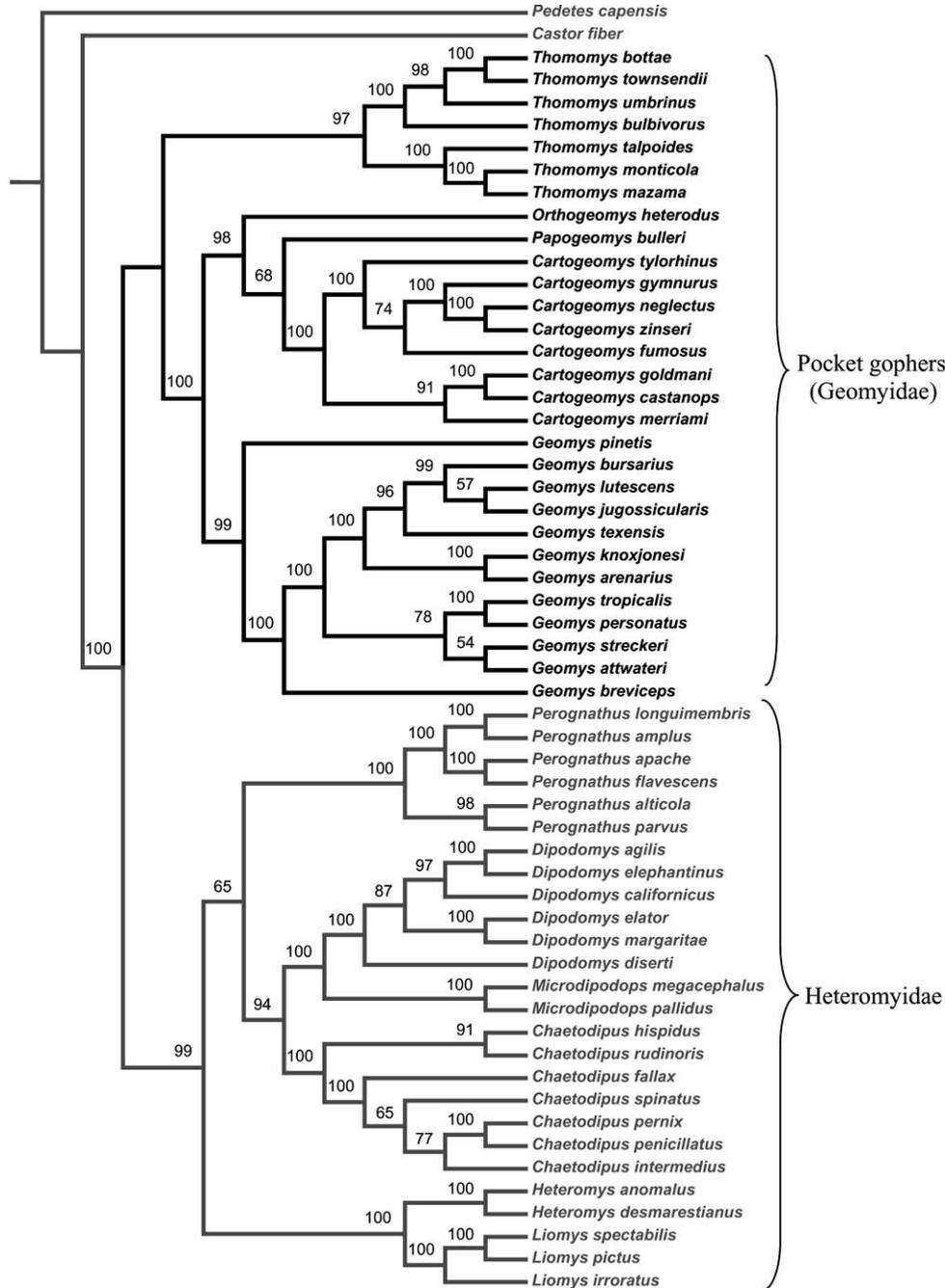


Fig. (2). Topology of the phylogenetic tree of pocket gophers and relatives. Non-subterranean are in gray. *Pedetes capensis* and *Castor fiber* are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

quences of the genera *Thomomys*, *Geomys*, *Pappogeomys*, *Orthogeomys* and *Cratogeomys* of the family Geomyidae; the sister family Heteromyidae was represented by sequences of the genera *Perognathus*, *Microdipodops*, *Heteromys*, *Dipodomys*, *Liomys* and *Chaetodipus* [35]. We set *Pedetes capensis* and *Castor fiber* as outgroup taxa. In the case of bathyergids, we obtained representative sequences of *Bathyergus*, *Cryptomys*, *Georchus* and *Heterocephalus*, and used *Hystrix africae australis* and *Thryonomys swinderianus* as outgroup taxa.

The phylogenetic reconstructions were done combining maximum likelihood (ML) and Bayesian approaches. Bayesian analyses were performed in MrBayes version 3.1.2 [36]. Four simultaneous Markov chains (three heated and one cold) were run for 200,000 generations, with random starting trees. Trees were sampled every 100 generations. The likelihood converged on a stable value by 10,000 generations in the three runs. Every analysis was checked for convergence. Of the resulting 2,001 trees, 500 were discarded as “burn-in”, and the remaining 1501 trees were summarized in 50% majority rule consensus trees. This consensus tree and the associated settings were used as a starting point for a heuristic ML search in Paup* [37], with 25 random additions of taxa, using the TBR branch-swapping algorithm. The resulting trees were thus selected as our working hypothesis of phylogenetic relationships (Figs. 1, 2 and 3). We solved the few polytomies found in the phylogenetic reconstruction of genus *Ctenomys* using the phylogeny published by Slamovits *et al.* [38]. Robustness of the resultant trees was established by performing 1,000 heuristic bootstrap replications and TBR branch-swapping.

Analysis of Synonymous and Nonsynonymous Substitutions

In protein-coding sequences, it is possible to infer positive selection by a rate of amino-acid substitution that exceeds the rate of neutral substitution. Variation in estimates of dN, dS and ω was explored using an ML approach as implemented in PAML version 3.15 [39]. There variation in ω 's was estimated: *i*) along different branches while holding the rates constant across codons [40], *ii*) across codons while holding rates constant along branches [41] and *iii*) simultaneously across codons and along lineages [42-44]. The last two approaches use Bayesian posterior probabilities to determine the likelihood that a given codon position has experienced positive selective pressure.

In the case of allowing distinct estimates of ω for different lineages (Branch Models), for each of the data sets we compared the following models: a) a null model with a single ω for all branches in the phylogeny; b) a full model in which all branches in the phylogeny have different ω 's, c) a reference model allowing different ω 's for each primary clade or branch of interest and d) a simple “ecological” models with one ω for all subterranean taxa and one for all non-subterranean taxa. All these models are specified in Table 2.

Tuco-tucos and relatives - the reference model used 5 distinct ω 's: subterranean coruros, the tuco-tuco clade, its basal branch, non-subterranean octodontines, and the non-subterranean spiny rats. The ecological model compared the ω of subterranean tuco-tucos and coruros to all others (non-subterranean groups).

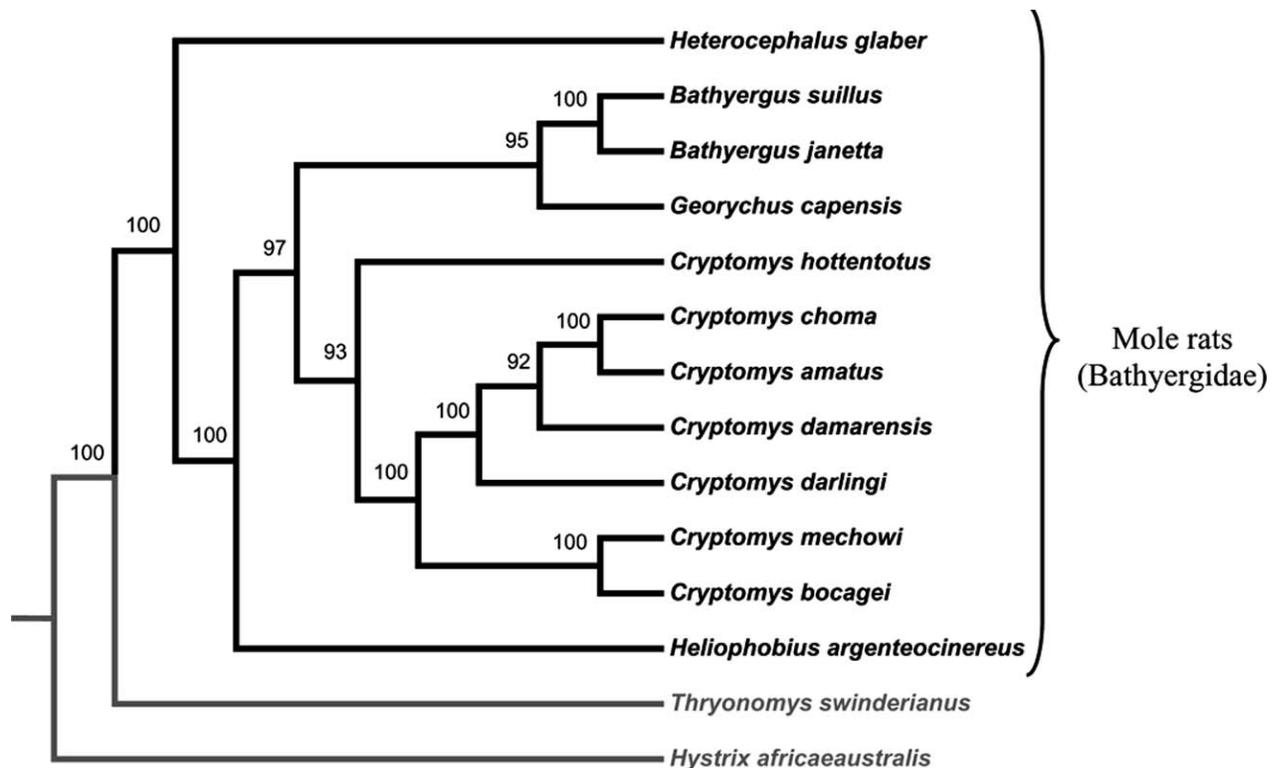


Fig. (3). Topology of the phylogenetic tree of mole rats and relatives. Non-subterranean taxa are in gray. *Thryonomys swinderianus* and *Hystrix africae australis* are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

Table 2. Summary of the Relationship Between Log-Likelihood Values, Number of Rate Parameters, and Likelihood Ratio Tests for Selected Comparisons of all Models Examined

A

Model	ω estimates	# Rate parameters	Ln(L)	-2 Δ Ln(L)	<i>p</i>
<i>Null</i>	0.0304	1	-9312.97	69.14	****
<i>Ecological</i>		2	-9278.39	20.60	****
Tuco-tucos + Coruro	0.0514				
Non-subterranean	0.0143				
<i>Reference</i>		5	-9268.10	62.46	n.s.
Spiny rats	0.0138				
Basal Tuco-tucos	0.0123				
Tuco-tucos	0.0549				
Octodontines	0.0143				
Coruro	0.0641				
<i>Full</i>	-	66	-9236.87		

B

Model	ω estimates	# Rate parameters	Ln(L)	-2 Δ Ln(L)	<i>p</i>
<i>Null</i>	0.0238	1	-20107.74	16.91	****
<i>Ecological</i>		2	-20099.28	6.69	*
Pocket-gophers	0.0294				
Non-subterranean	0.0200				
<i>Reference</i>		4	-20095.94	178.88	****
Basal Pocket-gophers	0.1277				
Crown Pocket-gophers	0.0287				
Heteromyids	0.0196				
Outgroup	0.0254				
<i>Full model</i>	-	111	-20006.49		

C

Model	ω estimates	# Rate parameters	Ln(L)	-2 Δ Ln(L)	<i>p</i>
<i>Null</i>	0.0495	1	-6491.12	37.17	****
<i>Ecological</i>		2	-6472.54	22.87	****
Bathyergids	0.0553				
Non-subterranean	0.0253				
<i>Reference</i>		3	-6483.97	75.41	****
Crown Bathyergids	0.0561				
Basal Bathyergids	0.0075				
Outgroups	0.0230				
<i>Full model</i>	-	26	-6446.26		

A) South American tuco-tucos, coruro, and allies, B) North American pocket gophers and allies, and C) African mole rats and allies. (*= $p < 0.05$, **= $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$). As models are nested, likelihood ratio tests compared a particular model with the next less complex model.

Pocket gophers and relatives - the reference model assigned 4 different ω 's: the crown geomyid clade, the basal geomyid branch, the heteromyids (sister taxa of geomyids), and all remaining non-subterranean branches. The rationale applied here was similar to the one used for the tuco-tucos

and coruros. The ecological model set a distinct ω for the subterranean lineages (pocket gophers) and compared this to the non-subterranean groups.

African mole rats and relatives - the reference model assigned 3 different ω 's: crown bathyergid clade, the basal

bathyerigid branch, and all remaining, non-subterranean lineages. The sister taxon of bathyerigids is poorly defined [45], so this case was not explored in more detail. The “ecological” model compares the bathyerigid clade to all others.

In the case of exploring variation across codons (Site Models), we compared the likelihood fit of several evolutionary models described by Yang *et al.* [41]: no variation across codons (M0), neutral model (M1a), selection model (M2), discrete distribution (M3), beta distribution (M7) and beta distribution + selection (M8). This approach uses Bayesian posterior probabilities to determine the likelihood that a given codon position has experienced positive selection ($\omega > 1$).

When exploring positive selection at individual sites along specific lineages, we used two variants of the Branch-site models (Model A and B) [42-44]. It assumes that different codon sites are subject to different levels of constraint or adaptive change, and this classification varies through the tree. The branches on the phylogeny are divided a priori into foreground and background lineages. Only foreground lineages may have experienced positive selection. The models assume four classes of sites: class 0 includes codons that are conserved throughout the tree ($0 < \omega_0 < 1$), class 1 includes codons that are evolving neutrally throughout the tree ($\omega_1 = 1$), and site classes 2a and 2b include codons that are conserved or neutral on the background branches, but evolve under positive selection on the foreground branches ($\omega_2 > 1$, estimated from the data). Model A of the model fixes $\omega_1 = 1$, whereas Model B estimates ω_1 from the data. In both cases, when $\omega_2 > 1$, the posterior probability that a codon belongs to class 2 is computed for each codon. Those with posterior probabilities > 0.95 are regarded as being under positive selection with high statistical confidence. Model A was tested against two possible null models: neutral Model M1a (test 1), and a model similar to A except that $\omega_2 = 1$ is fixed (test 2) [44]. The null model for Model B is Site Model M3 with 2 site classes. As PAML only allows two branch types, we chose to run the following comparisons for lineage variation: a) subterranean lineages (foreground branches) versus non-subterranean counterparts (background branches), and b) separate ω 's for the basal branch of subterranean lineages (foreground branch) and the remaining lineages (background branches).

The three datasets were analyzed independently with TreeSAAP software [15]. The baseml algorithm [39] was used to reconstruct ancestral character states at the nodes on the phylogenies described above. TreeSAAP evaluates the influence of selection in 31 amino acid properties (Table 3), grouping them into eight categories, from conservative to radical change. Only categories 6, 7 and 8, which are the most radical changes, were considered because they are unambiguously associated with molecular adaptation [20]. This approach aims to uncover physicochemical amino acid properties that have been affected by positive destabilizing selection, both overall and in specific amino acid sites. Following McClellan *et al.* [20], destabilizing selection is herein defined as selection that results in radical structural or functional shifts in local regions of the protein.

RESULTS

As indicated above, reference models allowed for variation in values of ω among groups of interest. In all cases,

Table 3. Amino Acid Properties for TreeSAAP

Abbreviation	Amino Acid Property
P_α	Alpha-helical tendencies
N_s	Average number of surrounding residues
P_β	Beta-structure tendencies
B_i	Bulkiness
B_r	Buriedness
R_F	Chromatographic index
P_c	Coil tendency
C	Composition
K°	Compressibility
pK'	Equilibrium constant
C_a	Helical contact area
H	Hydropathy
pH_i	Isoelectric point
E_l	Long-range nonbonded energy
F	Mean rms fluctuation displacement
M_v	Molecular volume
M_w	Molecular weight
H_{nc}	Normalized consensus hydrophobicity
V°	Partial specific volume
P_r	Polar requirement
P	Polarity
α_c	Power to be at the C-terminal
α_m	Power to be at the middle of the alpha-helix
α_n	Power to be at the N-terminal
μ	Refractive index
E_{sm}	Short-range and medium-range nonbonded energy
R_a	Solvent accessible reduction ratio
H_p	Surrounding hydrophobicity
H_t	Thermodynamic transfer hydrophobicity
E_t	Total nonbonded energy
P_t	Turn tendencies

reference models fitted significantly better than the null models (same ω for all branches). In turn, the full models (independent ω for each branch) had a significantly better likelihood score than other models tried, with the exception of South American tuco-tucos and relatives, for which reference and full models did not differ significantly. These results are summarized in Table 2.

In all cases, our results provide evidence for an accelerated rate of replacement changes relative to synonymous changes in subterranean lineages, relative to their non-subterranean relatives. The most striking example is that of South American octodontoids, where the estimated ω 's for the subterranean coruros and tuco-tucos in the reference model resemble each other and are several times higher than the nearly identical estimates obtained for all the non-subterranean branches. In fact, this five-parameter reference model can be simplified into a two-parameter ecological

Table 4. Positively Selected Codons Identified Using PAML (Branch-Site Model A), and the Corresponding Results Using TreeSAAP

position	Tuco-tucos + Coruro and relatives			Pocket Gophers and relatives			Mole Rats and relatives		
	PAML	TreeSAAP		PAML	TreeSAAP		PAML	TreeSAAP	
	Subt.	Subt.	N-Subt.	Subt.'s basal branch	Subt.	N-Subt.	Subt.	Subt.	N-Subt.
23	+	+	+						
46				+	+			+	
117	+	+							
249				+				+	
277							+	+	
295	+	+			+	+			
296	+	+			+			+	
306		+		+				+	
345	+	+	+		+	+		+	

Sites inferred to have evolved under positive directional selection (PAML) and positive destabilizing selection (TreeSAAP) are marked by +. Subt.= Subterranean, N-subt. = non-subterranean.

model that assigns an ω value to subterranean taxa and another ω to non-subterranean taxa without a significant loss of statistical fit. This two-parameter ecological model estimates that ω for subterranean groups are ~ 3.6 times greater than the corresponding value for non-subterranean lineages.

In North American pocket gophers, a two parameter ecological model, which assigned one ω to subterranean taxa and another ω to non-subterranean taxa, differs significantly from the null model. In this case, however, a four-parameter reference model is significantly better than the ecological model. In this reference model, the basal branch leading to pocket gophers had the highest ω value encountered in all our analyses, more than 6-fold higher than the lowest ω , corresponding to heteromyids, and more than 4-fold higher than that of the crown pocket gophers clade. For bathyergid rodents and relatives, the 3-parameter reference model indicated a higher ω for the bathyergids than either the basal branch or the outgroup taxa.

When ω was explored across codons, we found that in our three data sets (tuco-tucos and coruros, pocket gophers, African mole rats and their relatives) the best fitting model was the discrete model (M3 in PAML), which groups codons into 3 classes assigning ω 's from a discrete distribution to each class. In tuco-tucos and pocket gophers, we didn't find a class with $\omega > 1$; however, in the case of bathyergids we found ω 's > 1 (posterior probability > 0.99) for codons 241 (threonine) and 277 (alanine). In pocket gophers and tuco-tucos data matrices, position 277 is an alanine in all taxa (subterranean and non-subterranean); and position 241 is a threonine in some subterranean and non-subterranean taxa.

Branch-sites Model A aim at identifying positively selected codons. Its statistical significance can be assessed with test 1, which can show false positives [44], or with more conservative test 2. None of our branch-site models were significant using test 2. Positive results for Model A (p

< 0.001) using test 1 included: a) in all datasets, the distinction between subterranean and non-subterranean lineages, and b) in a model that distinguishes the basal branch of pocket gophers from the remaining lineages. Model B is tested against null model M3, and supported the following variation in ω ($p < 0.05$): i) subterranean taxa versus non-subterranean relatives in all datasets, ii) tuco-tucos' basal branch versus the remaining taxa, and ii) mole rats' basal branch versus the remaining taxa. Positions of cyt b found to be under selection were: i) 23, 117, 295, 296 and 345 in Model A comparing tuco-tucos and coruro versus their non-subterranean relatives, ii) 277 in case of Models A and B comparing mole rats and their non-subterranean relatives, and iii) 46, 249 and 306 in Model A comparing pocket gophers' basal branch and the remaining taxa (Tabel 4).

The TreeSAAP analyses identified three amino acid properties to be under positive destabilizing selection in our cyt b gene datasets ($p < 0.05$), both in the subterranean and

Table 5. Physicochemical Amino Acid Properties that have been Affected by Positive Destabilizing Selection in the Cytochrome b Gene Among all Groups Compared in this Study. Those Affected Exclusively in Subterranean Taxa are Underline

Tuco-tucos and Coruro	Pocket Gophers	African Mole Rats
P_a	P_a	P_a
pK'	pK'	pK'
α_c	α_c	α_c
<u>E_t</u>		<u>R_a</u>
<u>α_m</u>		<u>H</u>
<u>R_a</u>		<u>M_w</u>
		<u>V^o</u>

Table 6. Amino Acid Sites and Physicochemical Amino Acid Properties that have been Affected by Positive Destabilizing Selection. Sites Affected by Positive Destabilizing Selection Exclusively in Subterranean Taxa are Underline

Amino Acid Site	Tuco-tucos and Coruro	Pocket Gophers	African Mole Rats
MATRIX, N-TERMINUS			
4	<u>P_α</u>		
14		<u>pK'</u>	
19		<u>pK'</u>	
23	P _α		
29	<u>P_α</u>		
TRANSMEMBRANE, A-HELIX			
36			<u>pK'</u>
39			<u>pK'</u>
42 *	<u>Ca</u>	pK'	<u>pK'</u>
43		pK'	
46 *		<u>B_i, R_F, P_C, α_n, H_D</u>	<u>pK'</u>
52			<u>P_i</u>
RMEMBRANE, AB-LOOP			
57			<u>V^o, C₃, H_{nc}, E_{sm}</u>
59		<u>pK'</u>	
60			<u>P_α</u>
64		<u>P_α, P_C</u>	
67	<u>P_α</u>	P _α	P _α
69		<u>pK'</u>	
TRANSMEMBRANE, B-HELIX			
76			<u>P_α</u>
81		<u>α_m</u>	
82			<u>R_α</u>
84			<u>K^o, P_i</u>
85			<u>α_c</u>
87		<u>P_α</u>	
96		<u>R_α</u>	
MATRIX, BC-LOOP			
110			<u>P_α</u>
TRANSMEMBRANE, C-HELIX			
111 *	<u>α_c</u>		<u>K^o</u>
115	<u>pK'</u>		
117	<u>pK', R_α</u>		
123	<u>N_s, R_α</u>		
129		<u>R_α</u>	
INTERMEMBRANE, CD-LOOP			
142		<u>H_{nc}, μ</u>	
149			<u>E_i</u>
158	<u>P_α, α_n, H_D</u>		<u>K^o</u>
169			<u>P_α, P_i</u>
TRANSMEMBRANE, D-HELIX			
173 *	<u>P_α</u>	<u>P_α</u>	<u>P_α, P_i</u>
190 *		<u>P_α, P_b</u>	<u>P_α</u>
193 *		<u>P_b</u>	<u>P_α</u>
194		<u>P_α</u>	

(Table 6). Contd.....

Amino Acid Site	Tuco-tucos and Coruro	Pocket Gophers	African Mole Rats
MATRIX, DE-LOOP			
209		R_F	
212	α_c		α_c
214	α_c	α_c	α_c
215		E_{sm}	
216		α_c	
218		pK'	
TRANSMEMBRANE, E-HELIX			
224	α_m		
226		E_i	
229	pK'		
232 *	pK'	E_i	
233		pK'	
235		pK'	
236		pK'	
237		P_d	
238 *	P_d	E_i	
240 *	P_d	E_i	
241			P_d
243			R_d
TRANSMEMBRANE, EF-LOOP			
246			α_m
249			α_m
250		P_d	
256			α_m
257 *	P_d	P_d	
272			M_w
277			$V^o, M_w, pH_i, C_o, H, H_{nc}, E_{cm}, E_i$
278			α_m
281		B_i, α_m, H_p	
TRANSMEMBRANE, F-HELIX			
293		pK'	
295	P_b, E_i	pK'	
296 *	P_c, E_i, F, P_i	F	H
300		pK'	
302			pK'
303			F, P_b, pK'
304			pK'
306 *	N_s, pK', E_i		pK'
307	N_s, pK', E_i		
MATRIX, FG-LOOP			
309	$N_s, B_i, P_c, \alpha_m, H_p, P_i$		
316			pK'
TRANSMEMBRANE, G-HELIX			
320 *		pK'	pK'
327 *	pK'		pK'
328		pK'	
329		P_i	pK'
331			α_c
334		pK'	R_d
340			α_c
344		α_c	

(Table 6). Contd.....

Amino Acid Site	Tuco-tucos and Coruro	Pocket Gophers	African Mole Rats
TRANSMEMBRANE, H-HELIX			
345 *	α_m	α_c	α_m
348	<u>N_s, B_r, pK', H, E_i, R_{α}, H_p</u>		
349	<u>N_s, B_r, pK', H, E_i, R_{α}, H_p</u>		
353	<u>H</u>		
356	<u>N_s, R_{α}</u>		
357	<u>N_s, B_r, pK', H, E_i, R_{α}, H_p, H_i</u>		
361	<u>N_s, B_r, pK', H, E_i, R_{α}, H_p, H_i</u>		
362	<u>N_s, B_r, pK', H, E_i, R_{α}, H_p, H_i</u>		
368	<u>N_s</u>		
374	<u>α_c</u>		

*Amino acid sites with an asterisk are the ones that are under destabilizing selection in two or the three subterranean groups.

non-subterranean component: dissociation equilibrium constant (pK'), alpha-helical tendencies (P _{α}), and power to be at the C-terminal (α_c). Along the gene, subterranean mole rats and tuco-tucus shown more positively destabilizing selected properties than their non-subterranean relatives (see Table 5); this difference was not found between pocket gophers and heteromyids. Among those sites shown to have been under destabilizing selection exclusively in subterranean taxa, four were shared only between tuco-tucos and pocket gophers (codons 232, 238, 240 and 257), four between tuco-tucus and mole rats (42, 111, 306 and 327), five between pocket gophers and mole rats (46, 190, 193, 320 and 345), and only 2 among all subterranean groups (173 and 296). With the exception of site 257, all these sites are within transmembrane domains. (see Table 6 for additional details).

DISCUSSION

As indicated in the introduction, demonstrating positive directional selection at the molecular level has been difficult. In particular, only a few genes show clearly accelerated rates of replacement relative to synonymous changes to the point that ω exceeds 1 [46, 20]. This is not surprising given that a) only a few amino acids are involved in some well-known cases of adaptation at the molecular level [13] and b) positive directional selection may be concentrated on short periods of accelerated evolution relative to the total length of the branches linking the taxa under study ("episodic selection", see [47]). In the case of this study, phylogenetically oriented papers that produced the cyt b data had noted more synonymous than replacement changes, so it was known that such an extreme ($\omega > 1$) would not be observed.

Our examination of the possibility of positive selection in subterranean lineages was motivated by the fact that the colonization of the subterranean niche is energetically demanding. This case has the additional advantage that multiple independent colonizations of the niche can be explored for consistent features evolved as convergent evolution under similar selection pressures, relative to their non-subterranean relatives. In addition, analyses of variation in ω are now possible both across lineages and codons, using likelihood and Bayesian approaches. Finally, methods developed to evaluate the strength of destabilizing selection in cyt b, successfully used in comparing cetaceans and their relatives, can be used to obtain an independent evaluation of our adap-

tive hypothesis; importantly, such an evaluation can be repeated in each of the cases under consideration.

Our main results, discussed and qualified in detail below, are: a) four lineages of subterranean lineages show significantly higher values of ω relative to their non-subterranean counterparts; b) whereas most codons show $\omega < 1$, a small number of codons shows $\omega > 1$; and c) destabilizing changes in biochemical properties are ubiquitous in the phylogenies, but tend to concentrate on the subterranean lineages. Overall and taken collectively, these results are consistent with a hypothesis that the colonization of the subterranean niche creates a selective regime of positive, directional selection in the cyt b gene.

Significantly Higher ω in Subterranean Rodents Relative to their Non-Subterranean Counterparts

Despite great differences in the time elapsed since the colonization of the subterranean niche on the different continents, we detected significant increases in the relative rates of replacement substitutions in the evolution of cyt b of subterranean groups relative to their non-subterranean relatives. Both tuco-tucos and coruros colonized the subterranean niche relatively recently, whereas pocket gophers, colonized the subterranean much earlier. Tuco-tucos and coruros display a 3.5-fold higher ω relative to their non-subterranean counterparts while the basal branch of the gopher radiation has the highest ω observed in our analyses ($\omega = 0.1277$). This high value of ω early in the history of pocket gophers is followed by a return to a lower ω similar to that of outgroup taxa (Table 2). In contrast, the tuco-tucos did not return to a lower rate. A model separating the basal tuco-tuco branch from the rest of the tuco-tuco clade shows that the cause of the increase lies in the latter. We suggest that, in their relatively protracted history, pocket gophers underwent an early episode of increase in ω , whereas the process is ongoing in tuco-tucos and coruros. These changes in ω could be related to changes in the adaptive landscape associated with entering the subterranean niche.

Although none of our estimates of ω is greater than 1, the possibility remains that positive directional selection is the driving force behind the increase in ω . Finding amino acid replacements in excess of synonymous substitutions, globally or in specific regions, provides unequivocal evidence of positive selection at the molecular level. Nevertheless, Yang

[10] noted that this criterion could be excessively stringent, and suggested that statistically significant increments in ω 's could be suggestive of positive selection [48, 41]. Clearly, the subterranean genera examined in this study do not fit the most stringent requirements for demonstrating positive selection, as synonymous substitutions exceed replacement changes over all branches. However, *cyt b* interacts strongly with other mitochondrially and nuclearly encoded subunits of enzyme complexes of the electron transport chain, which could drive their coevolution. This process might occur through coordinated substitutions that change the functional constraint on interacting proteins by modifying a regime of purifying selection [49]. Unfortunately, this hypothesis is impossible to test with *cyt b* sequences only.

A Small Number of Codons Shows $\omega > 1$

We further explored variation in ω across codons along a subset of the branches leading to subterranean groups or their basal branch, and the few positions identified as being under positive selection. These results are consistent with the branch and the site models, because the effect of the acceleration observed in the branches leading to subterranean lineages was only evident when combining both site and branch variation in the analysis, and was diluted among a large majority of slowly evolving sites when we did not consider lineage variation. Apparently, particular codon sites are responsible for the significant increase in ω found in these independent subterranean radiations. Interestingly, our results show positive selection on codon 227 in the bathyergid dataset in agreement with a recent study which detected an important amino acid change in this codon, in two species with distinct metabolic requirements: an aquatic mammal (dugong) and a highland mammal (alpaca). da Fonseca *et al.* [21] have concluded that these amino acid changes are possible adaptations to the aquatic environment and the life at high altitude, respectively (although see [50]).

Destabilizing Changes in Biochemical Properties Tend to Concentrate on the Subterranean Lineages

Our results regarding rate variation were supported with a different approach to detect selection in amino acid sequences, namely to look at the magnitudes of property change of non-synonymous residues across a phylogeny, using TreeSAAP software [15]. Amino acid substitutions have a wide range of effects on a protein depending on the difference in physicochemical properties and location in the protein structure. This approach provides further resolution on the type of positive selection detected (directional or non-directional, stabilizing or destabilizing), and offers insights into how the identified selection affects the overall structure and function of the protein [20]. In this case, the results show a common evolutionary pattern of the *cyt b* gene associated with the conquest of subterranean habitat.

The results given by TreeSAAP are consistent with those from PAML in suggesting that certain codons are subjected to positive selection, particularly, but not exclusively, among subterranean lineages. In general, subterranean groups showed more selected properties and sites under positive destabilizing selection than non-subterranean relatives. Beside, as shown in Table 4, the sites that PAML detects as having been under positive selection are included among those detected by TreeSAAP. In this sense, the TreeSAAP

software seems to be more sensitive to detect selection under these conditions. This has been observed in other cases (e.g., da Fonseca *et al.* [20], Mc. Clellan *et al.* [21]). In the case of pocket gophers, PAML suggested a difference between the basal branch leading to them and the remaining taxa in that dataset. It is not possible to make a direct comparison of those results and analyses with TreeSAAP, that does not allow a distinction between specific branches.

Taken together, the different analyses point toward the action of positive, directional selection in the evolution of the *cyt b* gene, in association with the colonization of the subterranean environment in rodents. However, alternative explanations might be proposed on the basis of variation in metabolic rate, body mass, population size, and generation time among lineages [51-54]. In general, the aforementioned factors are expected to affect all types of substitutions and therefore, should not change the ω ratio of the *cyt b*. However, variation in metabolic rate might result in a different regime of purifying selection [55]. Spradling *et al.* [56] for instance, have shown that rates of nonsynonymous substitution in *cyt b* differ significantly among rodent genera, but these differences are not associated with variation in generation time, body size or metabolic rate. Smaller effective population size may decrease the effectiveness of purifying selection against slightly deleterious mutations, leading to accelerated rates of replacement substitutions. Regrettably, there is no clear way of distinguishing between directional selection and a relaxation of purifying selection when ω 's are less than 1. However, the results obtained with TreeSAAP offer evidence of directional positive selection, not just negative selection, either over the entire *cyt b* and in specific sites, driving destabilizing biochemical change in subterranean lineages.

CONCLUSION

In sum, the acceleration of replacement rates estimated in subterranean tuco-tucos, coruros and the early pocket gophers with respect to their relatives might be explained by changes in the selective regime in conjunction with the colonization of the hypoxic subterranean niche. A selectionist explanation is consistent with the pattern observed, both on the basis of examination of variation in ω and of the nature of biochemical changes. But alternative explanations, primarily the relaxation of purifying selection, cannot be firmly ruled out with the data at hand. These and other comparative analyses, however, will require additional work to generate the sequences and phylogenetic framework, still lacking in sufficient detail for many of these taxa [30].

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