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Research paper

Molecular adaptive convergence in the α-globin gene in subterranean octodontid rodents

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ABSTRACT

Tuco-tucos (*Ctenomys*) and related coruros (*Spalacopus*) are South American subterranean rodents. An energetically demanding lifestyle within the hypoxic/hypercapnic underground atmosphere may change the selective regime on genes involved in O_2 transport in blood. In addition, some species of tuco-tucos may be found at high altitude, thus facing additional reductions in changes O_2 availabily. We examined sequence variation in the alpha globin subunit gene of hemoglobine in these lineages, within a robust phylogenetic context. Using different approaches (classical and Bayesian maximum likelihood (PAML/Datamonkey) and alternatives methods (TreeSAAP)) we found at least 2 sites with evidence of positive selection in the basal branch of Octodontidae, but not in tuco-tucos. These results suggest some adaptive changes associated to fossoriality, but not strictly to life underground.

1. Introduction

Assessing the predictability of evolution is a central question in current evolutionary research. In a macroevolutionary framework, phylogenetically independent invasions of specialized niches by sets of relatively closely related species have proven to be powerful study systems. Among vertebrates, the independent invasion of high-altitude environments by multiple birds, the evolution of diving in multiple mammalian taxa or the independent invasion of the subterranean niche by rodents represent natural study systems to address these type of questions (see Lacey et al., 2000).

Subterranean rodents live most of their lives underground in their usually closed burrows, which are dark, hypoxic, hypercapnic, and thermally stable relative to their above-ground counterparts. From a biological standpoint, life underground imposes particular challenges, which have driven subterranean rodents to develop convergent morphological and physiological features (Nevo et al., 1999). An important constraint faced by subterranean mammals is the low availability of O_2 (hypoxia) and the excess of CO_2 (hypercapnia) in the subterranean environment (Darden, 1972; Arieli, 1990; Shams et al., 2005). Maximal CO_2 levels (6.1%) and minimal O_2 levels (7.2%) were recorded in mounds of *Spalax carmeli* in Israel (Shams et al., 2005). Models of diffusion gas exchange (Withers, 1978) and experimental data (MacLean, 1981) show that burrow atmospheres are generally hypoxic and hypercapnic (Buffenstein, 2000).

Under low O_2 partial pressure in the burrow atmosphere and facing the potential CO_2 perturbation of their blood acid-base balance, subterranean mammals are expected to avoid excessive energy expenditure in respiratory work. The mechanisms by which this is achieved are not fully understood, but apparently include several physiological adjustments in relation to the predicted values of mammals of similar body mass (predicted by Stahl, 1967) and at different levels (reviewed by Boggs et al., 1984, Buffenstein, 2000, Nevo et al., 1999). For example, oxygen carrying capacity is increased, facilitated by elevated hemoglobin concentrations, high intrinsic affinity for oxygen and more red blood cells (reviewed by Buffenstein, 2000).

There have been at least eight independent invasions of the subterranean niche by rodents. Among them, comparisons between the

Abbreviations: O_2, oxygen; CO_2, carbon dioxide; Hb, hemoglobine; Cl, cloride; α-globine, alpha-globine subunit gene of hemoglobine; β-globine, beta-globine subunit gene of hemoglobine; PAML, phylogenetic analysis by maximum likelihood (software); TreeSAAP, selection on amino acid properties using phylogenetic trees (software); MEME, mixed effects model of evolution (software); REL, random effects branch-site model (software); Mya, millon years ago; M asl., metres above sea level; SDS, sodium dodecyl sulfate; NaCl, sodium chloride; DNA, deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; $MgCl_2$, magnesium chloride; Ala, alanine; Gly, glycine; Asp, aspartic acid; Glu, glutamic acid; Asn, aspargine; Ser, serine; Thr, threonine; Leu, leucine

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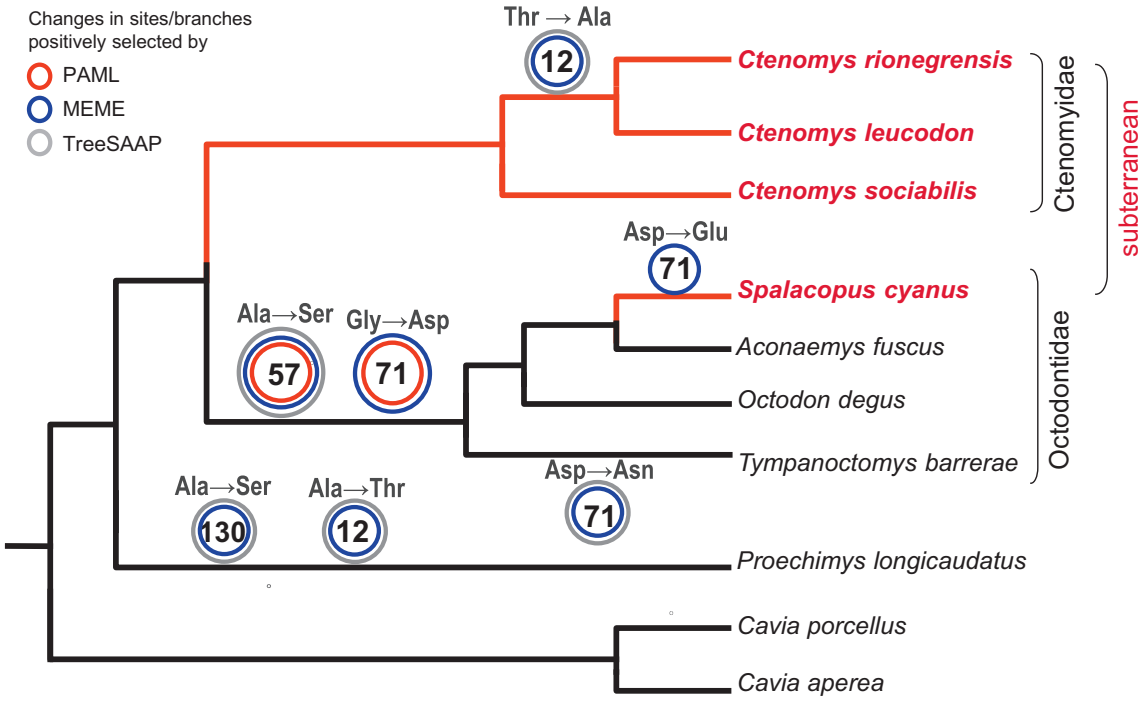

Fig. 1. Phylogenetic relationship among caviomorph rodents included in this study. Numbers are amino acid sites of α-globin gene identified to be under natural selection by different methods. Red, blue and gray circles, show sites identified as positively selected by PAML, MEME and TreeSAAP, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

South American sibling families Octodontidae (degus, coruros and allies) and Ctenomyidae (tuco-tucos) provide a unique opportunity to trace the evolution of adaptations related to life underground because of their phylogenetic affinities and their relatively recent occupation of the subterranean niche (Lessa et al., 2008). Burrowing for shelter and rearing young (fossoriality) are shared characteristics among these two families of rodents, but only two extant genera, *Ctenomys* (tuco-tucos) and *Spalacopus* (coruros) have acquired fully subterranean lifestyles and their associated anatomical and physiological adaptations (Fig. 1). These two South American genera of subterranean rodents are analogous to North American pocket gophers but have entered the subterranean niche more recently. Moreover, the sister groups of most other subterranean rodent lineages, if at all known, tend to be very divergent from the subterranean groups, but this is not the case with Octodontidae and Ctenomyidae which have diverged between 14 and 23 Mya (reviewed in Patton et al., 2015). Within Octodontidae, the coruro has evolved a fully subterranean life and acquired numerous morphological changes within the last 2 to 3 Mya (Lessa et al., 2008; Opazo, 2005). The family Ctenomyidae accumulated changes associated to subterranean life in a mosaic fashion along several lineages, in a longer process that has taken at least 8 Mya; the extant diversity of tuco-tucos includes > 63 nominal species (Patton et al., 2015) that diversified in the last 3.5 Mya (Verzi et al., 2010). In addition, phylogenetic relationships among the genera are well-established (e.g., Opazo, 2005; Upham and Patterson, 2012 and references therein), which facilitates the tracing of changes associated with the acquisition of subterranean adaptations onto an established phylogeny, enabling us to identify and discriminate adaptations restricted to subterranean lineages from more general ones associated with fossoriality.

Previous study suggested a link between niche shifts and weak directional (or episodic) selection at mitochondrial genes (Tomasco and Lessa, 2011, 2014; Silva et al., 2009). In this study we present a further approximation to explore the molecular underpinnings of adaptation to life underground in South American rodents. To do so, we investigated patterns of molecular evolution in the gene encoding for the α-subunit of hemoglobin of species in the genera *Octodon* and *Ctenomys*. Specifically, the goal of this study is to look for convergent or parallel changes in the α-globin genes of subterranean lineages relative to their fossorial counterparts, and explore whether these changes were driven by natural selection.

2. Materials and methods

2.1. Specimens examined

We selected 2 specimens from 10 caviomorph rodent species, including representatives of two related but independent subterranean lineages. Among these subterranean lineages were three species of tuco-tucos (*Ctenomys*) and the coruro (*Spalacopus cyanus*). Fossorial, but non-subterranean relatives were three Octodontid rodents (*Aconaemys fuscus*, *Octodon degus* and *Tympanoctomys barrerae*). We also add a non-fossorial spiny rat (*Proechimys longicaudatus*), and finally two species of *Cavia* (*C. porcellus* and *C. aperea*) which were used as outgroup. Because tuco-tucos comprise > 63 species and were considered one of the most rapidly speciating mammalian lineages (Patton et al., 2015), we chose a sample of 3 species from this group, in an attempt to capture the diversity of lifestyles and molecular differentiation and habitat features following Tomasco and Lessa (2011): *C. sociabilis, C. rionegrensis* and *C. leucodon.* First two species live on lowland, about 0 m asl, but *C. leucodon* occurs between 3800 and 4500 m asl. We also included sequences of *O. degus* and *C. porcellus* taken from GenBank (XM_003478407.2 and XM_004627947.1 respectively). Table 1 summarizes information about specimen voucher numbers and sampling localities of samples.

2.2. DNA extraction, amplification, sequencing and alignment

Total DNA extractions were made with SDS/proteinase K digestion/NaCl protein precipitation/alcohol precipitation of DNA (modified from Miller et al., 1988) from liver preserved in 95% ethyl-alcohol. The almost complete HBA gene was amplified using two pair of primers specially disigned for this study. One pair was HAF4 (5′

Table 1
Species and information of specimens examined.

Species and vouchers (GenBank acc. number)	Collection and procedence
Ctenomys sociabilis EAL545, EAL533 (MF169882, MF169881)	Donated by Eileen Lacey, University of California, Berkeley.
Ctenomys rionegrensis EV1064, EV1114 (MF169883, MF169884)	Laboratorio de Evolución, Facultad de Ciencias
Ctenomys leucodon MSB:Mamm:59654, NK14789 (MF16988, MF169886)	University of New Mexico, USA
Octodon degus 109,241, 109,251 (MF169887, MF169888)	Donated by F. Bozinovic, Universidad Católica de Chile
Spalacopus cyanus DG657 (MF169889) SSUC_Ma_00405 (MF169890)	Donated by G. D'Elía, Universidad Austral de Chile Universidad Católica de Chile
Aconaemys fuscus *GD1284, GD722* (MF169891, MF169892)	Donated by G. D'Elía, Universidad Austral de Chile.
Tympanoctomys barrerae AK13811 (MF169893) AO73 (MF169894)	Texas A & M University, USA. Donated by A.Ojeda. IADIZA-CCT, CONICET, Argentina.
Proechimys longicaudatus MSB:Mamm:57192 (MF16989)	University of New Mexico, USA
Cavia porcellus CP (MF169896) (LT548179.1)	Institut Pasteur, Montevideo Uruguay GenBank
Cavia aperea CA684 (MF169898) EMG1395 (MF169897)	Laboratorio de Evolución, Facultad de Ciencias Donated by E.González, MNHN, Montevideo, Uruguay.

TTCTGGTTCTGACACAGACTCAGrAAG-3′, forward) and HAR6 (5′ CTTAGCGGTATTTGGAAGTCAGCACG-3′, reverse), and a few sequences were amplified with combination N (5′ ACACCCTTCTGGTTCTGACA-3′, forward) and I (5′ AGACTTTATTCAAAGACCAAGAGGT-3′, reverse). Amplification was carried out in a total volume of 20 μl containing the following final concentrations of each constituent: 10 μl of DNA (≈ 0.4 μg/ml) used as a template, 1 × Taq Polymerase Buffer, 240 μM of each dNTP, 240 nM of each primer, 2 units of Taq Polymerase and 3 mM of $MgCl_2$. PCR amplifications were performed in a PXE0.2 Thermal Cycler (Thermo - Electron Corporation), by an initial denaturation of 3 min at 94 °C, followed by 40 cycles of 20 s of denaturation at 94 °C, 20 s of annealing at 62 °C and 20 s of extension at 72 °C, and a final extension of 5 min at 72 °C. In each reaction, the corresponding negative control was included. The amplified products were electrophoresed in 0.8% agarose gels (100 V, 20 min), the DNA bands were visualized after GoodView staining under UV light, and expected size was determined in relation to a 100 bp DNA size standard (GIBCO BRL). PCR products were purified and automatic sequencing from both ends was done by Macrogen. Inc. (http://www.macrogen.com), under BigDyeTM terminator cycling conditions in an ABI 3730xl Sequencer. All sequences were deposited in GenBank (see Table 1). Sequences obtained were edited with Proseq (Filatov, 2009), and all sequences were aligned by Clustal in MEGA6. The intron-exon structure and inspections of the lack of stop codons and in/dels that generate frameshift were checked.

2.3. Data analyses

The phylogeny considered for this study (Fig. 1) was obtained by pruning the comprehensive tree of octodontoid genera in Upham and Patterson (2012) (Honeycutt et al., 2003; Opazo, 2005) for setting the position of the genera, and the tree proposed by Parada et al. (2011) and Tomasco and Lessa (2011) for setting the relationships within the tuco-tucos (*Ctenomys*).

In general, methods for detecting adaptive molecular evolution in a phylogenetic context compare rates of synonymous (dS) and non-synonymous substitution rates (dN) in protein-coding genes, consider $dN/dS > 1$ as an evidence for Darwinian selection (for a review see Nielsen, 2005; Yang and Bielawski, 2000). The most conventional program for this purpose is codeml, implemented in the PAML package (Yang, 2009). codeml is conservative and detects pervasive natural selection among sites (Yang and Nielsen, 1998) and branches (Yang et al., 2000), given a tree and an alignment. But, because adaptive evolution frequently occurs in episodic bursts, localized to a few sites in a gene, and to a small number of lineages in a phylogenetic tree, the program incorporate the possibility of testing both simultaneously. A popular class of "branch-site" PAML provides a statistical framework to search for evidence of such episodic selection, but for computational tractability, it is necessary to partition the tree a priori into "foreground" branches, which are allowed to undergo diversifying selective bursts and "background" branches, which that are negatively selected or neutral (Yang and Nielsen, 2002; Yang et al., 2005; Zhang et al., 2005). Recently, new methods have been developed to detect branch and sites simultaneously using a more flexible framework relative to codeml: REL (Kosakovsky Pond et al., 2011) and MEME (Murrell et al., 2012). REL and MEME are implemented in the Datamonkey web server (http://www.datamonkey.org/), are more sensitive relative to PAML and use alternative approaches. REL evaluates variation in dN/dS along the alignment and then evaluates its likely placement on the tree, whereas MEME evaluates variation in dN/dS along branches, and then assesses which sites contribute to variation in dN/dS. In contrast to codeml, REL and MEME allow dS to vary among sites. In addition, TreeSAAP (Woolley et al., 2003), implements a different approach, comparing the observed distribution of physicochemical changes inferred from a phylogenetic tree with a distribution based on the assumption of the random amino acid replacement expected under strict neutrality. This test is developed in the program TreeSAAP and shows to be much more sensitive than previous ones. It has the advantage of giving information about the physicochemical property involved in amino acid. In this study, the approach was to apply all these complementary tests.

In PAML4.6, variation in the values for ω was estimated along different branches while holding rates constant across codons (Yang and Nielsen, 1998), across codons while holding rates constant along branches (Yang et al., 2000), and simultaneously across codons and along lineages (Yang and Nielsen, 2002; Yang et al., 2005; Zhang et al., 2005). The last two approaches use Bayesian posterior probabilities to determine the likelihood that a given codon position has experienced positive selective pressure. To obtain distinct estimates of ω for different lineages (Branch Models), we performed (i) a null model with a single ω for all branches in the phylogeny; (ii) a full model in which all branches in the phylogeny have different ω values, and (iii) models of intermediate complexity that allow different ω values for each clade or branch of interest (phylogenetic, e.g., by family, and/or "ecological", i.e., distinguishing subterranean and non-subterranean taxa). To explore rate variation across codons (site models), we compared the likelihood of fit of several evolutionary models described by Yang et al. (2000) and tested positive selection using the three likelihood ratio test (LRT) recommended in the PAML4 user manual (M1a-M2a, M7-M8 and M8a-M8 comparisons). When searching for positive selection at individual sites along specific lineages, we used two variants of the Branchsite model A and the LRT (Likelihood-ratio test) between them (Model A and Model A modified), also as recommended (Yang and Nielsen, 2002; Yang et al., 2005; Zhang et al., 2005). As PAML4.6 only allows two branch types, we chose to run comparisons of variation between lineages, considering different combinations of subterranean lineages (the foreground branches, namely, the coruro, tuco-tucos or both) versus their non-subterranean counterparts (background branches). For site and branch-site models, when the likelihood ratio test

was significant, the Bayes Empirical Bayes (BEB) was used to calculate posterior probabilities for site classes to determine which codon positions have experienced positive selection ($\omega > 1$). In all cases, PAML was run three times with different starting values of ω (0.4, 1 and 4), as recommended to check for multiple local optima, and the level for significance was 0.05.

In Branch-siteREL (Kosakovsky Pond et al., 2011) and MEME (Murrell et al., 2012) implemented in the Datamonkey server (http://www.datamonkey.org, Delport et al., 2010), ω variation was evaluated among lineages and sites simultaneously and the level for significance was 0.05 for the site and, conditional upon that, an Empirical Bayes Factor (EBF) > 20 for the branch or branches.

Significant physicochemical amino acid changes among residues in HBA protein-coding gen were identified by the algorithm implemented in the TreeSAAP 3.2 software package (Woolley et al., 2003). Non-synonymous changes were considered to be the result of positive, destabilizing selection (from now on, "radical changes") only if they met two stringent criteria: a) they were assigned to the most extreme categories of structural or functional changes (categories 6, 7 and 8 of Woolley et al., 2003); and b) they were significant at the $p < 0.001$. We ran ModelGenerator (Keane et al., 2006) to select a substitution model for each gene and reconstruct ancestral sequences in Baseml (Yang, 1997), and the model selected was REV (Saccone et al., 1990).

3. Results

We obtained sequences for a fragment of approximately 1.2 kbs, covering the three exons and two intervening introns of the HBA, but missing the last five codons of the 3rd exon, including the stop codon (Fig. 2, see also Fig. 1 in Tomasco et al., 2017). As expected, levels of variation in the alignment were higher in the introns relative to exons (data not shown). Phylogenetic relationships among the sequences in ML and Bayesian analyses do not deviate from the expectations based on organismal relationships.

Both, MEME and the branch-and-site approach in PAML identified 2 sites (codons 57 and 71) evolving under positive Darwinian selection in the stem branch of the octodontid tree (Fig. 1). In addition, MEME suggested that site 71 is evolving under positive selection in two additional branches, those leading to *T. barrerae and S. cyanus*. TreeSAAP yielded similar results, as they suggest that changes in 57 in the stem of the tree of octodontid α-globins and in site 71 in the branches leading to *T. barrerae* are adaptively significant with regard to physicochemical properties. These results are conservative, and Table 2 presents marginal results data that could be of interest for future studies (see also Figs. 2 and 3 in Tomasco et al., 2017 for MEME and TreeSAAP results, respectively). Branch-siteREL did not find any lineages at which a proportion of sites evolve with dN/dS > 1.

4. Discussion

Despite the large differences in the approaches used, all method agree in recognizing sites 57 and 71 as having been under the influence of weak and/or episodic positive selection in particular branches of the tree. Interestingly, the amino acid replacements involved in these sites are similar to those reported for other species of mammals as adaptations of the α-globin to hypoxia, as discussed below.

Evidence of positive selection was found on basal branch of octodontids at site 57, that involves a replacement of a hydrophobic and apolar amino acid Ala by a hydrophilic amino acid Ser. This site is adjacent to a highly conserved His among orders of mammals. The imidazole group of this aminoacid interacts with oxygen facilitating the reversible binding with protein and assists in stabilizing the bond $Fe—O_2$ (Storz and Moriyama 2008). This means that a single amino acid change could explain differences in the oxygen affinity facilitating loading and unloading of O_2. Traced on the phylogeny, this aminoacid replacement would be showing an adaptation to fossoriality basal to octodontids. It is expected that the fossorial also face periods of hypoxia in some instances, such as periods resting in the nest. A similar replacement was reported as an adaptation to highland hypoxia in *P. maniculatus,* i.e.: a replacement of Ala by Gly in site (Storz et al., 2007).

Site 71 codes for an amino acid involved in α helix adjacent to a highly conserved His, and presents three amino acid replacements on different branches. On the basal branch of octodontids Gly, a non polar aminoacid, is substituted by Asp, which is negatively charged. This replacement would change the net charge of the protein and possibly its tertiary structure. This substitution could also suggest a general adaptation to fossoriality. Within octodontids, there are two more substitutions. In the branch leading to *T. barrerae* Asp was replaced by Glu, which would have significantly changed the physicochemical properties of the protein. On the branch leading to the coruro *S. cyanus* Asp is replaced by Asn. Interestingly, this change has also been reported as an adaptation to hipoxia in *P. maniculatus.*

There are also other amino acid replacements that showed marginal values in the MEME that deserve discussion. Site 130 is marginally highlighted by MEME to be under positive selection on the branch leading to *P. longicaudatus*, in particular a replacement of a hydrophobic

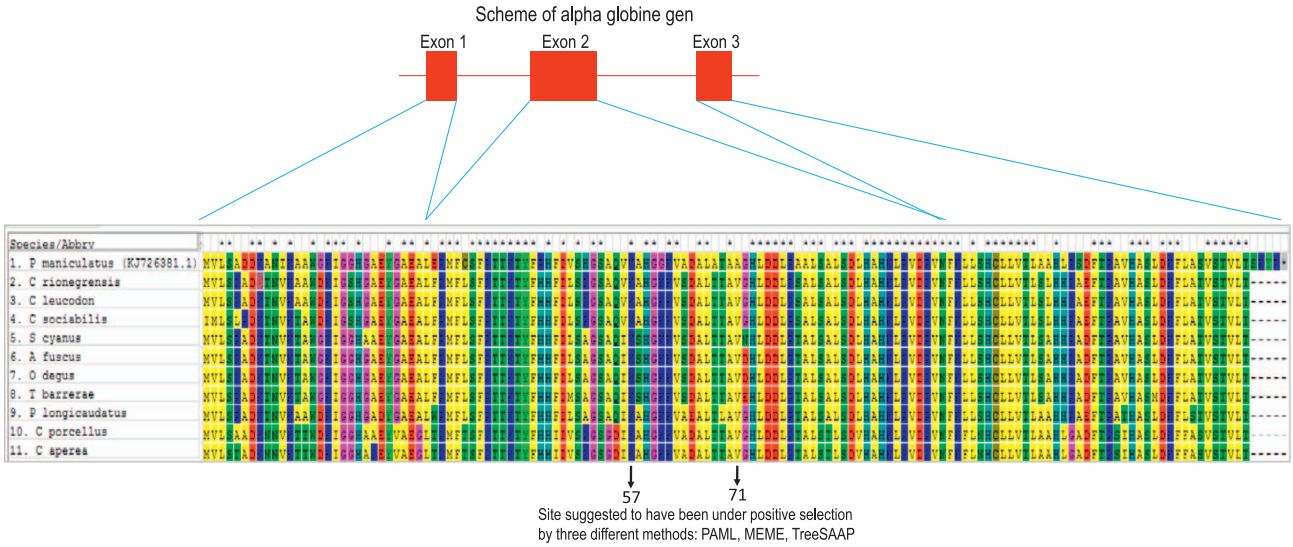

Fig. 2. Amino acid alignment of α-globine for all species analysed. A schematic representation of the structure of the gene is included, and also the sites that were found under positive selection by three different methods.

Table 2
Description of relevant sites that were showed by different methods to be under positive selection.

Site	Change	Branch under positive selection	Method with p value or post. probability (*)		
			PAML	MEME	TREESAAP
12	Thr > Ala	*C. leucodon* + *C. rionegrensis*	–	$p = 0.14$	–
		P. longicaudatus	–		–
57	Ala > Ser	basal octodontid	$p = 0.919$*	$p = 0.01$	$p < 0.001$
71	Gly > Asp	basal octodontid	$p = 0.838$*	$p = 0.04$	–
	Asp > Glu	*T. barrerae*	–		$p < 0.001$
	Asp > Asn	*S. cyanus*	–		$p < 0.001$
111	Ala > Leu	basal *Ctenomys*	–	$p = 0.10$	–
130	Ala > Ser	*P. longicaudatus*	–	$p = 0.07$	–

Ala by a hydrophilic Ser. Although the biological interpretation of this replacement would be under positive selection is beyond the scope of this paper, it is interesting to note that similar substitution is responsible for the relatively high Hb-O_2 affinity of the vicuña (*Vicugna vicugna*, i.e.: α130(H13)Ala > Thr), which introduces a polar hydroxyl group that inhibits Cl^- binding at the neighboring α131Asn residue (see Storz and Moriyama 2008 and references therein). The same replacement that found in *P. longicaudatus*. α130:Ala > Ser was found in elephant and it was proposed that the effect on O_2 affinity is the same that in vicuña (Weber 2007). Another site is 111 on the basal branch of *Ctenomys*, where Ala is replaced by Leu, involving two nucleotide substitutions. While both are hydrophobic amino acids, Leu presents a much larger and branched functional group and may produce steric impediment in the protein. The other is site 12 on the branch leading to the common ancestor of *C. rionegrensis* and *C. leucodon*. In this case a hydrophilic Thr was replaced by a hydrophobic Ala, certainly modifying the physiochemical properties of protein. None of these substitutions are found in *C. sociabilis*, a species that is sister to all other species of tuco-tucos sequenced so far (Parada et al., 2011; Pearson and Christie, 1985) and that keeps its burrows open and may thus face less hypoxic conditions than the other two species.

As stated above both sites found under positive selection in this study, site 57 and 71 of α globin, are involved in the very well-studied case of adaptation to hypoxia of *P. maniculatus*, in which the high- and low-affinity alleles differ in combinations of changes in sites 50, 57, 60, 64 and 71. We are not suggesting that substitution of found in our study have the same effects of mutations found in *P. maniculatus*. Indeed, we are aware that substitution are not the same, the genetic/protein backgrounds is key in determining the possible effect of the mutation, and more research is needed to determine the specific roles of selected substitutions in this group. Functional experiments on native Hb variants (e.g.: Revsbech et al., 2013) and protein engineering experiments based on site directed mutagenesis could reveal the phenotypic effects of these specific amino acid replacements (eg.: Natarajan et al., 2015, Cheviron et al., 2014), and structural analysis could reveal that epistasis for Hb-O_2 affinity and allosteric regulatory control may be attributable to indirect interactions between structurally remote sites (Natarajan et al., 2013).

These results suggest that the evolution of α globin in octodontoids was in part modeled by positive selection. However, we failed to find a direct association between subterranean lifestyle and molecular adaptation. Rather, the best candidate cases of adaptive substitutions were found at the base of octodontids, a family that includes one subterranean genus and several fossorial relatives. Typically subterranean tuco-tucos, including the high altitude *C. leucodon*, lacked these changes, although other sites of potential interest for future studies were noted. Further studies are needed to identify the real role of those amino-acid replacement selected, such as biochemical and physiological assays activity in different conditions. Also, the characterization of β globin gene and the study other globin genes deserve to be evaluated (Avivi et al. 2010). Finally, sampling the large number of tuco-tuco species would provide more statistical power and increase the ability to examine adaptive changes along multiple lineages.

5. Conclusion

The expectation of convergent evolution of HBA in fully subterranean tuco-tucos and coruros was not met by the data. Rather some putatively adaptive changes in the octodontid rodents appear to be associated with the evolution of fossoriality in general, and not to the evolution of fully subterranean habits. These changes could nonetheless pave the way for positive selection of subsequent changes in other genes. However, if we consider the evolution of α-globin at a higher hierarchical level, it is worth noting that the same sites and changes were found posited to be under positive selection in the α-globin of octodontoid rodents and in other rodent groups.

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