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Adaptaciones a la hipoxia e hipercapnia del nicho subterráneo en roedores octodontoideos.

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RESUMEN

La predominancia de la selección natural positiva como mecanismo evolutivo ha sido controversial desde un principio y lo es, en alguna medida, hasta nuestros días. Encontrar ejemplos de ésta a diferentes niveles de organización biológica es relevante en biología evolutiva. La evolución adaptativa de mamíferos subterráneos, es decir aquellos que viven y realizan la mayoría de sus actividades bajo tierra, es un ejemplo de evolución convergente en respuesta a restricciones similares. Entre los desafíos ambientales más importantes impuestos por este hábitat se encuentran la baja disponibilidad de O_2 (hipoxia) y el exceso de CO_2 (hipercapnia). En Sudamérica dos géneros de roedores octodontoideos, los tuco-tucos (*Ctenomys*) y el coruro (*Spalacopus*), han colonizado recientemente el nicho subterráneo y constituyen una oportunidad única para trazar la evolución de adaptaciones fisiológicas y moleculares, estudiándolas dentro de un marco temporal y filogenético conocido. En esta tesis se investigan algunas adaptaciones a la hipoxia e hipercapnia en roedores octodontoideos subterráneos desde diferentes perspectivas. A nivel fisiológico, se evalua la respuesta respiratoria frente a diferentes niveles de O_2 y CO_2 en el coruro y una especie fosorial, el degu (*Octodon degus*). En relación con los degus, los coruros muestran una respuesta más atenuada frente a la hipercapnia, es lo esperable para los animales subterráneos, mientras que es más acentuada a la hipoxia e hiperoxia. A nivel molecular, se analizan los patrones de evolución de genes codificados por genoma mitocondrial, comparando los dos linajes subterráneos con sus parientes cercanos no subterráneos, debido a que la hipoxia y/o hipercapnia podrían haber propiciado la evolución de adaptaciones en los genes implicados en la respiración celular. En particular: *i*) se secuencian y describen los genomas mitocondriales completos de 7 especies de estos roedores y se los compara con las dos especies de roedores histricognatos reportados en Genbank, *ii*) se aportan datos sobre las relaciones filogenéticas entre ellas y se estiman tiempos de divergencia entre linajes, *iii*) se evalúa la acción de la selección positiva en todos los genes codificantes de proteínas, y *iv*) se analiza con detalle la evolución del gen de la subunidad II de la citocromo oxidasa c (COX2), en un grupo más amplio de especies, con una mayor representación de especies de tucu-tucus. Los genomas mitocondriales de estos roedores no tienen apartamientos importantes en sus patrones de evolución con respecto a los de otros mamíferos. Su análisis apoya hipótesis filogenéticas previamente propuestas, aunque sugiere que la invasión del nicho subterráneo es anterior a lo previamente propuesto. Además, se encuentra en muchos, pero no todos los genes estudiados, una aceleración en las tasas de reemplazo aminocídico y una concentración de cambios de reemplazo desestabilizantes en los linajes subterráneos; el caso más extremo fue COX2. El mismo patrón fue encontrado en un estudio incluyendo los octodontoideos y varios linajes subterráneos adicionales, en el gen del citocromo b, también mitocondrial. El conjunto, los resultados pueden ser interpretados como una huella consistente de adaptaciones a la hipoxia en un contexto de fuerte selección purificadora.

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INTRODUCCION

Breve reseña histórica: neutralismo *vs.* seleccionismo

En su libro “El origen de las especies ….” (On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life) Charles Darwin (1859) propuso que todos los organismos sobre la tierra habrían evolucionado a partir de un único o unos pocos organismos primitivos por descendencia con modificación y que la principal fuerza de evolución sería la selección natural. La mayoría de los biólogos aceptaron la primera proposición mientras que la segunda fue controversial desde un principio y, en alguna medida, lo es hasta nuestros días (Nei 2005).

El neodarwinismo alcanzó su máximo apogeo en los 1950s y 1960s, y hasta ese momento se pensaba que casi todas las características morfológicas o fisiológicas habían evolucionado por selección natural. Esta situación comenzó a cambiar a partir de mediados de los 1960s cuando los primeros estudios moleculares con electroforesis y secuencias aminoacídicas de proteínas comenzaron a revelar propiedades interesantes de la evolución molecular, en particular: i) que la cantidad de polimorfismo intraespecífico era inesperadamente alta, ii) que la tasa de cambio evolutivo era relativamente constante y iii) que la cantidad de cambio era inversamente proporcional a la importancia de la proteína estudiada. En ese momento muchos evolucionistas pensaban que la variación intraespecífica debía ser mantenida por selección balanceadora.

En este contexto es que Kimura (1968) y King y Jukes (1969) proponen formalmente la teoría neutral de la evolución molecular, donde se plantea que la mayoría de los cambios evolutivos en las proteínas serían neutros y serían mantenidos en las poblaciones por deriva genética. Poco tiempo después Ohta (1973, 1974) propone una ampliación de la teoría anterior, reformulada más recientemente (Ohta 1992, 2002), llamada teoría cuasineutral. Propone que la mayoría de los cambios evolutivos no serían neutros estrictamente, sino cuasineutrales; es decir que serían débilmente deletéreos o ventajosos, con la condición de $|Ns| \leq 4$, siendo N el tamaño poblacional efectivo y s el coeficiente de selección para mutaciones en heterocigosis. Su destino dependería fundamentalmente del azar y, a diferencia de la teoría neutral original, de los coeficientes de selección y del tamaño poblacional. En poblaciones pequeñas estas mutaciones se comportarían como neutras mientras que en las poblaciones grandes el efecto de la

selección sería mayor. Sin embargo, para algunos autores esta teoría es esencialmente la misma que la teoría neutral original; de hecho, las mutaciones, incluso con valores |Ns| mayores se podrían considerar neutrales si N es grande (Nei, 2005).

Independientemente de las interpretaciones divergentes, estaba claro a principios de los 1980s que los grandes patrones de variación proteico dentro y entre las especies se ajustaban a las predicciones de la teoría neutral, por lo que la misma desafiaba la teoría sintética reinante. Esto no significa que todas las sustituciones aminoacídicas sean neutrales o cuasineutrales; deben existir sustituciones aminoacídicas que cambien la función de la proteína y sean seleccionadas. Estas son la fuente de adaptaciones y varias han podido ser identificadas, como se discutirá más adelante. La diferencia entre ambas teorías radica en la importancia relativa que tienen la deriva y la selección positiva en mantener y fijar la menor parte de la variación no deletérea que no es eliminada por la selección purificadora. El progreso más importante en el estudio de la evolución en ese período fue que la teoría neutral generó muchas predicciones sobre las distribución de frecuencias alélicas dentro de las poblaciones, y las relaciones entre la variación genética dentro y entre poblaciones, de manera que se podría evaluar la aplicabilidad de la teoría neutral a datos reales usando varios métodos estadísticos. En otras palabras, uno podría usar la teoría neutral o cuasineutral como hipótesis nula para el estudio de la evolución molecular (Nei 2005). Este tipo de estudio estadístico no se podía hacer antes de que la teoría neutral haya sido formulada.

Durante las últimas 40 años, se ha producido un enorme progreso en el estudio de las bases moleculares de la evolución fenotípica. Dos acontecimientos fueron claves. Por una parte, se hizo posible el trabajo con secuencias de ADN que son más informativas que las de proteínas. Gran parte del ADN no es traducido (como las regiones intergenómicas, intrones regiones flanqueantes, regiones reguladoras, entre otras) y el código genético es degenerado, por lo que una cierta proporción de sustituciones no resultan en sustituciones aminoacídicas. La variación genética en todas estas regiones puede ser estudiada únicamente examinando las secuencias de ADN. Por otro lado, en las últimas dos décadas ha sido posible acceder a la secuenciación de ADN a gran escala y la generación de secuencias de genomas completos, lo que ha permitido estudiar problemas clásicos a otra escala e identificar fenómenos y procesos antes inimaginables. Por ejemplo, mediante la comparación de genomas se han encontrado segmentos de

ADN de entre 1Kb a varias Mb de largo que difieren en el número de copias presentes en un organismo, a diferencia de lo "clásicamente" planteado para seres diploides (tener dos haplotipos de cada región autosómica, una por cromosoma). Esta variación en el número de copias de ADN parecería ser un fenómeno muy extendido y común entre los humanos (Sebat et al. 2004, Iafrate et al. 2004) y se generarían *de novo* durante las primeras fases del desarrollo (Bruder et al. 2008). Esta variación también se ha relacionado con varias enfermedades y otras cualidades de los organismos (http://www.sanger.ac.uk/humgen/cnv/). Las diferentes hipótesis sobre los procesos que generan y mantienen estas regiones están siendo actualmente evaluadas (Nozawa et al. 2007), e incluyen explicaciones de corte tanto neutralista como seleccionista. Más en general, la teoría neutral de la evolución molecular sigue siendo la hipótesis nula contra la cual evaluar, para cada caso particular, la acción de la selección positiva a nivel molecular (e.g.: Nei et al. 2010).

La larga controversia entre seleccionismo y neutralismo, y la necesidad de un estudio pormenorizado "caso a caso", indica que la comprensión de los mecanismos de evolución es fundamental y que la resolución del problema es extremadamente compleja. Actualmente, la selección natural es aceptada como mecanismo principal en la evolución de caracteres morfológicos, si bien el efecto de la deriva genética en los mismos también sigue siendo cuestionada (Nei 2005). La búsqueda de adaptaciones anatómicas, fisiológicas y de comportamiento es común, y existen numerosos ejemplos de la acción de la selección positiva en todos estos tipos de caracteres. Estos hallazgos no son banales y requieren confirmar la presencia de características que tengan una base heredable y aumenten la eficacia darwiniana de los individuos que las portan. Todas estas características fenotípicas (en un sentido amplio) tienen una base genética y por tanto la evolución fenotípica debería poder ser explicada por la evolución a nivel genómico. Incluso si se aceptarse una visión "neutralista", en que la mayoría de las sustituciones a nivel molecular son neutras o cuasineutras y su destino depende en mayor medida de la deriva genética, deberíamos ser capaces de encontrar las bases moleculares de la evolución fenotípica, es decir, aquellas sustituciones efectivamente adaptativas que se han fijado por selección positiva.

No es sencillo encontrar las bases genéticas de las características fenotípicas que han sido seleccionadas, es decir, mostrar evidencia de selección darwiniana positiva a

nivel molecular. Algunos ejemplos clásicos bien corroborados son la evolución de la hemoglobina (Hb) a bajos niveles de oxígeno en mamíferos de altitud, buceadoras y subterráneos, la evolución de las lisozimas en respuesta a la acidez estomacal en herbívoros, o de las opsinas (pigmentos oculares) para la visión en color de diferentes vertebrados (revisados en Nei 2005). La búsqueda de adaptaciones a nivel molecular asociadas a un cambio ecológico en un contexto filogenético conocido es de suma importancia para una teoría de la evolución completa.

Detección de adaptaciones moleculares

En las últimas dos décadas se ha prestado gran interés a la detección de selección positiva en genes codificantes de proteínas (Wong et al. 2004), aunque los casos en que la selección natural a nivel molecular ha sido puesta en evidencia son escasos (revisado por Endo et al. 1996, Yang y Bielawsky 2000, Ford 2002, Delport et al. 2009). La selección natural muchas veces opera sobre uno o unos pocos sitios aminoacídicos y estos sitios pueden ser identificados analizando la estructura cristalográfica de la proteína o mediante métodos estadísticos. En este período se han desarrollado varios métodos estadísticos para evaluar la teoría neutral y éstos podrían ser agrupados en seis categorías principales.

La primera categoría examina el patrón de distribución de los cambios nucleotídicos y detecta selección positiva o negativa evaluando desviaciones de la distribución esperada por neutralidad (Tajima 1989, Fu y Li 1993, Fu 1997, Simonsen et al. 1995). La segunda categoría de test consiste en considerar dos o más loci y examinar la consistencia de la variación dentro y entre especies (Hudson et al. 1987). De encontrar inconsistencia se invoca a la selección en uno o más loci de los estudiados. Estas primeras dos categorías son adaptaciones para ADN de pruebas anteriores para electroforesis de proteínas. La tercera clase de pruebas examina si la relación de cambios sinónimos y no-sinónimos dentro de las poblaciones (polimorfismos) es la misma que entre poblaciones (sustituciones) (McDonald y Kreitman 1991). La cuarta categoría usa la distribución de frecuencias alélicas de equilibrio (steady-state) de Wright (1938) bajo mutaciones irreversibles separadamente para sitios sinónimos y no-sinónimos (Sawyer y Hartl 1992). La quinta evalúa la relación de la tasa de cambios nucleotídicos sinónimos

(dS) con respecto a la de cambios no sinónimos (dN). La sexta y última categoría evalúa la calidad del cambio nucleotídico con respecto al efecto que este tiene sobre las propiedades fisicoquímicas de los aminoácidos que codifican (e.g.: Datta et al. 2010, Woolley et al. 2003). Hay también muchos otros tests, pero que no son usados tan frecuentemente (Kreitman 2000), por lo que no se consideraron en esta breve revisión.

En la práctica, todos estos métodos (excepto las dos últimas categorías) son dependientes de las asunciones que la población tiene tamaño constante a lo largo de su evolución. Si esta asunción es violada, la interpretación de los resultados se complica, y aún si las asunciones se cumplen aproximadamente, el poder estadístico de esos métodos es generalmente bajo (Nei 2005). En parte por esto es difícil obtener evidencia convincente de selección positiva (Kreitman 2000, Wright y Gaut 2005). Estas últimas dos categorías (evaluar dN/dS o las propiedades del cambio aminocídico) son las únicas que no requieren asunciones sobre la estructura poblacional y condiciones de equilibrio (Ford 2002), y al mismo tiempo permiten hacer comparaciones interespecíficas sin involucrar el nivel poblacional. Es por ello que serán las aproximaciones usadas en esta tesis. Estos tests tienen sus propias asunciones, claro está, por ejemplo sobre el sesgo en el uso de codones, la relación entre las tasas de transiciones y transversiones, entre otras, que de ser violadas, pueden producir resultados erróneos, pero estos problemas pueden ser evitados potencialmente usando un modelo de evolución apropiado al realizar el test (Yang y Bielawski 2000, Yang y Nielsen 2000). En los siguientes párrafos se hará una descripción somera de las pruebas que serán las utilizadas en los capítulos 3 y 4 de esta tesis.

En general, un exceso de cambios no sinónimos con respecto a los sinónimos en los genes codificantes de proteínas, es una muestra indiscutible de selección positiva a nivel molecular. La acción de la selección positiva ha sido demostrada de esta forma en varios sistemas clásicos (Li 1997), como el complejo principal de histocomatibilidad (Hughes y Nei 1988), la lisozima estomacal de primates (Messier y Stewart 1997), las lisinas espermáticas de abalones (Lee et al. 1995) y los genes del virus HIV humano (Yamaguchi- Kabata y Gojobori 2000), entre otros (revisado por Endo et al. 1996, Yang y Bielawski 2000, Ford 2002, Nei 2005, entre otros), así como en numerosos genes en diferentes organismos. Entre los casos mejor estudiados se encuentran varias proteínas involucradas en la reproducción (revisado por Swanson y Vaquier 2002, Yang y Bielawsky

2000), el sistema inmune (revisado por Nei 2005) y la auto-incompatibilidad en plantas (Takebayashi et al. 2003) y genes del virus HIV humano (Nielsen y Yang 1998), entre otros.

La idea subyacente a este método es que, como las mutaciones sinónimas son invisibles a la selección natural (es suficiente con que estén, como máximo, bajo un régimen estable de selección purificadora muy débil) mientras que las no sinónimas pueden estar bajo fuertes presiones selectivas, la comparación de las tasas de fijación de estos dos tipos provee una poderosa herramienta para evaluar la importancia de la selección natural en el proceso de evolución molecular. Las tasas dN y dS están definidas como el número de sustituciones no sinónimas y sinónimas por sitio, respectivamente, y su relación dN/dS (comúnmente llamada ω) es una medida de la presión de selección a nivel de la proteína. Un $\omega>1$ significa que las mutaciones no sinónimas han conferido una ventaja adaptativa a la proteína y se han fijado con mayor frecuencia que la sinónimas. Es decir, que los sitos con $\omega>1$ serían portadores de adaptaciones a nivel molecular. Los valores de $\omega=1$ y $\omega<1$, indicarían neutralidad estricta o selección purificadora, respectivamente. Los análisis de la relación en ω basados en filogenias han ayudado a descubrir patrones de evolución consistentes con episodios de selección direccional positiva.

Dos clases de métodos han sido propuestos para estimar dN y dS entre dos secuencias de nucleotídicas codificantes de proteínas. La primera de ellas incluye más de una docena de métodos intuitivos desarrollados desde la década de 1980 (Miyata y Yasunaga 1980, Nei y Gojobori 1986, Li et al. 1985, Li 1993, Pamilo y Bianchi 1993, Comeron 1995, Ina 1995, Yang y Nielsen 2000). Estos métodos implican los siguientes pasos: contar los sitios sinónimos (S) y no sinónimas (N) en los sitios de las dos secuencias, contar las diferencias sinónimas y no sinónimas entre las dos secuencias, y corregir para múltiples sustituciones en el mismo sitio. S y N se definen como el largo de la secuencia, multiplicado por la proporción de cambios sinónimos y no sinónimos antes que la selección actúe sobre la proteína (Ina 1995, Goldman y Yang 1994). La mayoría de estos métodos hacen suposiciones simplistas sobre el proceso de sustitución nucleotidica e implican un tratamiento *ad hoc* de los datos (Ina 1995, Yang y Nielsen 2000), por lo tanto, muchas veces se refiere a ellos como métodos aproximados (Yang y Bielawski 2000, Yang y Nielsen 2000). A pesar de que para muchos de ellos ha habido mejoras

sustanciales incorporando, por ejemplo, el sesgo transicional, el sesgo en el uso de codones ha sido ignorado (ver también Yang y Nielsen 2000) y puede tener efectos devastadores en la estimación de dN y dS (discutido en Yang y Bielawski 2000, entre otros).

La segunda clase de métodos utilizan modelos de máxima verosimilitud (ML) y se basan en modelos explícitos de sustitución entre codones (Goldman y Yang 1994, Muse 1996). Los parámetros en el modelo (como la relación transición/transversión y entre el dN y dS) son estimados a partir de los datos por ML, y se utilizan para el cálculo de dN y dS de acuerdo a su definiciones (Goldman y Yang 1994, Yang y Nielsen 2000, Yang 2001). Una característica importante del método es que el modelo se formula a nivel de las tasas instantáneas (donde no hay posibilidad de cambios múltiples) y el análisis lleva a cabo todas las tareas difíciles en un solo paso: la estimación de parámetros de mutación, la corrección para sustituciones múltiples, y la ponderación de los cambios entre los codones. Finalmente, se aplican pruebas estadísticas para comprobar si dN es significativamente mayor que dS. Para los métodos aproximados, se utiliza la aproximación dN-dS. Por ML, el test utiliza la relación de los valores de verosimilitud (llamado LRT, por su sigla en inglés *Likelihood Ratio Test*), y el doble de la diferencia entre el logaritmo de la verosimilitud cada modelo a contrastar, se compara con la distribución Chi cuadrado con tantos grados de libertad como la diferencia en el número de parámetros estimados en ambos modelos.

Utilizando la aproximación por ML, el parámetro ω puede ser estimado de diferentes formas: i) por “ramas”, en donde se estima ω para todo el gen (o parte del mismo) para cada rama dentro de una filogenia (Messier y Stewart 1997, Zhang et al. 1998, Yang 1998, Yang y Nielsen 1998; Kosakovsky Pond y Frost 2005a); (ii) por “sitio” que estima ω para diferentes sitios dentro de un gen a través de toda la filogenia (Nielsen y Yang 1998; Swanson et al. 2003, Suzuki 2004); y (iii) ‘‘rama-stio’’ estima ω para diferentes sitios en diferentes ramas a lo largo de una filogenia (Yang y Nielsen 2002; Forsberg y Christiansen 2003, Clark et al. 2003; Guindon et al. 2004). Alguno de estos modelos son los más utilizados hasta ahora, fundamentalmente aquellos implementados en el programa PAML (Yang 1997 y 2007).

Los modelos de sitios han sido los más utilizados (ver recuadro 1) y han tenido éxito en identificar codones específicos bajo selección, pero poco se sabe de las propiedades fisicoquímicas particulares que están siendo sujetas a selección. Esto sería importante, por ejemplo en el caso de genes virales, porque identificar los sitios específicos bajo selección considerando las propiedades fisicoquímicas bajo selección puede arrojar luz sobre la bioquímica subyacentes a la evasión mutacional de una respuesta inmune (e.g.: Yang et al. 2003). Con esta motivación Sainudiin et al. (2005) condujeron un estudio para detectar la acción de la selección positiva sobre propiedades fisicoquímicas en codones individuales usando máxima verosimilitud (siguiendo a Nielsen y Yang 1998) y generalizando el concepto de ω reemplazándolo por γ, la relación entre cambios que alteran y no alteran las propiedades, para explorar las propiedades fisicoquímicas que fueron blanco de la selección. Posteriormente, Wong et al. (2006) proponen una generalización del método anterior que permite detectar la acción de la selección natural sobre sitios específicos y/o sobre propiedades fisicoquímicas de interés, espeficiadas *a priori*.

De todas formas, el criterio de detectar selección positiva por medio de un exceso de cambios de reemplazo sobre cambios sinónimos, es muy conservador para usos prácticos (e.g.: Nozawa et al. 2009, McClellan et al. 2005, Yang y Bielawski 2000), porque los genes conservados casi siempre tienen más sustituciones sinónimas que no sinónimas aún si algunos sitios han sido influenciados por la selección positiva. Incluso, algunas clases de selección, como la selección balanceadora, no serían detectables con este método. Además, como generalmente el cambio adaptativo radica en uno o unas pocas sustituciones aminoacídicas, la comparación pareada entre secuencias, tiene poco poder porque promedia los valores de ω sobre todos los sitios y linajes. Los métodos para detectar selección a lo largo de linajes funcionan sólo si ω promediada sobre todos los sitios es > 1. De forma similar, el test de selección positiva por sitios funciona solo si ω promediada entre todos los linajes es > 1. Si la evolución adaptativa ocurre sólo en un breve período de tiempo y afecta unos pocos sitios aminoacídicos, ninguno de los métodos anteriores daría resultados significativos. En estos casos ω puede ser bajo y menor a 1, y no ser detectado por esos métodos (e.g.: Yokoyama y Takenaka 2004). Finalmente, los modelos que permiten evaluar la variación en ω a lo largo de ramas y sitios simultáneamente, deberían incrementar el poder de estos tests, pero en la práctica sólo son posibles de implementar con ramas establecidas *a priori* (Yang y Nielsen 2002,

Forsberg y Christiansen 2003, Clark et al. 2003), presentan una tendencia a generar falsos positivos (Nozawa et al. 2009), son computacionalmente costosos y con resultados aún difícilmente interpretables (Guindon et al. 2004,ver también Zhai et al. 2007). Por otro lado, valores altos de ω en algunos sitios, no necesariamente significan que haya ocurrido un cambio funcional en un gen. En hemoglobinas, que son conocidas por ser conservadas, la tasa de sustitución aminoacídica es mayor en la región superficial de la molécula que en el interior o en los grupos hemo, pero estos cambios parecen no afectar de manera apreciable su función (Massingham y Goldman 2005, ver también Yang et al. 2000). Finalmente, una vez que se detecta la selección positiva mediante un ω mayor a uno, esto no provee evidencia que sugiera la causa de esta selección, pudiéndose inferir una posible causa viendo el dominio afectado de la proteína. Es por eso que sería importante encontrar cambios funcionales en las proteínas que identificar sitios con valores de dN/dS altos (Gu 1999, 2001, Knudsen y Miyamoto 2001, Nam et al. 2005).

En virtud de estas dificultades, surgieron algunos modelos estadísticos alternativos que evalúan los cambios en las propiedades fisicoquímicas de los aminoácidos (Xia y Li 1998, McClellan y McCraken 2001, Pupko et al. 2003, Suzuki 2007, Datta et al. 2010). A pesar de que la comparación estadística formal entre cambios radicales y no radicales como prueba estadística de selección positiva está aún en discusión (Dagan et al. 2002, Hanada et al. 2007), algunos autores han propuesto métodos y evaluado sus cualidades (Hanada et al. 2007, Popadin et al. 2007). Entre los métodos que tienen en cuenta las propiedades aminoacídicas están los propuestos por Xia y Li (1998) y McClellan et al. (2005), que calculan la distribución esperada por azar de los cambios aminoacídicos posibles, en base a las diferencias fijas entre residuos, dada una propiedad amonoacídica particular. Comparando la distribución esperada con los cambios inferidos sobre una filogenia bien corroborada, es posible la detección de desviaciones significativas de las expectativas bajo neutralidad. Otra aproximación relacionada, es la propuesta por Pupko et al. (2003), que evalúa la existencia de desviaciones significativas de lo esperado por azar de la distancia fisicoquímica media a lo largo de un linaje o un subárbol. En todos ellos, la idea básica es detectar desviaciones significativas de un proceso consistente con la neutralidad. Por ejemplo, una tendencia a cambios radicales en una propiedad particular, es, en principio, inconsistente con la neutralidad. También Datta et al. (2010) han propuesto un modelo bayesiano jerárquico que permitiría determinar si un conjunto

Recuadro 1. Detección de selección natural positiva sobre codones.

Los modelos por sitio más utilizados hasta ahora son aquellos implementados en el programa PAML (Phylogenetic Analysis by Maximum Likelihood; Yang 1997 y 2007), y son derivados de los originalmente propuestos por Nielsen y Yang (1998) y Yang et al. (2000; ensayan modelos y distribuciones más realistas que las propuestas originalmente en 1998). Estos autores desarrollaron un método bayesiano usando una aproximación de máxima verosimilitud para detectar sitios posiblemente seleccionados positivamente. Aunque se critica que asume que las tasas relativas de sustituciones sinónimas y no-sinónimas son las mismas para todos codones positivamente seleccionados, lo que es poco realista, este modelo que ha sido y es ampliamente utilizado para detectar selección positiva a nivel molecular. Sin embargo, otros modelos han sido propuestos con el mismo fin, pero con menor éxito. Poco tiempo antes, Fitch y cols. (Fitch et al. 1997, Bush et al. 1999) usaron parsimonia para reconstruir las secuencias ancestrales, y contaron los cambios de cada codón a lo largo de las ramas del árbol. Ellos pusieron a prueba si la proporción de sustituciones sinónimas en cada sitio era mayor al promedio sobre todos los sitios. Poco después, Suzuki y Gojobori (1999) desarrollaron una aproximación más sistemática por Máxima Parsimonia intentando evitar las limitaciones de los anteriores e incorporando menos asunciones. Para cada sitio en la secuencia, ellos estimaron el número de sitios sinónimos y no sinónimos y las diferencias a lo largo de un árbol usando las secuencias ancestrales reconstruidas, y evaluaron si la proporción de sustituciones no sinónimas diferían de lo esperado bajo neutralidad ($\omega=1$). El criterio de Suzuki y Gojobori es más estricto que el de Fitch et al., porque la relación ω promediada entre todos los sitios es casi siempre menor a 1. Estos últimos dos métodos requieren muchas secuencias en el conjunto de datos para que haya cambios suficientes en los stios individuales, y han sido criticados porque su confiabilidad depende de la reconstrucción de las secuencias ancestrales, que es mayormente afectada en sitios bajo selección (Yang et al. 1995) o por el sesgo composicional, que es más extremo al considerar sitios individuales (Yang y Biealawski 2000). Además, las mejoras propuestas para el método de Suzuki y Gojobori (Su 2000 con cambios menores y Suzuki 2004 que incluye la inferencia bayesiana para la secuencia ancestral) han tenido poco éxito. Hace pocos años, el programa HiPhy (Kosakovsky Pond et al. 2005), ha implementado tres nuevos métodos que han comenzado a utilizarse con mayor frecuencia en los últimos años (Kosakovsky Pond y Frost 2005b, Kosakovsky Pond et al. 2006), e incluyen variación simultánea de las tasas de cambios sinónimas y no-sinónimas entre sitios. Más recientemente, otros autores han propuesto métodos similares pero en un marco completamente bayesiano (Huelsenbeck y Dyer 2004, Scheffler y Seoighe 2005, Ouyang y Liang 2007) para aprovechar la ventaja que brinda esta metodología al tener en cuenta la incertidumbre en los parámetros, incluyendo la topología del árbol, los largos de ramas y el modelo de sustitución de codones del ADN. Según sus autores, estos modelos completamente bayesianos, si bien implican un costo computacional mayor, serían más ventajosos que los anteriores al generar menos falsos positivos o negativos cuando las secuencias usadas son cortas o la divergencia entre ellas es muy alta o muy baja.

de propiedades aminoacídicas está siendo conservada o ha cambiado radicalmente a nivel del codón. El método propuesto por McClellan et al. (2005) ha sido implementado en el programa TreeSAAP (Woolley et al. 2003), ha sido usado en al menos una decena de estudios de selección y se ha evaluado su performance en comparación con otros (McClellan y Edison 2010, Maxwell et al. 2010).

Roedores subterráneos y adaptaciones a la hipoxia y/o hipercapnia

En todos los continentes, excepto en Australia y Antártida, existen 250 especies de roedores subterráneos aproximadamente (38 géneros, 6 familias según Begall et al. 2007). Estos animales están especializados en múltiples aspectos a su forma única de vida, desarrollando todas sus actividades vitales (forrajeo, apareamiento, etc.) casi exclusivamente bajo tierra dentro de cuevas construidas por ellos mismos. Otros animales construyen cuevas, pero realizan parte de sus actividades en superficie, y son llamados fosoriales. Por supuesto, existe un continuo entre ambas categorías. También, otros grupos de mamíferos como los topos (e.g.: Talpidae y Chrysochloridae), el topo marsupial (*Notoryctes typhlops*) y ciertos armadillos, entre otros (Begall et al. 2007), utilizan el ecotopo subterráneo.

La vida subterránea confiere ventajas y limitaciones frente a la vida en superficie, que imponen presiones selectivas particulares, esperándose que los roedores subterráneos compartan especializaciones no encontradas en los taxa de superficie (Buffenstein 2000, Nevo 1999). Específicamente, el ambiente subterráneo es oscuro, relativamente estable en temperatura y humedad, hipóxico e hipercápnico, y privado de la mayoría de las señales sensoriales disponibles en superficie. La cueva ofrece refugio de los predadores y de extremos climáticos, pero la excavación es energéticamente costosa, la producción primaria es relativamente baja y los recursos alimenticios escasos, además de impredecibles y con distribución en parches (Begall et al. 2007). Algunas de las adaptaciones morfológicas convergentes más llamativas que exhiben estos linajes, extremadamente distantes evolutivamente entre ellos, incluyen la forma compacta y cilíndrica del cuerpo, cola y cuello cortos, reducción de los ojos y oído externo, así como patas delanteras, parrilla costal y músculos asociados grandes y fuertes (Nevo 1999).

La evolución adaptativa de los mamíferos subterráneos involucra cambios estructurales y funcionales que son tanto regresivos (degenerativos) como progresivos (compensatorios) en la naturaleza (Nevo 1999). La evolución global convergente y en mosaico de los mamíferos subterráneos debido a restricciones y limitaciones similares es un ejemplo excelente de evolución por selección natural obtenido por métodos comparativos (Nevo 2001). Los mamíferos subterráneos nos informan acerca de la naturaleza de la radiación adaptativa, de la diversificación de especies en espacio y tiempo, de cómo los genotipos y fenotipos se forman, se pierden y convergen como si fuera un bricolaje de la selección natural.

A pesar de las llamativas características de estos animales, la biología de los mamíferos subterráneos se mantuvo por mucho tiempo sin estudiar, probablemente por los problemas asociados a su estilo de vida críptico y las técnicas de captura. Sin embargo, a partir de la revisión seminal de Eviatar Nevo en 1979 (Nevo 1979) y el informe de eusocialidad en la rata topo desnuda de Jennifer Jarvis en 1981 (Jarvis 1981), el descubrimiento por los mamíferos subterráneos en general y en roedores subterráneos en particular emergió (Begall et al. 2007). Desde entonces, el conocimiento de estos animales ha aumentado, habiéndose publicado posteriormente al menos cuatro libros de revisión sobre el tema: Nevo y Reig (1990), Nevo (1999), Lacey et al. (2000) y Begall et al. (2007).

Entre los desafíos ambientales más importantes impuestos por este hábitat se encuentra la baja disponibilidad de O_2 (hipoxia) y, especialmente, el exceso de CO_2 (hipercapnia) del ambiente subterráneo (Withers 1978, MacLean 1981 citados en Arieli 1990, Shams et al. 2005). Entre los animales que están adaptados a la hipoxia crónica (como los buceadores o aquellos que viven a alturas elevadas), los subterráneos son los únicos que están obligados a inhalar aire con elevadas concentraciones de CO_2 (Quilliam 1971). El intercambio gaseoso entre la cueva y el aire atmosférico depende de las propiedades de difusión del sustrato, influenciado por el tipo de suelo, la temperatura y humedad ambiente, etc. (Arieli 1979), y la baja ventilación que es alcanzada primariamente por el movimiento de los animales (Buffenstein 2000). Estos factores hacen que la composición gaseosa de las cuevas difiera considerablemente del aire exterior, aumentando esta diferencia con cualquier actividad realizada por sus habitantes. Esta

restricción es importante en sí misma, y más aún si se considera la alta demanda energética que implica la excavación necesaria para la vida bajo tierra y su concomitante requerimiento de O_2, así como la imposibilidad de acceder al aire exterior.

Las estrategias generales para el mantenimiento de un adecuado transporte de O_2 a los tejidos para un mamífero que enfrenta una situación de hipoxia/hipercapnia representan un compromiso entre el aumento en el aporte de O_2 y su consumo, por los gastos energéticos que implica (Nevo 1999). Por un lado, aumentar la presión parcial de O_2 (PO_2) alveolar incrementando la ventilación implica elevar el gasto energético, lo que requiere mayor consumo de O_2. Otra estrategia es aumentar la concentración de Hb en sangre de forma de transportar más O_2, pero esto aumenta la viscosidad de la sangre y por lo tanto la energía requerida para su propulsión. Las adaptaciones de los roedores subterráneos a la hipoxia e hipercapnia (revisado en Boggs et al. 1984, Nevo 1999, Buffenstein 2000) se evidencian a diferentes niveles, desde modificaciones en las propiedades de transporte de O_2 en sangre hasta la reconfiguración a nivel de células, órganos y tejidos.

Los estudios realizados hasta ahora muestran que las especies subterráneas *Thomomys bottae* (Lechner 1977) y *Spalax ehrembergi* (Arieli et al. 1977, Arieli y Nevo 1991), presentan mayor tolerancia a la hipoxia y/o hipercapnia, teniendo presiones críticas de O_2 (PO_2 ambiental límite por debajo de la cual el organismo no puede mantener su consumo necesario de O_2, y disminuye su metabolismo hasta morir) menores a lo esperado para mamíferos de superficie. Estos roedores alcanzan actividades metabólicas altas a baja PO_2 ambiental (Lovegrove 1989, Vleck 1979), a las que una rata de laboratorio no mantendría una tasa metabólica basal normal (Arieli y Kerem 1984). En estas especies, la ventilación en normoxia es baja (igual para *Talpa europea*, familia Talpidae, también subterránea) (Arieli et al. 1986a, Darden 1972, Quilliam et al. 1971) y existe una respuesta atenuada de aumento de la ventilación pulmonar frente a la hipoxia (Arieli y Ar 1979, Boggs et al. 1984). Una atenuación de la respuesta normal a la hipercapnia (aumento de la ventilación), se ha reportado tanto en mamíferos fosoriales como también en algunas aves fosoriales (Boggs et al. 1984), pero es más exagerada en los tres géneros subterráneos estudiados, los dos antes mencionados (Arieli y Ar 1979, Boggs et al. 1984, Darden 1972) y *Cryptomys hottentotus* (datos no publicados mencionados en Buffenstein, 2000). Por último, una combinación de hipercapnia e hipoxia,

que en mamíferos no subterráneos desencadenaría un aumento de la ventilación mayor a lo que cada uno de los estímulos haría por separado, puede ser tolerado por *S. erhembergi*, incluso continuando con sus actividades normales (Arieli et al. 1977).

Además, en varias especies subterráneas, como *T. bottae* (Chapmann y Bennett 1975, Lechner 1976), *T. eurpopea* (Orden Soricomorpha, antes Insectívora Jelkmann et al. 1981), *S. ehrembergi* (Arieli et al. 1986b), *C. hottentotus* (Broekman et al. 2006) y *Heterocephalus glaber* (Johansen et al. 1976) el hematocrito es alto y la concentración de Hb se encuentra en el límite mayor del rango para mamíferos terrestres, pero no es diferente de lo encontrado en mamíferos pequeños (Lee y Brown 1970), antílopes y venados (Pospisil et al. 1984). La afinidad por el O_2 de la Hb de muchas de estas especies es alta, y que puede lograrse por uno o combinaciones de los siguientes tres mecanismos: una alta afinidad intrínseca de la Hb (Johansen et al. 1976), un bajo nivel de 2,3-bifosfoglicerato (2,3DPG) en glóbulos rojos (Ar et al. 1977) o una reducida afinidad de la Hb con el 2,3DPG (Jelkmann et al. 1981), lo que también se ha encontrado para algunos mamíferos fosoriales (Arieli et al. 1986b, Boggs et al. 1984). Además, en 3 de estas especies (*T. bottae*, *T. umbrinus* (Lechner 1976), *S ehrembergi* (Ar et al. 1977) y en *Ctenomys talarum* (no publicado, citado por Arieli 1990) la concentración de mioglobina es mayor en el músculo esquelético pero no en el miocardio, en comparación con *Rattus novergicus* (Arieli 1990).

Otras adaptaciones morfológicas conocidas únicamente en especies del género *Spalax* incluyen la reducción de la distancia de intercambio gaseoso entre la sangre y los tejidos (Arieli 1990): i) incremento en la densidad capilar en el músculo esquelético, cardíaco y en los pulmones (Arieli y Arr 1981), ii) aumento del número relativo de mitocondrias en el músculo esquelético (Widmer et al. 1997) y iii) elevada concentración de mioglobina tisular y sanguínea (Ar et al. 1977, encontrado también para *T. bottae* (Lechner 1976) y *C. talarum* (citado en Arieli 1990). Además, en estas últimas especies (Ar et al. 1977, Arieli et al. 1986a) y en *T. europea* (Armsby et al. 1966) se ha encontrado una menor salida cardíaca y menor frecuencia cardíaca que lo esperado para su peso corporal en normoxia (Arieli et al.1986a).

Finalmente, las presiones parciales de CO_2 (PCO_2) sanguínea y tisular estimadas para *Spalax* y *T. bottae* (Arieli 1990) son mayores que las encontradas en mamíferos no

subterráneos (Ar et al. 1977), con valores que en estos últimos alterarían el equilibrio acido-base de la sangre. Existen al menos tres estrategias conocidas para sobrellevar las altas PCO_2. En algunas especies como *T. bottae* y *T. umbrinus*, esto no produce grandes cambios en el pH sanguíneo, por la alta concentración de Hb y fosfatos carbónicos en sangre (Chapman y Bennett 1975, Lechner 1976). Otras en cambio, como *S. erhenbergi* (Ar et al. 1977), y probablemente *C. hottentotus* (Quilliam et al. 1971, aunque ver vanAardt et al. 2007) toleran mayores diferencias en el pH sanguíneo y cuando éste disminuye la afinidad de la Hb por el O_2 aumenta llevando más O_2 a los tejidos (esto se explica por el "efecto Bohr"). Otro mecanismo se ha descrito recientemente para el *Scalopus aquaticus* (Talpidae), en donde la Hb tiene una menor afinidad por el O_2 (contrario a lo esperado para especies bajo hipoxia crónica) y es insensible al 2,3 difosfoglicerato, prácticamente ausente en los glóbulos rojos de esta especie (Campbell et al. 2010)

Por otra parte, hay autores que consideran que la hipoxia y la hipercapnia explicarían la observación de que la tasa metabólica basal o en reposo de mamíferos subterráneos es menor a lo esperado según tu tamaño corporal (revisado por Contreras y McNab 1990), y alcanza extremos en *H. glaber* (McNab 1979), como una forma de ahorro energético debido al gran costo de la excavación (Arieli 1990). Otra hipótesis alternativa propone que el factor desencadenante de este fenómeno sería evitar el sobrecalentamiento corporal y el ahorro energético asociado a la baja productividad del ambiente subterráneo (Arieli 1990). Evidencia adicional a favor de la primera hipótesis se obtiene al observar como la tasa metabólica va disminuyendo con la altitud en diferentes poblaciones de *S. cyanus* (Contreras 1986), en donde la subterranealidad se combina con la altitud para disminuir la PO_2 ambiental. Sin embargo, algunos especies como *S. ehrenbergi*, *T. talpoides* y *S. acuaticus* (McNab 1979) mantienen tasas metabólicas normales, y estudios recientes sobre el tema no han podido apoyar una u otra hipótesis en forma concluyente (Bozinovic et al. 2005, White 2003).

Las bases moleculares de estas adaptaciones están comenzando a ser dilucidadas. Los primeros estudios a nivel molecular fueron realizados sobre la secuencia de la Hb (cadenas cadenas α y β), y es bien conocida su adaptación a la hipoxia por altura en vertebrados (Weber 2007). En algunos mamíferos subterráneos ésta muestra una afinidad alta por el O_2. En *Talpa europea* la alta afinidad de la Hb se debe a un efecto reducido del 2,3-DPG, asociado a varios reemplazos aminoacídicos en la α-hélice

(Jelkmann et al. 1981, Nevo 1999). Una nueva adaptación a la hipoxia crónica ha sido descrita recientemente para *Scalopus aquaticus*, en donde la Hb tiene una menor afinidad por el O_2 (contrario a lo esperado para especies bajo hipoxia crónica) y es insensible al 2,3DPG prácticamente ausente en los glóbulos rojos de esta especie (Campbell et al. 2010), debido a un simple cambio de reemplazo en una cadena δ (δ136Gly-Glu).

Otros estudios fueron realizados posteriormente analizando la estructura primaria de la mioglobina, niveles del receptor del factor de crecimiento endotelial (VEGF), bicarbonatos urinarios y polimorfismos de la haptoglobina (revisados por Nevo 1999). Más recientemente, estudios de expresión de genes asociados a la tolerancia a la hipoxia en el género *Spalax* han encontrado diferencias entre sus especies más o menos tolerantes, y entre éstas y *R. novergicus* (Avivi et al. 2005 y 2006, Brodsky et al. 2005, Nasser et al. 2005, Polyakov et al. 2004, Shams et al. 2004, Sandwall et al. 2009, entre otros). Algunos de los genes analizados incluyen el VEGF, la heparanasa, genes supresores de tumores y asociados a la apoptosis (e.g.: Band et al. 2008, 2009, 2010), entre otros. Estos últimos estudios se han visto promovidos porque la respuesta celular frente a la hipoxia es semejante a la respuesta de células tumorales, por lo que entender la base molecular de dichas adaptaciones podría mejorar el desarrollo de nuevas modalidades terapéuticas para esta enfermedad (e.g.: Band et al. 2009). Además se ha sugerido que *Spalax* exhibe daño biomolecular bajo debido a la existencia de estrategias fisiológicas que le permiten evitar el daño oxidativo frente a niveles flucutantes de O_2 y CO_2, posiblemente por la mayor actividad de enzimas antioxidantes, principalmente de la superóxido dismutasa (Caballero et al. 2006).

Con respecto a las adaptaciones a la hipoxia, se puede deducir de la sección anterior que, si bien muchas características se han considerado como adaptaciones generales a la hipoxia e hipercapnia del ambiente subterráneo, las mismas sólo fueron evaluadas en pocas especies subterráneas, fundamentalmente de los géneros *Spalax* (mediterráneo), *Heterocephalus* (africano) y *Thomomys* (norteamericano). Además, pocas variables fueron estudiadas comparativamente en varias especies y los rangos de algunos parámetros se superponen con los de especies no subterráneas, no pudiéndose identificar un patrón general de la subterraneidad. Lo mismo ocurre con las PO_2 y PCO_2 de las cuevas, de los que hay registros formales para las especies *S. ehrenbergi* (Arieli 1979) y *T. bottae* (Darden 1972) (ver también Withers 1978, MacLean 1981 citado en

Buffenstein 2000). Además, es importante destacar que los grupos subterráneos estudiados son muy divergentes de sus hermanos no subterráneos (que a veces son desconocidos), dificultando la asignación de las diferencias encontradas a la vida subterránea.

Roedores caviomorfos como modelo de estudio

Se denomina informalmente *caviomorfos* al conjunto de roedores histricognatos (suborden Hystricognathi) sudamericanos. Su antigua clasificación como infraorden Caviomorpha se considera inapropiada debido a que es improbable que todos los roedores histricognatos sudamericanos representen una única radiación (Wilson y Reeder 2005). Sin embargo, varios trabajos moleculares apoyan la monofilia del grupo (e.g.: Nedbal et al. 1994, Huchon y Douzery 2001, Opazo 2005) y también su división en 4 superfamilias: Octodontoidea, Cavioidea, Chinchilloidea, y Erethizontoidea, propuestas originalmente por Woods (1982) (recuadro 2).

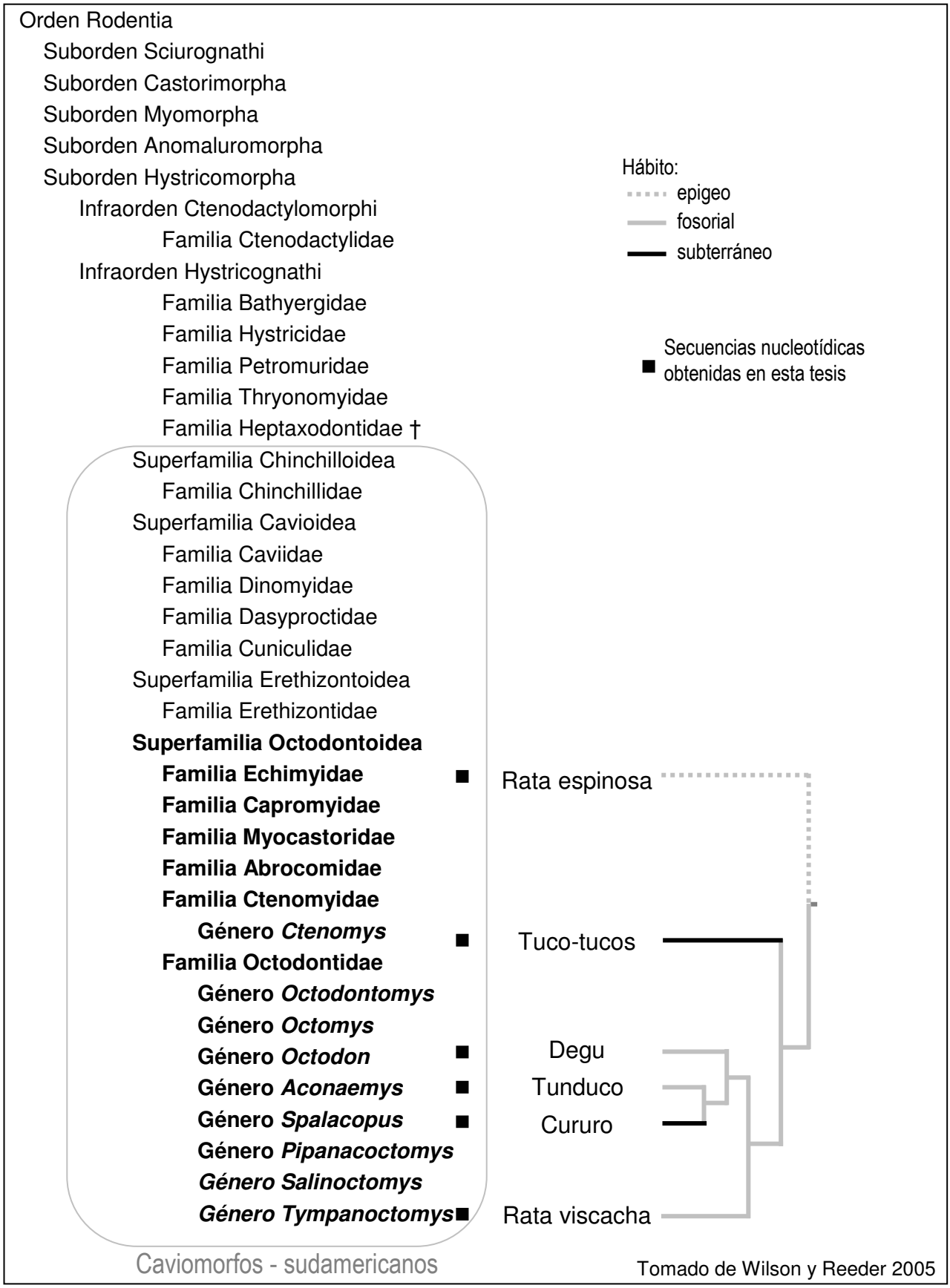
Los roedores caviomorfos son particularmente interesantes porque presentan características diferentes al resto de los roedores. En particular, son conocidos por presentar un período de gestación largo (desde 50 a 75 días), tamaño de camada bajo (entre 1 a tres crías) y crías altamente precociales, en relación a lo conocido en el resto de los roedores (Nowak 1991). A nivel molecular, presentan características particulares que condujeron en la década del 90 a cuestionar su estatus como roedores (Graur et al. 1991, Li et al. 1992), aunque estudios posteriores al 2000, con mayor representación de loci y taxa, reestablecieron el consenso sobre la monofilia del orden Rodentia (e.g.: Carleton et al. 2005, da Fonseca et al. 2008, Lin et al. 2002). Otro ejemplo particularmente llamativo de su diferenciación molecular es el gen de la Insulina: si bien su estructura y función es altamente conservada en mamíferos, en histricognatos presenta varias sustituciones aminoacídicas que tendrían consecuencias importantes en sus propiedades fisicoquímicas (Opazo et al. 2005). En particular en caviomorfos y durante la evolución de la superfamilia Octodontoidea habrían ocurrido en forma sucesiva cambios importantes, como la deleción de un aminoácido en la cadena B y dos inserciones en la cadena A (Opazo et al. 2005). Sin embargo, y a pesar de su importancia para la biología evolutiva,

los roedores caviomorfos no han sido estudiados en profundidad, a excepción de la especie modelo *Cavia porcellus*.

En Sudamérica, el nicho subterráneo ha sido colonizado recientemente por dos géneros de roedores caviomorfos: el género *Ctenomys* (los tuco-tucos) que presenta más de 60 especies vivientes, y el género monotípocio *Spalacopus* (conocido como coruro). Estos géneros pertenecen a las familias hermanas Ctenomyidae y Octodontidae, respectivamente (a veces clasificadas como subfamilias Ctenomyinae y Octodontinae dentro de la familia Octodontidae, Reig 1958, McKenna y Bell 1997 citados en Wilson y Reader 2005), compuestas principalmente de especies fosoriales. Dentro de la misma superfamilia, llamada Octodontoidea, existen otras cuatro familias con gran diversidad de hábitos de vida, incluyendo especies cursoriales y arborícolas. Esta superfamilia ha atraído la atención de los mastozoólogos por presentar una gran diversidad cromosómica, con números diploides que van desde 2n=10 (Anderson et al. 1987) a 2n=102 (Contreras et al. 1990).

Para comprender los patrones y procesos por los cuales la variabilidad de adaptaciones a la hipoxia e hipercapnia antes mencionada se originó y persiste, las familias hermanas Octodontidae y Ctenomyidae de roedores caviomorfos subterráneos representan una oportunidad única. Sería posible trazar la evolución de adaptaciones fisiológicas y moleculares, estudiándolas dentro de un marco temporal y filogenético conocido (recuadro 2). En primer lugar, las relaciones filogenéticas dentro y entre estas familias, y entre éstas y sus parientes más cercanos, están razonablemente establecidas (Opazo 2005 y citas allí). Lo mismo sucede con las correspondientes estimaciones de tiempos de divergencia, que son relativamente recientes si se los compara con los de otros grupos de roedores subterráneos. La divergencia estimada para la separación de las dos familias es entre 15 a 25 millones de años aproximadamente (Opazo 2005, Honeycutt et al. 2003, Gallardo y Kirsch 2001), y para la diversificación de Octodontoidea entre 15 y 30 millones de años aproximadamente (Opazo 2005, Huchon y Douzery 2001, Gallardo y Kirsch 2001). Por otro lado, estas familias hermanas se componen de especies estrictamente subterráneas y otras fosoriales. La comparación entre estos géneros subterráneos permitiría, a diferencia de los estudios tradicionales que comparaban

Recuadro 2. Clasificación del orden Rodentia indicando géneros utilizados en esta tesis.



subterráneos versus no-subterráneos, identificar y discriminar las adaptaciones propias de la subterranealidad estricta de aquellas que simplemente son adaptaciones más generales a la fosorialidad, en comparación con el resto de la superfamilia de hábitos epígeos

Objetivos generales y específicos.

En esta tesis se propone investigar algunas de las adaptaciones a la vida subterránea, en particular a la hipoxia e hipercapnia, en roedores octodontoideos subterráneos y no subterráneos, desde diferentes perspectivas.
En particular:

- a nivel fisiológico, se evaluará la respuesta de las variables respiratorias a diferentes niveles de O_2 y CO_2 del ambiente en dos especies
- a nivel molecular se analizarán los patrones de evolución de genes relacionados con la fosforilación oxidativa codificados por genoma mitocondrial

Organización de la tesis

Esta tesis ha sido organizada en 4 capítulos, cada uno de los cuales se corresponde con un manuscrito que fue o será enviado para su publicación en una revista internacional arbitrada. Al final de todos ellos se presenta una discusión integrada de los principales resultados obtenidos. En el capítulo 1, se aborda la problemática desde una perspectiva fisiológica, evaluando la respuesta de variables respiratorias a diferentes niveles de O_2 y CO_2 en dos especies de roedores octodontoideos, una subterránea y otra fosorial. En los capítulos 2 y 3 se estudia la evolución del genoma mitocondrial de 7 especies de roedores octodontoideos, incluyendo los dos linajes subterráneos y sus parientes cercanos no subterráneos. El genoma mitocondrial contiene 13 genes que codifican para subunidades proteícas de complejos involucrados en la fosforilación oxidativa, por lo que la baja disponibilidad de O_2 podría modificar las presiones selectivas en estos genes en los organismos subterráneos. En el capítulo 2, se describen los

genomas mitocondriales completos de las 7 especies de estos roedores, comparados con las únicas dos especies de roedores histricognatos reportados en Genbank, se aportan datos sobre las relaciones filogenéticas entre ellas y se estiman tiempos de divergencia entre linajes. En el capítulo 3 se evalúa la acción de la selección positiva en genes codificantes de proteínas del genoma mitcondrial. Finalmente, en el capítulo 4 se analiza con detalle la evolución del gen de la subunidad II de la citocromo oxidasa c, en un grupo más amplio de especies, con una mayor representación de especies de tucu-tucus.

Capítulo 1

Estrategias respiratorias comparadas entre roedores octodontoideos fosoriales y subterráneos para afrontar un ambiente hipóxico e hipercápnico.

El ambiente subterráneo presenta una atmósfera hipóxica e hipercápnica que ha llevado a la adquisición de adaptaciones de las especies que viven en este medio. A pesar del interés ecológico, fisiológico y evolutivo de esta situación, la información al respecto es escasa y fragmentada. En este capítulo se evaluó la respuesta ventilatoria frente a las presiones selectivas impuestas por el nicho subterráneo en dos especies de roedores octodontoideos cercanamente emparentados: el degu *Octodon degus* (degus, N= 5) y el coruro *Spalacopus cyanus* (coruro, N= 4). Esta última es subterránea y vive la mayoría de su vida bajo tierra, mientras que la primera es fosorial, es decir, construye y utiliza cuevas, pero éstas permanecen abiertas y realiza buena parte de sus actividades en superficie. Utilizando técnicas no invasivas en animales no anestesiados, se midieron el volumen corriente (V_T), la frecuencia respiratoria (f_R) y su producto el volumen inspiratorio por minuto (V_I) en respuesta a la ausencia de O2, normoxia (21% de O_2), diferentes niveles de hipoxia (de 5, 10 y 15 % de O_2), hiperoxia (100% de O_2) e hipercapnia (10% de CO_2). La respiración en normoxia de ambas especies es significativamente menor a lo esperado para su tamaño corporal (mayor f_R, y menor V_T y V_I), y difieren en las estrategias respiratorias frente a los diferentes desafíos. En particular los coruros muestran una respuesta mucho más acentuada a la hipoxia e hiperoxia, y más atenuada frente a la hipercapnia, que los degus. Si bien la respuesta atenuada a la hipercapnia en coruros es lo esperable para los animales subterráneos, la respuesta a la hipoxia es variable en éstos, y podría ser explicada por la hipoxia sostenida durante su desarrollo embrionario.

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ORIGINAL PAPER

Comparative respiratory strategies of subterranean and fossorial octodontid rodents to cope with hypoxic and hypercapnic atmospheres

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Abstract Subterranean rodents construct large and complex burrows and spend most of their lives underground, while fossorial species construct simpler burrows and are more active above ground. An important constraint faced by subterranean mammals is the chronic hypoxia and hypercapnia of the burrow atmosphere. The traits, regarded as "adaptations of rodents to hypoxia and hypercapnia", have been evaluated in only a few subterranean species. In addition, well-studied subterranean taxa are very divergent to their sister groups, making it difficult to assess the adaptive path leading to subterranean life. The closely related sister genera *Octodon* and *Spalacopus* of Neotropical rodents offer a unique opportunity to trace the evolution of physiological mechanisms. We studied the ventilatory responses of selected octodontid rodents to selective pressures imposed by the subterranean niche under the working hypothesis that life underground, in hypoxic and hypercapnic conditions, promotes convergent physiological changes. To perform this study we used the following species: *Spalacopus cyanus* (the subterranean coruros) and *Octodon degus* (the fossorial degus) from central Chile. Ventilatory tidal volume and respiratory frequency were measured in non-anaesthetized spontaneously breathing animals. Acute hypoxic challenges (O_2 1–15%) and hypercapnia (CO_2 10%) were induced to study respiratory strategies using non-invasive whole body pletismography techniques. Our results show that coruros have a larger ventilatory response to acute hypoxia as than degus. On the other hand, hypercapnic respiratory responses in coruros seem to be attenuated when compared to those in degus. Our results suggest that coruros and degus have different respiratory strategies to survive in the hypoxic and hypercapnic atmospheres present in their burrows.

Keywords Ventilatory response · Hypoxia · Hypercapnia · Octodontids · Rodents

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Introduction

The convergent evolution of subterranean mammals is one of the most fascinating and puzzling evolutionary phenomena (Nevo 1999). Eight lineages of rodents have invaded the subterranean niche independently (Lacey et al. 2000); these species live most of their lives underground in their usually closed burrows, and they are called collectively subterranean. Other lineages have species that construct and use burrows, but are more active above ground, and they are called fossorial. Life underground imposes particular selective pressures, which have driven subterranean rodents to develop convergent morphological and physiological features (Nevo 1999). In comparison, fossorial rodents are much less specialized than subterranean species.

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 northern Israel in the breeding mounds of *Spalax carmeli* in a flooded, poorly drained field of heavy clay soil with high water content (Shams et al. 2005). Gas interchange between burrows and atmosphere depends on the gas permeability properties of the soil, which is affected by such factors as temperature and humidity (Arieli 1979), and the limited ventilation is the result primarily of animal movements (Buffenstein 2000). These factors make burrow gas composition to differ considerably from atmospheric air, and any activity of the inhabitants increases such difference. Models of diffusion gas exchange (Withers 1978) and experimental data (MacLean 1981) show that, unless the soil is completely devoid of biotic substances, burrow atmospheres will always be hypoxic and hypercapnic relative to the surface atmosphere (Buffenstein 2000).

Under low O_2 partial pressure (pO_2) in the burrow atmosphere and facing the potential CO_2 perturbation of their blood acid–base balance, subterranean mammals are expected to show certain physiological mechanisms to avoid excessive energy expenditure in respiratory work. These mechanisms are not fully understood, but 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 1999). For example, the critical pO_2 levels in these mammals are lower (Arieli 1990; Arieli and Nevo 1991; Lechner 1977), and metabolic rates can be maintained at low pO_2 (Lovegrove 1989; Vleck 1979). Besides, ventilation in normoxia is lower (Arieli 1990; Boggs et al. 1998), and ventilatory response to hypoxia, hypercapnia, or both is attenuated (Arieli 1990; Boggs et al. 1998; Buffenstein 2000). Cardiac output and heart frequency are also lower in normoxia (Arieli 1990), and show a twofold increased response to hypoxia even at low temperatures (Arieli et al. 1986). Moreover, oxygen carrying capacity is increased, facilitated by elevated hemoglobin concentrations, high intrinsic affinity for oxygen and more red blood cells (reviewed by Buffenstein 2000). Furthermore, their myoglobin concentration is higher in blood and skeletal muscles; the capillary density in myocardium, skeletal muscles, and lungs is elevated (Arieli 1990), and they have a high relative amount of mitochondria in skeletal muscles (Widmer et al. 1997). Finally, they have high arterial and tissue pCO_2 (Arieli 1990), and they cope with it without altering the blood acid–base equilibrium by at least two known different mechanisms (Buffenstein 2000; Quilliam et al. 1971).

Other mechanisms are known only for species of the genus *Spalax* and are related to shortening of the diffusion distance, increase of the O_2 permeability constant (reviewed by Arieli 1990), and avoidance of oxidative damage under fluctuating O_2 and CO_2 levels by increasing the activity levels of antioxidant enzymes, such as superoxide dismutase (Caballero et al. 2006). Recent studies have also found differences in gene expression among subterranean species with different critical pO_2 (Avivi et al. 2006; Nasser et al. 2005; Polyakov et al. 2004). However, these mechanisms have been evaluated in only a few subterranean species from the genera *Spalax* (Mediterranean), *Heterocephalus* (African), and *Thomomys* (North American), and there are no comparative studies including several species. Besides, well-studied subterranean taxa are very divergent from their sister groups (if known), making it difficult to assess the adaptive path leading to subterranean life.

To understand the physiological strategies of subterranean mammals for coping with subterranean atmospheric conditions in comparison to less specialized forms, we examined the influence of experimental variations in hypoxic and hypercapnic conditions on respiratory tidal volume and respiratory frequency in response to different values of pO_2 and pCO_2, of the fully subterranean coruro *Spalacopus cyanus* (Rodentia: Octodontidae) and the fossorial degu *Octodon degus* (Rodentia: Octodontidae). Coruros are distributed from 30 to 37°S along the coast of Chile and also above 2,000 m sea level in the Andes range. Degus are diurnal rodents inhabiting the semiarid and Mediterranean environments of northern and central Chile to 2,000 m above sea level. In many areas of their range of distribution, both species overlap and are sympatric, but one inhabits above (degus) and the other below (coruros) the surface. We hypothesized that coruros show an attenuated response to hypercapnia and hyperoxia compared with the related degus, whereas the response to hypoxia is depressed in the latter.

The closely related sister genera *Octodon* and *Spalacopus* of Neotropical endemic rodents offer an opportunity to trace the evolution of physiological traits. The monotypic genus *Spalacopus* is strictly subterranean and shares convergent adaptations to underground life, while the genus *Octodon* has three burrowing species. Moreover, the phylogenetic relationships and estimations of divergence times are well established as relatively recent (reviewed by Opazo 2005), which would allow tracing the changes associated with the acquisition of subterranean adaptations along a known phylogeny, as well as identifying and discriminating such adaptations from more general ones associated with fossoriality.

Methods

Animals studied

Experiments were performed on five adults of the fossorial species *Octodon degus* ("degus"; all males between 194.4

and 217.8 g) and four adults of the subterranean species *Spalacopus cyanus* ("coruros"; 3 males and 1 female between 72.8 and 100.4 g). Degus and coruros were caught using Oneida Victor Traps and Tomahawk Live Trap traps, respectively, at a field station of the Universidad de Chile (33°23′S, 70°31′W, altitude 495 m) in Rinconada de Maipú, in the Metropolitan Region, Chile, between October 18th and October 21st, 2007. Traps did not injure the animals, all of which were non-reproductive and looked completely healthy. Animals were kept in a ventilated room in individual polycarbonate rat cages (45 × 23 × 21 cm) with a bedding of hardwood chips, water, and food (rabbit commercial pellet supplemented with apples, carrots, lettuce, and sunflower seeds) provided ad libitum, with a photoperiod of LD = 12:12 and ambient temperature set at 25°C. Animals were kept under these conditions approximately 10 days before measurements began. After measurements were made, animals were entered into collection of Pontificia Universidad Católica de Chile with the access numbers SSUC_Ma 00408–004012 and SSUC_Ma 00412–00415 for coruros and degus, respectively.

Measurements

The experimental protocol was approved by the Ethical Committee of the Facultad de Ciencias Biológicas of the Pontificia Universidad Católica de Chile. We measured tidal volume (V_T, ml), respiratory frequency (f_R, breaths per minute, BPM), minute inspiratory volume (V_I, ml/min) in response to different pO_2 (from anoxia to hyperoxia) and one pCO_2 level using a whole body pletismography system (Respiromax, Columbus Instruments, USA) in conscious normally breathing animals. The system includes a restrainer with a design that allows an unobtrusive, but secure seal around the animal's neck that isolates the head in the inhalation/exposure chamber. The calibration of the experimental setup was performed before every measure using the value of the barometric pressure at the level of Santiago city at each day of experiment. We measured the ventilatory responses elicited by poikilocapnic levels of pO_2 (5–670 Torr), maintained until the response was in a semi steady state (~10–20 s).

The experimental conditions sequentially tried were: (1) normoxia and normocapnia (room air), (2) anoxia (100% N_2 for 20 s), (3) 30 hypoxia (pO_2 5–100 Torr for 30 s), (4) hyperoxia (100% O_2 for 60 s), and (5) hypercapnia (air with 10% CO_2 for 30 s). Enough time was allowed between measurements until basal parameters were reached in normoxia. Animals were exposed to 100% O_2, which is the well known Dejours (1962) test. Because of its non-invasive nature, the Dejours test is used in human subjects and animals to assess the peripheral chemoreceptors contribution to ventilation. Exposure to hyperoxic (100% O_2) stimuli leads to ventilatory depression that occurs during the first few seconds of hyperoxia (15–20 s). This effect is primarily due to a decrease in the carotid body chemosensory activity. The magnitude of the ventilatory depression caused by hyperoxia is often used as an index of carotid body chemoreception sensitivity.

The hypoxic mixtures were obtained by mixing 100% O_2 and 100 N_2 using a flowmeter system (Dwyer Instruments, USA). Optimal values for hypoxic mixtures were measured using an oxygen analyzer (Omehda, USA). In all cases, experiments were performed at room temperature, between 25 and 27°C.

Statistical analysis

Respiratory frequency, tidal volume, and minute inspiratory volume are expressed as mean ± SEM unless otherwise stated. For comparisons between species, the respiratory data were normalized with respect to basal values. A two-way analysis of variance (ANOVA) was performed to seek for statistical difference between species and different pO_2 treatments. An unpaired t test with Welch's correction was performed to seek for statistical difference of hypercapnia and hyperoxia between species.

Results

Mean basal values of f_R, V_T, and V_I, and their response to different inspired pO_2 and hypercapnia are summarized in Table 1. Figure 1 shows the variation of the three variables to different pO_2 tried. Figure 2 shows these variables in response to 10% of CO_2.

Ventilation in normoxia

According with expectations regarding their larger body mass, degus have higher V_T (0.18 ± 0.04 ml) and V_I (40.60 ± 11.65 ml/min) than coruros (V_T = 0.12 ± 0.03 ml and V_I = 18.70 ± 4.57 ml/min). Interestingly, degus have also higher f_R than coruros (230.68 ± 34.52 and 157.65 ± 14.38 BPM, respectively). However, neither of these differences is statistically significant ($P > 0.10$ in all cases). Both species have significantly higher f_R and lower V_I and V_T than expected for their body mass according to Stahl (1967), compared to expected values f_R: 287 and 153%, V_T: 12 and 21%, V_I: 38 and 28%, in degus and coruros, respectively.

Effect of hypoxia

Both species respond to hypoxia by increasing f_R, V_T, and V_I. However, in degus, the ventilatory response to different

Table 1 Variation of respiratory variables in response to different pO_2 and pCO_2

	Normoxia	Anoxia	Hypoxia			Hyperoxia	Hypercapnia
	21% O_2	100%N_2	5% O_2	10% O_2	15% O_2	100% O_2	10% CO_2
Respiratory frequency							
Degus	230.68 ± 34.52	235.42 ± 6.64	201.22 ± 19.33	179.31 ± 17.05	166.11 ± 16.12	135.41 ± 20.10	205.5 ± 20.4
		[1.02 ± 0.01]	[1.06 ± 0.01]	[0.94 ± 0.02]	[0.93 ± 0.04]	[0.82 ± 0.10]	[1.24 ± 0.10]
Coruros	157.65 ± 14.38	173.78 ± 1.11	171.14 ± 1.11	158.91 ± 1.11	152.17 ± 1.07	90.43 ± 0.61	155.95 ± 1.03
		[8.50 ± 0.05]	[19.29 ± 0.04]	[11.27 ± 0.04]	[8.36 ± 0.03]	[17.21 ± 0.11]	[9.11 ± 0.05]
Tidal volume							
Degus	0.18 ± 0.04	0.21 ± 0.04	0.22 ± 0.04	0.17 ± 0.04	0.15 ± 0.04	0.09 ± 0.02	0.16 ± 0.03
		[1.26 ± 0.12]	[1.34 ± 0.10]	[1.31 ± 0.15]	[1.12 ± 0.05]	[0.73 ± 0.03]	[1.32 ± 0.16]
Coruros	0.12 ± 0.03	0.18 ± 0.04	0.17 ± 0.02	0.14 ± 0.03	0.13 ± 0.02	0.09 ± 0.02	0.15 ± 0.03
		[1.54 ± 0.10]	[1.51 ± 0.20]	[1.45 ± 0.17]	[1.12 ± 0.05]	[0.87 ± 0.11]	[1.21 ± 0.08]
Minute volume							
Degus	40.60 ± 11.65	49.46 ± 12.29	45.44 ± 12.14	32.31 ± 10.24	23.87 ± 8.28	13.83 ± 5.02	34.56 ± 7.97
		[1.28 ± 0.12]	[1.42 ± 0.12]	[1.23 ± 0.14]	[1.03 ± 0.05]	[0.60 ± 0.07]	[1.84 ± 0.10]
Coruros	18.70 ± 4.57	29.50 ± 4.34	28.40 ± 4.75	22.28 ± 4.01	18.90 ± 2.72	7.90 ± 2.01	22.65 ± 4.70
		[1.71 ± 0.19]	[1.64 ± 0.15]	[1.63 ± 0.26]	[1.20 ± 0.08]	[0.50 ± 0.03]	[1.26 ± 0.05]

In each cell, mean values of respiratory variables (respiratory frequency in BPM, tidal volume in ml, and minute volume in ml/min) ± SEM to different pO_2 and pCO_2, above, and below between brackets its mean response with respect to basal values ± SEM. Degus and coruros are *O. degus* and *S. cyanus*, respectively

pO_2 was lower (Fig. 1). We found a statistically significant difference between the V_I response (accounts for approximately 4.84% of the total variance, $F = 7.5_{(1,42)}$, $P = 0.009$) and treatments of different pO_2 (approximately 65.83% of the total variance, $F = 20.42_{(5,42)}$, $P < 0.0001$). Increments in V_T seem to be more evident than those in f_R, and the latter seem to be more important in coruros than in degus. In the case of V_T, pO_2 accounts for approximately 52.48% of the total variance and is extremely significant ($F = 10.84_{(5,41)}$, $P < 0.0001$) and species effect accounts for approximately 3.88% of the total variance and is marginal ($F = 4.01_{(1,41)}$, $P = 0.0519$). In the case of f_R, species effect accounts for approximately 1.54% of the total variance, but is not significant ($F = 1.98_{(1,41)}$, $P = 0.1673$), whereas pO_2 accounts for approximately 57.93% of the variance and is extremely significant ($F = 14.82_{(5,41)}$, $P < 0.0001$). Statistically significant interaction between species and pO_2 was only found for f_R (approximately 15.19% of the total variance $F = 3.89_{(5,41)}$, $P = 0.0056$), so the P values of species and pO_2 effects are more difficult to interpret in this case.

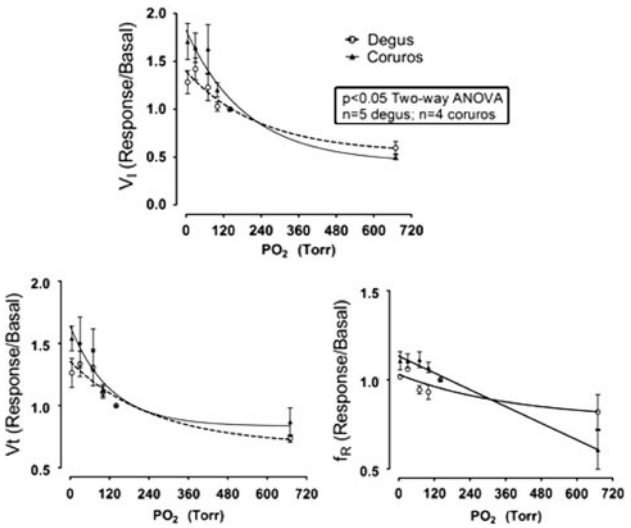

Fig. 1 Variation of the respiratory variables to different pO_2. All variables are represented as its mean response with respect to basal values ± SEM; f_R respiratory frequency in BPM; V_T tidal volume in ml; V_I in ml and minute volume. Degus and cururos are *O. degus* and *S. cyanus*, respectively

Effect of hypercapnia

Both species respond to hypercapnia by increasing both V_T and f_R. The response was large in degus than in coruros (Fig. 2). There was no difference between species in the response of V_T and f_R (with respect to basal values) to 10% of CO_2, whereas the response of V_I (with respect to basal values) was marginal ($t = 2$, $df = 5$, $P = 0.0847$).

Effect of hyperoxia

In response to 100% O_2, both species showed a decrease both in V_T and f_R (and consequently V_I), but the response was more accentuated in coruros than in degus, and

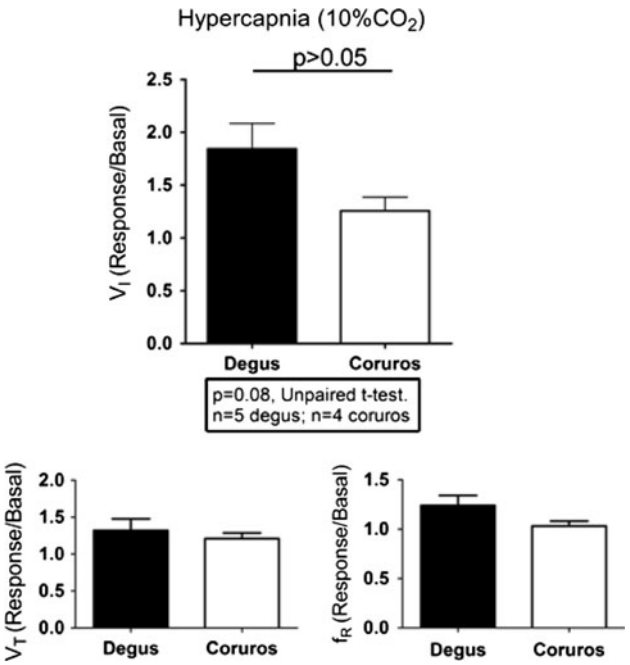

Fig. 2 Respiratory variables in response to 10% of CO_2. All variables are represented as its mean response with respect to basal values ± SEM; f_R respiratory frequency in BPM; V_T tidal volume in ml; V_I in ml and minute volume. Degus and cururos are *O. degus* and *S. cyanus*, respectively

specially by a reduction of f_R. However, neither of these differences between species is statistically significant ($P > 0.10$ in all cases).

Discussion

Mammalian respiration increases in response to hypoxia and/or hypercapnia. However, the ventilatory responses of high altitude, fossorial, or diving mammals may differ from this scheme (Buffenstein 2000; Ramírez et al. 2007; Nevo 1999). It is generally accepted that burrowing mammals show a reduced ventilatory sensitivity in particular to hypercapnia and possibly to hypoxia (Boggs et al. 1984).

This is the first time that a study addresses the ventilation response to hypoxia and hypercapnia in closely related octodontid rodents, including one fossorial species and one strictly subterranean species. Coruro (*S. cyanus*) was chosen because it is exposed to hypoxic and hypercapnic ambient in its complex burrows. Degus (*O. degus*) is a closely related species that maintains semifossorial habits and uses burrows, but it is cursorial with daily activity outside their burrows (Vásquez et al. 2002) and frequent access to open air. Our results show that both species have different ventilation responses to hypoxia and hypercapnia, suggesting different physiological adaptations to the hypoxic and hypercapnic environment.

As expected, given their larger body mass, degus have had higher observed values of V_T and V_I in normoxia than coruros, although they are not significant, possibly due to the small sample sizes used. Interestingly, both species had significantly higher f_R, and lower V_I and V_T in normoxia than the expected for their body mass (following allometric scaling proposed by Stahl 1967). This result agrees with the idea that the reduced ventilation in normoxia is a characteristic physiological response among fossorial mammalian species to cope with hypoxic and hypercapnic atmospheres. It is interesting to note that f_R is higher (1.5 and 3 times) than that predicted by allometry for coruros and degus, respectively. Subterranean species *T. bottae* and *S. ehrenbergi* display normoxic ventilation 80 and 70%, respectively, compared to the one predicted (Arieli and Ar 1979; Darden 1972). As suggested by Arieli and Ar (1979), if normal mammalian gas transport is maintained at a low ventilation rate, the potential remains to increase ventilation and, together with other physiological traits, to maintain normal gas transport in the hypoxic-hypercapnic atmosphere of the burrow. Low oxygen consumption, together with the low ventilation in normoxia found in these species, demands other mechanisms along the gas transport pathway to maintain a standard metabolic rate, as has been shown for other species at several levels (e.g., cardiovascular, hematological, and tissue levels). Only few complementary strategies have been reported for these species, and some others should be investigated. It has been reported that coruros have a basal metabolic rate (75–85% lower than expected) and critical pO_2 levels significantly lower than the aboveground counterparts (Contreras 1986). Although comparisons between coruros and degus have not been done, data suggest that the critical pO_2 in coruros (ranged from 79.4 to 91.9 Torr) is lower than in degus (139 Torr) (Morrison and Rosenmann 1975; Rosenmann and Morrison 1975; Contreras and McNab 1990). Data on resting metabolic rate reported in these studies are not easily comparable. On the other hand, hematocrit and the erythrocyte hemoglobin concentration are reported only for degus (48.8 ± 6% and 256 ± 4 g/L, respectively), but they did not show a particular pattern (Morrison et al. 1963). The low normoxic ventilation of coruros and degus may be an example of convergent evolution to the fossorial niche in rodents, which is more accentuated in subterranean life.

Contrasting the results from the two closely related species analyzed in this study, give clues that uncover not only the action of selective pressures of subterranean habitat but also difference in chemoreceptors sensibility. Coruros showed an attenuated response to hypercapnia and hyperoxia compared with degus, meanwhile the response to different levels of hypoxia seems to be depressed in the latter. This result strongly suggests that subterranean

rodents have a blunted ventilatory response to hypercapnia. It is generally accepted that burrowing mammals show a reduced ventilatory sensitivity in particular to hypercapnia and possibly to hypoxia (Boggs 1992; Boggs et al. 1984; Tenney and Boggs 1986). The "actual" respiratory pattern may be masked in these marginal significances due to the low numbers of individuals available for this study. Unfortunately, no complementary data on these species are available yet to support our findings. So, although these results must be viewed with caution, they suggest that adaptative physiological changes in response to extreme hypoxia, characteristic of the subterranean niche, have appeared recently in the lineage leading to *Spalacopus*, no longer than 2.5 millon years ago (Lessa et al. 2008). Previously studied subterranean rodents have deep roots that prevent us from determining the time elapsed from these acquisitions.

Reduced ventilatory response to hypoxia presented by degus in comparison to coruros is not the pattern expected initially, but it is not necessarily a characteristic of subterranean mammals, and is found in some burrowing animals. In studies on the echidna *Tachyglossus aculeatus* (Frappell et al. 1994), the armadillo *Dasypus novemcincus* (Boggs et al. 1998), and the wombat *Lasiorhinus latifrons* (Frappell et al. 2002), the hyperventilatory response is depressed under hypoxia. However, other species like the Syrian hamster *Mesocricetus auratus* (Walker et al. 1985), woodchuck *Marmota monax* (Boggs and Birchard 1989), and golden-mantled squirrel *Spermophilus lateralis* (Barros et al. 2001) and columbian ground squirrel *S. columbianus* (Milsom et al. 1986), which are all fossorial, show no difference from similar-sized epigeal species. In fossorial golden-mantled ground squirrel *Spermophilus lateralis*, a biphasic response to hypoxia of f_R has been reported, with an initial increase followed by a decline back to resting levels (Barros et al. 2001).

The fact that subterranean coruros have a more acute response to hypoxia than fossorial degus, which is in agreement with previous studies which show how persistent changes in the neural control system are generated based on prior experience (Mortola 2004). Chronic sustained hypoxia (pO_2 50–70 Torr) elicits plasticity in the carotid body chemoreceptors (Mitchell and Johnson 2003), with delayed effects on the central neural integration of carotid chemoafferent neurons that become more prominent as the duration of hypoxia is extended (Dwinell and Powell 1999; Powell et al. 1998). In the case of coruros, hypoxia may last long periods inside closed burrows, and the species show enhanced ventilatory response as the result of the potentiation of the carotid chemoreflex to this stimulus. However, hypoxia during the neonatal period affects adult ventilatory control, altering resting breathing patterns, and attenuating the hypoxic ventilatory response (Okubo and Mortola 1990). On the other hand, degus might tolerate a likely intermittent hypoxia throughout their lives, facing hypoxia only while resting at night. This stimulation may elicit plasticity via central neural mechanisms (Ling et al. 2001) with additional effects at the carotid body chemoreceptors (Prabhakar 2001; Rey et al. 2004), which may increase the short-term hypoxic ventilatory response (Ling et al. 2001) observed in the species.

The ventilatory response to changes in inspired CO_2 is an increase in ventilation, either in the rate or the depth of inhalation. This response as well as CO_2 sensitivity displays considerable interspecific variation (Boggs et al. 1998), but appears to be linked to habitat. Burrowing animals often encounter hypercapnia, and have on average a reduced sensitivity to CO_2 and reduced response to it compared to their non-fossorial or non-diving counterparts (reviewed by Boggs et al. 1984; Frappell et al. 2002), despite some exceptions (*Proechimys yonenagae:* Barros et al. 1998; *Spermophilus lateralis:* Garland et al. 1994). Increased tidal volume in response to CO_2 is a consistent response interspecifically within eutherian mammals (Barros et al. 1998; Frappell et al. 2002), with a few notable exceptions related to anatomical constraints which increase only the f_R (the armadillo *Dasypus novemcincus* (Boggs et al. 1998) and the bat *Phyllostomus discolor* (Walsh et al. 1996)) as explained by Frappell et al. 2002, or the subterranean *S. ehrenbergi* which simultaneously increase V_T and f_R (Arieli and Ar 1979). Wang and Warburton (1995) proposed that, in animals that employ the diaphragm muscle to shift the liver posteriorly and thereby expand the lungs, increasing ventilation by increasing tidal volume is less expensive than by increasing frequency. The response of coruros to CO_2 is similar to the general one reported for burrowing mammals: an attenuated increase in ventilation mediated primarily through changes in tidal volume. This result may support convergent evolution among subterranean rodents that have invaded the underground life independently. On the other hand, degus strongly respond to CO_2 by increasing both respiratory variables, and this pattern differs from predictions for fossorial mammals.

Response to hyperoxia was more attenuated in degus than in coruros. O_2-sensitive chemoreceptors are external to the brain in the carotid bodies (instead CO_2/pH-sensitive chemoreceptors are in the carotid bodies, but major sites are also distributed within the brain). The hyperoxic Dejours (1962) test has been extensively used to establish peripheral carotid body chemoreceptor sensitivity to oxygen. Coruros showed a dramatic decrease in f_R in response to 100% O_2 compared to degu's ventilatory response. These results suggest that cururos show an increase in response to acute hypoxia, due to an enhanced oxygen peripheral chemoreceptor sensibility. Unfortunately, the response to hyperoxia has not been studied in other

subterranean/fossorial taxa, and it is not possible to compare our results with other species.

Several limitations of these studies come from the way of working in this field. Comparisons with other species studied are difficult, because previous studies have been carried out under very different conditions of pO_2 and pCO_2, either in time or in concentration of each gas. It would be informative to standardize the results from different species to be able to compare and obtain general patterns. In this study, a non-invasive method was used, in which animals were not anesthetized during the experiments. This has the advantage of not altering stimulation and natural chemoreception, but it includes the effect of stress of animals and the possible acclimatization of the animal to the systems. These results cannot be directly compared with most of the previously reported data because they used anesthetized animals, so these kind of complementary studies in degus and coruros are needed. Another drawback of these studies is the lack of comparisons within a proper phylogenetic context. Most of the conclusions taken in previous studies came from comparing a fossorial species with a distant phylogenetically related laboratory rat. Finally, the separate effects of hypoxia and hypercapnia are important to characterize the response to a single stimulus. However, this approach cannot predict the effect of two stimuli together. In future studies, different levels of hypoxia and hypercapnia together may be used in order to assess the combined effect of both stimuli.

In conclusion, both degus and coruros have lower ventilation in normoxia than expected for their body mass, according to fossorial/burrowing habits, but they differ in their response to hypoxia, hyperoxia, and hypercapnia. Coruros, but not degus, exposed to hypercapnia showed a blunted hyperventilatory response, characteristic of fossorial mammals. Exposure to hypoxia resulted in greater hyperventilation in cururos than in degus, suggesting that cururos show a greater sensitivity to changes in oxygen concentration via peripheral chemoreceptors.

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Capítulo 2

Genomas mitocondriales de siete roedores octodontoideos y la edad de colonización del nicho subterráneo

La identificación de los mecanismos de evolución de los genomas mediante la genómica comparada es uno de los desafíos más importantes de la biología evolutiva. Por sus características, el genoma mitocondrial de los vertebrados ha sido ampliamente usado en estudios de filogenias y poblacionales, y su secuencia completa ha sido reportada en más de un centenar de especies. A pesar de su gran diversidad, la fauna neotropical ha sido subrepresentada. Los roedores caviomofos, particularmente los de la superfamilia Octodontoidea, son particularmente interesantes para la biología evolutiva porque son bastante divergentes en comparación con otros roedores e incluyen dos géneros que invadieron recientemente el nicho subterráneo de manera independiente (tuco-tucos, género *Ctenomys,* y el coruro, género *Spalacopus*). En este capítulo reportamos y anotamos el genoma mitocondrial de siete especies de roedores octodontoideos, y lo comparamos con genomas de roedores histricognatos previamente reportados, como el cobayo *Cavia porcellus* y el hystricognato africano *Thryonomys swinderianus.* Encontramos una sintenia completa entre estos genomas, y en acuerdo con las hipótesis filogenéticas previamente propuestas, corroborando que el tuco-tuco social (*Ctenomys sociabilis*) es la especie más divergente dentro del género. Los tiempos de divergencia estimados considerando diferentes puntos de calibración de acuerdo con el registro fósil, apoya estimaciones anteriores, pero sugieren una antigüedad mayor que la previamente propuesta para la invasión del nicho subterráneo al menos por parte de los tuco-tucos.

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Manuscript Draft

Manuscript Number:

Title: Complete mitochondrial genomes of seven caviomorph rodents and the age of the colonization of the subterranean niche by octodontoid rodents

Article Type: Research Paper

Keywords: Caviomorpha; mitochondrial genome; molecular dating

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Abstract: Identifying the mechanisms of genome evolution by comparative genomics is one of the important goals of modern evolutionary biologists. The mitochondrial genome of vertebrates has been widely used in phylogenetic and population studies. Complete sequences have been reported for hundreds of mammalian species, but despite high diversity, the Neotropical fauna is under represented. South American caviomorph rodents are particularly interesting for evolutionary biologists because they are divergent from other rodents, and include two genera that have recently invaded the subterranan niche independently (tuco-tucos, genus Ctenomys, and the coruro, genus Spalacopus). We report and annotate the mitochondrial genome of seven species of caviomorph rodents and compare them with two previously reported genomes of the caviomorph rodent Cavia porcellus and the African hystricognath rodent Thryonomys swinderianus. We found complete synteny between these genomes and an agreement with previous phylogenetic hypotheses proposed for these species. This finding confirms that the social tuco-tuco (Ctenomys sociabilis) is a divergent species within the genus. Estimated divergence times of clades, considering the different fossil calibration points, support most previous estimates but suggest an older age than previous estimates for the invasion of the subterranean niche.

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Opposed Reviewers:

29th October 2010

M. Goodman
Editor-in-Chief of the
Molecular Phylogenetics and Evolution

I am submitting two companion manuscripts, namely "**Mitochondrial genomes of seven caviomorph and the age of the colonization of the subterranean niche by octodontid rodents**" (TomascoLessa2010_1) and "**The evolution of mitochondrial genomes in subterranean caviomoph rodents: adaptation in a background of purifying selection**" (TomascoLessa2010_2), for consideration for their publication in *Molecular Phylogenetics and Evolution* as research articles. In line with Associate Editor Link Olson's suggestion, we are resubmitting them as new subtmissions after having each thoroughly reviewed by two native speakers of the English language (their names appear in the acknowledgments of these manuscripts). These studies are based on the same data set, generated as part of my dissertation, although its analysis and interpretation differ and would deserve, at least from my point of view, to be published separately.

Both studies are based on complete sequences of the mitochondrial genomes of seven South American caviomorph rodents, with special emphasis on subterranean lineages and their relatives. These rodents are particularly interesting for evolutionary biologists because they are fairly divergent from most other rodents, and include two genera that recently invaded the subterranean niche independently (tuco-tucos, genus *Ctenomys*, and the coruro, genus *Spalacopus*). In contrast to other subterranean rodents, which are very divergent from their sister groups (if known), these genera are younger and would make it easier to assess the adaptive path leading to subterranean life. In the first manuscript we describe these genomes and compare them with previously reported genomes (those of the guinea pig *Cavia porcellus* and the African cane rat *Thryonomys swinderianus*). We also carried out phylogenetic analyses and estimated divergence times of different clades, which allowed us to suggest an older age than early estimates for the invasion of the subterranean niche. In the second manuscript we examined the molecular evolution of 13 protein coding genes looking for footprints of positive selection. Our working hypothesis is that an energetically demanding lifestyle, coupled

with the hypoxic atmosphere characteristic of the subterranean environment, may change the selective regime of genes encoding proteins involved in cellular respiration. We used maximum-likelihood and Bayesian approaches (dS/dN ratio) and an alternative procedure that tests for positive selection on quantitative physicochemical amino acid properties. The results given by these two approaches are consistent with each other and suggest a link between weak directional or episodic selection at the molecular level and niche shift, in a background dominated by purifying selection.

In sum, these studies report original information about the molecular evolution of mitochondrial genome in caviomorph rodents.

Finally, all instructions for authors suggested in the webpage were taken into account when writing the manuscripts. Their submission for publication have been approved by the authors. These manuscripts have not been published and are not under consideration for publication elsewhere.

We would very much appreciate if you would consider these articles for publication in *Molecular Phylogenetics and Evolution*.

Yours sincerely,

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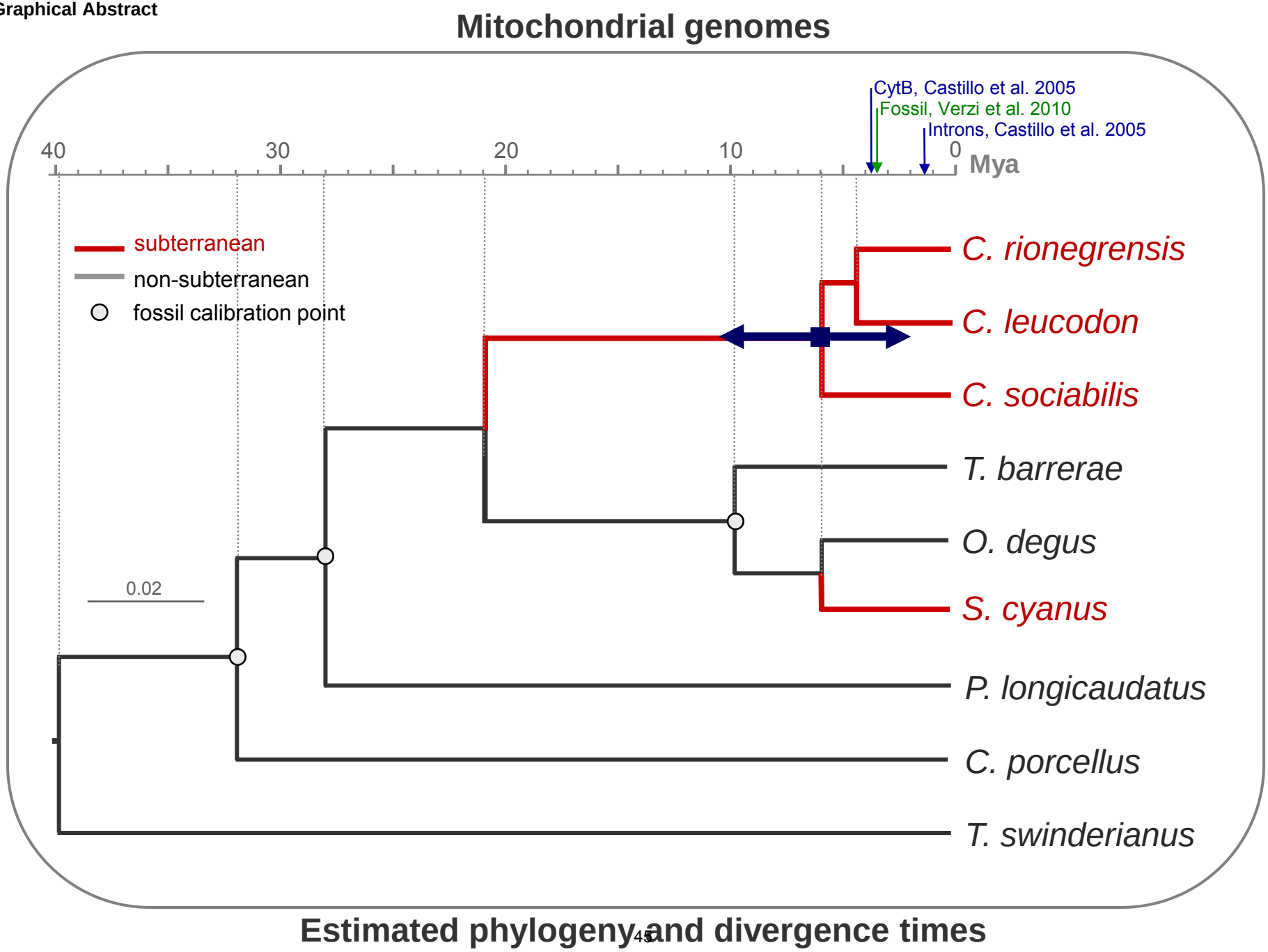
***Research Highlights**

- complete mitochondrial genomes of seven caviomorph rodents are reported
- these genomes are compared with two available hystricognath mitochondrial genomes
- phylogenies and divergence times are estimated
- subterranean niche colonization by octodontids may be older than previously estimated

*Graphical Abstract



Complete mitochondrial genomes of seven caviomorph rodents and the age of the colonization of the subterranean niche by octodontoid rodents

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Abstract

Identifying the mechanisms of genome evolution by comparative genomics is one of the important goals of modern evolutionary biologists. The mitochondrial genome of vertebrates has been widely used in phylogenetic and population studies. Complete sequences have been reported for hundreds of mammalian species, but despite high diversity, the Neotropical fauna is under represented. South American caviomorph rodents are particularly interesting for evolutionary biologists because they are divergent from other rodents, and include two genera that have recently invaded the subterranan niche independently (tuco-tucos, genus *Ctenomys,* and the coruro, genus *Spalacopus*). We report and annotate the mitochondrial genome of seven species of caviomorph rodents and compare them with two previously reported genomes of the caviomorph rodent *Cavia porcellus* and the African hystricognath rodent *Thryonomys swinderianus.* We found complete synteny between these genomes and an agreement with previous phylogenetic hypotheses proposed for these species. This finding confirms that the social tuco-tuco (*Ctenomys sociabilis*) is a divergent species within the genus. Estimated divergence times of clades, considering the different fossil calibration points, support most previous estimates but suggest an older age than previous estimates for the invasion of the subterranean niche.

Key words: Caviomorpha, mitochondrial genome, molecular dating

1. Introduction

Identifying the mechanisms of genome evolution by comparative genomics is an important goal of modern evolutionary biology. The mitochondrial genomes of vertebrates have been widely used in phylogenetic and population studies and have been completely sequenced and reported for about 300 species of mammals. However, the South American fauna is underrepresented with only 28 Neotropical mammals reported in GenBank.

Among Neotropical mammals, caviomorph rodents are of particular interest in to evolutionary biology. They were traditionally classified as Caviomorpha, an infraorder of South American hystricognaths, and show unique morphological and molecular features in comparison to other eutherian mammals. In 1991, Graur et al. proposed that caviomorphs should be reclassified as a separate order based on an analysis of the amino acid sequences of guinea pigs (see also Grauer et al., 1992; Li et al., 1992). This point of view was supported by several studies (D'Erchia et al., 1996; Reyes et al., 2000) while several papers supported rodent monophyly (Cao et al., 1994; Kuma and Miyata, 1994; Mouchaty et al., 2001; Robinson-Rechavi, 2000). Subsequent studies have expanded the representation of taxa and loci and restored the consensus among mammalian biologists that the Order Rodentia is monophyletic (Carleton et al., 2005; da Fonseca et al., 2008; Lin et al., 2002). The fact remains that caviomorph rodents have distinctive features, in particular at the molecular level, that deserve additional study. A conspicuous example is their divergent insuline gene (Opazo et al., 2005, and reference therein). Mitochondrial genomes of caviomorph rodents are only represented by that of the guinea pig,

Cavia porcellus; the only other known mitochondrial genome for hystricognath rodents is that of the African greater cane rat, *Thryonomys swinderianus*.

Caviomorph rodents have undergone an explosive diversification upon arrival into South America probably from Africa (Sallam et al., 2009). Two sister caviomorph families, Octodontidae and Ctenomyidae, invaded the subterranean niche independently. These rodents construct large and complex burrows, spend most of their lives underground and share convergent adaptations. In contrast to other subterranean rodents, that are very divergent from their sister groups (if known), these families are younger making it easier to assess the adaptive path leading to subterranean life. Attempts to calibrate radiation points within the caviomorphs have considered one or a few genes (Galewski et al., 2005; Honeycutt et al., 2003; Huchon and Douzery, 2001; Opazo, 2005). None of theser efforts paid special attention to subterranean lineages, such as the tuco-tucos (genus *Ctenomys*) which comprise more than 56 species and have been considered one of the most rapidly speciating mammalian lineages (Reig et al., 1990), or the coruro (monotypic genus *Spalacopus*) which lacks calibration. Molecular characterization of complete mitochondrial genomes of the subterranean representatives, and the possibility of dating these radiations events with new fossil findings (e.g.: Flynn et al., 2003; Verzi et al., 2010; Vucetich et al., 2010) would contribute to our knowledge about neotropical fauna and also shed light on the evolutionary processes underlying differentiation of subterranean life as well as the evolution of the mitochondrial genome itself.

Octodontidae and Ctenomyidae are members of the superfamily Octodontoidea, that also includes spiny rats (Echimyidae), which are primarily cursorial or arboreal (Nowak 1991). Estimated divergence times among families of Octodontoidea range between 15 and 30 Mya (Huchon and Douzery, 2001; Gallardo and Kirsch 2001; Opazo, 2005). We report, annotate and

describe the mitochondrial genome of seven species of octodontoid rodents. We also compare them with two genomes previously reported in GenBank that belong to the caviomorph *Cavia porcellus* and the African Hystricognath *Thryonomys swinderianus.* We found complete synteny between these genomes and an agreement with phylogenetic hypotheses proposed for these species. Our estimation of divergence times corroborates previous studies indicating an earlier age of origin for the subterranean genera *Ctenomys* and *Spalacopus*, which is in line with recent fossil reports. These findings provide a context for a new analyses of patterns of molecular evolution of the protein-coding mitochondrial genes of octodontids (Tomasco and Lessa, submitted).

2. Materials and Methods

2.1 Specimens Examined

We sequenced the complete mitochondrial genome of seven species of caviomorph rodents: three divergent species of the family Ctenomyidae (genus *Ctenomys*, known as tuco-tucos: *C. rionegrenisis*, *C. leucodon*, *C. sociabilis*), three species of family Octodontidae (the coruro *Spalacopus cyanus*, the degu *Octodon degus* and the red vizcacha rat *Tympanoctomys barrerae*), and one spiny rat, Family Echimyidae (the long-tailed spiny-rat *Proechimys longicaudatus*). Tuco-tucos comprise more than 56 species and have been considered one of the most rapidly speciating mammalian lineages (Reig et al., 1990): three species were chosen that are representative of the diversity of lifestyles and molecular differentiation (Castillo et al., 2005; Parada, 2007; Slamovits et al., 2001, and references therein). *C. rionegrensis* is a typical

representative of the genus. *C. leucodon* is a morphologically specialized species that lives at high altitude and was once suggested to be a distinct subgenus (*Haptomys*) by Osgood (1946). *C. sociabilis* is sister to all other species of the genus (Cutrera and Lacey, 2007; Parada, 2007) and is social. Information about voucher specimens is provided in Table 1.

2.2 DNA Extraction, Amplification, Sequencing, and contigs generation

Total DNA extractions were made with SDS/proteinase K/NaCl/alcohol precipitation (modified from Miller et al., 1988) from liver preserved in 95% ethyl-alcohol. The complete mtDNA of these seven species was amplified using a series of primers designed for this study (Table 1). A first set of primers were designed using conserved mtDNA regions of the two hystricognath rodents available in GenBank: *Cavia porcellus* (NC_000884) and *Thryonomys swinderianus* (NC_002658) to amplify 1 to 2 Kb length fragments which were sequenced from both ends. Based on these sequences, specific primers were designed to complete internal sequencing. Some additional primers that were used are Tuco06 (5´- GTGAAATGGAATTTTGTCTGA-3´, Wlasiuk et al., 2003), Tuco07(5´- ATTACAGCAATAGTAATAAT-3´ Wlasiuk et al., 2003), Tuco14A (5´-CCAATGTAATTTTTATAC-3´ Wlasiuk et al., 2003), TucoPro (5´- TTC TAA TTA AAC TAT TTC TTG - 3´ Tomasco and Lessa, 2007), TDKD (5´- CCT GAA GTA GGA ACC AGA TG -3´, Kocher et al., 1989) and MVZ 05 (5´-CGAAGCTTGATATGAAAAACCATCGTT-3´; Smith and Patton, 1993). Generally, 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, 1X Taq Polymerase Buffer, 240 µM of each dNTP, 240 nM of each primer, 2 units of Taq Polymerase and 4 mM of MgCl2. PCR amplifications were performed in a PXE0.2 Thermal Cycler (Thermo

– Electron Corporation), with an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 47 °C and 30 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. Some experiments were performed by with differing number of cycles, annealing temperatures or $MgCl_2$ concentrations. The amplified products were electrophoresed in 0.8% agarose gels (100 V, 20 min), the DNA bands were visualized after EtBr staining under UV light, and the expected size was determined in relation to a 100bp DNA size standard (GIBCO BRL). PCR products were purified using ethanol precipitation and automatic sequencing was done by Macrogen Inc. (http://www.macrogen.com), under BigDyeTM terminator cycling conditions in an ABI 3730xl Sequencer (Applied Biosystems).

Raw sequence files were assembled into contigs using the Phred-Phrap-Consed package software (http://www.phrap.org/phredphrapconsed.html#block_consed) (Ewing and Green, 1998; Ewing et al., 1998; Gordon, 2004; Gordon et al., 1998, 2001). Transfer tRNA analysis was conducted with tRNAscan-SE (Lowe and Eddy, 1997). Open reading frames between tRNAs were found and protein coding sequences were identified using BLAST searches (blastp) as implemented at the NCBI website (http://www.ncbi.nlm.nih.gov/). Alignments of ribosomal genes were made with MUSCLE (Edgar, 2004) and checked by eye. ACT-Artemis software (http://www.sanger.ac.uk/Software/ACT/) was used for final annotation of resulting genomes (Carver et al., 2005) which were deposited in GenBank with accession numbers HM544128 to HM544134.

2.3 Phylogenetic analyses

Phylogenetic reconstruction was implemented differentially depending on the kind of sequences analysed from the mitochondrial genome of 9 hystrocognath rodents: 7 were obtained in this

study and the two are available in GenBank: *Cavia porcellus* (NC_000884), and *Thryonomys swinderianus* (NC_002658) as outgroups. Two phylogenetic analyses were carried out. The first included 12 of the 13 protein-coding genes of the mtDNA and the second was restricted to non-coding protein genes. In the former case, the ND6 gene was excluded because it is encoded by the light-strand, which has a significantly different base composition from than the heavy-chain (Gibson et al., 2005). Multiple alignments of the concatenated sequences were generated with MUSCLE (Edgar, 2004). Gaps and ambiguous sites were removed, resulting in a total alignment of 10,775 nucleotides (≈ 3,591 codons). Bayesian inference methods with Markov chain Monte Carlo (MCMC) sampling were implemented in MrBayes (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) to assess phylogenetic relationships among the species. We used a General-Time-Reversible substitution model (Saccone et al., 1990) with the invariant sites and gamma options (five categories) after determining the optimal model of sequence substitution with ModelGenerator (Keane et al., 2006). One cold and three incrementally heated chains were run for 20,000 generations, and trees were sampled every 10 generations from the last 10,000 generated (well after the chain reached stationarity) and 1,000 trees were used for inferring Bayesian posterior probabilities (50% of burn-in fraction). We also implemented a Maximum Likelihood search in PhyML (Guindon and Gascuel, 2003) with a GTR substitution model and default options.

Another database was generated with sequencies from non-protein-coding sequences (rRNAs, tRNAs, replication origin, but excluding the control region), resulting in a total alignment of 4,288 nucleotides. Phylogenetic analysis was carried out under the criterion of maximum parsimony using PAUP (version 4.0 b10, Swofford, 2003), treating gaps as a fifth base. The relative support for each clade in the most parsimonious reconstructions was assessed by 50% majority rule consensus trees from 1000 bootstrap replicas. In addition, maximum-likelihood

searches were carried out using in PhyML (Guindon and Gascuel, 2003) with GTR substitution model and default options.

2.4 Estimation of age of radiation

The data base constructed with 12 of the 13 protein-coding genes of the mtDNA (excluding ND6 gene) was used to apply a relaxed molecular clock to infer the age of the nodes of the tree. We used BEAST v. 1.5.4 (Drummond and Rambaut, 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) method to estimate substitution rate and node ages. We used the GTR+gamma+invariant sites substitution model with four gamma categories, the data were partitioned by codon and we used the SRD06 Model (which has been found to provide a better fit for protein coding nucleotide data), and implemented a Yule branching rate prior, with rate variation across branches assumed to be uncorrelated and lognormally distributed (Drummond et al., 2006). Each final MCMC chain was run for 100000000 generations (Burnin 2000), with parameters sampled every 1,000 steps. Examination of MCMC samples using TRACER v. 1.5 (Rambaut and Drummond, 2007) suggested that the independent chains were each adequately sampling the same probability distribution; effective sample sizes for all parameters of interest were greater than 500/acceptable. Four direct fossil calibrations were used and treated as having a translated normal distribution. The data available based on the appearance of the first fossil of each clade/group are as follows. The oldest and most accepted caviomorph fossils are at least 31.5 Ma (Flynn et al., 2003, or perhaps even older Frailey and Campbell, 2004), and 29.5 Ma for the oldest octodontoid rodents without allocation to a particular family (Vucetich et al., 2010). The superfamily Octodontoidea is almost as old as Caviomorpha, and the early differentiation of the families Ctenomyidae and Octodontidae is at least 9.13 Ma (Verzi 1999; Vucetich et al., 1999; Zárate et al., 2007). We used 9.3 Ma as the upper limit for the Octodontinae radiation and 3.5 Ma

for the age of the genus *Ctenomys* (Verzi et al., 2010). We tried two identical runs, including and excluding the calibration point of the genus *Ctenomys*.

3. Results and Discussion

3.1 General description of mitochondrial genomes

In six of the seven species, the genomes were sequenced completely, while the genome of *C. rionegrensis* is reported without approximately 130 bp, placed on the end of the 16S gene and the beginning of Val tRNA. In agreement with previous studies (da Fonseca et al., 2008; Hassanin et al., 2009, etc.), we found complete synteny between these genomes and mtDNA of other mammals. We identified: i) the 13 protein coding genes encodes proteins involved in OXPHOS machinery: seven subunits of the NADH dehydrogenase or NADH ubiquinone oxidoreductase complex (ND: ND1, 2, 3, 4, 4L, 5 and 6), the CytB subunit of the ubiquinol cytochrome c oxidoreductase or cytBc1 complex (CytB), three subunits of the COX complex (COX1, 2 and 3), and two subunits of ATP synthase (ATPase: ATP6 and ATP8), ii) 2 subunits of ribosomal RNA (rRNA: 12S and16S) and iii) 22 tRNA. Percentage of similarity and particular difference in each gene are detailed in table 4. These sequenced mtDNA genomes vary in length from 16,860 bp in *T. barrerae* to 17,015 bp in *C. sociabilis* and the average nucleotide composition was 12.4% G, 23.5% C, 29.6% T and 34.5% A (Table 2), which is well within the range found in other mammals. These genomes are similar in size to those of *Cavia porcellus* (NC_000884: 16,801 pb) and *Tryonomys swinderianus* (NC_002658: 16,626 pb). Most of the size variation was due to differences in the length of the control region. Mitochondrial synteny

contrasts with numerous rearrangements of the nuclear present in Octodontoid rodents, which exhibit extensive chromosomal variation, (2n=10-102, Anderson et al., 1987; Contreras et al. 1990), which is nearly as great as the known variation for all mammals (Wilson y Reader, 2005).

The longest overlapping coding region is 51 bases and is shared between ATP6 and ATP8. Several shorter overlapping regions also exist, which is common in cavimorph rodents and in other mammalian genomes, including caviomorph rodents. The most used start codon for the protein-coding genes is ATG, but ATT, ATC and ATA are also used in ND2, ND3 and ND5. Although the last three are not the classical start codons in the mitochondrial code, they have all been reported as alternative initiation codon in other mammals. The genome of *C. porcellus* uses the codon GAG as the initiation codon of ND6 while other species use ATG. This may represent a sequencing error in the reported mithochondrial genome of *C. porcellus* (D'Erchia et al., 1996). The most used stop codon is TAA. Other stop codons are: AGA for CytB, AGG and TGA for COX1 (in *C. porcellus* and *S. cyanus*, respectively), TAG for ND1, ND2 (*P. longicaudatus*, *S. cyanus*, *O. degus*), ND6 (genus *Ctenomys* and *O. degus*) and ATP8 (*T. barrerae* and *O. degus*). Several stop codons share a base with the following gene and/or are incomplete (TAA in COX3, ND3, ND4). The gene coding ND5 in *T. barrerae* has three more codons than other species near the end that code for the amino acids F, L and T, and the total length of the protein would be 602 amino acids. Compared to the expected end of ATP8 in *C. porcellus*, *P. longicaudatus* ends three codons early, and *S. cyanus* one codon later (coding for Y). The COX1 has one codon less than that of *C. porcellus* at the 5' end in all species except in *P. longicaudatus*. Apart from these, no substantial differences in codon bias, gene length, intragenic spacer regions, or predicted

amino acid content were found relative to mitochondrial protein-coding genes of other eutherian mammals.

The control region of these species ranged from 774 bp in *C. leucodon* to 1,372 bp in *T. barrerae* (table 3). The three conserved sequence blocks (CSBs) described in other mammals (Walberg and Clayton, 1981) are also found in these species. This length variation was primarily due to differences in a complex tandem repeat region that is found between CSB I and CSB II; *C. leucodon* lacks this tandem repeat region and *C. sociabilis* has the longest repeat, followed by *C. rionegrensis*. The repeats are variable; and *C. rionegrensis* has the shortest and simplest pattern. The major repeat motif for each species is described in table 3; all are similar to that of *C. porcellus* (NC_000884, $(GT)_3ACG(CA)_2GAC$). Although the nature of the repeat motif was not analysed in detail, it may be phylogenetically informative. The control region sequence of *O. degus* obtained in this study is similar to two previously reported with 7 and 8 changes (AY007364.1 and AY007365.1, respectively), and is quite divergent from two others having 30 and 34 changes (AY007362. 1 and AY007363.1, respectively).

3.2 Phylogenetic reconstructions

Phylogenies (Figure 1) were congruent with each other and with previous phylogenetic hypotheses (Opazo, 2005; Parada, 2007). For protein coding genes, the families Octodontidae and Ctenomyidae as reciprocally monophyletic taxa (e.g. Honeycutt et al., 2003; Opazo 2005), and all nodes have high posterior probabilities (p=1) with values of non-parametric bootstrap greater than 97. Relationships among species within these two families agree with recent molecular phylogenetic studies (Opazo, 2005; Parada, 2007) and confirm the early divergence of *C. sociabilis* within *Ctenomys* (Cutrera and Lacey, 2007; Parada, 2007). Phylogenies obtained from non-protein-coding sequences show the same topology, though the relationship of *P.*

longicaudatus as sister to the clade formed by Octodontidae and Ctenomyidae is poorly supported. This relationship was captured in one of the two most parsimonious trees found (length = 2,964, consistency index CI = 0.76, and retention index RI = 0.58) with a bootstrap value less than 50%, and maximum-likelihood non parametric bootstrap support is 22%.

APPROXIMATE LOCATION OF FIGURE 1

3.3 Estimates of divergence times

Most of the estimations of divergence times except that for octodontids are older than fossil ages included as calibration points. Because previous estimates of divergence times differ from these, either in methodology, markers or species used, our results are not directly comparable. Nevertheless, it is possible to make some general comparisons. An advantage of the estimations reported in this study is the use of four calibration points, while previous ones were based on a single point, i.e. the first caviomorph around 31 Mya (except Honeycutt et al., 2003, who added a second one). A summary of these estimations is shown in figure 2.

APPROXIMATE LOCATION OF FIGURE 2

The divergence time for the Caviomorpha–Phyomorpha split was dated at 39.68 Mya (95% credibility interval 34.46 – 54.18), and is within the range of previously published estimates by Sallam et al. (2009, 36.1 ± 2.9 Mya), though older than estimates reported by Opazo (2005; 32.2 ± 2.4 Mya), and much more younger than estimates of Huchon and Douzery (2001, 46-63 Mya). The crown group of caviomorph rodents was dated at 31.68 Mya (95% credibility

interval 29.90 – 33.47), which overlaps with previously reported estimates by Opazo (2005, 33.8±1.8) and Galewski et al. (2005, 32.2±2.4 Mya), and is very close to dates estimated by Sallam et al. (2009, 30.7 Mya), but younger than that of Honeycutt et al. (2003, 35 – 40.4 Mya). Estimated divergence time among members of the superfamily Octodontoidea is 27.90 Mya (95% credibility interval 25.99 – 29.75), which agrees with estimation of Gallardo and Kirsch (2001, 25–30Mya) but older than the estimaterns made by Opazo (2005, 20.6±2.4 Mya) and Huchon and Douzery (2001, 15-18 Mya). Octodontidae and Ctenomyidae were considered either independent families or the tuco-tucos were placed as a subfamily (Ctenomyinae) of the Octodontidae (Pascual et al., 1965; cited in Lessa and Cook, 1998). In agreement with previous studies (e.g.: Huchon and Douzery, 2001; Honeycutt et al., 2003; Opazo, 2005), our results support the ranking of ctenomyids at the familial level. The estimated divergence between Ctenomyidae and Octodontidae was placed during the middle Miocene around 20.74 Mya (95% credibility interval 13.88 – 26.99), which agrees with previous estimates of Opazo (2005, 15±2.1Mya), Honeycutt et al. (2003, 16.7–22.5 Mya) and Gallardo and Kirsch (2001, 25 Mya). Diversification of the three species of Octodontidae was estimated to occur 9.56 Mya (95% credibility interval 7.69 – 11.43), which coincides estimates made by Opazo (2002, 7.79±1.55Mya), Honeycutt et al. (2003, 5 – 14.1 Mya) and Gallardo and Kirsch (2001, 7 – 10 Mya). The split of *Octodon degus* and *Spalacopus cyanus* would have occurred around 5.73 Mya (95% credibility interval 2.64 – 8.57), an estimation a little older than those of Opazo (2005, 4.28 ±1.08Mya), Gallardo and Kirsch (2001, 4 -5 Mya) and Honeycutt et al. (2003, 1.1 -5.3). All estimates did not change substantially with the inclusion of the oldest fossil of *Ctenomys* as a calibration point.

The age of the genus *Ctenomys* was estimated around 5.88 Mya (95% credibility interval 2.08 – 10.84), much older than estimates made by Castillo et al. (2005) with CytB and intron data (3.7 and 1.3 Mya, respectively). When the oldest fossil of *Ctenomys* (3.5 Mya) is a calibration point, estimates of divergence of this genus are younger: 4.01 Mya (95% credibility interval 2.48 – 5.58). This genus has been considered one of the most rapidly speciating mammalian lineages (Reig, 1970, 1989; Reig and Kiblisky, 1969; Reig et al., 1990) partially because the age of the genus has been estimated at approximately 1.8 million years (Reig et al., 1990). More recently, however, Verzi et al. (2010) reported *Ctenomys* fossils >3 Mya. The general trend based on molecular data and fossil findings points to an older tuco-tuco radiation than envisioned by Reig et al. (1990) and others. A full evaluation of the case of *Spalacopus* would require molecular data on the closely related genus *Aconaemys*. However, available data also point to a somewhat older date of divergence of *Spalacopus* than earlier estimates.

In sum, the invasion of the subterranean niche in octodontoid rodents still represents a relatively young evolutionary experiment. However, there is growing evidence that, at least in the case of tuco-tucos, it predates the Pleistocene.

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Figure 1. Phylogenetic reconstrucion of mitochondrial genomes of nine hystricognath rodents. Black numbers are non parametric bootstrap values obtained by Maximun-likelihood, either for protein conding genes (left) and non proteing conding sequences (right in cursive); gray numbers are 50% majority rule bootstrap values obtained by Maximun parsimony.

Figure 2. Divergence times estimated for nine hystricognath rodents. Divergence times estimated with 12 of the 13 protein-coding genes of the mtDNA applying a relaxed molecular clock in BEAST. Divergence ages are expressed in million years before present and white circles indicate the calibration points. 95% credibility intervals are given in the text.

Figure_1

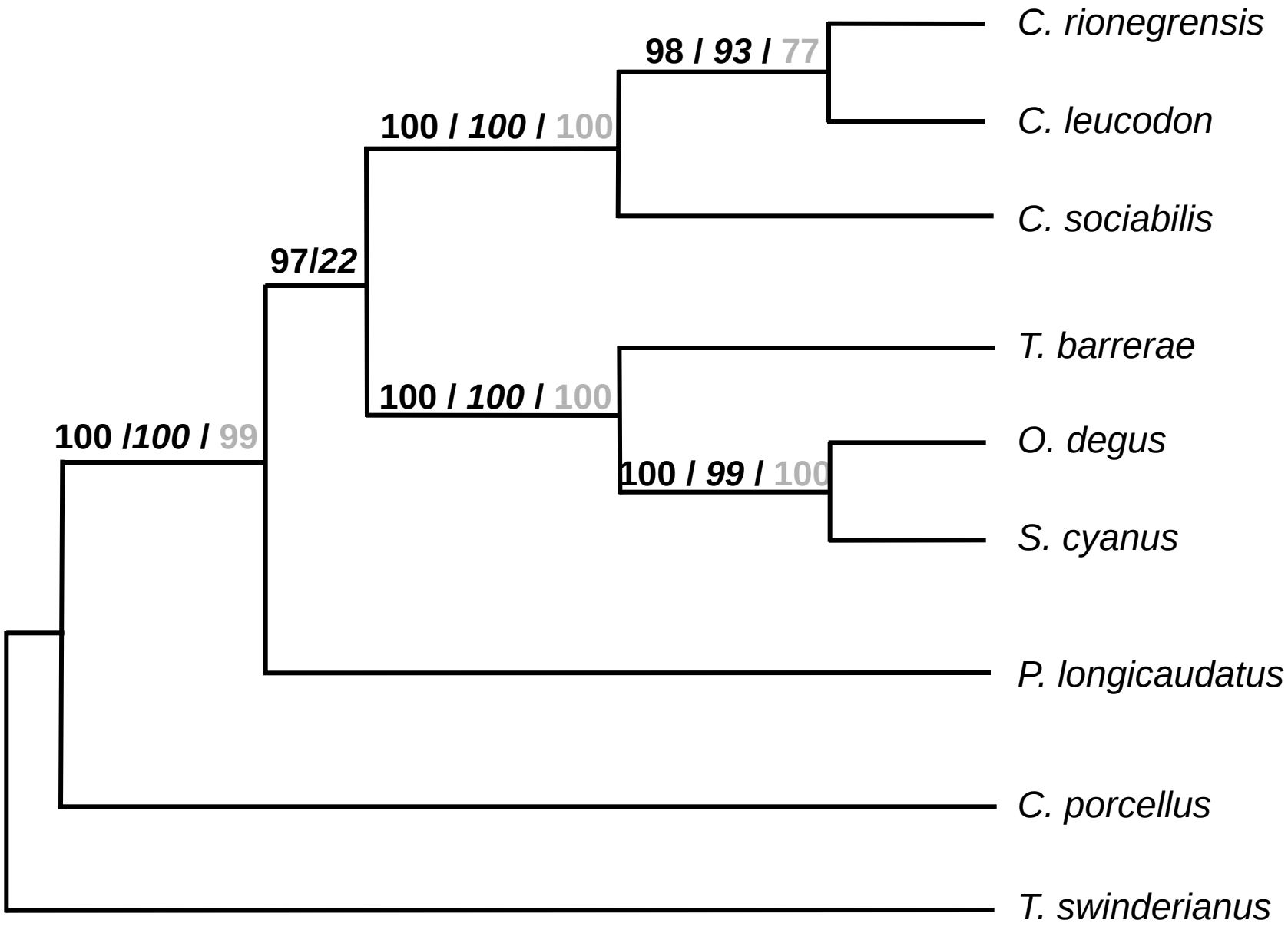
C. rionegrensis
98 / 93 / 77
C. leucodon
100 / 100 / 100
C. sociabilis
97/22
T. barrerae
100 / 100 / 100
O. degus
100 /100 / 99
100 / 99 / 100
S. cyanus
P. longicaudatus
C. porcellus
T. swinderianus

Figure_2

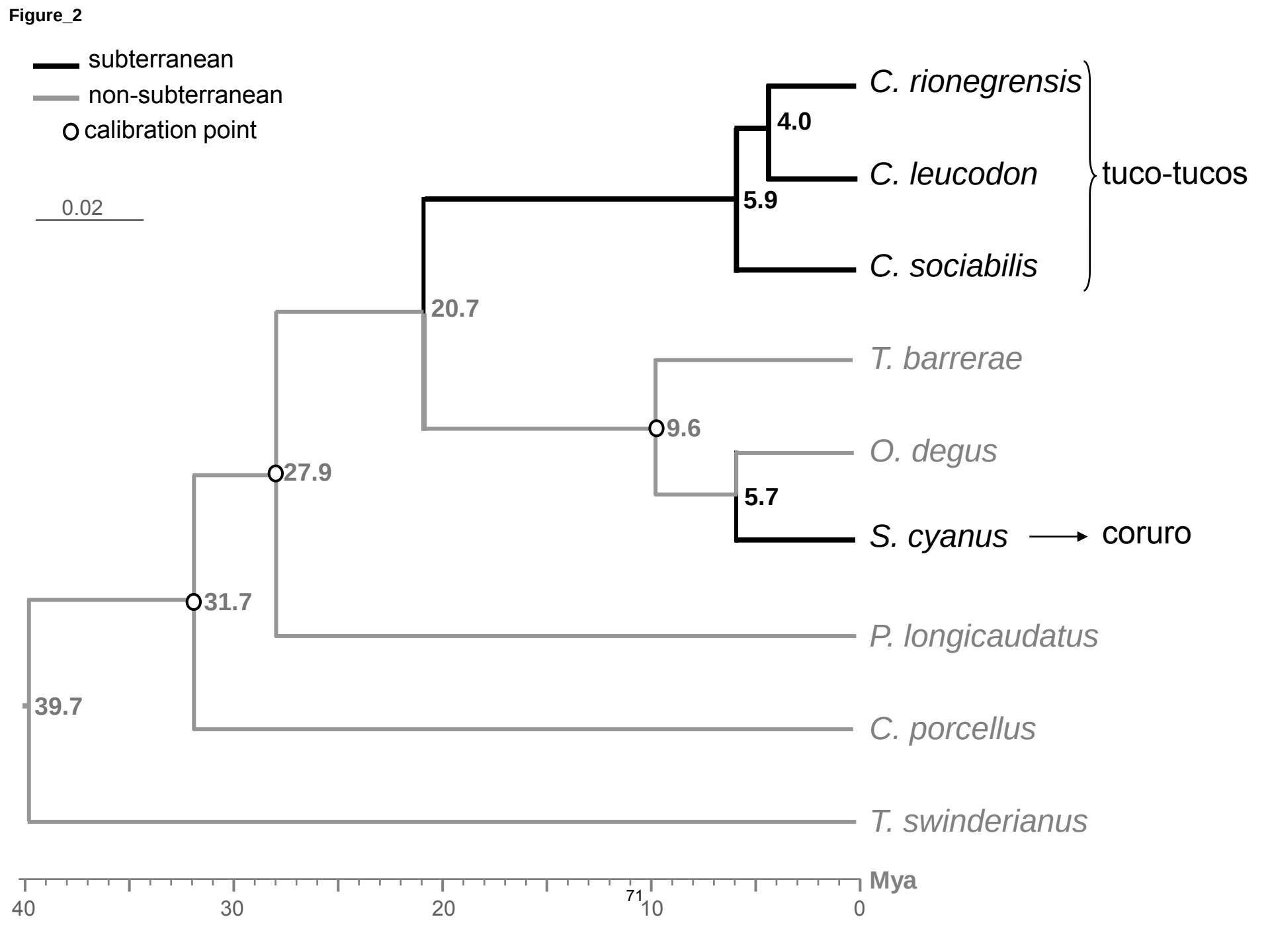
subterranean
non-subterranean
calibration point
0.02
C. rionegrensis
C. leucodon
C. sociabilis
tuco-tucos
4.0
5.9
20.7
T. barrerae
9.6
O. degus
27.9
5.7
S. cyanus
coruro
31.7
P. longicaudatus
39.7
C. porcellus
T. swinderianus
Mya
40
30
20
10
0

Table 1. Species and voucher information of specimens examined.

Species and voucher	**Collection and procedence**
Ctenomys sociabilis EAL545	MVZ, University of California, Berkeley. Reserva Nacional Nahuel Huapi, Neuquén Argentina 40º 57’ S, 71º 03’ W
Ctenomys rionegrensis EV1064	Laboratorio de Evolución, Facultad de Ciencias Estancia “El Abrojal”, Departamento de Río Negro, Uruguay 33° 01’ 75’’ S, 57° 53’ 96’’ W
Ctenomys leucodon MSB:Mamm:59654	University of New Mexico 14 km SW San Andrés de Machaca, La Paz, Bolivia 17° S, 69° 4’ 0.012’’ W
Octodon degus 109241	Donated by Francisco Bozinovic Parque Nacional Fray Jorge, IV Región de Coquimbo, Chile 30º 38’ 75’’ S, 71º 39’ 17’’ W
Spalacopus cyanus DG657	Donated by Guillermo D´Elía Comuna “Lo Barnichea”, Región Metropolitana, Chile 33º 19’ 33’’S, 70º 17’ 08’’ W
Tympanoctomys barrerae AK13811	Texas A&M University Mendoza, Argentina
Proechimys longicaudatus MSB:Mamm:57192	University of New Mexico 27 Km SE of Santa Cruz, Bolivia 17° 58’ S, 63° 03’W

Table 2. Primers designed for the amplification of mitochondrial fragments of caviomorphs rodents. Sequences are reported in the 5´-3´sense. Name: numbers indicate 3´end position in the genome of *Cavia porcellus* (NC_000884), H and L indicate if sequence is complementary to the light or heavy strand, respectively.

Name	sequence 5´-3´
MF 33 Ho	gaataaaaaaataagacgagaag
MF 37 Ht	aaagcaaggcactgaaaatg
MF 186 H	aggagcygrtatcaagcacac
MF 272 L	gtcaaactttcgtttattgcttaatt
MF 298 H	aattaagcaataaacgaaagtttgac
MF 350 L	tttattaattagggttaatcg
MF 503 L	tggggtatctaatcccagttt
MF 1610 H	agcagccatcaattaagaaagcgtt
MF 1696 L	ttactaatattaacattatytcttc
MF 1986 L	ggtcaggataccgcggccgtt
MF 2426 L	cctgatccaacatcgaggtcg
MF 2512 H	aatccaggtcggtttctatcta
MF 3050 H	tattcatyctagctacatcaag
MF 3081 L	gcgtatttrgartttgatgctca
MF 3865 H	tcgggcccataccccgaaaatgttg
MF 4074 L	attattgatgcngttgcttg
MF 4356 H	tactmacatgacaaaaaatygc
MF 4969 L	agrgctttgaaggcyctyggtct
MF 5065 H	atcaracrctttarttaagctaaac
MF 5219 H	gaagctgctcctttgaattkgc
MF 5398 L	gttcctactatdccdgctcawgc
MF 5672 Ha	tcatctatarttgaagccgg
MF 5672 Hb	tcatcwatagtmgaagcwgg
MF 6127 H	acataytattcaggwaaaaaaga
MF 6401 L	caatrtytartgadgagtt
MF 6610 H	aggctcattyatytctctcacagc
MF 6710 H	ttyccwcaacayttyytagg

MF 6879 L	grgggttcaattccttcct
MF 7333 H	ggmcaycaatgataytgaag
MF 7383 H	gaactwamyttygactchtatata
MF 7475 L	agtacrtcttctgatgaratta
MF 7787 H	taatgaaatgccacarytaga
MF 7944 L	gthggtgtratraaaraggmraata
MF 7997 L	ataryrgggaayataataat
MF 8187 L	gatarytgngtagtkggggt
MF 8789 H	atatwccartgatgacgmga
MF 8927 L	cctagttctggwgtaggdgcta
MF 9234 H	acaggatttcayggaytacaygt
MF 9289 L	ccraartggtgtttagakgtraa
MF 9832 H	gaatatggtarttagtttaa
MF 9852 H	taaaaacaaatgatttcg
MF 10161 L	ggtaagataatttttarcattgtag
MF 10178 Ha	taaatctactacaatgctaaaaa
MF 10178 Hb	taaacttattacaatgctaaaaa
MF 10985 H	tatgaggaataatcataactag
MF 10988 H	tatgaggaataattataactag
MF 11023 H	cgccaaacagacttaaaatccct
MF 11112 L	tgtaaggccgtgtgcrattatta
MF 11426 L	ggtagctttcctcgttgtgttg
MF 11438 H	caacacaacgaggaaagctacc
MF 11578 H	tagtttaacnaaaacattagattgt
MF 11638 L	tgttaaactataattacag
MF 11691 L	gttcctaagacyaatggattacttct
MF 11725 L	acttttatttggagttgcacc
MF 11797 L	ggtaaggtaagggtagttarggtta
MF 11874 L	gttggrattaggctgaggaaaaagga
MF 12144 L	agttggaatargttgttwgctgt
MF 12251 H	gatcagatgctaatacagcagc
MF 12700 H	tcaaaaaaatcgtagcattctc
MF 12871 L	attcctgttarggcwaggcttccaa
MF 13836 L	cartatcctgagacrtgagg

MF 14378 L	atyartcawccgtartttacgt
MF 15876 H	ctyctcgctccgggcccata
MF 16441 Ho	atcccaaaaaacaataatcac
MF 16601 Ht	gtatctattaacaaaccccc

Table 3. General characteristics of sequenced mtDNA. CR: control region.

		New complete mtDNAs sequenced						
		Length in bp			Nucloetide frequencies (%)			
Taxon	Acc. Number	Total	D-loop	Main repeat motif of CR	T	C	A	G
C. sociabilis	MH544129	17,015	1,545	TAYACACG	28.6	24.7	32.1	14.6
C. leucudon	MH5441131	16,216	744	none	30.7	22.1	35.2	12.1
C. rionegrensis	MH544130	16,721	1,474	TATACACG	30.5	22.7	34.7	12.1
O. degus	MH544134	16,786	1,329	TATACACACACG(T)	30.1	22.7	34.9	12.2
S. cyanus	MH544133	16,832	1,357	TACACAYG	28.5	24.8	34.1	12.6
T. barrerae	MH544132	16,860	1,382	CATGTACACMG	28.5	24.6	34.5	12.3
P. longicaudatus	MH544128	16,817	1,373	TATACACASS	29.4	24.2	33.6	12.8
Average		16,750	1,320		29.6	23.5	34.5	12.4

Table 4. Nucleotide composition and evolutionary divergence between sequences. Mean pearwise absolute number of differences betwen species of: Ct: *Ctenomys*; Oc: Octodontidae; Ct:Oc: *Ctenomys* and Octodontidae; Ct-Oc/P: *Ctenomys* or Octodontidae and *P. longicaudatus*; Ct-Oc/C: *Ctenomys* or Octodontidae and *C. porcellus*; all together. * sequencie of *C. rionegrensis* incomplete; rRNA mean value for rRNAs genes; tRNA: mean values for all tRNA genes, P-cd: mean values for all protein coding genes; mean: general mean value for all genes.

	Nucleotide frequencies (%)				Mean pearwise number of differences					
gene	**T(U)**	**C**	**A**	**G**	***Ct***	**Oc**	**Ct/Oc**	**Ct-Oc/P**	**Ct-Oc/C**	**All**
12S	24.6	20.7	38.0	16.7	23.7	49.7	84.3	105.7	132.7	90.8
***16S**	25.9	18.9	38.7	16.4	87.0	99.3	211.7	229.6	268.2	210.1
Ala	27.5	23.0	36.2	13.3	10.0	6.0	14.3	15.2	19.0	14.3
Arg	33.9	13.6	42.1	10.4	1.7	3.3	5.0	6.5	9.0	5.8
Asn	25.2	22.9	35.9	16.1	5.3	2.0	9.6	7.2	11.4	8.3
Asp	37.9	13.2	38.6	10.3	0.7	3.7	12.3	8.7	9.1	8.6
Cys	27.8	24.1	28.4	19.8	0.7	3.3	4.0	2.7	4.1	3.3
Gln	28.0	22.7	38.6	10.7	4.0	4.0	8.1	7.2	8.9	7.2
Glu	26.6	19.2	40.8	13.4	3.3	4.0	10.3	5.8	11.4	8.2
Gly	29.9	18.5	35.2	16.3	4.0	7.0	12.0	12.3	14.0	11.2
His	35.2	12.9	41.4	10.5	2.7	4.0	5.4	7.3	5.9	5.5
Ile	32.1	12.8	37.4	17.6	1.3	5.3	3.8	3.7	5.9	4.2
Met	24.6	27.5	29.0	18.8	1.3	2.7	4.0	4.7	3.4	3.6
Leu	28.2	22.0	32.1	17.7	2.0	0.7	2.7	1.5	4.3	2.5
Leu2	29.1	14.1	38.6	18.2	1.3	2.7	3.0	3.7	3.0	2.9
Lys	30.2	17.5	38.1	14.2	2.7	6.7	7.0	7.5	12.7	8.0
Phe	28.5	16.4	38.9	16.2	3.3	2.0	7.0	4.2	6.3	5.3
Pro	24.6	24.4	39.4	11.6	3.3	5.3	13.2	9.8	12.4	10.4
Ser	23.7	26.1	34.8	15.4	3.3	3.3	7.2	6.7	9.4	6.8
Ser2	30.2	22.2	33.0	14.7	4.7	4.7	9.9	6.8	10.7	8.3
Thr	27.3	17.7	38.8	16.2	2.7	4.0	8.0	7.3	14.0	8.4
Trp	28.2	17.7	37.5	16.6	4.0	8.3	10.6	9.8	14.3	10.4
Tyr	35.1	19.1	32.2	13.6	1.3	3.3	5.3	6.3	18.0	8.1
***Val**	**23.9**	**23.9**	36.1	16.1	7.0	4.7	14.2	14.0	10.0	11.2
ATP6	**32.3**	25.7	31.5	10.4	29.3	24.7	46.7	52.5	61.0	47.3
ATP8	29.9	23.8	40.7	5.7	13.3	15.0	22.6	34.3	36.4	26.8
CytB	29.7	27.0	30.8	12.6	36.3	39.3	58.8	57.7	71.4	57.2
COX1	**32.8**	21.5	29.3	16.4	25.3	39.7	41.8	41.5	49.0	41.5
COX2	29.9	22.3	34.9	12.9	16.7	24.7	24.3	23.8	36.0	26.4
COX3	**31.2**	24.7	29.7	14.4	12.7	23.0	27.2	29.5	47.6	30.8
ND1	30.3	26.8	32.2	10.7	22.0	36.7	52.2	57.0	74.6	53.9

ND2	29.3	26.4	36.6	7.7	37.3	71.3	100.7	105.7	154.4	105.3
ND3	**33.7**	24.9	31.8	9.6	32.0	67.0	80.9	71.5	98.1	76.5
ND4	31.4	25.3	33.8	9.6	150.0	209.7	288.3	315.2	359.0	288.5
ND4L	**35.4**	21.7	31.7	11.1	39.3	56.0	69.9	78.2	87.6	71.3
ND5	31.2	25.3	33.3	10.1	195.7	308.3	417.7	438.5	533.1	415.5
ND6	22.8	**26.2**	42.7	8.2	44.0	75.3	96.7	100.8	148.9	102.7

Capítulo 3

La evolución de los genomas mitocondriales en roedores caviomorfos subterráneos: adaptación en un contexto de selección purificadora.

Los tuco-tucos sudamericanos (género *Ctenomys*) y su pariente cercano el coruro (género *Spalacopus*) son linajes de roedores que colonizaron el nicho subterráneo de manera independiente. Un estilo de vida demandante desde el punto de vista energético asociado al ambiente hipóxico en hipercápnico característico del nicho subterráneo podría cambiar los regímenes selectivos de los genes que codifican para las proteínas involucradas en la respiración celular. Examinamos la evolución molecular de los 13 genes codificantes de proteínas del genoma mitocondrial de siete especies de roedores caviomorfos, incluyendo estos dos géneros subterráneos y sus parientes cercanos no subrerráneos. Usando aproximaciones bayesianas y de máxima verosimilitud, estimamos la tasa de sustituciones sinónimas (dS) y no sinónimas (dN). En todos los genes excepto las subunidades 6 y 8 de la ATPase, encontramos una relación entre sustituciones no sinónimas y sinónimas ω (dN/dS) significativamente mayor en los grupos subterráneos con respecto a sus parientes no subterráneos, aunque estos ω no fueron mayores a 1. Usando un procedimiento alternativo que evaluar la existencia de selección positiva a partir las propiedades fisicoquímicas de los amionoácidos involucrados en los reemplazos, encontramos que los cambios desestabilizantes en las proteínas se enconuentran en toda la filogenia, pero tienden a concentrarse en los linajes subterráneos. También encontramos cambios convergentes entre los géneros subterráneos usados en este estudio y otros mamíferos adaptados a la hipoxia. Los resultados obtenidos por las dos aproximaciones son consistentes y sugieren un nexo entre selección direccional positiva débil o episódica a nivel molecular y la colonización del nicho subterráneo, en un contexto de fuerte selección purificadora.

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Title: The evolution of mitochondrial genomes in subterranean caviomoph rodents: adaptation against a background of purifying selection

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Abstract: Two rodent lineages have independently colonized the subterranean niche, South American tuco-tucos (genus Ctenomys) and related coruros (genus Spalacopus). The energetically demanding lifestyles of these species coupled with hypoxic atmospheres characteristic of the subterranean environments may have altered the selective regimes on genes that encoding cellular respiration related proteins. Here, we examined the molecular evolution of 13 protein coding genes in the mitochondrial genome of seven caviomoph rodents, including these two subterranean genera 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 subterranean groups with respect to their non-subterranean counterparts in 11 of 13 genes, although no ω ratios were larger than 1. An alternative procedure was used to test for positive selection on quantitative physicochemical properties. Destabilizing changes in biochemical properties were found to be ubiquitous across phylogenies, but concentrated in the subterranean lineages. Convergent changes were also found between subterranean genera used in this study and other hypoxia adapted mammals. The results of this study suggest a link between nich shifts and weak directional (or episodic) selection at the molecular level over a background of purifying selection.

14th February 2011

Carey Krajewski
Associate Editor
Molecular Phylogenetics and Evolution

I am submitting the revised version of the manuscript titled "**The evolution of mitochondrial genomes in subterranean caviomoph rodents: adaptation in a background of purifying selection**" for consideration for its publication in *Molecular Phylogenetics and Evolution* as a research article.

First of all, I would like to thank, on behalf of the two authors of the manuscript, the positive comments and contributions of Reviewers 1 and 2, which helped us make this manuscript notoriously better. All minor comments and suggestions were accepted and incorporated in the new version and all the information required by Reviewers was also included. I would like to comment some of these modifications in order to facilitate the reassessment of the new version of the manuscript.

The manuscript have been thoroughly reviewed by two native speakers of the English language (Joseph Cook and Jolene Rearick, and they are thanked in the acknowledgements section), and I carefully checked the text to supress jargon and telegraphic statements. Any further suggestion on this regard will be very welcome.

Following suggestions of Reviewer #1:

- we made our best effort to complete the description of methods, and added all the information requested. We included a new paragraph with detailed information about phylogenetic analyses and some clarifying comments which answer the questions about topologies used in different analyses. We also included comments about how the branch site model was implemented, how many times PAML was run and the corresponding starting omega values.

- we added a sentence in the discussion section related to the exclusion of species of the genus *Aconaemys* from the analyses and how this might affect interpretation of the results.
- we modified tables 2 and 3, changing the order and content of two columns related to "amino acid change", in order to made them more interpretable. In the new version we reported the residues involved in the amino acid change only for relevant branches.
- we accepted all minor changes, added missing references and corrected typographic mistakes.

We would also like to explain why we did follow a few suggestions.

Reviewer #1:

We did not include any new sentence about "alternative ways to infer positive selection" (when ω-values are not greater than one) in the Materials and Methods section. We believe the issue is sufficiently addressed in the introduction and discussion.

We kept the name "coruro" following Wilson and Reader (2005), the standard general reference on mammal species of the world. Specialists will have no problem with either name, and non-specialists will find "coruro" more easily than "cururo".

We did not describe "intermediate models" implemented in branch models. Since they gave no significant results, the information seems to be irrelevant to readers and, besides, such models are difficult to describe. I guess the point is to mention that we gave alternative models a chance to show something relevant—they did not.

Reviewer #2:

Reviewer #2 suggests the presentation of "raw data", such as "base pair and amino acid alignments" and "3-D structures". These files can be directly downloaded from GenBank and they seem to be uninformative in that way. In case you requested it, we would be glad to prepare and upload as supplementary material one or several files with the alignments (nucleotide or aminoacidic) of each gene separately or all together. We also could prepare 3-D structures of proteins from cytochrome bc1 complex where important sites are highlighted. At this point, however, little is gained by checking such presentations of the data.

In sum, we believe we have a much improved version of the manuscript that effectively addresses the excellent comments of both reviewers and that we are justified in the cases of the two suggestions we did not follow.

I thank again for the suggestions made and look forward to your answer.

Yours sincerely,

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The evolution of mitochondrial genomes in subterranean caviomoph rodents: adaptation against a background of purifying selection.

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Abstract

Two rodent lineages have independently colonized the subterranean niche, South American tuco-tucos (genus *Ctenomys*) and related coruros (genus *Spalacopus*). The energetically demanding lifestyles of these species coupled with hypoxic atmospheres characteristic of the subterranean environments may have altered the selective regimes on genes that encoding cellular respiration related proteins. Here, we examined the molecular evolution of 13 protein coding genes in the mitochondrial genome of seven caviomoph rodents, including these two subterranean genera 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 subterranean groups with respect to their non-subterranean counterparts in 11 of 13 genes, although no ω ratios were larger than 1. An alternative procedure was used to test for positive selection on quantitative physicochemical properties. Destabilizing changes in biochemical properties were found to be ubiquitous across phylogenies, but concentrated in the subterranean lineages. Convergent changes were also found between subterranean genera used in this study and other hypoxia adapted mammals. The results of this study suggest a link between nich shifts and weak directional (or episodic) selection at the molecular level over a background of purifying selection.

key words: positive selection, mtDNA, Caviomorpha, subterranean niche, purifying selection

1. Introduction

Mitochondrial DNA (mtDNA) has long been used for phylogenetic reconstruction, phylogeography and population historical inference (Avise, 2000; Chapple et al., 2009). It has often been assumed mtDNA evolves according to the neutral theory due to correlations between global rates of mtDNA evolution, metabolic rate and generation time (Martin, 1995; Martin and Palumbi, 1993). Thus, little attention devoted to molecular adaptation of mitochondrially encoded proteins though variation in protein coding genes of the mitochondria that are involved in oxidative phosphorylation (OXPHOS) can directly influence metabolic performance (da Fonseca et al., 2008). Despite strong functional constraints, mtDNA may be subject to positive directional selection in specific cases greater energy requirements or limited oxygen availability.

Many studies provide support for instances of adaptive selection in mammalian mitochondrial protein coding genes. Sequence variation in CytB has been correlated with ecological differences among chromosomal races of blind mole-rats (*Spalax ehrenbergi*, Nevo 1999) and the metabolic shift in cetaceans relative to their terrestrial ancestors (McClellan et al. 2005). Increasing metabolic demands of an expanding cerebral cortex were suggested by Grossman et al. 2004 to have driven co-adaptation among subunits of OXPHOS proteins during primate evolution, while mitochondrial amino-acid polymorphisms in humans have been shown to improve aerobic capacity and adaptation to different thermal environments (Ballard and Whitlock, 2004; Blier et al., 2001; Dalziel et al., 2006; Grossman et al., 2004; Jobson et al., 2004). In addition, adaptation in COX and CytB genes has been suggested due to increased evolutionary rates in anthropoids (as opposed to other mammals, Andrews et al. 1998 and Adkins and Honeycutt 1994) and in mammal species adapted to unusual oxygen

requirements (da Fonseca et al., 2008; Di Rocco et al., 2006; Luo et al., 2008; Shen et al., 2010; Xu et al., 2005).

Due to their functional importance, purifying selection is the dominate force in the evolution of mitochondrial genes; however, weak and/or episodic positive selection may occur in this background of strong purifying selection if selective pressures shift such as might be experienced when oxygen availability decreases.

Fossorial rodents constitute an ideal system to test hypotheses about adaptive evolution driven by ecological shifts. The subterranean niche is characterized by high levels of carbon dioxide and low levels of oxygen (Buffenstein, 2000). Those stresses in addition to the high energy requirements associated with burrowing (Vleck, 1979) suggest that proteins involved in respiration likely experienced positive directional selection in response to their entry into the fossorial habitat. Under this hypothesis, accelerated rates of replacement relative to silent substitutions ($\omega=dN/dS$) in these genes are expected in subterranean organisms with respect to their non-subterranean sister taxa. The sister families Octodontidae and Ctenomyidae provide a unique opportunity to trace the evolution of adaptations related to digging (Lessa et al., 2008). Burrowing for shelter and rearing young is the rule among these families of rodents but only two living lineages, *Ctenomys* (tuco-tucos) and *Spalacopus* (coruros), have recently evolved fully subterranean habits and associated adaptations. Spiny rats (Echimyidae) are largely cursorial and arboreal and represent a suitable outgroup to the Ctenomyd-Octodontid clade (e.g.: Honeycutt et al. 2003, Opazo 2005). Phylogenetic relationships among genera are well established (Opazo, 2005 and references therein) (figure 1), which would allow tracing the changes associated with the acquisition of subterranean adaptations onto a known phylogeny, as well as identifying and discriminating such adaptations from more general ones associated with fossoriality.

APPROXIMATE LOCATION OF FIGURE 1

Da Silva et al. (2009) found a significantly higher ω ratio in the CytB among independent lineages of subterranean rodents (tuco-tucos, coruros, pocket gophers and mole rats) when compared with their above-ground relatives, suggesting a link between directional selection in this gene and the niche shift to life underground. However, this evidence is also consistent with alternative neutralist explanations, such as relaxation of purifying selection due to reduction of population sizes in subterranean lineages. Mitochondrial genes are completely linked in mammals and thus all share a single evolutionary history. If relaxation of purifying selection due to smaller population sizes is the cause of increased ω in subterranean lineages, the pattern should be observed in all protein coding genes. Alternatively, an adaptive process might have affected several, but not necessarely all of these genes. Different patterns of rate variation among lineages are expected if positive selection is applied episodically across the genome. An examination of the pattern of evolution in mitochondrial genomes may thus provide a test of these alternative hypotheses.

Our goal was to test the hypothesis that adaptation to energetically demanding lifestyles, in particular those associated with limited oxygen availability, may involve weak or episodic adaptive change in proteins linked to the OXPHOS, including those encoded by the mtDNA. To examine this possibility, we analyzed 13 protein coding genes of mtDNA from two related but independent subterranean lineages of caviomorph rodents (tuco-tucos and the cururo). We then compared variation in these lineages with that of their non subterranean counterparts to search for two independent footprints of positive natural selection coincidence with niche shifts.

2. Materials and Methods

We selected 13 proteing coding genes from the complete mtDNA of seven caviomorph rodents species, representatives of two related but independent subterranean lineages. Among these were three species of tuco-tucos (*Ctenomys*) and the coruro (*Spalacopus cyanus* HM544133), two non-subterranean allies (*Octodon degus* HM544134 and *Tympanoctomys barrerae* HM544132), and a spiny rat as an outgroup (*Proechimys longicaudatus* HM544128). Tuco-tucos are comprised of more than 56 species and are considered one of the most rapidly speciating mammalian lineages (Reig et al., 1990). We represent this group with 3 species in an attempt to capture the diversity of lifestyles and molecular differentiation contained within this genus (Castillo et al., 2005; Parada, 2007; Slamovits et al., 2001 and references therein). *C. rionegrensis* HM544130 is typical of the many low elevation, asocial species of the genus. *C. leucodon* HM544131 is a specialized high altitude (>4000m) species that was once suggested to be a distinct subgenus (*Haptomys*) by Osgood (1946). *C. sociabilis* HM544129 is sister to the clade that includes all other *Ctenomys* species (Cutrera and Lacey, 2007; Parada ,2007), is social and lives in open burrows (Pearson and Christie, 1984).

The tree topology considered in all the analyses is shown in figure 1. This phylogeny was constructed using 12 of the 13 protein-coding genes of the mtDNA genome of 9 hystrocognath rodents: 7 obtained in this study and the two available in GenBank: *Cavia porcellus* (NC_000884), and *Thryonomys swinderianus* (NC_002658) considered as outgroup. These two taxa are too divergent to be used in the analysis of positive selection. For this purpose we used the spiny rat *P. longicaudatus*,which was confirmed as sister to the remaining octodontoids used in this study. The ND6 gene was excluded because it is encoded

by the light-strand which has a significantly different base composition from the heavy-chain (Gibson et al., 2005). Multiple alignments of the concatenated sequences were generated with MUSCLE (Edgar, 2004). Gaps and ambiguous sites were removed, resulting in a total alignment of 10,775 nucleotides (≈ 3,591 codons). Bayesian inference methods with Markov chain Monte Carlo (MCMC) sampling were used as implemented in MrBayes (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). We used a General-Time-Reversible substitution model (Saccone et al., 1990) with the invariant site plus gamma options (five categories) after determining the optimal model of sequence substitution with ModelGenerator (Keane et al., 2006). One cold and three incrementally heated chains were run for 20,000 generations, and trees were sampled every 10 generations from the last 10,000 generated (well after the chain reached stationarity) and 1,000 trees were used for inferring Bayesian posterior probabilities (50% of burn-in fraction). All nodes of this phylogeny have high posterior probabilities (p=1), and the topology agrees with previous phylogenetic hypotheses proposed for these species (e.g.: Opazo, 2005; Parada et al., *in press*) and also with those obtained by other methods (data not shown).

Variation in estimates of dN, dS and ω was explored using an ML approach as implemented in PAML4 (Yang, 2007). Variation in ω´s was estimated: *i)* along different branches while holding the rates constant across codons (Yang and Nielsen, 1998), *ii)* across codons while holding rates constant along branches (Yang et al., 2000) and *iii)* 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. In the case of allowing 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's, and iii) different intermediate models allowing different ω's for each clade or branch of interest (either phylogenetic and/or "ecological", namely subterranean or non-subterranean taxa). In the case of exploring variation across codons (Site Models), we compared the likelihood fit of several evolutionary models described by Yang et al. (2000) and testeded positive selection using the three likelihood ratio test (LRT) recommended in the PAML4 user manual (M1a-M2a, M7-M8 and M8a-M8 comparisons). When exploring positive selection at individual sites along specific lineages, we used two variants of the Branch-site model A and the LTR between them (Model A and Model A modified) also as recommended (Yang and Nielsen, 2002; Yang et al., 2005; Zhang et al., 2005). As PAML only allows two branch types, we chose to run comparisons of variation between lineages considering different combinations of subterranean lineages (foreground branches, namely, coruro, tuco-tucos or both) versus nonsubterranean counterparts (background branches). For site and branch-site models, the Bayes empirical Bayes (BEB) was used to calculate posterior probabilities for site classes to determine which codon positions have experienced positive selection ($\omega>1$) when the likelihood ratio test was significant. In all cases, PAML was run three times with different omega starting values (0.4, 1 and 4, respectively), as recommended to check for multiple local optima, and the α level of significance was 0.05.

Significant physicochemical amino acid changes among residues in mitochondrial protein coding genes were identified by the algorithm implemented in TreeSAAP software (Woolley et al., 2003). TreeSAAP which compares the observed distribution of physicochemical changes inferred from a phylogenetic tree with a distribution based on the assumption of completely random amino acid replacement expected under strict neutrality. Nucleotide nonsynonymous changes are classified into eight categories. Only categories 6, 7

and 8, which exhibited the most radical changes based on the observed numbers of amino acid replacements significantly differing from the null model ($P < 0.001$), were considered to be potentially affected by directional selective pressures (positive-destabilizing selection). We ran ModelGenerator (Keane et al., 2006) to select a substitution model for each gene to reconstruct ancestral sequences from a tree generated in Baseml (Yang, 1997). The selected models were: a) TN (Tamura and Nei, 1993) for ND3, b) HKY (Hasegawa et al., 1985) for ATP6, ATP8, ND4L and ND6, and c) REV (Saccone et al., 1990) for the remaining genes.

3. Results

3.1 Variation in ω=dN/dS

The Branch Models yielded the most interesting PAML results. Full models (12 independent ωs, one for each branch) were significantly better than null models (a single ω for all branches), for all genes except ND3 and ATP8. In the cases of ND1, ND2, ND4L and COX2 these full models could be simplified to two-parameter "ecological" models that assigned one ω value to subterranean taxa and another ω to non-subterranean taxa without a significant loss of statistical fit. In the remaining genes, full models could be simplified to other alternatives (Table 1).

Although the pattern of variation among lineages in substitution rates varied substantially across genes and no ω values greater than one were estimated for any gene, ω values were higher for subterranean than non-subterranean taxa (with the exception of ATP8). The most striking example is that of COX2 in the coruro, with an estimated ω almost 30 times that of nonsubterranean branches. Estimations of ω in subterranean taxa were mostly higher in the coruro than in tuco-tucos, except for ND4L, APT6, COX1 and COX3; in the latter two genes, the inverse pattern is found (if the tuco-tuco clade includes the basal branch). A

summary of results for three parameter models, which allow one ω for each of the subterranean lineages – tuco-tucos and coruro – and another for nonsubterranean taxa, is shown in table 1.

When variation in ω was explored across codons, we found that in most genes the discrete model (M3 in PAML) was the best, which groups codons into 3 classes assigning ω from a discrete distribution to each class. However, we found models of selection significantly better than the null only in ATP8 (using M1a-M2a, M7-M8 and M8a-M8 comparisons), and we found ω > 1 (posterior probability = 0.978) for codon 41 (Y). We also found the same model of selection was significantly better than the null in COX3 gene (M7-M8 comparison), but no specific codons were found to be under positive selection. The Branch-sites Model A aims to identify positively selected codons. Its statistical significance can be assessed with test 1 (which can show false positives, Zhang et al., 2005), or with more conservative test 2. None of our branch-site models was significant using test 2.

3.2 Changes of properties of aminoacids

Significant physicochemical amino acid changes among residues in mitochondrial protein coding genes were identified with TreeSAAP. There are more radical amino acid property changes in the tips (~ 77%) relative to the interior branches (~ 23%) of the tree, and grouped branches leading to subterranean taxa retain more than 50% of radical amino acid property changes. Branches leading to non-subterranean relatives (excluding the outgroup) contain 28%. All genes except ND3 show amino acid properties under positive-destabilizing selection. Proteins varied in the fraction of strong positively selected amino acid properties per site, from 0.03 in COX3 to 0.38 in ATP8 (supplementary material). The pattern of selected properties was different between subterranean and non subterranean taxa in six of the proteins analyzed (ATP6, ND2, ND4, ND4L, CytB and ND5) (supplementary material).

Table 2 shows the sites (or adjacent sites) infered to have been subjected to positive selection independently in subterranean lineages (coruro and tuco-tucos). Most of the genes have one site in this situation, except ND4L with two sites, and genes coding COX1 and ND5 with three sites each. Interestingly, in some cases (like site 46 of CytB or COX1, or site 50 in ATP6) the same property has been selected in tuco-tucos and coruros, but resulting in different amino acid replacement. Site 41 of ATP8 was found to be under positive-destabilizing selection in branches leading to the coruro, and was also detected by PAML. As will be discussed in the following section, some of the sites found under positive-destabilizing selection by TreeSAAP in this dataset were previously found to be under selection (da Fonseca et al., 2008; Da Silva et al., 2009; Luo et al., 2008).

4. Discussion

Purifying selection predominates in the evolution of mtDNA. However, it is possible that weak and/or episodic positive selection occurs in this background of strong purifying selection when a shift is made to a greater energy demanding lifestyle or reduced availability of oxygen (e.g. Shen et al., 2010). We examined the possibility of weak and/or episodic positive selection in subterranean lineages because colonization of the subterranean niche involves these characteristics and may impact the function of mitochondrial genes. Among octodontids, two independent and relatively recent colonizations of the subterranean niche can be explored for consistent features of convergent evolution under similar selection pressures, relative to their nonsubterranean relatives. In addition, analyses of variation in ω can be complemented with methods developed to evaluate the strength of destabilizing selection on biochemical amino acid properties.

Our main results are: *1*) most genes show increased ω in subterranean relative to nonsubterranean lineages, *2*) patterns of variation among lineages in substitution rates vary across genes, *3*) destabilizing changes in biochemical properties are ubiquitous in the phylogenies, but tend to concentrate within the subterranean lineages and *4*) some convergent changes found here among independent subterranean genera were suggested by previous studies of lineages adapted to oxygen-limited lifestyles. 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 protein coding mtDNA genes, but that positive selection in likely episodic in a background of purifying selection.

4.1 Acceleration of ω in subterranean lineages

Twelve genes (all except ATP8) show increased ω in subterranean lineages relative to nonsubterranean ones. In several genes, simpler models can capture most of variation of the full model without a significant loss of fit, similar to the results of Da Silva et al., (2009) for CytB. Likelihood values tended to be significantly higher when separating the coruro from tuco-tucos (in ATP6, COX2, ND1, ND5 and ND6). In general the coruro shows higher ω values than the tuco-tucos. A similar contrast has been found in morphological adaptations for digging: the coruro has accumulated them quickly in a short period of time, relative to a more protracted process in tuco-tucos (Lessa et al., 2008).

We further explored variation in ω across codons with the sites model (Yang et al., 2002), and only one codon position, 41, was identified as being under positive selection in ATP8. This result is consistent with the fact that the effect of the acceleration in few sites was diluted among a large majority of slowly evolving sites. Interestingly, this site and adjacent ones were also found to be positively selected by TeeSAAP (Table 2).

4.2 Pattern of ω variation differs among lineages in all genes

When ωs are smaller than 1, the study of a single gene does not allow us to reject alternative, non-selectionist explanations of rate variation, such as a relaxation of purifying selection, variation in metabolic rate, body mass, population size, and generation time among lineages (Bromham et al., 1996; Li et al., 1996; Martin 1995; Martin and Palumbi 1993). However, under a model of relaxation of purifying selection the same pattern of variation among lineages is expected in all genes. In contrast with these expectations this study found a) most, but not all genes have greater ω's in subterranean lineages; and b) in many, but not all cases, they are greater in coruros than in tuco-tucos.

Three examples illustrate the large variation found across genes and lineages in this study. In COX2, coruros show a ω almost 30 times greater than that of non-subterranean lineages, and 11 times higher than tuco-tucos. In contrast, ATP6 shows an increase in ω in tuco-tucos, but not in the coruro. Finally, ATP8 shows significantly reduced, not increased ω, in both subterranean genera.

4.3 Destabilizing changes are concentrated on subterranean lineages

Our results regarding ω were also supported using a different approach to detect selection in amino acid sequences, namely to examine the magnitude of property change of non-synonymous residues across a phylogeny using TreeSAAP (Woolley et al., 2003). The results of this analysis were consistent with those from PAML, also suggesting that there is a concentration of codons subjected to positive selection, particularly (but not exclusively) among subterranean lineages. In general, subterranean groups showed more selected

properties and sites under positive desestabilizing selection than non-subterranean relatives. In addition, the single site that PAML detected as having been under positive selection was also included among those detected by TreeSAAP. In this sense, the TreeSAAP software seems to be more sensitive to detect selection under these conditions (even using a highly conservative criterion of $p< 0.01$), as has been observed in other cases (e.g., da Fonseca et al., 1998; Da Silva et al., 2009; Mc. Clellan et al., 2005).

The amount of changes relative to branch length is higher in subterranean lineages, namely the branches within the tuco-tucos and leading to the coruro, except in *C. sociablis* (figure 2). These results seem to indicate a concentration of destabilizing changes in biochemical properties on the subterranean lineages. The estimated number of positively selected changes per 0.01 units of branch lengths is almost 5 in these subterranean lineages, while the remaining values were not greater than 3.1. Interestingly, *C. sociabilis*, which has a quite behaviorally distinctive from other species of tucu-tucos is the most divergent species of the genus (Cutrera and Lacey, 2007; Parada, 2007). This species is social and always maintains the burrows open (Pearson and Christie, 1985), thereby preventing significant departures of O_2 and CO_2 concentrations relative to the above ground conditions. These results seem to indicate a concentration of destabilizing changes in biochemical properties on the subterranean lineages. Differences in number of genetic changes in subterranean genera also agree with observations regarding morphological adaptations to life underground. The lineage leading to the coruro has adapted to a fully subterranean life, acquiring numerous morphological changes in some 2.2 million years. In contrast, ctenomyids (family Ctenomyidae) have accumulated changes associated with subterranean life in a mosaic fashion along several lineages, in a process that has taken at least 8 million years as documented in the fossil record (Lessa et al., 2008). However, a more thorough evaluation of the case of the coruro would require molecular data on the closely related genus *Aconaemys*.

APPROXIMATE LOCATION OF FIGURE 2

4.4 Convergent changes among subterranean lineages

In particular codon sites, properties involved in radical physicochemical amino acid changes are the same in independent subterranean lineages, although not always involving the same amino acid replacement. Convergent amino acid substitution in subterranean lineages was found in only 19 out of 3,591 sites analysed. The same amino acid substitution in subterranean lineages was found in sites 406 and 483 in COX1, 165 in COX2, 153 in ND1, 156 in ND2, 105 in ND4, and 424 in ND5. However the same property was selected due to different amino acid replacement in site 46 of CytB, 46 of COX1, 50 of ATP6, 42 of COX3, 4 and 21 of ND4L and 272 in ND5. For instance, in site 46 of COX1 the Alpha-helical tendency (Pα) has been selected in the basal branch of tuco-tucos, which changed from arginine to threonine as well as in the branch leading to the coruro, which changed to serine. Both amino acids are polar and neutral residues, whereas arginine is neutral but nonpolar. Similar situations were observed in site 46 of CytB and site 50 of ATP6.

Interestingly, our results show positive selection on some of the same codon sites found previously in studies of mammalian subterranean radiations or instances of similar metabolic requirements (Table 3). For instance, Da Silva et al. (2009) detected an important amino acid change in codon 46 of CytB in subterranean pocket gophers and bathyergids, as did we. We also found an amino acid substitution in codon 6 of ND6, like that found by Luo et al. (2008) in pikas, In this paper it was also suggested the ND6 codon 6 replacement and others replacement could potentially function in modulation of mitochondrial complexes and electron transport efficiency under cold and hypoxic conditions. We found codons 5 and 6 of

ND6 to be under positive selection in coruro and two species of tuco-tucos (Tables 5 and 6). The remaining codon variation that matched those found in previous studies (Table 3) mostly belong to the well studied CytB and COX.

5. Conclusion

Taken together, our two analyses point toward the action of positive, directional selection in the evolution of several mitochondrial genes in association with the colonization of subterranean environments in rodents. To explain this, we propose a scenario of episodes of positive selection against a background of negative selection, as we ruled out the relaxation of purifying selection as a plausible explanation of observed patterns. Directional positive selection may be acting either at specific sites or regions of mitochondrial genes, driving destabilizing biochemical change in subterranean lineages. Finally, we agree with recent studies which suggest that the evolution of mitochondrial protein genes could be associated with metabolic adaptations to low O_2 environment (da Fonseca et al., 2008; Da Silva et al., 2009; Luo et al., 2008; Shen et al., 2010).

Adaptation to hypoxia/hypercapnia are known to occur at several levels, from modifications in blood's O_2- transporting properties, to a high tolerance of decreased O_2 availability entailing reconfigurations at both the organ and cellular levels (reviewed by Boggs et al., 1984; Buffenstein, 2000; Nevo, 1999). However, these adaptations to hypoxia have been evaluated only in few subterranean species from the genera *Spalax* (Mediterranean), *Heterocephalus* (African) and *Thomomys* (Northamerican), and there are no comparative studies including several species. In addition, well-studied subterranean taxa are very divergent from their sister groups (if known), making it difficult to assess the adaptive path leading to subterranean life. Closely related sister families *Octodontidae* and

Ctenomyidae offer an ideal opportunity to trace the evolution of these traits, and this is the first step in this direction.

The molecular basis of hypoxia tolerance is starting to be known in hemoglobin, myoglobin and vascular endothelial growth factor (reviewed by Nevo, 1999). Several nucleotide changes responsible for physiological adaptation to high altitude in vertebrates have also been documented (reviewed by Weber, 2007). Many genes associated with the cellular response to hypoxia have been examined in *Spalax*, including those coding for vascular endothelial growth factor receptors, heparanase, tumor suppressor genes and apoptosis genes, among others (e.g.: Avivi et al., 2006; Band et al., 2008, 2009, 2010; Sandwall et al., 2009). Evolutionary adjustments to hypoxia likely require coordinated changes in interacting proteins or subunits, thereby necessitating coordinated changes in several unlinked genes (Blier et al., 2001; Rand et al., 2004; Storz et al., 2009). Subtle genetic variations could have generated substantial physiological changes, and be positively selected for, and still be undetectable by classical methods. It has also been shown that simple mutations in mitochondrial tRNA genes can also influence physical performance in humans (Rankinen et al., 2006) and mice (Moreno-Loshuertos et al., 2006), suggesting that tRNA polymorphisms may influence the rate of mitochondrial protein synthesis, as well as translation fidelity. Several substitutions in mitochondrial tRNA are reported among species included in this study, and their potential role in OXPHOS performance merits further assessment in future experiments. Expanding this study in term of function and nuclear-mitocondrial interactions will be possible in this new genomic era (Hassanin et al., 2009).

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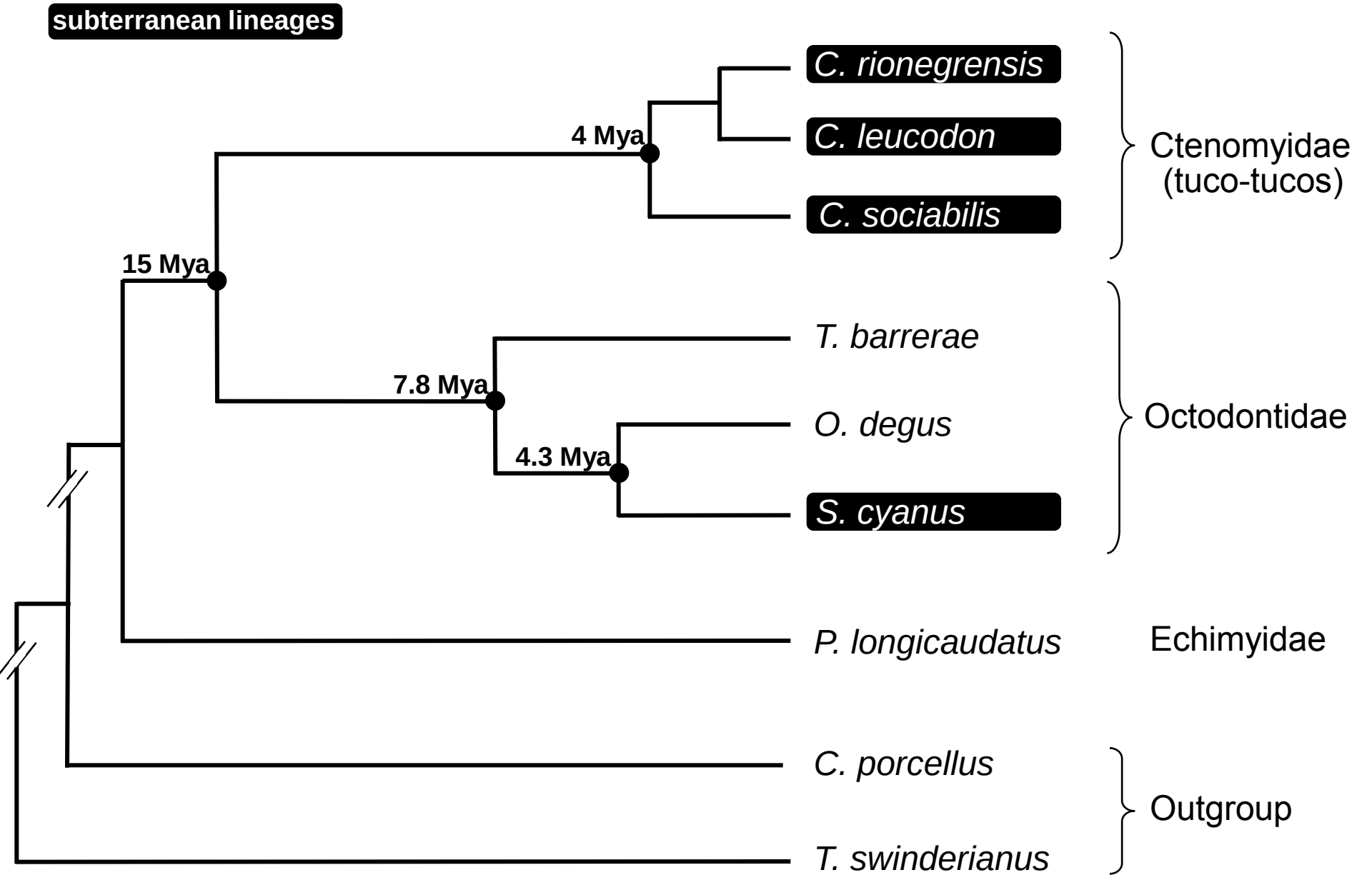
Figure 1. Phylogenetic relationship among hystricognath rodents included in this study. Divergence times are taken from Opazo (2005) and Verzi (2002). Mya: Millon years ago.

Figure 2. Amount of significant physicochemical amino acid changes among residues in mitochondrial protein coding genes (identified by TreeSAAP) along each branch of the tree. Only terminal branches are named. The estimated number of positively selected changes per 0.01 units of branch lengths are shown under each species name. Linear regression and regression coefficient are shown.


subterranean lineages
C. rionegrensis
C. leucodon
C. sociabilis
4 Mya
15 Mya
T. barrerae
7.8 Mya
O. degus
4.3 Mya
S. cyanus
P. longicaudatus
C. porcellus
T. swinderianus
Ctenomyidae
(tuco-tucos)
Octodontidae
Echimyidae
Outgroup

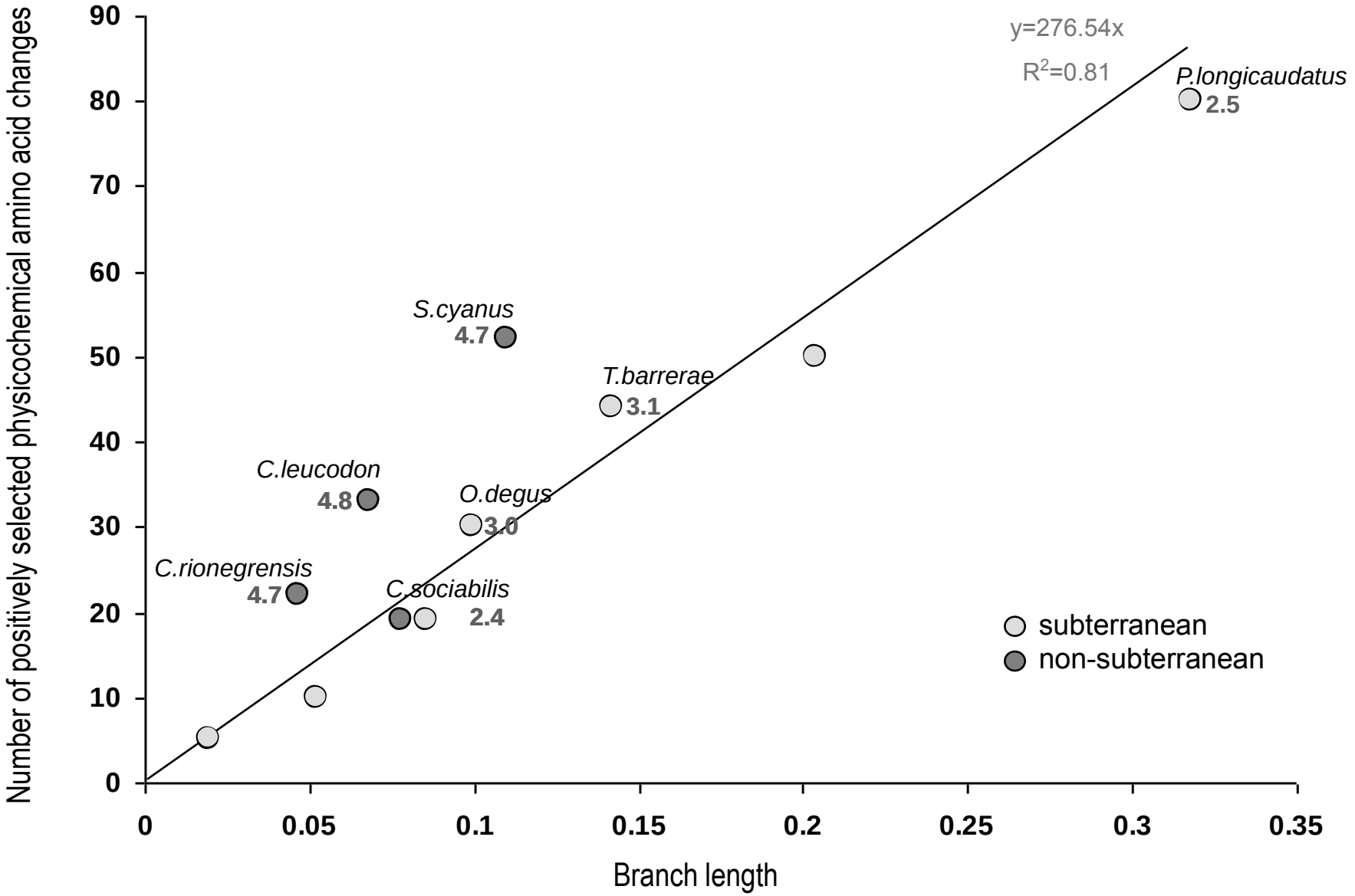

Number of positively selected physicochemical amino acid changes
Branch length
y=276.54x
R2=0.81
P.longicaudatus
2.5
S.cyanus
4.7
T.barrerae
3.1
C.leucodon
4.8
O.degus
3.0
C.rionegrensis
4.7
C.sociabilis
2.4
subterranean
non-subterranean
0
10
20
30
40
50
60
70
80
90
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35

Table 1. Estimation of ω values from PAML4 using 3 rate parameters models. ATP: ATP synthase subunits; CytB: cytochrome b; COX: cytochrome c oxidase subunits; ND: NADH dehydrogenase subunits. Fonts indicates values of ratio between subterranean and non-subterranean allies: in *cursive* ratio <0; normal letter: 1< ratio ≤3; bold: 3<ratio ≤10; bold and underlined: ratio >10. Selected model for each gene: most simple model to which the full model (one ω per branch) can be simplified: Null, a single ω for all branches; 2P, one ω for subterranean and another for nonsubterranean taxa; 7P and 8P, one ω to all subterranean branches and the others taxa vary freely; *: basal branch of tuco-tucos considered as subterranean. ---: value not estimated.

		Estimated ω values			
		Subterranean		Non-subt	Selected
gene	# codons	Tuco-tucos	Coruro		model
ATP6	225	**0.09**	*0.02*	0.02	Full
ATP8	62	*0.21*	*0.25*	0.47	Null
CytB	378	**0.05**	**0.06**	0.01	Full
COX1	510	**0.02**	0.01	0.01	8P
COX2	225	**0.03**	**<u>0.19</u>**	0.01	2P
COX3	260	0.03	0.02	0.01	8P
ND1	318	**0.02**	0.07	0.01	2P*
ND2	346	**0.06**	0.14	0.04	2P
ND3	114	0.06	---	0.05	Null
ND4	97	0.05	0.06	0.03	7P
ND4L	458	**0.07**	0.04	0.02	2P*
ND5	602	0.07	0.13	0.05	Full
ND6	176	0.07	**0.20**	0.03	7P

Table 2. Sites under positive-destabilizing selection in independent subterranean lineages (*Spalacopus* and *Ctenomys*). pK´: Equilibrium constant; αm: Power to be at the middle of the alpha-helix; Br: Buriedness; R_F: Chromatographic index; H: Hydropathy; E: Long-range nonbonded energy; Rα: Solvent accessible reduction ratio; pH_i: Isoelectric point; F: Mean r.m.s. fluctuation displacement; P: Polarity; Pα: Alpha-helical tendencies; Rα: Solvent accessible reduction ratio; P_t: Turn tendencies; Et: Total nonbonded energy; Pr: Polar requirement; αn: Power to be at the N-terminal; Ns: Average number of surrounding residues; Hp: Surrounding hydrophobicity; αc: Power to be at the C-terminal. Branches labeled as "Int Octodontids" and "Int *Ctenomys*" correspond to those leading to last common ancestors of *O. degus* and *S. cyanus* and of *C. rionegrensis* and *C. leucodon*, respectively. * site found to be under selection in previous studies (1: Da Silva et al., 2009; 2: da Fonseca et al., 2008; 3: Luo et al., 2008.); additional information in table 3.

				Amino acid change		
Gene	**Site**	**Branch**	**Property**	**from**	**to**	
ATP6	50	*S. cyanus* Basal *Ctenomys*	*pK´*	I	V	
ATP8	40	*S. cyanus*	Ns, αn, Hp	S	L	
	41	Int.Octodontids *S. cyanus* *T. barrerae*	αm	Y H Y	H Y H	
	42	*T. barrerae* Basal *Ctenomys*	Ns, αn, Hp Ns, Hp	L	S Q	
CytB	46	Basal *Ctenomys* *S. cyanus*	*pK´*	I	L V	***1**
COX1	46	Basal *Ctenomys* *S. cyanus*	*Pα*	A	T S	
	406	*C. sociabilis* *S. cyanus*	*αc*	D	N	***2**
	483	*C. leucodon* *S. cyanus*	*Pα*	M	T	***2**
COX2	165	*C. sociabilis* *S. cyanus*	*pK´*	V	I	
COX3	42	Basal *Ctenomys* *C. leucodon*	*pK´*	L I	I V	***2**
ND1	153	Basal *Ctenomys*	*pK´*	I	V	

		S. *cyanus* C. rionegrensis		V	I	
ND2	156	*C. sociabilis* *S. cyanus*	*pK´, EI, Rα*	T	I	
ND4	105	Basal *Ctenomys* *S. cyanus*	*RF*	F	S	
ND4L	4	Basal *Ctenomys* *S. cyanus* *C. leucodon*	*pK´* *Rα*	I V	V M T	
	21	Basal *Ctenomys* S. cyanus	*pK´*	I	M V	
ND5	272	*C. sociabilis* *S. cyanus*	*Pα*	T	A M	
	424	Basal *Ctenomys* *S. cyanus*	*Pα*	T	M	
	519	Basal *Ctenomys* Int. *Ctenomys*	P *Am, RF*	L H	H T	
	520	*S. cyanus*	*Pα*	T	A	
ND6	5	*C. leucodon* *S. cyanus*	*pK´*	I	V	
	6	*C. rionegrensis*	*pK´*	V	I	***3**

Table 3. Posible sites under positive-destabilizing selection in independent subterranean lineages of this study (*Spalacopus* and *Ctenomys*) and previous ones. pK´: Equilibrium constant; αm: Power to be at the middle of the alpha-helix; Br: Buriedness; R_F: Chromatographic index; H: Hydropathy; E: Long-range nonbonded energy; Rα: Solvent accessible reduction ratio; pH_i: Isoelectric point; F: Mean r.m.s. fluctuation displacement; P: Polarity; Pα: Alpha-helical tendencies; Rα: Solvent accessible reduction ratio; P_t: Turn tendencies; Et: Total nonbonded energy; Pr: Polar requirement; αn: Power to be at the N-terminal; Ns: Average number of surrounding residues; Hp: Surrounding hydrophobicity; αc: Power to be at the C-terminal. * site found to be under selection in previous studies using PAML4; 1: Da Silva et al., 2009, 2: da Fonseca et al., 2008, 3: Luo et al., 2008, 4: Shen et al., 2010.

				Amino acid change		Previous studies		
Gene	**Site**	**Branch**	**Property**	**to**	**from**	**Lineage**	**Property**	**Ref.**
CytB	4	*T. barrerae* *P. longicaudatus*	*pK´*	I	L	Subt. Caviomorphs	pK´	1
	14	*Basal Ctenomys*	*pK´*	I	V	Subt. pocket gophers	pK´	1
	*42	*Basal Ctenomys*	F	M	G	Subt. Caviomorphs Subt. pocket gophers	Ca pK´	1
		P. longicaudatus	*pK´*		I	Subt. mole rats Flying bats*	pK´	4
	46	*Basal Ctenomys*		I	L	Subt. pocket	Bi, RF, Pc, αn,	1
		S. cyanus	*pK´*		V	Subt. mole rats	pK´	1
		P. longicaudatus						1
	115	*C. leucodon*	*pK´*	I	M	Subt. Caviomorphs	pK´	1
	*117	*C. sociabilis*	*pK´*	V	I	Subt. Caviomorphs*	pK´, Rα	1
	226	*C. sociabilis*	*pK´*	I	V	Subt. pocket gophers	EI	1
	232	*C. rionegrensis*	*pK´*	V	I	Subt. Caviomorphs Subt. pocket gophers	pK´ EI	1
	236	*C. leucodon*	*pK´*	M	I	Subt. pocket gophers	pK´	1
	237	*Basal Ctenomys*	*pK´*	L	I	Subt. pocket gophers	Pα	1
	243	*P. longicaudatus*	*pK´*	V	I	Subt. pocket gophers	Rα	1
	*295	*C. sociabilis*	*pK´*	V	I	Subt. Caviomorphs* Heteromyds Subt. pocket gophers	Pc, Ei, F, Pt F F	1
	*306	*Basal Ctenomys*	*pK´*	M	I	Subt. Caviomorphs Subt. mole rats Subt. Pocket	Ns, pK, Ei pK´	1
	327	*O. degus*	*pK´*	I	A	Subt. Caviomorphs Subt. mole rats	pK´ pK´	1
	356	*P. longicaudatus*	*pK´*	V	I	Subt. Caviomorphs	Ns, Rα	1

	368	*C. leucodon*	F	S	L	Subt. Caviomorphs	Ns	1
COX1	406	*C. sociabilis* *S. cyanus*	*αc*	D	N	Mammals		2
	408	*T. barrerae*	*Pα*	T	A	Hypoxia tolerant Pika		3
	483	*C. leucodon* *S. cyanus*	*Pα*	M	T	Mammals		2
COX3	42	*Basal Ctenomys*	*pK*	L	I	Mammals		2
ND6	6	*C. rionegrensis*	*pK*	V	I	Hypoxia tolerant Pika		3

14th February 2011

Response to Reviewers

First of all, I would like to thank, on behalf of the two authors of the manuscript, the positive comments and contributions of Reviewers 1 and 2, which helped us make this manuscript notoriously better. All minor comments and suggestions were accepted and incorporated in the new version and all the information required by Reviewers was also included. I would like to comment some of these modifications in order to facilitate the reassessment of the new version of the manuscript.

Following suggestions of Reviewer #1:

- we made our best effort to complete the description of methods, and added all the information requested. We included a new paragraph with detailed information about phylogenetic analyses and some clarifying comments which answer the questions about topologies used in different analyses. We also included comments about how the branch site model was implemented, how many times PAML was run and the corresponding starting omega values.
- we added a sentence in the discussion section related to the exclusion of species of the genus *Aconaemys* from the analyses and how this might affect interpretation of the results.
- we modified tables 2 and 3, changing the order and content of two columns related to "amino acid change", in order to made them more interpretable. In the new version we reported the residues involved in the amino acid change only for relevant branches.
- we accepted all minor changes, added missing references and corrected typographic mistakes. These include items from introduction (item 1), materials and methods (item 6), acknowledgments (items 1 and 2), Figure 1, Table 2 (items 1, 5 and 6) and Table 3.

We would also like to explain why we did follow a few suggestions.

Reviewer #1:

We did not include any new sentence about "alternative ways to infer positive selection" (when ω-values are not greater than one) in the Materials and Methods section. We believe the issue is sufficiently addressed in the introduction and discussion.

We kept the name "coruro" following Wilson and Reader (2005), the standard general reference on mammal species of the world. Specialists will have no problem with either name, and non-specialists will find "coruro" more easily than "cururo".

We did not describe "intermediate models" implemented in branch models. Since they gave no significant results, the information seems to be irrelevant to readers and, besides, such models are difficult to describe. I guess the point is to mention that we gave alternative models a chance to show something relevant—they did not.

Reviewer #2:

Reviewer #2 suggests the presentation of "raw data", such as "base pair and amino acid alignments" and "3-D structures". These files can be directly downloaded from GenBank and they seem to be uninformative in that way. In case you requested it, we would be glad to prepare and upload as supplementary material one or several files with the alignments (nucleotide or aminoacidic) of each gene separately or all together. We also could prepare 3-D structures of proteins from cytochrome bc1 complex where important sites are highlighted. At this point, however, little is gained by checking such presentations of the data.

In sum, we believe we have a much improved version of the manuscript that effectively addresses the excellent comments of both reviewers and that we are justified in the cases of the two suggestions we did not follow.

I thank again for the suggestions made and look forward to your answer.

Yours sincerely,

Ivanna Tomasco

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***Research Highlights**

- evolution of mitochondrial genes dominated by purifying selection (ω ratio<1)
- 2 approaches suggest a link between weak/episodic selection and niche shift
- significantly higher ω ratio in mitochondrial genes of subterranean groups.
- significant physicochemical changes concentrated in subterranean lineages.
- some changes shared with others groups adapted to hypoxia

*Graphical Abstract

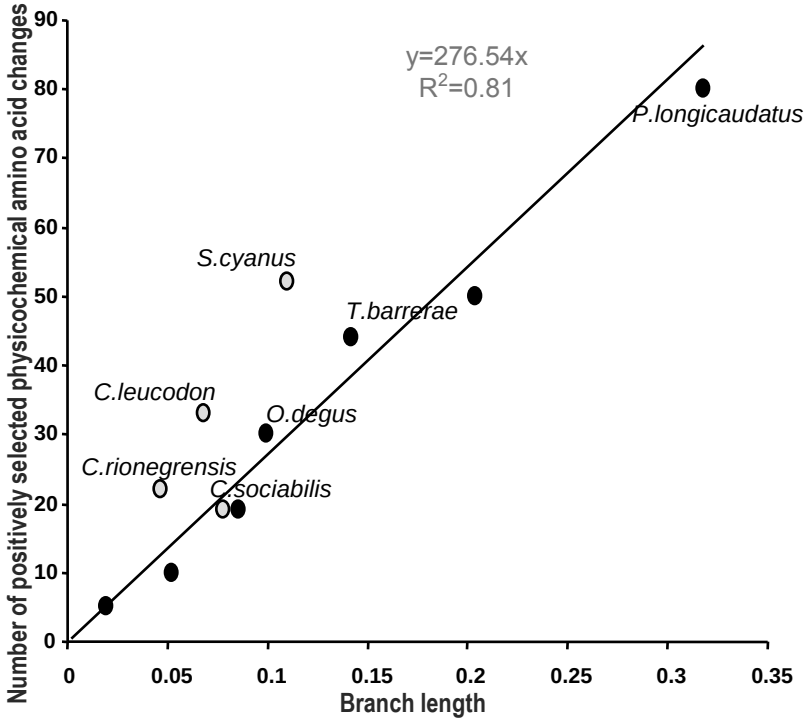
Underground life (hypoxia, hypercapnia)

Known adaptations at different levels

Adaptative change in proteins involved in cellular respiration

13 encoded by **MITOCHONDRIAL GENOME**

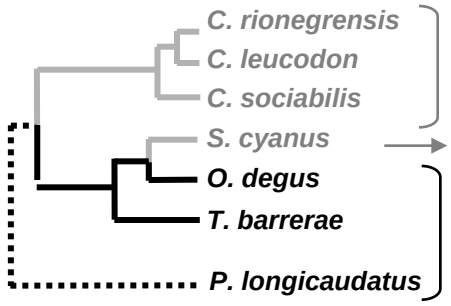
destabilizing changes in biochemical properties tend to concentrate on the subterranean (o) lineages.



two approaches suggest a link between weak directional or episodic selection at the molecular level and niche shift, in a background of purifying selection

significantly higher ω ratio (dN/dS) in the subterranean groups

subterranean
non-subterranean
outgroup



Estimated ω values

	ATP6	ATP8	CytB	COX1	COX2	COX3	ND1	ND2	ND3	ND4	ND4L	ND5	ND6	gene
	225	62	378	510	225	260	318	346	114	97	458	602	176	#codons
tuco-tucos	0.09	0.21	0.05	0.02	0.03	0.03	0.02	0.06	0.06	0.05	0.07	0.07	0.07	subt.
coruro	0.02	0.25	0.06	0.01	0.19	0.02	0.07	0.14	---	0.06	0.04	0.13	0.20	
non-subt	0.02	0.47	0.01	0.01	0.01	0.01	0.01	0.04	0.05	0.03	0.02	0.05	0.03	non-subt.

ω subterranean / ω non-subterranean

ratio <0,122< ratio ≤3; 3< ratio ≤10; 3< ratio ≤10.

Supplementary Material

Click here to download Supplementary Material: supplementary material.pdf

Capítulo 4

Evolución acelerada de la COX2 en linajes subterráneos de roedores caviomofos.

Los tuco-tucos sudamericanos (género *Ctenomys*) y su pariente cercano el coruro (género *Spalacopus*) son linajes de roedores que colonizaron el nicho subterráneo de manera independiente. Un estilo de vida demandante desde el punto de vista energético asociado al ambiente hipóxico en hipercápnico característico del nicho subterráneo podría cambiar los regímenes selectivos de los genes que codifican para las proteínas involucradas en la respiración celular. Como el O_2 es el último aceptor de electrones en el proceso catalizado por el complejo de la citocromo oxidasa c, las modificaciones en la actividad y/o estructura de esta proteína podrían contribuir a la adaptación a la vida bajo tierra. Examinamos la posibilidad de que la selección positiva débil o episódica afecten la evolución del gen de la subunidad II de la citocromo oxidasa c en estos linajes de roedores subterráneos en comparación con sus parientes cercanos. Usando aproximaciones bayesianas y de máxima verosimilitud, estimamos las tasas de sustituciones sinónimas (dS) y no sinónimas (dN). Encontramos una relación de las tasas de sustituciones ω (dN/dS) significativamente mayor en los grupos subterráneos con respecto a sus parientes no subterráneos, aunque estos ω no fueron mayores a 1. Usando un procedimiento alternativo que evalúa la existencia de selección direccional positiva a partir las propiedades fisicoquímicas de los amionoácidos involucrados en los reemplazos, encontramos que los cambios desestabilizantes en las proteínas se concentran en las ramas terminales de los linajes subterráneos, especialmente en aquella que conduce al coruro. También encontramos cambios convergentes entre los géneros subterráneos usados en este estudio y otros mamíferos adaptados a la hipoxia. Los resultados obtenidos por las dos aproximaciones son consistentes y sugieren un nexo entre selección direccional positiva débil o episódica a nivel molecular y la colonización del nicho subterráneo, en un contexto de fuerte selección purificadora.

Accelerated evolution of COX2 in subterranean lineages of caviomorph rodents

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Abstract

South American tuco-tucos (genus *Ctenomys*) and related coruros (genus *Spalacopus*) 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. Since oxygen is the ultimate electron acceptor in a process catalyzed by cytochrome c oxidase complex, modification of this enzyme structure and/or activity may contribute to adaptation to subterranean life relative to their close allies. We have examined the possibility that weak and/or episodic positive selection affects the evolution of the subunit II of cytochrome c oxidase gene in these subterranean lineages. 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 the subterranean groups with respect to their non-subterranean counterparts, althought ω's remain smaller than 1. We also found that destabilizing changes in biochemical properties are concentrated on the tip branches leading tosubterranean lineages, especially in that leading to the coruro. These two approaches are consistent with each other and suggest a link between weak directional or episodic selection at the molecular level in this gene and niche shift, in a background of purifying selection.

Introduction

Although mitochondrial DNA (mtDNA) has often been assumed to evolve neutrally, it encodes for proteins involved in oxidative phosphorylation (OXPHOS) that can directly influence metabolic performance. So, despite strong functional constrains, mtDNA may be subject to positive directional selection in cases, for example, of energy demanding lifestyles and/or limited availability of oxygen (e.g.: Grossman et al. 2004, McClellan et al. 2005, Fontanillas et al. 2005, Dalziel et al. 2006).

Mitochondrial subunits of the cytochrome c oxidase complex (COX) have gained particular interest in the study of molecular adaptations since the demonstration of an acceleration of their rate of substitution during the radiation of anthropoid primates (Adkins and Honeycutt 1994, Andrews and Easteal 2000, Schmidt et al 2004). These findings were attributed to an adaptive process related to the emergence of a larger, energy-dependent neocortex in anthropoid lineages (Grossman et al. 2001). COX is a well known multimeric complex involved in the terminal oxidative step of energy metabolism; specifically, it catalyes the transfer of electrons from reduced cytochrome c (a nuclear protein) to oxygen. Subunits I, II and III (COX1, COX2, and COX3, respectively) are encoded in the mitochondrial genome and the remaining ten in the nucleus (Capaldi et al. 1983). COX1 and COX2, make up the catalytic core involved in electron transport, whereas COX3 is believed to play a structural or regulatory role (Brunori et al. 1987; Tzukihara 1996).

We have focused on COX2, one of the core catalytic subunits of COX. COX2 is involved in electron transfer from cytochrome c and plays a role in substrate/product channeling, and COX assembly, stability, and regulation. Recent studies heve detected

positive selection acting in COX2 on taxa that had clearly experienced significant changes in their metabolic needs, such as in high-performance fish (Dalziel et al. 2006). Among mammals, adaptations in COX2 have been suggested in species adapted to unusual oxygen requirements, like diving in cetaceans, flying in bats, and life at high altitudes (Xu et al. 2005, da Fonseca et al. 2008, Luo et al. 2008), and other extreme environments (Di Rocco et al. 2006).

Fossorial rodents constitute an ideal study system to test hypotheses about adaptive evolution driven by important ecological shifts.The subterranean niche is characterized by high levels of carbon dioxide and low levels of oxygen (Buffenstein 2000) and implies high energy requirements associated to burrowing (Vleck 1979). So, it is likely that proteins involved in respiration may have evolved under positive directional selection in response to habitat requirements. Particularly, the sister families Octodontidae and Ctenomyidae provide a unique opportunity to trace the evolution of adaptations related to digging (Lessa et al. 2008). Burrowing for sheltering and rearing is the rule in these rodents but only two extant lineages, *Ctenomys* (tuco-tucos) and *Spalacopus* (coruro), have recently evolved fully subterranean habits. Phylogenetic relationships among genera are relatively well established (Opazo 2005 and references therein), making it possible to trace changes associated to the acquisition of subterranean adaptations along a known phylogeny (Lessa et al. 2008).

Recently, Tomasco and Lessa (submited) found accelerated rates of replacement substitution in most protein-coding mitochondrial genes of subterranean tuco-tucos and coruros with respect to their non-subterranean close relatives, suggesting a link between directional selection acting on the mitochondrial genome and niche shift to live underground. The most extreme variation was found in COX2, in which coruros showed a

ratio between rates of synonymous and nonsynonymous substitutions (ω) almost 30 times greater than that of non-subterranean lineages, and 11 times higher than that of tuco-tucos. However, tuco-tucos comprise more than 56 living species (Reig et al., 1990) and were represented only by 3 species. A more dense representation of this genus is necesary to discover positively selected codon sites in this gene and more in general, for a better characterization of COX2 evolution. Here, we present results obtaied with two different approaches: a) examining the variation in ω values across lineages and codons, using the program PAML4 (Yang 1997) and b) accumulation of radical changes at the aminoacid level, using the TreeSAAP (Woolley et al. 2003).

Materials and Methods

Specimens Examined

We obtained the complete COX2 sequences of several species of octodontoid rodents, including representatives of two related, but independent, subterranean lineages (28 species of tuco-tucos – *Ctenomys* - and the coruro *Spalacopus cyanus*), three non-subterranean allies (*Aconaemys fuscus*, *Octodon degus* and *Tympanoctomys barrerae*), and a spiny rat (*Proechimys longicaudatus* Family Echimyidae) as an outgroup (see table 1). 28 species of *Ctenomys* where chosen to represent the known diversity of the genus (Parada et al. 2007): *C. mendocinus (*from Las Heras), *C. flamarioni*, *C. australis* and *C. porteusi* from the main mendocinus group; *C. mendocinus (*from Tupungato) and *C. rionegrensis* from the secondary mendocinus group; *C. opimus and C. scagliari* from the opimus-fulvus group; *C. occultus,, C. latro* and *C. tucumanus* from the chaco group; *C. goodfellowi* and *C.*

boliviensis from the boliviano-matogrosense group; *C. llathu* and *C. frater* from the *boliviano-paraguayo group; C. pundt* and *C. talarum* from the pundti-talarum group; *C. pearsoni*, *C. torquatus* from the torquatus group; *C. haigi*, *C. magellanicus* and *C. sericeus* from the patagonic group; and non-grouped species *C. sociabilis*, *C. steinbachi*, *C. ita*, *C. minutus, C. leucodon*, *Ctenomys* sp. (19MH, previously reported as *C. talarum* but posible new species M.S. Mora et al. 2007). Unless mentioned, all sequences and deposited in GenBank with accession number from HM636472 to HM636497, as detailed in table 1.

DNA extraction, amplification, sequencing and alignement

Total DNA extractions were made with SDS/proteinase K digestion/NaCl protein presipitation/alcohol precipitation of DNA (modified from Miller et al., 1988) from liver preserved in 95% ethyl-alcohol. The complete COX2 was amplified using primers MF7766L and MF6710H (Tomasco and Lessa, submited). 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, 1X Taq Polymerase Buffer, 240 µM of each dNTP, 240 nM of each primer, 2 units of Taq Polymerase and 4 mM of MgCl2. PCR amplifications were performed in a PXE0.2 Thermal Cycler (Thermo – Electron Corporation), by an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 47 °C and 30 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 EtBr staining under UV light, and expected size

was determined in relation to a 100bp DNA size standard (GIBCO BRL). PCR products were purified and automatic sequencing was done by Macrogen. Inc. (http://www.macrogen.com), under BigDyeTM terminator cycling conditions in a Sequencer ABI 3730xl.

Data analyses

The phylogeny considered for this study (figure 1) was obtained by Maximun Likelihood search in PhyML (Guindon and Gascuel 2003) with HKY substitution model (best fitted model obtained with ModelGenerator, Keane et al. 2006), and the algorithm approach of simultaneous NNI (Guindon and Gascuel, 2003) starting from a neighbor joining tree. This phylogeny compares well to those reported previously (Castillo et al. 2005, Parada 2007, Slamovits et al. 2001 and references therein).

Variation in estimates of dN, dS and its ratio (ω= dN/dS) was explored using an Maximum Likelihood approach as implemented in PAML4 (Yang 1997). Variation in ω´s was estimated: *i)* along different branches while holding the rates constant across codons (branch models, Yang and Nielsen 1998), *ii)* across codons while holding rates constant along branches (site models, Yang et al. 2000) and *iii)* simultaneously across codons and along lineages (Branch-site models, 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 has evolved under positive directional selection. In the case of branch models, we assessed 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's, and iii) intermediate models allowing different ω's for clades or branches (clades and/or

"ecological" groups, namely subterranean or non-subterranean). In the case of site models, we compared several evolutionary models described by Yang *et al.* (2000) and tested for positive selection using the three likelihood ratio test (LRT) recommended in PAML4 user manual (M1a-M2a, M7-M8 and M8a-M8 comparisons). We used two variants of the Branch-site model A (Model A and modified Model A) and compared them with a LTR as recommended in PAML user guide (Yang and Nielsen 2002, Yang et al. 2005, Zhang et al. 2005). As PAML only allows two branch types, we chose to run comparisons for lineage variation considering different combinations of subterranean lineages (foreground branches) versus nonsubterranean 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 determine which codons that have experienced positive selection ($\omega>1$). In all cases, the α level of significance was 0.05.

Significant physicochemical amino acid changes in mitochondrial protein coding genes were identified by the algorithm implemented in TreeSAAP (Woolley et al. 2003), which compares the observed distribution of physicochemical changes inferred from a phylogenetic tree with a distribution based on the assumption of random amino acid replacement expected under strict neutrality. Nonsynonymous changes are classified into eight categories. The most radical changes (categories 6, 7 and 8) for which the observed numbers of amino acid replacements in the data set is significantly different from the null model ($P < 0.001$) are considered as being potentially resulting from positive-destabilizing selection. We ran ModelGenerator (Keane et al. 2006) to select a substitution model (HKY + gamma (Hasegawa et al. 1985)) for TreeSAAP and calculate ancestral sequences from the tree using Baseml (Yang 1997).

Results

Variation in ω

The most interesting results of PAML were obtained with Branch Models. The most complex model (63 independent ωs, one for each branch) fitted significantly better ($p<0.01$) than the the null model. No estimated ω values were greater than 1, and highest ω's were those of the basal branch of the coruro-*Aconaemys* clade and some within tuco-tucos (figure 1). Several intermediate models were examined and a summary of the results for the most relevant ones is shown in table 2. In all cases, separating the tuco-tuco clade from its basal branch results in significantly higher lnL values. The complex model can be simplified without a significant loss of statistical fit to models allowing one ω for subterranean lineages and another for nonsubterranean taxa (see table 2). The 2-ω model with highest lnL assigns one ω for the basal branch of coruro+*Aconaemys* clade and other for the remaining taxa. The ω value of COX2 in the subterranean taxa is between 3.5 and 5 times the one estimated for the nonsubterranean branches, and reaches even higher values in some branches in 3 and 4 parameter models. The highest ω value was estimated for basal branch of coruro-*Aconaemys* clade, followed by the coruro and the tuco-tucos clade.

The best fitting codon model was a discrete model (M3 in PAML) that groups codons into 3 classes. However, our branch-site models were significant using test 2 (Zhang et al. 2005), recommended to reduce the number of false positives.

TreeSAAP results

Significant physicochemical amino acid changes among residues in mitochondrial COX2 gene were identified by TreeSAAP and their distribution on the tree is shown in figure 1. The property selected in all cases was the Equilibrium constant (ionization of COOH). In some codons, this property shows evidence of positive selection independently in different branches: twice in sites 38, 211, 214 and 143, and 8 times in 165.

In all cases, the replacements implied nonpolar and neutral amino acids. Interestingly, in most cases the same property has been selected implying the same codon transitions: site 38 from CTT (Val) to ATT (Ile); site 143 from GTC (Val) to ATC (Ile); site 165 from CTT (Val) to ATT (Ile). Site 214 is inferred to have evolved twice from Val to Ile, but involving different codons. In site 211 the same property has been selected but implying different amino acid replacements (from Ile to Val in *C. magellanicus* and to Met in the coruros).

Discussion

As OXPHOS is essential for energy metabolism, purifying selection dominates the evolution of mtDNA. In particular, the mtDNA-encoded COX subunits are the most conserved genes in mitochondrial genomes (Luo et al. 2008; da Fonseca et al. 2008). However, it is possible that weak and/or episodic positive selection occurs in this background of strong purifying selection, in association with a shift to greater energy demanding lifestyles or limited availability of oxygen (e.g. Shen et al. 2010). Since oxygen is the ultimate electron acceptor in a process catalyzed by COX, it seems possible that

modification of its structure and/or activity contributes to adaptation to hypoxia (Luo et al. 2008). We have examined this possibility in two independent and relatively recent colonizations of the subterranean niche known to have ocurred among octodontoid rodents.

Our main results, discussed and qualified in detail below, are: *1*) COX2 shows increased ω in subterranean relative to nonsubterranean lineages, *2*) destabilizing changes in biochemical properties occurred mainly on terminal branches of the phylogeny leading to subterranean lineages *3*) some of these changes parallel those suggested by previous studies of lineages adapted to oxygen-limited lifestyles. Taken collectively, these results are consistent with the hypothesis that the colonization of the subterranean niche creates a selective regime of positive, directional selection in COX2, but that positive selection is likely episodic in a background dominated by purifying selection.

COX2 shows increased ω in subterranean lineages relative to nonsubterranean ones. Thus, simple models allowing for variation in ω between subterranean and non-subterranean lineages can capture most of variation of the full model without a significant loss of fit. Da Silva et al. (2009) recently found a similar pattern in the cytochrome b (CytB) of several lineages of subterranean rodents, including tuco-tucos and the coruro. In general, the coruro and the branch leading to the coruro-*Aconaemys* clade show higher ω values than tuco-tucos. A similar contrast has been found in morphological adaptations for digging: the coruro has accumulated numerous changes in a short period of time, relative to a more protracted process in tuco-tucos (Lessa et al. 2008). We found that the basal branch leading to the coruro-*Aconaemys* clade shows signs of destabilizing changes in some codons, in conjunction with increased ω. It is possible that adaptation to hypoxia preceed the differenciation of this genera, and Aconaemys still be able to live under an a hypoxic

environment. The coruro and Aconaemys offer an important opportunity to examine the transition from fossorial tu fully subterranean life. However, a much better characterization of their lifestyles and phyisiology is needed.

As purifying selection predominates in the evolution of mtDNA, it is expected that all estimated ωs values are smaller than 1. These results do not allow to reject alternative explanations of rate variation, such as a relaxation of purifying selection, variation in metabolic rate, body mass, population size, and generation time among lineages (Martin and Palumbi 1993; Martin 1995; Bromham et al. 1996; Li et al. 1996). However, under relaxation of purifying selection the same pattern of variation among lineages is expected in all genes. In contrast, Tomasco and Lessa (submitted) found that most, but not all, mitochondrial protein-coding genes have greater ω's in subterranean lineages in many of these cases as in COX2, they are greater in coruros than in tuco-tucos.

Furthermore, our results regarding ω 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 (Woolley et al. 2003). As COX2 is essential for energy metabolism, it is not surprising to find that most amino acid changes are highly conservative and involve residues with similar properties (e.g., Ile ↔ Val and Ile →Met). However, a as shown by TreeSAAP, some of them tend to desestabilize the Equilibrium constant property. These results are consistent with those from PAML in suggesting that there is a concentration of codons subjected to positive selection, particularly among subterranean lineages, especially in the branch leading to the coruro, and also in some tip branches of the tucu-tucos clade. Beside, four branches that PAML detects as accelerated show sites under positive selection using TreeSAAP. In this

sense, the TreeSAAP software seems to be more sensitive to detect selection under these conditions (even using a highly conservative criterion of $p< 0.01$), as it has been observed in other cases (e.g., da Fonseca et al. 1998; Mc.Clellan et al. 2005; Da Silva et al. 2009; Marques et al. 2006; Porter et al. 2007; Tomasco and Lessa submitted).

The same property (Equilibrium constant) seems to be selected independently in subterranean lineages, and sometimes on the same codon sites, including parallel amino acid substitutions. For example this property was selected simultaneously in codon sites 214 and 143 on the branches leading to *C. ita* and *C. opimus.* The same happened in site 211 in branches leading to the coruro and *C. magellanicus*. Interestingly, TreeSAAP detected destabilizing selection in sites 92 and 214 in octodontids (figure 1), in correspondence with findings of Da Fonseca et al. (2008). These authors found that both sites are posibly under positive selection; site 92 was highly variable and in contact with nuclear subunit VIB and the K216E mutation (charge inversion) was observed only in cetaceans, a group with similar metabolic requirements.

A recent study of high-performance fish (Dalziel et al. 2006) detected positive selection acting on COX2. Among mammals, adaptations in COX2 has been suggested in species adapted to unusual oxygen requirements, such as cetaceans, bats, and species living at high altitudes (Xu et al. 2005; da Fonseca et al. 2008; Luo et al. 2008) and other extreme enviroments (Di Rocco et al. 2006). In this study, like in these cases, the highly variable sites are located on the mitochondrial transmembrane domain or at the interface between mitochondrial and nuclear-encoded subunits. Although the changes found in mitochondrial COX2 do not encompass residues associated to the electron transfer, we speculate that these amino acid substitutions could change the structure COX2, thereby modifying the

conformation of COX complex and altering its function. In fact, COX assembly in mammals has been shown to be very sensitive to small changes in amino acid sequences not necessarily involved in the catalytic function (Barrientos et al., 2001). They also could be associated with compensatory mutations in the nuclear subunits (co-evolution), as suggested by Rand et al. (2004). Although the role of COX in adaptation to hypoxia remains unclear, these amino acid changes may have functional implications and thus be part of the adaptive response improving aerobic capacity (Blier et al. 2001; Grossman et al. 2004). Ultimately, the adaptive significance of these replacements needs to be tested by physiological experiments.

We advocate that studying the action of positive selection at the molecular level, specially in cases were purifying selection predominates, should not simply rely on traditional approaches that are known to be unsuccessful in most cases. Tests of selection base don dN/dS are known to be conservative (e.g.:. Shen et al. 2010, McCellan et al. 2004, Yang y Bielawski 2000). Indeed, this and other studies have found additional clues of the action of positive selection in the distribution of radical changes in the properties of aminoacids. The contrast between subterranean and non-subterranean rodents is guided by ecological considerations which, in turn, suggest adaptive scenarios that may be subjected to analyses. Thus, the same contrasts between subterranean taxa and their non-subterranean counterparts have been found across diverse taxa in CytB (Da Silva et al., 2009), across many genes in the mitochondrial genome of selected taxa (Tomasco and Lessa, submitted), and, in this study, in a more detailed survey of COX2. These analyses of mitochondrial genes and genomes pave the way to the study of the nuclear counterparts of the functional units of mitochondrial respiration.

The branch leading to the coruro shows four sites under positive-destabilizing selection, followed by those leading to the outgroup and *C. tucumanus* (3 sites each). These results were also partialy suggested by PAML, particularly in the case of the basal branch of coruro-*Aconaemys* clade, and branches leading to *C. boliviensis* and *C. torquatus* (figure 1). As will be discussed in the following section, some of the sites found to be under positive-destabilizing selection by TreeSAAP in this dataset, are close to sites previously found to be under selection (da Fonseca et al. 2008, Dalziel et al. 2006, Tomasco and Lessa, submitted).

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Figure 1. Phylogenetic reconstruction of COX2 and summary of results obtained with PAML and TreeSAAP. Numbers above nodes are non parametric bootstrap values obtained in Maximun-likelihood analyses. Branches with estimated ω values higher than

0.1 using PAML are indicated in bold. Significant physicochemical amino acid changes identified by TreeSAAP are indicated as numbers in boxes along corresponding branch.

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Table 1. Species and voucher information of specimens examined. *: sequences generated by previous studies. Acronyms indicate the collection in which vouchers are deposited: NK University of New Mexico; AK Texas A&M University; CA, EV, FC and PNG Laboratorio de Evolución, Universidad de la República; C-Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", CB donated by Claudio Bidau; SV donated by Sergio G. Vincon, GD donated by Guillermo D'Elía, 19MH, LE05, MH52, 59R, donated by Matías S. Mora.

Species	**Voucher**	**GenBank acc.**
Spalacopus cyanus	GD657	MH544133*
Aconaemys fuscus	GD1284	HM636483
Octodon degus	109241	MH544134*
Tympanoctomys barrerae	AK13811	MH544132*
Proechimys longicaudatus	MSB:Mamm:57192	MH544128*
C. mendocinus (Tupungato)	FC5521	HM636491
C. mendocinus (Las Heras)	CB333	HM636494
C. flamarioni	CH8	HM636484
C. australis	MH52	HM636476
C. porteusi	LE05	HM636485
C. rionegrensis	EV1064	MH544130*
C. opimus	MSB:Mamm:55377	HM636472
C. scagliari	C-04696	HM636486
C. occultus	C-04685	HM636496
C. latro	C-04679	HM636487
C. tucumanus	C-04670	HM636495
C. goodfellowi	NK13029	HM636474
C. boliviensis	MSB:Mamm:211396	HM636488
C. llathu	MSB:Mamm:3959	HM636473
C. frater	MSB:Mamm:57188	HM636475
C. pundti	C-04042	HM636482
C. talarum	59R	HM636477
C. pearsoni	CA722	HM636480
C. torquatus	CA564	HM636492
C. haigi	SV62	HM636489
C. magellanicus	PNG365	HM636493
C. sericeus	SV45	HM636490
C. sociabilis	EAL545	MH544129*

C. steinbachi	MSB:Mamm:55369	HM636497
C. ita	MSB:Mamm:63387	HM636481
C. minutus	MSB:Mamm:55367	HM636478
C. leucodon	MSB:Mamm:59654	MH544130*
Ctenomys sp.	19MH	HM636479

Table 2. Results from analyses of ω variation along different branches using PAML4. Only most relevant models tried are shown: null model (a single ω for all branches), full model (one ω per branch), and most simple models that are significantly better than the null model ($p<0.001$) and to which the full model can be simplified. Shaded boxes indicates grouped taxa and corresponding ω value. Ratio highest/lowest is the ratio of **ω** values between subterranean with higher ω and non-subterranean allies. ---: no corresp.

Taxa	**Estimated ω values**								
P. longicaudatus	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01
Basal tuco-tucos							0.03	0.03	0.01
Crown tuco-tucos		0.03	0.04	0.04		0.03			0 - 0.76
Basal coruro+*Acon*					0.11	0.08		0.08	0.26
A. fuscus									0.04
(coruro) S. cyanus							0.09		0.08
O. degus									0.01
T. barrerae									0.01
O. degus								0.01	0.01
Basal Octdontidae									0.01
lnL	-4041	-4030	-4026	-4025	-4022	-4022	-4028	-4025	-3991
Ratio highest/lowest	---	**3.5**	**4.6**	**4.8**	**5.0**	**10.5**	**9.1**	**9.5**	---
# paramters	**1**	**2**				**3**		**4**	**63**

DISCUSIÓN

La respiración es un proceso fisiológico indispensable para la vida de los organismos aeróbicos, cuyo fin es liberar energía por la oxidación de nutrientes para producir ATP usando O_2 como aceptor último de los electrones. Implica todos los procesos, desde la obtención del O_2 hasta la liberación del CO_2 que se genera como subproducto y es tóxico en altas concentraciones para la mayoría de los seres vivos. Los ambientes con bajas concentraciones de O_2 (hipoxia) representan un gran desafío fisiológico para los animales por generar severas restricciones al metabolismo aeróbico. Asimismo, aquellos ambientes con altas concentraciones de CO_2 (hipercapnia), dificultan su eliminación y podrían alterar el equilibrio acido-base de la sangre en vertebrados. Las adaptaciones a la hipoxia y/o hipercapnia son variadas se evidencian a varios niveles, desde cambios en los patrones venilatorios, hasta sutiles modificaciones de las proteínas involucradas en cualquier etapa del proceso. Ambos niveles fueron abordados por esta tesis. Todos estos cambios tienen su correlato genético, y deberíamos ser capaces de identificarlos con las herramientas disponibles. Sin embargo, esta tarea no siempre es sencilla. En algunos casos, se conocen los genes que codifican una proteína clave, por ejemplo, la Hb, y es posible identificar cambios nucleotídicos subyacentes a la adaptación (revisado en Li 1997), como la mayor afinidad de ésta por el O_2 (e.g.: Jelkmann et al. 1981,). En otros casos, las características implicadas son más complejas y posiblemente de regulación poligénica, como los patrones venilatorios, lo que dificulta descubrir las bases moleculares de la adaptación.

El nicho subterráneo es particularmente hipóxico e hipercápnico y sus habitantes se encuentran adaptados a sobrevivir en esas condiciones. Muchas adaptaciones morfológicas y fisiológicas a la hipoxia/hipercapnia han sido descritas para mamíferos subterráneos (e.g.: Nevo 1999, Boggs et al. 1984), mientras que a nivel molecular la información es mucho más limitada, si no desconocida, a excepción de unos pocos genes en uno o dos linajes. Esta tesis intenta identificar adaptaciones a la hipoxia y/o hipercapnia provocadas por la vida bajo superficie en los roedores caviomorfos. Estos roedores presentan características particularmente llamativas para estudiar adaptaciones a la hipoxia/hipercapnia, y sin embargo, son prácticamente desconocidos en este aspecto.

Tres de los cuatro capítulos de esta tesis se han centrado en el estudio de estas adaptaciones a nivel molecular. La hipótesis de trabajo ha sido que la hipoxia, la hipercapnia o ambas podrían haber propiciado la evolución de adaptaciones en los genes

implicados en la respiración celular, como los codificados por el genoma mitocondrial. En este sentido, se presentan resultados consistentes con la acción de la selección natural positiva en genes mitocondriales de estos roedores asociada a la invasión del nicho subterráneo. En el capítulo 3 esta hipótesis se ha evaluado para todos los genes mitocondriales codificantes de proteínas, encontrándose en muchos, pero no todos ellos, una aceleración en las tasas de reemplazo aminocídico y una concentración de cambios de reemplazo desestabilizantes en los linajes subterráneos, los tucu-tucos y el coruro, en comparación con sus parientes no subterráneos. El gen que mostró un patrón más llamativo fue el de la subunidad 2 de la citocromo oxidasa (COX2) en el coruro, con un ω estimado casi 30 veces mayor que la de los linajes no subterráneos, por lo que fue estudiada con mayor detalle en el capítulo 4, principalmente cubriendo la diversidad dentro de los tuco-tucos, confirmando el resultado anterior. Por otro lado, en un estudio anterior (Da Silva et al. 2009), también reportamos una aceleración en las tasas de reemplazo y de cambios desestabilizantes en diferentes taxa subterráneos, incluyendo los caviomorfos pero no limitado a ellos, en el gen del citocromo b, también mitocondrial (CB). En ninguno de los casos anteriores encontramos valores de ω mayores a 1, lo que sería evidencia indiscutible, pero muy exigente y difícilmente alcanzable, de selección positiva (e.g.:. Nozawa et al. 2009, McCellan et al. 2005, Yang y Bielawski 2000). Este resultado es consistente con lo esperado para genes que por su importancia funcional están sometidos a una fuerte presión de selección purficadora (Stewart et al. 2008) (capítulos 2, 3 y 4). Como se corrobora en el capítulo 2, los genomas mitocondriales de estos roedores no tienen apartamientos importantes en sus patrones de evolución con respecto a los de otros mamíferos. Su análisis apoya hipótesis filogenéticas previamente propuestas, aunque sugiere que la invasión del nicho subterráneo es anterior a lo previamente sugerido.

El mismo contraste entre taxones subterráneas y sus parientes no-subterráneos se han encontrado en diversos taxa en CB (Da Silva et al. 2009), a través de muchos genes del genoma mitocondrial de caviomorfos (capítulo 3) y en un estudio más detallado de COX2 (capítulo 4), lo que puede ser interpretado como una huella consistente de adaptaciones a la hipoxia en un contexto de fuerte selección purificadora. Esta conclusión es particularmente interesante debido que, en general, se asume que la evolución del genoma mitocondrial se ajusta a la teoría neutral. Finalmente, el conjunto de estos resultados sugiere que sería importante estudiar otros genes, como aquellos genes

nucleares que codifican las el resto de las subunidades que componen los complejos proteicos implicados en la fosforilación oxidativa. Ello permitiría investigar la coevolución de genes asociados funcionalmente (Doan et al. 2004, Grossman et al. 2004).

El estudio de la variación en los patrones ventilatorios en respuesta a diferentes concentraciones de O_2 y CO_2 realizado en esta tesis es un aporte sobre las adaptaciones fisiológicas de estos roedores a la hipoxia e hipercapnia, donde la información era prácticamente inexistente. Confirma que los coruros presentan, en comparación con los degus, una respuesta atenuada a la hipercapnia (y no así a la hipoxia) característica de los mamíferos subterráneos, y una mayor sensibilidad a cambios en la presión parcial de O_2 a través de los quimiorreceptores periféricos (capítulo 1). Estos resultados confirman la presencia de adaptaciones convergentes entre roedores subterráneos con respecto a la respuesta ventilatoria a la hipercapnia, pero no a la hipoxia (Boggs et al. 1984).

Las bases moleculares de estas adaptaciones ventilatorias están lejos de ser conocidas pero este resultado sugiere, al menos como primera aproximación, el análisis de los genes que codifican los quimiorreceptores periféricos antes mencionados en búsqueda de huellas de selección positiva, y posiblemente convergente, entre diferentes roedores subterráneos. Las adaptaciones fisiológicas encontradas en combinación con las dataciones halladas en el capítulo 2, sugieren que las mismas se habrían adquirido en el linaje que conduce al coruro en un período no mayor a 6 millones de años, lo que es mucho menor a la divergencia de otros roedores subterráneos estudiados. Con la información disponible es imposible discriminar si estas adaptaciones serían exclusivas del coruro o compartidas con su pariente más cercano, el género *Aconaemys*. A nivel nucleotídico, la evolución de la CO2 sugiere que la selección positiva habría actuado previo a la diferenciación de ambos géneros, en la rama basal del clado coruro-*Aconaemys*. En este sentido, la ventaja del estudio de roedores caviomofos es la presencia dos invasiones relativamente recientes al nicho subterráneo, cuyas relaciones filogenéticas con sus parientes no subterráneos más cercanos son bien conocidas. Esto permitiría comenzar a descifrar el ritmo con el que estas adaptaciones fisiológicas y morfológicas se han adquirido.

Los cambios ocurridos en los genes mitocondriales estudiados serían un aspecto particular de las adaptaciones a la hipoxia/hipercapnia relacionadas con la

subterranealidad, pero no darían cuenta de todas las posibles adaptaciones, ni en particular los cambios en los patrones ventilatorios reportados. Es muy probable que los patrones ventilatorios no estén regulados por los genes del genoma mitocondrial, cuyos productos proteicos son bien conocidos en estructura y función, y se encuentran confinados a la mitocondria, por lo que ambos hallazgos no están directamente relacionados. Las adaptaciones a la hipoxia y a la hipercapnia en estos roedores seguramente involucren otras características anatómicas y fisiológicas todavía desconocidas, algunas de las cuales podrían ser las reportadas previamente para otros linajes subterráneos. Sus bases genéticas comienzan apenas a ser exploradas y podría incluir "genes candidatos" sugeridos en esta tesis.

Perspectivas

Esta tesis en un aporte al estudio de los mamíferos subterráneos, sobre los cuales se cuenta con información incompleta así como al conocimiento de los roedores caviomofos en particular. Entre otras cosas, se muestra la presencia adaptaciones fisiológicas convergentes a la hipoxia en los coruros, se presentan posibles adaptaciones a nivel de genes del genoma mitocondrial en cururos y tucu-tucos, y se sugieren posibles genes candidatos que sería interesante evaluar si han evolucionados o no bajo selección positiva, y tal vez convergente, en roedores caviomorfos y subterráneos en general. Sin embargo, esta tesis pretende ser una primera aproximación al estudio de las adaptaciones a la hipoxia. A continuación se proponen algunos estudios complementarios que permitirían alcanzar una visión más amplia e integrada del tema.

Estudio de fisiología repiratoria

Para comprender los patrones y procesos por los cuales esta variabilidad fenotípica se originó y persiste, y las respuestas fisiológicas a la selección natural, es necesaria una comparación de la respuesta de las variables fisiológicas que confieren adaptaciones a la hipoxia/hipercapnia dentro de un marco temporal y filogenético conocido. De esta manera sería posible trazar la evolución de determinadas adaptaciones fisiológicas sobre la filogenia, e identificar cómo o cuándo surgieron. En este sentido el estudio presentado en el capítulo 1 podría ser complementado estudiando la respuesta de

variables fisiológicas frente a diferentes concentraciones de O_2 y CO_2 en al menos una especie representante los géneros *Ctenomys* y *Aconaemys*. El primero son los tuco-tucos y constituyen el otro género de roedores caviomofos subterráneos, y el segundo es un género fosorial más próximo al coruro (e.g., Honeycutt et al. 2003). La inclusión de los tuco-tucos es clave para evaluar la presencia o no de convergencia adaptativa en los caviomofos dentro del mismo contexto filogenético y, más en general, para ampliar la comparación con el resto de los roedores subterráneos. La inclusión de *Aconaemys* es relevante dado que permitiría trazar con mayor precisión la adquisición de las adaptaciones fisiológicas encontradas en el coruro, como fue realizado en la evolución de la COX2. En estos ensayos, también sería deseable probar desafíos combinados de hipoxia e hipercapnia, que son los que realmente enfrentan los animales en su ambiente.

Dado que la hipótesis de trabajo es que la vida subterránea, en condiciones de hipoxia e hipercapnia, propicia cambios fisiológicos convergentes, sería deseable corroborar la composición gaseosa de las cuevas en estas especies subterráneas. Con ese objetivo, un relevamiento preliminar registró un 18% de O_2 en cuevas de coruro (el aire atmosférico tiene aproximadamente un 21% de O_2), aunque probablemente sea mayor. Además, la información existente sobre el resto de las variables fisiológicas relacionadas a la respiración en roedores octodontoideos, como parámetros hematológicos, pO_2 crítica, tasas metabólicas, entre otras, es muy fragmentaria e incompleta, haciendo imposible su comparación (Morrison et al. 1963, Morrison y Rosenmann 1975, Rosenmann y Morrison 1975, Contreras y McNab 1990, Arieli 1990), por lo que sería deseable evaluar estos parámetros en al menos 1 especie de cada uno de los géneros de tuco-tucos, coruro, degus y *Aconaemys*. De esa manera se podría evaluar las adaptaciones a la hipoxia de forma integral, bajo un “diseño experimental” estratégico, incluyendo los contextos temporal y filogenético conocidos. Finalmente, otras adaptaciones podrían ser estudiadas en este contexto, como la actividad de enzimas antioxidantes en la glándula de Harder, como ha a sido sugerido para *Spalax ehrenbergi* como adaptación a la hipoxia (Caballero et al. 2006)

Adaptación en proteínas respiratorias

Los resultados obtenidos con los genes codificados por el genoma mitocondrial sugieren, por un lado, analizar su variación en otros linajes subterráneos independientes

(análogo al trabajo de Da Silva et al. 2009 pero con todos los genes mitocondriales) y por otro, el estudio de los genes de las subunidades proteicas con las que han coevolucionado (Rand et al. 2004, Grossman et al. 2004). Recientemente, algunos tests han sido sugeridos para evaluar coadaptación, por ejemplo mediante la teoría de "Información Mutua"[1] (e.g.: Dunn et al. 2008, Buslje et al. 2009), aunque por el momento requieren volúmenes de información importante.

Asimismo, estos resultados motivan el interés en ampliar esa perspectiva y evaluar la existencia de cambios adaptativos en otras proteínas relacionadas con el metabolismo aeróbico y el sistema de transporte del O_2, en particular en los genes de las cadenas alfa y beta de la Hb. Además de las adaptaciones a la hipoxia de la Hb en mamíferos subterráneos, existe abundancia evidencia de que cambios en Hb juegan un papel importante en la respuesta adaptativa a la vida en ambientes hipóxicos (ver Perutz 1983, Storz 2007, Storz et al. 2008, Winslow 2007, y referencias allí citadas). Entre éstas, existen ajustes fisiológicos de corto plazo que involucran cambios transitorios en la expresión génica, como por ejemplo incrementos en el número de eritrocitos en sangre, o cambios en el ritmo respiratorio, y otras modificaciones de más largo impacto que involucran cambios en la estructura de la Hb (Weber 2007). En particular, es común que mamíferos y aves que viven en ambientes de alta montaña posean Hbs con mayor afinidad por el O_2 en relación con grupos que no viven en ambientes tan extremos. La evidencia acumulada hasta ahora ha logrado relacionar cambios en un número reducido de residuos de las cadenas de la Hb con modificaciones en las propiedades bioquímicas y funcionales de esta proteína en respuesta a la altura en anfibios (Weber et al. 2002), aves (Jessen et al. 1991, McCracken et al. 2009 a, b), y mamíferos (Reynafarje et al. 1975, Storz et al. 2007 y 2009). Los roedores caviomorfos constituyen un modelo sumamente atractivo para el estudio de adaptaciones a la hipoxia ya que, además de presentar dos invasiones independientes del nicho subterráneo, incluyen especies con un amplio rango de variación altitudinal que va de 0 a ~ 4000 msnm. Ambas condiciones disminuyen las concentraciones de O_2 disponible para respirar (Nevo 1999).

Con respecto a las pruebas para evidenciar evolución adaptativa a nivel molecular, es evidente que se hace necesario probar alternativas más sensibles que el parámetro ω,

[1] La teoría de información mutua es generalmente aplicada para predecir correlaciones posicionales en alineamientos múltiples de secuencias aminoacídicas, posibilitando la identificación precisa de sectores que resultan de la covariación entre algunos de los sitios de una proteína

fundamentalmente en el caso de fuerte selección purificadora de fondo, selección episódica, etc. (e.g.:. Nozawa et al. 2009, McClellan et al. 2005, Yang y Bielawski 2000). Varios tests alternativas han sido sugeridos en los últimos años, que evalúan las consecuencias de los cambios en relación con sus propiedades aminoacídicas (Sainudiin et al. 2005, Wong et al. 2006, Xia y Li 1998, McClellan y McCraken 2001, Pupko et al. 2003, Suzuki 2007, Datta et al. 2010) y uno de ellos fue utilizado en esta tesis (McClellan et al. 2005, Woolley et al. 2003). Si bien sus autores aseveran que estos tienen una mayor eficiencia en detectar sitios seleccionados que otros métodos, esto debería ser examinado más cuidadosamente con datos reales y simulaciones, lo que es de esperar ocurra en los próximos años (i.e.: McClelland y Edison 2010). Asimismo, sería interesante incluir en el análisis variación en los cambios sinónimos ya que éstos no son necesariamente neutros y podrían afectar la eficiencia en la traducción o el plegamiento de una proteína debido a que algunos codones se traducen más eficientemente (e.g.: Carlini y Wolfgang 2003, Carlini 2004, Kahali et al. 2007) o alteran lugares de señalamiento (e.g.: Pagani et al. 2005) (ver también Wagner 2008). Más en general, sería sumamente interesante incluir el análisis de genes no codificantes de proteínas, debido a que la selección positiva también puede actuar sobre genes ribosomales y los tRNA, ambos ignorados en esta tesis, y pueden tener consecuencias sobre la performance energética (Rankinen et al. 2006, Moreno-Loshuertos et al. 2006). Finalmente, en un estudio donde se sugiere la evolución adaptativa en ciertos linajes, uno podría inferir las proteínas ancestrales, que pueden ser sintetizadas y examinadas en el laboratorio (Golding y Dean 1998, Chang y Donoghue 2000).

Para terminar, los resultados obtenidos en esta tesis, en particular las secuencias de los genomas mitocondriales completos obtenidos, han sido subutilizados y su análisis preliminar sugiere su utilidad para posteriores estudios. Por ejemplo, la variabilidad en el repetido presente en la región control del genoma mitocondrial de estos roedores, así como la información proveniente de los tRNAs y los rRNA, mostraron evidencia de tener información filogenética. Sin embargo, estas regiones fueron excluidas del análisis filogenético por carecer de modelos de sustitución apropiados implementados en los programas de análisis filogenético comúnmente usados. El uso de estas regiones podría resolver casos controvertidos dentro de los octodóntidos, como es el caso de las relaciones más basales dentro de los tuco-tucos (e.g.: Parada 2007, Castillo et al. 2005, Slamovits et al. 2001), entre otros.

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APÉNDICE

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 cytochome 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 tucu-tucus showed more sites whose amino acid properties may be under positive selection in the cytochome 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], althought 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 oxigen 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 oxigen-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)	*Ctenomys talarum*	AF370699	Pocket gophers (Geomyidae)	*Thomomys bottae*	U65263
	Ctenomys rionegrenses	AF119114		*Thomomys townsendii*	U65282
	Ctenomys mendocinus	AF007062		*Thomomys umbrinus*	U65288
	Ctenomys australis	AF370697		*Thomomys bulbivorus*	AF155867
	Ctenomys pearsoni	AF119108		*Thomomys talpoides*	U65291
	Ctenomys maulinus	AF370702		*Thomomys monticola*	U65292
	Ctenomys tuconax	AF370684		*Thomomys mazama*	AF215806
	Ctenomys magellanicus	AF370690		*Orthogeomys heterodus*	U65300
	Ctenomys coyhaiquensis	AF119112		*Papogeomys bulleri*	L11900
	Ctenomys haigi	AF007063		*Cartogeomys tylorhinus*	AF302183
	Ctenomys tucumanus	AF370691		*Cartogeomys gymnurus*	AF302179
	Ctenomys argentinus	AF370680		*Cartogeomys neglectus*	AF302174
	Ctenomys latro	AF370704		*Cartogeomys castanops*	AF302171
	Ctenomys leucodon	AF007056		*Cartogeomys zinseri*	AF302170
	Ctenomys sp. "ITA"	AF007047		*Cartogeomys fumosus*	AF302165
	Ctenomys sp. "MINUT"	AF007053		*Cartogeomys goldman*	AF302176
	Ctenomys sp. "MONTE"	AF007057		*Cartogeomys merriami*	AF302160
	Ctenomys steinbachi	AF007044		*Geomys pinetis*	AF158698
	Ctenomys goodfellowi	AF007051		*Geomys bursarius*	AF158697
	Ctenomys boliviensis	AF007037		*Geomys lutescens*	AY393950
	Ctenomys lewisi	AF007049		*Geomys jugossicularis*	AY393949
	Ctenomys frater	AF007046		*Geomys texensis*	AY393965
	Ctenomys conoveri	AF007055		*Geomys knoxjonesi*	AY393947
	Ctenomys fulvus	AF370687		*Geomys arenarius*	AY393935
	Ctenomys opimus	AF007042		*Geomys tropicalis*	AY393970
Octodontidae	*Spalacopus cyanus*	AF007061		*Geomys personatus*	AY393959
	Octodon degus	AF007059		*Geomys streckeri*	AY393968
	Octodontomys gliroides	AF370706		*Geomys attwateri*	AY393938
	Tympanoctomys barrerae	AF007060		*Geomys breviceps*	AF158689
Spiny rats (Echimyidae) Outgroup	*Makalata didelphoides*	L23363	Heteromyidae	*Perognathus longuimembris*	U65302
	Mesomys hispidus	L23385		*Perognathus amplus*	U65301
	Proechimys simonsi	U35414		*Perognathus apache*	AY926412
	Euryzygomatomys spinosus	U34858		*Perognathus flavescens*	AY926411
	Trionomys paratus	U35165		*Perognathus alticola*	AY926413
African mole rats and relatives				*Perognathus parvus*	AY926407
Mole rats (Bathyergidae)	*Heterocephalus glaber*	AF155870		*Dipodomys agilis*	U65303
	Bathyergus suillus	AF012242		*Dipodomys elephantinus*	AY926374
	Bathyergus janetta	AF012241		*Dipodomys californicus*	AY926368
	Georychus capensis	AF012243		*Dipodomys elator*	AY926376
	Cryptomys hottentotus	AF012239		*Dipodomys margaritae*	AY926370
	Cryptomys choma	AF012234		*Dipodomys diserti*	AY926381
	Cryptomys amatus	AF012233		*Microdipodops megacephalus*	AF172833
	Cryptomys damarensis	AF012223		*Microdipodops pallidus*	AY926361
	Cryptomys darlingi	AF012232		*Chaetodipus hispidus*	AF172832
	Cryptomys mechowi	AF012230		*Chaetodipus rudinoris*	AY926397
	Cryptomys bocagei	AF012229		*Chaetodipus fallax*	AY926402
	Heliophobius argenteocinereus	U87527		*Chaetodipus spinatus*	AY926398
Outgrup	*Thryonomys swinderianus*	AJ301644		*Chaetodipus pernix*	AY926395
	Hystrix africaeaustralis	X70674		*Chaetodipus penicillatus*	AY926388
				Chaetodipus intermedius	AY926389
				Heteromys anomalus	DQ168468
				Heteromys desmarestianus	DQ168467
				Liomys spectabilis	DQ168550
				Liomys pictus	DQ168535
				Liomys irroratus	DQ168501
			Outgroup	*Pedetes capensis*	AJ389527
				Castor fiber	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 subtitution 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-

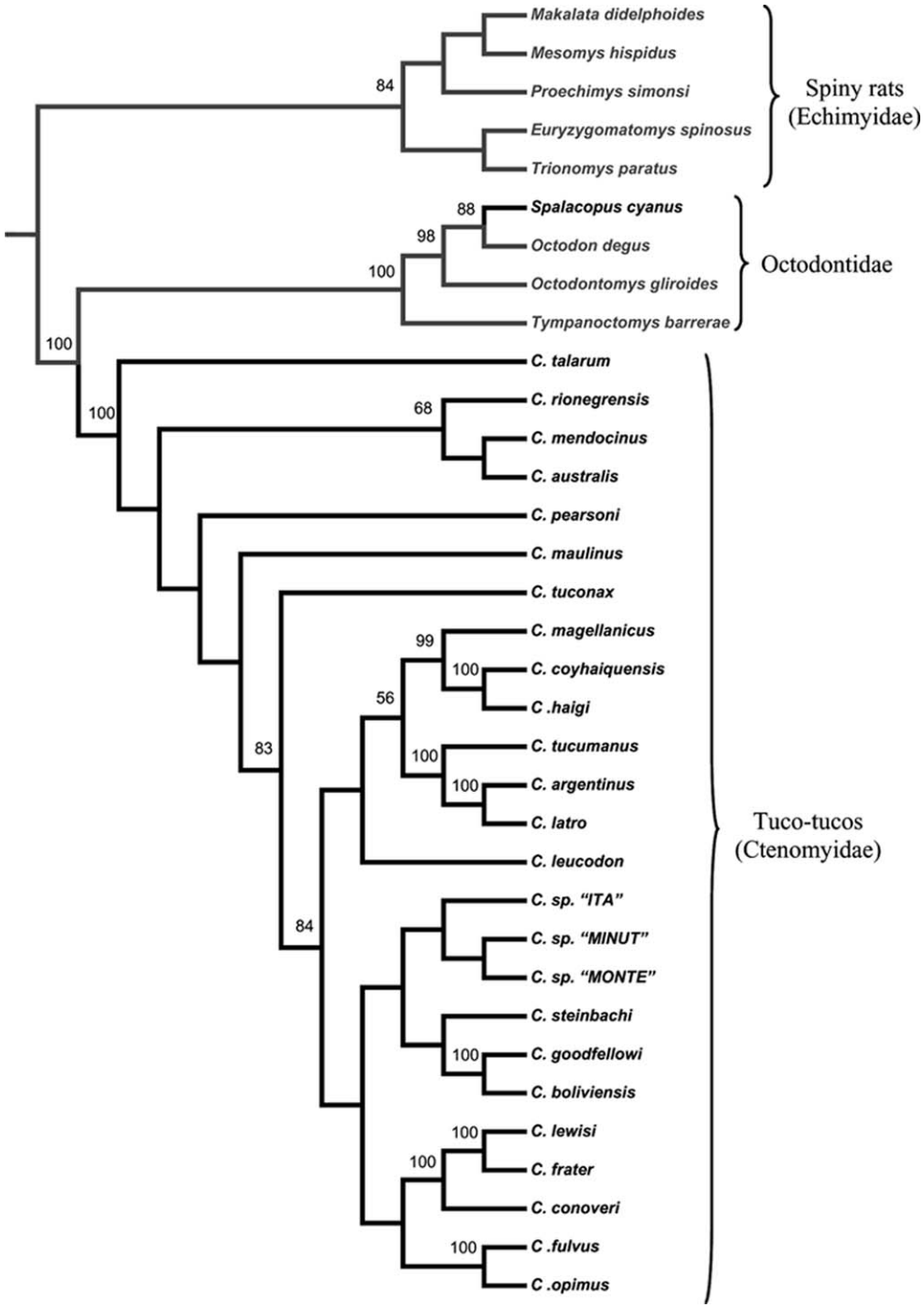

Fig. (1). Topology of the phylogenetic tree of tuco-tucos, coruro and relatives. (*C.* = *Ctenomys*). Non-subterranean taxa are in gray. Spiny rats are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

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 raleted 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 relted families of South American rodents: a) Ctenomyidae, consisting of species of the subterranean genus *Ctenomys*; b) Octodontidae, which includes a single subterranean specie (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-

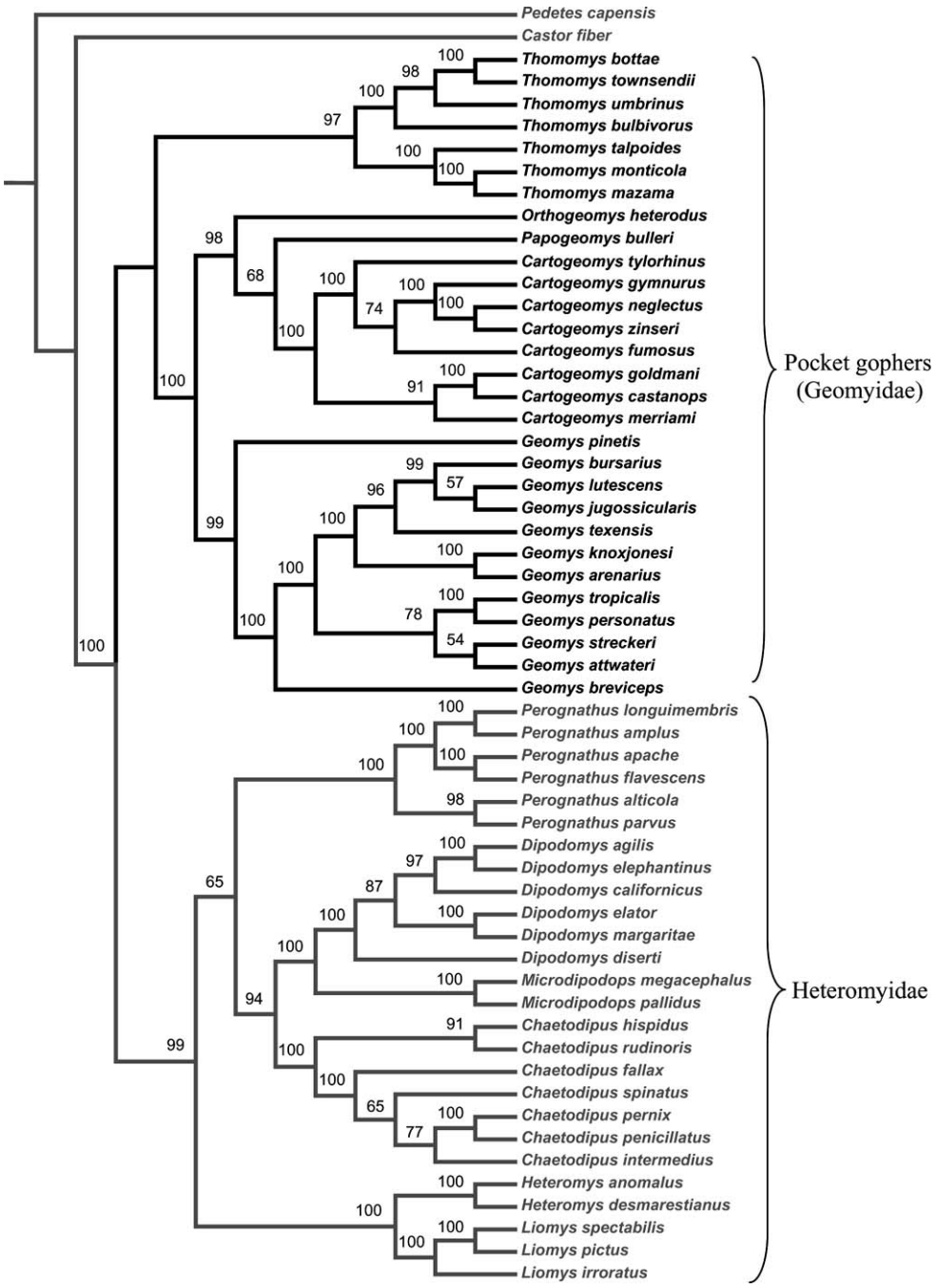

Fig. (2). Topology of the phylogenetic tree of pocket gophers and relatives. Non-subterranean are in gray. *Pedetes capensis* and *Castor fiber* are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

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 *Chaeotodipus* [35]. We set *Pedetes capensis* and *Castor fiber* as outgroup taxa. In the case of bathyergids, we obtained representative sequences of *Bathyergus*, *Cryptomys*, *Georychus* and *Heterocephalus*, and used *Hystrix africaeaustralis* 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).

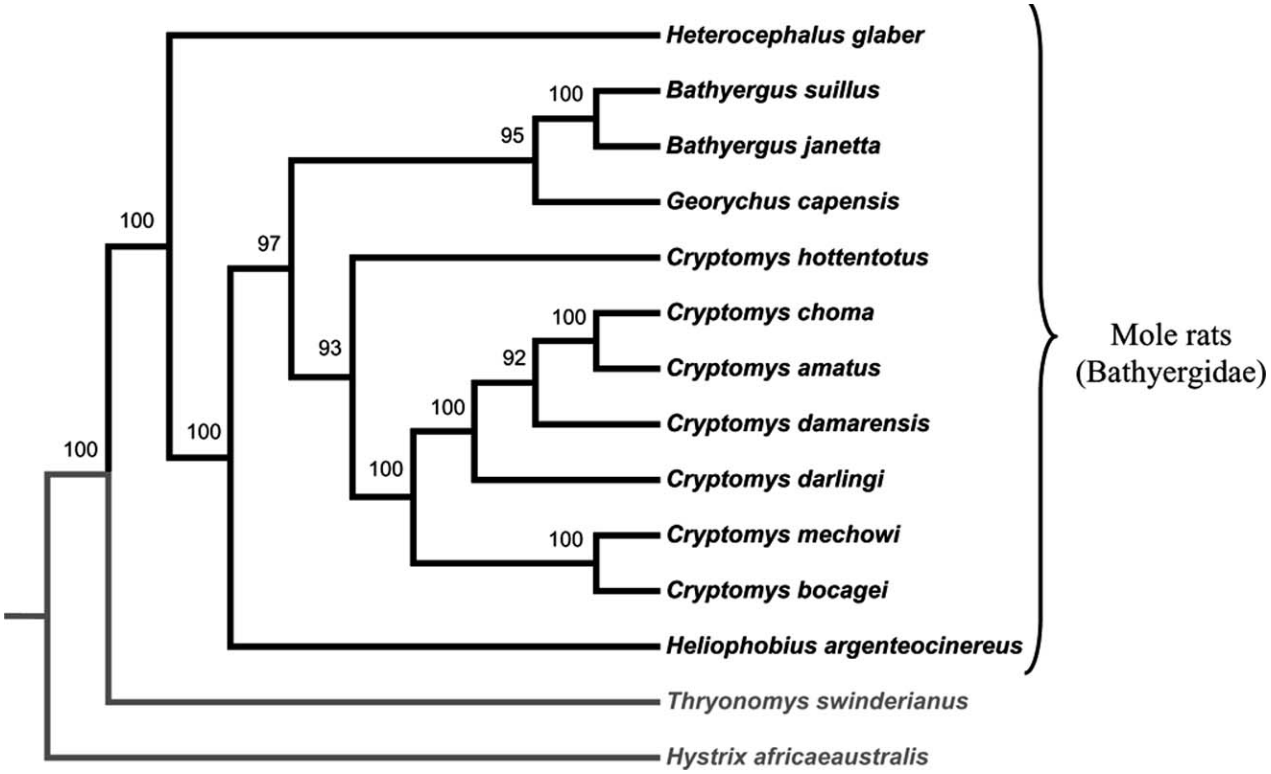

Fig. (3). Topology of the phylogenetic tree of mole rats and relatives. Non-subterranean taxa are in gray. *Thryonomys swinderianus* and *Hystrix africaeaustralis* are designated the outgroup. Numbers above the nodes are the bootstrap values > 50%.

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)	-2Δ Ln(L)	*p*
Null	0.0304	1	-9312.97	69.14	****
Ecological		2	-9278.39	20.60	****
Tuco-tucos + Coruro	0.0514				
Non-subterranean	0.0143				
Reference		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				
Full	-	66	-9236.87		

B

Model	ω estimates	# Rate parameters	Ln(L)	-2Δ Ln(L)	*p*
Null	0.0238	1	-20107.74	16.91	****
Ecological		2	-20099.28	6.69	*
Pocket-gophers	0.0294				
Non-subterranean	0.0200				
Reference		4	-20095.94	178.88	****
Basal Pocket-gophers	0.1277				
Crown Pocket-gophers	0.0287				
Heteromyids	0.0196				
Outgroup	0.0254				
Full model	-	111	-20006,49		

C

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

bathyergid branch, and all remaining, non-subterranean lineages. The sister taxon of bathyergids is poorly defined [45], so this case was not explored in more detail. The "ecological" model compares the bathyergid 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 distibution + selection (M8). This approach uses Bayesian posterior probabilities to determine the likelihood that a given codon position has experienced positive selection ($\omega > 1$).

When exporing 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 diferent 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 unambigously 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.

Table 3. Amino Acid Properties for TreeSAAP

Abbreviation	Amino Acid Property
P_α	Alpha-helical tendencies
N_s	Average number of surrounding residues
P_b	Beta-structure tendencies
B_l	Bulkiness
B_r	Buriedness
R_F	Chromatographic index
P_c	Coil tendencie
C	Composition
K^o	Compressibility
pK´	Equilibrium constant
C_a	Helical contact area
H	Hydropathy
pH_i	Isoelectric point
E_l	Long-range nonbonded energy
F	Mean rmsfluctuation displacement
M_v	Molecular volume
M_w	Molecular weight
H_{nc}	Normalized consensus hydrophobicity
V^o	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_α	Solvent accessible reduction ratio
H_p	Surrounding hydrophobicity
H_t	Thermodynamic transfer hydrophobicity
E_t	Total nonbonded energy
P_t	Turn tendencies

RESULTS

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

	Tuco-tucos + Coruro and relatives			Pocket Gophers and relatives			Mole Rats and relatives		
	PAML	TreeSAAP		PAML	TreeSAAP		PAML	TreeSAAP	
position	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 higer 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 compareing 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_α	P_α	P_α
pK´	pK´	pK´
$\underline{\alpha_c}$	α_c	α_c
$\underline{E_l}$		$\underline{R_\alpha}$
$\underline{\alpha_m}$		$\underline{H}$
$\underline{R_\alpha}$		$\underline{M_w}$
		$\underline{V^o}$

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	$\underline{P_{\alpha}}$		
14		$\underline{pK´}$	
19		$\underline{pK´}$	
23	P_{α}		
29	$\underline{P_{\alpha}}$		
TRANSMEMBRANE, A-HELIX			
36			$\underline{pK´}$
39			$\underline{pK´}$
42 *	$\underline{Ca}$	pK´	$\underline{pK´}$
43		pK´	
46 *		$\underline{B_l, R_F, P_c, \alpha_n, H_p}$	$\underline{pK´}$
52			$\underline{P_t}$
RMEMBRANE, AB-LOOP			
57			$\underline{V^o, C_a, H_{nc}, E_{sm}}$
59		$\underline{pK´}$	
60			$\underline{P_{\alpha}}$
64		$\underline{P_{\alpha}, P_c}$	
67	$\underline{P_{\alpha}}$	P_{α}	P_{α}
69		$\underline{pK´}$	
TRANSMEMBRANE, B-HELIX			
76			$\underline{P_{\alpha}}$
81		$\underline{\alpha_m}$	
82			$\underline{R_{\alpha}}$
84			$\underline{K^o, P_c}$
85			$\underline{\alpha_c}$
87		$\underline{P_{\alpha}}$	
96		$\underline{R_{\alpha}}$	
MATRIX, BC-LOOP			
110			$\underline{P_{\alpha}}$
TRANSMEMBRANE, C-HELIX			
111 *	$\underline{\alpha_c}$		$\underline{K^o}$
115	$\underline{pK´}$		
117	$\underline{pK´, R_{\alpha}}$		
123	$\underline{N_s, R_{\alpha}}$		
129		$\underline{R_{\alpha}}$	
INTERMEMBRANE, CD-LOOP			
142		$\underline{H_{nc}, \mu}$	
149			$\underline{E_t}$
158	$\underline{P_{\alpha}, \alpha_n, H_p}$		$\underline{K^o}$
169			$\underline{P_{\alpha}, P_t}$
TRANSMEMBRANE, D-HELIX			
173 *	$\underline{P_{\alpha}}$	$\underline{P_{\alpha}}$	$\underline{P_{\alpha}, P_t}$
190 *		$\underline{P_{\alpha}, P_b}$	$\underline{P_{\alpha}}$
193 *		$\underline{P_b}$	$\underline{P_{\alpha}}$
194		$\underline{P_{\alpha}}$	

(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_l	
229	pK´		
232 *	pK´	E_l	
233		pK´	
235		pK´	
236		pK´	
237		P_α	
238 *	P_α	E_l	
240 *	P_α	E_l	
241			P_α
243			R_α
TRANSMEMBRANE, EF-LOOP			
246			α_m
249			α_n
250		P_α	
256			α_n
257 *	P_α	P_α	
272			M_w
277			V^o, M_w, pH_i, C_a, H, H_{nc}, E_{sm}, E_t
278			α_m
281		B_l, α_n, H_p	
TRANSMEMBRANE, F-HELIX			
293		pK´	
295	P_b, E_l	pK´	
296 *	P_c, E_l, F, P_t	F	H
300		pK´	
302			pK´
303			F, P_b, pK´
304			pK´
306 *	N_s, pK´, E_l		pK´
307	N_s, pK´, E_l		
MATRIX, FG-LOOP			
309	N_s, B_l, P_c, α_n, H_p , P_t		
316			pK´
TRANSMEMBRANE, G-HELIX			
320 *		pK´	pK´
327 *	pK´		pK´
328		pK´	
329		P_t	pK´
331			α_c
334		pK´	R_α
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	$\underline{\alpha_c}$	$\underline{\alpha_m}$
348	$\underline{N_s, B_r, pK', H, E_l, R_\alpha, H_p}$		
349	$\underline{N_s, B_r, pK', H, E_l, R_\alpha, H_p}$		
353	$\underline{H}$		
356	$\underline{N_s, R_\alpha}$		
357	$\underline{N_s, B_r, pK', H, E_l, R_\alpha, H_p, H_t}$		
361	$\underline{N_s, B_r, pK', H, E_l, R_\alpha, H_p, H_t}$		
362	$\underline{N_s, B_r, pK', H, E_l, R_\alpha, H_p, H_t}$		
368	$\underline{N_s}$		
374	$\underline{\alpha_c}$		

*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 tucu-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 tucu-tucus and pocket gophers (codons 232, 238, 240 and 257), four between tucu-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 cleary accelerated rates of replacement relative to synonymous changes to the point that ω exceeds 1 [46, 20]. This is not suprising 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 adaptive 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 purifing selection [49]. Unfortunately, this hypothesis is impossible to test with cyt b sequences only.

A Small Number of Codons Shows ω > 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 subterraneous 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 considered 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 comon 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 desestabilizing 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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