Journal of Heredity 2007:98(3):243–249
doi:10.1093/jhered/esm013
Advance Access publication April 8, 2007

Cytochrome *b* Pseudogene Originated from a Highly Divergent Mitochondrial Lineage in Genus *Rupicapra*

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Abstract

We have identified a nuclear pseudogene (numt) of cytochrome *b* (*cytb*) in chamois. The comparison of a fragment of 402 nucleotides of *cytb* and the pseudogene between the 2 species *Rupicapra rupicapra* and *Rupicapra pyrenaica* allowed direct measurement of relative rates and patterns of evolution. Mitochondrial genes evolved 7 to 12 times faster than their nuclear counterparts. Substitutions in the nucleus include a frameshift and a stop codon. Phylogenetic analysis of nuclear and mitochondrial lineages on *Rupicapra* and related species showed that the nuclear branch evolved as a functional mitochondrial gene until the split of the 2 species of chamois and as a typical pseudogene later on. We propose that the pseudogene originated from a highly divergent mitochondrial lineage that did not persist in the mitochondrion and transposed to the nucleus in a time close to speciation. The concurrence of highly differentiated lineages at speciation points to hybridization between highly divergent populations.

Mitochondrial pseudogenes integrated in the nuclear genomes (numt's) are known from many taxa (reviewed in Bensasson et al. 2001). The use of universal primers to amplify mitochondrial sequences lead in many cases to the unwanted finding of nuclear paralogous of mitochondrial genes. More recently, numt's were detected in scans of sequenced eukaryotic genomes (Richly and Leister 2004; Schmitz et al. 2005). The numt's come in many sizes, from all types of mitochondrial sequences, and bear different degrees of similarity to their nuclear counterparts. Their abundance varies in different eukaryotic taxa (http://pseudogene.net). When transferred to the nucleus, mitochondrial sequences become nonfunctional pseudogenes and can be used to study the rates and patterns of neutral mutation in the nucleus (Li et al. 1981; López et al. 1997). The numt's show a higher proportion of nonsynonymous substitutions in protein-coding genes and increased transversion bias than the functional mitochondrial copies (Sunnucks and Hales 1996; Bensasson et al. 2000). In vertebrates, but not in insects (Pons and Vogler 2005), numt's have been found to exhibit a lower nucleotide substitution rate compared to their mitochondrial counterparts (López et al. 1994; Arctander 1995; Lü et al. 2002). Study of numt's also provides information about their own origin and the evolutionary histories and phylogenetic relationships of taxa, given that they represent a lineage that evolved in parallel to the mitochondrial DNA (mtDNA) sequence.

Here we present the comparison of a fragment of *cytb* and its nuclear counterpart in the same individuals of the 2 species of chamois, *Rupicapra rupicapra* and *Rupicapra pyrenaica*. Our results allowed to draw conclusions about rates and patterns of pseudogene evolution and showed that the nuclear copy originated from a lineage different from the one that persisted in the mitochondria.

Materials and Methods

Samples and DNA Extraction

A total of 8 samples, 4 of the species *R. pyrenaica*, from the Cantabrian Mountains (CBW20 and CBW22 from Somiedo in the west; CBE4 and CBE5 from Aller in the east), and 4 of *R. rupicapra*, from the Alps (ALW1 was from Val di Susa in the west; ALE1, ALE2, and ALE5 were from Tarvisio in the east), were included in the study. Samples CBW20 and CBW22 were recently collected by gamekeepers and consisted of a small piece of fresh liver preserved in 96% ethanol. The other 6 samples corresponded to individuals included in a previous study (Pérez et al. 2002).

Two methods were used to isolate DNA for amplification from fresh liver tissue. First, total DNA was extracted with Chelex, following Estoup et al. (1996). Second, genomic DNA enriched with mtDNA was also extracted

using the alkaline lysis method of Tamura and Aotsuka (1988).

Amplification and Cloning of Polymerase Chain Reaction Products

In this study, we used the cytochrome *b* universal primers L14724 (5′-CGAAGCTTGATATGAAAAACCATCG-TTG-3′) and H15149 (5′-AAACTGCAGCCCCTCAGAA-TGATATTTGTCCTCA-3′) (Irwin et al. 1991) to amplify a 486-bp polymerase chain reaction (PCR) product. Conditions were as follows: 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster city, CA) in 20-μl reactions, 1 μM of each primer, 250 μM of each deoxynucleoside triphosphate, 1× PCR Gold Buffer (Applied Biosystems), and 2.5 mM MgCl2. Cycling parameters were performed using 1 cycle of 95°C for 12 min, followed by 30 cycles, each of 95°C for 15 s; 64°C for 30 s; and 72°C for 1 min; followed by 72°C for 10 min. PCR products were electrophoresed along with size standards in 2% agarose gel in 1× tris-borate-EDTA and visualized by UV. Products of amplification reaction that produced a single strong band of the expected size were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK), and they were directly cloned into the pMOSBlue vector (Amersham Biosciences) and transformed into MOSBlue competent cells according to the supplier's specification. Clones were screened for inserts of the expected size by PCR amplification with the universal primers M13 and T7. Sequences, mitochondrial and nuclear, were identified by restriction of the PCR product with *Bsm*I.

Sequence Determination and Analysis

For sequencing, plasmid DNA was prepared for selected clones (Sambrook et al. 1989). Sequences were determined for both strands using the T7 and M13 universal primers. The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing, and samples were run on an Applied Biosystems (ABI) 310 automated DNA sequencer.

Cytb sequences of species of the tribe caprini (Ropiquet and Hassanin 2005) were retrieved from GenBank. Sequences obtained in this study have been submitted to the GenBank database with accession numbers EF158826–158841.

The nucleotide sequences were edited and aligned manually with Se-Al v2.0a11 (Rambaut 1996). Analyses of sequences were executed with MEGA vs2 (Kumar et al. 2004) and DnaSP 4.0 (Rozas et al. 2003).

Distance matrices and phylogenetic relationships were computed with PHYLIP vs3.5c (Felsenstein 1993), MEGA, and PAUP* (Swofford 2000), using tree approaches. For neighbor-joining (NJ) tree, Jukes–Cantor distances were used. The maximum parsimony (MP) analysis was carried out using the branch and bound algorithm. Maximum likelihood (ML) analysis was done using HKY85 model, after testing and comparing several models, with parameters estimated from the data set. The reliability of the nodes in each of the analysis was assessed by 1000 bootstrap replications. Substitution rates in the lineages, mitochondrial and nuclear, were compared by the relative rate test, using *Capra hircus* as outgroup, with the program RRTree (Robinson-Rechavi and Huchon 2000).

Table 1. Absolute number of mutations, sequence divergence, and estimated number of synonymous (K_s) and nonsynonymous (K_a) substitutions per 100 sites in mitochondrial (*cytb*) and nuclear sequences (nu*cytb*)

	cytb	nu*cytb*	*cytb* versus nu*cytb*
No. of haplotypes	2	4	
No. of mutations	15 (1 shared)	5	19 fixed
Synonymous	13	2	14
Mean divergence	8.57	1.25	27.69
K_s	8.34	0.52	25.63
K_a	0.38	0.25	2.15

Results

nu*cytb* Detection and Nucleotide Substitution Pattern

PCR amplification with the universal primers L14724 and H15149 eventually produced haplotypes with an insertion of an adenine at position 50 of the coding DNA sequence. This haplotype represents a nonfunctional copy of the gene and was presumed to correspond to a nuclear pseudogene. From then on, restriction with *Bsm*I was used to identify haplotypes with the insertion (that lack a target for the enzyme) or without it. To check the possibility that the insertion-bearing haplotype is a nuclear pseudogene, DNA was extracted from 2 fresh liver samples (CBW20 and CBW22) with 2 different methods, extraction with Chelex to obtain DNA total and the alkaline lysis to recover mainly mtDNA. Four amplification products from each sample and DNA extraction were analyzed. As expected, every product amplified from the alkaline lysis DNA extractions corresponded to the functional (mitochondrial) copy of the gene, whereas the 2 copies (functional and nonfunctional) were obtained from total DNA, indicating that the nonfunctional copy corresponded to a nuclear pseudogene. Both sequences, mitochondrial (*cytb*) and nuclear (nu*cytb*), of 6 additional individuals were obtained by amplification, cloning, and analysis of at least 4 clones from each one. If the 2 sequences, nu*cytb* and the *cytb*, were not represented in these clones, additional copies were examined until they were obtained.

A total of 8 pairs of sequences (*cytb* and nu*cytb*), 4 from the species *R. pyrenaica* and 4 from the species *R. rupicapra*, were studied. Analysis of sequences revealed 4 haplotypes among the 8 sequences bearing the nucleotide insertion and hence corresponding to the nuclear copy (2 in *R. pyrenaica* and 3 in *R. rupicapra*, 1 shared) and among the mitochondrial sequences 2 haplotypes, one common to the 4 individuals of *R. rupricapra* and the other common to the 4 individuals of *R. pyrenaica*. Overall, a total of 38 substitutions were observed in 37 sites (Table 1) in the region corresponding to the first 402 nucleotides of cytochrome *b*. The average divergence between nuclear and mitochondrial copies of the gene was 27.69

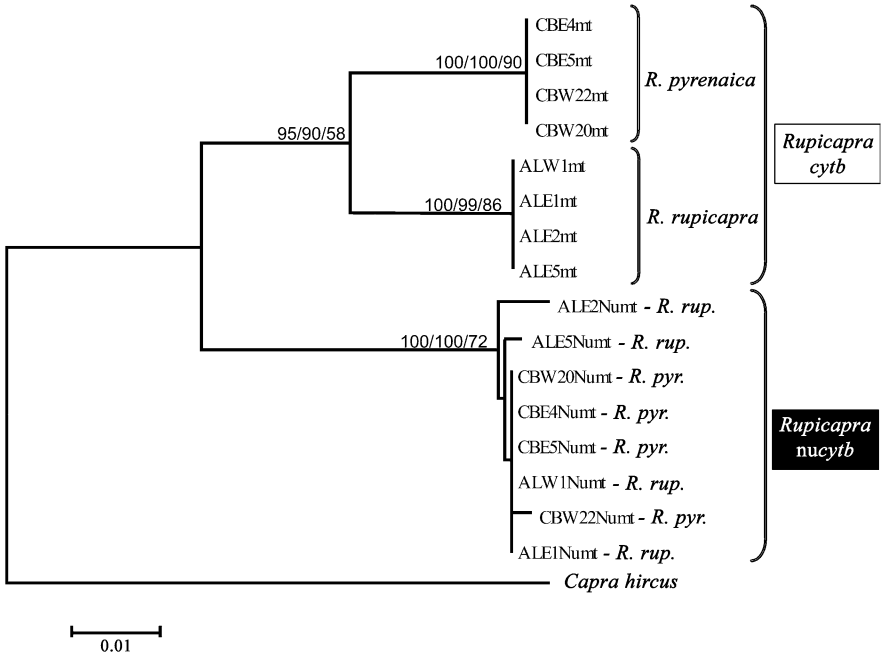

Figure 1. NJ tree with Jukes–Cantor distance of 402-bp sequences of cytochrome *b* gene and pseudogene. Numbers at nodes indicate bootstrap values for the NJ/MP/ML phylogenetic analysis, respectively.

substitutions, and 19 of these differences were fixed. A total of 15 mutations were observed among mitochondrial sequences, with an average of 8.57 substitutions between pairs of individuals. The ratio between synonymous and nonsynonymous mutations was 22.09. Only 5 mutations (one of them shared with the *cytb* group) were observed in the nu*cytb* sequences with an average of 1.25 substitutions between pairs of individuals. Three of the 5 mutations were nonsynonymous ($K_s/K_a = 2.09$) including a transversion that produced a stop codon.

Phylogenetic Analysis of the nu*cytb* and *cytb* Sequences

Phylogenetic trees of the 16 sequences were obtained by different methods using the sequence of *C. hircus* as outgroup, with identical results considering NJ, MP, or ML analysis. The insertion on pseudogene copies was eliminated from the analysis. All phylogenetic trees (NJ, MP, and ML) unambiguously supported 2 monophyletic clades, with very strong bootstrap support, one consisting of the nuclear copies and the other consisting of the mitochondrial ones (Figure 1). The 2 mitochondrial clades were also clearly revealed by any method while the grouping of the nuclear sequences varied and had low support with any method. It is clear that the pseudogene originated before the split of *R. rupicapra* and *R. pyrenaica*, and hence, comparison of the estimated number of substitutions per site between the 2 species either between sequences of the pseudogene (0.31%) or the mitochondrial gene (3.83%) (Table 2) provides an estimation of the relative evolutionary rates in the nucleus and the cytoplasm. The comparison gives a rate 12.27 times higher for the mitochondrial sequence. Alternatively, the average number of substitutions per site within mitochondrial (8.57) or nuclear sequences (1.25) can be used to obtain an estimation of the relative rate that in this case is 6.86.

Figure 2 shows a probably evolutionary scheme for the nuclear pseudogene (nuR), the functional mitochondrial gene of *Rupicapra* (mtR), and the mitochondrial gene of *Capra*, where A denotes the most recent common ancestor of sequences nuclear and mitochondrial of *Rupicapra*, O denotes the point of transposition to the nucleus, and Snu and Smt denote the split of nuclear and cytoplasmic sequences into the lineages *R. pyrenaica* and *R. rupicapra*. The number of substitutions per site in the branches leading from A to the pseudogene and to the mitochondrial *cytb* gene of chamois was similar ($P = 0.83$ in the relative rate test). This result contrasts with the observation of different evolutionary rates in the mitochondrion and the nucleus based on comparison between the 2 species of chamois.

Alternative explanations to this discrepancy are that the pseudogene originated before the divergence between *Capra* and *Rupicapra* and the outgroup rooting is wrong or that the pseudogene originated from a mitochondrial lineage quite different to the one that at last became established in *Rupicapra*. In order to discern between these 2 alternatives, we compared sequences of chamois with multiple mitochondrial sequences of *cytb* from species of the tribe caprini sensu lato (Ropiquet and Hassanin 2005). All phylogenetic trees support that the pseudogene originated after the split of the branch leading to *Rupicapra*. A parsimony analysis that included all the species in the tribe resulted in multiple equally parsimonious trees with a low consistence index (0.49)

Table 2. Jukes–Cantor distances (%)

	C. hircus	*R. pyrenaica cytb*	*R. rupicapra cytb*	*R. pyrenaica* nuc*ytb*
R. pyrenaica cytb	12.123			
R. rupicapra cytb	11.831	3.827		
R. pyrenaica nuc*ytb*	11.904	7.379	7.105	
R. rupicapra nuc*ytb*	12.050	7.242	7.173	0.312

because of the quick accumulation of third-position transitions that results in saturation. By including in the analysis only the most closely related genera, that is, *Naemorhedus*, *Ovibos*, and *Oreamnos*, only one tree with 110 steps and a consistency of 0.79 was recovered. This tree again places the pseudogene on the branch leading to *Rupicapra*. Branch lengths were estimated by the average pathway method (Nei and Kumar 2000). The nucleotide sites with base-pair differences between sequences of chamois are presented in Figure 3, along with homologous sequences from the tribe caprini. There were 40.5 steps from the most recent common ancestor (A in Figure 2) in the clade *Rupicapra*. Substitutions in the branches, mitochondrial and nuclear, before and after the split of *R. pyrenaica* and *R.rupicapra* were inferred from the most parsimonious tree, and the corresponding Jukes–Cantor distances are presented over the branches in Figure 2. The ancestral branch in the nuclear clade (A-Snu) is equal or longer than the branch in the mitochondrial clade (A-Smt). Inspection of substitutions assigned to each branch shows that the 8 substitutions unambiguously assigned to A-Smt were synonymous (5 transitions T-C and 2 transitions A-G). Eleven substitutions were assigned to the branch A-Snu and they were 5 transitions T-C and 6 transitions G-A. Five of the mutations were nonsynonymous (see Figure 3), which nevertheless are synonymous to one or another of the species

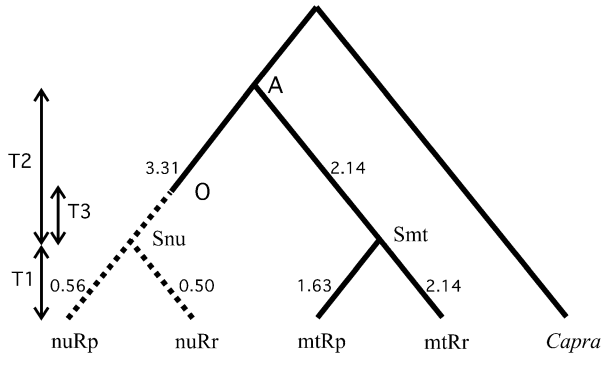

Figure 2. Plausible phylogenetic tree for cytochrome *b* gene and pseudogene. A is the most recent common ancestor of mitochondrial and nuclear sequences, O indicates the translocation of *cytb* to the nucleus, and Snu and Smt represent the split of *Rupicapra pyrenaica* and *Rupicapra rupicapra* nuclear and mitochondrial sequences. Percent substitutions in each branch were estimated from parsimony following Jukes–Cantor.

of the tribe and hence could accumulate in a functional copy of the gene. The rates and patterns of evolution in the nuclear and mitochondrial clades after the divergence of the 2 species were strikingly different. Mitochondrial sequences clearly evolved faster than nuclear ones, and only one out of the 12 substitutions unambiguously assigned to the external branches of mitochondrial sequences was nonsynonymous. On the contrary, 3 out of the 4 substitutions in the nuclear branches were nonsynonymous, different from the *cytb* sequence of caprini species, and include a stop codon. This substitution profile is compatible with mutations accumulated in a nonfunctional copy.

Discussion

Two types of sequences from each of 8 individual chamois have been obtained by standard PCR-sequencing protocol of the *cytb* gene. The sequences were inferred to be a functional mitochondrial gene and a nuclear pseudogene from selective extraction of mtDNA and study of substitution patterns. Our results add to the multiple reports of unwanted amplification of nuclear pseudogenes in phylogenetic studies of close related species (Arctander 1995; López et al. 1997; Pons and Vogler 2005; Schmitz et al. 2005; Kim et al. 2006). Phylogenetic inferences will be seriously distorted if nuclear sequences are included in the analysis, and it is therefore mandatory to follow recommendations given elsewhere (Bensasson et al. 2001; Thalmann et al. 2004) to avoid their unnoticed inclusion. On the other hand, numt sequences represent molecular "fossils" and are potentially useful for phylogenetic studies to be used as outgroups (Zischler et al. 1995; Hay et al. 2004).

The numt's can be used to compare rates and patterns of mutation in the nucleus and the mitochondria. In this study, the mitochondrial sequences were 6.86 times more divergent than the nuclear ones. This ratio is larger (12.27) if we compare average number of substitution between the 2 species of *Rupicapra* for mitochondrial or nuclear sequences. Our data are consistent with the general conclusion that mitochondrial sequences evolve 5–10 times faster than do the nuclear ones in mammals (Brown et al. 1979; Arctander 1995; Zischler et al. 1995; Lü et al. 2002; Kim et al. 2006). The ratio of synonymous to nonsynonymous substitutions in the mitochondrion was 22 showing that mutations were subject to purifying selection. In the pseudogene, this ratio was about 10 times lower and the pattern of nucleotide substitutions reflected the trend of spontaneous mutations accumulating under neutrality as in previous studies (López et al. 1994; Arctander 1995; Bensasson et al. 2001).

Divergence between a pair of sequences (d) is commonly expressed as a linear function of time (T), $d = 2\lambda T$, where λ is the absolute rate of substitution expressed as base-pair divergence per year. The time of divergence between the 2 clades of mitochondrial sequences ($T1$ in Figure 2) can be estimated applying the mean evolutionary rate of the cytochrome *b* for the family Bovidae, 0.63% substitutions per million years (MY) (Hassanin and Douzery 1999), and the distances in

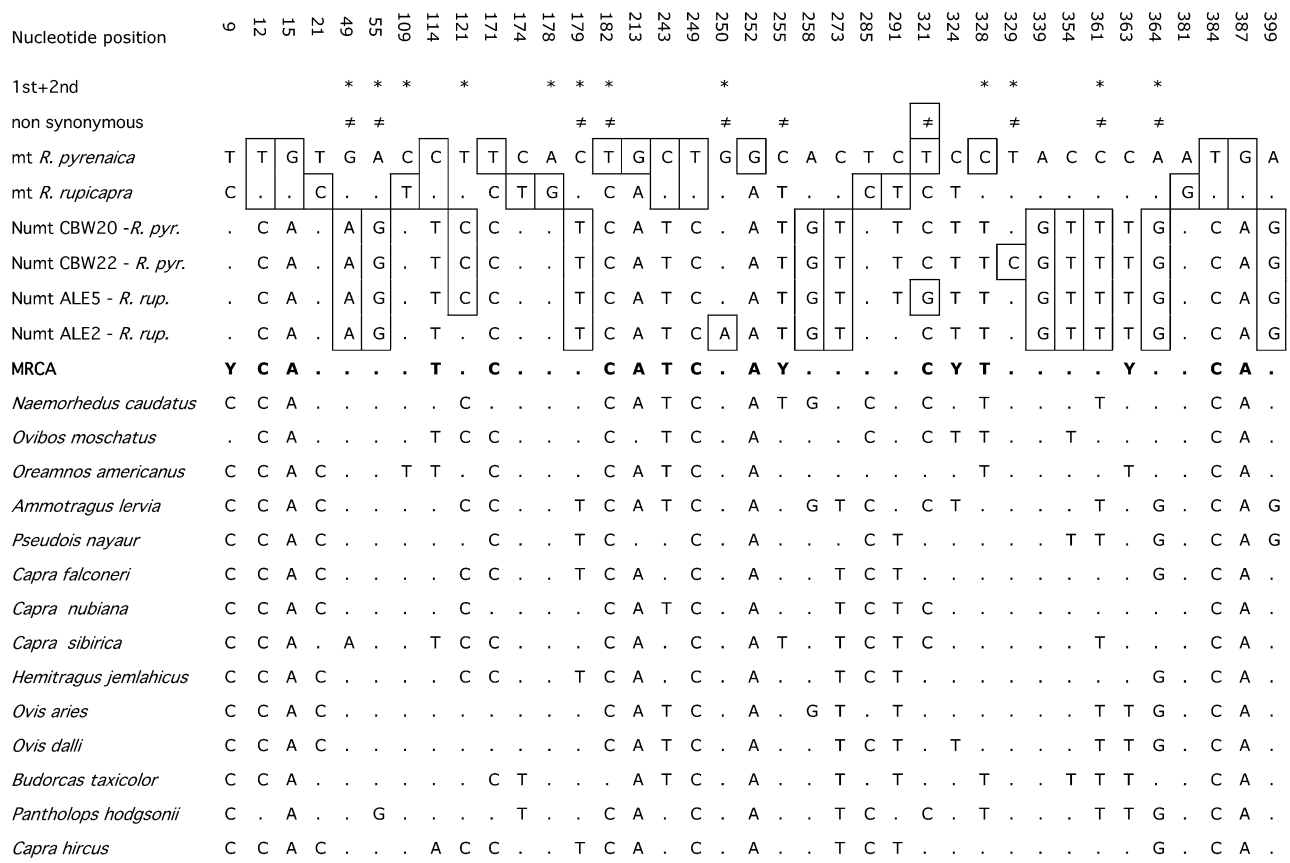

Figure 3. Variable sites in 402 bp of *cyth* in *Rupicapra* gene and pseudogene along with homologue sites in the tribe caprini. Nucleotide positions are indicated with reference to the functional *cyth* gene. Inferred changes relative to the mitochondrial and nuclear most recent common ancestor (MRCA) are boxed. Changes at the first and second codon positions (*) as well as nonsynonymous changes (≠) are indicated.

Figure 2, as 3.0 MY. Nuclear sequences have a common origin as is shown by phylogenetic analysis of substitutions and by the presence of the same insertion in all of them. Hence, the mitochondrial sequence leading to the present-day pseudogene must have been translocated to the nucleus before speciation at any time between A and Snu in Figure 2. The time elapsed between A and Snu (*72*) is equal to the time between A and Smt and can be estimated from the number of substitutions accumulated in the mitochondrial clade as dA−Smt/λmt that gives 3.4 MY. Therefore, the clade that at last was transposed to the nucleus as a pseudogene originated 6.4 MY before present. These estimates of divergence time are about 4 times shorter if the more general rate of mitochondrial gene divergence in vertebrates, 2.5% substitutions per MY (Hasegawa et al. 1985), is used.

The internal nuclear branch (A-Snu) would be expected to be shorter than the mitochondrial one (A-Smt), given that from the time of transposition to the nucleus (O) it evolved at a rate 5 to 10 times lower. Nevertheless, it is longer than the branch of the mitochondrial clade. The observation of long internal branches leading to the common ancestor of pseudogenes and mitochondrial genes is frequent and has been interpreted in different ways (reviewed in Bensasson et al. 2001). First, there are a number of problems associated to phylogenetic analysis. The type and rate of sequence variation in numt's can be complicated by the difficulty in identifying sister lineages across taxa and by the effect of the different rates of evolution that could lead to saturation in the mitochondrial branches and not in the nuclear ones (Bensasson et al. 2001; Schmitz et al. 2005). Alternative interpretations are that new mtDNA sequences that arrive to the nucleus can experience a mutation rate that is initially high (López et al. 1997), for example, as a result of the methylation of CG sites that would increase the rate of G-A transitions (Zischler et al. 1998). Numt branches could also be longer because the mitochondrial sequence that was integrated in the nucleus might have belonged to a mitochondrial lineage that is now extinct or unsampled (Sunnucks and Hales 1996). In this study, comparison of nu*cyth* with functional *cyth* denotes that before speciation it evolved as a functional gene both in rate and pattern of substitution except for the insertion. Patterns of evolution unexpected under neutrality are usually observed in studies of mitochondrial pseudogenes (López et al. 1994; Sunnucks and Hales 1996; DeWoody et al. 1999; Lü et al. 2002). The evolution of the nuclear sequences after speciation is typical of a pseudogene.

Comparable results were found by Lü et al. (2002) in the genus *Nycticebus* where changes in a *cytb* pseudogene accumulated at different rates and patterns before and after speciation. They interpreted that the pseudogene originated from a duplication event in the mitochondrial genome of a common ancestor, diverged in the mitochondria as a functional copy, and then inserted into the nuclear genome and pseudogenized at a time close to the speciation date. As the authors argue, no duplication in the mitochondria has been observed in vertebrates. A stronger argument against this interpretation is that the transposition of a mitochondrial sequence to the nucleus is thought to occur primarily through the release of mitochondrial sequences into the cytoplasm and their posterior translocation and integration in the nucleus (Blanchard and Schmidt 1996; Bensasson et al. 2001). In this context, it is difficult to explain the simultaneous loss of the duplicate sequence from the mitochondrion and its acquisition by the nucleus. We think that the most plausible interpretation is that a pseudogene originated from a mitochondrial lineage highly divergent from the one finally established in the genera. The observation of a relatively recent transposition to the nucleus of a sequence highly divergent from the present-day mitochondrial sequences can be regarded as the outcome of populations that evolved in isolation and hence diverged for mitochondrial sequences and later became in contact and hybridize. It can be speculated that the numt originated at the time of hybridization that should be close to the date of speciation. The nuclear sequences have a single origin, and their presence in both species together with the absence of the ancestral mitochondrial copy points to a strong genetic drive. The sequences, mitochondrial and nuclear, must have been subjected to lineage sorting, and the copy that at last persisted in the mitochondrion was from a lineage different that the ancestor of the pseudogene.

Acknowledgments

We thank Montserrat Aguadé and Trinidad Pérez for their critical reading of the manuscript. José Francisco Quirós (Guardería de Caza, Principado de Asturias) provided fresh samples of liver. This study was supported by grant MEC-04-CGL2004-06010 from the Ministerio de Educación y Ciencia.

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Received May 1, 2006
Accepted December 5, 2006

Corresponding Editor: Stephen J. O'Brien