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Molecular phylogeny and evolution of the extinct bovid *Myotragus balearicus*

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Abstract

Myotragus balearicus was a dwarf artiodactyl endemic to the Eastern Balearic Islands, where it evolved in isolation for more than 5 million years before becoming extinct between 3640 and 2135 cal BC (calibrated years BC). Numerous unusual apomorphies obscure the relationship between *Myotragus* and the extant Caprinae. Therefore, genetic data for this species would significantly contribute to the clarification of its taxonomic position. In this study, we amplify, sequence, and clone a 338-base pair (bp) segment of the mitochondrial cytochrome *b* (cyt *b*) gene from a >9 Kyr *Myotragus* subfossil from la Cova des Gorgs (Mallorca). Our results confirm the phylogenetic affinity of *Myotragus* with the sheep (*Ovis*) and the takin (*Budorcas*). In each tree, the *Myotragus* branch is long in comparison with the other taxa, which may be evidence of a local change in the rate of evolution in cyt *b*. This rate change may be due to in part to an early age of first reproduction and short generation time in *Myotragus*, factors that are potentially related to the extreme reduction in size of the adult *Myotragus* as compared to the other Caprinae.

Keywords: *Myotragus balearicus*; Molecular systematics; Cytochrome *b*; Generation time; Evolution

1. Introduction

Myotragus balaericus (Bate, 1909) was a dwarf bovid endemic to the Eastern Balearic Islands or Gymnesics (Mallorca, Menorca, Cabrera, and sa Dragonera) in the Western Mediterranean Sea (Alcover et al., 1981). The ancestor of *Myotragus* probably colonized Mallorca while the Mediterranean was desiccated, between 5.7 and 5.35 million years ago (Mya) during the Messinian (Clauzon et al., 1996). When the Gibraltar Strait re-opened, the ancestor of *Myotragus* became trapped on the islands, where it evolved in isolation until going extinct between 3640 and 2135 cal years BC (Ramis and Alcover, 2001). The timing of this extinction coincides with the first records of human settlement on the island (Burleigh and Clutton-Brock, 1980), which suggests a human role in the extinction of *Myotragus* (Alcover et al., 1999a,b).

Morphologically, *Myotragus* was quite distinct from the other Caprinae (see Fig. 1). It is the smallest Caprinae known: the largest adult specimens found would not have reached more than 45–50 cm from the ground to the shoulder, and probably weighed no more than 50–70 kg, while neonatal weight has been estimated to around 700–900 g (Bover and Alcover, 1999a). In addition to its small size, *Myotragus* had eyes in a frontal position, a monophiodontic incisiform dentition with constantly growing incisor in both jaws (Bover and Alcover, 1999b), and modified limb bones that most likely restricted it to slow locomotion (Alcover et al., 1981; Quetglas and Bover, 1998; Sondaar, 1977). These and other automorphies have obscured the relationship of *Myotragus* with the other Caprinae.

Traditionally, the subfamily Caprinae has been divided into four tribes: Rupicaprini (including the genera

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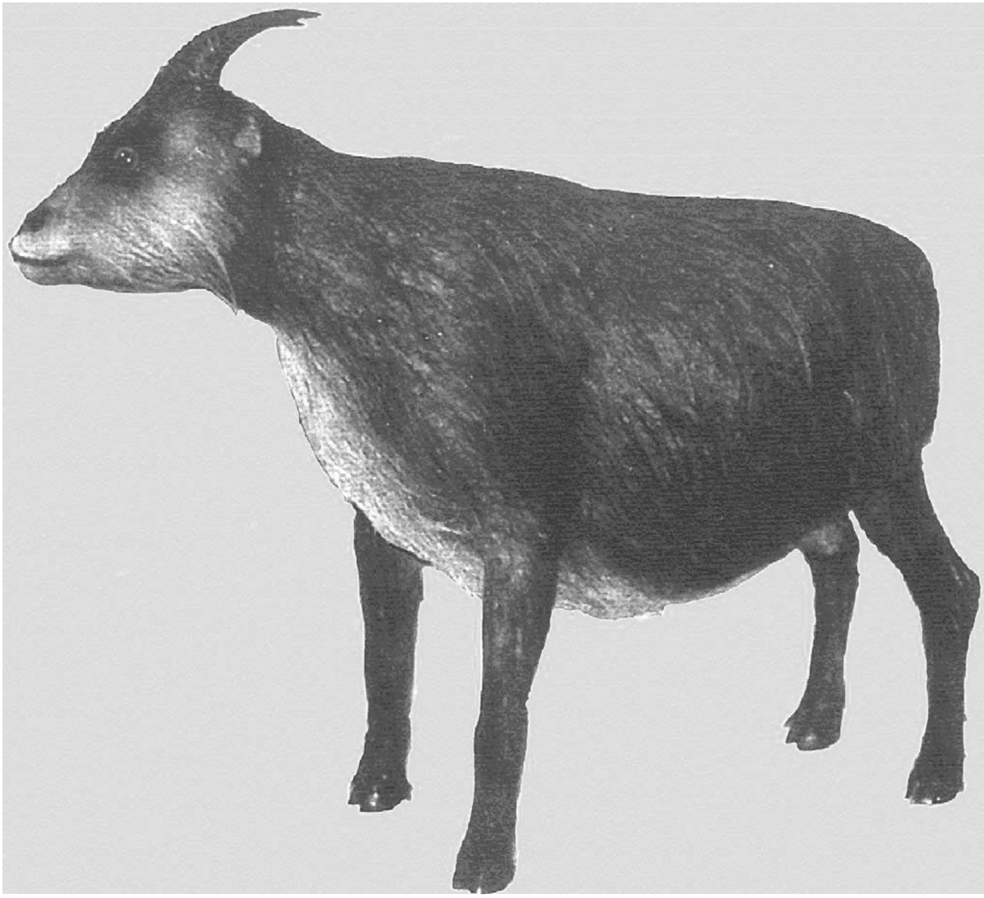

Fig. 1. Reconstruction of *Myotragus balearicus*.

Rupicapra, *Oreamnos*, *Capricornis*, and *Nemorhaedus*), Ovibovini (*Ovibos* and *Budorcas*), Caprini (*Ovis*, *Capra*, *Pseudois*, *Hemitragus*, and *Ammotragus*), and Saigini (*Saiga* and *Pantholops*) (Nowak, 1991; Simpson, 1945). However, recent morphological and molecular studies have questioned these relationships (Chikuni et al., 1995; Gatesy et al., 1997; Geist, 1987; Gentry, 1980, 1992; Groves and Shields, 1996; Hassanin et al., 1998a,b; Hassanin and Douzery, 1999; Thomas, 1994). For example, two recent molecular studies have suggested that *Saiga* should actually be placed in the Antelopinae, rather than in the Caprinae (Gatesy et al., 1997; Hassanin et al., 1998a). The accuracy of the other tribal classifications has also remained a matter of debate. Most modern molecular studies (Groves and Shields, 1996, 1997; Hassanin and Douzery, 1999) have found only three stable clades into the Caprinae: *Capra* and *Hemitragus*, *Capricornis*, *Ovibos*, and *Nemorhaedus*, and *Ovis* and *Budorcas*.

Myotragus is most often placed in the tribe Rupicaprini, most closely related to *Nemorhaedus* and *Capricornis* (Nowak, 1991; Simpson, 1945), however, this relationship has been challenged (Gentry, 1992) and remains problematic. To clarify the issue, we use ancient DNA (aDNA) techniques to recover small fragments of mitochondrial DNA (mtDNA) from the remains of two *Myotragus* specimens. In an earlier study, we recovered a short (55-bp) fragment of cyt *b* from a 4770–4400 cal BC 2σ (UtC 5171) year old *Myotragus* individual found in the cave site Cova Estreta (Pollença, Mallorca) (Lalueza-Fox et al., 2000). Although the fragment was short, the authenticity of the results was supported by cloning of PCR products, phylogenetic analysis, and independent replication, as is required in aDNA research (Cooper and Poinar, 2000; Hofreiter et al., 2001). Preliminary phylogenies generated using this 55-bp fragment suggested a close relationship between *Myotragus* and *Budorcas*, as has previously been suggested based on morphological evidence (Andrews, 1915). Like

Table 1
Primer sequences used in the study

L-14,899 5′-ATCCTAACAGGCCTATTCCT-3′
H-14,955 5′-ACCATAGTTTACATCTCGGC-3′
L-14,942 5′-CAACAACAGCATTYTCYTCTG-3′
L-14,983 5′-CTATGGCTGAATTATCCG-3′
L-15,062 5′-CGAGGCCTGTACTACGGATC-3′
H-15,071 5′-CCGATGTTTCATGTTTCTAGGA-3′
H-15,238 5′-AACTGAGAATCCGCCTCAG-3′

L and H refer to the light and heavy strands, respectively, and numbers refer to the 5′ position in the Anderson et al. (1981) human mtDNA sequence.

other molecular studies, these results did not support the traditional tribal divisions within the Caprinae. Unfortunately, however, the advanced state of decay of the *Myotragus* specimen used in the analysis made it impossible to generate any additional molecular data, which would have been necessary to confirm the phylogenetic placement of *Myotragus*. In this study, we extract and amplify 338 bp of cyt *b* from a *Myotragus* sample not used in the previous study. We use these data to construct a molecular phylogeny for the Caprinae, and to confirm the phylogenetic position of *Myotragus* within the subfamily.

2. Materials and methods

2.1. DNA extraction, amplification, cloning, and sequencing

A left tibia (Accession No. MNIB 60173) from Cova des Gorgs (Escorca, Mallorca) was chosen for analysis based on its unusually good macroscopic preservation. The medial portion of the sample was used for DNA analysis, while the proximal portion was sent for radiocarbon dating. The remainder of the sample is currently held in the vertebrate collection in the Museu de la Naturalesa de les Illes Balears (MNIB, Palma de Mallorca). Despite the superior preservation of the sample, the bone yielded a radiocarbon date of 7750–7585 cal BC 2σ (Beta 143117), approximately 3000 years older than the bone used in the previous study.

In Barcelona, DNA was extracted from approximately 1 g of bone. The sample was powdered and decalcified overnight in 10 ml of 0.5 M EDTA, followed by an overnight incubation in 1 ml of 10% SDS, 0.5 ml of 1 M Tris–HCl, and 100 μl of 1 mg/ml proteinase K. The sample was then extracted using phenol/chloroform techniques and desalted with Centricon 30 microconcentrators (Amicon). Extraction procedures were performed in an isolated pre-PCR area with positive air pressure. Appropriate controls were used in each step of the analysis, adopting the standard precautions of aDNA studies (Cooper and Poinar, 2000; Handt et al., 1994).

PCR amplifications were carried out in 25 μl reactions with 1 μl of extract, 1 U EcoTaq and 1× buffer (EcoGen), 2 mg/ml BSA, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, and 1 μM primers. PCR products were visualized in low melting point agarose gels, and bands representing positive results were excised, melted in 150–200 μl of water, and reamplified. Primers used in the study are listed in Table 1.

The primer pairs L14,899/H15,071, L14,942/H15,071, L14,983/H15,071, and L15,062/H15,238 yielded positive amplifications and were sequenced on an ABI 377a DNA sequencer (Applied Biosystems). To detect nuclear copies or errors potentially introduced by template damage, two fragments (L14,942/H15,071 and L15,062/H15,238) were cloned using a Sure Clone Ligation Kit (Pharmacia) according to the manufacturer's instructions.

To ensure the authenticity of the result, DNA was extracted, amplified, cloned, and sequenced from a separate bone sample from the same *Myotragus* specimen at the Ancient Biomolecules Centre in Oxford, England. The procedure was similar to that described above (details can be found in Barnes et al., 2002) except a high-fidelity enzyme (Platinum Taq *Pfu* Hi-Fi, Gibco-BRL) was used in the amplification.

2.2. Phylogenetic analyses

Cyt *b* sequences were obtained from GenBank for all of the genera within the Caprinae, and all of the species

Table 2
Extant Caprinae species and subspecies included in this study

Genus	Species	Subspecies	Common name	Accession No.
Saiga	*tatarica*		Saiga	U17864
Nemorhaedus	*caudatus*		Chinese goral	U17861
Capricornis	*crispus*		Japanese serow	D32191
Oreamnos	*americanus*		Mountain goat	U17863
Rupicapra	*pyrenaica*		Pyrenean chamois	AF034726
Ovibos	*moschatus*		Arctic muskox	U17862
Budorcas	*taxicolor*	*taxicolor*	Takin	U17868
Budorcas	*taxicolor*	*bedfordi*	Golden takin	U17867
Ammotragus	*lervia*		Barbary sheep	AF034731
Pseudois	*nayaur*		Blue sheep	AF034732
Hemitragus	*jemlahicus*		Himalayan tahr	AF034733
Capra	*hircus*		Domestic goat	U17866
Ovis	*aries*		Domestic sheep	U1734731
Ovis	*aries*	*musimon*	Mouflon	D84203
Ovis	*ammon*	*darwini*	Argali	AF034727
Ovis	*dalli*	*dalli*	Dall's sheep	AF034728
Ovis	*vignei*		Urial	AF034729
Pantholops	*hodgsoni*		Chiru	AF034724

Bos taurus (V00654) was used as outgroup.

and subspecies of *Ovis* and *Budorcas* for which the genetic data were available. In total, 19 extant taxa were used in the analyses (Table 2).

Maximum likelihood (ML) phylogenetic analyses were performed using PAUP 4.0b8 (Swofford, 1998). Likelihood ratio tests (Huelsenbeck and Krandall, 1997) were used to determine the simplest model that could not be rejected in favor of a more complex model. Analyses were performed using a genetal time reversible (GTR) model of nucleotide substitution (six substitution types) and codon partitioning, which allows for different rates of substitution between first, second, and third codon positions. Full-heuristic searches were performed twice with TBR branch swapping and a re-estimation of parameters between the searches. Local stability of the tree was evaluated using 500 full-heuristic ML bootstrap replicates, with TBR branch swapping and starting trees generated by neighbor-joining (NJ). Because codon partitioning cannot be used in bootstrap analysis within PAUP, parameters were estimated using the GTR model with four variable rates of substitution and a proportion of invariable sites. Analyses were performed initially using a single representative species from each of the genera within the Caprinae. Because of the advanced state of decay of the *Myotragus* specimen, we were unable to generate more than 338 bp of cyt *b*. The analyses were performed both excluding the site that were unavailable for *Myotragus* and including all 1143 bp of cyt *b* and treating the data unavailable for *Myotragus* as missing sites. To further explore the relationship between *Myotragus*, *Ovis*, and *Budorcas*, additional species within *Ovis* and *Budorcas* were included and the analysis was performed as above.

Maximum parsimony (MP) and NJ trees from a distance matrix (with the Kimura two-parameters model) were also generated using PHYLIP v3.4 (Felsenstein, 1991), using both the entire cyt *b* gene and the short 338-bp fragment.

3. Results

3.1. Authenticity of the Myotragus sequence

Ancient DNA procedures are highly susceptible to contamination by modern, exogenous DNA, and therefore necessary precautions must be taken to ensure the authenticity of the results (Cooper and Poinar, 2000). Accordingly, DNA sequences were derived independently in two dedicated ancient DNA laboratories. These sequences matched exactly, and cloning procedures indicated that no nuclear mitochondrial copies were amplified. In total, 338 bp of cyt *b* were amplified and sequenced for *Myotragus*. Because of the advanced state of decay of the sample, the 338 bp was amplified in several overlapping fragments, and in each case the overlapping sequences matched exactly. Additionally, two of the fragments overlapped with the previously derived *Myotragus* sequence (Lalueza-Fox et al., 2000) (see Fig. 2).

The majority of the substitutions observed in the *Myotragus* sample are not shared by the other Caprinae, are in the third codon position, and do not result in any amino acid changes. Of the 13 third position changes, however, two do result in amino acid substitution: at nucleotide position 15,020, isoleucine changes to valine, and at position 15,095 valine becomes methionine. It has been suggested, however that these amino acid replacements will not result in significant changes in the secondary structure of the protein encoded (Hassanin et al., 1998b). Therefore, it is unlikely that the changes observed in the *Myotragus* sequence can be attributed to random template damage.

When compared against other bovid sequences already deposited in GenBank, *Myotragus* closely matched several of the extant Bovids, however, it did not appear to be closely related to the bovids previously identified as potential phylogenetic relatives. The 338-bp *Myotragus* sequence shares from 86–89% of positions with *Ovis*, *Budorcas*, *Rupicapra*, *Oreamnos*, *Capra*, *Hemitragus*, *Ovibos*, *Capricornis*, and *Nemorhaedus*. Fewer sites are shared with genera in the Hippotraginae and Antilopinae, which are two subfamilies closely related to the Caprinae.

3.2. Cloning results

In Barcelona, six clones were sequenced from the L14,942/H15,071 fragment, as were nine from the L15,062/H15,238 fragment (Fig. 3). Within the first fragment (130 bp), four single substitutions and two multiple substitutions were detected. For the second fragment (177 bp) eight single substitutions and two multiple substitutions were found. The error rate (/1000 bp, with multiple substitutions weighed as single mutations) was similar among the clones of both fragments (7.69 for the first, and 5.65 for the second fragment). In Oxford, eight clones of the L14,983/H15,701 fragment were sequenced. Four single and no multiple substitutions were detected, giving an error rate of 5.61/1000 bp. All of the error rates observed were significantly higher than error rates observed in molecular studies utilizing modern DNA, which on average detect 2–3 errors/1000 bp (Cooper et al., 2001). Among the errors observed, two were found in more than one clone: at nucleotide position 15,181, four of nine clones read A, while the remaining five were read as T. At nucleotide position 15,223, five clones were read as T, while the remaining four were C. These substitutions were not observed in the direct sequencing results, however, and were therefore not included in the putative consensus sequence.

```
             14900
               |
Capricornis    GCA ATA CAC TAC ACA TCC GAT ACA ACG ACA GCA TGT TCT TCT GTA ACA CAC ATT TGC CGA
Nemorhaedus    ... ... ... ... T.. ... ..C ... ..A ... ... ... ... ... ... ... ... ..C ... ...
Oreamnos       ... ... ... ... ... ... ..C ... ..A ... ... ..C ..C ... ... ... ... ..C ..T ...
Rupicapra      ... ... ... ... ... ... ... ... G.A ... ... ..C ..C ... ... ..C ... ... ... ...
Ammotragus     ... ... ... ... ... ... ..C ... .TA ... ... .TC ..C ... ... G.C ... ..C ... ...
Budorcas       ... ... ... ... ... ... ... ... ..A .T. ... ..C ..C ... ... G.C ... ... ..T ...
Ovibos         ... ... ... ... ... ... ..C ... ..A ... ... .TC ... ... ... ... ... ... ... ..G
Pseudois       ... ... ... ..T ... ... ..C ... .TA ... ... .TC ..C ... ..G ..T ... ..C ... ..G
Hemitragus     ... ... ... ... ... ... ..C ... .TA ... ... .TC ... ... ... ..C ... ... ... ...
Capra          ... ... ... ..T ... ... ..C ... .TA ... ... .T. ..C ... ... ..T ... ... ..T ...
Ovis           ... ... ... ..T ... C.. ..C ... ..A ... ... .TC ..C ... ... ..C ... ... ... ...
Pantholops     ... ... ... ... ... ..T ... ... ..A ... ... .TC ... ... ... ..C ... ... ... ...
MYOTRAGUS      ... ... ... ..T ... ... ..C ... ..A ... ... ..C ..C ... ..C G.C ..T ... ... ...

Saiga          ... ... ... ... ... G.T ..C ... G.A ... ... .TC CAC ... ..T ... ... ..C ... ...
Bos            ... ... ... ... ... ... ..C ... ..A ... ... ..C ..C ... ..T ..C ..T ..C ... ...
Human          ..C ..G ... ... T.. C.. ..C G.C T.A ... ... ..C ..C ... A.C G.C ..T ..C AC. ...

Capricornis    GAC GTA AAC TAC GGC TGA ATT ATC CGA TAT ATA CAC GCA AAC GGA GCA TCA ATA TTC TTT
Nemorhaedus    ... ... ... ..T ..T ... ... ... ... ..C ... ... ... ... ... ... ... ... ..T ..C
Oreamnos       ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ..T ..C ... ..T ..C
Rupicapra      ..T ... ... ... ... ... ..C ... ... ..C ... ..T ... ..T ... ... ... ... ..T ..C
Ammotragus     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ...
Budorcas       ... ... ... ..T ... ... ..C ... ... ... ..G ... ..G ... ... ... ... ... ..T ...
Ovibos         ..T ... ... ... ... ... ..C ..T ... ... ... ... ... ... ... ... ... ... ... ..C
Pseudois       ..T ..C ... ..T ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C
Hemitragus     ..T ... ..T ..T ... ... ..C ... ... ..C ... ... ... ... ... ... ... ... ... ...
Capra          ..T ... ..T ..T... ... ..C ... ... ..C ... ... ... ... ... ... ... ... ... ...
Ovis           ... ..G ... ..T ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ..T ...
Pantholops     ..T ..T ... ..T ... ... ... ..T ... ..C ... ... ... ... ... ... ... ... ..T ...
MYOTRAGUS      ... ... ... ..T ... ... ... ... ... ... ... ..T ... ... ... ... ..C ... ..T ..C

Saiga          ... ..C ... ..T ... ... ... ... ... ... ... ..T ... ... ... ... ..C ... ... ...
Bos            ... ..G ... ... ... ... ..C ... ... ..C ... ... ... ... ... ..T ... ..G ..T ...
Human          ... ... ... ... ... ... ..C ... ..C ..C C.T ... ... ..T ..C ..C ... ..G ..T ...

Capricornis    ATC TGC CTA TTC ATA CAC GTA GGA CGA GGC CTA TAC TAC GGA TCG TAC ACT TTC TTA GAA
Nemorhaedus    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... .C. ..G
Oreamnos       ... ... ... ..T ... ... ... ... ... ... ... ..T ..T ... ..A ..T ..C ..T ... ...
Rupicapra      ... ... ... ... ... ..T ... ... ... ... ... ..T ... ... ..A ... ... ..T C.. ...
Ammotragus     ..T ... ... ... ... ... ... ... ... ... ... ..T ... ... ..A ... ... ..T C.. ...
Budorcas       ... ... ... ..T ..G ..T ... ... ... ... ..G ..T ... ... ..A ... ..C ... ... ...
Ovibos         ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ...
Pseudois       ... ... ... ... ... ..T ... ..G ... ... ... ..T ... ... ..A ..T ..C ..T C.. ..G
Hemitragus     ... ... ..G ... ... ..T A.C ... ... ... ... ..T ... ... ..A ... ..C ... C.. ...
Capra          ... ... ... ... ... ..T A.C ... ... ..T ... ..T ..T ... ..A ..T ..C ..T C.. ...
Ovis           ... ... ... ..T ..G ..T ... ... ... ... ... ..T ..T ... ..A ..T ..C ... C.. ...
Pantholops     ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ..A ... .TC ... ... ..G
MYOTRAGUS      G.. ... ... ..T ..G ... ..G ... ..G ... ... ... ... ... ..A ... ... ... C.. ...

Saiga          ... ... ... ... ... ... ... ... ... ... ..T ..T ... ... ..C ..T ..A ... C.. ...
Bos            ... ... T.. .AT ..G ... ... ... ... ... T.. ..T ... ..G ..T ... ... ..T C.. ...
Human          ... ... T.C ..T C.G ... A.C ..G ... ... ... ... ... ... ..A .TT CT. .A. ... ...

Capricornis    ACA TGG AAC ATC GGG GTA ATT CTC CTA CTC ACA GCG ATA GCC ACA GCA TTC ATA GGC TAT
Nemorhaedus    ... ..A ..T ..T ... ... ..C ..T ... ... ... A.A ... ..T ... ... ... ..G ..T ..C
Oreamnos       ... ..A ... ..T ..A ... ..C ... T.. ..T ... A.A ... ... ..G ... ... ... ... ...
Rupicapra      ... ..A ... ... ..A ... ..C ... ... ... ... A.A ... ... ... ..G ..T ..G ... ..C
Ammotragus     ... ..A ..T G.A ..A ..G ..C ... ... T.. G.. A.A ... ... ... ... ... ... ..A ...
Budorcas       ... ..A ... ... ..A ... ... ... ... T.T G.. A.A ... ... ... ... ... ... ... ...
Ovibos         ... ..A ... ..A ... ... ... ..T ... ... .T. A.A ... ..T ... ... ... ... ... ...
Pseudois       ... ..A ... ... ..A A.C ... ... ... T.. G.. A.A ... ... ... ... ... ... ... ..C
Hemitragus     ... ..A ... ..T ..A ... ..C ... ... ... ... A.A ... ... ... ... ... ... ... ...
Capra          ... ..A ... ..T ..A ... ..C ... ..G ... G.G A.A ..G ... ... ... ... ... ... ...
Ovis           ... ..A ... ... ..A ... ..C ... ... T.T G.G A.A ... ... ... ... ... ... ... ...
Pantholops     ... ..A ... ... ... ... ..C ... ... T.T G.. A.T ... ... ... ... ... ... ... ...
MYOTRAGUS      ... ..A ... ... ..A A.. ..C ... ... T.. ... A.A ... ..T ... ... ... ... ..T ..C

Saiga          ... ..A ... ..T ..A ..T ... ..T ... T.. G.. A.A ... ... ... ... ... ... ..A ...
Bos            ... ..A ..T ..T ..A ... ..C ..T ..G ... ... .TA ... ... ... ..T ... ... ..A ..C
Human          ..C ..A ..T ... ..C ..T ..C ... ..G ... G.. A.T ... ..A ... ..C ... ... ... ...
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Fig. 2. *Myotragus* mtDNA cyt *b* sequence alignment between nucleotide positon (np) 14,900 and np 15,237. *Myotragus* (*Myotragus balearicus*), *Capricornis* (serow), *Budorcas taxicolor taxicolor* (takin), *Nemorhaedus* (goral), *Oreamnos* (Mountain goat), *Rupicapra* (chamois), *Ovibos* (Arctic muskox), *Hemitragus* (Himalayan tahr), *Capra* (goat), *Ovis aries* (sheep), *Saiga* (Saiga), *Pantholops* (chiru), and *Bos* (cow); subspecies of *Budorcas* and *Ovis* are not shown, although have been included in the phylogenetic tree. Dots indicate sequence identity. Sequences of human and cow are displayed as a contamination controls.

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Capricornis     GTC CTA CCA TGA GGA CAA ATA TCA TTC TGA GGG GCT ACA GTT ATT ACC AAC CTC CTC TCA
Nemorhaedus     ... ... ... ... ... ... ... ... ... ... ... ..C ... ..C ..C ... ..T ... ... ...
Oreamnos        ... ... ... ... ... ... ... ... ... ... ..A ... ... ..C ..C ..T ... ... ... ...
Rupicapra       ... ... ... ... ... ..G ... ... ... ... ... ..C ... ..C ..C ... ..T ... ... ...
Ammotragus      ... ..G ... ... ... ..G ... ... ... ... ... ..A ... ..C ..C ... ... ..T ... ...
Budorcas        ... T.. ... ... ... ... ... ... ..T ... ..A ..A ... ..C ... ... ... ... ... ...
Ovibos          ... ... ... ... ... ... ... ... ... ... ..A ... ... ..C ..C ..T ... ... ... ...
Pseudois        ... T.G ... ... ... ... ... ... ..T ... ... ..A ... ..C ..C ... ... ..T ... ...
Hemitragus      ... T.. ... ... ... ..G ... ... ... ... ... ..A ... ..C ..C ... ... ..T ... ...
Capra           ..T T.. ... ... ... ... ... ... ... ... ... ..A ... ..C ..C ..T ..T ..T ... ...
Ovis            ... T.. ... ... ... ... ... ... ... ... ..A ..A ... ... ... ... ... ... ..T ...
Pantholops      ..T T.. ... ... ... ... ... ... ... ... ..A ..A ... ..A ... ... ... ... ..T ...
MYOTRAGUS       ..T T.. ... ... ... ... ... ..T ..T ... ..A ..A ..C ... ..C ... ... ... ... ...

Saiga           ... ... ... ... ... ... ... ... ... ... ..A ..A ... ..C ..C ... ..T ... ..T ...
Bos             ... ... ... ... ... ... ... ... ... ... ..A ..A ... ..C ..C ... ... ... T.A ...
Human           ... ..C ..G ... ... ... ... ... ... ... ... ..C ... ..A ... ..A ... T.A ..A ..C


                                                                    15237
                                                                    |
Capricornis     GCA ATC CCA TAC ATT GGC ACA AAC CTA GTC GAA TGA AT
Nemorhaedus     ... ..T ... ..T ... ... ... .G. ... ... ... ... ..
Oreamnos        ... ... ... ... ..C ... ... ... ... ... ... ... ..
Rupicapra       ... ..T ... ..T ... ... ... .G. ... ... ... ... ..
Ammotragus      ... ... ... ... ... ... ... G.. ..G ... ... ... ..
Budorcas        ... ... ... ... ... ... ... ... ... ..T ..G ... ..
Ovibos          ... ... ... ... ..C ... ... ... ... ... ... ... ..
Pseudois        ... ... ..C ..T ... ... ... ..T ... ... ... ..G ..
Hemitragus      ... ..T ... ..T ..C ... ... ... ... ... ... ... ..
Capra           ... ... ... ..T ... ... ... ... ... ... ... ... ..
Ovis            ... ..T ... ..T ... ... ... ... ... ... ... ... ..
Pantholops      ... ... ... ... ... ... ... G.. ... ... ... ... ..
MYOTRAGUS       ..T ... ... ... ... ..A ..C ... ..C ..A ... ... ..

Saiga           ... ... ... ..T ..C ... ... G.. ... ..A ... ... ..
Bos             ... ... ... ... ..C ... ... ..T T.. ... ... ... ..
Human           ..C ... ... ... ... ..G ... G.. ... ..T C.. ... ..
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Fig. 2. (*continued*)

3.3. Phylogenetic analyses

Of the 338 bp sequenced for *Myotragus*, 109 sites were polymorphic within the Caprinae. A total of 122 substitutions were observed, of which 96 were transitions and 26 were transversions. The base composition was as follows: 32.1% A, 25.5% C, 15.9% G, and 26.5% T.

Fig. 4 depicts the ML tree derived for the Caprinae using all of cyt *b* and treating the unavailable data as missing sites for *Myotragus*. Majority rule bootstrap consensus values are given for nodes with greater than 50% support. The analysis excluding all but the 338 bp available for *Myotragus* resulted in no significant change in the tree topology. The branching of *Myotragus* cannot be resolved, and three species (*Ovis*, *Budorcas*, and *Myotragus*) form a trichotomy. The analysis performed using only a single representative of each of the genera resulted in similar tree topology to that in Fig. 4, however resulted in higher bootstrap support values. In the *Ovis* + *Budorcas* + *Myotragus* clade, *Myotragus* appears to be more closely related to *Budorcas*, however the bootstrap support is fairly low (50%). As less data were available for *Myotragus*, these low bootstrap values are not unexpected.

When the analysis was performed excluding *Myotragus*, three clades were fairly well supported by bootstrap resampling: *Capra* + *Hemitragus* + *Pseudois*, *Capricornis* + *Nemorhaedus* + *Ovibos*, and *Ovis* + *Budorcas* (data not shown). These relationships were maintained both in MP and in NJ trees. Some intermediate nodes, however, including *Oreamnos*, *Pantholops*, *Rupicapra*, and *Ammotragus*, remained unstable throughout the analyses. The problematic taxonomic positions of these taxa have been previously reported (e.g., Groves and Shields, 1996; Hassanin and Douzery, 1999).

4. Discussion

Our results indicate that *Myotragus* is closely related to extant Caprinae species, and in particular to *Ovis* and *Budorcas*. The lack of resolution of the basal branches of the trees may be indicative of a quick initial radiation of the Caprinae, as has been suggested by Vrba (1985). If this is the case, the phylogenetic relationships of these species may be difficult to accurately resolve. It has also been suggested that the functional constraints of protein-coding genes like cyt *b* will make these genes prone to high levels of homoplasic events, particularly for transitions at third codon positions that do not result in amino acid changes, and that this saturation may obscure phylogenies derived from these genes (Hassanin and Douzery, 1999). By partitioning the analysis to allow each codon position a distinct substitution pattern, we decrease the effect of multiple substitutions and the difference in the substitution rate between codon positions allowing for a more accurate phylogeny of the Caprinae.

```
L14899>GCAATACACTATACATCCGACACAACAACAGCATGCTCCTCTGTCGCCCATATTTGCCGAGACGTAAACTATGGCTGAATTATCCGATACATACATGCAAACGGAGCATCCATATTTTTCGTCTGCCTATTTATGCACGTGGGACGGGGCCTATACTACGGATCATACACTT
Seq1   ............................................................................................................................................................................<H15071
Seq2                                                                                  L14983>......................................................................................
Clone1C                                                                                      ....................................................A.................................
Clone2C                                                                                      ......................................................................................
Clone3C                                                                                      ..........................................................T...........................
Clone4C                                                                                      ......................................................................................
Clone5C                                                                                      ......................................................................................
Clone6C                                                                                      .................................................T.T..................................
Clone7C                                                                                      ......................................................................................
Clone8C                                                                                      ......................................................................................
Seq3                                       L14942>.................................................................................................................................
Clone1A                                           .................................................................................................................................
Clone2A                                           .......................................................................................................A.........................
Clone3A                                           ............................C....................................................................................................
Clone4A                                           ..........................................................................................................T.....T................
Clone5A                                           ..................................................T.............................................................T................
Clone6A                                           ..........................................................................................................T.............T........
Seq4                                                                                                                                                                L15062>........
Seq5                                                                                                                                                                       ........
Clone1B                                                                                                                                                                    ........
Clone2B                                                                                                                                                                    ........
Clone3B                                                                                                                                                                    ........
Clone4B                                                                                                                                                                    ........
Clone5B                                                                                                                                                                    ........
Clone6B                                                                                                                                                                    ........
Clone7B                                                                                                                                                                    ........
Clone9B                                                                                                                                                                    ........
Clone10B                                                                                                                                                                   ........


                                                                                                                     15181                                     15223
                                                                                                                     |                                         |
        TCCTAGAAACATGAAACATTGGAATAATCCTCCTATTCACAACAATAGCTACAGCATTCATAGGTTACGTTTTACCATGAGGACAAATGTATTTTTGAGGAGCAACCGTTATCACCAACCTCCTCTCAGCTATCCCATACATTGGAACCAACCTCGTAGAATGAAT<H15238
Seq4    ......................................................................................................................................................................
Seq5    ......................................................................................................................................................................
Clone1B .............................................................................................................A........................................................
Clone2B .......................................................................................................................................................T..............
Clone3B ..........................................................................................................................G...........................................
Clone4B ......................................G...............................A.............................T.................................................................
Clone5B .............................................................................................................A.........................................T..............
Clone6B .............................................................................................................A........................................................
Clone7B .............................................................................................................A.........................................T..............
Clone9B .....................................................................................................C.................................................T..............
Clone10B...................C............................................................................................T......................................T..............
```

Fig. 3. DNA sequences of clones used to generate a consensus for the *Myotragus* sequence between np 14,900 and 15,237, compared to direct sequences of 14,899–15,071, 14,942–15,071, 14,983–15,071, and 15,062–15,238. Dots indicate identity to the *Myotragus* consensus sequence. A and B correspond to clones obtained in Barcelona and C correspond to clones from Oxford; sequences 1–5 correspond to direct sequences from different PCR amplifications. Additional clones for the L14,899/H14,955 fragment can be found in Lalueza-Fox et al. (2000).

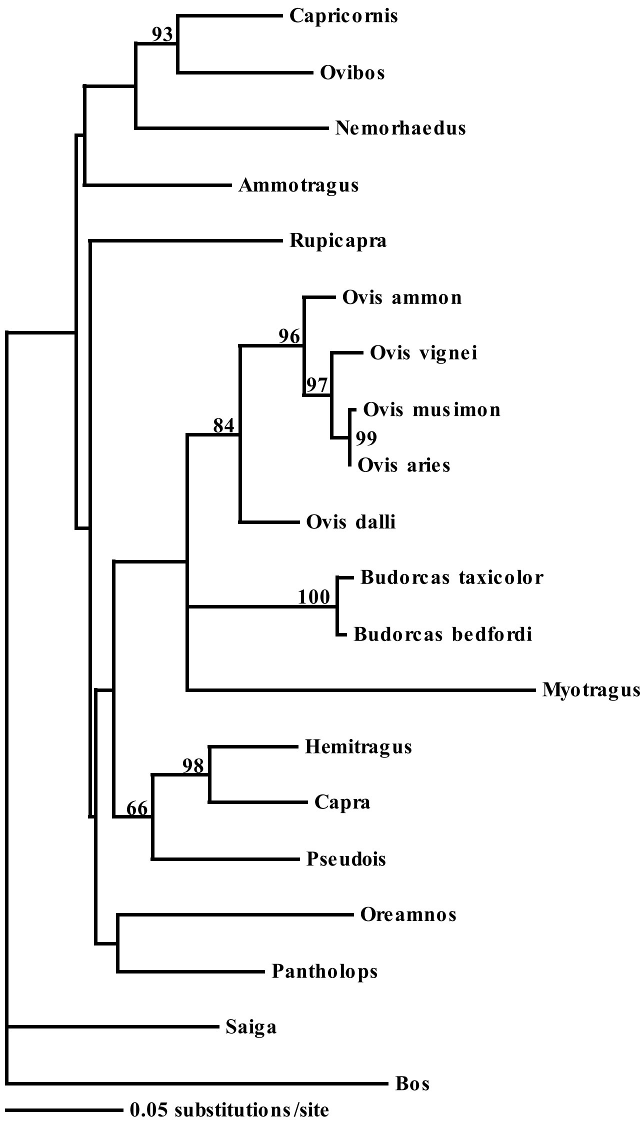

Fig. 4. ML tree generated for *Myotragus* and the extant species of the Caprinae, including all *Budorcas* and *Ovis* species and subspecies and *Bos* as the outgroup, for the entire cyt *b* gene. Nodes with bootstrap support values greater than 50% are shown.

Phylogenetically, it is interesting to note that *Myotragus* does not seem to be a close relative of *Rupicapra*, its closest geographic neighbor (*R. pyrenaica* is distributed along the mountains of Northwestern Spain, the Pyrenees, and the Apennines of central Italy). In contrast, *Myotragus* seems to be closely related to *Budorcas*, a bovid that inhabits some mountainous areas of Nepal, China, and Bhutan, with elevations between 1200 and 3650 m. Therefore, the present-day geographic distribution of the Caprinae does not help to explain their phylogeny. Not surprisingly, traditional phylogenies based predominantly on morphological traits such as body size and horn shape fail to suggest a relationship between *Myotragus* and *Budorcas*, the smallest and one of the largest Caprinae, respectively (takin males can weigh more than 300 kg). As Groves and Shields (1997) point out, body size is expected to have evolved (either increasing or decreasing) independently in separate Caprinae lineages, and is therefore not a useful phylogenetic marker. The present case is even more striking, since dwarfism in *Myotragus* may be linked to its island endemism (Alcover et al., 1981).

Some authors (e.g., Andrews, 1915; Marcus, 1998) have previously described morphological similarities between *Myotragus* and *Budorcas*. These traits include the general shape of several bones: robust and strait femora (slightly slender in *Budorcas*), iliac wings of the pelvis in similar position, similar insertion area in the deltoid crest of the humeri and similar metacarpal proportions. However, whether these morphological similarities are indicative of phylogenetic relationships, or of convergent evolution resulting from isolation in similar ecological habitats remains unanswered.

Our results do not support a Rupicaprine tribe, which has traditionally included *Myotragus*, *Capricornis*, *Nemorhaedus*, *Rupicapra*, and *Oreamnos* (Nowak, 1991). As has been suggested (e.g., Gatesy et al., 1997; Groves and Shields, 1996, 1997; Hassanin and Douzery, 1999) a reclassification of the tribes within the subfamily Caprinae is obviously necessary.

Likelihood ratio tests performed against the molecular clock showed that the *Myotragus* lineage did not evolved in a clock-like manner. The long branch of *Myotragus* with respect to the other Caprinae may be the result of a faster substitution rate in this species. This different rate may be related to an early age of first reproduction and a shorter generation time in *Myotragus* than in other bovids. In mammals, the generation and gestation time are usually related to the body size (see, for instance, Martin and MacLarnon, 1985). *Myotragus* is the smallest Caprine known: although the majority of the specimens would have stood to 45 or 50 cm at the shoulder, adult specimens have been found that reached only 22 cm in height. The ratio of neonate to adult *Myotragus* weight has been estimated to be only 2%, again the smallest ratio ever described in bovids (Bover and Alcover, 1999a).

Although the reproductive strategy and gestation time in *Myotragus* are currently unknown, it seems clear that long-term evolution in complete isolation in the Eastern Balearic Islands not only produced an extreme reduction in body size, but also may have led to differences in other aspects of the animal's natural history. Together with insular dwarf elephants, hippopotami, and deer, *Myotragus* represents one of the best known examples of insular dwarfism (Alcover et al., 1981). From the correlation between generation time and body size recorded in mammals (Martin and MacLarnon, 1985) a reduction both in the age of the first reproduction and in the generation time would seem to be the expected result of such a dwarfism process. These changes provide a testable explanation for the high-substitution rates recorded for *Myotragus* in our study.

Despite notable differences in size, morphological traits, and geographical range between these species, our data strongly suggest a taxonomic relationship between *Myotragus*, *Budorcas*, and *Ovis*. Even with the addition of the *Myotragus* data, however, we were unable to resolve the problematic taxonomic classification of the remainder of the Caprinae. The addition of slowly evolving nuclear genes as well as additional morphological and paleontological evidence will be necessary to solve this longstanding problem.

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