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MITOCHONDRIAL CYTOCHROME *B* SEQUENCE DIVERGENCE AMONG SPANISH, ALPINE AND ABRUZZO CHAMOIS (GENUS *RUPICAPRA*)

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ABSTRACT - We have studied genetic divergence and phylogenetic relationships of Alpine, Spanish and Abruzzo chamois (genus *Rupicapra)* by sequencing a region **of** 330 nucleotides within the mitochondrial DNA cytochrome *b* gene (mtDNA cyt *b*). These sequences were aligned with additional homologous sequences of Caprinae: Japanese serow, Chinese goral, Canadian mountain goat, Mishmi takin, muskox, Sardinian mouflon and domestic goat. Results suggest that, using representatives of the Bovini as outgroups, the Caprinae constitute a monophyletic clade. However, inferred phylogenetic relationships among and within tribes of Caprinae were poorly defined and did not reflect current evolutionary and taxonomical views. In fact, the Asian Rupicaprini goral and serow constituted a strongly supported clade, which included the muskox, while the takin grouped with *Ovis*. Therefore, the monophyly of Ovibovini was not supported by cyt *b* sequences. Species of *Rupicapra* joined a strongly supported monophyletic clade, which was distantly related to the Asian rupicaprins and *Oreamnos*. Therefore, the monophyly of the Rupicaprini was not supported by these cyt *b* sequences. There were sister species relationships within *Rupicapra,* Spanish and Alpine chamois and the Abruzzo chamois (*Rupicapra pyrenaica ornata*) was strictly related to the Spanish chamois *(Rupicapra pyrenaica parva*), as previously suggested by allozyme data and hiogeographic reconstructions.

Key words: Rupicapra pyrenaica parva, Rupicupra pyrenaica or-nata, *Rupicapra rupicapra,* Caprinae, genetic divergence, mitochondrial DNA, cytochrome *b*.

INTRODUCTION

Some aspects of phylogenetic relationships and systematics of the subfamily Caprinae are controversial. Three living tribes (Rupicaprini, Ovibovini and Caprini; Grubb, 1993) are universally recognized, but their monophyly have been repeatedly questioned, after re-evaluation of fossil records (Gentry, 1990; 1992), quantitative morphometrics (Gentry, 1992), biochemical (Hartl et al., 1990; Randi et al., 1991) *and* molecular (Chikuni et al., 1995; Groves and Shields, 1996) findings.

The chamois (genus *Rupicapra)* **is** an evolutionary advanced rupicaprin taxa (Geist, 1987), only distantly related to the other Rupicaprini both from a morphologic (Gentry, 1990) and genetical (Hartl et al., 1990; Randi et al., 1991) point of view. Based on the admittedly poor fossil records, Masini

and Lovari (1988) suggested that the Rupicaprini originated during the Miocene in Asia. *Rupicapra* is supposed to have originated recently in the Villafranchian (2.5 million years ago) and evolved in west Eurasia during middle and late Pleistocene (Geist, 1987; Masini and Lovari, 1988).

Present west European populations of *Rupicapra* have probably relictual distributions, restricted to the Cantabrian mountains and the Pyrenees, the Alpine range, and central Italian Apennines. Recently all the existing populations of chamois were classified in the single polytypic species *Rupicapra rupicapra*, with nine (Dolan, 1963) or ten (Knaus and Schroeder, 1983) subspecies. A taxonomical revision of *Rupicapra* by Lovari and colleagues (Lovari and Scala, 1980; Scala and Lovari, 1984; Nascetti et al., 1985; Masini and Lovari, 1988), recommended the inclusion of the Iberian and Apennine populations in a new species *R. pyrenaica*, with three subspecies: *R. p. pyrenaica* and *R. p. parva* (the Spanish chamois), and *R. p. ornata* (the Abruzzo chamois). A close relationship between Spanish and Abruzzo chamois were suggested by Lovari (1985) based on behavioral observations supported by genetic analyses of allozyme electrophoretic variability (Nascetti et al., 1985).

Although most studies at population level have used mtDNA restriction fragment analyses (Hammer et al., 1995), more detailed information about the genetic consequences of recent speciation events and geographic subdivision of genetic variability among conspecific populations can be obtained by nucleotide sequencing (Wenink et al., 1996). In this paper we aim to compare nucleotide sequences of a portion of the mitochondrial DNA cytochrome *h* gene of *R. rupicapra, R. p. parva* and *R. p. ornata*. DNA sequence divergence among *Rupicapra* will be evaluated within the framework of molecular evolution of the Caprinae.

Material and Methods

DNA Sequencing

Tissue and blood samples were collected from two samples of Spanish (*Rupicapra pyrenaica parva*), Abruzzo *(R. p. ornata)* and Alpine *(R. rupicapra)* chamois. Total DNA was extracted from about 20 mg of minced tissues in 550 ml of lysis buffer (0.05M Tris/HCl, pH 8.0, 0.02M EDTA, 1% SDS, 0.2M NaCl, 1% b-mercaptoethanol), and digested overnight with 0.2 mg/ml proteinase-k. After RNAse treatment, 350 ml of 5M NaCl were added and proteins were pelleted by centrifugation (15,000 rpm, 30 min). Surnatants were cleaned by phenol:chloroform:isoamyl alchool extractions, and DNA was ethanol precipitated and resuspendend in TE. DNA was collected from 100 ml of whole blood, lysed by adding 1 ml of chilled sterile water. Nuclei and white cells were pelleted (15,000 rpm, 5 min) and resuspended in *550* ml of lysis buffer, then DNA was extracted following the same procedure described for tissues.

The entire mtDNA cyt *b* was amplified by polymerase chain reaction (PCR) using primers L1 (14464) = 5' - CGA AGC TTG ATA TGA AAA ACC ATC GTT G - 3', and H1 (15707) = 5' - GTC TTC AGT TTT TGG TTT ACA AGA C - 3', of which 5' ends bind to nucleotides nos. 14,464 and 15,707 of the bovine tRNA-Glu and tRNA-Thr genes (Anderson et al., 1982). Amplifications were performed using a 9600 Perkin Elmer thermocycler, with the following protocol: 94°C for 2 min; 94°C for 15 sec, 50 - 55 °C for 15 sec, 72°C for 1 min (30 cycles), 72°C for 10 min. PCR products were purified by 1.5% low-melting agarose gel and agarase (Boehringer) digestion. All sequences were obtained by double-strand DNA cycle sequencing using DTaq Sequenase (Amersham) and the following primers: L1, L2 (14520) = 5'- AAC ATC CGA AAA TCA CAC CC - 3', L3 (14583) = 5'- CCA TCC AAC ATC TCA GCA TGA TGA AA - 3'. Mitochondrial DNA cyt *h* se-

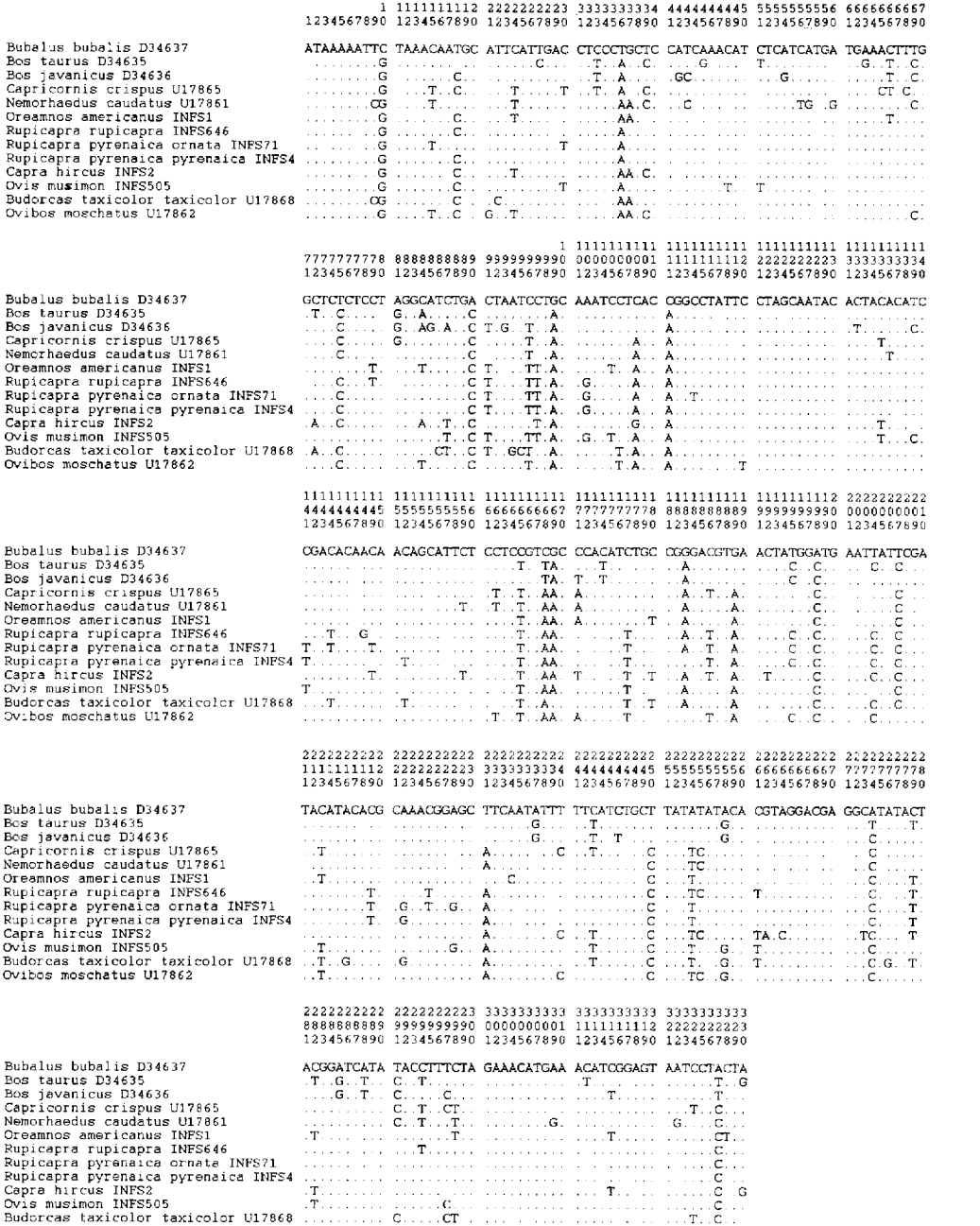

Figure 1. Alignment of 330 nucleotides of mtDNA cyt *b* sequences (L-strand) of 10 taxa of Caprinae and three Bovini. These sequences include the 5' portion of the of cyt *b*, from position no. 14,544 to 14,874. Nucleotide positions are mapped using the bovine sequence published by Anderson *et al.* (1982).

Table 1. Pairwise estimated % DNA distances (Tamura and Nei, 1993) among 13 taxa of the Bovini.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13
1 - *B. bubalis*	00	13	13	15	13	12	12	13	11	16	13	16	16
2 - *B. taurus*		00	10	15	16	16	15	16	15	16	17	19	17
3 - *B. javanicus*			00	15	17	15	17	18	15	20	15	19	16
4 - *C. crispus*				00	8	11	11	12	12	13	11	13	7
5 - *N. caudatus*					00	11	11	12	12	14	15	14	9
6 - *O. americanus*						00	9	11	9	11	9	12	12
7 - *R. rupicapra*							00	4	3	10	9	11	11
8 - *R. p. ornata*								00	3	13	9	12	13
9 - *R. p. parva*									00	11	8	10	11
10 - *C. hircus*										00	12	14	14
11 - *O. musimon*											00	11	15
12 - *B. t. taxicolor*												00	13
13 - *O. moschatus*													00

quences of other taxa of Ruminantia were obtained from the GenBank and used for phylogenetic analyses: cattle *(Bos taurus),* banteng (*B. javanicus*), and water buffalo (*Bubalus bubalis*) (Kikkawa *et al.,* 1995 and unpubl.; GenBank accession nos. D34635, D34636, D34637); Mishmi takin (*Budorcas taxicolor taxicolor),* muskox *(Ovibos moschatus),* Chinese goral *(Nemorhaedus caudatus),* Japanese serow *(Capricornis crisps),* (Groves and Shields, 1996; U17868, U17862, U17861, U17865). Moreover, we have added new cyt *b* sequences of the domestic goat (*Capra hircus),* and Sardinian muflon (*Ovis gmelini).*

Data Analysis

Phylogenetic analyses of the aligned sequences were performed with neighbor-joining (NJ; Saitou and Nei, 1987), and maximum parsimony (MP; Swofford, 1993) procedures using MEGA (Kumar *et al.,* 1993) and PAUP (Swofford, 1993) computer programs. NJ trees were computed using Tamura and Nei's (1993) DNA distances. Parsimony analyses, excluding uninformative substitutions, were performed through heuristic searches with TBR and MULPARS options in use, and with 10 random-additions of sample sequences. Support of clusters and clades was evaluated by bootstrap (Felsenstein, 1985), as a percentage of group recurrence based on 1,000 bootstrapped replications, with both **MEGA** and PAUP.

RESULTS

We sequenced a region of 330 nucleotides of mtDNA cyt *b* gene of Spanish, Abruzzo and Alpine chamois. These sequences were aligned with corresponding sequences of three species of Bovini and other Caprinae (Fig. 1). The Tamura and Nei (1993) pairwise distances among the sequences are shown in Table 1. This distance matrix was used to build the NJ tree (Fig. *2).* The MP tree, obtained by PAUP with heuristic searches randomly repeated 10 times, was identical to the NJ tree (not shown). In both distance and parsimony analyses representatives of the Bovini *(Bostaurus, B. javanicus* and *Bubalus bubalis*) were considered as

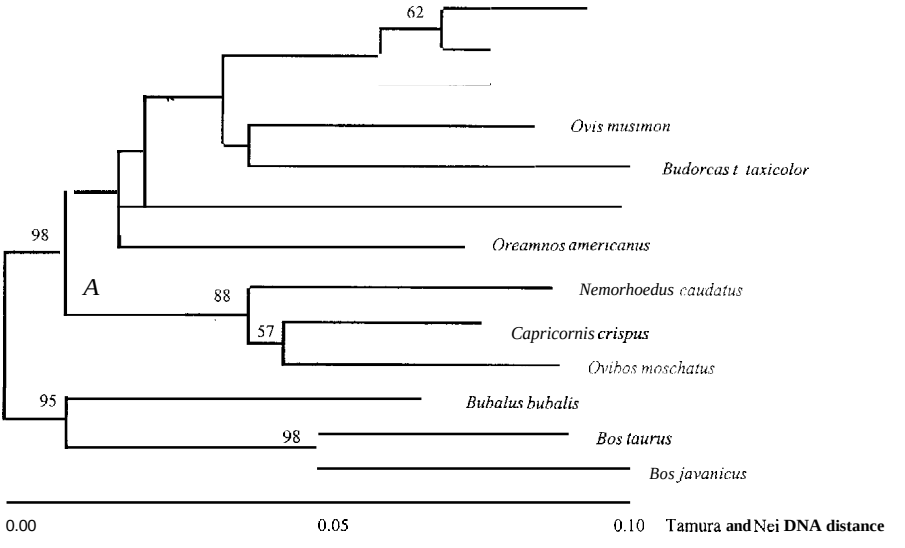

Figure 2. Neighbor-joining tree computed using Tamura and Nei (1993) DNA distances and the cyt *b* sequences of Caprinae showed in Fig. 1. The tree was rooted using three species of Bovini as an outgroup. Branch lengths are indicated and correspond to the estimated percentage nucleotide divergence among taxa. Bootstrap values of the groups which occurred with frequencies greater than 50% after 1,000 bootstrap runs are indicated.

outgroups of the Caprinae, according to morphological (Gentry, 1992) and molecular findings (Allard et al.. 1992; Cronin et al., 1996).

Our results indicate the existence of two clearly defined lineages: a) the Asian Rupicaprini *Capricornis* and *Nemorhaedus*, which group with *Ovibos;* b) a cluster which groups the three *Rupicapra* taxa. *Ovis* is weakly linked to *Budorcas*; *Capra* and *Oreamnos* are intermediate between the Asian Rupicaprini and *Rupicapra*. Bootstrapping supports the clade of the Asian Rupicaprini with *Ovibos* (88%) and the *Rupicapra* clade (95%), but topological relationships of ***Ovis,*** *Budorcas* and *Capra* are weak (Fig. ***2).*** The Spanish and Alpine chamois are sister lineages, and the Abruzzo chamois is strictly related to the *pyrenaica* lineage.

Genetic distance among the Caprinae range from 15% sequence divergence among *Ovis*, *Ovibos* and *Nemorhaedus*, to 3% sequence divergence among *R. p. parva* and *R. p. ornata*. The Spanish and Alpine chamois have about 4% sequence divergence. It should be observed that most of the sequence divergence among *ornata* and *parva* is attributed to the long branch which leads to *ornata* in the NJ tree (Fig. ***2).*** This branch is four times longer than its sister branch leading to *parva* and is the consequence of apparent heterogeneous rate of molecular evolution in the *ornata* lineage.

DISCUSSION

Partial nucleotide sequences of the mtDNA cyt *b* gene suggest that the Asian Rupicaprini *Nemorhaedus* and *Cupricornis*

represent a basal evolutionary lineage among the Caprinae, in accordance with current evolutionary reconstructions and taxonomy (Geist, 1987). The genus *Rupicapra* is monophyletic. It is weakly related to *Ovis, Capra*, and *Or-enmnos,* but is only distantly related to the Asian Rupicaprini. The deeper genetic gap in *Rupicapra* is between the two species *R. rupicapra* and *R. pyrenaica*. The Abruzzo chamois *R. p. ornata* is more strictly related to the Spanish than to the Alpine chamois, as previously suggested by allozyme analyses and biogeographic reconstructions (Nascetti et al., 1985; Masini and Lovari. 1988). Therefore, this mtDNA cyt *b* phylogeny suggests that the Rupicaprini may not be treated as a monophyletic group and, consequently, it cannot be assumed that all living Rupicaprini are ancestors of the Ovibovini and Caprini (Randi *et al*., 1991). The cluster of ancestral Asian Rupicaprini excludes *Oreamnos* and *Rupicapra*.

Morphometrical analyses (Gentry, 1992) showed that *Rupicapra* has a unique combination of skeletal traits and does not group with the other Rupicaprini. but is linked to the Caprini. The monophyly of the Ovibovini tribe is not supported, because *Ovibos* is linked to the Asian Rupicaprini and *Budorcas* to *Ovis* (Groves and Shields, 1996). From a skeletal point of view, the Ovibovini appears as a monophyletic group (Gentry, 1992), probably as a consequence of the evolutionary convergence of morphological traits (Groves and Shields, 1996). The great genetic divergence among *Capra* and *Ovis* could potentially disrupt the monophyly of the Caprini tribe. These results are in accordance with allozyme and other mtDNA sequence data (Hartl et al.. 1990: Randi et al., 1991: Groves and Shields. 1996). Moreover, mitochondrial phylogenies of Caprinae are substantially supported by sequences of autosomal k-casein genes (Chikuni et al., 1995: Cronin et al., 1996).

Molecular evolution of the cyt *b* of Caprinae exhibits heterogeneous substitution rates at different nucleotides, as shown by branches of different lengths in the **NJ** tree (Fig. 2). Heterogenous rates can be due to different substitution rates among evolutionary lineages or to the genetic consequences of population bottlenecks and extended periods of low effective population size. Heterogeneous rates can bias the estimation of genetic distances and the reconstruction **of** phylogenetic trees. In particular, the long branch associated with the *ornata* lineage could be a consequence of the segregation of an ancestral mtDNA haplotype in the Abruzzo population, which was isolated for centuries and underwent a recent dramatic bottleneck (Lovari, 1989). The genetic effects of population bottlenecks were described by Nascetti *et al.* (1995), which found the Abruzzo chamois population to be monomorphic at 25 allozyme loci.

The combined effects of isolation and bottleneck could have fixed an ancestral mtDNA haplotype in the Abruzzo chamois population, a haplotype which could predate the time of reproductive isolation of the population. Mitochondrial DNA polymorphisms were shown by Hammer et *al.* (1995) in some Alpine chamois populations. Biased estimation of inter- and intra-population sequence variability, and the possible retention of an ancestral mtDNA haplotype in the Abruzzo chamois population, make any estimate of divergence time and any taxonomical conclusion from this preliminar data set inadvisable.

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REFERENCES

Allard, M.W., Miyamoto, M.M., Jareki, L., Kraus, F. and Tennant, M.R., 1992. DNA systematics and evolution of the artiodactyl family Bovidae. Proc. Natl. Acad. Sci. USA, 89: 3972-3976.

Anderson, **S.,** De Bruijn, H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, Y.G., 1982. Complete sequence of bovine mitochondrial DNA: Conserved features of the mammalian mitochondrial genome. J. Mol. Biol., 156: 683-717.

Chikuni. K., Mon. Y.. Tabata, T., Saito, M., Monma, M. and Kosugiyama, M.. 1995. Molecular phylogeny based on the kappa-casein and cyt *b* sequences in the mammalian suborder Ruminantia. J. Mol. Evol., 41: 859-866.

Cronin, M.A., Stuart, R., Pierson, B.J. and Patton, J.C., 1996. K-casein gene phylogeny of higher ruminants (Pecora, Artiodactyla). Mol. Phylogenet. Evol.. 6: 295-311.

Dolan, J.M., 1963. Beitrag zur systematischen Gliederung des Tribus *Rupicaprini* Simpson 1945. Zeitschrift fur zoologische Systematik und Evolutionsforschung, 1: 31 1-407.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39: 83-791.

Geist. V.. 1987. On the evolution of the Caprinae. In: **H.** Soma (ed.), The biology and management of *Capricornis* and related mountain antelopes. Croom Helm, London, pp. : 3-40.

Gentry, A.W., 1990. Evolution and dispersal of the African Bovidae. In: G.A. Bubenik and AB. Bubenik (eds.), Horns, pronghorns and antlers: Evolution, morphology, physiology and social significance. Springer-Verlag. New York, pp.: 195-230.

Gentry, A.W., 1992. The subfamilies and tribes of the family Bovidae. Mammal. Rev. 22: 1-32.

Groves. P. and Shields, G.F., 1996. Phylogenetics of the Caprinae based on cytochrome *b* sequences. Mol. Phylogenet. Evol., 5: 467-476.

Grubb, P., 1993. Order Artiodactyla. In: D.E. Wilson and D.M. Reeder (eds.), Mammal species of the world. A taxonomic and geographic reference. Smithsonian Institution Press, Washington and London, pp.: 377-414

Hammer, S., Nadlinger, K. and Hartl, G.B., 1995. Mitochondrial DNA differentiation in chamois (genus *Rupicapra*): implications for taxonomy, conservation and management. Acta Theriologica Suppl., 3: 145-155.

Hartl, G.B., Burger, H., Willing, R. and Suchentrunk, F., 1990. On the biochemical systematics of the Caprini and Rupicaprini. Biochem. Syst. Ecol., 18: 175-182.

Kikkawa, Y., Amano, T. and Suzuki, H.. 1995. Analysis of genetic diversity of domestic cattle in East and Southeast Asia in terms of variations in restriction sites and sequences of mitochondrial DNA. Biochem. Genet., 33: 51-60.

Knaus, W. and Schröder, W., 1983. Das Gamswild. Paul Parey, Hamburg und Berlin.

Kumar. S., Tamura, K. and Nei, M., 1993. MEGA: Molecular Evolutionary Genetic Analysis, Version 1.0. The Pennsylvania State University, University Park, PA 16802.

Lovari, S., 1985. Behavioural repertoire of the Abruzzo Chamois *Rupicapra pyrenaica ornata* (Ncumann, 1899) (Artiodactyla: Bovidae). Saugetierkundliche Mitteilungen, 32: 113-136.

Lovari, S.. 1989. L'evoluzione del camoscio appenninico. Le Scienze, 247: 46-55.

Lovari. S. and Scala, C., 1980. Revision of *Rupicapra* genus. 1. A statistical re-evaluation of Couturier's data on the morphometry of six subspecies of chamois. Boll. Zool., 47: 113-124.

Masini, F. and Lovari. S., 1988. Systematics, phylogenetic relationships, and dispersal of the chamois (*Rupicapra* spp.). Quaternary Research; 30: 339-349.

Nascetti, G., Lovari. S., Lanfranchi, P., Berducou, C., Mattiucci, S., Rossi, L. and Bullini, L., 1985. Revision of *Rupicapra* genus. III. Electrophoretic studies demonstrating species distinction of chamois populations of the Alps from those of the Apennines and Pyrenees. In: S. Lovari (ed.), The biology and management of mountain ungulates. Croon Helm, London, pp.: 57-62.

Randi, E., Fusco, G.. Lorenzini, R., Toso, S. and Tosi, G., 1991. Allozyme divergence and phylogenetic relationships among *Capra*, *Ovis* and *Rupicapra* (Artiodactyla, Bovidae). Heredity, 67: 281-286.

Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Scala, C. and Lovari. S., 1984. Revision of *Rupicapra* genus. II. A skull and horn statistical comparison of *Rupicapra rupicapra oi-nata* and *R. rupicapra pyrenaica*. Boll. Zool.. 51: 285-294.

Swofford, D.L., 1993. PAUP: Phylogenetic Ananlysis Using Parsimony, Version 3.1. Illinois Nat. Hist. Survey, Champaign, Illinois.

Tamura, K. and Nei, M., 1993. Stimation of the number of nucleotidc substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol.. 10: 512-526.

Wenink, P.W., Baker, A.J., Rosner, H.-U. and Tilanus, M.G.J., 1996. Global mitochondrial DNA phylogeography of holarctic breeding dunlins (*Calidris alpina*). Evolution, 50: 318-330.