*Journal of Heredity* 2009:100(1):47–55
doi:10.1093/jhered/esn074
Advance Access publication September 15, 2008

# Cytochrome *b* Phylogeography of Chamois (*Rupicapra* spp.). Population Contractions, Expansions and Hybridizations Governed the Diversification of the Genus

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## Abstract

The chamois provides an excellent model for exploring the effect of historical and evolutionary events on diversification. We investigate cytochrome *b* (*cytb*) sequences in the 10 recognized subspecies of *Rupicapra* classified within 2 species: *Rupicapra pyrenaica*, with the subspecies *parva*, *pyrenaica*, and *ornata*, and *Rupicapra rupicapra*, with *cartusiana*, *rupicapra*, *tatrica*, *carpatica*, *balcanica*, *asiatica*, and *caucasica*. A fragment of 349 bp of the *cytb* was sequenced in 189 individuals. We identified 3 *cytb* lineages: Clade West in Iberia and Western Alps; Clade Central in the Apennines and the Massif of Chartreuse; and Clade East present in populations to the east of the Alps. The 2 proposed species were polyphyletic; the clades West and Central are represented in both, whereas the Clade East is restricted to *R. rupicapra*. In contrast to the current systematic, *cytb* phylogenies suggest the classification of the 10 subspecies of chamois into a single species, *R. rupicapra*. Phylogeny and geographical distribution of the 3 lineages show the effects of limited latitudinal range expansions, contractions, and hybridizations among highly divergent lineages, along with a major role of the glacial ice sheets of the Alps and the Pyrenees as barriers to gene flow, on the diversification of extant taxa.

**Key words:** *chamois, ice ages, mtDNA, phylogeography, Rupicapra, taxonomy*

## Introduction

The study of genetic differentiation among geographical populations of related taxa allows past historical and evolutionary events leading to current phylogeographic structure to be inferred (Avise et al. 1987). An increasing number of studies based on DNA polymorphism provide information about the influence of the Pleistocene glaciations on present species distribution (Avise et al. 1998; Taberlet et al. 1998; Hewitt 2004).

Chamois (genus *Rupicapra*) are mountain ungulates of the subfamily Caprinae, presently distributed over most of the medium to high-altitude mountain ranges of Southern Europe, the Balkans, and the Near East. Paleontological evidence shows that the Rupicaprini originated during the Miocene in Asia and that *Rupicapra* spread to Europe during the middle Pleistocene (Masini and Lovari 1988). There are 10 distinct geographical populations of chamois that have been recognized as subspecies (Couturier 1938; Dolan 1963). The geographical distribution of living chamois is presented in Figure 1. In recent years, geographical populations have been grouped into 2 species on the basis of morphological and behavioral characters, *Rupicapra*

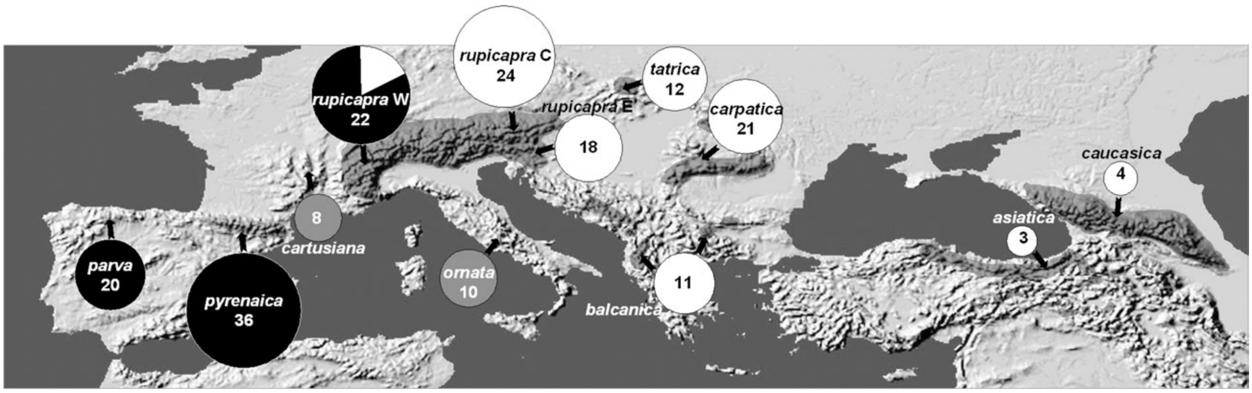

**Figure 1.** Present distribution of chamois, genus *Rupicapra* (based on Masini and Lovari 1988), and sampling locations. The numbers in the pie charts correspond to sample size. The proportion of *cytb* clades, West, Central, and East are represented in black, gray, and white, respectively.

*pyrenaica* (with the subspecies *parva*, *pyrenaica*, and *ornata*) from southwestern Europe and *Rupicapra rupicapra* (with the subspecies *cartusiana*, *rupicapra*, *tatrica*, *carpatica*, *balcanica*, *asiatica*, and *caucasica*) from northeastern Europe (Grubb 1993). Analysis of genetic variation for allozyme loci (Nascetti et al. 1985), minisatellites (Pérez et al. 1996), and restriction fragment length polymorphisms of mitochondrial DNA (Hammer et al. 1995) showed a considerably higher divergence between populations of the 2 proposed species than between populations within the same species. Microsatellite analysis of 8 of the 10 proposed subspecies showed a clear differentiation between every pair of populations and clearly separates 2 groups corresponding to the 2 proposed species of chamois (Pérez et al. 2002). Microsatellite differentiation was ascribed to expansions and contractions within limited geographic regions during Pleistocene glacial–interglacial periods, with the alpine barrier having played a substantial role in West–East differentiation.

The mitochondrial DNA is suitable to study the geographical separation of populations of different geographical origin as well as close related species due to its high substitution rate and its maternal mode of inheritance (Avise et al. 1987). The geographical distribution of separated mtDNA clades allows the study of historical, demographic, and dispersal events. In addition, differentiation between mtDNA sequences can be used to date the separation among phylogenetic groups, assuming a coincidence between the split of the molecular lineages and the speciation event. Comparison of a limited number of mtDNA sequences of the 2 proposed species of chamois lead to estimations of separation time around 1.5 million years (MY) (Lalueza-Fox et al. 2005; Ropiquet and Hassanin 2005; Rodríguez et al. 2007), but the study of microsatellites has given much more recent dates for separation (Pérez et al. 2002).

This paper presents the comparison of a fragment of cytochrome *b* (*cytb*) in populations of chamois across its geographical range. We sequenced a fragment of 349 bp in 189 individuals comprising the 10 recognized subspecies. The data were used to investigate genetic relationships among the proposed species and subspecies of *Rupicapra* and to contribute to understand the influence of Pleistocene glaciations on differentiation.

## Materials and Methods

### Population Samples

A total of 189 specimens, including the 10 recognized subspecies of the genus *Rupicapra*, were collected from locations across its geographical range (see Figure 1). Ninety-seven individuals were the same as the ones genotyped for microsatellites in a previous study (Pérez et al. 2002). In general, samples were either muscle, liver, or skin preserved in 96% ethanol, but 16 out of 21 samples of *carpatica* were teeth, 2 of the 3 samples of *asiatica* were hairs, and the samples of *balcanica*, obtained from accidentally dead or poisoned animals, were diverse (bone, salted skin, or hair).

### DNA Extraction, Amplification, and Sequencing

DNA isolation and sequencing was performed either in the laboratory of Vienna (Austria) or in the laboratory of Oviedo (Spain) using different methods. A shared subset of 33 samples (including low-quality samples) was sequenced in both laboratories.

*Vienna Laboratory ($n$ = 83)*

DNA was extracted following the protocol described in the Genetic Analysis Manual (LI-COR, Inc. 1999). The sample lysated was subjected to standard phenol/chloroform extraction and DNA precipitation procedure (Sambrook et al. 1989). The obtained DNA was utilized in the polymerase chain reaction (PCR) using universal primer set, L14724 5′-CGAAGCTTGATATGAAAAACCATCG-TTG-3′ and H15149 5′-AAACTGCAGCCCCTCAGAAT-GATATTTGTCCTCA-3′ (Kocher et al. 1989; Irwin et al. 1991), to amplify a 424-bp fragment of the mitochondrial genome corresponding to map position 14129–14552 relative to *Capra hircus* (AF533441). PCR was performed on an Eppendorf Mastercycler gradient in a 50-μl reaction mix containing 2.5 mM $MgCl_2$, each dNTP at 200 μM, each primer at 25 pM, 1.25 U of *Taq* polymerase (Promega, Madison, WI), the reaction buffer at 1× concentration supplied by the manufacturer, and <1 μg of the extracted DNA as matrix. After denaturation step of 2 min at 94 °C, 35 PCR cycles with 30 s at 94 °C, 30 s at 50 °C, and 45 s at 74 °C followed by a final extension step of 10 min at 74 °C.

For cloning, the PCR products the pGEM-T Easy cloning kit (Promega) was used following the manufacturer's instructions. The plasmids were isolated with the NucleoSpin Plasmid purification kit (Macherey-Nagel, Dueren, Germany) and cycle sequenced (Epicentre Biotechnologies, Madison, WI) with IRD700 and IRD800 labeled M13 primers (MWG Biotech, Ebersberg, Germany) on a LI-COR 4200 automated sequencer.

### Oviedo Laboratory (n = 106)

DNA from soft tissue was extracted with Chelex, following Estoup et al. (1996). DNA from bone or teeth was extracted from 1-g powdered material following Cattaneo et al. (1995) and purified further with Chelex (Pérez et al. 2002). A 349-bp fragment of the mitochondrial gene encoding *cytb* (map position 14200–14548 relative to *C. hircus*, AF533441) was amplified, using primer pairs (mtCYTB forward 5′-ACCCACTAATAAAAATTGTAAACAACG-3′ and mtCYTB reverse 5′-CAGAATGATATTTGTCCTCATGGT-3′). These specific primers had to be designed to avoid the casual amplification of a nuclear pseudogene that has been previously described (Rodríguez et al. 2007). The PCRs were performed in a final volume of 20 μl containing 2 μl (~ 40–70 ng) of DNA, 0.5 μM of each primer, 1× PCR Buffer, 200 μM of each dNTP, 2.5 mM MgCl2, and 0.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany). Amplification was carried out in a 9700 thermal cycler (Applied Biosystems, Foster City, CA) with an initial step of 3 min at 94 °C, 30 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72° followed by 10 min at 72 °C. PCR products were electrophoresed along with size standards in 2% agarose gel in 1× Tris–borate–EDTA and visualized by UV.

The PCR amplified products were purified with the Exo-SAP-IT kit (USB Corporation, Cleveland, OH) and sequencing reactions were performed with the previous designed primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing products were purified with isopropanol precipitation and sequenced in an ABI 310 Genetic Analyzer (Applied Biosystems). The raw sequence data were analyzed using the ABI Prism DNA Sequencer Analysis software v3.4.1.

## Data Analysis

The mitochondrial *cytb* gene sequences were aligned with the pseudogene Numt*cytb* sequences of chamois (Rodríguez et al. 2007) using the CLUSTALW (Thompson et al. 1994) multiple alignment program of BioEdit (Hall 1999) and the 349-bp fragment common to both laboratories was retained for analysis. All generated haplotypes were submitted to National Center for Biotechnology Information GenBank. Analyses of sequences were executed with MEGA3 software package (Kumar et al. 2004) and DnaSP 4.0 (Rozas et al. 2003). Analysis of differentiation between populations was performed with the software package ARLEQUIN 3.11 (Excoffier et al. 2005). Haplotype diversity ($h$) and nucleotide diversity ($\pi$) were estimated for each sample. Differentiation between populations was investigated by an analysis of molecular variance (AMOVA), which takes into account variation in haplotype frequencies among populations as well as distance between haplotypes. Kimura-2 parameter (Kimura-2P) model of nucleotide substitution was used. The analogous of Wright's (1965) $F$ Statistics were also computed. The significance level of population subdivision was determined by 10 000-fold nonparametric permutation procedure (consisting in permuting haplotypes, individuals, or populations among individuals, populations, or groups of populations) also implemented in the Arlequin software. The evolutionary genetic relationships between subspecies were analyzed with a Neighbor-Joining (NJ) tree computed with PHYLIP 3.67 (Felsenstein 2005) from average number of pairwise differences among populations obtained under the Kimura-2P model of nucleotide substitution.

Distance matrices and phylogenetic relationships between haplotypes were computed with MEGA and PAUP* (Swofford 2000). First, we have chosen not to use sophisticated models of nucleotide substitution for analyzing phylogenies because differences in genetic estimates of distances is low when one is studying closely related sequences. In addition, statistical prediction based on a model with many parameters is subject to more errors than simple ones (Nei and Kumar 2000). A NJ tree based on Kimura-2P distance was constructed with MEGA. The reliability of the nodes was assessed by 1000 bootstrap replicates (Felsenstein 1985). The topology of the tree was further investigated by maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP. The MP analysis was performed using the branch and bound algorithm. ML was performed under the model of nucleotide substitution HKY86 with no among-site rate variation, which was chosen from the results of MODELTEST 3.7 (Posada and Crandall 1998). Trees were rooted with the most common sequence of the *cytb* nuclear pseudogene previously identified (nu*cytb*, GenBank accession number EF158834; Rodríguez et al. 2007). The nuclear pseudogene of *cytb* arose more recently than the *Rupicapra* divergence from *Capra*. In these cases, nuclear mitochondrial pseudogenes (numts) have been proposed to be ideal for rooting a fast-evolving mitochondrial phylogeny because they are relics of ancient mtDNA (Bensasson et al. 2001). They have changed little from the ancestral state of mtDNA sequence initially transposed to the nucleus, given that the nuclear mutation rate is lower than the mitochondrial mutation rate (Bensasson et al. 2001). The 50% majority rule consensus tree was obtained and the reliability of the nodes

**Table 1.** Estimates of diversity for *cytb* sequences of chamois

| Species | Subspecies | Area | Number of genotyped individuals | Number of haplotypes | Number of sites polymorphic | Gene diversity ($h$) ± SD | Nucleotide diversity ($\pi$) ± SD |
|---|---|---|---|---|---|---|---|
| *Rupicapra pyrenaica* | | | | | | | |
| | *parva* | | 20 | 3 | 4 | 0.4158 ± 0.1157 | 0.003209 ± 0.002436 |
| | *pyrenaica* | | 36 | 3 | 2 | 0.4079 ± 0.0862 | 0.001236 ± 0.001268 |
| | *ornata* | | 10 | 2 | 1 | 0.2000 ± 0.1541 | 0.000575 ± 0.000874 |
| *Rupicapra rupicapra* | | | | | | | |
| | *cartusiana* | | 8 | 1 | 0 | 0 | 0 |
| | *rupicapra* | | 64 | 7 | 16 | 0.7584 ± 0.0349 | 0.017333 ± 0.009274 |
| | | W | 22 | 4 | 14 | 0.6970 ± 0.0529 | 0.013757 ± 0.007792 |
| | | C | 24 | 4 | 3 | 0.6920± 0.0554 | 0.002523 ± 0.002041 |
| | | E | 18 | 3 | 2 | 0.5033± 0.1027 | 0.001541 ± 0.001496 |
| | *tatrica* | | 12 | 2 | 1 | 0.4848 ± 0.1059 | 0.001392 ± 0.001446 |
| | *carpatica* | | 21 | 4 | 4 | 0.5857 ± 0.1026 | 0.002384 ± 0.001976 |
| | *balcanica* | | 11 | 1 | 0 | 0 | 0 |
| | *asiatica* | | 3 | 2 | 1 | 0.6667 ± 0.3143 | 0.001916 ± 0.002387 |
| | *caucasica* | | 4 | 2 | 4 | 0.5000 ± 0.2652 | 0.005776 ± 0.004838 |
| Total | | | 189 | 20 | 2.917 | 0.9041 ± 0.0086 | 0.021819 ± 0.011313 |

SD, standard deviation. The data of the subspecies *Rupicapra rupicapra rupicapra* are provided both for the pooled sample and for the individual samples West (W), Central (C), and East (E).

in each of the analysis was assessed by 1000 bootstrap replicates.

Evolutionary relationships between the 20 haplotypes were also analyzed by a Median-Joining network (Bandelt et al. 1999) constructed with NETWORK 4.2 (Fluxus Technology Ltd., Suffolk, UK). This method differs from traditional ones by allowing extant haplotypes to occupy internal nodes. The parameter $\varepsilon$ was set to 0 to obtain the minimum spanning network.

## Results

### *cytb* Diversity

The *cytb* alignment (349 nucleotides, 189 individuals) showed 20 haplotypes. New haplotypes were numbered consecutively as they were first identified. During the experimentation, the numbers 14 and 15 were assigned to haplotypes shown to correspond to a pseudogene later on (Rodríguez et al. 2007). Thus, the numbers of haplotypes in the present study are from 1 to 22, except for the numbers 14 and 15 (GenBank accession numbers EU836150–EU836169). The 33 samples sequenced in both laboratories gave identical results. Haplotypes were defined by 27 polymorphic sites including 27 substitutions of which 23 were transitions and 4 transversions, 21 were synonymous, and 6 nonsynonymous. Haplotype diversity ($h = 0.9041 \pm 0.0086$) as well as nucleotide diversity ($\pi = 0.0218 \pm 0.0113$) and mean number of pairwise differences (7.61 ± 3.57) were high. The *cytb* diversity (Table 1) was higher in chamois from the Alps (*Rupicapra rupicapra rupicapra*) than elsewhere and was extremely low for populations from the Balkans (*Rupicapra rupicapra balcanica*) and Massif of Chartreuse (*Rupicapra rupicapra cartusiana*) and, to a lesser extent, from the Apennines (*Rupicapra pyrenaica ornata*). In particular, the nucleotide diversity was very high in *R. r. rupicapra* sampled from Val di Susa in the western side of the Alps.

Nucleotide differences among the 10 recognized subspecies of chamois account for 76.97% variation. This variation was significantly partitioned using AMOVA (Table 2) into the component between species that accounts for the 44.34% and the component between subspecies within species that accounts for the 32.64% of variation for nucleotide diversity. The variation within subspecies is greatly due to the differentiation between alpine subpopulations that accounts for the 57.34% of the variation within subspecies.

Differentiation between chamois populations occupying the 12 studied areas was analyzed with a NJ tree computed from the average number of pairwise differences. Populations split into 3 clear groups (Figure 2). Chamois sampled from Val di Susa, in the western Alps (*rupicapra*W, in the figure), which are of the species *R. rupicapra* group with the populations from the Cantabrian Mountains (*parva*) and the Pyrenees (*pyrenaica*), both of the species *R. pyrenaica*. The samples from the Apennines (*R. p. ornata*) and the Massif of Chartreuse (*R. r. cartusiana*) form a second group, and the samples from Central and West Alps are very close to

**Table 2.** AMOVA for *cytb* sequence variation (Kimura-2P model) among species and subspecies of the chamois

| Groups | Degrees of freedom | Percentage of variation | Fixation indices | $P$ |
|---|---|---|---|---|
| Among species | 1 | 44.34 | 0.44336 | 0.01662 |
| Among subspecies within species | 8 | 32.64 | 0.58634 | <0.0001 |
| Within subspecies | 179 | 23.03 | 0.76974 | <0.0001 |

Probability values refer to variance components and fixation indices and were generated by comparing observed values against the distribution of 10 000 random permutations.

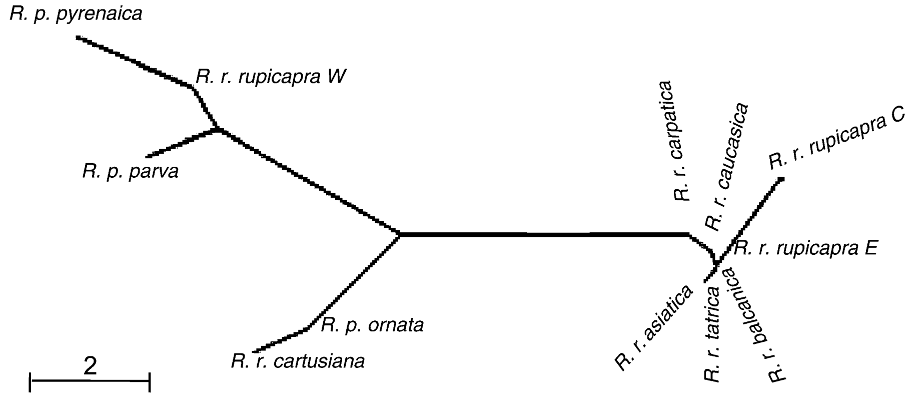

**Figure 2.** Unrooted NJ tree showing relationships between population samples based on average number of pairwise differences obtained under the Kimura-2P model of nucleotide substitution of *cytb* for chamois. Scale bar represents molecular distance as number of pairwise differences.

the populations of eastern Europe and Asia and with them form a group. This group, with low differentiation, includes all the populations of the nominal species *R. rupicapra* but *R. r. cartusiana*. Partition of nucleotide differentiation among these 3 groups using AMOVA explains 77.19% of variation.

## Phylogenetic Relationships among *cytb* Haplotypes

Phylogenetic trees obtained with different methods NJ, MP, or ML), using the sequence of the *cytb* pseudogene (Rodríguez et al. 2007) as outgroup, split the haplotypes in 3 main groups (Figure 3). These 3 clades thereafter named Clade West, Clade Central, and Clade East were similarly revealed

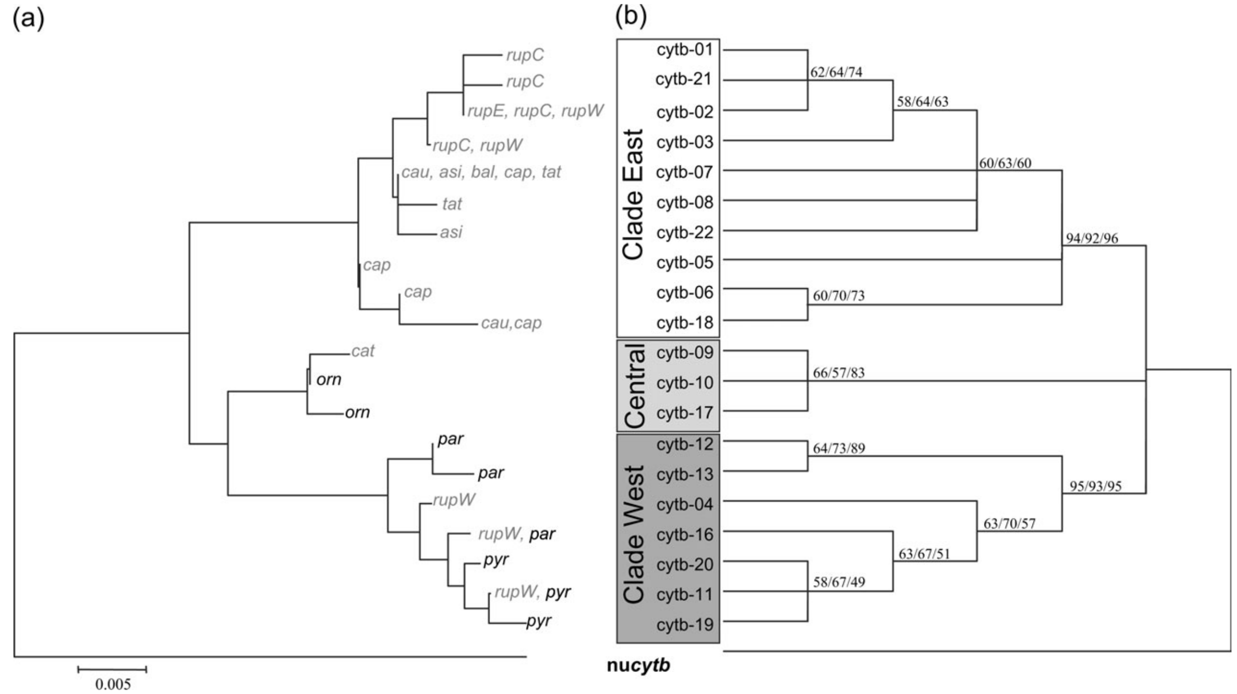

**Figure 3.** (**a**) NJ tree showing phylogenetic relationships between haplotypes obtained from number of substitutions per nucleotide under the Kimura-2P model. (**b**) Consensus topology tree of *cytb* haplotypes showing bootstrap support for MP, ML, and NJ analysis (respectively) at the nodes. The abbreviated name of the populations in which haplotypes were sampled is given in gray for populations of *Rupicapra rupicapra* (*cat*, *cartusiana*; *rupW*, *rupicapra*W; *rupC*, *rupicapra* C; *rupE*, *rupicapra*E; *tat*, *tatrica*; *cap*, *carpatica*; *bal*, *balcanica*; *asi*, *asiatica*; and *cau*, *caucasica*) and in black for populations of *Rupicapra pyrenaica* (*par*, *parva*; *pyr*, *pyrenaica*; and *orn*, *ornata*). Clades of haplotypes are indicated. Scale bar represents substitutions per nucleotide position.

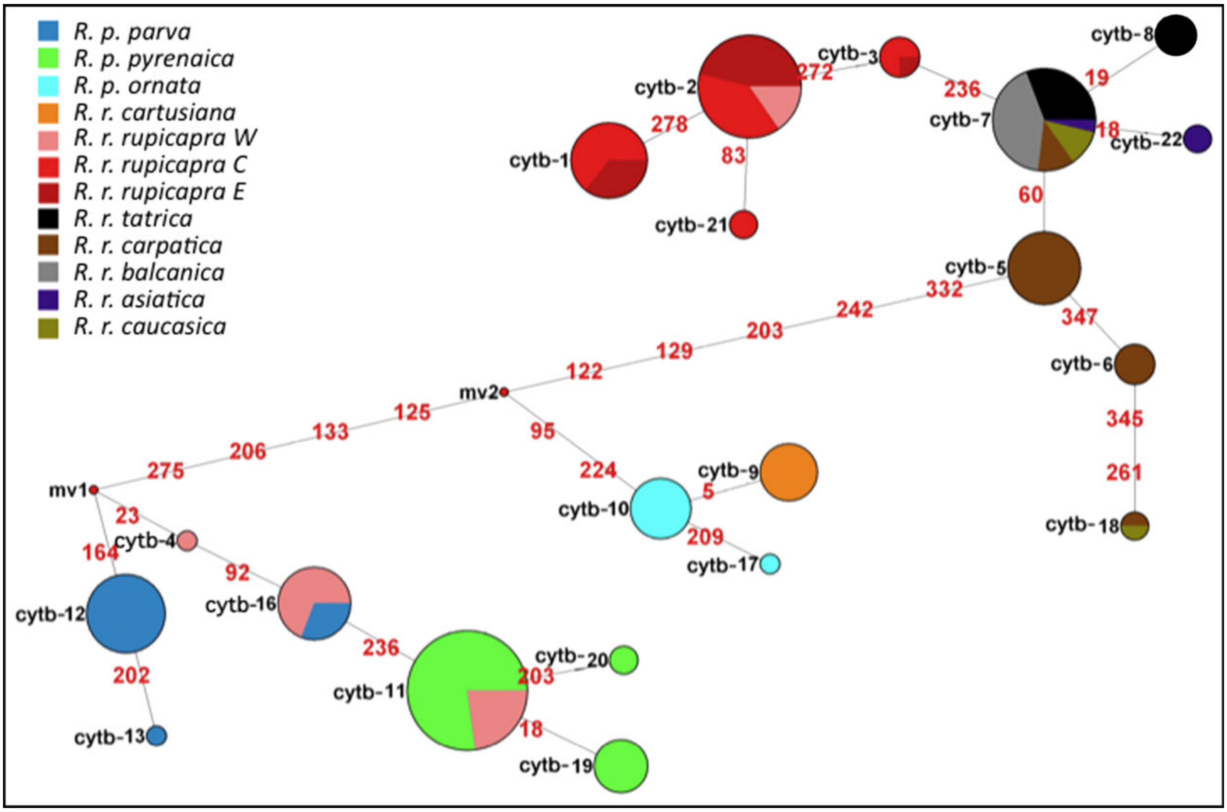

**Figure 4.** Network of *cytb* haplotypes of chamois and their geographic distribution. Pie areas correspond to haplotypic frequencies. The proportions accounted for by the different subspecies are represented in different colors.

by the Network analysis, displayed in Figure 4 that shows, in addition, the geographical distribution and frequency of the *cytb* haplotypes. The distribution of the 3 main clades across Europe is synthesized in Figure 1. The Clade West was represented by 7 haplotypes sampled from the Iberian Peninsula and the West Alps. The Clade Central is only represented by 3 haplotypes sampled from the Apennines and the Massif of Chartreuse. Finally, the Clade East is the most diverse and is represented by 10 haplotypes, sampled from Central Europe, the Balkans, Turkey, and the Caucasus.

Pairwise divergence per nucleotide ($d$) is commonly expressed as a linear function of time ($T$), $d = 2\lambda T$, where $\lambda$ is the absolute rate of substitution expressed as base-pair divergence per year. The time of divergence between the 3 clades of mitochondrial sequences have been tentatively estimated applying the mean evolutionary rate of the *cytb* for the family Bovidae, 0.63% substitutions per MY (Hassanin and Douzery 1999). The resulting time of divergence between the clades West and Central was 2.1 MY and between clades Central and East was 2.3 MY. The estimated divergence between the clades West and East was 3.1 MY. 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.

## Discussion

At present, chamois populations are classified into 2 species *R. pyrenaica* and *R. rupicapra* (Grubb 1993). Nevertheless, the taxonomy of the genus has been object of continuous revisions. Camerano (1914) distinguished the species *Rupicapra ornata* besides the 2 currently accepted ones. Later on, the 10 accepted subspecies were considered within a single species by Couturier (1938) and Dolan (1963) and more recently they have been grouped into 2 species *R. pyrenaica* and *R. rupicapra* (Nascetti et al. 1985).

Microsatellite analysis of 8 of the 10 proposed subspecies clearly separate 2 groups corresponding to the 2 recognized species of chamois (Pérez et al. 2002), offering some support for this classification, but obviously, information on additional markers was needed. Mitochondrial DNA data provide information about phylogeny that is frequently used to diagnose species using the phylogenetic species concept (PSC). The evolutionary significant units, essentially equivalent to species under the PSC (Vogler and DeSalle 1994), have been defined as groups of individuals reciprocally monophyletic for mtDNA alleles and also differing significantly for the frequency of alleles at nuclear loci (Moritz 1994).

Although based in a short sequence, we show here that the 2 proposed species of *Rupicapra*, *R. pyrenaica* and

*R. rupicapra*, were polyphyletic for *cytb*, and the clades West and Central are represented in both proposed species, whereas the Clade East is restricted to *R. rupicapra*. It is interesting that *R. r. cartusiana* groups with *R. p. ornata*. The subspecies cartusiana was not characterized for microsatellites but has been found to differ from several *R. r. rupicapra* populations on the basis of electrophoretic enzymatic variation (Pemberton et al. 1989). Additional analyses are needed to clarify the relationships between these 2 subspecies. The population from the West Italian Alps (*R. r. rupicapra*W) is polyphyletic with 1 haplotype (cytb-02) belonging to the Clade East and 2 haplotypes (cytb-11 and cytb-16) belonging to the Clade West. The *cytb* Clade West was identified in 16 individual alpine specimens that were otherwise shown to cluster together with the populations from the East Alps, the Tatra, the Carpathians, the Balkans, and the Caucasus for microsatellite markers (Pérez et al. 2002). This provides evidence of past hybridization between the 2 proposed species. In conclusion, in contrast to the current classification of the genus *Rupicapra* into 2 species, *cytb* phylogenies suggest to classify the 10 subspecies of chamois into a single species, *R. rupicapra*, as considered in Couturier (1938) and Dolan (1963).

Overall, the genus *Rupicapra* exhibits levels of diversity comparable to that found in other genera of Artiodactyla in Europe (Randi et al. 1998, 2004; Hundertmark et al. 2002). The subspecies *ornata* and *cartusiana*, which were classified, respectively, as vulnerable and endangered (Caprinae Specialist Group 2006), showed very low diversities, what could be attributed to their reduced population size. The subspecies *ornata* was nearly extinct early in the 20th century and in the late 1940s (Lovari 1985) and recovered to 800 animals by 2003 (Boitani et al. 2003). The subspecies *cartusiana* lives in the isolated mountain system of Chartreuse at the western edge of the French Alps. The population passed by the critical size of about 200 individuals in 1980 and recovered to about 770 individuals by 1997 (Roucher 1999). In the opposite side, the subspecies *rupicapra*, represented nowadays by many thousands of individuals, has very high levels of diversity. The great deal of diversity at the nucleotide level in this population can be attributed to admixture of different lineages colonizing the Alps after glacial ages as has been suggested (Schaschl et al. 2003).

Following Masini and Lovari (1988), the chamois, or its direct ancestor, may have reached the European region as a late immigrant during the early or middle Pleistocene, probably from southwest Asia. The first fossils of *Rupicapra* are recorded from the middle Pleistocene of France, together with *Hemitragus* and *Ovis*, and a few remains of Rissian age have been found in the Pyrenees, the Italian Alps, the Apennines, and Hungary. The distribution of present-day *cytb* haplotypes shows an East–West differentiation compatible with a westward dispersion along the mountain chains of the Alpine system. The identification of an older mitochondrial lineage as a nuclear pseudogene in both recognized species was interpreted as evidence of an old hybridization between 2 highly differentiated populations in the origin of present chamois (Rodríguez et al. 2007). The finding of this, now extinct, mitochondrial lineage suggests that there were at least 2 waves of immigration of chamois precursors into Europe. The genetic divergence (7%) between the lineage fossilized in the nucleus and the one in the mitochondria of present-day chamois placed the split long before the separation of the present-day clades.

The distribution of live *cytb* lineages can be related to isolation of populations during the glaciations and to recolonization routes. The ice sheets in the Alps and the Pyrenees during glacial maxima must have constituted barriers that greatly limited the contact between populations already showing a pattern of isolation by distance previous to the main glacial periods. The Clade West was presumably isolated to the south of the Pyrenees in the Iberian Peninsula; the Clade Central must correspond to the isolation of chamois most likely between the Pyrenees and the Alps; and the Clade East should have been isolated to the east of the Alps. The present-day distribution of the main clades is likely a reflection of isolation of populations already differentiated, followed by postglacial recolonization that was mainly limited by the Alpine barrier. The Clade West in all probability recolonized the West Alps and met there with the lineage coming from the East that occupied most of the Alps. The populations that constitute the Clade Central either were isolated because the glacial maxima or the population to the South recolonized the western Alps and was again split later on.

It is interesting to compare these data with the structure obtained from the analysis of microsatellite variation over the same samples (Pérez et al. 2002). In that case, genetic distances between pairs of populations depended on geographical distance denoting a genetic flow among contiguous populations and only the barrier of the Alps could be noted. The somewhat contrasting pictures offered by the 2 kinds of markers can be related to their different mode of evolution. Microsatellite markers narrate the recent phylogenetic history of tens or thousands of years, whereas mitochondrial ones shed light on the deeper phylogenetic history (Emerson and Hewitt 2005). The extent of *cytb* difference suggests that the 3 main clades diverged for some 1–3 MY, that is, prior to the beginning of the strong climatic oscillations of the Quaternary. These data concur with other studies on comparative phylogeography in Europe (Taberlet et al. 1998) in that the divergence between lineages can be explained in the context of divergence among the 3 areas (subsequently refuges) by isolation by distance. The subsequent range contractions and expansions due to climatic oscillations may have eliminated haplotypes present in glacial areas. The posterior postglacial recolonization shows a suture zone in the Alps where the differentiated clades West and East meet and hybridize.

The data presented here suggests that historical events of population contractions, expansions, and hybridizations among highly divergent lineages marked the diversification of the genus *Rupicapra*.

## Funding

Ministerio de Educación y Ciencia (CGL2004-06010) and the Theodor-Koerner-Research Award 2003 (to S.H.).

## Acknowledgments

Anita Haiden (Vienna) and Ana Corao (Oviedo) provided excellent technical assistance. For collaboration and help in collecting chamois samples, the authors are indebted to the following institutions: the Regional Governments of Principado de Asturias (Consejeria de Agricultura); Aragón (Diputación General de Aragón); the hunting wardens from Asturias and Aragón; Camino Real Hunting; as well as the following people: Javier Pérez-Barbería, Carlos Nores, L. Rossi, Juan Carlos del Campo, Juan Bejar, Paloma Barracina, H. Papaioannou, M. Brown, Dr W. Gasienica-Byrcyn, Dr T. Skalski, J. Meana, S. Erceg, P. Veinberg, J. Badridge, Alvaro Mazón, Michal Adamec, Richard Kraft, Juan Herrero, Athanassios I. Sfougaris, and Friederike Spitzenberger. We thank Sara de Albornoz for the correction and improvement of the article's English usage. We are also indebted to 3 anonymous referees for valuable suggestions they made to improve the manuscript.

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**Received March 13, 2008**
**Accepted August 18, 2008**

**Corresponding Editor: Jose Lopez**