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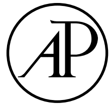

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Phylogeography of chamois (*Rupicapra* spp.) inferred from microsatellites

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Abstract

Evolutionary relationships among populations of chamois (*Rupicapra* spp.) across their current range from the Caucasus to the Cantabrian Mountains were investigated. The allelic variation in 23 microsatellite loci was assessed in eight geographical populations, recognised as subspecies of the two closely related species *R. pyrenaica* and *R. rupicapra*. Analysis of variance in allele frequencies (*Fst*, statistics) and in repeat numbers (*Rst*, statistics) showed these data to be highly structured. Two genetic distances between pairs of populations, *Ds* and $(\delta\mu)^2$, were computed and phylogenetic trees were constructed. Similar patterns were produced by the different statistics. All trees indicate a deep divergence between the two recognised species, which is compatible with archaeological data that place their split in the Riss–Würm interglacial period. Genetic distances between pairs of populations are highly correlated with geographical distance. This suggests that the history of the genus during Pleistocene glacial-interglacial periods was dominated by expansions and contractions within limited geographic regions, leading to alternate contact and isolation of contiguous populations. In addition, the alpine barrier has played a substantial role in West–East differentiation. © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

Study of the 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 species expansions and contractions (Avise et al., 1998; Hewitt, 1996; Taberlet et al., 1998).

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 recognised as subspecies (Couturier, 1938 and Dolan, 1963; cited in Masini and Lovari, 1988). The geographical distribution of living chamois, as well as population sizes given by Masini and Lovari (1988), presented in Fig. 1. Today, at least three subspecies have been drastically reduced in size: there are about 2000 individuals of *R. r. caucasica* (Jason Badridge, pers. comm.); the populations of *R. r. balcanica* from Greece and Bulgaria have declined severely over the last few years (Haritakis Papaioannou and Michael Brown, pers. comm.) and the population of *R. r. tatrica* has been reduced to about 200 individuals (Wojciech Gasienica Byrcyn, pers. comm.). In recent years, geographical populations have been grouped into two species on the basis of morphological and behavioural characters, *Rupicapra pyrenaica* (with the subspecies *parva*, *pyrenaica*, and *ornata*) from south-western Europe and *R. rupicapra* (with the subspecies *cartusiana*, *rupicapra*, *tatrica*, *carpatica*, *balcanica*, *asiatica*, and *caucasica*) from north-eastern Europe. Analysis of genetic variation of a limited number of populations for allozyme loci (Nascetti et al., 1985) and RFLPs of mitochondrial DNA (Hammer et al., 1995) showed a considerably higher divergence between populations of the two proposed species than between populations within the same species. This was interpreted as support for the two species distinction.

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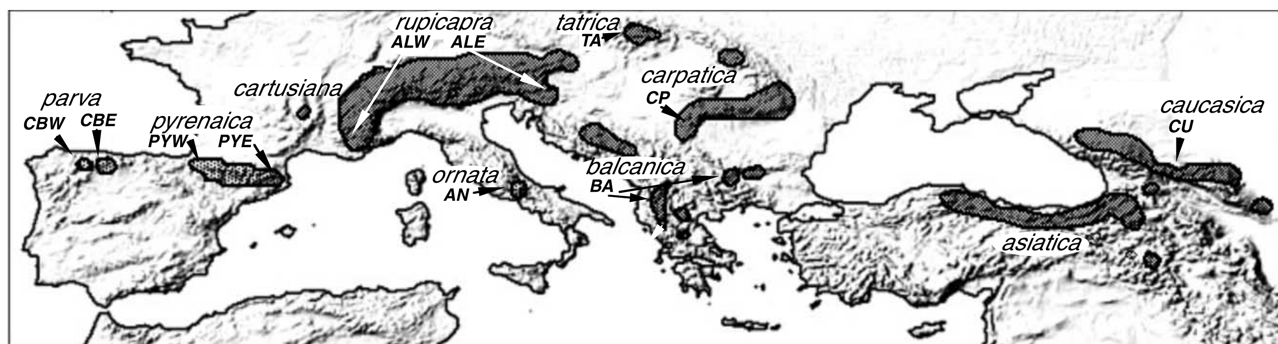

Rupicapra pyrenaica		*Rupicapra rupicapra*			
parva	(6.000)	*cartusiana*	(100)	*balcanica*	(25.000)
pyrenaica	(25.000)	*rupicapra*	(450.000)	*caucasica*	(13.000)
ornata	(350)	*tatrica*	(900)	*asiatica*	(unknown)
		carpatica	(2.500)		

Fig. 1. Present distribution and population sizes of the genus *Rupicapra* (based on Masini and Lovari, 1988). Sampled locations are marked with an arrowhead and labelled with the abbreviations used throughout.

Microsatellites have been found to be very useful in the study of phylogenetic relationships among populations within species or between closely related species (Bowcock et al., 1994; Forbes et al., 1995; Goldstein et al., 1995; MacHugh et al., 1997; Paetkau et al., 1997; Polziehn et al., 2000; Richard and Thorpe, 2001). In this paper, we use microsatellite polymorphisms to investigate the genetic variation in chamois across its geographical range. We screened 25 bovine and caprine microsatellite loci, amplifying a specific product in chamois (Pérez et al., 2000) in 145 individuals comprising eight different subspecies. The data were used to quantify levels of genetic variability within local populations as well as to investigate genetic relationships among the proposed species and subspecies to gain insights into *Rupicapra* phylogeny. The results were related to the fossil record and the influence of Pleistocene glaciations on the population dynamics.

2. Materials and methods

2.1. Population samples

Our objective was to collect at least 20 samples from each location across the geographical range of the genus *Rupicapra*. The protected status of most populations, together with other practical difficulties, meant that we were unable to obtain any samples from the massif of Chartreuse (*R. r. cartusiana*) or from Turkey (*R. r. asiatica*) and that we obtained only a small number of samples from some other populations. It has been shown that increasing the number of loci has a larger and more important effect on the sampling variance than increasing the sample size (Shriver et al., 1995; Takezaki and Nei, 1996). Therefore, the study was based on a large number of loci to counterbalance the small number of samples of some populations. Samples were collected from 1995 to 2001. For large populations, where hunting is allowed, samples were of either muscle or skin preserved in 96% ethanol by gamekeepers or teeth from skulls sent to taxidermists. For protected populations, samples were obtained from accidentally dead or poisoned animals; tissues as well as their conservation method were diverse (bone, salted skin, muscle in ethanol, and muscle in formalin) and were sent by biologists. A total of 145 samples were collected from 11 locations (Fig. 1).

2.2. Laboratory analysis

Two methods were used to isolate DNA for amplification. DNA from soft tissue was extracted with Chelex, following Estoup et al. (1996). DNA from bone or teeth was extracted from 1 g powered material following Cattaneo et al. (1995) and purified further with Chelex. After DNA precipitation, the pellet was resuspended in 250 μl sterile water and 50 μl was transferred to a new tube and 450 μl Chelex 10% was added. The mixture was incubated at 60 °C for 2 h. After testing the quality and quantity of the DNA in a minigel, 2–5 μl appropriate dilution (1/10–1/40) was used to perform each PCR reaction.

The microsatellite markers used in this study included 14 caprine and 11 bovine loci, amplifying specific products in chamois. PCR conditions were as described in Pérez et al. (2000). PCR products were electrophoresed in 6% denaturing polyacrylamide gels and visual-

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ised by silver staining (Promega). Sequencing reactions of pUC18 (silver sequence DNA sequencing system, Promega) were used as standard markers to assign the allele size. For loci with many alleles, additional gels, in which individuals were ordered according to their previously determined allele size, were run to check further genotypes.

2.3. Statistical analysis

Multilocus individual genotypes were arranged in a matrix of 25 loci per 145 individuals. For three individuals (1 from Tatra, 1 from the Balkans, and 1 from the Caucasus), multilocus genotypes were incomplete because some loci could not be amplified.
Weinberg equilibrium for each locus and for each geographic area, as well as the test of disequilibrium for pairs of loci, was performed using GENEPOP on the web (http://wbiomed.curtin.edu.au/genepop/; Raymond and Rousset, 1995). The sequential Bonferroni procedure was applied to correct the significance level for multiple comparisons (Sokal and Rohlf, 1995). In each population, every locus was tested for departure from Hardy–Weinberg by the "exact HW test" (Weir, 1996). The algorithm used to estimate the exact *P* value was a Markov-chain method, with the defaults recommended by the authors. Global tests across loci for each population or across populations for each locus were constructed using Fisher's method. Linkage disequilibrium was tested for all possible pairs of loci in each population and globally for each pair of loci across populations. Observed and expected heterozygosities were also calculated with GENEPOP.

Differences in the extent of genetic variation between studied areas were tested by an ANOVA of the number of heterozygous loci per individual, a variable indicating heterozygosity (Weir, 1996). Non-HW loci were excluded; therefore, the values were based on 23 loci. Comparisons among all possible pairs of samples were carried out using Student's *t* test and the significance level was corrected by the sequential test of Bonferroni.

The allele-sharing distance between every pair of individuals (Bowcock et al., 1994) was calculated using the calculator at http://www.biology.ualberta.ca/jbrzusto/, and a neighbour-joining tree (Saitou and Nei, 1987) was constructed from the resulting distance matrix.

The genetic structure of the populations was analysed by both Wright's F-statistics (Weir, 1996), based on differences in allele frequencies, and by Rst-statistics (Slatkin, 1995), based on differences in the allele size. *Rst* is more appropriate for studying the levels of genetic variation under the stepwise mutation models thought to apply to microsatellites. The proportion of genetic variation, both in allele frequencies or in allele sizes (without standardisation), accounted for by the different phylogenetic levels was analysed by a hierarchical analysis of variance (Weir, 1996) with the aid of the FSTAT 2.9.1 (Goudet, 2000). Bootstrapping over loci was used to obtain confidence intervals for values of *Fst* (15,000 bootstraps).

Recent studies have tested the performance of different genetic distance measures in resolving the evolutionary relations of closely related populations or species from microsatellite data (Paetkau et al., 1997; Takezaki and Nei, 1996). The results have shown that Nei's standard distance, *Ds* (Nei, 1972) and $(\delta\mu)^2$, specifically developed for microsatellite loci, (Goldstein et al., 1995) performed well. *Ds* is more appropriate for studying the fine-scale population differentiation, while $(\delta\mu)^2$ is better for resolving the relationships among very distinct populations and closely related species and for estimating evolutionary times. Consequently, we calculated both distances, *Ds* and $(\delta\mu)^2$. The GENDIST program in PHYLIP 3.5c (Felsestein, 1993) was used to obtain *Ds*, while MICROSAT (Minch, 1995) was used to calculate $(\delta\mu)^2$. UPGMA and Neighbour-Joining trees were produced with the NEIGHBOR program from PHYLIP 3.5c. Bootstrapping gene frequencies over loci were achieved with SEQBOOT from PHYLIP 3.5c for *Ds* and with MICROSAT for $(\delta\mu)^2$. These multiple data sets were used to obtain consensus trees with the CONSENSE program, in PHYLIP 3.5c. Three diagrams were obtained with TreeViewPPC 1.6 (Page, 2000). Distances were based on 20 loci; two loci in broad HW disequilibrium (see Section 3) and three monomorphic loci were excluded.

The relation between genetic and geographical distances was analysed by regression of $(\delta\mu)^2$ on the distance, in hundreds of kilometres, between pairs of studied areas. The significance of the association was tested by a Mantel test performed with GENEPOP on the web. This program computes significance by determining the distribution of the Spearman Rank correlation coefficient under the null hypothesis of independence and comparing the observed value with this distribution. We used 100,000 permutations to determine the rejection zone of the correlation.

3. Results

3.1. Within-population data

The number of heterozygotes for loci SR-CRSP07 and INRA040 had already been shown to be much lower than expected in the Cantabrian population (Pérez et al., 2000). Further analysis of these two loci showed that SR-CRSP07 may be X-linked; no male, out of 33, showed more than one allele, while 17 out of the 49 known females were heterozygotes. Locus INRA040 has non-amplifying alleles, the Cantabrian population showed a deficit of heterozygotes but amplification products were obtained for all the individuals analysed. However, it was impos-

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sible to amplify INRA040 from two individuals from the Pyrenees, from three individuals from the Alps or from any of the 17 individuals from the Carpathians. We conclude that INRA040 has one or more non-amplifying alleles that are highly frequent in the species *R. rupicapra*. These two loci were excluded from posterior analysis. Another three loci, SR-CRSP02, BM1824, and ILSTS008, were monomorphic.

Deviations from Hardy–Weinberg equilibrium were tested for every combination of locus per study area showing polymorphism and with a sample size higher than 5. Significant deviations were observed for one, CP-SR-CRSP13, out of the 146 populations per loci combinations tested and for two loci, SR-CRSP14 and SR-CRSP15, when global tests across populations were performed ($\alpha = 0.05$). In the three cases, there was heterozygote deficiency that could arise due to non-amplifying alleles or to population subdivision. In the case of SR-CRSP14, non-amplifying alleles may be the most plausible explanation because there is a deficit of heterozygotes in populations that are in clear equilibrium for other loci. The other two are probably due to population subdivision. Global tests across loci show a deficit of heterozygotes for populations CP and BA (Table 1). Disequilibrium between pairs of loci was non-significant in every comparison.

In total, 179 alleles were detected across the 23 loci and the 11 study areas. This gives a mean number of alleles per locus of 7.78. The mean number of alleles per locus in each population is 3.22. This mean is biased downwards by the populations with very small sample sizes. The total number of alleles in the two species was 132 for *R. pyrenaica* and 148 for *R. rupicapra*.

Observed heterozygosities were, in general, slightly lower than expected, indicating a general excess of homozygotes, which in the Carpathians and the Balkans was significant ($P < 0.001$). An analysis of variance of individual observed heterozygosities revealed differences between populations ($F_{10,131} = 9.88$; $P = 3.4 \times 10^{-12}$). Populations from the Alps, the West-Pyrenees, and the East-Cantabrian Mountains are significantly more variable ($\alpha = 0.05$) than populations from the Apennines, the Carpathians, the Caucasus, and the West-Cantabrian Mountains. Differences between the pairs AN–TA, AN–PYE, AN–CBW, PYE–ALW, and ALW–BA were also significant.

3.2. Among-population data

A neighbour-joining tree of 142 individuals (3 individuals with incomplete multilocus genotypes were excluded) based on allele sharing (Fig. 2) shows striking differences between populations pertaining to different species. The individual from the Apennines and one of the individuals from the Balkans do not group so closely with their specific group.

Species-private alleles, 30 for *R. pyrenaica* and 47 for *R. rupicapra*, were found in 17 loci (Fig. 3). Of these, only one locus, SR-CRSP06, can be considered diagnostic, six alleles were found in *R. pyrenaica*, ranging in size between 143 and 153 base pairs, while *R. rupicapra* has only two alleles of sizes 139 and 141 bp. Among the species-private alleles, 33 were also population specific (Table 1). It may be noted that the population of the Apennines, with only one sampled individual, showed two private alleles.

Analysis of variance indicates a significant structure of the data. The percentages of the genetic variance accounted for by differences between species, between study areas, and within study areas were 29.85, 11.78, and 58.37 for frequencies and 44.90, 9.08, and 46.02 for allele sizes, respectively. Every pairwise comparison of genetic differentiation between studied areas was significant ($\alpha = 0.05$, data not shown).

Pairwise genetic distances, *Ds* and $(\delta\mu)^2$ (Table 2), were highly correlated ($r = 0.92$). For distantly related pairs of populations, $(\delta\mu)^2$ increases more than *Ds*.

Table 1
Descriptive statistics for each population over all loci

Study area	Abbreviation	*n*	LP	*A*	PA	*P*-HW	He	Ho
Cantabrian-West	CBW	19.00	0.68	3.00	2	0.1856	37.91	36.16
Cantabrian-East	CBE	21.00	0.76	3.72		0.3800	44.92	45.13
Pyrenees-West	PYW	24.00	0.76	4.44	12	0.1069	44.40	44.02
Pyrenees-East	PYE	17.00	0.76	4.16		0.2278	43.01	39.13
Apennines	AN	1.00	0.08	1.08	2	–	–	8.70
Alps-West	ALW	18.00	0.83	4.08	2	0.3373	50.77	47.58
Alps-East	ALE	11.00	0.83	3.71		0.1143	48.15	46.25
Tatra	TA	2.91	0.79	2.29	1	0.7848	46.96	42.75
Carpathians	CP	17.00	0.79	3.04	3	0.0007	37.78	30.69
Balkans	BA	6.78	0.74	3.09	5	0.0005	48.76	35.61
Caucasus	CU	6.43	0.58	2.83	6	0.1733	37.74	32.09

n, mean number of individuals typed per locus (it equals the number of individuals sampled in each population, except for the samples TA, BA, and CU for which some amplifications failed due to bad quality of the DNA sample); LP, proportion of polymorphic loci; *A*, mean number of alleles per locus; PA, number of private alleles; *P*-HW, exact *P* value associated with the Hardy–Weinberg equilibrium; He, expected heterozygosity in %; Ho, observed heterozygosity in %.

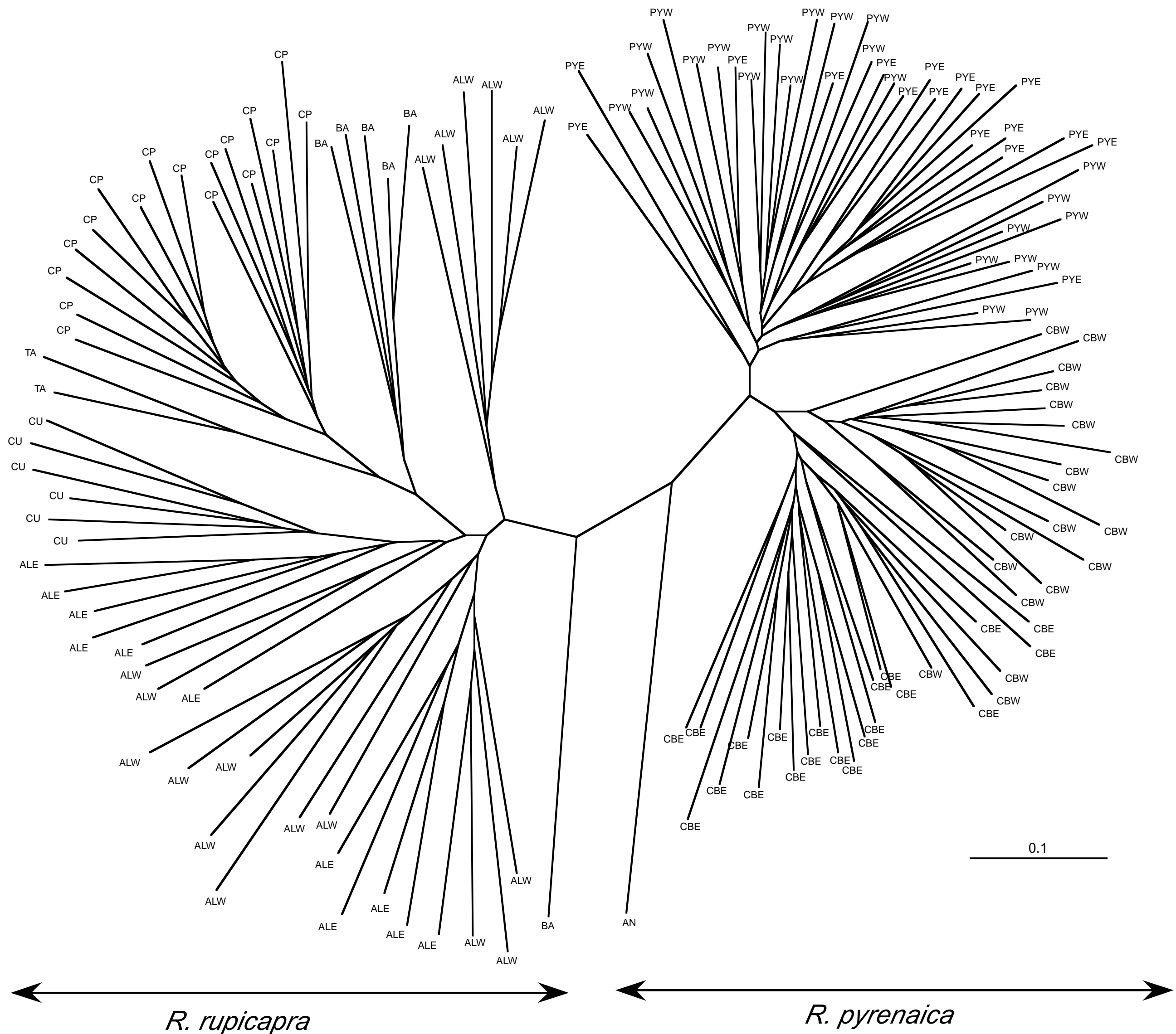

Fig. 2. Neighbour-joining tree of individuals based on allele-sharing distance for multilocus genotypes.

Neighbour-joining trees (Fig. 4) were constructed from matrixes of both genetic distances, *Ds* and $(\delta\mu)^2$. Populations group into two main clusters representing the two proposed species *R. pyrenaica* and *R. rupicapra*. There is close agreement between trees based on *Ds* or $(\delta\mu)^2$ that only differ in the branches of the Caucasica population. In the tree based on $(\delta\mu)^2$, the populations from the Alps, the Carpathians, and the Tatra, group close together and the Caucasica population is more distantly related than in the tree based on *Ds*.

The neighbour-joining tree of $(\delta\mu)^2$ distance recapitulates the geographical distribution areas of chamois. Pairwise $(\delta\mu)^2$ distances were represented against estimated geographical distances between pairs of study areas (Fig. 5). There is a clear correlation ($r = 0.66$) between both measures ($P = 0.00034$, one-tailed Mantel test). It can be observed that the relationships between genetic and geographical distances (the regression coefficients) are equal whether at the level of pairs of populations within species ($b = 0.15 \pm 0.06$) or in pairs of populations between species ($b = 0.17 \pm 0.03$). The intercepts of the two regression lines are 1.59 ± 0.80 for pairs of study areas within species and 6.93 ± 0.69 for pairs between species (t Student $= 5.07$, df $= 51$, $P = 2.8 \times 10^{-6}$). Therefore, mean genetic distances adjusted by geographical distance are larger for interspecific comparisons.

4. Discussion

For the 23 autosomal standard microsatellite loci assayed (Pérez et al., 2000), 20 were polymorphic in the genus *Rupicapra*, with a mean of 8.80 alleles per locus.

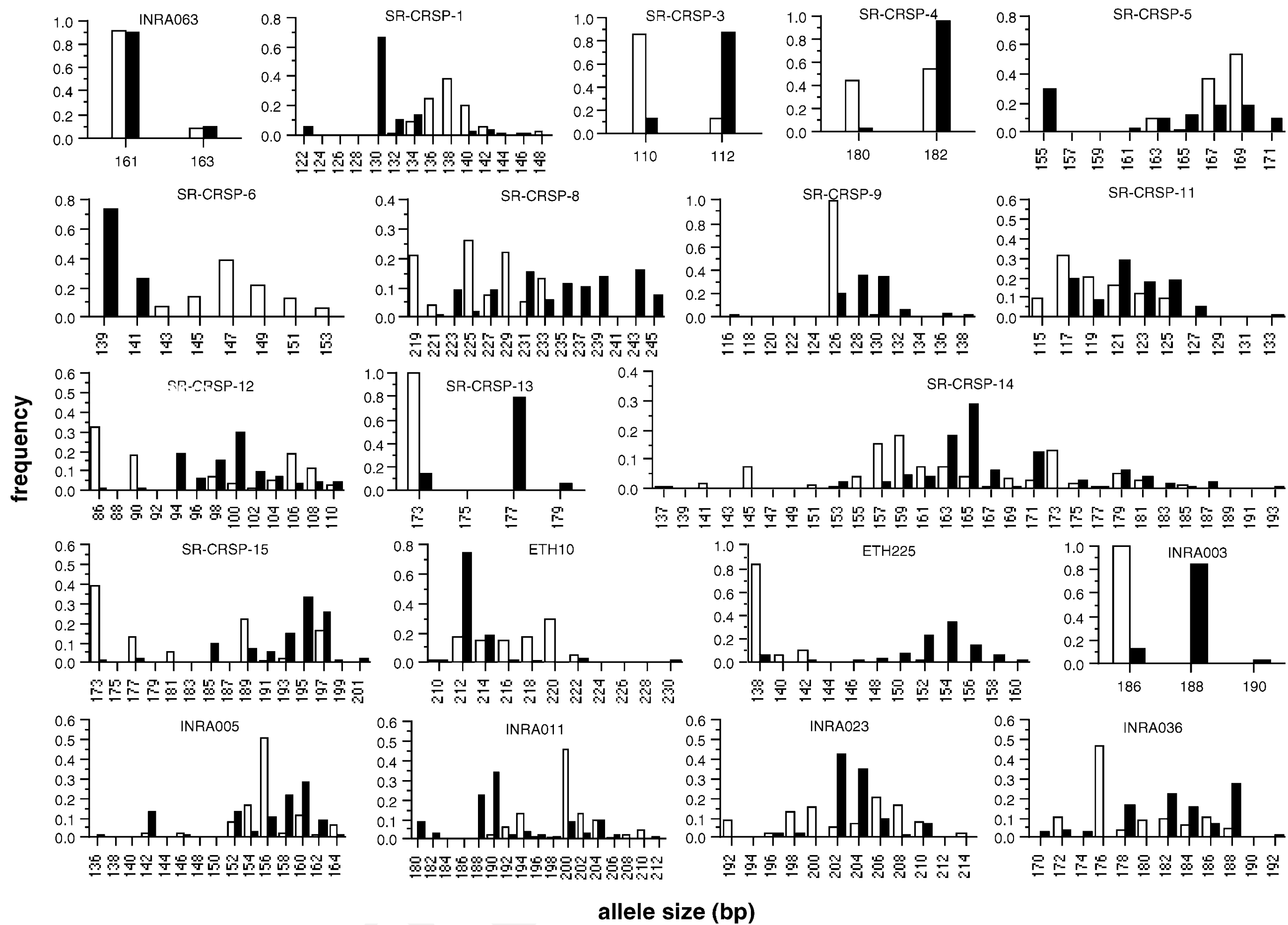

Fig. 3. Allele frequency distributions for polymorphic loci in *R. pyrenaica* (white bars) and *R. rupicapra* (black bars).

Table 2
Pairwise genetic distances between study areas: *Ds* above diagonal, $(\delta\mu)^2$ below

	CBW	CBE	PYW	PYE	AN	ALW	ALE	TA	CP	BA	CU
CBW		0.16	0.25	0.28	0.82	0.99	1.06	1.18	1.46	1.01	1.25
CBE	0.66		0.26	0.29	0.79	0.89	1.02	1.24	1.58	1.03	1.25
PYW	1.64	2.10		0.08	0.77	0.97	1.03	1.20	1.65	1.13	1.34
PYE	2.78	1.88	1.82		0.90	0.91	1.04	1.24	1.64	1.08	1.34
AN	6.89	5.93	7.32	10.10		0.85	0.89	1.37	1.37	1.24	1.01
ALW	8.30	6.72	8.72	8.50	7.32		0.15	0.39	0.33	0.27	0.22
ALE	9.37	8.19	9.02	9.60	8.48	0.55		0.38	0.37	0.28	0.25
TA	10.10	8.14	11.99	10.72	9.40	2.01	2.14		0.34	0.36	0.48
CP	12.64	10.51	13.76	12.83	9.99	1.56	2.05	1.53		0.37	0.38
BA	8.42	6.36	10.00	9.35	7.49	2.47	2.70	2.04	2.53		0.39
CU	15.64	13.36	14.30	14.01	12.66	3.71	4.43	6.27	2.63	5.63	

Rectangles highlight interspecific comparisons.

Chamois populations have three to four alleles per locus and expected heterozygosities between 38% and 51%. These estimates of population genetic diversity are low when compared with values reported for other wild or domestic artiodactyls (e.g., Bancroft et al., 1995; Fickel and Reinsch, 2000; Forbes et al., 1995; MacHugh et al., 1997) and comparable to reported diversities in North American red deer, wapiti (Polziehn et al., 2000) and in vicuña (Kadwell et al., 2001). The relatively low levels of diversity in our study may be explained by the bias to high variability in the choice of microsatellite loci in the original species (Pepin et al., 1995). This bias would not affect homologous species, where microsatellite loci could have smaller or altered repeats (Forbes et al., 1995). Differences among heterozygosities seem to reflect population sizes. The large Alpine populations showed the largest values,

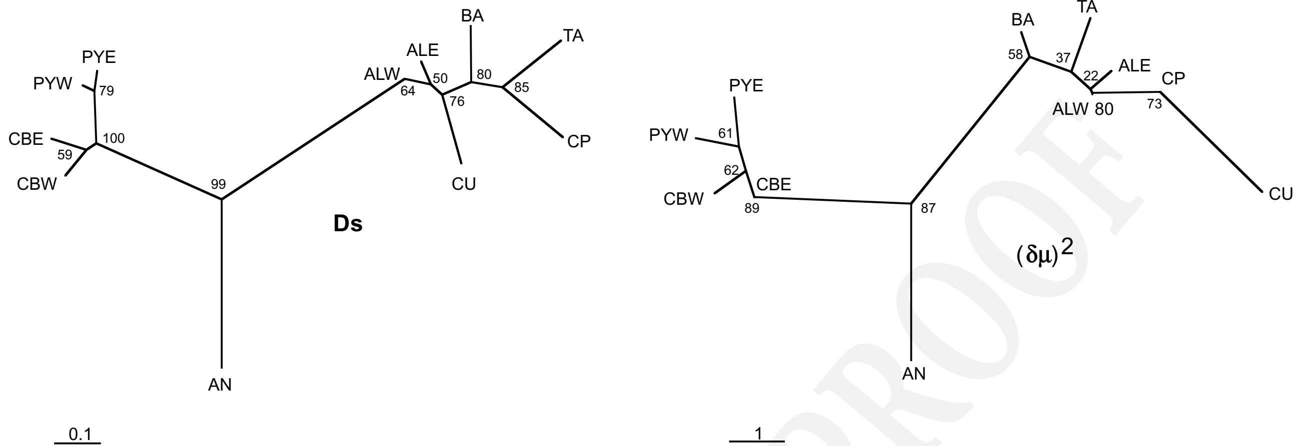

Fig. 4. Neighbour-joining trees summarising phylogeographic relationships between populations based on genetic distances *Ds* and $(\delta\mu)^2$. Bootstrap values indicating the degree of support of each branch point are shown besides the node as the number of replicates, out of 100, in which the cluster was formed.

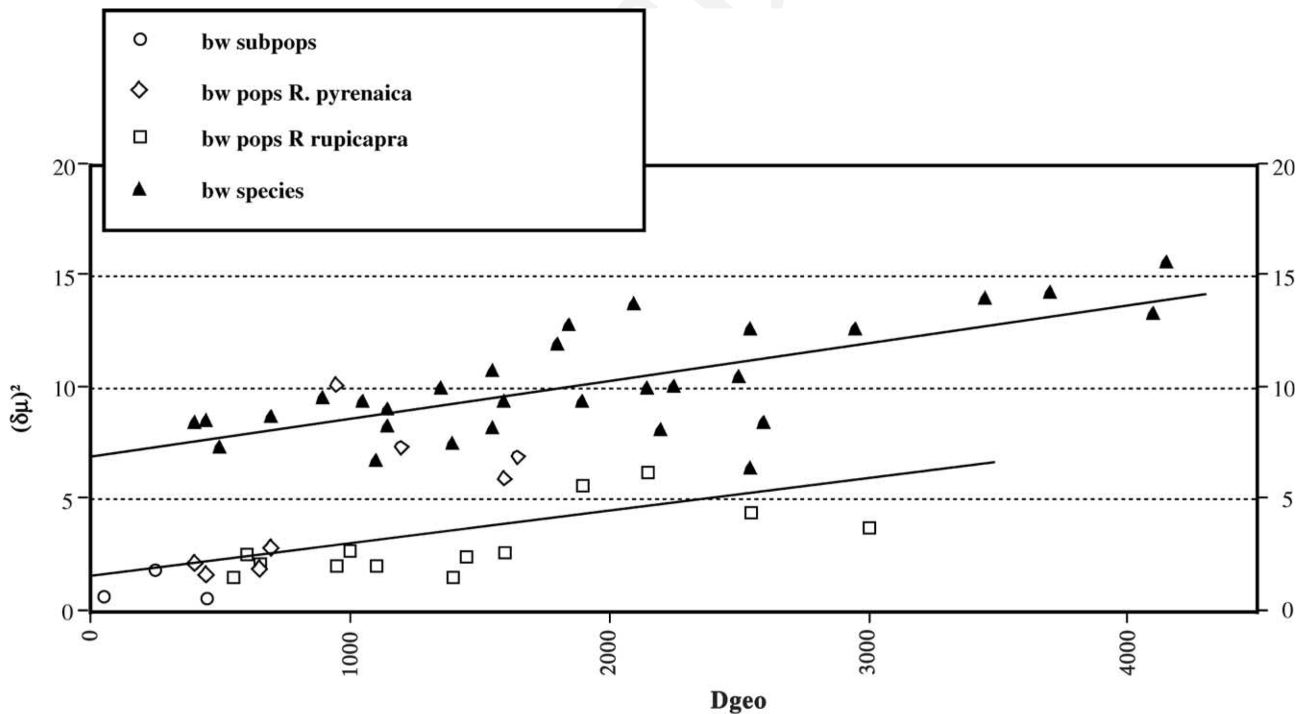

Fig. 5. Regression of $(\delta\mu)^2$ values on geographical distance.

353 while populations of reduced size such as those of the 354 Apennines and the Carpathian Mountains were more 355 homozygous. Worth noting is the low diversity of the 356 Caucasica population, which may be related to the re-357 cently suffered drastic reduction in population size.

358 Observed heteozygosities were generally lower than 359 expected, reflecting the spatial structuring of popula-360 tions. Population structuring into subpopulations is re-361 vealed further by the differentiation between areas 362 within mountain ranges. Moritz (1994) has suggested 363 that regions with significantly different allele frequen-364 cies, and hence, with low migration rates are important 365 from a local management perspective and defined them 366 as management units (MUs). From this viewpoint, it 367 may be noted that local subpopulations of chamois 368 within a mountain range are significantly different from 369 one another and it is therefore important to prevent 370 local declines to avoid the loss of genetic variability.

371 Geographically isolated populations of the different 372 mountain ranges were ascribed to 10 subspecies. These 373 10 subspecies were considered either within a single 374 species by Couturier (1938) and Dolan (1963) (cited in

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Masini and Lovari, 1988) or they have more recently been grouped into two species *R. pyrenaica* and *R. rupicapra* (Lovari and Scala, 1980; Nascetti et al., 1985). Microsatellite analysis of 8 of the 10 proposed subspecies showed a clear differentiation between every pair of populations. Trees based on genetic distances, either individual band-sharing distances or between population distances, clearly separate two groups corresponding to the two proposed species of chamois. The single individual from the Apennines is closer to the Pyrenaica group than to Rupicapra, but assignment is not clear. It can also be noted that this individual is homozygous for two private alleles. Camerano (1914) distinguished the species *R. ornata* besides the two currently accepted ones. Our data offer some support for this classification, but obviously additional samples have to be analysed to clarify the phylogenetic relationships of Apennine chamois. Furthermore, it may be noted that the current genetic constitution of the Apennine chamois may have been largely determined by extreme genetic drift. The subspecies was nearly extinct early in the 20th century and in the late 1940s (Lovari, 1985) and today is composed of a reduced number of individuals.

There was substantial genetic variation between species, 30% for allele frequencies and 45% for allele sizes. Variation between studied areas within species accounted for approximately 10% of the genetic variance, irrespective of whether *Fst* or *Rst* estimates were used. In most cases but not always, genetic distances reflected a larger differentiation among pairs of populations in different mountain ranges, or proposed subspecies than between subpopulations. The lowest Nei's standard genetic distance between proposed subspecies ($Ds = 0.22$ for the pair ALW–CU) was greater than the largest *Ds* value within mountain ranges. When $(\delta\mu)^2$ is considered, three distances (CBW–PYW, ALW–CP, and TA–CP) are lower than the value of 1.82 observed between the two Pyrenean populations. Genetic distances can be compared with microsatellite-based values reported for other pairs of proposed subspecies. For example, Nei's genetic distance between isolated bear populations ranged between 0.4 and 1.5 (Paetkau et al., 1999), while distances among populations of bighorn sheep ranged between 0.17 and 1.38 (Forbes and Hogg, 1999). *Ds* distances between subspecies of chamois (0.22–0.90) are at the lower end of these values and are comparable to *Ds* between proposed subspecies of the North American deer, wapiti ($Ds = 0.18$–0.69; Polziehn et al., 2000) and between North American populations of grey wolves ($Ds = 0.13$–0.67; Roy et al., 1994). In contrast with the observation of Forbes and Hogg (1999) in bighorn sheep, $(\delta\mu)^2$ reveals more differentiation. Though not dramatic, those between pairs of proposed subspecies are greater than differences between subpopulations of the same subspecies. In addition, there is the certainty of actual discontinuities between populations in different mountain ranges. Taking these factors altogether, it might be proposed that, though not very differentiated, populations in different mountain ranges have undergone significant independent evolution sensu Moritz (1994), and hence, they could be considered evolutionarily significant units (ESUs) or subspecies. However, this matter cannot be solved by microsatellite analysis alone and must be the object of mtDNA analysis.

The level of divergence between populations can be compared with archaeological data. The Rupricaprinae originated in Asia during the late Miocene. Masini and Lovari (1988) proposed that the chamois, or its direct ancestor, may have reached Europe as a late immigrant during the early or middle Pleistocene and moved westward, along the mountain chains of the Alpine System. They related its arrival to a cold climatic phase, marked by the arrival in Europe of waves of taxa from cold or open environment. The *Rupricapra* genus is thought to have evolved during the middle and late Pleistocene in West-Eurasia. In the middle Pleistocene, chamois occurred in the same geographic area that living species currently occupy.

Genetic distances are correlated with distances between mountain chains, which is fully compatible with the Asiatic origin of *Rupicapra* and the European colonisation westward along the mountain ranges. The close association of genetic and geographical distance implies that populations differentiated "in situ" and no major migrations occurred after the initial colonisation. Within this general scenario, it was shown that, for equally distant areas, mean genetic distances between the pairs *R. pyrenaica*–*R. rupicapra* are greater than within species, implying that there was an additional barrier to gene flow between the two taxonomic groups. This observation is in agreement with the proposed split of the *Rupicapra* genus into *R. pyrenaica* and *R. rupicapra*. Divergence times (Fig. 6) were calculated from $E(\delta\mu)^2 = 2\beta\tau$, where β is the mutation rate and τ is the time of generation (Goldstein et al., 1995). We lack an estimate for the mutation rate of the microsatellite loci studied and have used the average mutation rate of 5.6×10^{-4}, calculated for 15 microsatellite loci in humans (Weber and Wong, 1993). The generation time in chamois was estimated as 6.24 years/generation (Gaillard, 1992). Separation times were calculated from the UPGMA tree, based on the $(\delta\mu)^2$ distance. It should be noted that these estimates have a considerable error due to the error of the distance itself, on the one hand, and to the added error in the mutation rate estimate, on the other. This error may be large because microsatellite mutation rates differ between loci and between species (i.e., Di Rienzo et al., 1998; Vázquez et al., 2000). The estimated divergence time between the two putative species, *R. rupicapra* and *R. pyrenaica*, is 57,000 years. Our estimate is lower than the 280,000 years of separation estimated from mean nucleotide divergence among mitochondrial RFLP haplotypes of three sub-

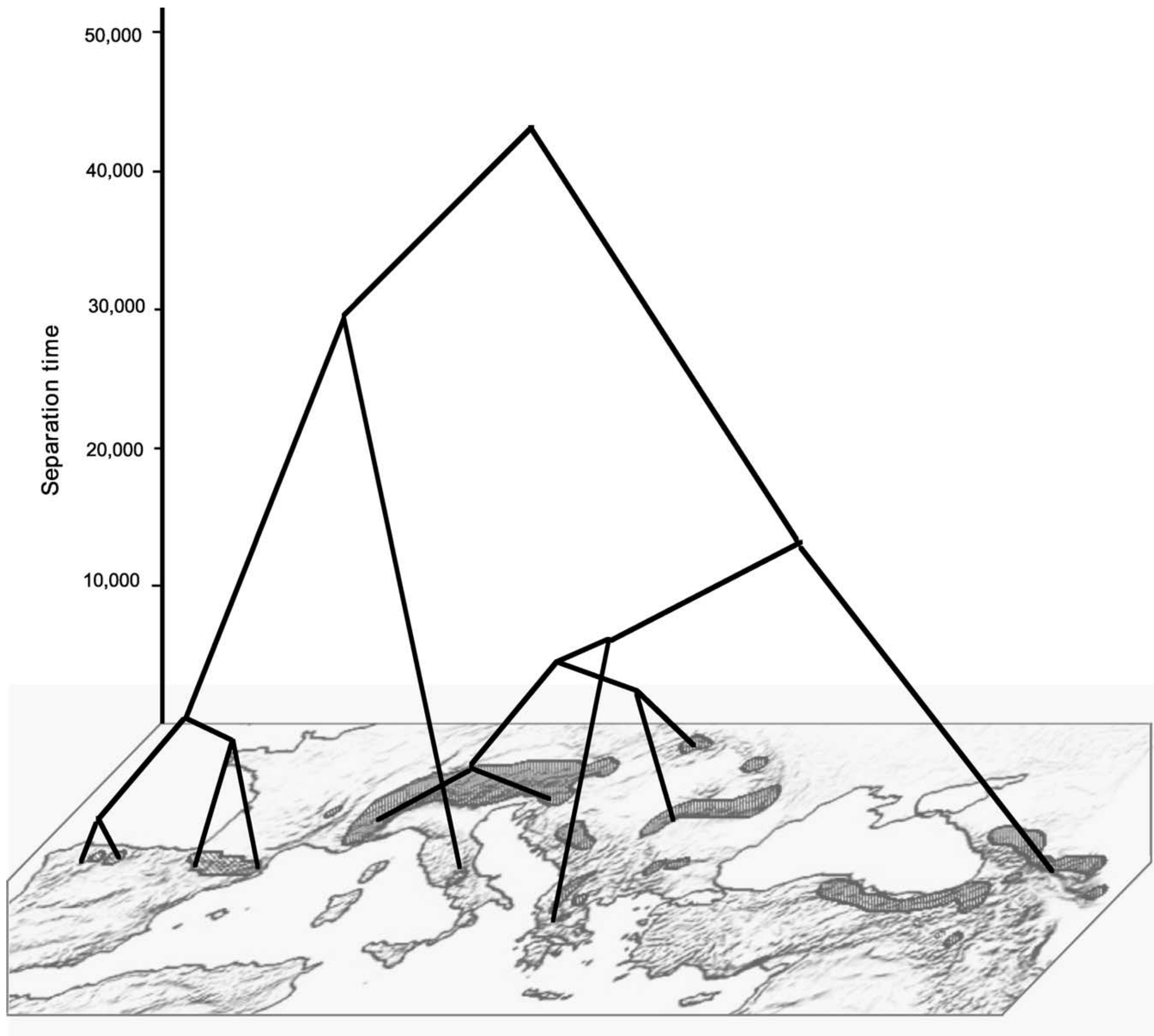

Fig. 6. Phylogeography of chamois.

species (Hammer et al., 1995). On the basis of the fossil record, Masini and Lovari (1988) placed the split during the interglacial period Riss–Würm, somewhere in the middle of the two genetic distance-based estimates.

Genetic distances between pairs of populations within species mostly depend on the geographical distance of the pairs being considered. Masini and Lovari (1988) proposed that *R. rupicapra* evolved in Eastern Europe or Asia Minor during a time of geographic isolation and then spread again to Western Europe during Würm II. In the said case, no association between genetic and geographical distance needs to occur in the interspecific comparisons. Our results suggest instead that the history of the genus during Pleistocene glacial-interglacial periods was dominated by expansions and contractions within limited geographic regions, leading to alternate contact and isolation of contiguous populations. Finally, the warm climate of the Holocene had rendered the populations definitively isolated occupying the top of the different mountain ranges.

The phylogeography of chamois may be compared with other taxa in Eurasia for which a small degree of congruence was found (Hewitt, 1996; Taberlet et al., 1998). In general, the northern regions were colonised from Iberian and Balkan refugees and the alpine barrier often isolated Italian lineages. Contrary to the general trend, Rupicapra differentiated without major migrations, presumably because it is a cold-tolerant species. As in other taxa, the alpine barrier has played a substantial role in West–East differentiation that led to the two species of the genus. Some studies have reported the evolution in isolation during Pleistocene periods of climatic fluctuations (Hundertmark et al., 2002; Leonard et al., 2000; Paulo et al., 2001). Our results also point to isolation

by distance as the primary agent for differentiation in chamois.

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