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Short Note

The chamois (*Rupicapra* cf. *pyrenaica*) in central Italy: what ancient DNA tells us

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Abstract

The Apennine chamois (*Rupicapra* cf. *pyrenaica*) is a very endangered mountain mammal. At the beginning of the 20th century, only a small population survived in the Abruzzo, Lazio and Molise National Park (Central Italy) and, despite its reintroduction in different Apennine massifs and an increased census size, its genetic variability is the lowest among bottlenecked mammals. The ancient DNA analysis of a skull dated back to ≈3000 cal yr BP allowed us to describe a new haplotype belonging to the mitochondrial Central Clade (including Chartreuse and Apennine populations) but never found in extant chamois. This result underlines that the demographic collapse of Apennine populations, which probably started in the Pleistocene, was combined with an ever-increasing genetic erosion in gradually smaller and isolated populations.

The chamois (*Rupicapra* spp.) is a mammal distributed in the main Eurasian mountain ranges, from the Caucasus to the Iberian Peninsula, and currently classified into two species: *Rupicapra rupicapra* and *Rupicapra pyrenaica* (Corlatti et al., 2011). The former is distributed in central and eastern Europe, Anatolia and Caucasus, and includes seven subspecies (*cartusiana*, *rupicapra*, *tatrica*, *carpatica*, *balcanica*, *asiatica*, *caucasica*). The latter is distributed in southwestern Europe and includes three subspecies (*parva*, *pyrenaica*, *ornata*) (Fig. 1). The Italian Peninsula is currently inhabited by the Alpine chamois (*R. r. rupicapra*), widespread in the Alps, and by the Apennine chamois (*R. p. ornata*), in central Apennine (Aulagnier et al., 2008; Herrero et al., 2008).

Genetic analyses performed with microsatellites and mitochondrial DNA (mtDNA) revealed the presence of three major clades distributed in Western (Clade W), Central (Clade C) and Eastern Europe (Clade E) (Fig. 1) that would diverge during Quaternary climatic oscillations (Rodríguez et al., 2010). The expansion of chamois populations to lower altitudes during glacial periods and their contraction to high altitudes during interglacials, together with the presence during glacial maxima of Alps and Pyrenees glacial sheets acting as barriers, have shaped the current distribution of *Rupicapra* spp. Clades (Rodríguez et al., 2010). In particular, the evolutionary history reconstructed from mtDNA data (Rodríguez et al., 2010; Pérez et al., 2014) showed that chamois colonised Europe from the east during the late Pliocene/Early Pleistocene, determining the first split between Clade mtE (corresponding to *R. rupicapra*) and Clades mtW/mtC (corresponding to *R. pyrenaica*) (≈1.9 mya). Clades mtW and mtC diverged ≈1.2 mya and, following the expansion of Clades mtW and mtE towards the Western Alps, the Clade mtC was splitted into two isolated groups (≈0.09 mya), currently represented by Chartreuse (*R. r. cartusiana*) and Apennine (*R. p. ornata*) populations (Rodríguez et al., 2010; Pérez et al., 2014). In the case of *R. r. cartusiana*, analysis performed using nuclear markers grouped it within the Clade E, highlighting an ancient male-mediated hybridization between different populations (Rodríguez et al., 2010; Pérez et al., 2013, 2017). In addition, very low values of genetic diversity for *R. p. ornata* proved the occurrence of past isolation and bottleneck events (Rodríguez et al., 2010). In fact, at the beginning of the 20th century, the Apennine population consisted of no more than 30 individuals, survived in the Abruzzo, Lazio and Molise National Park (Central Italy) (see Lovari, 1989). Only recently, the implementation of conservation and reintroduction policies has allowed the recovery of the Apennine population, currently amounting to ≈3000 individuals (Vv.Aa., 2018).

For some authors, the extant Apennine chamois population (*R. p. ornata*) might derive from individuals introduced in the 18th century from Spain (see Masseti and Nappi, 2007). However, the anthropochorous origin of Apennine chamois disagrees with documentary (De Marchi, 1599; Costa, 1839) and archaeological evidences (Masseti and Salari, 2012, 2017). The historical distribution of the genus *Rupicapra* in Italy is documented by the recovery of fossil and subfossil bones, very common in Pleistocene and Holocene archaeological deposits (Masseti and Salari, 2012, 2017). Many remains are in very poor conservation conditions, preventing the distinction between *R. rupicapra* and *R. pyre-*

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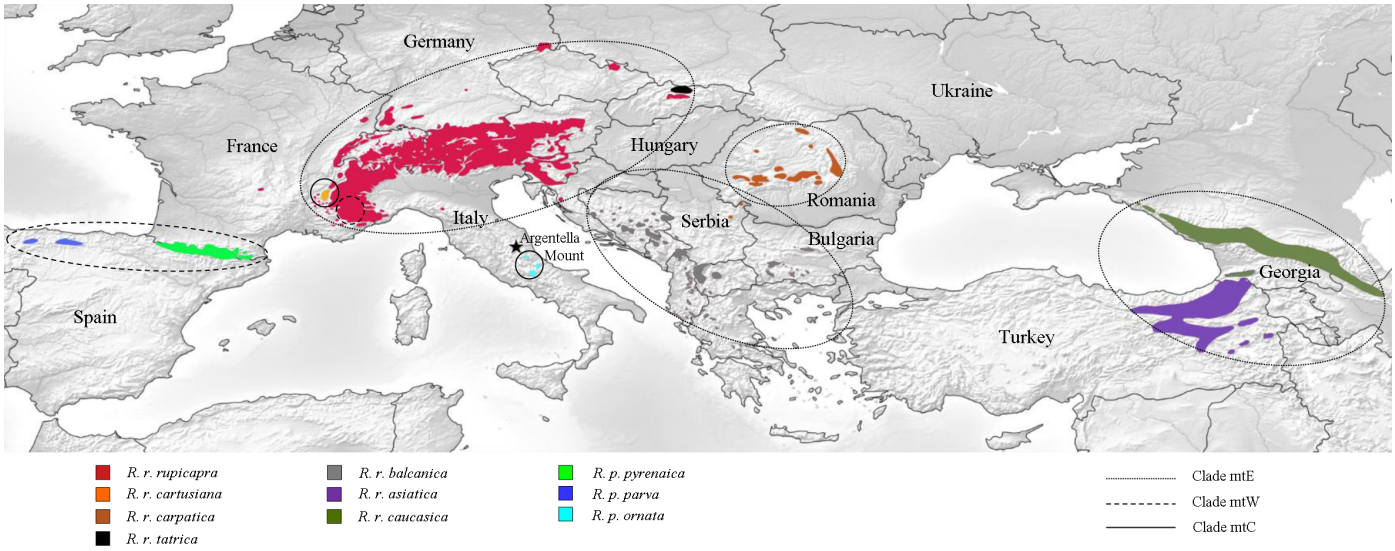

Figure 1 – Maps showing the geographic distribution of the extant subspecies of the genus *Rupicapra* and their attribution to mitochondrial clades. Distribution data were downloaded from the IUCN Red List (http://www.iucnredlist.org) and mitochondrial clades were represented as proposed by Rodríguez et al. (2010). The discovery site of the ancient skull analysed in this study is indicated with a black star.

naica. However, the best-preserved bone samples seem to confirm the past presence of *R. rupicapra* in Northern Italy and of a "*pyrenaica*-like" chamois in Central-Southern Italy (see Salari et al., 2014; Masseti and Salari, 2017).

Here we analyse an ancient skull of chamois, recovered in a cave in the Sibillini Mountains (Central Apennines). In this area the chamois went probably extinct between 17th and 18th centuries, and only recently reintroduced using *R. p. ornata* individuals from the Abruzzo, Lazio and Molise National Park (Rossetti et al., 2015). Morphometric and genetic analyses of the specimen were carried out to identify it at subspecies level and to demonstrate the loss of genetic diversity in the Apennine chamois population.

The studied specimen is a partial skull (Fig. 2) found in 1978 in a cave at 1500 m a.s.l. on the south-east slope of Argentella Mount (Fig. 1; Ripa Grande, Sibillini Mountains, Italy) (Pedrotti, 1983). Description of the skull and morphometric measurements were taken according to Salari et al. (2014) and compared with data from other studies (Camerano, 1914, 1916; Salari et al., 2014). The age of death of this animal was estimated according to Pérez-Barbería (1994). Bone powder, drilled from dental alveoli, was used to estimate by radiocarbon dating the age of chamois remains. The analysis was carried out at "CEnter of applied physics, DAting and Diagnostic" (University of Salento, LE, Italy) using the Accelerator Mass Spectrometry (AMS) method (Calcagnile et al., 2005) and the calibrated age was obtained using OxCal Ver. 3.10 software (Reimer et al., 2013).

DNA extraction and PCR set up were performed in a dedicated laboratory using standard precautions to work on ancient DNA (aDNA) (Willerslev and Cooper, 2005). A tooth (left M^2) was sampled and decontaminated prior to DNA extraction to reduce the presence of exogenous DNA and PCR inhibitors (Rohland and Hofreiter, 2007). The outer surface of the sample was removed using sandpaper, the tooth immersed in bleach for 5 min, rinsed twice with ultrapure sterile water and exposed to UV 30 min per side. The whole tooth was digested overnight in a waterbath as proposed by (Rodrigues et al., 2018). Genomic DNA was extracted using a modified silica-spin column method (Yang et al., 2004, 2008) and eluted in 100 µl of ultrapure sterile water.

A ≈500 base pairs (bp) sequence of the mitochondrial DNA control region (mtDNA CR) was obtained by amplifying seven overlapping fragments. Species-specific primer pairs were designed (Tab. 1) using the software Primer3Plus (Untergasser et al., 2012) and the complete mtDNA genome of *R. p. ornata* (GenBank accession number KJ184173) as a reference sequence (Pérez et al., 2014). PCRs were carried out using the amplification protocol proposed by Splendiani et al. (2016). The annealing temperature was set at 54 °C for the RpoCR5 primer pair and at 55 °C for all the others. PCR products were checked on 2% agarose gel stained with GelRed™ (Biotium). All amplicons were purified by exoSAP-IT™ (Thermo Scientific) and sequenced in both directions using an automated sequencer, ABIPRISM 3730XL (Applied Biosystems). Negative controls were added during both extraction and amplification phases with the aim to detect contamination. A second DNA extraction and amplification was performed, from a sample of bone powder, in order to validate the result.

The sequence obtained was aligned in CLUSTALW (Larkin et al., 2007) with all the CR haplotypes described for the genus *Rupicapra* (Rodríguez et al., 2010). The alignment was checked by eye and manually edited on BioEdit (Hall, 1999). In order to visualize the relationship between our haplotype and those previously described (Rodríguez et al., 2010), a haplotype network was built using the software Network 5 (Fluxus Technology Ltd., www.fluxus-engineering.com). Gaps were ignored. Firstly, a Reduced-Median network (with r=2) was run and, secondly the definitive Median-Joining network (with ε=0) was carried out to obtain a simplified output (Rodríguez et al., 2010).

The skull was radiocarbon dated to 3065–2864 cal yr BP (2847+36 ^{14}C yr BP). The incompleteness of the skull (Fig. 2a) prevented the ob-

Table 1 – Primer pairs designed and used to amplify the mtDNA control region of chamois ancient sample.

Primer name		Sequence 5' to 3'	Product length
RpoCR1	F	TCAACACCCAAAGCTGAAGTT	131 bp
	R	TGTTGTGTGTTTGAAAGTTTTAGTGA	
RpoCR2	F	TCAAGAGCCTTCCCAGTATTAAA	105 bp
	R	ACTTATGCGGTGGGTGCAT	
RpoCR3	F	AAGCCTCCCACCCTACAAAC	118 bp
	R	TGGGGTAAGCCATGTAATGC	
RpoCR4	F	TGCATTAATGTAATACAAATGTGGT	134 bp
	R	GCGGAGGGCAGATCATTTA	
RpoCR5	F	AGTGCATTACATGGCTTACCC	123 bp
	R	CACTTCCGGTACCCGCTTAT	
RpoCR6	F	ATTAAATGATCTGCCCTCCGC	125 bp
	R	GGGATATGCATGTTGACAAGG	
RpoCR7	F	CGTACATGGCACATGAGGTC	105 bp
	R	AAGCGGGTTGCTGGTTTC	

Abbreviations: F=forward primer, R=reverse primer, bp=base pair

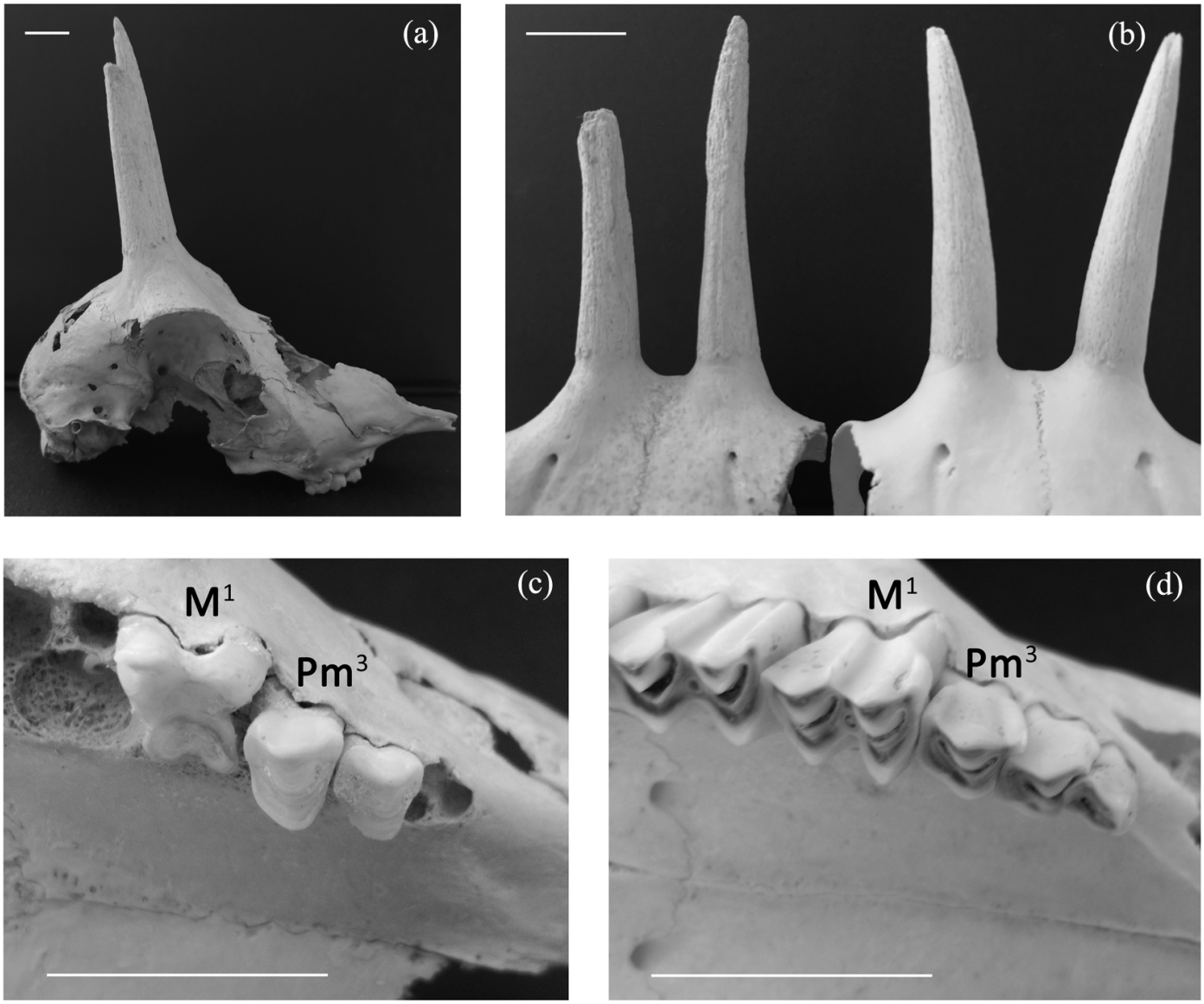

Figure 2 – The skull of the Ripa Grande chamois a) right lateral view; b) comparison between the Ripa Grande chamois (on the left) and Alpine chamois (on the right) horn cores, in frontal view; c) Ripa Grande chamois and d) Alpine chamois right maxillary teeth to show the level of wearing in the subfossil specimen. M^1, first upper molar; Pm^3, third upper molar. Scale bar=2 cm.

servation of the ethmoidal vacuity, usually open in *R. rupicapra* and fully closed in *R. pyrenaica*. On the other hand, the horn-core section is ellipsoidal at the base with a slightly medio-lateral compression that evolves to a sub-triangular section with posterior base at the apex (Fig. 2b), as usual in *R. pyrenaica* (see Masini, 1985; Masini and Lovari, 1988). The same applies to the ratio between the antero-posterior diameter at the base of the horn-core, confirming that the specimen from Ripa Grande falls within variability range of the living Apennine chamois (Tab. 2). Teeth are represented by Pm^{1-3} on both sides, M^{1-2} on the left and only M^1 on the right maxilla. The level of teeth erosion indicates that the Ripa Grande chamois was a very old individual (>12 years, due to the extensive obliteration of infundibulum on Pm^3 and M^1, compare Fig. 2c, d). The length of the molar row (Tab. 2) suggests that the specimen was of small to medium in size.

The analysis of aDNA allowed the amplification of a 417 bp sequence of the mtDNA CR from the Ripa Grande chamois (GenBank accession number MK880130). The multiple alignment and the Median Joining network (Fig. 3) allowed the classification of the ancient sample as a new haplotype related to haplotypes of the subspecies *R. r. cartusiana* (CR23, CR24, CR25) and *R. p. ornata* (CR22) (Rodríguez et al., 2010).

Our analysis confirmed the observations of previous studies which attributed the skull to a *R. p. ornata* individual from Holocene (Masini, 1985; Masini and Lovari, 1988). The radiocarbon dating performed on the chamois skull from Ripa Grande placed it in the Bronze Age (≈3000 yr BP), when climatic conditions in central Italy were characterized by cooling events coeval with cooling cycles in the North Atlantic waters (Giraudi, 2005). Morphological analysis indicated that the skull resembles a "*pyrenaica*-like" individual, despite the strong climatic oscillations should have promoted local phenotypic adaptations. This phenotypic conservatism is expected to occur in Quaternary large

Table 2 – Measurements (in mm) of the sub-fossil chamois from Ripa Grande (Sibillini Mountains, Marche, Italy) compared to living populations (data from Camerano, 1914, 1916).

Skull parameters	Ripa Grande	*R. p. ornata*	*R. pyrenaica*	*R. rupicapra*
LMR	36.1	35.4 (36.7) 39.2	38 (40.5) 43	-
HCL	92.3	70 (120.2) 141	60 (87.0) 100	62 (87.4) 124
APD	18.7	19 (25.4) 29	15 (18.5) 22	13 (20.7) 27
TD	16.1	17.5 (21.4) 24.5	14.5 (16.9) 19.5	12 (18.4) 26
MD	8.8	10 (11.9) 14	8 (9.0) 10	9 (16.0) 21
TD/MD ratio	1.83	1.80	1.88	1.15

LMR: length of the molar row; HCL: horn-core length; APD: antero-posterior horn-core diameter; TD: transverse horn-core diameter; MD: minimum distance at the base of the horn-core.

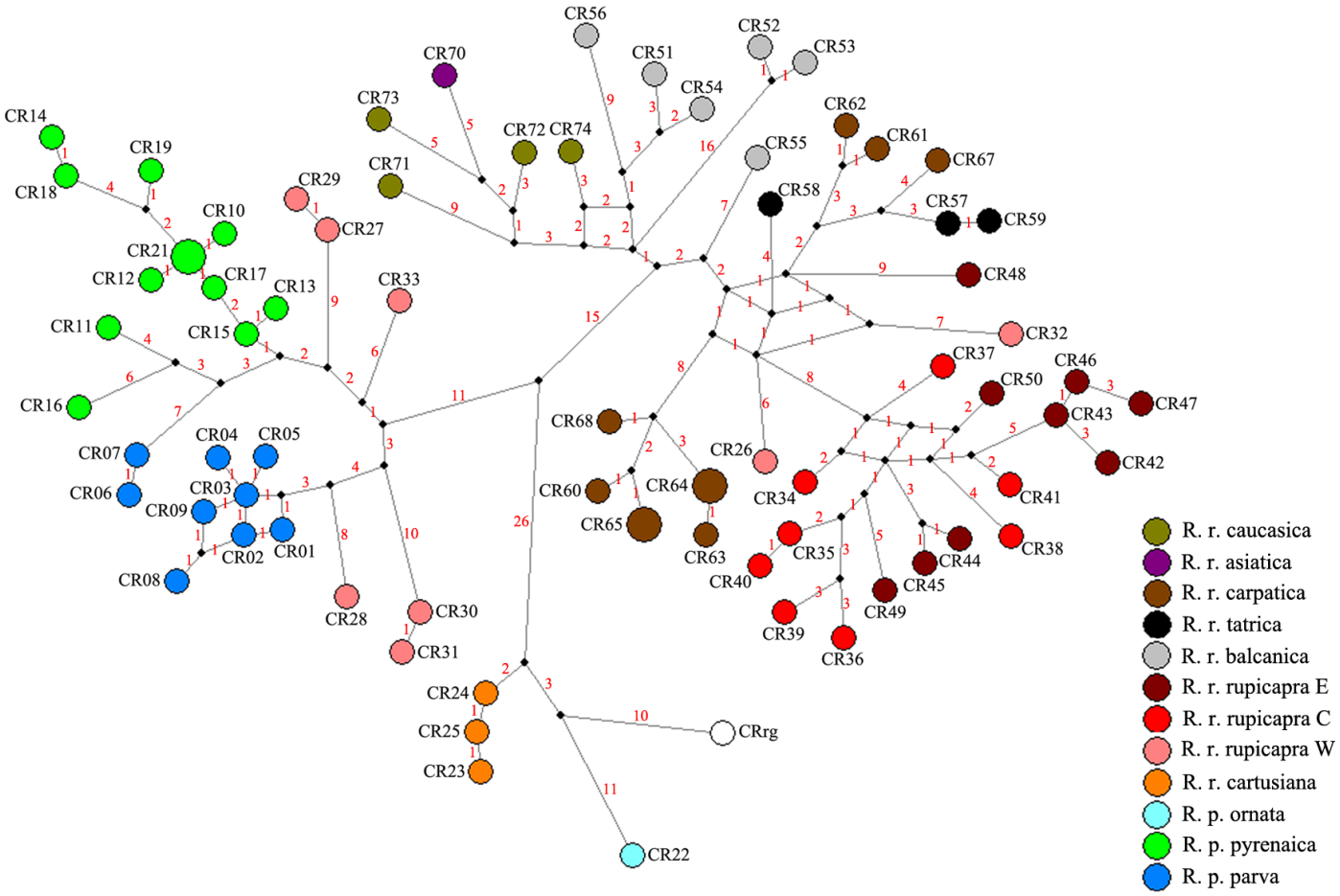

Figure 3 – Median-Joining network showing the relationship between all the mtDNA control region sequences from Rodríguez et al. (2010) and the one obtained in this study (CRrg). The different colours indicate different subspecies of chamois (see Rodríguez et al., 2010) except for the new ancient haplotype coloured in white.

mammals (Raia et al., 2011). The strong tooth wear revealed that this chamois was an adult, whereas measurements on preserved portions of the skull indicated a medium-small sized individual. The uniqueness of the specimen did not allow us to evaluate if the small size might be related to the consequences of genetic drift acting in an isolated population, or if the animal studied was simply a minus-variant individual.

The comparison of mtDNA CR sequence obtained from our sample with homologous sequences of living individuals of the genus *Rupicapra* indicates that the ancient chamois belonged to a new haplotype clearly related with those of the Clade mtC. The strict relationship between Ripa Grande haplotype and that observed so far within the subspecies *R. p. ornata* allowed us to confirm, together with morphological and previous zooarchaeological evidences (Salari et al., 2014; Masseti and Salari, 2017), the natural presence of a "*pyrenaica*-like" chamois in central Italy since ancient times, providing a further evidence to exclude the hypothesis of an anthropochorous introduction of Apennine chamois (Masseti and Nappi, 2007).

In addition, this result is perfectly in agreement with the evolutionary history of the genus *Rupicapra* reconstructed using mtDNA, with both subspecies (*R. r. cartusiana* and *R. p. ornata*) considered belonging to the Clade mtC (Rodríguez et al., 2010). The Chartreuse and Apennine populations started to diverge in the late Pleistocene (Pérez et al., 2014), as consequence of the split of the Clade mtC caused by the expansion of Clades mtW and mtE in Western Alps (Rodríguez et al., 2010; Pérez et al., 2014). The identification of a new haplotype related to the one currently described for the subspecies *R. p. ornata* proves the loss of genetic diversity, occurred as a consequence of a drastic reduction in size of the Apennine chamois population. The population size diminution that has affected the Apennine chamois over the centuries is probably the result of the anthropic pressure and Holocene climate changes. The human consumption of this species has been demonstrated since the Pleistocene, when hunter-gatherers hunted different species of ungulates (Bertini Vacca, 2012). Also competition with sheep, widespread in central Italy since the 7th millennium BP (Barker, 1981), and diffusion of epizootic diseases (Corlatti et al., 2011) may have contributed to chamois diminution. In central Italy, the drastic reduction in population size led to a genetic collapse of the Apennine chamois as demonstrated by the analysis of both mtDNA and microsatellites that highlighted for *R. p. ornata* the lowest values of genetic diversity observed so far among all bottlenecked mammal populations (Rodríguez et al., 2010).

The analyses carried out here, even if on a single specimen, show that the study of archaeological remains could have a great importance to resolve taxonomic and conservation questions of the genus *Rupicapra* in Italy. The aDNA analysis would allow the unquestionable identification of bone remains and consequently make possible the reconstruction of the original distribution range and the evolutionary history of chamois populations of the Italian Peninsula. In addition, the analysis of a large number of ancient samples would permit the direct evaluation of the loss of genetic diversity as a consequence of local extinction or size reduction occurred in the past in chamois populations.

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