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First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning

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Abstract

Two experiments have been performed to clone the bucardo, an extinct wild goat. The karyoplasts were thawed fibroblasts derived from skin biopsies, obtained and cryopreserved in 1999 from the last living specimen, a female, which died in 2000. Cytoplasts were mature oocytes collected from the oviducts of superovulated domestic goats. Oocytes were enucleated and coupled to bucardo's fibroblasts by electrofusion. Reconstructed embryos were cultured for 36 h or 7 d and transferred to either Spanish ibex or hybrid (Spanish ibex male × domestic goat) synchronized recipients. Embryos were placed, according to their developmental stage, into the oviduct or into the uterine horn ipsilateral to an ovulated ovary. Pregnancy was monitored through their plasmatic PAG levels. In Experiment 1, 285 embryos were reconstructed and 30 of them were transferred at the 3- to 6-cells stage to 5 recipients. The remaining embryos were further cultured to day 7, and 24 of them transferred at compact morula/blastocyst stage to 8 recipients. In Experiment 2, 154 reconstructed embryos were transferred to 44 recipients at the 3- to 6-cells stage. Pregnancies were attained in 0/8 and 7/49 of the uterine and oviduct-transferred recipients, respectively. One recipient maintained pregnancy to term, displaying very high PAG levels. One morphologically normal bucardo female was obtained by caesarean section. The newborn died some minutes after birth due to physical defects in lungs. Nuclear DNA confirmed that the clone was genetically identical to the bucardo's donor cells. To our knowledge, this is the first animal born from an extinct subspecies.

Keywords: Bucardo; Interspecies; Nuclear transfer; Spanish ibex; Wild goat

1. Introduction

The bucardo (Pyrenean ibex; *Capra pyrenaica pyrenaica*) was one of the four subspecies of the Spanish ibex (Cabra Montés—*Capra pyrenaica*) identified in 1910 by Cabrera [1] according to morphological characteristics. Two subspecies of Spanish ibex are at

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present free-living in Spain: *Capra pyrenaica victoriae*, living in Central and Northwest Spain and *Capra pyrenaica hispanica* living both in the South and Eastern Spanish Mediterranean Mountains. The Portuguese ibex (*Capra pyrenaica lusitanica*) became extinct in 1892 [1]. The bucardo population was abundant in the Pyrenees, but decreased very quickly along the last two centuries supposedly due to high hunting pressure. In the second half of the 20th century only a scarce population was living in the National Ordesa Park situated in the Spanish Central Pyrenees. All the *in situ* attempts to stop the declining of the population by natural methods, such as strategies of food supply, were ineffective. As a consequence, in 1989, the EU and the local Aragon Government underwent a Project to capture all available population of bucardos, with the aim to multiply them by assisted reproduction in captivity, but at this time only three old females and no males were living. Genetic studies of this population showed an extremely low variability in the MHC [2], which may in part explain the bucardo's decline. Natural hybridization of the three remaining females with *C. p. hispanica* fertile males was attempted. Although the levels of faecal estrogens and progesterone indicated that pregnancies took place in two females (Alabart, unpublished data), no live kids were observed. In 1999 only one bucardo female of about 12-year old was living. In a final attempt to preserve the bucardo's genetic resources, we captured this last specimen and cells from a skin biopsy were obtained, multiplied and kept frozen in liquid nitrogen. This animal was *in situ* released just after biopsy and died in 2000. Therefore, the Spanish Government recently declared the bucardo extinct [3]. It is also currently listed as extinct by the IUCN Red List.

Previous studies on interspecies nuclear transfer (NT) followed by embryo transfer into domestic recipients resulted on live offspring, both in gaur (*Bos gaurus*) [4] and mouflon (*Ovis orientalis musimon*) [5]. It has not been proved that the obtained clones can reach the adult stage. The cloned Gaur survived for only few days and the cloned mouflon survived for at least 7 months [6], but no more information is available to our knowledge. In spite of this, we attempted a similar approach for the bucardo, using the cryopreserved cells, since cloning is the only possibility to avoid its complete disappearance. This paper presents the results obtained in this study.

2. Materials and methods

Embryos were reconstructed by fusion of epithelial bucardo's cells and enucleated oocytes of domestic goats, and transferred to pure Spanish ibex or hybrids (Spanish ibex × domestic goats).

Unless otherwise specified, all materials were obtained from Sigma–Aldrich. All experimental procedures are in accordance with the current European Directive 86/609/EEC (DOCE number 358).

2.1. Preparation of karyoplasts

Donor cells were derived from a skin biopsy of the last specimen and grown from explants as described in Ref. [7]. The cells grown out of the explants were trypsinized and seeded in new culture dishes after 2 weeks (passage 1). In the following steps, cells were passaged at subconfluency and a portion was frozen for long-term storage. Fibroblasts were characterized by indirect immunofluorescence using an anti-vimentin antibody (V9 clone; Chemicon). All cultures were conducted in DMEM supplemented with 10% foetal calf serum at 38.5 °C and 5% CO_2 in humidified air. Nuclear transfer experiments were performed using cells at passage 3, which were maintained confluent for at least 72 h in DMEM–10% FCS before NT. Cells for NT were collected by trypsinisation and kept in suspension in culture medium at room temperature for 20–120 min before being transferred to the manipulation chamber.

2.2. Superovulation and collection of oocytes

Mature oocytes were collected from domestic goats that were superovulated using highly purified porcine FSH and LH (Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liège, Belgium). Thirty adult, mixed breed goats were synchronized by 45 mg fluorogestone acetate (FGA) sponges (Intervet) during 11 d. Superovulation was achieved with 6 intramuscular doses of pFSH (4, 4, 2, 2, 2 and 2 mg) at 12 h intervals, starting 48 h before sponge withdrawal. Cloprostenol (75 μg; Estrumate, Schering-Plough) was administered at the first FSH injection and two doses of 66 μg pLH were applied at the 5th and 6th FSH injections. Ovulations were synchronized by an intravenous injection of 50 μg of LHRH (SIGMA, L-7134) applied 32 h after sponge withdrawal. Oocytes were collected under general anaesthesia by retrograde flushing of the oviducts with Dulbecco's Modified PBS, supplemented with BSA (2 g l^{-1}), 28–34 h after LHRH injection [8]. Oocytes were transferred to M199 medium supplemented with 10% FCS. Oocytes with attached cumulus cells were denuded by exposure to 0.5 mg ml^{-1} hyaluronidase in M199–HEPES (20 mM)

for 5 min at room temperature followed by gentle pipetting with a small-bore pipette. Denuded oocytes were incubated in M199 medium supplemented with 10% FCS and 0.5 μg ml^{-1} Hoechst 33342 for 20 min before enucleation.

2.3. Nuclear transfer

Enucleation was performed in M199 supplemented with cytochalasin B (5 μg ml^{-1}) and Hoechst dye under an inverted microscope equipped with micromanipulators and epifluorescence. A single fibroblast was injected into the perivitelline space of each enucleated oocyte. Fusion was achieved by application of 2 DC pulses of 2.0 kV cm^{-1} for 50 μs each in 0.3 M mannitol supplemented with 100 μM calcium and magnesium. Fused embryos were incubated in M199 with 10% FCS for 2 h. Activation was then performed by incubation in the presence of ionomycin 5 μM for 5 min. Activated embryos were incubated in M199 with 10% FCS containing 2 mM 6-dimethylaminopurine and cytochalasin B (5 μg ml^{-1}) for 4 h [9]. Reconstructed embryos were co-cultured *in vitro* with Vero cells in microdrops for 36 h (Experiment 2) or 7 d (Experiment 1) in B2 medium (Laboratoire CCD, Paris, France) containing 2.5% FCS.

2.4. Embryo transfer

Embryos were transferred to either pure Spanish ibex ($n = 17$) or to hybrid (Spanish ibex male × domestic goat; $n = 40$) females kept in captivity. Only a part of the viable embryos obtained were transferred, at each experiment depending on the number of ovulating recipient goats available and/or on the morphological quality of the embryos. In Experiment 1 the reconstructed embryos were cultured for 36 h after fusion. Thirty of those cleaved at 3- to 6-cell stage were transferred into the oviduct of 5 recipients (6 embryos per recipient). The remaining embryos were further cultured to day 7 postfusions and 24 of them were transferred into the uterus of 8 recipients at compact morula/blastocyst (3–4 embryos per recipient). In Experiment 2, only embryos cleaved at 36 h postfusion were transferred into the oviduct of 44 recipients at the 3- to 6-cell stage (3–6 embryos per recipient).

Recipients were synchronized using FGA sponges (45 mg; Intervet) for 11 d plus 400 IU of equine chorionic gonadotropin (Intervet) and 50 μg of Cloprostenol 48 h before sponge withdrawal. In the recipients receiving compact morula/blastocysts (Experiment 1), sponges were removed from recipients 40 h later than from oocyte donors to compensate for the slower development of in vitro cultured NT embryos. Embryos were placed ipsilateral to an ovulated ovary using a Tom Cat™ catheter (Sherwood Medical). Pregnancy diagnosis and monitoring was performed fortnightly from about 45 d after transfer to the end of pregnancy, by external ultrasonography and determination of the plasmatic concentration of PAG (pregnancy-associated glycoprotein). Recipients with PAG levels greater than 1 ng/ml were considered pregnant.

2.5. Pregnancy-associated glycoprotein (PAG) analysis

PAG was assayed by the method detailed previously in domestic goats [10]. In the case of standards, 100 μl of the added buffer were replaced by 100 μl plasma from non-pregnant Spanish ibex females to mimic samples and minimize non-specific interferences.

2.6. Chromosome complement

Chromosomal analysis was performed in the Centro de Análisis Genéticos (CAGT), located in Zaragoza, Spain. The number of chromosomes in Pyrenean ibex was determined by conventional Giemsa–trypsin–Giemsa (GTG)-banding in 15 metaphase spreads obtained from blood lymphocytes that were cultured in supplemented RPMI 1430 medium.

2.7. Mitochondrial and nuclear DNA analyses

Analyses were carried out in the Servei Veterinari de Genética Molecular (Universidad Autónoma de Barcelona, Spain). DNA was isolated by standard procedures from donor cells, kidney of the clone, and blood of the recipient and of the oocyte donor goats. A 653 bp mitochondrial D-loop fragment was amplified and sequenced as described previously [11]. Sequences have been submitted to GenBank with the following accession numbers: DQ839377 (bucardo donor cells), DQ839376 (newborn bucardo clone; oocyte donor goat), DQ839374 (hybrid recipient goat) and DQ839375 (control oocyte donor goat). Nine microsatellite markers previously tested in the Spanish ibex [2], were used to test the authenticity of the clone: CSSM66, ETH10, ETH152, ETH225, ILSTS0005, INRA0005, INRA0037, INRA032, and INRA063.

2.8. Pathological studies

The main organs were weighed and photographed. In the case of lung, a standard histopathological analysis was

Table 1
Results transferring embryos at the 3- to 6-cell stage into the oviduct and embryos at blastocyst stage into the uterus (Experiment 1).

Stage	Reconstructed embryos	Cleaved embryos at 36 h (%)[a]	Cultured embryos	Blastocysts (%)	Transferred embryos	No. of recipients	Pregnant recipients (45 d)
3- to 6-cell[b]	285	151 (53.0)	–	–	30[b]	5	2
Blastocysts			121	79 (65.3)	24	8	0

[a] Only embryos with at least 3 cells were considered.
[b] Most of the transferred embryos were at the 4-cell stage.

carried out. Physical body measurements were carried out according to standard procedures in goats [12].

2.9. Statistical analysis

Pregnancy rates were compared by Yates' corrected Chi-square test. PAG levels were compared by the Mann–Whitney test. These tests were carried out using the SAS statistical package [13].

3. Results

After the third passage of the original source of cells, the population was characterized as a homogeneous population of fibroblasts. At confluence, the cells stopped dividing and they were used for nuclear transfer after a period of time at this stage.

In a first series, a group of reconstructed NT embryos was transferred either at the 3- to 6-cells stage in the oviduct or at compact morula/blastocysts stage in the uterus of synchronized recipients (Experiment 1). In this first experiment, pregnancies were achieved only when embryos were transferred at the 3- to 6-cells stage. So, additional attempts using only this stage were performed in four trials (Experiment 2).

In Experiment 1, 301 oocytes were obtained from the 30 operated domestic goats. The mean ovulation rate and oocyte recovery were 12.9 and 79.1 %, respectively. From a total of 285 reconstructed embryos, 209 cleaved after 36 h of culture and 151 (53.0%) reached at least 3 cells. Thirty of the embryos were transferred to the oviduct of recipients. The 121 remaining embryos were cultured to day 7 postfusion and 79 (65.3%) reached the compact morula/blastocyst stage. The 24 morphologically better embryos were transferred to uterus of recipients (Table 1).

In Experiment 2 (Table 2), 497 embryos were reconstituted in four trials and 235 of them (47.3%) cleaved beyond 2-cells stage after 36 h. The 154 morphologically better embryos at the 3- to 6-cells stage were transferred to 44 recipients.

In the compact morula/blastocyst transferred recipients (Experiment 1), no pregnancies were observed. Pregnancy diagnoses were positive in 14.3% (7/49) of recipients receiving 3- to 6-cells embryos (Experiments 1 and 2). Pregnancy rates of pure Spanish ibex and hybryds goats were similar (11.8% vs. 12.5%, respectively; NS).

Pregnancy terminated spontaneously before day 75 in 6 recipients as confirmed by the PAG profiles. Within these recipients, no significant differences were found in PAG values between pure wild and hybrid females (Fig. 1a). In one hybrid goat, pregnancy continued normally until term, displaying levels of PAG about 70 times higher than recipients that terminate their pregnancy prematurely (Fig. 1b). At day 162 postfusion, we performed a caesarean section based on a pregnancy length of about 162 d in *C. p. hispanica* [14,15]. One bucardo female weighing 2.6k was obtained alive, without external morphological abnormalities. Although

Table 2
Results transferring embryos at 3- to 6-cell stage into the oviduct (Experiment 2).

Trials	Reconstructed embryos	Cleaved embryos at 36 h (%)[a]	Transferred embryos (%)[b]	No. of recipients	Pregnant recipients (45 d)	Pregnant recipients (term)
1	107	52 (48.6)	44 (84.6)	11	1	1
2	127	75 (59.1)	24 (32.0)	6	0	0
3	99	45 (45.5)	24 (53.3)	9	3	0
4	164	63 (38.4)	62 (98.4)	18	1	0
Total	497	235 (47.3)	154 (65.5)	44	5	1

[a] Only embryos with at least 3 cells were considered.
[b] Most of the transferred embryos were at the 4-cell stage.

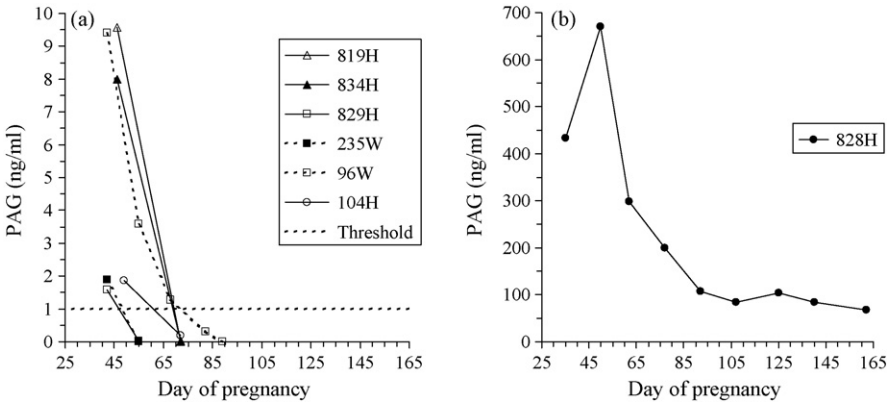

Fig. 1. Concentrations of pregnancy-associated glycoprotein (ng/ml) during pregnancy in goats terminating their pregnancy prematurely (a), and in a hybrid recipient goat delivering a living clone (b). Solid lines: hybrid recipient goats; dashed lines: wild recipient goats; triangles: Experiment 1, morulas; closed circle: Experiment 2, trial 1; squares: Experiment 2, trial 3; open circle: Experiment 2, trial 4; threshold: cut-off PAG value to consider a goat pregnant (1 ng/ml).

Table 3
Biometric parameters of the newborn bucardo female.

Anatomical measurements (cm)		Weight of organs (g)	
Body length	31	Live weight at birth	2,601
Wither height	36	Genital tract	1.4
Rump height	37	Ovaries	0.093
Chest depth	13	Kidney, inc. fat (right)	16.3
Chest width	7.6	Kidney, inc. fat (left)	15.1
Chest girth	28	Kidney (right)	12.7
Shoulder width	8	Kidney (left)	11.7
Rump width	8	Spleen	2.5
Rump length	9	Adrenals	1.0
Fore shin circumference	5.4	Brain, inc. cerebellum	86.8
Hind shin circumference	5.4	Liver	66.6
Head width	7.5	Heart	26.6
Head length	14	Lungs	65.2
Cranium length	10		
Face length	6		

the newborn displayed a normal cardiac rhythm as well as other vital signs at delivery (i.e., open eyes, mouth opening, legs and tongue movements), it suffered from severe respiratory distress after delivery and died some minutes afterwards. At necropsy, we observed atelectasis in the left lung and the occurrence of a supplementary lobe that had its own pleural investment, which was not connected with the tracheobronchial tree. This additional lobe occupied the majority of the thorax virtual space. The rest of the observed organs were normal in appearance (Table 3). No more pathological alterations were observed, either in anatomopathological examinations or in standard haematological studies.

The nuclear DNA study performed with 9 microsatellite markers showed that the newborn bucardo was genetically identical to the bucardo's donor cells and different from the hybrid surrogate mother, the domestic oocyte donor (#113; Table 4) and from

Table 4
Microsatellite analysis of the cloned bucardo, donor cells, recipient and oocyte donor goats. Values represent fragment sizes for both alleles from each microsatellite locus.

Marker	NBF	ABF	SHG	ODG #113	CDG #75424
	Band 1/Band 2	Band 1/Band 2	Band 1/Band 2	Band 1/Band 2	Band 1/Band 2
CSSM66	225/225	225/225	187/219	195/209	209/211
ETH10	199/209	199/209	199/209	207/209	205/205
ETH152	209/209	209/209	199/207	199/199	199/199
ETH225	145/145	145/145	145/147	147/147	147/147
ILSTS0005	176/176	176/176	176/178	178/178	174/186
INRA0005	139/139	139/139	139/141	141/141	137/141
INRA0037	104/104	104/104	104/104	103/103	103/104
INRA032	130/130	130/130	130/142	134/136	134/138
INRA063	153/153	153/153	153/163	163/165	165/165

NBF: newborn bucardo female; ABF: somatic nuclear donor cells from the adult bucardo female; SHG: surrogate hybrid goat; ODG #113: the oocyte donor domestic goat; CDG #75424: a control domestic goat.

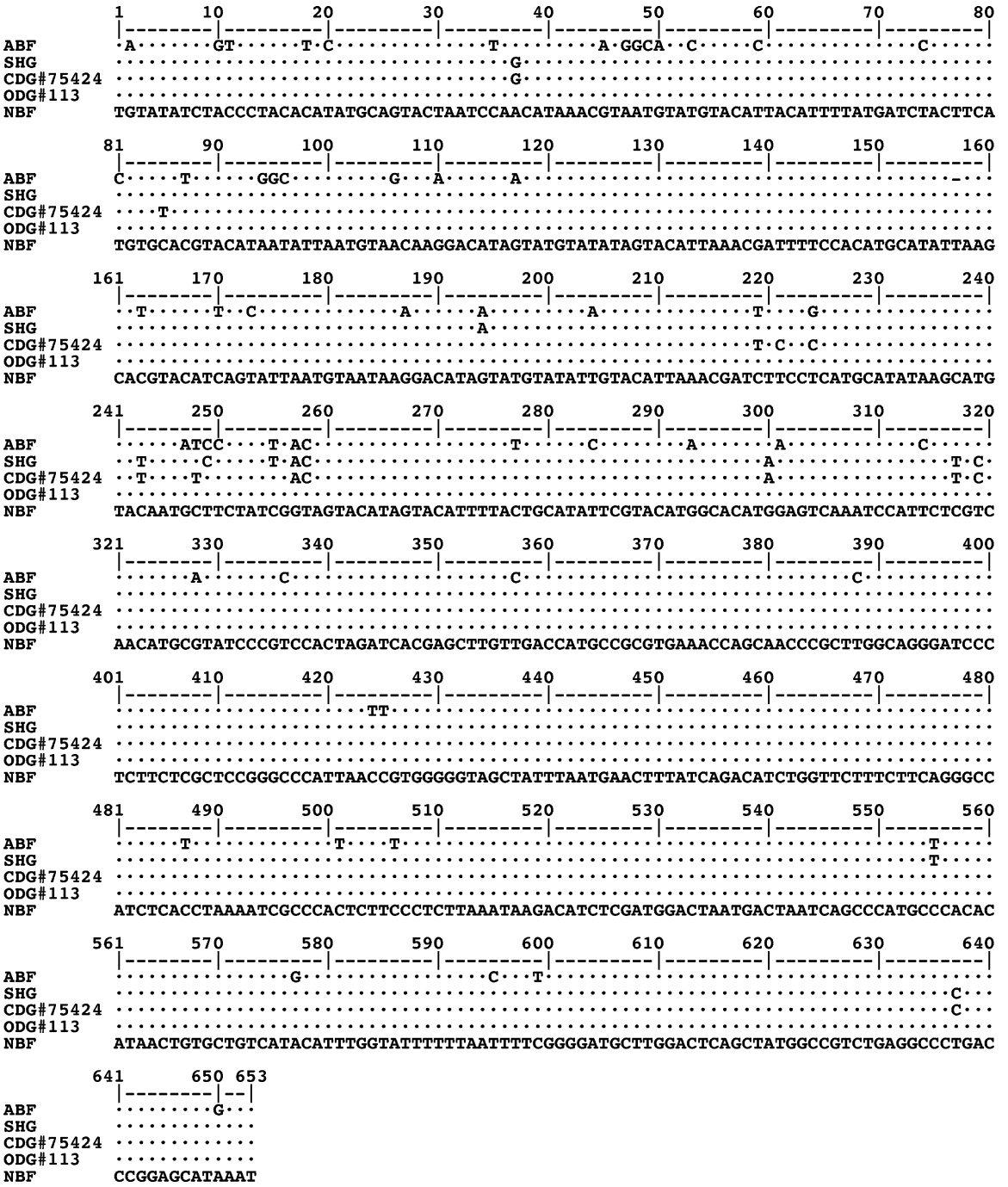

Fig. 2. Mitochondrial DNA analysis of the newborn bucardo female (NBF; the clone), somatic nuclear donor cells from the adult bucardo female (ABF), the surrogate hybrid goat (SHG), the oocyte donor goat (ODG #113), and a control domestic goat (CDG #75424). Sequence alignment of a 653 bp fragment in the D-loop region. Dots indicate identity with the clone (NBF).

another oocyte donor domestic goat included as control (#75424; Table 4). Mitochondrial DNA studies revealed 100% identity only between the newborn bucardo female and its oocyte donor domestic goat, as expected (Fig. 2).

4. Discussion

Many attempts have been achieved to multiply endangered species of ruminants by NT embryos transferred to domestic females. Some of them, such

as argali [16] and banteng [17], resulted in pregnancies. In the case of gaur [4] and mouflon [5], pregnancies went on to term. In these interspecific cloning experiments, both, the oocyte donors and surrogate mothers were females from domestic species whose phylogenetic distance and pregnancy duration were close to the karyoplast donors. On the other hand, in our study although the different Spanish ibex subspecies have identical number of chromosomes as the domestic goat ($2n = 60$), genomic DNA studies showed that the bucardo was a monophyletic subspecies [2]. Even though the phylogenetic closest subspecies to bucardo is the Spanish ibex, *C. p. hispanica*, we used domestic goats as oocyte donors due to the difficulty to capture and maintain wild goats in captivity in sufficient numbers.

Oocytes were obtained surgically from the oviduct after a superovulatory treatment. Although a great number of oocytes can be obtained either by ovum pick-up or from slaughterhouse ovaries, we used *in vivo* matured oocytes since it has been reported that *in vitro* culture leads to a sub-optimal maturation resulting in a loss of embryo viability after fertilization [18]. The treatment, consisting in a combination of decreasing doses of highly purified FSH with an injection of LH at the end of the progestagen treatment, has been successfully used in superovulation of domestic goats for embryo transfer purposes [19]. The association of an injection of LHRH, 32 h after the sponge removal, improved the synchronization of the ovulations and the homogeneity of the recovered oocytes.

A percentage of reconstructed embryos did not develop after cleaving as previously reported in domestic goats after nuclear transfer from fibroblasts [20]. In addition, the rate of embryos reaching the blastocyst stage in the present study are similar to that reported in the interspecific cloning of the mouflon [5].

The use of domestic goats as recipients was rejected because its length of gestation (150 d) is quite different from Spanish ibex (162 d) [21]. In previous studies we found that the Spanish ibex embryos transferred to domestic goats resulted in pregnancies to term only when the ibex embryo shared the uterus with a domestic goat embryo [14]. On the other hand, hybrids (Spanish ibex × domestic goat female) were able to bring successfully to term transferred Spanish ibex embryos [15]. In the present study we used both, Spanish ibex and hybrids as foster-mothers, resulting in similar pregnancy rates between genotypes. Since hybrids are less stressful and cheaper, present results suggest the suitability of this genotype as recipients of the NT bucardo embryos.

In our experiment, reconstructed embryos were cultured *in vitro* for 36 h or 7 d before transfer. In goats, NT embryos are usually transferred to recipients at day 2 (4-cell stage) [22,23]. However, *in vitro* culture to the blastocyst stage permits better embryo selection, transferring a lower number of embryos per recipient. Moreover, embryo transfer at the compact morula–blastocyst stage to the uterine horn is easier and faster than to the oviduct after laparotomy. Finally, previous experiments performed in domestic goats showed that the number of born kids from NT embryos transferred at blastocyst stage were significantly higher than at the 4-cells stage (6.2 vs. 0.7 kids born per 100 transferred embryos, respectively) [9]. Unlike what was expected, in the present work no pregnancies were achieved using blastocyts. Nevertheless our results do not prove a better efficiency of the oviductal transfer, since our experiment is not comparable with that previously reported [9] in terms of experimental animals or techniques. It is possible that one cause of embryo mortality was a lack of synchronization between the physiological stage of the embryo and the recipient uterus, as there is scarce information about the characteristics of the sexual cycle of the Spanish ibex. We know that it is a seasonal polyestric species whose oestrus appears every 19.6 d as mean [21], but no information is available about the endocrinology along the cycle.

Six recipients lost the embryos before day 75. Some authors reported early abortions in cow and sheep, frequently associated with functional deficiencies occurring at the onset of placentation [24]. The seventh recipient maintained pregnancy to term, displaying high levels of plasmatic PAG, mainly in the early pregnancy. Similar results have been recorded in interspecific embryo transfer between domestic goats and Spanish ibex [14]. Although the role of PAG in recipients of NT embryos it is not known, it has been reported that in pregnancies of NT bovine embryos high PAG levels are not associated with pregnancy loss and do not preclude a normal full term development in this species. In fact, recipients with higher PAG levels have more chances to go to term [25].

The delivered kid was genetically identical to the bucardo, disregarding the influences of mitochondrial DNA from the oocyte of the domestic donor and of the uterine environment of the hybrid recipient. The newborn displayed serious respiratory distress and died few minutes after the caesarean section. In other species, postnatal losses at birth or during the first few days of life are associated with prolonged gestation, dystocia or occurrence of large offspring syndrome [26]. The birth weight of the cloned *C. p. pyrenaica*

(2.6k) did not exceed the mean birth weight plus 2 standard deviations (2.9k) reported for *C. p. hispanica* (1.9 ± 0.5k; mean ± S.D.) [21], thus suggesting that the large offspring syndrome did not occur and that the birth weight of the clone can be considered normal. At necropsy all the organs appeared normal except lungs. Physical defects in the lungs as well as in other organs have also been reported in neonatal cloned sheep that failed to survive [27].

To our knowledge, this is the first animal born from an extinct subspecies and the first successful interspecies adult somatic cell nuclear transfer in the Capra genus. Our results show that enucleated oocytes from domestic goats support mitotic cleavage of bucardo karyoplasms and that the bucardo somatic cells stored in liquid nitrogen can be reprogrammed to achieve embryo development and offspring. The efficiency of this process is quite similar to that attained in interspecific cloning experiments in others species. Moreover, this study demonstrates that the use of hybrid foster mothers may be considered to be used in projects undertaken to rescue endangered or extinct species.

At present it can be assumed that cloning is a not very effective way to preserve endangered species, because the complexity to handling the experimental wild animal and the insufficient knowledge on both, the cellular mechanisms involved in the technique and on the reproductive characteristics of the animals [28–30]. However, in species as bucardo, cloning is the only possibility to avoid its complete disappearance. The present work encourages to appropriately store somatic tissues and cells of all endangered species or suitable animals, as they may be useful for future cloning-based conservation programs.

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