Complete Replacement of the Mitochondrial Genotype in a *Bos indicus* Calf Reconstructed by Nuclear Transfer to a *Bos taurus* Oocyte

Flávio V. Meirelles,* Vilceu Bordignon,† Yeda Watanabe,* Michelle Watanabe,‡ André Dayan,‡ Raysildo B. Lôbo,* Joaquim M. Garcia‡ and Lawrence C. Smith†

†*Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Quebec J2S 7C6, Canada,* **Departamento de Genética, Faculdade de Medicina-USP, Ribeirão Preto, SP 14049-900, Brazil and* ‡*Departamento de Reprodução Animal, Faculdade de Ciências Agrárias e Veterinárias-UNESP, Jaboticabal, SP 14884-900, Brazil*

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ABSTRACT

Due to the exclusively maternal inheritance of mitochondria, mitochondrial genotypes can be coupled to a particular nuclear genotype by continuous mating of founder females and their female offspring to males of the desired nuclear genotype. However, backcrossing is a gradual procedure that, apart from being lengthy, cannot ascertain that genetic and epigenetic changes will modify the original nuclear genotype. Animal cloning by nuclear transfer using host ooplasm carrying polymorphic mitochondrial genomes allows, among other biotechnology applications, the coupling of nuclear and mitochondrial genotypes of diverse origin within a single generation. Previous attempts to use *Bos taurus* oocytes as hosts to transfer nuclei from unrelated species led to the development to the blastocyst stage but none supported gestation to term. Our aim in this study was to determine whether *B. taurus* oocytes support development of nuclei from the closely related *B. indicus* cattle and to examine the fate of their mitochondrial genotypes throughout development. We show that *indicus:taurus* reconstructed oocytes develop to the blastocyst stage and produce live offspring after transfer to surrogate cows. We also demonstrate that, in reconstructed embryos, donor cell-derived mitochondria undergo a stringent genetic drift during early development leading, in most cases, to a reduction or complete elimination of *B. indicus* mtDNA. These results demonstrate that cross-subspecies animal cloning is a viable approach both for matching diverse nuclear and cytoplasmic genes to create novel breeds of cattle and for rescuing closely related endangered cattle.

ACCORDING to the classical Linnean nomenclature there are two types of cattle, *Bos indicus* (humped or zebu) and *Bos taurus* (humpless). Sequence comparisons of a hypervariable region of their mitochondrial DNA (mtDNA) have shown that the *taurus* and Asian *indicus* breeds differ on average by ~8%, suggesting that divergence occurred between 0.2 and 1 million years ago (Loftus *et al.* 1994). It is likely that mtDNA polymorphisms played a significant role through natural selection in the adaptation of different cattle types to regional environmental conditions. Since mtDNA is inherited almost exclusively through the maternal lineage, mitochondrial genotypes can be exchanged among interfertile species by continuous backcrossing to purebred males and by gradually modifying nuclear genes at each generation without altering the matrilineal mitochondrial genotype (Gyllensten *et al.* 1985). This gradual replacement occurred during the introduction of *B. indicus* breeds to America when imported Indian bulls were mated to indigenous cattle of European origin to produce the current American zebu carrying mostly *B. taurus* mtDNA (Meirelles *et al.* 1999).

A faster means of replacing the mtDNA genes in animals is to perform oocyte reconstruction by nuclear transfer. Analysis of Dolly, the first sheep cloned from a somatic cell (Wilmut *et al.* 1997), indicated that the mtDNA differed from that of the progenitor mammary gland cell, indicating that its mitochondria were derived entirely from the ewe from which the host oocyte was obtained (Evans *et al.* 1999). Cattle clones have recently been shown to contain minute amounts of mtDNA originating from the cell used as the nuclear donor, indicating that low levels of mitochondrial heteroplasmy may occur in some nuclear transfer progeny derived from somatic cells (Steinborn *et al.* 2000). Larger amounts of donor-derived mtDNA have been observed in blastomere-derived clones, possibly due to the quantity of donor cell cytoplasm present at reconstruction (Steinborn *et al.* 1998; Hiendleder *et al.* 1999; Takeda *et al.* 1999). The latter reports describe experiments using nuclear and cytoplasm donor animals with similar nuclear and mitochondrial genetic systems.

Previous attempts to use *B. taurus* oocytes as hosts to transfer nuclei from unrelated species led to development to the blastocyst stage but none supported gestation to term. Since viable human xenomitochondrial

Corresponding author: Lawrence C. Smith, Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, 3200, rue Sicotte CP 5000, Saint-Hyacinthe, PQ J2S 7C6, Canada. E-mail: smithl@medvet.umontreal.ca

cybrids can be established harboring mtDNA from gorillas and chimpanzees, but not from farther apart primate species (Kenyon and Moraes 1997; Barrientos *et al.* 1998), it is possible that incompatibility among the nuclear and mitochondrial genetic systems is partially responsible for the developmental arrest observed in cross-species reconstructed embryos. The current study was designed to establish whether nuclei from *B. indicus* cattle can support development after reconstruction using host oocytes from a *B. taurus* breed. The second goal was to determine the segregation patterns of *B. indicus* mtDNA at different stages during development to term. We demonstrate that viable embryos and offspring can be obtained after cross-subspecies embryo reconstruction and that the percentage of *B. indicus*-derived mtDNA decreases during development. Moreover, we show that a stringent mtDNA segregation step occurs during preimplantation development leading, in most cases, to the partial or complete elimination of donor cell-derived mtDNA.

MATERIALS AND METHODS

Source of oocytes and embryos: *B. taurus* oocytes were obtained by postmortem follicular aspiration of ovaries from Holstein cows slaughtered at a local abattoir and transported in saline at 35° to the laboratory within 2 hr after slaughter. Follicles with diameters between 2 and 8 mm were punctured with a 19-gauge needle and cumulus oocyte complexes (COCs) with several layers of cumulus cells and homogeneous cytoplasm were washed in HEPES-buffered tissue culture medium (TCM-199; GIBCO BRL, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal calf serum (FCS; GIBCO). Groups of 20 COCs were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 50 μg ml^{-1} luteinizing hormone (Ayerst, London, Ontario, Canada), 0.5 μg ml^{-1} follicle stimulating hormone (Folltropin-V; Vetrepharm, St.-Laurent, PQ, Canada), 1 μg ml^{-1} estradiol 17-β (Sigma, St. Louis), 22 μg ml^{-1} pyruvate (Sigma) and 50 μg ml^{-1} gentamicin (Sigma). After 24 hr of maturation *in vitro*, oocytes were *in vitro* fertilized (IVF) using standard protocols. Briefly, COCs were placed in 50-μl drops of Tyrode's medium, supplemented with 0.6% bovine serum albumin (BSA; Fraction V, Sigma), lactate, pyruvate, gentamicin, and 10 μg ml^{-1} of heparin (Parrish *et al.* 1986). Frozen-thawed spermatozoa were washed and centrifuged through a Percoll gradient and diluted at 10^6 live spermatozoa ml^{-1}.

Oocytes carrying *B. indicus* mtDNA were obtained by *in vivo* follicular aspiration of Nellore cows with registered pedigree (Pure of Imported Origin) and confirmed mtDNA typing was performed by restriction analysis (Meirelles *et al.* 1999). Briefly, leucocyte-derived genomic DNA was extracted from blood samples using standard protocols (Sambrook *et al.* 1989) and three different regions of the mtDNA were amplified by PCR to ascertain that only animals carrying mtDNA of *B. indicus* origin were used in this study. COCs were recovered by ultrasound-guided follicular aspiration using an echographic apparatus with a 5-mHz convex probe. After recovery, COCs were selected and transported to the laboratory for *in vitro* maturation and fertilization with frozen semen from a *B. indicus* bull using procedures as described above. After fertilization, presumed zygotes were transferred to 50-μl drops of Menezo's B2 medium (MB2; Pharmascience, Paris) supplemented with 10% FCS in the presence of bovine oviductal epithelial cells. All the cultures above were performed in drops under equilibrated mineral oil at 39° in a humidified atmosphere of 5% CO_2 in air.

Microsurgical reconstruction and embryo transfer: Oocyte reconstruction was performed using standard procedures. At 30 hr after maturation, *B. taurus* oocytes were stripped of cumulus cells by vigorous shaking and selected for the presence of a first polar body. Parthenogenetic activation was achieved by exposure of *in vitro* matured oocytes to 5 μM ionomycin (Sigma) for 4 min in TCM-199 HEPES-buffered medium, supplemented with 2 mg/ml^{-1} BSA, and washed and cultured for an additional 2 hr for second polar body extrusion (Powell and Barnes 1992). Activated oocytes were enucleated at the telophase II stage by removing a small portion of cytoplasm surrounding the position of the second polar body as described previously (Bordignon and Smith 1998). *B. indicus* embryos were transported to the micromanipulation laboratory and a single blastomere derived from morula at day 5 after IVF was injected into the perivitelline space of the enucleated *B. taurus* oocyte. The resulting couplet was placed in a 0.3 M mannitol (Sigma) solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.5-kV electrical pulse of 70 μsec. After electrical stimulation, oocytes were washed in PBS and cultured in MB2 in the presence of bovine epithelial oviductal cells. Successfully reconstructed zygotes were cultured for 7 days to the blastocyst stage. Reconstructed embryos were recovered at different stages during development for mtDNA analysis. Some blastocysts were transferred to synchronized recipient heifers to obtain fetuses and live offspring after gestation to term.

mtDNA analysis of embryos, fetuses, and offspring: The content of *B. indicus* and *B. taurus* mtDNA in all samples was analyzed by PCR-restriction fragment length polymorphism. Reconstructed oocytes, embryonic blastomeres, and whole morula or blastocysts were isolated, washed in saline, placed in 10 μl of autoclaved water and stored at −70° until PCR amplification. Fetal and offspring tissues were digested with pronase before extraction of genomic DNA using standard protocols (Sambrook *et al.* 1989). The mtDNA ratios were assessed from 746-bp PCR amplification fragments comprising the 16SRNA gene of the mtDNA (positions 1694 to 2440, according to Anderson *et al.* 1982). The oligonucleotides used for PCR amplification were BosmtF3: 5′-GCCCGAAACCA GACGAGCTAC-3′ and BosmtR3: 5′-CCTCGTGCGGCCATT CATACAA-3′. The PCR reaction mixture consisted of 100 pmol and 120 pmol of the above oligonucleotide primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 μM each dGTP, dATP, dTTP, and CTP (dNTP; Pharmacia, Piscataway, NJ), and 1.25 units of *Taq* polymerase (Pharmacia) in a volume of 50 μl. PCR was performed for 35 cycles, each consisting of denaturation for 45 sec at 94°, annealing for 55 sec at 58°, and extension for 120 sec at 72° using a thermocycler (TwinBlock EasyCycler, Ericomp, San Diego). The amplified fragments were digested for 2 hr at 37° with 5 units of *Hpa*I added directly to 20 μl of the PCR product. The amplified region contains one nonspecific (*B. taurus* and *B. indicus*) *Hpa*I site at position 2218 and one *B. indicus*-specific *Hpa*I site at position 2043. Therefore, *Hpa*I digestion yielded two bands of 522 bp and 224 bp in *B. taurus* mtDNA and three bands of 347 bp, 224 bp, and 175 bp in *B. indicus* mtDNA. Although nondigested fragments (746 bp) were rare, these could be separated from digested fragments and, therefore, did not interfere with the quantification of *B. taurus* and *B. indicus* mtDNA. *Hpa*I digestion products were loaded onto 1% agarose gels and stained with ethidium bromide. When *B. indicus* mtDNA was not detected in agarose gels, a further sample was separated on 10% acrylamide gels and stained using the

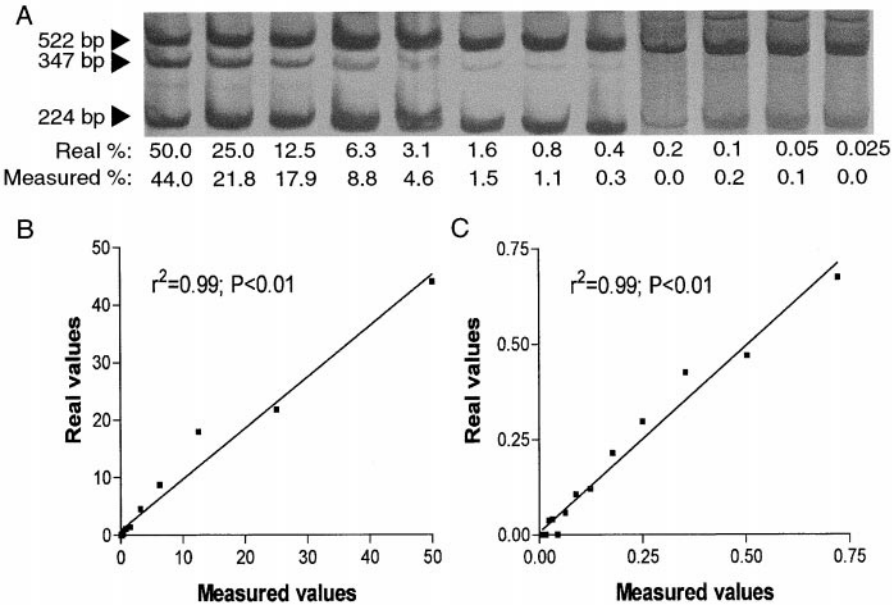

FIGURE 1.—(A) Silver-stained acrylamide gel showing decreasing ratios of *B. indicus:B. taurus* mtDNA. Values are provided for amount loaded on the gel (Real %) and measurements obtained by densitometry analysis (Measured %). The staining procedure utilized allowed densitometric and visual identification of *B. indicus* specific band at 0.05% and higher concentrations. Regression analysis indicates a high and significant coefficient of determination (r^2) using both (B) percentage and (C) transformed data (arc sine of proportions).

silver staining method. Visual assessment of the silver-stained gel allowed the detection of <1% *B. indicus* mtDNA in the amplified samples (Figure 1), indicating that the level of sensitivity of our technique was below 1%. Ratios of *B. indicus:B. taurus* mtDNA content were calculated by densitometric quantification of the 347-bp (*B. indicus*) and 522-bp (*B. taurus*) bands and corrections for molecular weights (Image Gauge 3.12; Fuji Photo Film, Tokyo).

RESULTS

***B. indicus* mtDNA content in nuclear transfer embryos:** Oocytes were recovered from three purebred Nellore cows by ultrasound-guided follicular aspiration in which the *B. indicus* origin of mtDNA had been verified. Oocytes were matured, fertilized, and cultured *in vitro* to obtain morula-stage donor embryos for nuclear transfer. A total of 110 reconstructed oocytes were produced by the fusion of *B. indicus* blastomeres to enucleated oocytes carrying *B. taurus* mtDNA. Sixty-one embryos were recovered at different stages of development and analyzed either as whole embryos (one-cell, morula, and blastocysts) or disaggregated and analyzed as single blastomeres (two- to eight-cell stages). Fifty-three percent of the reconstructed embryos cleaved at 24 hr and 21% developed to morula/blastocyst stage at day 7 after nuclear transfer. Similar rates of development are obtained routinely from within *B. taurus* reconstructed embryos, indicating that the development to blastocyst was not affected by *indicus:taurus* reconstructions.

In general, no changes were observed in the proportion of *B. indicus* mtDNA during the period in which embryos were cultured to the eight-cell stage, indicating a lack of segregation of blastomere-derived mitochondrial genotypes during cleavage to the fourth cell cycle (Figure 2). With the exception of an outlier embryo, a consistent decrease in the percentage of *indicus* mtDNA was observed after the eight-cell stage. Closer analysis of mtDNA ratios among morula and blastocysts indicated the occurrence of a genetic drift at this stage of development where, with the exception of one blastocyst that contained 63% *indicus* mtDNA, most embryos contained little or no *indicus* mtDNA (Figure 2 inset). These results indicate that passage from eight cells to morula in reconstructed embryos involves a change in mtDNA populations leading to a stringent mitochondrial genetic drift.

***B. indicus* mtDNA content in reconstructed fetuses**

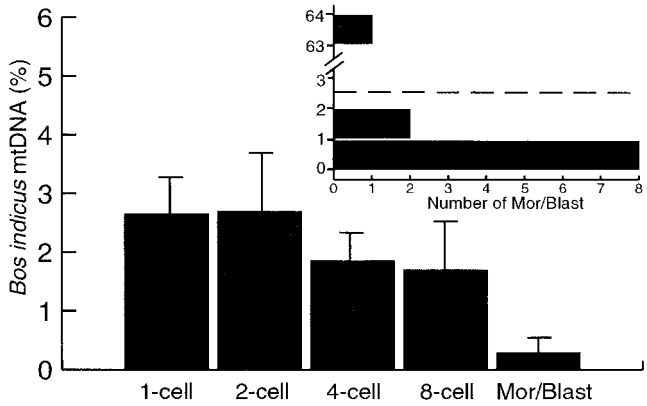

FIGURE 2.—Levels of mtDNA heteroplasmy in reconstructed embryos at different stages of early development. Bars represent the average percentage content of *B. indicus*-derived mtDNA and vertical lines are standard errors of the means. The inset graph shows the distribution of morula- and blastocyst-stage reconstructed embryos according to *B. indicus* mtDNA content. The dashed horizontal line indicates the amount of *B. indicus* mtDNA present at the time of oocyte reconstruction. Note that most morulae and blastocysts have either no (homoplasmic) or very little *indicus* mtDNA whereas one blastocyst contained 63% *indicus* mtDNA.

and offspring: A total of eight reconstructed blastocysts were transferred to two recipient heifers to generate gestations. One recipient was slaughtered at 55 days of gestation to obtain fetal tissue for analysis, whereas another recipient was allowed to carry gestation to term to obtain live offspring. The fetus was recovered from the uterine horn and dissected to obtain both extraembryonic and embryonic tissues. On average 2.7% mtDNA of *B. indicus* origin was present in extraembryonic tissues (0.9% in chorium, 1.8% in allantois, 2.5% in amnion, and 4.7% in the umbilical cord) and embryonic tissues (0.6% in brain, 0.8% in lungs, and 3.6% in heart). The average amount of donor cell-derived mtDNA observed at 55 days of gestation was similar to that introduced at nuclear transfer in reconstructed oocytes, suggesting a random genetic drift of mitochondrial genotypes during development. Moreover, these data indicate that *B. taurus* mtDNA remains viable throughout early fetal development but is not selectively replicated or eliminated when exposed to a *B. indicus* nuclear genotype.

Zebulon, the first calf to be produced by reconstruction among different cattle types, was obtained by caesarean section at 288 days of gestation and showed all the phenotypic characteristics of a naturally bred Nellore calf (Figure 3A). Although healthy and normal at birth, Zebulon showed a minor deviation of the nasal septum (Figure 3B) and a persistent urachus, which was surgically corrected at 4 weeks after birth. It is unclear whether these features are natural occurrences or anomalies associated with the technique of embryo reconstruction. Biopsies obtained from several extra-embryonic and embryonic organs and tissues were analyzed to determine mtDNA content. Using a screening procedure able to detect up to 0.05% mtDNA, none of the tissues examined contained detectable traces of *B. indicus* mtDNA (Figure 3C), indicating that the mitochondria from the donor cell were eliminated at some stage during embryo or fetal development. Since most embryos examined contained between 0 and 0.005% *B. indicus* mtDNA at the morula and blastocyst stages, it is likely that Zebulon originated from the transfer of a blastocyst containing none or very few *B. indicus* mtDNAs.

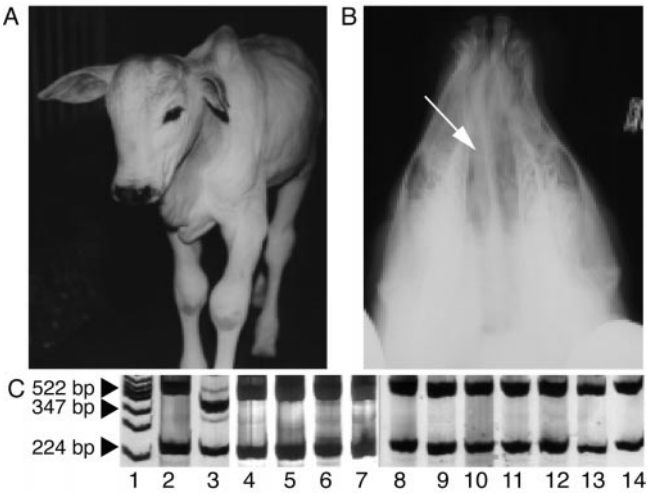

FIGURE 3.—(A) Zebulon, the first Zebu calf born from a reconstructed embryo derived by fusion of *B. indicus* morula-stage blastomere to a *B. taurus* enucleated oocyte. (B) X-ray image of the head of the calf showing a slight deviation of the nasal septum (arrow); (C) Silver-stained polyacylamide gel showing the mitochondrial DNA derived by PCR amplification and digestion with *Hpa*I. Lanes show (1) molecular marker, (2) *B. taurus*, (3) *B. indicus*, (4) umbilical cord, (5) cotyledon, (6) chorium, (7) allantois, (8) leukocytes, (9) muscle, (10) lymph node, (11) spleen, (12) reticulum, (13) bladder, and (14) peritoneum. No trace of *B. indicus* mtDNA was observed in any tissue analyzed either visually or by densitometry.

DISCUSSION

Herein we report the first successful nuclear transfer across subspecies barriers and present Zebulon, a cloned Nellore calf carrying exclusively *B. taurus* mitochondria derived from the host oocyte. Previous attempts to reconstruct animals between species have led to limited success. In mice, reciprocal exchange of pronuclei among zygotes from *Mus caroli and M. musculus* species led to developmental failure during preimplantation (SOLTER *et al.* 1985). In cattle, enucleated host oocytes have been fused to fibroblast cells from sheep, pig, monkey, and rat in an attempt to assess the viability after cross-species zygote reconstruction (DOMINKO *et al.* 1999). Although initial reprogramming of the donor nucleus occurred, including nuclear swelling and completion of first mitotic cleavage and blastocyst formation, all cross-species embryos transferred to suitable recipients failed to establish pregnancies. In this study neither blastocyst development nor pregnancy establishment were affected with *B. indicus:taurus* reconstructed embryos, indicating not only normal embryonic and fetal viability of cross-subspecies reconstructed embryos but also that *B. indicus* nuclear-encoded replication factors can successfully bind to and replicate *B. taurus* mtDNA.

Indian-derived *B. indicus* and *B. taurus* mtDNA diverge by ~5% in the control region (D-loop) and by 8% in the most hypervariable region of the D-loop (LOFTUS *et al.* 1994). However, putative sequences that allow the binding of nuclear-encoded factors to mtDNA are highly conserved between *B. indicus* and *B. taurus* mtDNA, suggesting compatible interaction between *B. indicus* nuclear-encoded *trans*-acting factors and the *B. taurus* mtDNA replicative machinery. Although the cause of the shift to homoplasmy during embryogenesis is unknown, it is possible that only a limited pool of mitochondria undergoes replication at this stage and that it makes up the majority of mtDNAs at the morula and blastocyst stages. Since no change in the total amount of mtDNA is observed until after the blastocyst

stage (Piko and Taylor 1987), it is tempting to propose that the shifts in ratios of mtDNA are caused by a turnover of mitochondria. Mitochondrial turnover could occur through the replication of a small pool of mtDNAs contained within metabolically active organelles concomitant with the elimination of nonreplicating organelles. Such a phenomenon could lead to a genetic bottleneck, which would explain the homogeneity of mitochondrial genotypes in most animals regardless of the high mutation rate observed in mtDNAs (Brown *et al.* 1979). Nonetheless, it remains to be determined whether this preimplantation mitochondrial genetic drift occurs in naturally occurring heteroplasmic lineages in cattle and other mammals.

Different patterns of segregation stringency have been observed in mouse heteroplasmic matrilineages obtained by karyoplast and cytoplast transfer using polymorphic mtDNA from the NZB strain (Jenuth *et al.* 1996; Meirelles and Smith 1997). Indeed, segregation patterns in a heteroplasmic lineage throughout a 15-generation period have indicated stable maintenance of heteroplasmy levels (our unpublished results). Analysis of mtDNA ratios during early development indicated that karyoplast-derived mitochondria were preferentially replicated at the blastocyst stage, suggesting that the perinuclear positioning of mitochondria in the reconstructed zygote may lead to replicative advantage during early embryogenesis (Meirelles and Smith 1998). Mitochondrial replicative advantage has also been observed in heteroplasmic mice derived using mtDNA from the C57 and RR strains (Takeda *et al.* 2000). In the latter, however, RR mtDNA was preferably replicated regardless of the nuclear background, suggesting a pattern similar to the one reported in this study. A reciprocal experiment in which *B. taurus* cells are fused to enucleated *B. indicus* oocytes will be necessary to determine whether *B. taurus* mtDNA has a replicative advantage over *B. indicus* mtDNA regardless of the nuclear background. Such a replicative advantage during the intromission of *B. indicus* cattle into Africa could explain the prevalence of *B. taurus* mtDNA in the African Zebu cattle (Loftus *et al.* 1994).

This investigation supports the feasibility of closely related cross-subspecies nuclear transfer to rescue endangered species. However, it will nevertheless be necessary to ascertain that the mtDNA of the endangered species is also rescued to derive offspring with the original mitochondria. A strategy to accomplish this may be to microinject mitochondria obtained from somatic cells into reconstructed oocytes, as shown to produce heteroplasmic mice (Irwin *et al.* 1999). Moreover, since most segregation has occurred by the morula stage, blastocyst biopsy could be performed to screen and select heteroplasmic embryos before transfer to surrogate females. Nonetheless, cattle carrying mtDNA from a different species may have certain advantages over animals with homoplasmic genotypes. If so, the creation of novel synthetic cattle breeds with complementary mitochondrial and nuclear systems, as described in this article, may be used to improve both the productivity and adaptability of domestic animals.

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