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The Fibroblast Cryobank of Wild Mammals of Kazakhstan and Interspecies Somatic Cell Nuclear Transfer of Snow Leopard (*Panthera uncia*)

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Simple Summary

Living cells stored in liquid nitrogen can keep the genetic material of rare animals for decades and may later be used in research or to produce embryos. Freezing, however, kills part of the cells, and species differ in how well their cells survive it. We froze skin cells (fibroblasts) of 19 wild mammal species from Kazakhstan by the same method and counted live cells before and after thawing. We also measured the proliferation rate of the cells in culture before freezing and after thawing. Cells of small rodents survived best, while cells of large animals such as deer (*Cervus elaphus bactrianus* Lydekker, 1900), saiga (*Saiga tatarica* Linnaeus, 1766), kulan (*Equus hemionus* Pallas, 1775), and porcupine (*Hystrix indica* Kerr, 1792) survived worst, even though more of their cells were alive before freezing. Thawed cells of all species could still multiply, although more slowly than before freezing. Thawed snow leopard (*Panthera uncia* Schreber, 1775) cells were then transferred into domestic cat oocytes, and in preliminary experiments, almost a third of the reconstructed embryos developed to the morula stage. The results help to build a cryobank of wild animal cells and suggest that freezing protocols for some species need to be adjusted.

Abstract

Cryobanks of living somatic cells support ex situ conservation, but little is known about how cells of wild mammals from Central Asia tolerate freezing. We compared the viability of skin fibroblasts from 272 individuals of 19 species and subspecies (11 families) before and after programmable slow freezing with 1.5 M of dimethyl sulfoxide (DMSO), using propidium iodide staining and image-based cytometry. Viability fell from 83.1 ± 1.8% to 48.0 ± 1.6% (species means ± SEM). Post-thaw viability was highest in hamsters, voles, and murid rodents and lowest in the porcupine, deer, and kulan, and pre-freeze viability did not predict it. Thawed cells of all species proliferated but doubled 2–9 h more slowly ($p < 0.001$). Snow leopard (*Panthera uncia* Schreber, 1775) fibroblasts were used as nuclear donors for interspecies handmade cloning with domestic cat oocytes. Bisection of 238 oocytes yielded 162 cytoplasts (68.1%), 33 of 78 triplets fused (42.3%), and 10 of 33 embryos (30.3%) developed to the morula stage by day 5. A single DMSO protocol allowed all

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19 taxa to be banked, but post-thaw viability was low in several species, and the protocol needs species-specific optimization.

Keywords: cryopreservation; fibroblasts; cell viability; dimethyl sulfoxide; image-based cytometry; snow leopard; *Panthera uncia*; handmade cloning; interspecies nuclear transfer; cryobank

1. Introduction

Field conservation of rare species is increasingly supported by keeping their genetic material ex situ. Genome resource banks store gametes, embryos, tissues, and cell cultures, and this reserve can be used to manage diversity in small populations [1,2]. Living cell cultures can be expanded after thawing, so they offer more than deoxyribonucleic acid (DNA) samples or fixed tissue [3,4]. Such collections should be built while animals of rare species are still available for sampling [4,5].

Skin fibroblasts suit this purpose well. A small piece of ear skin can be taken during capture or a veterinary examination, the cells are isolated by explant culture, and they grow readily in vitro [6–8]. Cryopreserved fibroblasts supply nuclei for nuclear transfer, including transfer between species [9,10]. With domestic cat oocytes as recipients, this approach produced cloned African wildcat kittens and embryos of other wild felids [11–13]. Fibroblasts are also the starting material for induced pluripotent stem cells, which have been derived from the snow leopard as well [14,15]. Somatic cell banks are regarded as one of the main tools for conserving the gene pool of wild cats [16,17].

Freezing damages cells in two ways. If cooling is too fast, ice forms inside the cell; if it is too slow, the cell suffers from osmotic stress and high salt concentration [18,19]. The optimal cooling rate depends on cell size and on membrane permeability to water and cryoprotectant [19–21], and these properties vary among species. Species differences in cryotolerance are well documented for spermatozoa [22]. In wild mammals, fibroblasts of different species have been shown to respond differently to the same cryoprotectant solutions [23] and to grow at different rates in culture [24], but comparisons of many taxa under one protocol are still rare.

In Kazakhstan, a cryobank of somatic cells of wild animals is being built by the Laboratory of Cryobiology and Germplasm Biobank of Wild Animals at the Institute of Zoology. Among mammals, we have previously studied the viability of saiga and snow leopard fibroblasts under different cryopreservation methods [25,26], of Bukhara deer (*Cervus elaphus bactrianus* Lydekker, 1900) fibroblasts [27], and of Altai lynx fibroblasts [28], and developed methods for cryopreserving snow leopard fibroblasts [29,30]. The snow leopard is listed in the Red Data Book of Kazakhstan and as Vulnerable on the Red List of the International Union for Conservation of Nature (IUCN) [31]. We use it as a model both for cryopreserving somatic cells of large felids and for using these cells in interspecies cloning.

The aim of this work was to compare the viability of skin fibroblasts of 19 species and subspecies of wild mammals of Kazakhstan before and after programmable slow freezing with dimethyl sulfoxide (DMSO) and to relate differences in cell survival to taxonomic affiliation. Using the snow leopard as an example, we also tested whether cryopreserved fibroblasts can serve as nuclear donors for interspecies cloning.

2. Materials and Methods

2.1. Animals and Sampling

Fibroblasts were obtained from ear skin of 272 individuals of 19 mammalian species and subspecies from 11 families and 6 orders (listed in Section 3.2). Some of these taxa also appear in the literature under other names: *Allactaga elater* for *Scarturus elater*, *Felis manul* for *Otocolobus manul*, and *C. hanglu bactrianus* for *Cervus elaphus bactrianus*. Samples of 17 taxa came from wild populations in Kazakhstan, and those of the Amur leopard (*Panthera pardus orientalis* Schlegel, 1857) and sika deer (*Cervus nippon* Temminck, 1838) from animals kept at Almaty Zoo. Each animal provided one skin sample and one primary culture, which was grown to passage 3. The set included the snow leopard (IUCN category Vulnerable, VU) [31], Amur leopard (Critically Endangered, CR), kulan and saiga (Near Threatened, NT), as well as the Turkestan lynx (*Lynx lynx isabellinus* Blyth, 1847), Pallas's cat (*Otocolobus manul* Pallas, 1776), Bukhara deer, and Indian crested porcupine (*Hystrix indica* Kerr, 1792), which are listed in the Red Data Book of Kazakhstan. The Turkestan lynx is treated here as *Lynx lynx isabellinus*, following the subspecies classification of Central Asian lynx based on morphometric and genetic data [32]. Collection of tissue samples from rare and endangered vertebrates for the cryobank under program BR18574058 was approved by the Committee of Forestry and Wildlife of the Ministry of Ecology and Natural Resources of the Republic of Kazakhstan (letter No. 27-3-16/3987-KLKhZhM of 28 April, 2023). Under the Law of the Republic of Kazakhstan "On the Protection, Reproduction and Use of Wildlife," sampling without removal of animals from the wild is classed as general use of wildlife and requires no further permit (Articles 23 and 26).

2.2. Reagents and Equipment

High-glucose Dulbecco's modified Eagle's medium (DMEM), tissue culture medium 199 (TCM-199), L-glutamine, penicillin-streptomycin solution, fetal bovine serum (FBS), trypsin 1:250, dimethyl sulfoxide (DMSO), hyaluronidase, pronase, cytochalasin B, Hoechst 33342, phytohemagglutinin (PHA), calcium ionophore, 6-dimethylaminopurine (6-DMAP), sodium bicarbonate, sodium pyruvate, calcium lactate, epidermal growth factor, cysteine, demecolcine, mineral oil, mannitol, polyvinyl alcohol, and gentamicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). We also used 60 mm Petri dishes and four-well plates (Nunc, Roskilde, Denmark), 25 and 75 cm^2 culture flasks (Sigma-Aldrich), disposable pipettes, 1.5 mL cryovials (Sarstedt, Nümbrecht, Germany), 50 mL tubes (Corning, Corning, NY, USA), and the Tali Viability Kit-Dead Cell Red (Life Technologies, Carlsbad, CA, USA). Cells were handled in a class II biosafety cabinet (Esco, Singapore) and cultured in a CB 56 carbon dioxide (CO_2) incubator (Binder, Tuttlingen, Germany). Cells and oocytes were examined under an Axiovert 40 CFL inverted microscope with a digital camera and Stemi 305 and Stemi SV stereomicroscopes (Carl Zeiss, Jena, Germany), AxioCam Erc 5s (Carl Zeiss, Jena, Germany). Other equipment included MiniSpin (Eppendorf, Hamburg, Germany) and ELMI (Riga, Latvia) centrifuges, a Kryo 360-3.3 programmable freezer (Planer, Sunbury-on-Thames, UK), a Tali image-based cytometer (Invitrogen, Carlsbad, CA, USA), and a CF-150/B cell fusion instrument (BLS, Budapest, Hungary).

2.3. Fibroblast Isolation and Culture

Skin samples were transported to the laboratory in DMEM with 20% FBS and 1% penicillin-streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) at 20–24 °C. They were washed twice in Dulbecco's phosphate-buffered saline (DPBS) and cut into fragments of about 1 × 1 mm, which were placed on the bottom of 25 cm^2 flasks and covered with DMEM containing 10% FBS, 2 mM of L-glutamine, and 1% penicillin-streptomycin. Explants were cultured in the CB 56 CO_2 incubator (Binder) at 37 °C, 5% CO_2, and 85% humidity,

and the medium was changed every 5 days. Cell outgrowth and the formation of a growth zone were monitored under the Axiovert 40 CFL inverted microscope (Carl Zeiss). When a monolayer had formed, the cells were detached with warm 0.25% trypsin (1:250) for 7–10 min at 37 °C, the enzyme was inactivated with medium containing FBS, and the cells were washed by centrifugation, resuspended in fresh medium and split 1:2 [33]. Third-passage cultures were frozen for the comparison of species. Cells were harvested for freezing at about 80–90% confluence. Cell identity was judged by morphology only: the cultures consisted of spindle-shaped cells, although epithelial-like cells could still be present at passages 2–3 (Section 3.1). Contamination was checked by transferring an aliquot of culture medium to general and selective liquid media, including Sabouraud Dextrose Broth (Condalab, Madrid, Spain); no fungal contamination and no acidophilic bacteria were detected.

2.4. Programmable Slow Freezing

Fibroblasts were suspended at 2 × 10^6 cells/mL in DPBS containing DMSO [34] at a final concentration of 1.5 M and 10% FBS and dispensed into 1.5 mL cryovials (1 mL per vial; 10 cryovials per animal). After 2 h of equilibration at +5 °C, the cryovials were cooled in the programmable freezer at 1 °C/min to −40 °C and then at 4 °C/min to −85 °C (Figure 1), transferred to liquid nitrogen and stored in Dewar vessels at −196 °C. Cells were frozen in independent runs of the programmable freezer, each containing cultures of one or several species, and were stored for 6 months before thawing.

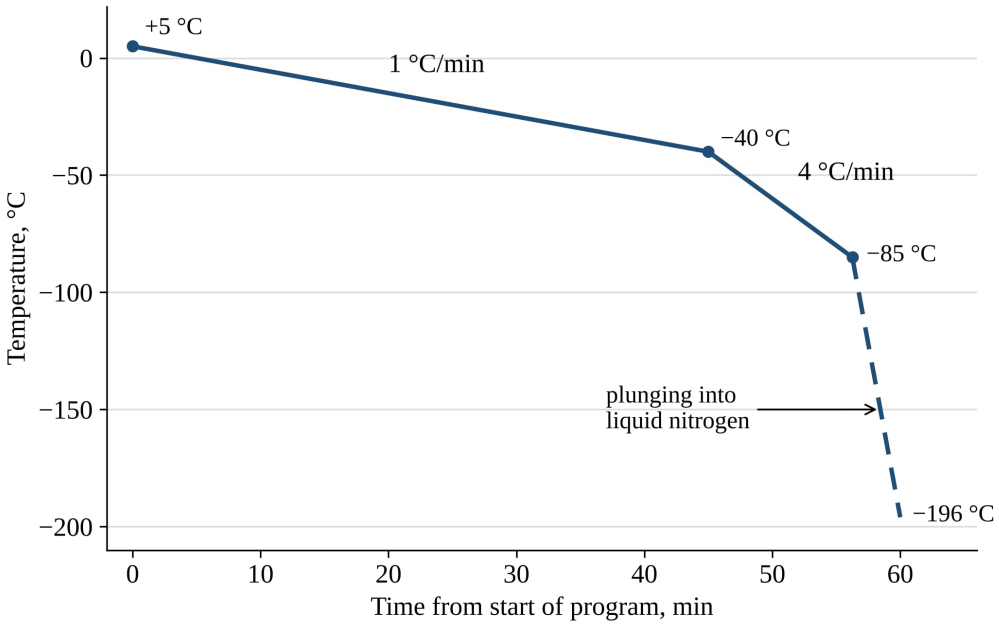


Figure 1. Cooling program used for slow freezing of fibroblasts in the programmable freezer. Solid line, controlled cooling at 1 °C/min from +5 to −40 °C and at 4 °C/min to −85 °C; dashed line, plunging of cryovials into liquid nitrogen (−196 °C).

2.5. Thawing, Viability and Proliferation Assessment

Cryovials were thawed for 5 min in a 37 °C water bath. The cryoprotectant was removed by washing in a 20-fold volume of DMEM and centrifugation (160 *g*, 10 min), and the cells were resuspended in complete culture medium (DMEM with 10% FBS, 2 mM of L-glutamine, and 1% penicillin-streptomycin) [33]. Viability was measured in third-passage cultures with the Tali image-based cytometer. A suspension containing 1 × 10^5 to 1 × 10^7 cells/mL was used; 100 µL of it were mixed with 1 µL of Tali Viability Kit—Dead Cell Red reagent (propidium iodide; Invitrogen), gently vortexed and incubated

for 5 min at room temperature in the dark. Then, 25 µL of the stained suspension were loaded onto a Tali Cellular Analysis Slide (T10794; Invitrogen), and 20 fields per slide were imaged in bright-field and red fluorescence (530 nm excitation, 585 nm longpass emission) and analyzed automatically with the built-in viability assay, which counts all cells and the dead (red) cells and calculates the percentage of live cells [35]. Propidium iodide exclusion measures membrane integrity only and does not show whether cells can attach and proliferate. Viability was expressed as the proportion of live cells before freezing (V_{pre}) and after thawing (V_{post}). Each sample was measured three times in the Tali cytometer (Invitrogen) (technical replicates), and the SEM was calculated from these technical replicates, not from the number of animals. Some thawed snow leopard cells were replated in DMEM with 10% FBS to check whether they could attach and proliferate in a flask; growth of non-cryopreserved and frozen-thawed cells of all species was compared as described below.

Proliferation of non-cryopreserved and frozen-thawed fibroblasts was compared in all 19 taxa. Non-cryopreserved cells were taken from the same third-passage cultures immediately before freezing, and frozen-thawed cells after slow freezing and thawing as described above. One culture (one animal) per species was used. Cells were seeded in 24-well plates (Nunc) at 1×10^4 cells/mL in DMEM with 10% FBS, 2 mM of L-glutamine, and 1% penicillin-streptomycin and cultured at 37 °C and 5% CO_2. Every 24 h for 7 days (0–168 h), cells from three wells per group were detached with 0.25% trypsin, counted, and recorded; each well was counted once. Growth curves were plotted from the mean counts at each time point.

The population doubling time (PDT) was calculated with the Doubling Time Computing tool [36] using Equation (1):

$$PDT = \frac{T \times ln2}{ln\left(\frac{X_e}{X_b}\right)} \quad (1)$$

where T is the incubation time (h), and X_b and X_e are the numbers of cells at the beginning and at the end of that time. PDT of non-cryopreserved and frozen-thawed cells was determined for 15 species.

2.6. Recipient Oocytes

Domestic cat ovaries were obtained at a veterinary clinic after routine ovariectomy performed under the Almaty city program for humane control of stray animals (2021–2024), which provided free sterilization for about 9000 dogs and cats. The ovaries ($n = 28$) from 14 domestic cats were transported in TCM-199 with 15 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich), 15 mM of $NaHCO_3$, 0.36 mM of pyruvate, 1 mM of glutamine, 2.2 mM of calcium lactate, 25 ng/mL epidermal growth factor, and 0.4% FBS. Cumulus–oocyte complexes (COCs) were released by slicing the ovaries in DPBS under the Stemi 305 stereomicroscope. For in vitro maturation (IVM), groups of 7–8 COCs were placed in 500 µL of medium in four-well plates under mineral oil and cultured for 24 h at 38 °C and 5% CO_2. The maturation medium was TCM-199 with 2.2 mM of calcium lactate, 0.36 mM of pyruvate, 2 mM of glutamine, 1.12 mM of cysteine, and 0.3% FBS. After maturation, oocytes were graded under the stereomicroscope as A, round, with an intact membrane and dark, evenly granulated ooplasm; B, with slightly uneven or paler ooplasm or a slightly irregular shape; or C, shrunken, with fragmented or vacuolated ooplasm. Only oocytes with an extruded first polar body (metaphase II) were bisected, and only grade A and B oocytes were used. Cumulus cells were removed with 1 mg/mL hyaluronidase with gentle pipetting, and 0.04 µg/mL demecolcine was used for induced enucleation.

2.7. Enucleation by Handmade Cloning

Cytoplasts were prepared by handmade cloning (HMC) [37]. This procedure had previously been used in our laboratory for Kazakh argali (*Ovis ammon collium*) [38] and was adapted for the present study. The zona pellucida of mature oocytes was removed with pronase (3.3 mg/mL, 60 s). The oocytes were then bisected with a microblade (Ultra Sharp Splitting Blade; Bioniche, Belleville, ON, Canada) under the Stemi SV stereomicroscope in T20 medium (TCM-199 with 20% FBS) containing 5 µg/mL cytochalasin B. The cut was guided by the polar body, which lies next to the metaphase plate. Both halves of each oocyte were stained with Hoechst 33342 and examined briefly under ultraviolet light; halves without visible chromatin were taken as cytoplasts, and halves containing chromatin were discarded. Each oocyte could therefore yield at most one cytoplast.

2.8. Donor Cell Preparation, Fusion, and Activation

Snow leopard fibroblasts from one male (cell line SLM003) cryopreserved by programmable slow freezing were thawed, diluted 1:5 with DMEM, pelleted (300 *g*, 7–10 min), and grown in DMEM with 20% FBS at 37 °C and 5% CO_2 for 21–22 days until they formed a monolayer. These donor cultures were set up separately from the replating test described in Section 2.5 and were seeded at a lower density, which is why they needed 21–22 days to reach confluence, compared with 7 days in Section 3.5. The cells were detached with 0.25% trypsin (7–10 min), washed, and allowed to settle in a drop of T2 medium (TCM-199 with 2% FBS). Cytoplasts in groups of 10 were dipped for 5 s in 500 µg/mL PHA in T2 and attached one by one to fibroblasts. The pairs were equilibrated for 10 s in fusion medium (0.3 M mannitol, 0.01% polyvinyl alcohol) and placed in a fusion chamber (Microslide for electrofusion, model 450; BTX, San Diego, CA, USA) connected to the CF-150/B fusion machine. An alternating current (AC) field of 0.6 kV/cm and 700 kHz aligned them along the electrode with the fibroblast facing away from the wire, after which a single 9 µs direct current (DC) pulse of 2.0 kV/cm was applied. For the second fusion, five large cytoplasts were placed in a row in a fusion drop and drawn into a capillary one after another with the fused pairs (cytoplast, pair, cytoplast, pair, and so on), keeping them spaced apart. The pairs and cytoplasts were transferred to the electrode, the AC voltage was raised to 9 V, a DC pulse was applied, and the AC field was switched off. The reconstructed embryos were then collected into a drop of T20 medium. Fusion was checked under the stereomicroscope after each pulse; a triplet was counted as fused when the fibroblast and both cytoplasts had merged into one round structure.

One hour after fusion, the embryos were activated with 2 µM of calcium ionophore in T20 for 5 min and then incubated in medium with 2 mM of 6-DMAP. No activation-only (parthenogenetic) controls, no intraspecies controls with domestic cat fibroblasts, and no molecular confirmation of the snow leopard origin of the embryonic nuclei were included in these experiments.

2.9. Embryo Culture

Reconstructed embryos were cultured for 7 days at 38 °C in IVC-1 medium (in vitro culture medium 1: TCM-199 with 2.2 mM of calcium lactate, 0.36 mM of pyruvate, 2 mM of glutamine, 1.12 mM of cysteine, gentamicin, and 0.3% FBS) in well-of-the-well microwells in the submarine incubation system under 5% oxygen (O_2), 5% CO_2, and 90% nitrogen (N_2) [39]. Embryos were examined on days 2, 3, and 5. Cleavage was recorded on day 2, and the four-cell stage was recorded on day 3. A morula was defined as a compact embryo of 16–32 blastomeres. Morulae were recorded on day 5, when every embryo was classified as a morula, a cleaved embryo arrested before the morula stage, or an uncleaved or degenerated embryo. Development was expressed as the proportion of fused reconstructed

embryos. Embryo development was assessed by the number of blastomeres because zona-free reconstructed embryos have no perivitelline space and cannot be graded by the usual criteria, such as the perivitelline space or the evenness of cleavage.

2.10. Statistics

For each species, we calculated the mean (M) and standard error of the mean (SEM) from technical replicates (repeated cytometer measurements); the SEM therefore de-scribes measurement variability, not variation between animals. The family mean was the unweighted mean of species values, and its error was as shown in Equation (2):

$$SEM_{family} = \frac{\sqrt{\sum_{i=1}^{k} SEM_i^2}}{k}, \quad (2)$$

where k is the number of species in the family.

Viability loss was expressed in percentage points (pp; $\Delta = V_{pre} - V_{post}$), and the share of surviving cells as the recovery index $K = V_{post}/V_{pre} \times 100\%$. K expresses the proportion of membrane-intact cells after thawing relative to that before freezing. It is not an absolute recovery of viable cells because cells lost during washing and centrifugation are not counted, and it does not reflect attachment or proliferation. The decline in viability across species was tested with the Wilcoxon signed-rank test. The relationship between pre-freeze and post-thaw viability was assessed with Pearson (r) and Spearman (ρ) correlation coefficients. Differences were considered significant at $p < 0.05$. Calculations were done in Python 3 with SciPy 1.17 [40].

PDT of non-cryopreserved and frozen-thawed cells of each species was compared with Welch's t-test from the means and SD of three wells. Growth curves were compared by two-way analysis of variance (ANOVA) with freezing and time (24–168 h) as factors, calculated from the means and SD of three wells per time point. Across species, PDT and maximum cell density before and after freezing were compared with the Wilcoxon signed-rank test, and the relationships of the mean reduction in cell number after freezing and of PDT with K with the Spearman coefficient (ρ).

Replication. The experimental unit and the type of replicate differed between assays.

Viability assay: the biological replicate was the animal. Each animal provided one primary culture, which was frozen at the third passage; the number of animals per species (n) is given in Section 3.2 (272 individuals of 19 taxa in total). Each sample was measured three times in the Tali cytometer (technical replicates; 20 fields per slide were analyzed automatically), and the SEM in the viability tables of Sections 3.2 and 3.3 was calculated from these technical replicates, so it describes measurement variability, not variation between animals.

Growth curves and PDT: one culture (one animal) per species was used, and both non-cryopreserved and frozen-thawed cells were taken from it. At each time point three wells per group were counted (technical replicates), and the SD in the growth curves and PDT figures of Section 3.5 refers to these wells. Because the growth curves came from one culture per species, the comparison of non-cryopreserved and frozen-thawed cells within a species reflects variation between wells, not between animals.

Nuclear transfer: donor cells came from one male snow leopard (cell line SLM003) and oocytes from 14 domestic cats, so all nuclear transfer results derive from a single donor cell line.

3. Results

3.1. Establishing Cultures

Cells began to migrate from explants no earlier than day 6 (most often on days 8–10; Figure 2a) and formed a growth zone as they divided (Figure 2b,c). After 10–12 days the monolayer covered about a third of a 25 cm^2 flask, and in snow leopard cultures, it became confluent by day 28 (Figure 2d). Trypsinization for 7–10 min detached the monolayer almost completely. The cultures grew faster with every passage, and epithelial-like cells, noticeable at the second passage, had nearly disappeared by the fifth. Cultures were obtained from all 19 species.

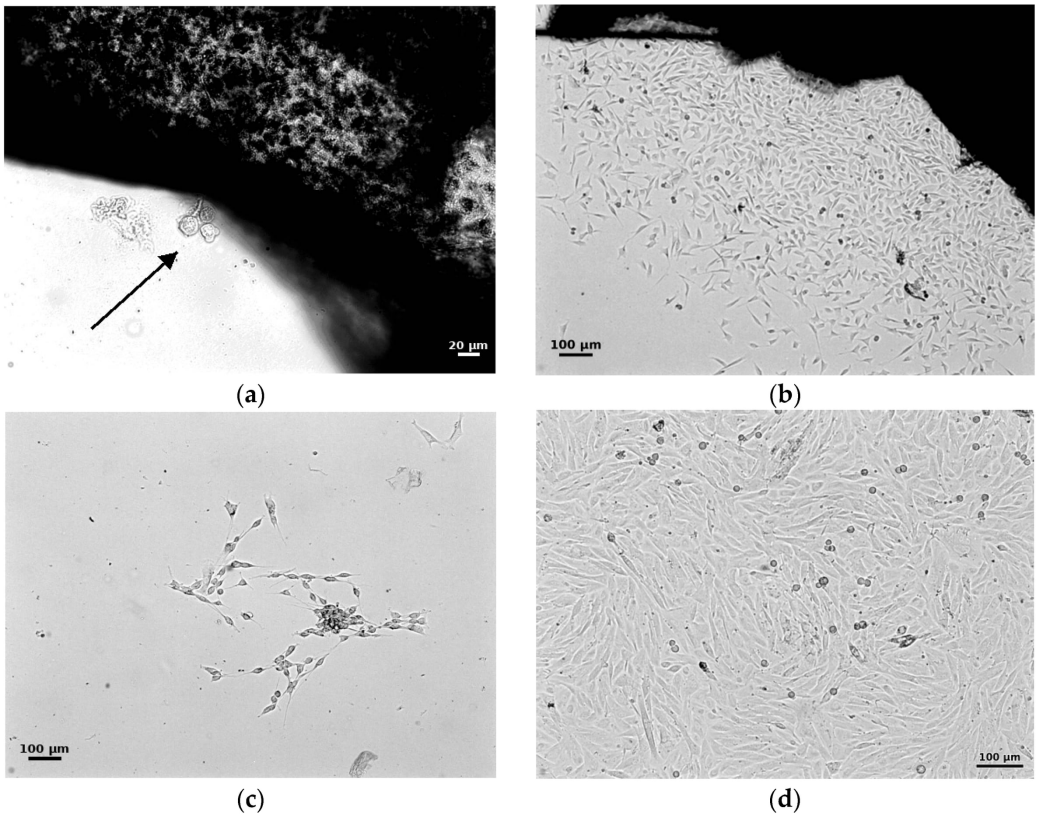


Figure 2. Outgrowth of fibroblasts from snow leopard skin explants. (**a**) A single fibroblast migrating from the explant (arrow); (**b**) outgrowth and proliferation of fibroblasts from the explant on day 14; (**c**) an islet of dividing fibroblasts on day 14; (**d**) fibroblast monolayer on day 28. Scale bars: 20 µm (**a**) and 100 µm (**b**–**d**).

3.2. Viability Before Freezing

Before freezing, the proportion of live cells ranged from 67.5 ± 7.1% in the kulan to 97.6% in the snow leopard and saiga, with a species mean of 83.1 ± 1.8% (Table 1). It was highest in felids (mean 90.7 ± 2.2%) and the saiga and lowest in the kulan (67.5%) and in gerbils and jirds (72.8–80.3%). Figure 3a shows a representative measurement of snow leopard cells before freezing.

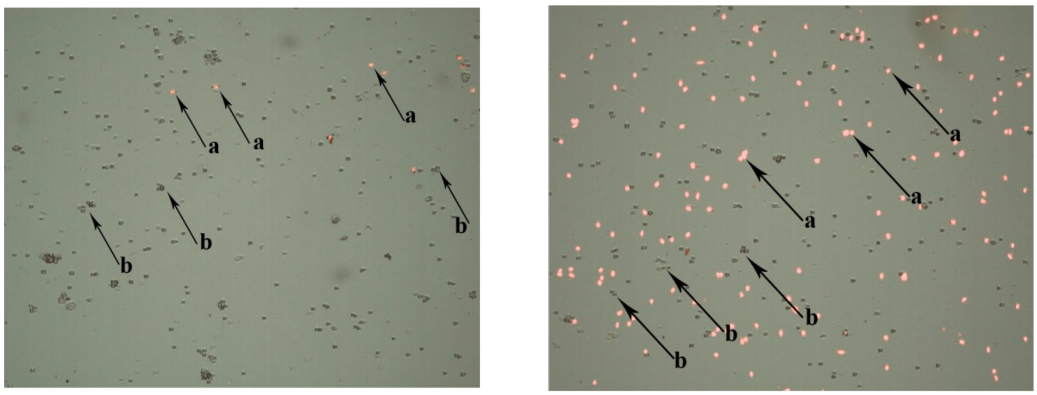
Table 1. Viability of skin fibroblasts of wild mammals before and after programmable slow freezing with 1.5 M DMSO (M ± SEM).

No.	Species (Subspecies)	*n*	Before Freezing, %	After Thawing, %	K, %	Origin
OrderEulipotyphla. Family Erinaceidae						
1.	Long-eared hedgehog *Hemiechinus auritus* Gmelin, 1770	12	75.4 ± 8.3	48.7 ± 7.4	64.6	wild
	Family mean (1 sp.)	12	75.4 ± 8.3	48.7 ± 7.4	64.6	
Order Lagomorpha. Family Leporidae						
2.	Tolai hare *Lepus tolai* Pallas, 1778	15	77.7 ± 8.7	47.2 ± 7.4	60.7	wild
	Family mean (1 sp.)	15	77.7 ± 8.7	47.2 ± 7.4	60.7	
Order Rodentia. Family Cricetidae						
3.	Gray dwarf hamster *Cricetulus migratorius* Pallas, 1773	22	84.7 ± 7.2	54.9 ± 6.3	64.8	wild
4.	Root vole *Microtus oeconomus* Pallas, 1776	26	80.7 ± 8.2	57.5 ± 5.6	71.3	wild
	Family mean (2 sp.)	48	82.7 ± 5.5	56.2 ± 4.2	68.0	
Family Muridae						
5.	Great gerbil *Rhombomys opimus* Lichtenstein, 1823	19	72.8 ± 8.6	54.6 ± 5.9	75.0	wild
6.	Tamarisk jird *Meriones tamariscinus* Pallas, 1773	14	78.8 ± 7.3	53.8 ± 5.5	68.3	wild
7.	Libyan jird *Meriones libycus* Lichtenstein, 1823	16	80.3 ± 8.4	56.1 ± 6.8	69.9	wild
8.	House mouse *Mus musculus* Linnaeus, 1758	18	78.5 ± 8.5	58.9 ± 5.9	75.0	wild
	Family mean (4 sp.)	67	77.6 ± 4.1	55.8 ± 3.0	72.0	
Family Dipodidae						
9.	Small five-toed jerboa *Scarturus elater* Lichtenstein, 1825	11	81.2 ± 7.3	49.3 ± 7.5	60.7	wild
	Family mean (1 sp.)	11	81.2 ± 7.3	49.3 ± 7.5	60.7	
Family Hystricidae						
10.	Indian crested porcupine *Hystrix indica* Kerr, 1792	12	87.4 ± 7.3	34.2 ± 7.8	39.1	wild
	Family mean (1 sp.)	12	87.4 ± 7.3	34.2 ± 7.8	39.1	
Order Carnivora. Family Mustelidae						
11.	Least weasel *Mustela nivalis* Linnaeus, 1766	5	87.4 ± 7.2	51.4 ± 6.9	58.8	wild
	Family mean (1 sp.)	5	87.4 ± 7.2	51.4 ± 6.9	58.8	
Family Felidae						
12.	Pallas's cat *Otocolobus manul* Pallas, 1776	3	87.9 ± 4.3	43.9 ± 11.1	49.9	wild
13.	Turkestan lynx *Lynx lynx isabellinus* Blyth, 1847	3	89.5 ± 5.2	44.5 ± 10.2	49.7	wild
14.	Snow leopard *Panthera uncia* Schreber, 1775	15	97.6 ± 0.4	49.3 ± 3.5	50.5	wild
15.	Amur leopard *Panthera pardus orientalis* Schlegel, 1857	1	87.7 ± 5.9	45.7 ± 8.9	52.1	zoo
	Family mean (4 sp.)	22	90.7 ± 2.2	45.8 ± 4.5	50.6	

Table 1. *Cont.*

No.	Species (Subspecies)	*n*	Before Freezing, %	After Thawing, %	K, %	Origin
Order Perissodactyla. Family Equidae						
16.	Kulan *Equus hemionus* Pallas, 1775	3	67.5 ± 7.1	39.6 ± 6.5	58.7	wild
	Family mean (1 sp.)	3	67.5 ± 7.1	39.6 ± 6.5	58.7	
Order Artiodactyla. Family Cervidae						
17.	Bukhara deer *Cervus elaphus bactrianus* Lydekker, 1900	25	89.1 ± 6.7	37.4 ± 3.5	42.0	wild
18.	Sika deer *Cervus nippon* Temminck, 1838	2	77.5 ± 7.3	39.6 ± 5.2	51.1	zoo
	Family mean (2 sp.)	27	83.3 ± 5.0	38.5 ± 3.1	46.2	
Family Bovidae						
19.	Saiga antelope *Saiga tatarica* Linnaeus, 1766	50	97.6 ± 0.7	44.5 ± 7.1	45.6	wild
	Family mean (1 sp.)	50	97.6 ± 0.7	44.5 ± 7.1	45.6	

Note: *n*, number of individuals; *K*, relative recovery index (V_{post}/V_{pre} × 100%). Family mean is the unweighted mean of species values; family SEM was calculated using Equation (2). SEM was calculated from technical replicates (repeated cytometer measurements), not from the number of animals.



(**a**) (**b**)

Figure 3. Representative measurement of snow leopard fibroblasts stained with propidium iodide in the Tali cytometer: (**a**) before freezing (98% live cells in this sample); (**b**) after thawing (44% live cells in this sample). Arrows: **a**, dead cells (red fluorescence); **b**, live cells. Species means are given in Table 1.

3.3. Viability After Thawing

After thawing, the proportion of live cells fell in every species (Wilcoxon test, $W = 0$, $p < 0.001$; a representative snow leopard sample is shown in Figure 3b). It ranged from 34.2 ± 7.8% in the Indian crested porcupine to 58.9 ± 5.9% in the house mouse, with a mean of 48.0 ± 1.6%. Losses ranged from 18.2 pp in the great gerbil to 53.2 pp in the porcupine and 53.1 pp in the saiga, with a mean of 35.2 ± 2.7 pp.

At the family level (Table 2, Figure 4), hamsters and voles (Cricetidae, 56.2 ± 4.2%) and murids (Muridae, 55.8 ± 3.0%) did best. Mustelids, jerboas, hedgehogs, hares, and felids were intermediate (45.8–51.4%), with bovids close to them (44.5%). Fibroblasts of equids (39.6%), cervids (38.5 ± 3.1%), and porcupines (34.2%) survived worst.

Table 2. Fibroblast viability by family (in decreasing order of post-thaw viability).

Family	Species	*n*, Individuals	Before Freezing, %	After Thawing, %	Δ, pp	K, %
Cricetidae	2	48	82.7 ± 5.5	56.2 ± 4.2	26.5	68.0
Muridae	4	67	77.6 ± 4.1	55.8 ± 3.0	21.7	72.0
Mustelidae	1	5	87.4 ± 7.2	51.4 ± 6.9	36.0	58.8
Dipodidae	1	11	81.2 ± 7.3	49.3 ± 7.5	31.9	60.7
Erinaceidae	1	12	75.4 ± 8.3	48.7 ± 7.4	26.7	64.6
Leporidae	1	15	77.7 ± 8.7	47.2 ± 7.4	30.5	60.7
Felidae	4	22	90.7 ± 2.2	45.8 ± 4.5	44.8	50.6
Bovidae	1	50	97.6 ± 0.7	44.5 ± 7.1	53.1	45.6
Equidae	1	3	67.5 ± 7.1	39.6 ± 6.5	27.9	58.7
Cervidae	2	27	83.3 ± 5.0	38.5 ± 3.1	44.8	46.2
Hystricidae	1	12	87.4 ± 7.3	34.2 ± 7.8	53.2	39.1

Note: n, number of individuals; Δ, viability loss (Vpre−Vpost), pp, calculated from unrounded means; K, recovery index.

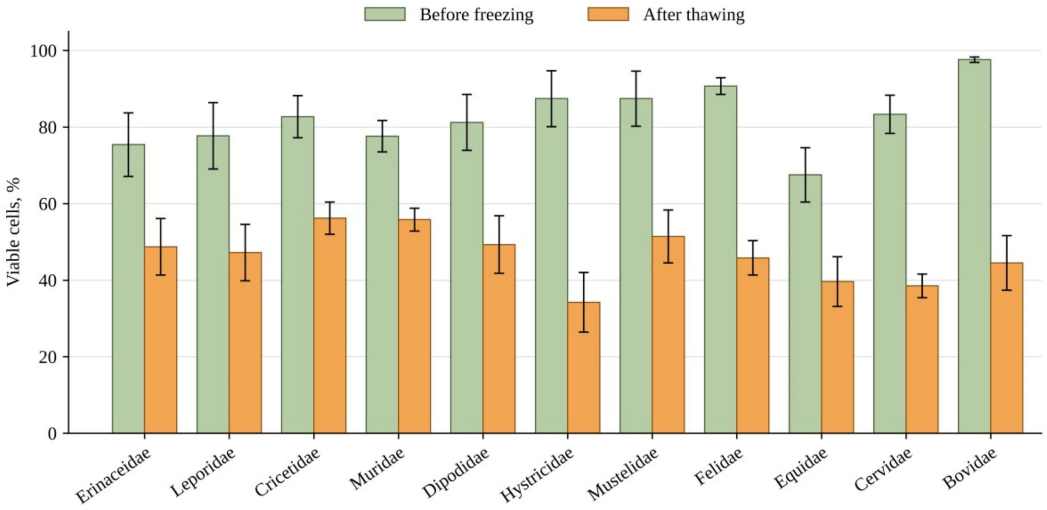


Figure 4. Proportion of viable fibroblasts before freezing and after thawing by family (M ± SEM).

3.4. Recovery Index

K ranged from 39.1% in the porcupine to 75.0% in the great gerbil and house mouse, with a mean of 58.3 ± 2.5% (Figure 5). In small rodents other than the jerboa, it exceeded 64%; in felids, it stayed around 50%; and in the saiga, Bukhara deer, and porcupine, it did not rise above 46%. High initial viability did not guarantee good survival. Snow leopard and saiga cultures had 97.6% live cells before freezing, yet their *K* values were only 50.5% and 45.6%, and in absolute terms, they did not exceed any of the small rodents.

Pre-freeze viability did not correlate with post-thaw viability ($r = -0.20$, $p = 0.42$; $\rho = -0.24$, $p = 0.31$). It was negatively correlated with the recovery index ($r = -0.63$, $p = 0.004$), but largely because V_{pre} is the denominator of K, so we do not attach separate biological meaning to this relationship.

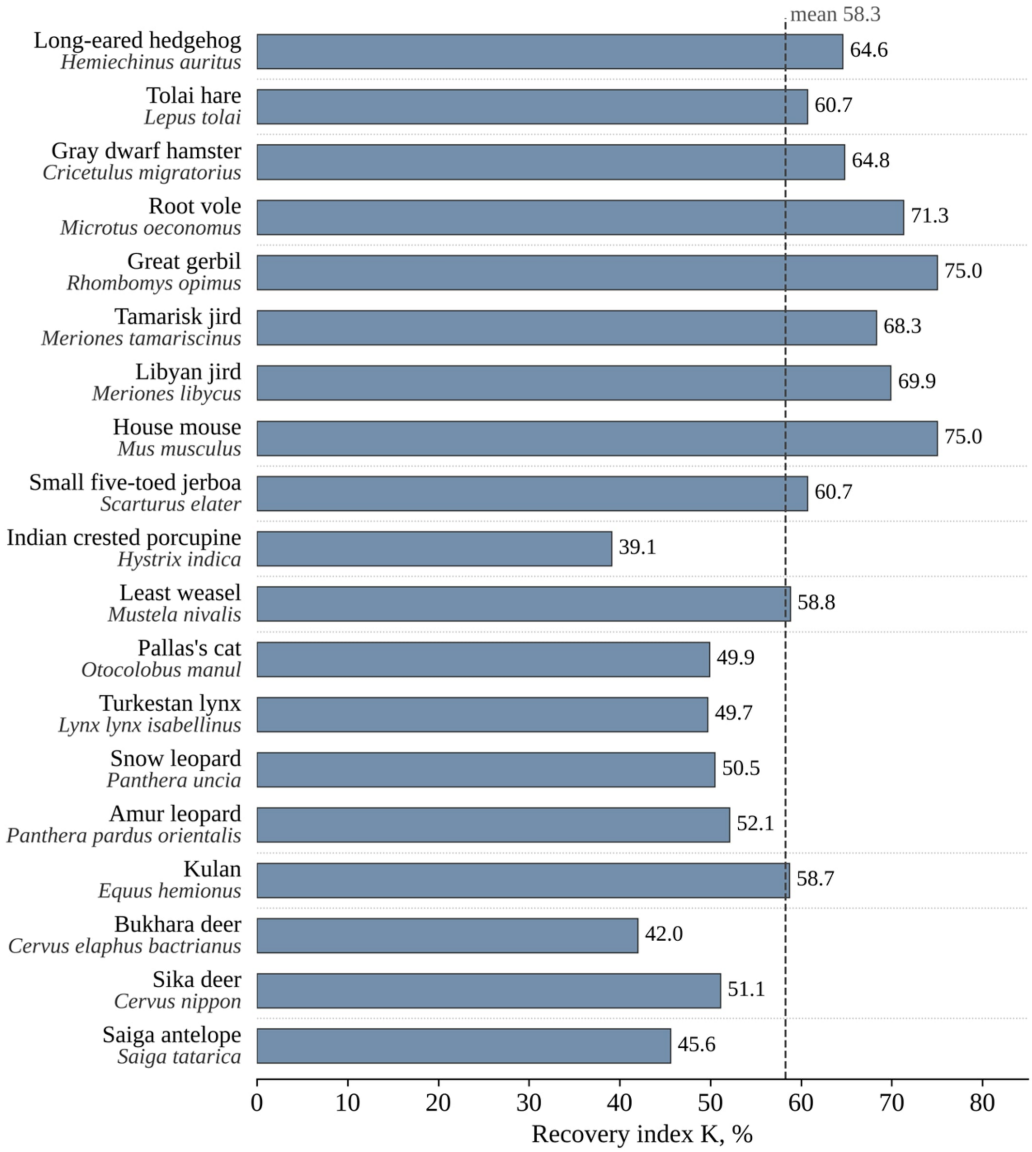


Figure 5. Recovery index (K) of fibroblasts in the species studied. The dashed line shows the mean of 19 species. Species are listed in the order of Table 1.

3.5. Proliferation of Non-Cryopreserved and Frozen-Thawed Fibroblasts

Fibroblasts of all 19 taxa proliferated after thawing (Figures 6 and 7). Growth curves of non-cryopreserved and frozen-thawed cells had the same shape: a lag phase during the first 24 h, exponential growth to 72–96 h, a maximum at 96 h in most species (at 120 h in the least weasel, in non-cryopreserved Amur leopard cells and in frozen-thawed kulan cells), and then a decline in cell number to 168 h. This corresponds to the lag, exponential, and plateau phases of a typical growth curve [33], followed by a decline. Frozen-thawed cultures had fewer cells than non-cryopreserved ones at every time point after seeding: on average, 12.6% fewer (7.9–16.3% depending on the species). The maximum density was $43.6 \pm 1.3 \times 10^3$ cells/mL after thawing and $48.9 \pm 1.5 \times 10^3$ cells/mL before freezing (means of 19 species ± SEM; Wilcoxon test, $p < 0.001$). Two-way ANOVA showed effects of time ($p < 0.001$) and of freezing ($p \leq 0.049$) in every species and no time × freezing interaction ($p \geq 0.61$), so freezing lowered the number of cells without changing the shape of the curve. Within species, the maximum density differed significantly only in Pallas's cat (54 vs. 41×10^3 cells/mL, $p = 0.047$), whose frozen-thawed cells stopped growing after 72 h. The

reduction in cell number after freezing was larger in species with a lower recovery index ($\rho = -0.57$, $p = 0.011$).

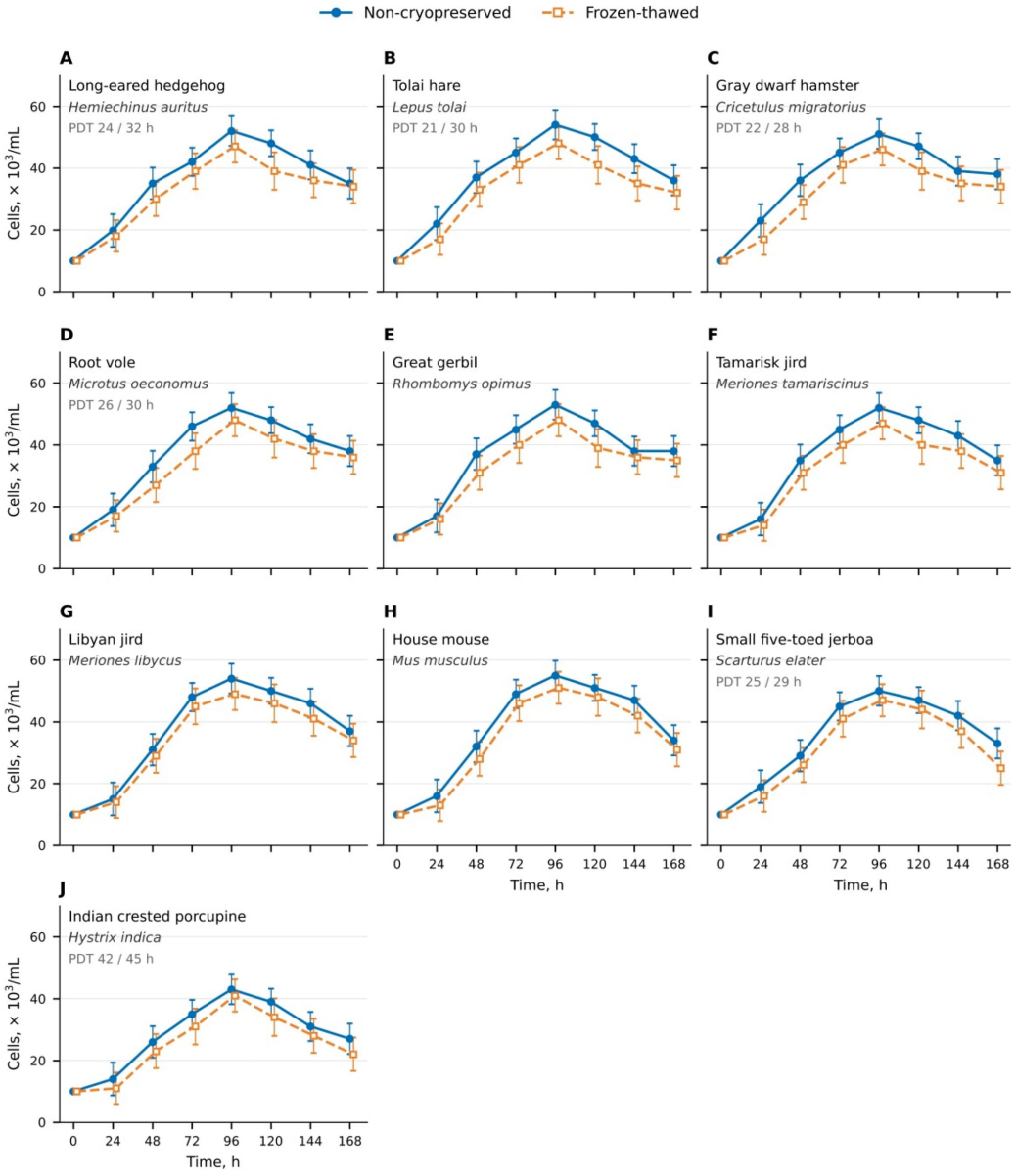


Figure 6. Growth curves of non-cryopreserved and frozen-thawed skin fibroblasts of Eulipotyphla, Lagomorpha and Rodentia: (**A**) *Hemiechinus auritus*; (**B**) *Lepus tolai*; (**C**) *Cricetulus migratorius*; (**D**) *Microtus oeconomus*; (**E**) *Rhombomys opimus*; (**F**) *Meriones tamariscinus*; (**G**) *Meriones libycus*; (**H**) *Mus musculus*; (**I**) *Scarturus elater*; (**J**) *Hystrix indica*. Cells were seeded at 1×10^4 cells/mL (10×10^3 cells/mL at 0 h). Values are means ± SD of three wells per time point; PDT before/after freezing is given in the panels where it was determined; species are shown in the order of Table 1. No difference was observed for PDT values between groups ($p > 0.05$).

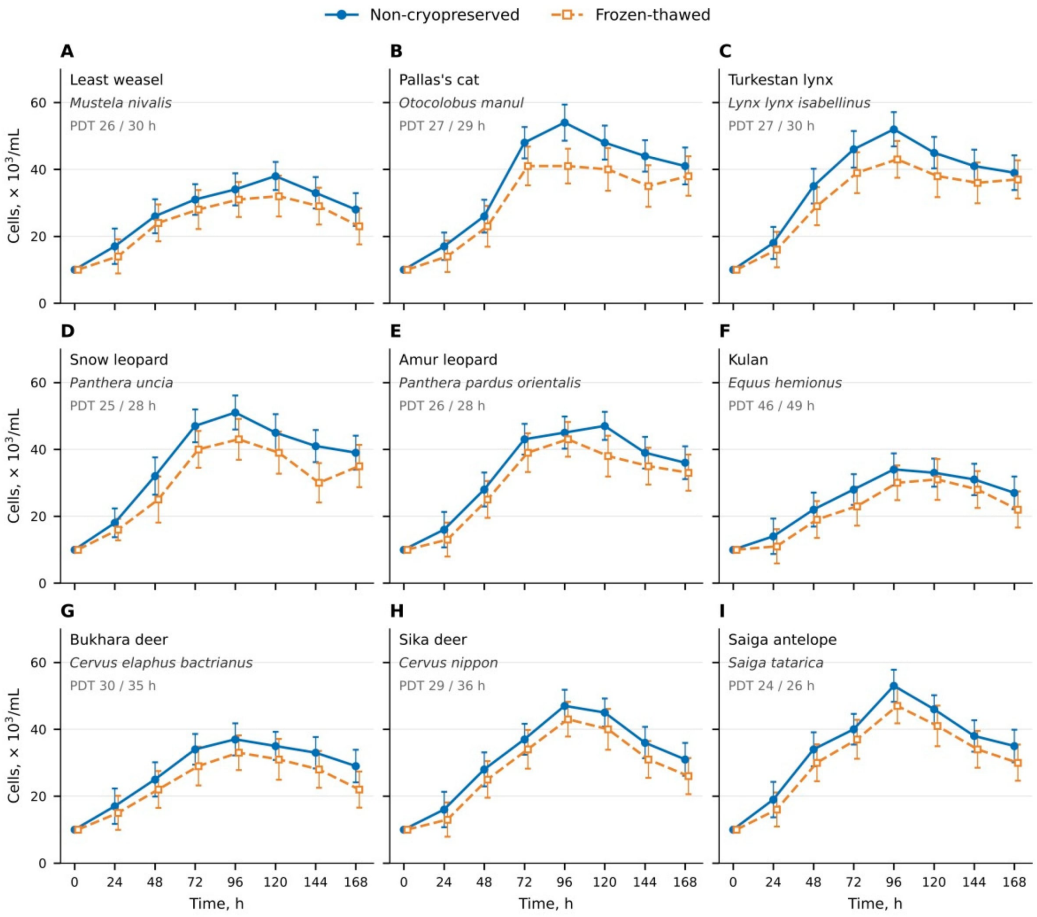


Figure 7. Growth curves of non-cryopreserved and frozen-thawed skin fibroblasts of Carnivora, Perissodactyla and Artiodactyla: (**A**) *Mustela nivalis*; (**B**) *Otocolobus manul*; (**C**) *Lynx lynx isabellinus*; (**D**) *Panthera uncia*; (**E**) *Panthera pardus orientalis*; (**F**) *Equus hemionus*; (**G**) *Cervus elaphus bactrianus*; (**H**) *Cervus nippon*; (**I**) *Saiga tatarica*. Cells were seeded at 1×10^4 cells/mL (10×10^3 cells/mL at 0 h). Values are means ± SD of three wells per time point; PDT before/after freezing is given in each panel. No difference was observed for PDT values between groups ($p > 0.05$).

PDT of non-cryopreserved fibroblasts ranged from 21 h in the tolai hare to 46 h in the kulan, and PDT of frozen-thawed fibroblasts from 26 h in the saiga to 49 h in the kulan (Figure 8). Within species, the difference was not significant (Welch's *t*-test, $p = 0.10$–0.67), but PDT after thawing was longer in all 15 species by 2–9 h (28.0 ± 1.8 vs. 32.3 ± 1.7 h; Wilcoxon test, $p < 0.001$). The largest increases were in the tolai hare (9 h), long-eared hedgehog (8 h), and sika deer (7 h), and the smallest (2–3 h) in felids, the saiga, porcupine, and kulan. Before freezing, PDT was longest in the porcupine and kulan (42 and 46 h) and 21–30 h in the other species. Species with a shorter PDT before freezing had a higher *K* ($\rho = -0.54$, $p = 0.037$).

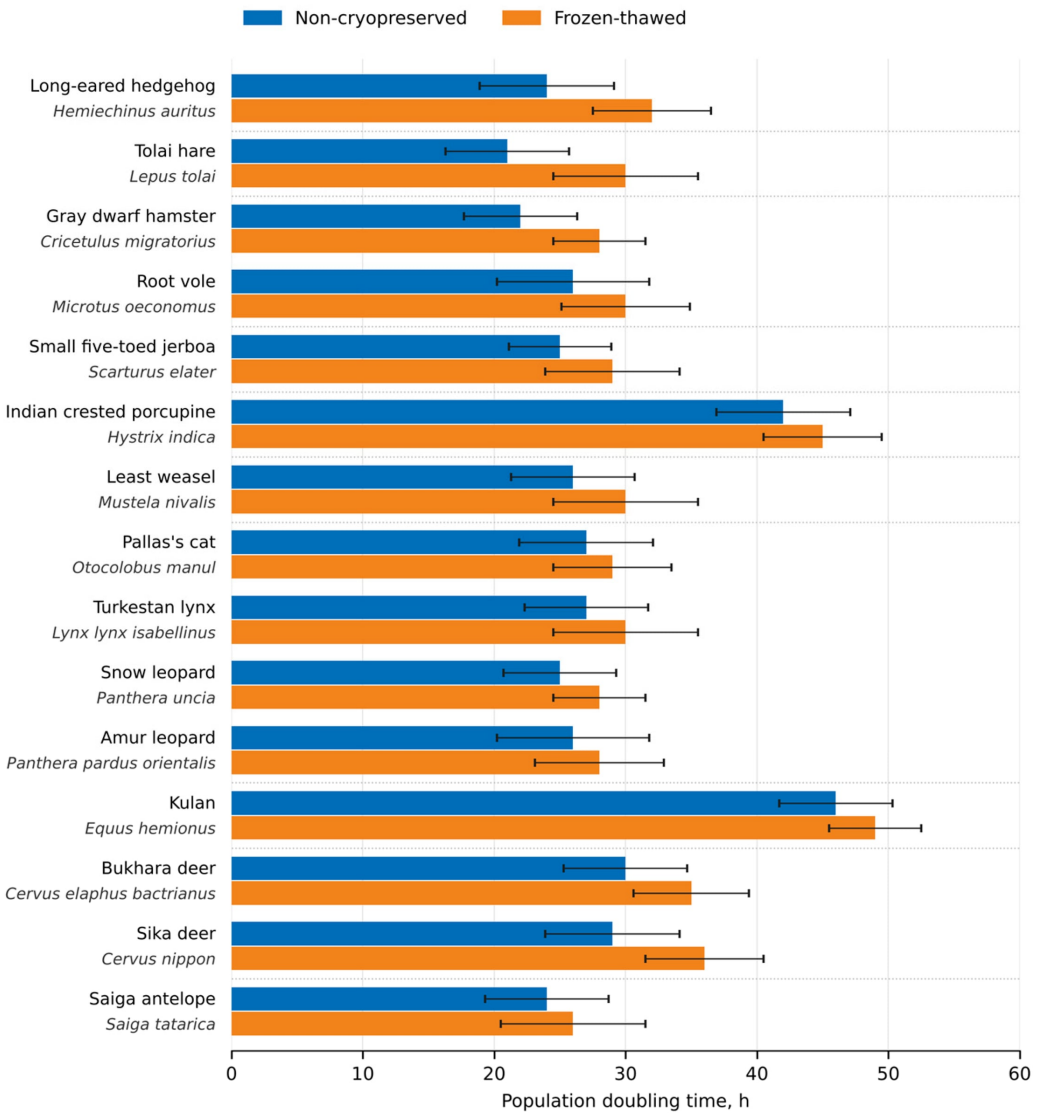


Figure 8. Population doubling time (PDT) of non-cryopreserved and frozen-thawed fibroblasts (mean ± SD, $n = 3$); species are listed in the order of Table 1. No difference was observed for PDT values between groups within any species (Welch's t-test, $p > 0.05$); across the 15 species, PDT was longer after thawing (Wilcoxon test, $p < 0.001$).

Thawed snow leopard fibroblasts replated in DMEM with 10% FBS (seeding density as in Section 2.5) attached to the substrate and formed a confluent monolayer within 7 days, which was then passaged. The cells had typical fibroblast morphology, the culture contained no epithelial or dead cells, and growth accelerated after the next passage. Thus, although only about half of the snow leopard cells had intact membranes after thawing, the surviving cells attached and grew.

3.6. Interspecies Nuclear Transfer

We recovered 662 COCs from 28 ovaries of 14 cats (Figure 9a–c). After 24 h of IVM, 238 oocytes (36.0%) reached metaphase II (MII) (Figure 9d). Hyaluronidase at 1 mg/mL removed the cumulus gently and effectively. With 0.04 µg/mL demecolcine, a metaphase spindle formed, and the first polar body was extruded.

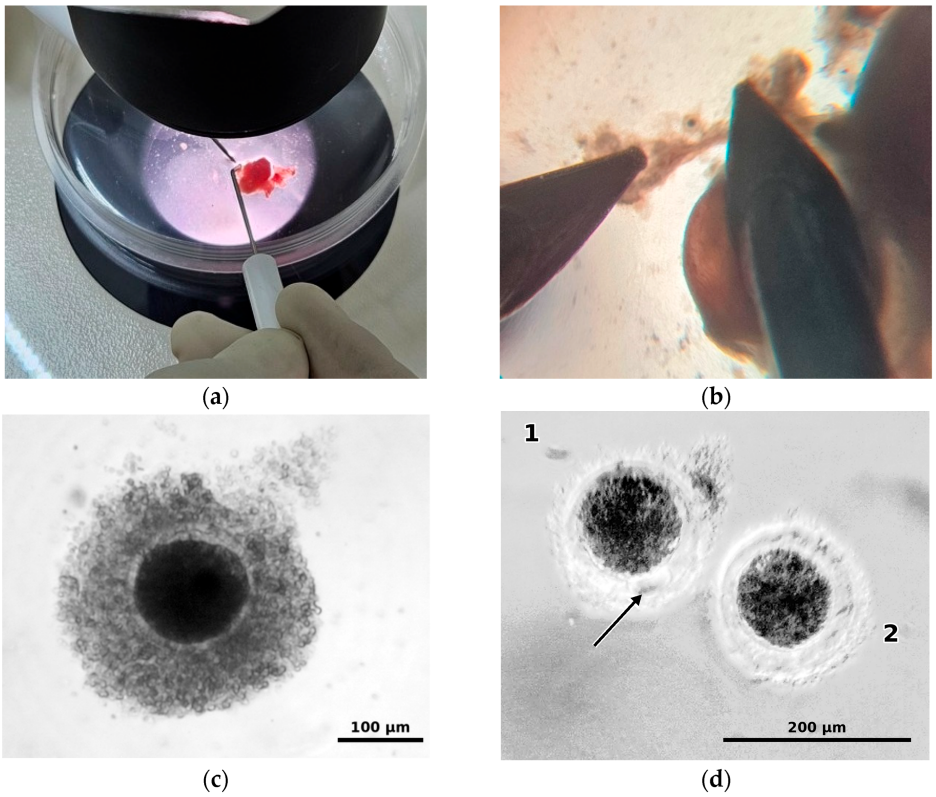


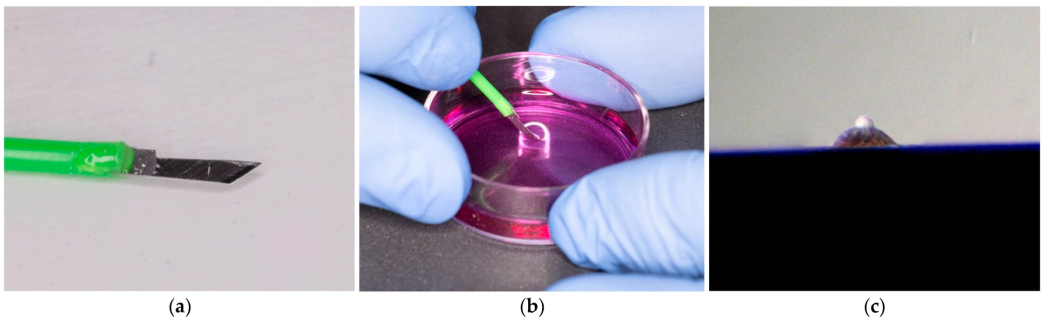
Figure 9. Recovery and maturation of domestic cat oocytes. (**a**,**b**) Slicing of an ovary under a stereomicroscope to release cumulus–oocyte complexes; (**c**) cumulus–oocyte complex; (**d**) oocytes after IVM: (**1**) metaphase II oocyte with an extruded polar body (arrow) and (**2**) immature oocyte. Scale bars: 100 µm (**c**) and 200 µm (**d**).

Bisection of 238 oocytes yielded 162 cytoplasts (68.1%) (Table 3; Figure 10). Each oocyte gave two halves, of which at most one was free of chromatin; no usable cytoplasts were obtained from the other 76 oocytes. Each triplet needed two cytoplasts, so 162 cytoplasts were used to produce 78 cytoplast–fibroblast–cytoplast triplets (156 cytoplasts); the remaining 6 were not used. Cytoplasts were attached to fibroblasts with 500 µg/mL PHA (Figure 11a,b), and after electrofusion with a 2.0 kV/cm pulse, 33 of 78 cytoplast–fibroblast–cytoplast triplets fused (42.3%) (Figure 11c–f). The reconstructed embryos were cultured individually in well-of-the-well microwells (Figure 12a); cleavage began by day 2, and four-cell embryos were seen by day 3 (Figure 12b). On day 5, 10 of 33 embryos (30.3%) were at the morula stage (Figure 12c); the others had arrested at the 2- to 8-cell stages, had not cleaved or had degenerated, and no blastocysts formed (Table 3).

Table 3. Results of interspecies nuclear transfer of snow leopard fibroblasts into domestic cat oocytes by handmade cloning.

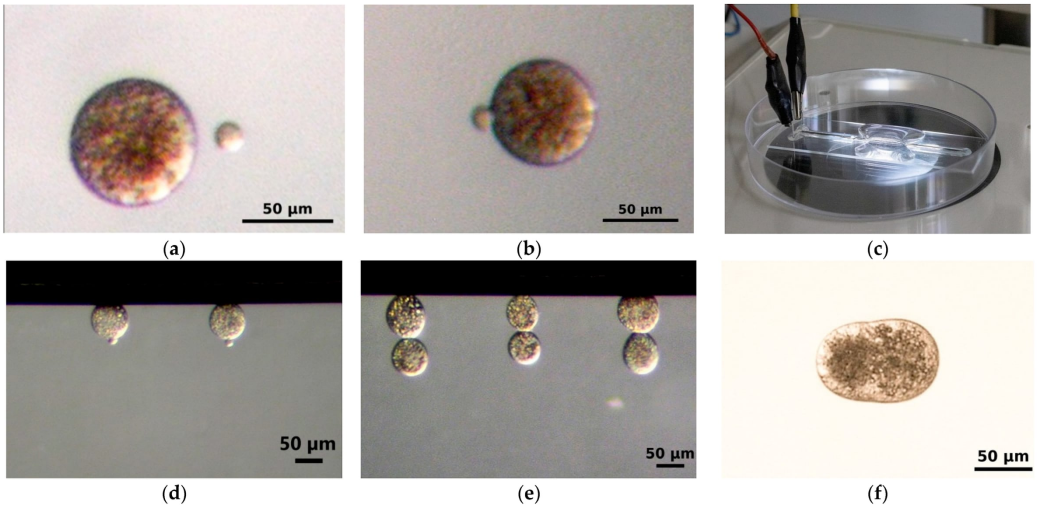
Stage	Conditions	*n*	Result
Oocyte maturation	TCM-199 with 0.3% FBS, 24 h, 38 °C	662 COCs	238 MII oocytes
Enucleation	Zona removal with pronase, bisection with a microblade	238 oocytes	162 cytoplasts (68.1%)
Fusion	Cytoplast–fibroblast–cytoplast triplets; DC pulse 2.0 kV/cm, 9 µs	78 triplets	33 fused (42.3%)
Culture (7 days)	IVC-1 medium, well-of-the-well, SIS (5% O_2)	33 embryos	10 morulae (30.3%)

Note: COCs, cumulus–oocyte complexes; MII, metaphase II; DC, direct current; FBS, fetal bovine serum; IVC-1, in vitro culture medium 1; SIS, submarine incubation system.



(**a**) (**b**) (**c**)

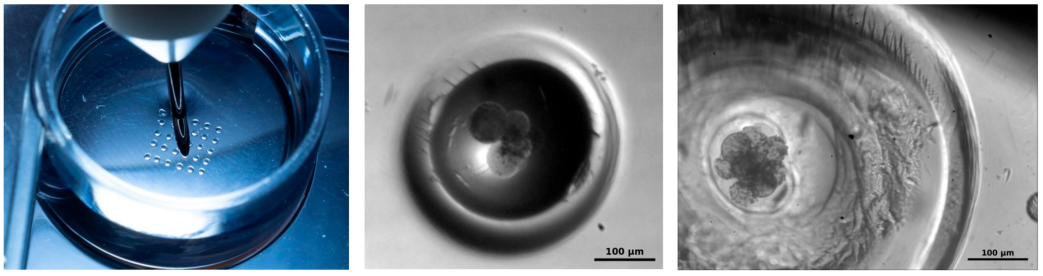
Figure 10. Enucleation of domestic cat oocytes by handmade cloning. (**a**) Ultra Sharp Splitting Blade used for bisection; (**b**,**c**) bisection of zona-free oocytes with the microblade.



(**a**) (**b**) (**c**)

(**d**) (**e**) (**f**)

Figure 11. Electrofusion of domestic cat cytoplasts with snow leopard fibroblasts. (**a**) Cytoplast and fibroblast; (**b**) cytoplast–fibroblast pair after PHA treatment; (**c**) BTX model 450 fusion chamber with fusion medium; (**d**) cytoplast–fibroblast pairs attached to the electrode; (**e**) cytoplast–fibroblast-cytoplast triplets in the fusion chamber; (**f**) reconstructed embryo after fusion and treatment with calcium ionophore. Scale bars: 50 µm (**a**,**b**,**d**–**f**).



(**a**) (**b**) (**c**)

Figure 12. Culture of reconstructed embryos in the submarine incubation system. (**a**) Preparation of a culture dish with well-of-the-well microwells; (**b**) four-cell embryo in a microwell on day 3; (**c**) morula on day 5. Scale bars: 100 µm.

No activation-only or domestic cat nuclear-transfer controls were run, and the origin of the embryonic nuclei was not confirmed. These results therefore show that reconstructed embryos developed to the morula stage, but not that this development was directed by the snow leopard nucleus.

4. Discussion

Programmable freezing with 1.5 M of DMSO kept viable fibroblasts of all 19 taxa, including those for which we had no previous data on somatic cell cryopreservation. This is convenient for building a collection: the same procedure can be applied to every species, and material from different animals is processed in the same way. On average, however, fewer than half of the cells had intact membranes after thawing, and in the saiga and porcupine, the losses exceeded 50 pp, so the protocol leaves room for improvement.

Part of the loss is related to the state of the cultures. Cells should be frozen in the logarithmic growth phase with high initial viability [33], whereas in the kulan, gerbils, and hedgehog, it was below 80%. In snow leopard cultures, we noticed that viability drops when the culture becomes overgrown before harvesting. The simplest remedy is to passage cells according to confluence rather than on a fixed schedule. Propidium iodide staining immediately after thawing also reflects only membrane integrity [35], and *K* does not include cells lost during washing, so neither value shows how many usable cells a cryovial contains. In jaguar fibroblasts, viability that appeared reduced right after thawing no longer differed from the control once the cultures reached confluence [41], and thawed snow leopard cells in our study likewise recovered and formed a monolayer within a week. Growth curves showed the same for all 19 taxa: frozen–thawed cells attached and proliferated, the curves kept their shape, and cell numbers were on average 13% lower than in non-cryopreserved cultures, with PDT 2–9 h longer. Species with lower *K* also showed a larger reduction in cell number, so propidium iodide staining and growth curves gave consistent results.

Fibroblasts of hamsters, voles, and murid rodents tolerated freezing best, and those of the porcupine, cervids, and kulan worst, although pre-freeze viability did not predict post-thaw viability. Two explanations are possible. The proliferative capacity of cultured fibroblasts differs among species [42,43], and small rodents control cell division and senescence differently [44], so their cultures may contain more actively dividing and more resilient cells. Our growth data support this in part: species with a shorter PDT before freezing had a higher *K*. In addition, the optimal cooling rate depends on cell size and membrane permeability [19,20], and a program cooling at 1 °C/min may be close to optimal for some species but not for others. Sampling conditions may also play a part: ungulates, felids, and the porcupine were more often sampled in the field after immobilization, and their samples took longer to reach the laboratory.

Felids deserve particular attention because they include the rarest species in our set. For slow freezing of jaguar fibroblasts, about 70% live cells on average have been reported [41], more than the 44–49% we obtained for the snow leopard, manul, lynx, and Amur leopard. In Neotropical cats, the best cryoprotectant solution differed even between closely related species [23], and jaguar fibroblasts tolerated ethylene glycol worse than DMSO [45]. The cryoprotectant and cooling rate for felid cells therefore have to be checked species by species, and vitrification, which we have already tested on snow leopard fibroblasts [26,30], is one of the options. Proliferation of felid cells was little affected by freezing: PDT of snow leopard fibroblasts was 25 h before and 28 h after freezing, and that of the other felids 26–30 h, close to the 28 h reported for thawed Bengal tiger fibroblasts [7] and 24 h for Siberian tiger fibroblasts [8].

Cells are banked so that they can be used later, for example, as nuclear donors. Interspecies transfer of wild felid fibroblast nuclei into domestic cat oocytes has already produced cloned African wildcat kittens [11–13]. In the sand cat, cryopreservation of donor cells was shown to affect the outcome of nuclear transfer [46], so post-thaw survival is directly linked to the usefulness of cells for cloning. In our experiments, snow leopard fibroblasts thawed after programmable freezing and grown for 3 weeks fused with domestic cat cytoplasts, and almost a third of the reconstructed embryos reached the morula stage.

These embryo data are preliminary. We did not run activation-only controls, so we cannot exclude that some morulae arose from cytoplasts that retained maternal chromatin and developed parthenogenetically, although every half was checked by Hoechst staining before use. We also had no intraspecies domestic cat controls and did not confirm the snow leopard origin of the embryonic nuclei. These controls and molecular identification of the nuclei are needed before the developmental competence of the donor cells can be judged.

Manual bisection gave cytoplasts from 68.1% of oocytes, and the method itself does not require expensive micromanipulators [37]. Fusion remains a weak point: 42.3% of cytoplast–fibroblast–cytoplast triplets fused, whereas about 85% has been reported for conventional nuclear transfer in felids [11]. Interspecies embryos carry mitochondria of the recipient species, and interspecies cloning of felids is still inefficient in vivo [9,13]. The next steps are therefore to improve fusion and activation and to follow embryo development to the blastocyst stage, with the controls listed above.

The study has limitations. The number of individuals varied widely among species (from one Amur leopard to 50 saigas), and the estimates for Felidae and Cervidae include Zoo-housed species represented by one individual (Amur leopard) and two individuals (sika deer). Nuclear transfer was carried out with cells of one species, without activation-only or intraspecies controls, and embryo development was followed only to the morula stage. Finally, propidium iodide exclusion does not show whether cells can attach and divide [35,41], which is why we added growth curves for all 19 taxa. Cells intended for nuclear transfer and reprogramming also need karyotype control [7,8], which was not done in this study.

5. Conclusions

Fibroblast cultures were established from ear skin of 19 species and subspecies of wild mammals of Kazakhstan from 11 families and stored in liquid nitrogen in the cryobank of the Institute of Zoology. After programmable freezing with 1.5 M of DMSO, fibroblast viability was 34.2–58.9% (mean 48.0 ± 1.6%) against 67.5–97.6% before freezing. Cells of small rodents tolerated freezing best, and those of the Indian crested porcupine, deer, kulan, and saiga tolerated it worst. Pre-freeze viability did not predict post-thaw viability. Frozen-thawed fibroblasts of all 19 taxa proliferated in culture: their growth curves kept the normal shape, but cell numbers were about 13% lower and PDT 2–9 h longer than before freezing. The banked cells therefore remain usable for culture, although freezing slows their growth.

Cryopreserved snow leopard fibroblasts attached and proliferated after thawing and could be used to reconstruct embryos with domestic cat cytoplasts. In handmade cloning, cytoplast yield was 68.1%, 42.3% of cytoplast–fibroblast–cytoplast triplets fused after a 2.0 kV/cm pulse, and 10 of 33 reconstructed embryos reached morula stage by day 5. Without activation-only and intraspecies controls and confirmation of the origin of the embryonic nuclei, these results are preliminary. A single DMSO protocol allowed fibroblasts of all 19 taxa to be banked, but post-thaw viability below 60% in every species, and below 45% in seven of them, shows that the protocol needs to be optimized for individual species.

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Institutional Review Board Statement: The study was approved by the Local Ethics Committee of the Institute of Zoology (protocol No. 5, 15 July, 2022; protocol No. 3, 30 November, 2023). Sampling of rare and endangered species was approved by the Committee of Forestry and Wildlife of the Ministry of Ecology and Natural Resources of the Republic of Kazakhstan (letter No. 27-3-16/3987-KLKhZhM of 28 April, 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: Author Yerzhan Toishibekov is employed by the company Embryo Technology Labs. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

6-DMAP	6-dimethylaminopurine
AC	alternating current
ANOVA	analysis of variance
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
CR	Critically Endangered (IUCN Red List category)
DC	direct current
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMC	handmade cloning
IUCN	International Union for Conservation of Nature
IVC-1	in vitro culture medium 1
IVM	in vitro maturation
K	recovery index
M	mean
MII	metaphase II
N_2	nitrogen
NT	Near Threatened (IUCN Red List category)
O_2	oxygen
PDT	population doubling time
PHA	phytohemagglutinin
pp	percentage points

SD	standard deviation
SEM	standard error of the mean
SIS	submarine incubation system
T2, T20	TCM-199 with 2% and 20% fetal bovine serum
TCM-199	tissue culture medium 199
V_{pre}, V_{post}	proportion of live cells before freezing and after thawing
VU	Vulnerable (IUCN Red List category)

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