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# Perpetuating biological diversity in threatened indigenous livestock breeds by creating biorepositories for the purpose of assisted reproductive technologies*

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**The current paper presents efforts undertaken to generate biorepositories of cryogenically protected male gametes, embryos, and somatic cell lines aimed at their subsequent use for a broad spectrum of the strategies related to experimental and applied embryology. Creating repositories of biological materials derived from rare indigenous breeds of selected livestock species (sheep, goats, cattle and pigs) seems to be highly justified in view of the pivotal role of the Department of Reproductive Biotechnology and Cryoconservation (DRBC), the National Research Institute of Animal Production (NRIAP). This role is reflected in the maintenance of biodiversity as a result of NRIAP activities focused on restoration and stabilization of the effective population size of critically endangered or endangered autochthonic livestock breeds that are characterized by extremely high or relatively high inbreeding rates. Moreover, this paper also seeks to provide the state-of-the-art method and an overview of applying biorepositories with cryopreserved germplasm-carrier biological materials and somatic cell lines to a wide variety of *in vitro* embryo production (IVP) and assisted reproductive technologies (ARTs) targeted at not only standard and microsurgical *in vitro* fertilization (IVF), but also cloning by somatic cell nuclear transfer (SCNT).**

**KEY WORDS: biorepositories / embryos / endangered livestock breeds / male gametes / somatic cell lines**

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It is beyond any doubt that a variety of countries at the participation of public and non-public companies focused on biodiversity conservation have decided to protect genetic resources of their autochthonic livestock breeds. A report by Wood *et al.* [2018] has demonstrated that efforts aimed at *in situ* protection of rare native breeds of farm animals should be predictive and thereby alterations in the environmental conditions need to be considered within programs relevant to the conservation of genetic resources. Both intensifying the production and establishing new high-genetic merit or highly productive livestock breeds or lines can tremendously destabilize the global diversity of domesticated animals [Lauvie *et al.* 2011]. That is why, many countries, including Poland, develop special programs related to the conservation of livestock breeds. In Poland indigenous breeds are under *in situ* protection that is preferable and financially supported by the government. The reason to base farm animal conservation on the *in situ* method was connected with the need to restore threatened populations as well as their dynamic nature, which allows successive generations of animals to be adapted to changing environmental conditions.

The application of novel approaches to ARTs appears to be strongly justified especially in regard to endangered, critically endangered or disappearing mammalian species listed in the Red List of Threatened Species, whose the newest version was published by the International Union for Conservation of Nature and Natural Resources (World Conservation Union; IUCN) in 2019. In Poland the Red List was first published in 1992. Taking into consideration the Red List foundations, the Food and Agriculture Organization of the United Nations (FAO) initiated three assessments of the risk status of livestock species within the framework of the World Watch List – Domestic Animal Diversity 1993, 1995 and 2000. According to FAO data, the number of endangered livestock breeds facing extinction has reached 17% as measured to the total number of approximately 8800 farm animal species existing in agricultural environment niches.

Effective population size ($N_e$) has been found to be a crucial parameter for determination of genetic diversity in indigenous livestock populations and prediction of inbreeding rate [Leroy *et al.* 2020, Polak *et al.* 2021]. The effective population size is the size of an ideal population (i.e., one that meets all the Hardy-Weinberg assumptions) that would lose heterozygosity at a rate equal to that of the observed population. Because the transmission of genes from one generation to the next is fundamentally a demographic process, the size of the population is an integral part of almost any population genetics analysis. Put differently, genetic drift is directly related to population size (a small population displays an enhanced genetic drift, whereas its large counterpart – a diminished genetic drift). The triangle of genetic drift, mutation and migration is the big three for the purposes of understanding a neutral theory approach to population genetic structure. In general, $N_e$ is less than the census population size ($N_c$; the actual number of animals present). The rate of genetic drift is not really proportional to $N_c$; it is rather proportional to $N_e$. In an ideal population of sexually reproducing specimens, $N_e$ will be equal to $N_c$. An ideal population is characterized

by the following parameters: 1) There are equal numbers of males and females, all of whom are able to reproduce; 2) All individuals are equally likely to produce offspring, and the number of offspring that each produces varies no more than expected by chance; 3) Mating is random; and 4) The number of breeding individuals is constant from one generation to the next. Most deviations from the afore-mentioned characteristics will reduce the effective population size. There are two types of $N_e$ designated as variance effective population size and inbreeding effective population size. The former focuses on changes in genetic variance, on consequences for the offspring generation and hence naturally leads to consideration of inter-population divergence. The latter focuses on changes in heterozygosity, on consequences for the parental generation, and hence naturally leads to consideration of the level of the inbreeding coefficient within populations [Leroy *et al.* 2020]. It is also worth highlighting that the effective population size is negatively correlated with the degree of inbreeding. If the effective population is size less than or equal to 50, a livestock breed is considered to be critically endangered and it requires to be protected *in situ* and *ex situ*. In turn, if the effective population size is less than or equal to 200 and simultaneously higher than 50, a livestock breed is shown to be endangered and efforts targeted at its *in situ* and *ex situ* conservation are mandatory [Leroy *et al.* 2020]. Moreover, if the effective population size is less than or equal to 1200 and simultaneously higher than 200, a livestock breed is found to be vulnerable and efforts aimed at its monitoring for *in situ* and *ex situ* conservation are required [Leroy *et al.* 2020]. Taking into consideration such rare native sheep breeds as Olkuska, Blackhead, Wrzosówka and the Old Type of Merino that together are covered by the program targeted at the conservation of genetic resources, the parameters of their effective population size range from approximately 277 to nearly 1107 and thus the aforementioned breeds are characterized by vulnerable status and the lowest extent of inbreeding [Polak *et al.* 2021]. In turn, the parameters of effective population size measured for the Carpathian goat and Polish Red cattle breeds subjected to *in situ* and *ex situ* protection oscillate around 199 and 170, respectively. For these reasons, the status of the aforementioned cattle breed is considered to be endangered and related to the relatively high inbreeding rate [Polak *et al.* 2021]. Furthermore, the effective population size estimated for indigenous pig breeds undergoing *in situ* protection fluctuates between approximately 80 (Złotnicka Spotted breed) and above 144 (Puławska breed). That is why, this finding predisposes these pig breeds to be endangered, which is reflected in their relatively high inbreeding rates [Polak *et al.* 2021].

## A broad spectrum and background of creating cryopreserved biological materials deposited in repositories at DRBC of NRIAP

To protect *ex situ* a variety of biological materials, including germplasm-based materials (male gametes – Fig. 1 and 2; embryos – Fig. 3) and somatic cell lines (Tab. 1) recovered from Polish indigenous breeds of selected livestock species (sheep, goats, cattle and pigs), their biorepositories were established at DRBC of

NRIAP. The structural and organizational units of DRBC provide facilities to store germplasm-carrier biological materials (semen, oocytes, embryos) derived from such livestock species as goats, pigs, horses, sheep and cattle. These materials, which are cryogenically stored in compliance with the EU regulations, are recovered from Polish indigenous breeds of the above-mentioned farm animal species covered by *in situ* protection programs.

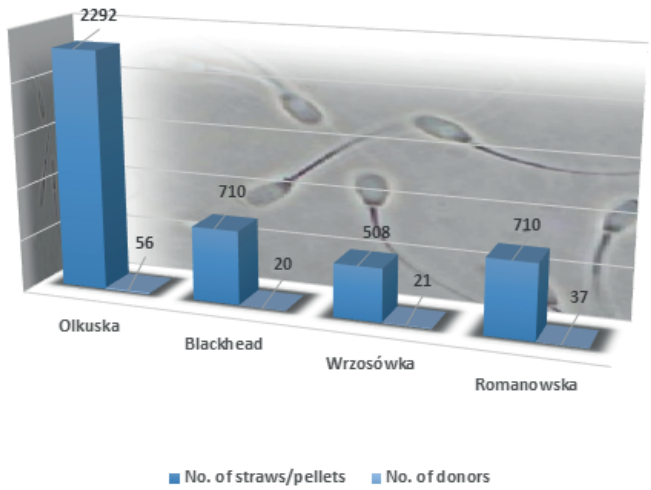


Fig. 1. DBRC biorepositories of cryopreserved semen derived from threatened indigenous sheep breeds.

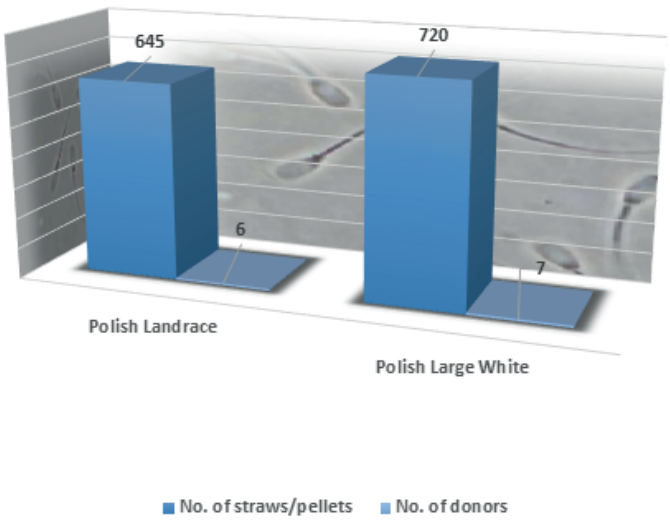


Fig. 2. DBRC biorepositories of cryopreserved semen derived from selected native pig breeds.

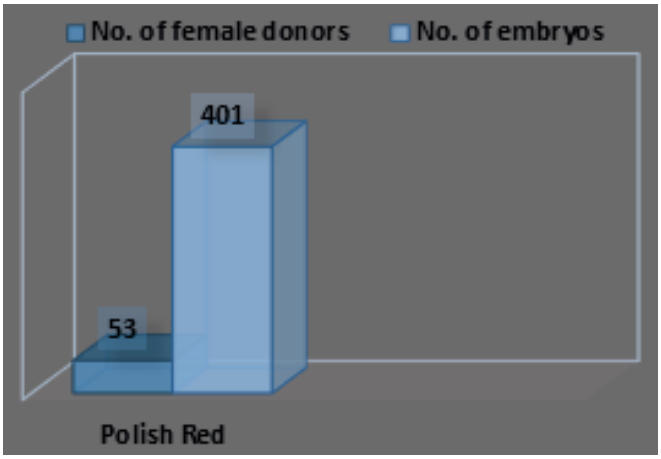


Fig. 3. DBRC biorepositories of cryopreserved embryos derived from the Polish Red cattle breed.

**Table 1.** DBRC biorepositories of cryopreserved somatic cells derived from threatened indigenous breeds of selected livestock species

| Species | Breed name | The type and provenance of somatic cell lines | Number of somatic cell lines | Number of female donors | Number of male donors | Quality of cell lines estimated by: | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cryosurvival rate | Adhesion/attachment to the substratum and trypsin-mediated detachment | Proliferative activity | Capabilities to reach a total confluency and undergo post-passage population doublings |
| Cattle | Polish Red | Mitotically stable cell lines of cutaneous dermal fibroblasts | 45 | 5 | - | Excellent* | Excellent* | Excellent* | Excellent* |
| Pigs | Puławska | | 71 | 10 | 1 | | | | |
| | Złotnicka Spotted | | 22 | 6 | - | | | | |
| Sheep | Polish Wrzosówka | | 15 | 3 | 3 | | | | |
| | Romanowska | | 32 | 3 | 3 | | | | |
| | Old Type of Merino | | 22 | 3 | 3 | | | | |
| Goats | Carpathian | | 13 | 3 | 3 | | | | |

* Quality designated as excellent denotes that the rate of fibroblast cells displaying detailed quality-related parameters was higher than or equal to 95%.

Within the framework of biodiversity protection programs, generating biorepositories of germplasm-carrier biological materials (male gametes and *in vivo*- or *in vitro*-fertilized embryos) by their cryogenic preservation can be used as an auxiliary strategy not only to maintain populations of protected mammalian species living in the wild, but also to re-establish livestock breeds and re-introduce them into anthropogenic agricultural ecosystems that are disappearing or are significantly decreasing in numbers.

An innovative tool of SCNT-mediated ART appears to be indispensable to perpetuate long-term *ex situ* and/or *in situ* conservation of biodiversity in indigenous breeds of various livestock species, including near threatened, vulnerable, endangered, critically endangered, vanishing and even extinct farmed animal breeds. The latter are especially susceptible to the phenomena of: 1) strongly intensified bottleneck effects stemming from an extremely attenuated genetic drift; 2) rapid genetic erosion resulting

in highly diminished intra-population and inter-specimen genotypic variability; and 3) a subsequent drastic population collapse. For all the above-mentioned reasons, establishment and subsequent cryopreservation of genetically stable or permanent primary cell cultures and somatic cell lines derived from different tissue explants/biopsy specimens provide an alternative or complementary research approach in relation to gamete and embryo freezing or vitrification procedures. It encompasses efficient approaches that enable to cryogenically protect genetic resources derived from endangered farm animal species from extinction and to successfully retain the *ex situ* and/or *in situ* conservation of their biodiversity.

**Applicability of sperm cryopreservation methods and semen biorepository-based ARTs developed and optimized by DBRC to retain biological diversity of threatened autochthonic livestock breeds**

Sperm cryoconservation plays a pivotal role in managing and preserving genetic resources of livestock species and breeds. It should be emphasized that the cryopreservation methods of germplasm-based biological materials (semen and embryos) collected from cattle seem to be the most advanced and the most efficient. For this reason, they are applied on a large scale to the reproductive biotechnology and *ex situ* conservation of genetic resources. Over the recent years, DBRC has carried out intensive research on increasing cryoconservation efficiency of semen derived from other farm animal species [Trzcińska and Bryła 2015, Gogol *et al.* 2019]. To ensure high quality of frozen-thawed semen derived from male specimens of livestock species, males and their ejaculates intended to be cryopreserved are subjected to seminological selection. Optimally spermatological selection of semen to be frozen can be accomplished when the condition of large size of animal population has been met. For small and vanishing populations of threatened livestock species covered by biodiversity conservation programs, it is not possible to choose the high-genetic merit male specimens, whose semen displays high cryoresistance- and cryosurvival-related parameters. Therefore, such efforts have been undertaken by DBRC to elaborate efficient methods for cryopreservation of semen regardless of its spermatological quality.

Several studies have examined cryodamage in spermatozoa of different species [Yeste 2016]. Cryopreservation protocols bring about such stressors as cold shock and oxidative attack affecting the structural and functional properties and biochemical, biophysical and molecular characteristics of plasma membranes, which result in reduced survival rates and freezability, leading to the onset and progression of molecular mechanisms underlying programmed cell death. The oxidative attack of free oxygen radicals (i.e., reactive oxygen species; ROS) on the cell membranes contributes to irreversible reduction in the fluidity, subsequent impermeabilization and terminal biodegradation of plasma membranes [Len *et al.* 2019]. The incidence of these processes can be decreased or limited by supplementation of freezing extenders with exogenous antioxidants.

On the one hand, the efforts of DBRC are focused on elaborating the qualitative and quantitative composition of extenders intended for freezing of semen especially derived from such livestock species as pigs, whose spermatozoa are susceptible to cryopreservation-related detrimental effects. On the other hand, DBRC undertakes efforts to develop reliable and feasible methods of assessing quality of sperm cells subjected to cryopreservation. Due to long-term storage of semen samples, it is inevitable that the stored material must be extensively assessed for structural, functional and molecular quality-related parameters, because an efficient practical use of the stored biological material depends on its initial quality. The basic criterion for the selection of ejaculates for cryopreservation is the assessment of fresh sperm motility, being the most important indicator of ejaculate quality and an the same time a crucial predictor of semen freezability. It is worth highlighting that, according to previous investigations [Rath *et al.* 2009, Knox 2015, Yeste 2017], only boar ejaculates with minimal quality parameters at 80% of all the motile spermatozoa and at 80% viable spermatozoa are classified as suitable to be cryopreserved. So far, a variety of attempts have been made to determine markers of ejaculate suitability for freezing. Seminal plasma proteins and some other compounds have been used as markers of fertility in bulls [Moura *et al.* 2006] and semen freezability in rams [Zalazar *et al.* 2016]. For boar semen, the protein HSP90AA1 was designated as an estimator of spermatozoon capability to withstand the cold shock. A reduction in relative abundance of that protein has been found to be related to enhanced sensibility/vulnerability to cold shock [Casas *et al.* 2010]. Furthermore, Vilagran et al. [Vilagran *et al.* 2014] demonstrated that the relative abundance of voltage-dependent anion channels type 2 (VDAC2) appears to be suitable for determining the capacity of boar ejaculates to be cryogenically protected and to display cryotolerance. In contrast to previous studies, in which the standard levels of minimal quality parameters at 80% of all motile spermatozoa and at 80% viable spermatozoa have been considered, the results of a study by Trzcińska and Bryła [2021] confirmed that fresh semen with sperm motility below 70% can be successfully cryopreserved in compliance with the assumptions and guidelines of patent no. Pat.228192 granted to NRIAP [Trzcińska and Bryła 2018] and a high cryosurvival rate can be accomplished. As a consequence, fresh boar semen characterized by: 1) spermatozoon motility below 70%; 2) a low percentage of viable sperm cells with apoptotic-like changes within plasmalemma ultrastructure; and simultaneously 3) a low percentage of viable spermatozoa with biodestructed acrosome membranes, exhibits a high cryosurvival rate. Cumulatively, the results of investigations by Trzcińska and Bryła [2021] showed that predicting suitability of porcine semen for cryopreservation based only on sperm motility turns out to be insufficient, especially in view of inter-specimen variability in this livestock species. Therefore, this indicates the requirement of applying other semen evaluation methods, including fluorocytochemical approaches applied to assess the spermatozoon plasmalemma and acrosome integrities. Moreover, these approaches allow for rapid detection of ultrastructural and functional changes within boar sperm cells. This latter

facilitates estimation of efficient semen freezing. Finally, this is of great importance not only for experimental and applied spermatology research, but also pig breeding practice.

Additionally, stress preconditioning of spermatozoa prior to their cryopreservation is an innovative approach for cryogenic protection of semen samples [Horváth *et al.* 2016]. The results of other investigations [Pribenszky *et al.* 2011, Bryła and Trzcińska 2020] provided a strong scientific justification that exposure of boar spermatozoa to high hydrostatic pressure (HHP) before freezing is a new technological solution proposed for sperm cryopreservation. Our research has proved that treatment of porcine semen with HHP at 35 MPa protects spermatozoa against cryo-biodegradation during freezing, thus enhancing remarkably the cryosurvival rate of exposed semen samples.

Another study by Trzcińska and Bryła [2015] demonstrated that, irrespective of the type of the cryoprotective medium applied, freezing and thawing procedures do not trigger internucleosomal DNA fragmentation related to late apoptotic events in boar spermatozoa. A similar result was stated for bovine spermatozoa undergoing cryopreservation [Martin *et al.* 2004]. In turn, Fraser *et al.* [2011] reported that freezing and thawing procedures accelerate destabilization processes in the chromatin structure, resulting in the disintegration of double DNA strands characterized by high susceptibility to oligonucleosomal fragmentation, independently of the sperm provenance, the type of cryprotective medium and the variant of semen packaging. Nonetheless, differences between studies may have been caused by inter-specimen variability in the semen-specific response to oxidative stress during the cryopreservation process. Moreover, the research differences may have also resulted from various methods applied to identify the incidence of oligonucleosomal DNA fragmentation.

The use of novel strategies in semen quality assessment mediated by induced photon emission (chemiluminescence) to quantify the frequency of lipid peroxidation in the plasma membrane of boar spermatozoa has provided evidence showing that supplementation of semen freezing extenders with butylated hydroxytoluene (BHT) improves biochemical and molecular quality-related sperm parameters post freezing/thawing. The protective effect of this antioxidant is attributed to two biochemical, biophysical and molecular mechanisms: 1) the incorporation of this compound into phospholipid bilayers of the sperm plasma membranes, making them more fluidic and permeant and protecting them from biodestruction; and 2) reduction of the biodestructive potential of lipid peroxyl radicals by their bioconversion to hydroperoxides. Our investigation has proved the protective effect of BHT supplementation on such functional quality-related parameters of post-thawed boar semen as sperm motility, viability, and acrosomal integrity [Trzcińska *et al.* 2015]. As a result, the highest reproductive performance of inseminated gilts (farrowing rate and litter size around 87% and 11 piglets, respectively) was indicated following cryopreservation of semen in an extender enriched with 1 mM BHT. These findings

have also confirmed that the supplementation of the cryoprotective medium with BHT successfully enhances the fertilizing ability of post-thawed boar spermatozoa. It is also noteworthy that our research aimed at cryopreservation of spermatozoa extended with a BHT-enriched cryoprotective medium has led to patent no. Pat.228192 awarded to NRIAP for the invention entitled: ”Extender for cryoconservation of boar semen and the procedure of semen freezing” [Trzcińska and Bryła 2018].

The approaches developed and optimized in DBRC to cryogenically protect semen collected from commercial native pig breeds such as Polish Large White and Polish Landrace also facilitate the use of cryopreserved semen for other purposes. These purposes encompass both protection of genetic resources and preservation of not only selected genetic merit, but also productivity/performance-related quantitative traits within the framework of breeding programs for rare indigenous Polish pig breeds such as the Puławska, Złotnicka Spotted and Złotnicka White. The creation of biological material collections of boar semen appears to be one of the most valuable tools aimed to genetically protect these native pig breed subpopulations from a reduction in intra-population and inter-specimen genotypic variability, their extinction or closure of pig farms due to a wide and non-controlled spread of African swine fever virus (ASFV) pathogens. It is also noteworthy that NRIAP has recently undertaken efforts leading to the establishment of the Semen Collection and Cryopreservation Centre (SCCC) certified according to the requirements of the Regional Veterinary Inspectorate (RVI) in Poland. Within the framework of structural, functional and organizational activities of the RVI-certified SCCC, research and development work aimed at cryogenic protection of boar semen samples will be realized on a large scale. These will be collected from male specimens of Polish native pig breeds and subsequently extended with BHT-enriched cryoprotective media in compliance with the assumptions and guidelines of the aforementioned patent no. Pat.228192 [Trzcińska and Bryła 2018].

Summing up, cryogenically protected semen recovered from threatened autochthonic pig breeds such as the Puławska, Złotnicka Spotted and Złotnicka White can be used for a wide spectrum of the *in vitro* embryo production (IVP) procedures. The IVP strategies are advanced ARTs that include three indispensable steps, i.e.: *in vitro* meiotic maturation (IVM) of oocytes, their IVF or reconstruction by somatic cell nuclear transfer (SCNT), and *in vitro* culture (IVC) of fertilized or cloned embryos [Glanzner *et al.* 2018, Nguyen et al. 2020, Skrzyszowska and Samiec 2021]. Similar to other ART such as classic artificial insemination, IVF frequently requires using cryopreserved/thawed spermatozoa and either can be accomplished by standard gamete co-incubation or can be assisted microsurgically by intracytoplasmic sperm injection (ICSI) into metaphase II (MII)-stage oocytes [Salamone *et al.* 2017, Fowler *et al.* 2018, Magata *et al.* 2019, Zuo *et al.* 2020]. In livestock species the effectiveness of ICSI-based IVP is largely dependent on activating the embryonic developmental program in metaphase II-stage oocytes, which can be induced by biological/physiological stimuli (intraooplasmic spermatozoon penetration) or by physical/chemical agents used for artificial stimulation of ICSI-fertilized oocytes

[Ashibe *et al.* 2019, Ressaissi *et al.* 2021]. The outcome of IVF/ICSI-based IVP is also determined by capabilities for epigenetic reprogramming of parental (maternal and paternal) genomes in extracorporeally fertilized embryos [Kropp *et al.* 2017, Diao *et al.* 2018, Takeda *et al.* 2019]. Additionally, it is worth highlighting that not only the intergenomic communication between nuclear and mitochondrial compartments [Tsai and St. John 2018, Zuidema and Sutovsky 2020], but also the frequency of programmed cell death (apoptosis and/or autophagy) [Jin *et al.* 2016, Rodriguez *et al.* 2019, Ramos-Ibeas *et al.* 2020] appear to be determinants affecting efficacy parameters in IVF/ICSI-mediated IVP of livestock species embryos.

**Feasibility and reliability of generating biorepositories comprised of cryopreserved somatic cell lines to be used as a source of nuclear donor cells for SCNT-mediated ARTs**

The elaboration of somatic cell cloning-based ARTs in livestock species and the establishment of somatic cell banking aimed at the recovery of endangered mammalian breeds and species threatened with extinction turns out to be indispensable [Li *et al.* 2009, Bai *et al.* 2010, Liu *et al.* 2008, 2011, Hossein *et al.* 2021, Lira *et al.* 2022]. The variability of animal genetic resources is a pivotal determinant of retaining biodiversity in domesticated and wild-living mammalian species. To the best of our knowledge, if these genetic resources are not protected from extinction, they will be lost forever. On the one hand, creation of genetic resources in the form of somatic (e.g., fibroblast) cell lines derived from Polish autochthonic livestock species seems to be a suitable and feasible research model for the maintenance of biodiversity in rare native breeds. On the other hand, generation of such genetic resources for the purposes of animal cloning by intra- or inter-species SCNT appears to be a powerful and helpful novel tool [de Queiroz Neta *et al.* 2018, Borges and Pereira 2019, Borges *et al.* 2020, Silva *et al.* 2021, Praxedes *et al.* 2021]. Therefore, such efforts need to be undertaken to ensure a thorough identification of factors determining mitotic activity, genetic stability, ex vivo expansion-related lifespan, replicative senescence as well as onset and progression of apoptotic or autophagic events in extracorporeally established nuclear donor cell lines (NDCLs). Recognizing the above factors might give rise to enhancement of molecular differentiability and epigenomic reprogrammability of somatic cell nuclei in SCNT-derived oocytes and corresponding cloned embryos [Wu *et al.* 2019, Jeong *et al.* 2020, Sun *et al.* 2020, Wiater *et al.* 2021*a*]. The aim of such procedures was not only to perpetuate the inheritance of desirable genes within herds of endangered or critically endangered native breeds, but also to diminish the bottleneck effects of inter-population genetic drift, decrease intra-population inbreeding and, as a consequence, increase intra- and inter-population genotypic variability among the vanishing indigenous livestock breeds. Furthermore, the intensification of the processes promoting inter-specimen genetic diversity can contribute to enhancement of individual and population resistance or herd immunity to exceptional stressors

(e.g., adverse environmental and climatic conditions or abnormal thermic, pathogenic, epidemic and pandemic shocks) [Leroy *et al.* 2016].

It is worth noting that efficient methods of establishing and cryogenically preserving mitotically stable dermal fibroblast cell lines of such endangered indigenous livestock breeds as the Puławska and Złotnicka Spotted pigs, Polish Red cattle, Polish Wrzosówka, Romanowska and Old Type of Merino sheep and Carpathian goats have been developed at DBRC. For those reasons, the biorepositories of cryopreserved somatic cell lines of the above-mentioned threatened autochthonic breeds have been created within the framework of DBRC (Table 1). So far, these biorepositories have provided the source of NDCLs for efficient production of somatic cell-cloned embryos of selected livestock species (pigs and cattle) [Samiec *et al.* 2020]. The efficiency of generating SCNT-derived embryos with the use of Polish Red cattle cutaneous fibroblast cells (CFCs) determined by their *ex vivo* capabilities to develop to the blastocyst stage (30.1%) was comparable to or remarkably higher than that

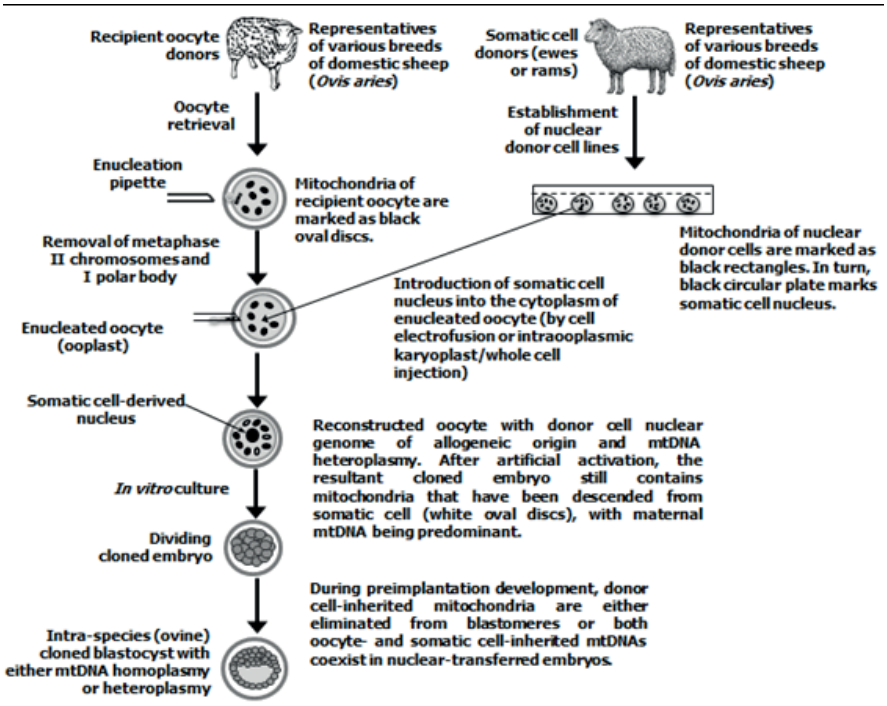


Fig. 4. Possible scenarios of extranuclear inheritance of the mitochondrial genome in cloned sheep embryos generated by intra-species SCNT. Molecular mechanisms underlying the distribution of nuclear donor somatic cell (NDSC)- and nuclear recipient ooplasm (NRO)-descended mtDNA fractions have not yet been fully elucidated in intra-species (ovine) cloned embryos. A vast majority of ovine SCNT-derived embryos at the blastocyst stage are characterized by the occurrence of homoplasmic mtDNA copies that have been inherited from enucleated oocytes (ooplasts), while the other cloned sheep embryos display the presence of heteroplasmic mtDNA copies stemming from both NDSC and NRO.

reported by other investigators [Glanzner *et al.* 2018, Zhou *et al.* 2019, Xu *et al.* 2019]. Analogously, the outcomes of cloned embryos generated with the use of Puławska or Złotnicka Spotted pig CFCs to complete their *in vitro* development to the blastocyst stage (Puławska breed: 34.1% and Złotnicka Spotted breed: 27.9%, respectively) were similar to or remarkably higher than those reported in other investigations [Zhang *et al.* 2012, Qu *et al.* 2020, Nguyen *et al.* 2021]. Cumulatively, the efficacy of intra-species (intra- or inter-breed) somatic cell cloning in farm animals is largely determined by: 1) the origin and molecular quality of the established NDCLs [Lee *et al.* 2019, Zhang *et al.* 2019, Gorczyca *et al.* 2021, Wiater *et al.* 2021*b*]; and 2) frequency of pro-apoptotic events in nuclear donor somatic cells and SCNT-derived embryos [Chi *et al.* 2017, Jeong *et al.* 2020, Gorczyca *et al.* 2021]. This efficacy is also affected by: 3) epigenomic reprogrammability of donor cell nuclei in SCNT-derived oocytes and corresponding embryos [Sampaio *et al.* 2020, Wang *et al.* 2020, Wiater *et al.* 2021*a*]; and 4) molecular communication between nuclear and mitochondrial DNA genomes in SCNT-derived oocytes and corresponding cloned embryos [Takeda 2019, Magalhães *et al.* 2020, Samiec and Skrzyszowska 2021] (Fig. 4).

**Comprehensive summary, further research directions and targets**

Following transition from basic to applied research, the strategies used for generating biorepositories comprised of germplasm-carrier biological materials and somatic cell lines followed by their use for IVF/ICSI- or SCNT-based IVP and ARTs could be characterized by increasing applicability not only in animal production, but also interdisciplinary research [Ruiz *et al.* 2013, Menezes *et al.* 2017, Salamone *et al.* 2017, Thongphakdee *et al.* 2020, Hildebrandt et al. 2021]. Therefore, the aforementioned strategies could contribute to: 1) conservation of genetic resources and establishment of genetic reserves of near-threatened, vulnerable, endangered, critically endangered and vanishing native breeds; 2) restoration and multiplication of subpopulations of disappearing and rare conservative breeds of livestock species in order to perpetuate biological diversity and to enhance intra-population and inter-specimen genetic variability; and 3) de-extinction and reintroduction of extinct breeds into the anthropogenic agricultural ecosystem niches, in the case of ancestors of some primitive livestock breeds exhibiting persistent resistance to adverse environmental and climatic conditions [Moulavi *et al.* 2017; Comizzoli *et al.* 2019, Lueders and Allen 2020, Marzano *et al.* 2020]. Furthermore, the applicability of SCNT-mediated cloning in a variety of farm animal species may lead to enhancement of genetic and productive merit in different breeds [Smits *et al.* 2012, Selokar *et al.* 2014, Skrzyszowska and Samiec, 2021].

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