EX SITU CONSERVATION AND GENETIC RESCUE OF ENDANGERED POLISH CATTLE AND PIG BREEDS WITH THE AID OF MODERN REPRODUCTIVE BIOTECHNOLOGY – A REVIEW

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Abstract

The development and optimization of reproductive biotechnology – specifically semen cryopreservation, spermatological diagnostics, and intraspecies cloning by somatic cell nuclear transfer (SCNT) – have become essential techniques to conserve the genetic resources and establish genetic reserves of endangered or vanishing native Polish livestock breeds. Moreover, this biotechnology is necessary for perpetuating biological diversity and enhancing genetic variability as well as for restoring and reintroducing breeds into anthropogenic agricultural ecosystems. On the one hand, the purpose of our paper is to interpret recent efforts aimed at the *ex situ* conservation of native cattle and pig breeds. On the other, it emphasizes the prominent role played by the National Research Institute of Animal Production (NRIAP) in maintaining biodiversity in agricultural environmental niches. Furthermore, our paper provides an overview of the conventional and modern strategies of the banking and cryopreservation of germplasm-carrier biological materials and somatic cell lines, spermatological diagnostics, and semen-based and SCNT-mediated assisted reproductive technologies (ARTs). These are the most reliable and powerful tools for *ex situ* protection of the genetic resources of endangered breeds of livestock, especially cattle and pigs.

Key words: livestock species/breeds, *ex situ* conservation, germplasm-carrier biological materials, assisted reproductive technologies, somatic cell cloning

The gradual intensification of artificial animal breeding has become necessary because a lack of genetic diversity is threatening the existence of certain native Polish livestock species and breeds. A variety of native farm animals have adapted to different niches in anthropogenic agricultural ecosystems, which cover a broad spec-

trum of climatic and environmental conditions. Furthermore, these breeds are highly resistant to disease, have long lifespans, high reproductive fertility and prolificacy, and produce high-quality milk and meat. Polish breeds can also feed on natural grasslands (Szarek et al., 2004; Litwińczuk et al., 2012; Gurgul et al., 2019), which gives an opportunity to develop and protect scenic landscapes. The optimal use of native breeds that are well acclimated to regional conditions is of great importance to small farms in south-eastern Poland.

In many countries, the protection of native genetic resources is an important part of biodiversity conservation. Wood et al. (2018) showed that programmes of *in situ* protection of rare native farm breeds should be predictive, and alterations to the environment must be considered. Both intensified production and the genetic establishment of new highly productive breeds or lines can destabilize the diversity of domesticated animals, which is why special livestock conservation programmes have been developed. The main risks related to extensive and ecological breeding of the herds of native endangered low-productive livestock breeds encompass intensification and globalization of production, which give rise to a diminishment in the size of local subpopulations and, as a consequence, to a dwindling of genetic reservoirs of threatened indigenous breeds (Lauvie et al., 2011). In Poland, native breeds are under *in situ* protection, which is financially supported by the Ministry of Agriculture and Rural Development and the European Agricultural Fund for Rural Development (EAFRD) under the framework of the Rural Development Programme (RDP). Farm animal conservation is based on the *in situ* method because it allows threatened animals to adapt to changing environmental conditions. The application of artificial reproductive technology (ART) seems especially justified in the context of the Red List of Threatened Species, the newest version of which was published in 2019 by the International Union for Conservation of Nature and Natural Resources (World Conservation Union; IUCN). In Poland, it was first published in 1992. Based on the Red List, the UN's Food and Agriculture Organization (FAO) assessed the risk status of livestock species within the framework of the World Watch List – Domestic Animal Diversity in 1993, 1995 and 2000. According to the FAO data, endangered livestock breeds account for 17% of approximately 8800 farm animal species existing in agricultural environment niches.

For both medium- and long-term conservation of genetic resources, the complementarity of the *in situ* and *ex situ* methods appears to be necessary. Within the framework of biodiversity protection programmes, *ex situ* conservation of germ-plasm-carrier biological material (male and female gametes and *in vivo*- or *in vitro*-fertilized embryos) by cryogenic preservation can be used as an ancillary strategy to maintain the population of protected animals in the wild and re-introduce farm breeds that have significantly decreased in numbers.

Endangered species are especially susceptible to genetic drift and genetic erosion, which can lead to population collapse. Therefore, to perpetuate the long-term *ex situ* or *in situ* conservation of biodiversity in native livestock species, animal cloning by somatic cell nuclear transfer (SCNT) appears to be unavoidable. The cryopreservation of genetically stable or permanent primary cell cultures and somatic cell lines from different tissue explants/biopsates provides a research alternative to gamete

and embryo freezing or vitrification. Such efforts are focused not only on the rescue of endangered species and breeds but also on retaining breed-related biodiversity.

Assessing the genetic diversity and estimating the endangered status of native cattle and pig breeds

An effective population size (N_e) has been found to be a crucial estimator for determining genetic diversity in native livestock populations and predicting the extent of inbreeding (Leroy et al., 2020; Polak et al., 2021). The size of an ideal population (i.e., one that meets all the Hardy–Weinberg assumptions) would be one that loses heterozygosity at a rate equal to that of the observed population. There are two types of N_e : one focuses on changes in genetic variation in offspring, which naturally leads to the consideration of inter-population divergence; the other focuses on changes in parental heterozygosity, which leads to consideration 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 size is less than or equal to 50, a breed is considered to be critically endangered and requires *in situ* and *ex situ* protection. If the size is between 50 and 200, the breed is endangered and conservation efforts are inevitable (Leroy et al., 2020). Taking into account all the native Polish cattle breeds in the programme relevant to the conservation of genetic resources, the effective population size of White-backed cattle oscillates around 38 and so is critically endangered and has the highest extent of inbreeding (Litwińczuk et al., 2012; Sawicka-Zugaj et al., 2018; Polak et al., 2021). The effective population size for other native Polish breeds that are subject to *in situ* protection ranges from approximately 67 (Polish Red and White) and 77 (Polish Black and White) to more than 170 (Polish Red). For these reasons, the status of these cattle breeds is considered to be endangered and has a relatively high inbreeding rate (Szarek et al., 2004; Litwińczuk et al., 2012; Cieślińska et al., 2019; Gurgul et al., 2019; Polak et al., 2021). Furthermore, the effective population size estimated for native pig breeds undergoing *in situ* protection fluctuates between approximately 80 (Złotnicka Spotted) to more than 144 (Puławska). Therefore, this finding predicts that these pigs will become endangered, and this is reflected in their relatively high rate of inbreeding (Szulc et al., 2011; Babicz et al., 2020; Polak et al., 2021).

The efforts of the NRIAP to implement strategies of germplasm-carrier biological material banking to perpetuate the genetic diversity of native Polish livestock breeds

At the United Nations Conference on Environment and Development (UNCED) in June 1992 in Rio de Janeiro, 167 countries signed the “Convention on Biological Diversity” (<https://sustainabledevelopment.un.org/outcomedocuments/agenda21>), which Poland subsequently ratified. For those reasons, the Ministry of Agriculture and Rural Development (MARD) delegated tasks to the NRIAP related to the coordination of activities leading to the protection of genetic resources and the collection and cryogenic preservation of biological materials from a variety of livestock.

It is noteworthy that the NRIAP collected native bull semen early and stored it at the Central Semen Bank (CSB), now called the Bank of Biological Material. Established in the 1960s, its main objective was to protect young bull semen from all insemination stations in Poland cryogenically. Moreover, MARD ordered the CSB to assess and classify this semen for insemination all over Poland. Over nearly 60 years, the CSB biorepository of bull semen increased, and as a result a unique collection of bovine germplasm-carrier biological material was created (Table 1). A vital element of this collection is that the semen was collected from bulls that had not been cross-bred. The aim of such procedures was to not only perpetuate the inheritance of desirable genes within endangered or critically endangered native breeds but also to diminish the bottleneck effects of inter-population genetic drift and reduce inbreeding, thereby increasing intra- and inter-population genotypic variability. Furthermore, increased genetic diversity contributes to enhanced individual or herd immunity to exceptional stressors (e.g., adverse environmental and climatic conditions or abnormal thermic, pathogenic, epidemic and pandemic shocks).

Veterinary legislation related to the collection and storage of bull semen was necessary because of biological materials recovered before Poland joined the EU in 2004. The oldest semen samples in pellets and straws that had been cryopreserved since the 1960s did not meet current identification requirements. Therefore, this unique collection originating from native breeds can only be used for artificial insemination in Polish breeding programmes.

To protect a variety of biological materials *ex situ*, especially germplasm-based material derived from different livestock species, a second bank, the National Bank of Biological Material (NBBM), was established in 2014 within the NRIAP. In its structure and organization, the NBBM can store germplasm-carrier biological materials (semen, oocytes, embryos) from goats, pigs, horses, sheep and cattle. These materials, which are cryogenically stored in compliance with European restrictions, are recovered from native Polish breeds of the above-mentioned species undergoing *in situ* protection.

The extent of cryopreserving germplasm-carrier biological material from different livestock species is comprehensively characterized within the framework of programmes that protect the genetic resources of a variety of breeds. These are available at <http://www.bioroznorodnosc.izoo.krakow.pl>.

Until now, bull semen collected from all rare native breeds has been stored and cryogenically preserved at the NBBM (Table 1). Moreover, the collection of germplasm-carrier biological materials has been enriched by the NBBM both with the embryos of endangered native pig breeds (e.g., Puławska and Złotnicka Spotted) vitrified according to the method developed in the NRIAP (Gajda and Smorąg, 2002) and with cryopreserved embryos of endangered native cattle breeds such as Polish Red, Polish Black and White and Polish Red and White (Table 1).

Table 1. The NRIAP collection of germplasm-carrier biological materials (semen and embryos) originating from Polish endangered native breeds of selected live-stock species

BANK OF BIOLOGICAL MATERIAL							
Species	Breed name	Material type	Number of samples	Number of male donors	Number of female donors	First year of collection	Last year of collection
Cattle	POLISH RED		48211	127	–	1966	2003
	POLISH BLACK AND WHITE	semen	9522	16	–	1973	1985
	POLISH RED AND WHITE		5457	21	–	1974	2001
	WHITE-BACKED		50	1	–	2007	2007
	NATIONAL BANK OF BIOLOGICAL MATERIAL						
Cattle	POLISH RED		12035	56	–	2006	2018
	POLISH BLACK AND WHITE		3995	20	–	2009	2020
	POLISH RED AND WHITE	semen	4669	21	–	2008	2019
	WHITE-BACKED		2109	37	–	2005	2019
	POLISH RED		18	2	2	2020	2020
Pigs	POLISH BLACK AND WHITE	embryos	3	1	1	2020	2020
	POLISH RED AND WHITE		10	2	3	2020	2020
	PULAWSKA		308	5	26	2017	2020
	ZŁOTNICKA SPOTTED	embryos	34	2	3	2018	2018

Table 2. The NRIAP biorepositories of cryopreserved somatic cells originating from Polish endangered native breeds of selected livestock species

Species	Breed name	Material type	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 PIGS	POLISH RED	Permanent cell lines of ear	45	5	–	Excellent***	Excellent***	Excellent***	Excellent***
	PULAWSKA	explant-derived	25	5	1				
	ZŁOTNICKA SPOTTED	dermal fibroblasts	22	6	–				

*As has been described in the study by Samiec et al. (2020).
**Cytogenetic assessment has confirmed a normal ploidy (euploidy) of evaluated cell lines as has been determined by the lack of karyotype changes such as aneuploidy or polyploidy (Samiec et al., 2020).
***Quality designated as excellent denotes that the rate of fibroblast cells displaying detailed quality-related parameters was higher than or equal to 95%.

To sum up, the cryopreservation methods of germplasm-based biological materials (semen and embryos) from cattle seem to be the most advanced and the most efficient. For this reason, they are applied on a large scale to reproductive biotechnology and *ex situ* conservation of genetic resources. Over the last years, the NRIAP has carried out intensive research on increasing cryoconservation efficiency of semen derived from other farm animals (Trzcińska and Bryła, 2015; Gogol et al., 2019). To ensure a high quality of frozen–thawed semen ejaculates intended for cryopreservation are subjected to seminological selection. A spermatological selection of semen, which is supposed to be frozen, can be accomplished whether or not a large of animal population size has been met. For small and vanishing populations of threatened livestock undergoing biodiversity conservation programmes, it is not possible to choose high-genetic merit male specimens, whose semen displays high cryoresistance- and cryosurvival-related parameters. Therefore, efforts have been undertaken at the NRIAP to elaborate efficient methods for the cryopreservation of semen regardless of spermatological quality.

NRIAP efforts to implement semen cryopreservation and seminological diagnostic strategies to perpetuate the genetic diversity of native livestock breeds in Poland

Sperm freezing plays a pivotal role in managing and preserving the genetic resources of livestock species and breeds, but several studies have examined cryo-damage in the sperm of different species (Yeste, 2016). The cryopreservation protocols bring about such stressors as cold shock and oxidative attack, which affect the structural and functional properties of plasma membranes and their biochemical, biophysical and molecular characteristics. The result is diminished survival rates and freezability, leading to the onset and progression of molecular mechanisms programmed for cell death. The oxidative attack of free oxygen radicals (i.e., reactive oxygen species; ROS) on cell membranes contributes to irreversible reduction in fluidity and the subsequent impermeability and terminal biodegradation of plasma membranes (Len et al., 2019). The incidence of these processes can be decreased or limited by supplementing freezing extenders with exogenous antioxidants.

The research activities of the NRIAP are focused on elaborating the qualitative and quantitative composition of extenders especially for the sperm of pigs, which are susceptible to the detrimental effects of cryopreservation. The NRIAP develops reliable and feasible methods of assessing the quality of cryopreserved sperm cells because semen samples in long-term storage must be extensively assessed for structural, functional and molecular quality-related parameters.

A novel strategy to assess semen quality, induced photon emission (chemiluminescence) has quantified the frequency of lipid peroxidation in the plasma membrane of boar sperm, and therefore has provided evidence that supplementing semen freezing extenders with the antioxidative agent butylated hydroxytoluene (BHT) improves the biochemical, biophysical and molecular quality-related parameters of frozen/thawed sperm. The protective effect of BHT is attributable to two biochemical, biophysical and molecular mechanisms. First, the incorporation of this compound into the phospholipid bilayers of the sperm plasma membranes makes them more

fluid and permeable and protects them from biodestruction. Second, it reduces biodestructive lipid peroxy radicals by converting them to hydroperoxides. The study by Trzcińska et al. (2015) proved this protective effect by supplementing a lactose-egg yolk extender with 1 mM BHT and 3% glycerol and applying it to post-thawed boar semen: total sperm motility (80.9%), viability (56.7%), and acrosomal integrity (60.1%). As a result, the highest reproductive performance of inseminated gilts (farrowing rate and litter size oscillating around 87% and 11 piglets, respectively) was indicated following the cryopreservation of semen in a BHT-enriched extender. These findings also confirmed that the supplementing of the cryoprotective medium with BHT successfully enhanced the fertilizing ability of post-thaw boar sperm. It is also noteworthy that our research into extending cryopreservation of sperm with a BHT-enriched cryoprotective medium led to the NRIAP's receiving a patent (No. PL228192-B1) for an invention entitled, "Extender for cryoconservation of boar semen and the procedure of semen freezing" (Trzcińska and Bryła, 2018).

The basic criterion for selecting ejaculate for cryopreservation is the assessment of fresh sperm motility, which is the most important indicator of quality and a crucial predictor of semen freezability. According to the previous investigations (Rath et al., 2009; Knox, 2015; Yeste, 2017), only boar ejaculate having minimal quality parameters of 80% motility and 80% viability are classified as cryopreservable. In contrast to previous studies, the results of the study by Trzcińska and Bryła (2021) confirmed that fresh semen having motility below 70% can be successfully cryopreserved in compliance with the assumptions and guidelines of NRIAP patent No. PL228192-B1 (Trzcińska and Bryła, 2018) with a high cryosurvival rate. As a consequence, fresh boar semen characterized by sperm motility below 70%, a low percentage of viability with apoptotic-like changes within the plasmalemma ultrastructure, and a low percentage of viable sperm with biodestroyed acrosome membranes exhibits a high cryosurvival rate. Cumulatively, the results of the investigation by Trzcińska and Bryła showed that predicting the suitability of porcine semen for cryopreservation based only on sperm motility turns out to be insufficient, especially when taking into account inter-specimen variability in this species. Therefore, other methods of semen evaluation are needed, including fluorocytochemical approaches to assess the plasmalemma and acrosome integrity of the sperm. Moreover, it would allow for the rapid detection of ultrastructural and functional changes within boar sperm cells, which would not only help estimate efficient semen freezing but also improve breeding.

In general, the approaches that have been developed and optimized in the NRIAP are targeted at cryopreserving semen from boars of commercial native pig breeds such as Polish Large White and Polish Landrace. Moreover, the aforementioned efforts can also be used to protect the genetic resources of threatened or critically endangered breeds such as Puławska, Złotnicka Spotted and Złotnicka White. These strategies also permit the preservation not only of selected genetic merit but also of productivity and performance traits within the framework of breeding programmes intended for rare native pig breeds. The creation of biological material collections of boar semen appears to be one of the most valuable tools to protect these subpopulations from diminishing because of intra-population and inter-specimen geno-

typic variability, or the spread of the African swine fever virus (ASFV). Notably, the NRIAP recently undertook to lead the establishment of the Semen Collection and Cryopreservation Centre (SCCC), which has been certified according to the requirements of Poland's Regional Veterinary Inspectorate (RVI). Within the framework of structural, functional and organizational activities, research and development at the SCCC is aimed at the cryogenic protection of boar semen samples on a large scale. These samples will have been collected from native pig breeds and extended with BHT-enriched cryoprotective media in compliance with the specifications of the previously mentioned patent, No. PL228192-B1.

Importance of somatic cell banking and SCNT-based cloning for genetic rescuing and maintaining the genetic diversity of Polish native livestock breeds – the efforts of the NRIAP undertaken in these research fields

The development and optimization of somatic cell cloning-based reproductive biotechnology in domesticated animal species and the establishment of somatic cell banking to preserve endangered breeds and species from extinction turns out to be indispensable (Andrabi et al., 2007; Arat et al., 2011; Song et al., 2019; Wang et al., 2020 a). The variability of animal genetic resources is a pivotal determinant of perpetuating the biodiversity in domesticated and wild species.

First, creating somatic (e.g., fibroblast) cell lines from native Polish livestock is a suitable and feasible genetic research model for maintaining biodiversity in rare native breeds. Second, the generation of such genetic resources for cloning by intra-species somatic cell nuclear transfer (SCNT), which includes intra-subspecies, cross-subspecies and intra- or inter-breed variants, and inter-species SCNT (including intra- and inter-family and intra- and inter-genus variants) appears to be powerful and helpful (Wells et al., 1998; Oh et al., 2008; Folch et al., 2009; Gómez et al., 2009; Liu et al., 2010; Wani et al., 2017; Veraguas et al., 2020). Therefore, such efforts must focus on giving not only comprehensive explanations of underlying biological mechanisms, but also detailed identifications of intrinsic molecular factors that affect proliferative activity, genetic stability, cytophysiological longevity, replicative senescence, physiological ageing, and the apoptotic or autophagy-dependent cell death of cultured nuclear donor cells. This will lead to the epigenetic reprogramming of their cell nuclei both in the host cytoplasm of SCNT-derived oocytes and in the blastomeres of the corresponding cloned embryos (Samiec et al., 2013; Gómez and Pope, 2015; Wang et al., 2020 b; Tunstall et al., 2018; Wu et al., 2019; Jeong et al., 2020; Sun et al., 2020).

Efficient methods of establishing and cryogenically preserving genetically stable cutaneous fibroblast primary cell cultures and cell lines of such endangered native livestock breeds as Polish Red cattle, Puławska and Żłotnicka Spotted pigs have already been developed at the Department of Reproductive Biotechnology and Cryoconservation of the NRIAP. For those purposes, internal biorepositories of cryopreserved somatic cells were created (Table 2) to generate somatic cell-cloned embryos of the aforementioned rare native Polish breeds (Samiec et al., 2020). The efficiency of generating bovine nuclear-transferred (NT) embryos reconstructed with Polish Red cattle ear explant-derived dermal fibroblast cells (EE-DFCs) to reach the blasto-

cyst stage *in vitro* (30.1%) was comparable to, or remarkably higher than, that reported by other investigators (Qu et al., 2017; Glanzner et al., 2018; Xu et al., 2019; Zhou et al., 2019). Analogously, the capabilities of porcine NT embryos reconstructed with Puławska or Żłotnicka Spotted pig EE-DFCs to complete their extracorporeal development to the blastocyst stage (Puławska: 34.1% and Żłotnicka Spotted: 27.9%, respectively) were similar to, or considerably higher than, those noticed in other studies (Glanzner et al., 2018; Zhao et al., 2018; Taweechaipaisankul et al., 2019; Jeong et al., 2020; Qu et al., 2020).

Figure 1. Patterns of cytoplasmic inheritance of mitochondrial genome copies in bovine cloned embryos generated by intra-species somatic cell nuclear transfer (SCNT) into enucleated oocytes. So far, the fate of nuclear donor cell-inherited mtDNAs has not been recognized in intra-species cloned embryos. In the majority of bovine SCNT-derived embryos, mitochondrial genome fractions primarily stem from nuclear recipient oocytes, whereas in their other counterparts, mtDNA molecules seem to be descended heteroplasmically (Author of Figure: Marcin Samiec)

To sum up, the efficiency of intra-species and inter-species somatic cell cloning of domesticated mammals is affected, to a great extent, by three factors: the provenance of frozen/thawed and *ex vivo*-expanded nuclear donor cell lines (NDCLs)

that provide genetic resources from different livestock species or breeds (Samiec and Skrzyszowska, 2010; Li et al., 2013; Olivera et al., 2016; Lee et al., 2019), the morphological, cytogenetic, cytophysiological and molecular quality parameters of the NDCLs (Samiec et al., 2013, 2019; Zhang et al., 2019; Wiater et al., 2021); and the subsequent incidence of programmed (apoptotic or autophagic) cell death in the development of cloned embryos (Chi et al., 2017; Samiec et al., 2015; Jeong et al., 2020). This efficiency is also dependent on such molecular mechanisms as the epigenetic ability of the nuclear donor cell-inherited genome to be reprogrammed in SCNT-derived oocytes and result in cloned embryos (Samiec and Skrzyszowska, 2018; Deng et al., 2020; Sampaio et al., 2020; Wang et al., 2020 b) and the intergenomic crosstalk between nuclear and mitochondrial DNA fractions in the host cytoplasm of nuclear recipient oocytes and the blastomeres of the developing cloned embryos (Srirattana et al., 2011; Samiec and Skrzyszowska, 2021; Takeda, 2019; Xu et al., 2019; Magalhães et al., 2020) (Figure 1).

Conclusions and future goals

Beyond any doubt, the strategies used both for 1) germplasm-carrier biological material banking and semen cryopreservation, diagnostics and assisted reproductive technologies and 2) for somatic cell banking and intra-species somatic cell cloning have a large application potential for the Polish economy, science and interdisciplinary research. These strategies could lead to the conservation of genetic resources and establishment of the genetic reserves of endangered or vanishing native breeds, restoration and multiplication of subpopulations of disappearing and rare conservative livestock breeds to maintain biodiversity genetic variation, and the reintroduction into the anthropogenic agricultural ecosystem niches breeds that are the ancestors of some primitive breeds characterized by persistent resistance to adverse environmental and climatic conditions (Wells et al., 1998; Arat et al., 2011; Selokar et al., 2014; Magalhães et al., 2020). Moreover, practically applied research into the cloning of different livestock breeds could serve to achieve other tangible benefits, including improved genetics (fertility and prolificacy) and production (increased milk and meat yield).

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Received: 22 II 2021

Accepted: 10 VI 2021