CRYOBANKING OF ANIMAL GENETIC RESOURCES: THE PHILIPPINE EXPERIENCE

Annabelle S. Sarabia & Libertado C. Cruz
Philippine Carabao Center
Department of Agriculture
National Headquarters and Gene Pool
Science City of Muñoz, Nueva Ecija, Philippines

Introduction

The past century has marked a net loss of diversity resulting from the increased rate of extinction of breeds and varieties. The alarming decline in biological diversity, particularly in animal genetic diversity, due to the rapidly growing human populations imposing pressures on the ecosystems, such as environmental destruction, habitat conversion and fragmentation and pollution, prompted conservation biologists to develop strategies to conserve and maintain genetic diversity. There are several approaches to conserve biodiversity and the reason for conservation will depend on what approach to adopt. Theoretically, there are three types of conservation measures, namely, (1) *in situ* conservation refers to the continuous use of the animal under its natural environment; (2) *ex situ in vivo* conservation is when live animal population are kept outside of their normal environment or not kept under normal management condition (e.g., zoological parks or government farms); and (3) *ex situ in vitro* conservation is external to the living animal in an artificial environment, under cryogenic conditions including the cryoconservation of embryos, semen, oocytes, somatic cells or tissues having the potential to reconstitute live animals in the future (FAO, 2007).

To date, the general trend of animal genetic resource (AnGR) conservation is *in situ* since this is more practical and less costly. However, this type of conservation is going on a relatively slow pace because of the complexity of maintaining live animals. The field of *in vitro* conservation is getting more attention with its wider application in the field of AnGR management and conservation. Semen freezing and cryopreservation has been successfully done and practiced since 1950's. Currently, cryopreservation techniques are commonly used in the storage of various tissues to include oocytes, embryos, somatic cells, primordial germ cells and others of laboratory and farm animals of interest. Hence, cryobanking would be the best alternative choice in AnGR conservation.

State of the Genetic Resource Conservation in the World

The recent publication of FAO (2007) on The State of the World's Animal Genetic Resources for Food and Agriculture is extensively covering the wide array of issues on the global state of livestock biodiversity. The loss in domestic animal diversity is estimated to be two breeds per week. The process of breed homogenization was attributed to factors like (a) pressure to adopt improved breeds and standardized production and breeding systems due to high performance and productivity; and (b) loss of traditional livelihoods and cultural diversity due to lack of resources resulting from the encroachment of agriculture or population pressure. As a result, the number of

indigenous livestock breeds has declined rapidly during the 20th century (Ilse Kohler-Rollefson, 2001). About 1/3 of more than 7,000 livestock breeds (including poultry) registered in the FAO global database are threatened by extinction (FAO/UNEP, 2000).

The current data analysis of the reports submitted by 169 member countries to FAO (2007) showed that the total number of breed records in the Global Databank has increased tremendously since the publication of World Watch List for Domestic Animal Diversity (WWL-DAD) (FAO/UNEP, 2000). The report analysis covered the period of 1999-2006. There was a marked increase in animal (mammalian and avian) breed population, though the percentage of breed from which the population data are available has decreased tremendously (Table 1). According to the report, the large discrepancy between the number of breed entries and the number which population data are available is in part accounted for since the latest data entered were extracted from country reports. However, there were reports that mentioned about the existence of the breed but do not include the details of the population size. Hence, this information is far from complete but the only available global data.

Table 1. Status of information recorded in the Global Databank for AnGR

Year of Analysis	Mammalian Species		Avian Species		Countries covered
	No. of national breed populations	% with population data	No. of national Breed populations	% with population data	
1993	2,719	53	-	-	131
1995	3,109	73	863	85	172
1999	5,330	63	1,049	77	172
2006	10,512	43	3,505	39	182*

*No data recorded for Andorra, Brunei Darussalam, Gaza Strip, Holy See, Liechtenstein, Marshall Islands, Federated State of Micronesia, Monaco, Nauru, Qatar, San Marino, Singapore, Timor-Leste, united Arab Emirates, West bank, Western Sahara
Source: FAO (2007)

In terms of species diversity, only about 40 of the 50,000 known avian and mammalian species have been domesticated. On a global scale, five species of agricultural importance are widely distributed and in large numbers, namely, cattle, sheep, chickens, goats and pigs. A total of 7,616 breeds were reported (6,536 local breeds and 1,080 transboundary breeds) and 690 of these breeds are classified as extinct. The risk status of the animal genetic resource was analyzed and a total of 1,491 breeds (20%) are considered at risk. With the introduction of the transboundary breed classification in the 2006 report, the assessment of the trends in genetic erosion cannot be directly compared among the risk status category from the 1999 report. What is alarming is that of the 45% of the newly reported local breeds are either at risk or already extinct. The report actually did not capture the genetic dilution of the local breeds by uncontrolled crossbreeding, which according to experts is the major threat to AnGR diversity.

Awareness of threats to diversity of the AnGR becomes a stimulus to many countries to institute policies and programs towards protection and conservation of their endowed animal resources. Based on FAO (2007) reports, of the 148 contributing countries, 52%

indicated presence of *in vivo* (Includes both *in situ* and *ex situ in vivo*) conservation measures and 37% has *in vitro* conservation programs. Countries mentioned with established genebanks were Japan, India, the Nordic countries, France, the Netherlands, Poland, the Czech Republic and Hungary. These genebanks store cryopreserved semen from all the main species, and embryos from cattle, sheep and goats. Few of them store poultry and horse semen. Tissue DNA samples are collected in the main species. Genebanks are initiated by governments or NGOs supported by universities and/or research institutions. In developed countries, there is a strong collaboration between genebanks and the animal breeding industry and breeders' associations in the collection of genetic materials. In developing countries that institute in vitro measures, collection and storage are limited to semen of local breeds (cattle, sheep, goat, water buffalo, etc.) at private or government institutions.

A conservation program could only be realized through collaborative efforts among stakeholders. These include among others, national governments, institutes for research and education (universities), NGOs, animal breeders' associations, farmers and pastoralists, part-time farmers and hobbyists, and breeding companies. Each of these stakeholders has a role to play in crafting and implementing their country's conservation measures.

It is worth mentioning though that in recent years, interest in the local animal genetic resource conservation rises due to the realization that local breeds may be able to compete with improve breeds in productivity within the context of their respective production systems (Intercooperation, 2000; Kebede, 2000), local breeds may harbor genes for resistance against diseases, genetic diversity in domesticated species with respect to adaptation to change is innate in traditional breeds, and in the context of "sustainable livelihood" approaches to development, local livestock is an important contributor to rural welfare and poverty alleviation (Anderson, 2000).

Still many countries in Africa, eastern parts of Europe and the Caucasus region, the Near and Middle East, Central and South Asia, and the Caribbean has yet to establish their national conservation programs (FAO 2007). These parts of the world are endowed with rich diversity of AnGR but ironically, their values are not recognized by the national authorities. Conservation programs are of national concern and national governments have responsibilities to address these concerns.

Why Cryobanking?

Experienced have shown that physical acquisition of genetic material is relatively quick and provides an important reserve of genetic resources that can be used for wide variety of conservation and research interests (McClintock et al., 2007). Although, many claimed that the gene banking approach is costly. However, it was demonstrated by the same team that the recurrent storage costs for cryobanked samples are manageable and in some countries, significantly less than *in situ* conservation particularly if the intention is for long-term storage (in terms of centuries). The initial establishment of the AnGR bank facility may be relatively high but if investments are amortized over a long period of time (say 20 or may be 200 years), then the cost will turn out to be low.

McClintock et al. (2007) emphasized that cryopreserved collections of genetic resources have multi-uses to wit, (a) reconstituting populations in the event of national need, (b)

reintroduction of genetic variability into breeds whose genetic base needs to be broadened or to reintroduce genes that may have been lost or have suffered a reduction in frequency, (c) development of new breeds or composite populations for either research or industry use, and (d) a source of material for genetic studies. Advances in genomics and informatics will intensify the capability of the system. Hwang (2008) pointed out that cryobanking is to SAVE as save work, including time and money, assurance of self-cell supply, validated cells, tested and qualified, and emergency, as a back-up supply. He further stressed that by cryobanking of cells we could save from expense of shipping the samples since it requires negligible space and weight, the aging of cells can be arrested, variable genetic materials can be stored and evolution could be possible at a shortest time.

There are numerous cryobanking technologies available for AnGR conservation. Cryotechniques are available from a range of tissues like blood, somatic cell to gametes (sperms and oocytes) or embryos and primordial germ cells (precursors of oocyte and sperm) and enabling technologies like programmable straw fillers, high precision programmable freezers, etc. At the low cost end, straws could be manually filled and cryopreservation can be performed in a disposable Styrofoam box with a high degree of repeatability. In some instances, sample collection and cryopreservation are being done in the field using mobile equipment in a mobile van. Sampling techniques are also readily available from existing gene banks.

Accordingly, the limits to AGRB activity may depend not in science but in politics. We have seen the extent of human control over genetics but it will remain to be seen if we have the will to bring about genetic resource management and bring under control the loss of habitats and their species. Unless this activity is positively chosen, destruction of the planet will keep on apace and no scientific discovery will reverse it (Watson and Holt, 2003).

Reproductive Biotechnology in Cryobanking

There had been tremendous advancement in the use of biotechniques in the fields of animal breeding, reproduction and molecular genetics in recent years. Reproductive biotechnologies like artificial insemination (AI), multiple ovulation embryo transfer (MOET), in vitro fertilization embryo transfer (IVEP), and somatic cell nuclear transfer (SCNT) embryo transfer have accelerated livestock improvement programs by speeding up genetic progress, reduce risk of disease transmission and expand number of animals that can be bred from a superior parents. Likewise there is rapidly advancing progress in the field of species characterization based on molecular markers and marker assisted selection (MAS), providing exciting opportunities in AnGR management (FAO, 2004). However, the utility of these technologies varies from country to country and among regions depending on economic and technical capabilities.

The success and usefulness of animal genome resource bank (AGRB) depend on assisted reproduction procedures or reproductive biotechniques, which include tools such as artificial insemination (AI), embryo transfer (ET), and in vitro fertilization (IVF) among others. These procedures are further enhanced by other techniques, such as intracytoplasmic sperm injection (ICSI). With the current advances in the manipulation of cells in culture, primitive germ cells are cultured to maturity for subsequent use in AGRB. Furthermore, with the development in cloning technique, individuals may be

generated from tissue cells in storage, thereby extending the concept of AGRB from simple reproductive germ cells and embryos to any valuable cell population or tissue.

Advances in cryogenic storage make it possible to store variety of cells for long periods of time. Biomaterials can be stored at -196^{o}C in a liquid nitrogen tank and the length of storage time becomes indefinite provided the liquid nitrogen is well maintained. Cryogenic storage techniques can be summarized as deep-freezing of sperm and oocytes; deep-freezing of embryos; and storage of genes as DNA (Pala, 2004).

Reproductive and molecular biotechnologies coupled with cryogenics are vital components of cryobanking. The commonly cryopreserved genetic materials for utilization and future use include the following:

Gametes (sperm and oocytes). Cryopreservation of semen is possible in all domestic animals including poultry (chickens, ducks, geese). However, this technique is specie-specific but general procedures are well-documented. Deep-freezing of slaughtered/abattoir-based or ovum-picked-up (OPU) mature oocytes and stored for prolonged periods prior to in vitro fertilization are possible in some mammalian livestock species. Collection in situ and in vivo (OPU) and cryopreservation of immature and unfertilized oocyte have been made possible. However, unfertilized oocytes are difficult to cryopreserve than embryos because the haploid female gamete is highly susceptible to chilling injury and cryoprotectant toxicity (Parks and Ruffing, 1996). This susceptibility is thought to be associated with the unique characteristics of mature oocytes, namely, large size and the presence of a meiotic spindle and cortical granules in the ooplasm (Candy et al., 1994). Two methods of freezing of oocytes are available, slow-freezing procedure and ultra-rapid freezing or vitrification procedures. The second procedure was developed to limit the damage to the oocyte due to chilling injuries or toxicity of the cryoprotectants. However, cryoconservation of oocytes to make it useful for the preservation of AnGR poses as a challenge and has yet to be validated on a large scale. Coulter (1992) reported that manipulation of spermatozoa provides opportunities for sperm sexing, the introduction of foreign DNA into oocytes and the formation of transgenic animals. Using these techniques and the new ones, one can control the structure of a newborn herd in the future and direct it to the needs of the future time (Pala, 2004).

Embryos. Deep-freezing of almost all mammalian embryos is successful. When thawed and transferred to recipient females effectively produce a progeny. In contrast to avian, hatched chicks have not been successfully obtained from eggs that have been frozen and thawed. This is may be because of the huge amount of lipid present in the vitellus (FAO, 2007). Embryos can be collected in vivo and/or produced in vitro through IVF or SCNT. A variety of protocols to freeze and thaw embryos have been done similar to oocytes. In slow freezing approach, equilibration of cryoprotectants and solutes between the medium surrounding the embryo and its intracellular components occurs slowly, thus limiting the risks of membrane rupture due to intracellular ice formation. Upon thawing, embryos are transferred to recipient females with or without removal of the cryoprotectant. Success rate of the procedure depends on the species, genetic origin, source (in vivo or in vitro) and stage of development of the embryos. Embryos cryoconserved at an early stage of their development result in lower parturition rates compared to a more advanced stage (Massip, 2001). Fast freezing (vitrification) involve ultra-high cooling and freezing of embryos in small amount of suspending medium in which cryoprotectant and other solutes (sugars) are in high concentrations. Survival

rates in sheep and goat embryos were 59 and 64%, respectively, using the pulled-straw vitrification technique (Cognie et al., 2003).

Somatic cells and somatic cell cloning. Since the first successful creation of Dolly the sheep, this technique has been shown to work in most mammals tested but not successfully demonstrated in avian species. The technique is a very attractive option for cryoconservation of AnGR. However, the success rate is very low, thus, turning out to be an expensive exercise. The main advantage of the technique is the choice of selecting exactly the animal to conserve, and later reconstitute a population of clones. Collection of somatic cells is easier than embryo collection, thus, samples could be extensively collected from field populations. But due to its uncertainty to produce live animals from the cryoprserved cells, it is unlikely to be the priority in species where cryoconservation of gametes and embryos is well developed. However, it could be a prudent back up where cryoconservation of gametes and embryos is not feasible or has low success rates (ERFP, 2003).

Deoxyribonucleic acid (DNA). If breed becomes extinct, one can bring the stored DNA into the active gene pool of the species by insertion of the DNA into the embryos of another breed of the same species (Pala, 2004). Animal species can be regenerated from the cryopreserved embryos and cell lines, but not from preserved DNA, due to limitations in technology. Animal DNA banks are constructed to preserve AnGR as a complementary conservation strategy. Animal DNA samples could be taken from preserved purified high molecular weight DNA and biological samples for DNA extraction, such as fresh blood, muscle, kidney, heart and hairs. Fresh tissues are preserved at -80^{o}C and DNA at 4^{o}C -20^{o}C or -80^{o}C. The storage of tissue samples rather than isolated DNA is a better preservation means to reduce DNA damage (Xiangyu and Zhang, 2006). Dry purified DNA can be preserved at room temperature for many years (Ryder et al., 2000). Frequent monitoring of the quality of preserved semen samples is required.

AnGR Cryobanking in the Philippines

The general effort to carry out conservation of livestock genetic diversity in the Philippines is of recent development. In fact, the National Biodiversity Strategies and Action Plan for the Philippines was only developed in 1997 and was subsequently revised in 2002 under the umbrella of the FAO.

Conservation of indigenous livestock species are largely through identification of communities where these animals are utilized for certain purposes and incentives are provided so that this in-situ system are sustained. This effort is complemented with establishment of ex-situ herds at the government institutional farms. Species of animals in ex-situ farm are shown below:

Species	Institutions
Beef Cattle	BAI
Native Chicken	BAI, SCUs
Native Carabao	PCC
Native Swine	BAI
Native Goat	BAI

BAI – Bureau of Animal Industry
PCC – Philippine Carabao Center
SCU – State College & Universities

Conservation efforts in water buffalo is by far the more organized with the establishment of the Philippine Carabao Center. Gene Pools of the indigenous breed are established in three major islands and are kept as Open Nucleus Herd (ONH). Gene Pool of introduced breeds, essentially the murrah breed are established in separate locations, utilizing mostly the network of 13 Centers of the Philippine Carabao Center (PCC).

In addition to indigenous stocks of water buffalo in in-situ and ex-situ facilities, semen are collected from top performing bulls at the PC Gene Pool and stored frozen. New semen donors are identified and are sent to semen laboratory for training and semen collection per yearly.

Semen from top performing bulls of the breed are also collected. About 2000 doses of frozen semen are stored from each bull, while the rest of the semen are utilized for AI. The same are also being carried out in the introduced breeds of beef cattle and goat at the institutional facilities. The extent of semen collection and banking is shown in Table below:

Table 2: Inventories of Frozen Semen at the Cryobank in the Philippines, 2008

	No. of Bull	Dose (Frozen Semen)
Water Buffalo		
Philippine Carabao	3	6,445
Murrah (Island Born)	55	69,409
Murrah (Bulgarian Sourced)	4	839
Sub-total	**61**	**76,693**
Cattle		
Brahman	5	18,876
Belmont red		7,759
Brangus		876
Holstein-Friesian		1,694
Indo Brazil		631
Nelore		5,049
Simbrah		1,673
Genepool		2,010
Sub-total		**38,568**
Goat		537
Sheep		700
Total		**116,498**

The establishment of reproductive biotechnology at PCC in 1996 facilitated for cryopreservation of in-vivo and in-vitro derived embryos of water buffaloes. Slaughter house obtained oocytes of native stocks are also collected, frozen and stored in the bank. Likewise, somatic cells of outstanding females, both the native and introduced breeds form part of the material collections at the bank.

Starting in 2008, after the issuance of Administrative Order No. 9, Series of 2008, PCC embarked an organized embryo collection and cryopreservation in goats. Efforts are now also underway so that all the domesticated livestock species are covered in the cryobanking and genetic characterization. This also include an endangered species, the Tamaraw, found only in an island in the Philippines, the Mindoro Island.

References

Anderson, S. 2000. Livelihoods and animal genetic resources: how can people's conservation contribute to poverty reduction? Paper presented at the International Conference and Workshop on Local Livestock Breeds for Sustainable Rural Livelihoods, held in Udaipur and Sadri, India, 1–4 November 2000. Cited by Kohler-Rollefson, I. (2001).

Candy, C.J., M.J. Wood, D.G. Whittingham, J.A. Merriman and N. Choudhury. 1994. Cryopreservation of immature mouse oocytes. Human Reproduction 9: 1738-1742.

Cognie, Y., G. Baril, N. Poulin and P. Mermillod. 2003. Current status of embryo technologies in sheep and goat. Theriogenology 59(1): 171-188.

Coulter, G.H. 1992. Bovine spermatozoa in vitro – a review of storage, fertility estimation and manipulation. Theriogenology 38(2): 197-207.

ERFP. 2003. Guidelines for the constitution of national cryopreservation programmes for farm animals, by S.J. Hiemstra, ed. Publication No. 1 of the European Regional Focal Point on Animal Genetic Resources.

FAO. 2004. The State of Food and Agriculture 2003-04. Agricultural Biotechnology – meeting the needs of the poor? Rome.

FAO. 2007. The State of the World's Animal Genetic Resources for Food and Agriculture, edited by Barbara Rischkowsky & Dafydd Pilling. Rome. 524 p

FAO/UNEP. 2000. World watch list for domestic animal diversity, 3rd Edition, edited by B. Scherf. Rome.

Hwang, Shia-Min. 2008. Cryobanking of animal cells. http://www.angrin.tlri.gw.tw/2008 Cryobanking/cryobanking of animal cells.pdf

Intercooperation. 2000. Capitalizing on experience. Indo-Swiss Cooperation in Livestock Development in India. Bern. Cited by Kohler-Rollefson, I. (2001).

Kebede, W.A. 2000. Do smallholder farmers benefit more from cross-bred (Somali x Anglo-Nubian) than from indigenous goats? University of Göttingen. (Ph.D. thesis). Cited by Kohler-Rollefson, I. (2001).

Kohler-Rollefson, I. 2001. Community Based Management of Animal Genetic Resources – with Special Reference to Pastoralists. In: Community-Based Management of Animal Genetic Resources. Proceedings of the Workshop held in Mbabane, Swaziland, 7-11 May 2001. pp 13-25.

Massip, A. 2001. Cryopreservation of embryos of farm animals. Reproduction in Domestic Animals 36(2): 49-55.

McClintock, S., E. Groeneveld, S. Hiemstra and H. Blackburn. 2007. Cryobanking in Animal Genetic Resources. http://www.fao.org/ag/againfo/programmes/en/genetics/ documents/Interlaken/sidevent/3-4/McClintock.pdf

Pala, A. 2004. Genetic conservation of livestock and factor analysis. Applied Ecology and Environmental Research 2(1): 135-141.

Parks, J.E. and N.A. Ruffing. 1996. Factors affecting low temperature survival of mammalian embryos. Theriogenology 37: 59-73.

Ryder, O.A., A. McLaren, S. Brenner, YP. Zhang and K. Benirschke. 2000. Preservation of DNA from endangered species. Science 288: 725-727.

Soule, M. 1991. Conservation: Tactics for a constant crisis. Science 253: 744-750.

Watson, P.F. and W.V. Holt, ed. 2003. Cryobanking the Genetic Resource: Wildlife conservation for the future? CRC Press: USA. 443 p.

Wildt, D.E., W.F. Rall, J.K. Critser, S.L. Monfort and U.S. Seal. 1997. Genome Resource Banks: Lining collections for biodiversity conservation. BioScience 47(10): 1-20.

Xiangyu, J. and YP. Zhang. 2006. DNA banking of animal genetic resources. In: DNA banks – providing novel options for genebanks? Topical Reviews in Agricultural Diversity, by de Vicente, M.C. and Andersson, M.S. (eds.). International Plant Resources Institute, Rome, Italy. 84 p.