Cryopreservation and Reconstitution Technologies: A Proposal to Establish A Genome Resource Bank For Threatened Australian Amphibians

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Table of Contents

<center>**RECOMMENDATIONS**</center>

Recommendation 1.1
Establishment of an Amphibian Genome Resource Bank:
A National Amphibian Genome Resource Bank (GRB) be established for threatened Australian amphibians.

The most effective approach to establish the GRB would be to exploit available infrastructure, management systems and governance procedures in existing institutions. These include museums, zoos, and research institutions (universities and CSIRO). Museums should add collections of frozen specimens, thereby adhering to (and indeed ingeniously amplifying) their archival and conservation mission.

Cooperative arrangements need to be forged between the various parties involved in achieving conservation of wild populations. GRBs could also be developed under the umbrella of the intensive species management strategies that are developing in zoos, within Species Survival Plans and Taxon Action Groups.

Actions and Comments
1. Tenders should be sought meeting a set of minimal criteria under a number of basic headings; (a) scientific capacity, (b) physical capacity, (c) governance and management, and (d) research capacity.
2. Preference should be directed to publicly funded and managed facilities whose charter is to research, interpret and protect the natural heritage of Australia. State Museums and Zoos are the most obvious facilities.
3. A GRB that is held as a national repository would be best placed to achieve its short and long-term objectives if it was associated with an active research facility. This linkage may involve collaborative efforts among several different institutions.
4. Minimum criteria: <u>Governance</u> - best practice standards of data storage, systems management, security, risk assessment, OHS procedures, maintenance and alarm back-up in case of unexpected events. <u>Technical capacity</u> to cryopreserve sperm in the field and transport it back to central repository. Ability to support and conduct cell culture cryopreservation of cell lines. Some technical functions may be best achieved in a research facility that is associated with the storage facility.
5. Construction of a list of priority species and populations to be placed in the initial GRB. This list should be derived from the list of nationally endangered species, and from discussions with state conservation agencies and field ecologists.
6. Consideration of the value of extending the concept to a broader range of native fauna. The primary criteria should be conservation status and the opportunity to access material and appropriate knowledge to cryopreserve suitable cells and tissues.
7. Investigate Linkages (MoU, cooperative storage, research) with international efforts in GRBs such as the British Frozen Ark project, and the US Monell frozen Tissue Collection.
8. Pursue Sperm Storage of prioritised frog species as a priority. Follow an adaptive research approach to ensure that the sperm of chosen species are optimally cryopreserved (freeze -> thawed -> fertilization capacity). It should be recognized that cryopreserving sperm can store most if not all the genetic diversity of a species. Storing the sperm of about 5 individuals from most populations captures some 90% of the genetic variation at the nuclear gene level. Rare alleles will be less well represented.

9. The issue of sex determination (male or female heterogamety needs to be determined separately for each taxon), in the collection to preserve the female germ line. Most frogs do not have heteromorphic chromosomal sex determining mechanisms so sex determining mechanism cannot be determined by a simple chromosome preparation. There is some evidence that sex can be determined by environmental conditions (temperature).

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Establish a program of studies of reproductive and cryobiology to optimize the function of an Amphibian Genome Resource Bank:
Conduct strategically directed studies of the reproductive and cryobiology of amphibians to make an Amphibian Genome Resource Bank directly useful in conservation programs and to optimize its operations and overall effectiveness.

The aim is to make the GRB directly useful. Proof of concept is needed to gain public acceptance.

Actions (Major Research Directions)
1. Focus early actions on optimizing protocols for the storage and use of sperm from selected species such as the Corroboree Frog and several rainforest species where genetic diversity is currently being lost or depleted.
2. Focus research on the storage and retrieval of totipotent diploid cells (blastocyst cells). Proof of concept is needed for the use of these cells to retrieve functional adults.
3. Protocols for the storage and retrieval of ovarian cells and cultures of ovarian tissue. Need to prove that immature oocytes can be used to reconstitute adult individuals.
4. Experiments to enucleate donor oocytes and perform associated nuclear transfers in native amphibian taxa.

Recommendation 1.3
Scale of a Amphibian Genome Resource Bank:
An Amphibian Genome Resource Bank for threatened amphibians should aim to sample and access samples from across the distribution range of a species and include isolated populations where possible.

Sampling strategy should include common and widespread species. The potential use and long term value of such a collection should not be overlooked. The collections in natural history museums provide significant value and interpretation of our national heritage. In the face of unexpected effects such as global climate change an accessible repository of genetic material may prove to be invaluable to future investigators in ways that we cannot fully anticipate at this time.

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Captive colonies:
It is high desirable to preserve the diversity represented within individuals to retain the genetic diversity of species held as captive populations. A plan needs to be developed for procedures and protocols of sampling from captive colonies held for conservation purposes. The plan should be part of the proposed national amphibian genome resource bank. Acquisition and storage of samples of sperm from males should be a priority. Cryostorage enables the diversity present in a founder to be reintroduced into a lineage at any time and for genetic diversity (heterozygosity) to be retained. Therefore, implementation of the plan to sample and store genetic material from captive colonies should include establishment of protocols to re-introduce stored genes back into captive colonies.

Recommendation 2.1
Develop an Action Plan for an Australian Threatened Amphibian Genome Resource Bank. The Action Plan would be a written document that contains explicit information that justifies the bank on the basis of both *in situ* and *ex situ* conservation.

It should contain:
- Relevant information on species biology (life history and natural reproduction).
- Numbers in the wild and in captivity.
- Accessibility of these animals for donating to the GRB (State agencies are responsible for the conservation of native animals and some consideration would need to be devoted to their role in such a plan).
- Type and amount of materials to be collected, to achieve the genetic management targets.
- A consideration of security issues. We suggest a 3-way division of stored genomes of threatened species; 1) an in perpetuity repository for use only when a species approaches extinction; 2) for routinely managed living animals both *in situ* and *ex situ*, and 3) containing biomaterials for research.
- Technical Aspects: collection, storage, use, research needs, funding, proprietary issues.
- Safety/security
- Database management

Recommendation 2.2
A realistic approach to establishing a GRB is to use facilities that already exist, or alternatively to implement small targeted initiatives dealing with species of special interest.

We recognise that to be effective and useful GRBs are one component in a multi-faceted program to conserve species. Cooperative arrangements need to be forged between wildlife managers, ecologists, museums, zoos, universities, and possibly private industry such as human fertility clinics.

Museums could add collections of frozen germ plasm, thereby adhering to (and indeed ingeniously amplifying) their archival and conservation mission. Government funded institutions such as Museums and public zoos have the advantage of providing a guarantee of safekeeping and security (appropriate stewardship).

Genome Resource Banks could also be developed under the umbrella of the intensive species management strategies that are developed by zoos under established processes such as Species Survival Plans and Taxon Action Groups

Preamble

"Biological diversity is the key to maintaining life as we know it"
(Wilson 1992)

The Convention on Biological Diversity, agreed at the United Nations' Earth Summit in 1992 explicitly recognizes the link between biodiversity conservation and sustainable development. It is in the spirit of the convention on biological diversity that the Amphibian GRB is conceived. Article 9 of the Convention deals specifically with *ex-situ* conservation measures.

Article 9. *Each Contracting Party shall, as far as possible and as appropriate, and predominantly for the purpose of complementing in-situ measures:*
(a) Adopt measures for the ex-situ conservation of components of biological diversity, preferably in the country of origin of such components;
(b) Establish and maintain facilities for ex-situ conservation of and research on plants, animals and micro- organisms, preferably in the country of origin of genetic resources;
(c) Adopt measures for the recovery and rehabilitation of threatened species and for their reintroduction into their natural habitats under appropriate conditions;
(d) Regulate and manage collection of biological resources from natural habitats for ex-situ conservation purposes so as not to threaten ecosystems and in-situ populations of species, except where special temporary ex-situ measures are required under subparagraph (c) above; and
(e) Cooperate in providing financial and other support for ex-situ conservation outlined in subparagraphs (a) to (d) above and in the establishment and maintenance of ex- situ conservation facilities in developing countries.

Section 1

The Problem of Amphibian Declines

1.1 Extinction

In 1980 the distinguished biologist E.O.Wilson observed,

"The worst thing that can happen - is not energy depletion, economic collapse, limited nuclear war, or conquest by a totalitarian government. As terrible as these catastrophes would be for us, they can be repaired within a few generations. The one process ongoing in the 1980s-90s that will take millions of years to correct is the loss of genetic and species diversity by the destruction of natural habitats. This is the folly our descendants are least likely to forgive us."

The loss of species and genetic diversity is a major feature of the biodiversity crisis (Sala *et al* 2000), a crisis that is predicted to escalate with the impact of global warming (Thomas *et al* 2004). Despite the recognition amongst biologists of the significance of extinction, actions to slow or halt species loss are widely recognized as being ineffectual. One problem not widely appreciated, that we detail below with an example from the global decline of amphibians, is that extinction often occurs while scientists are searching for the causal agent. We argue the need for contingency strategies to save genetic and species diversity once there is indication that a species is vulnerable. We develop our approach for Australian amphibians because of the rapid declines in this group, however the methods and strategy we propose are widely applicable to many organisms.

It is now generally accepted amongst conservation biologists that the amphibia are experiencing a major worldwide extinction crisis, which has been underway for the last twenty years. The World Conservation Union reported the first Global Amphibian Assessment (GAA) in the journal Science in late 2004 (*Stuart et al*, 2004), summarising data from 500 scientists and examining threats for all 5743 described amphibian species. The results demonstrate that **32% of amphibians are threatened** which is far more than for birds (12%) or mammals (23%). Of those threatened 7.4% are listed as critically endangered. Of considerable concern are the 122 species that can no longer be found ("possibly extinct" not formally "extinct" until exhaustive surveys are completed), which include nine Australian species, the majority of which disappeared in the past two decades. Focusing on threatened species, the GAA considered three groups based on the cause of decline. The largest number (207 species) were classified under the category of "enigmatic decline" i.e., *"declining, from pristine habitats, for reasons not fully understood",* although they point out that disease and climate change are cited as the most common causes. The percentage of enigmatic-decline species increased with increasing extinction risk, which suggests that the factors causing decline are driving species towards extinction particularly rapidly. The GAA notes that the geographical extent of enigmatic declines is likely to have been underestimated, particularly in the tropics, where amphibians have been insufficiently monitored.

The GAA concludes that "enigmatic-decline" species present the greatest challenge for conservation, *"because there are currently no known techniques for ensuring their survival in the wild"* and the only option currently available is captive breeding. We consider complimentary approaches to assist captive breeding and husbandry (*ex situ* methods) as an insurance against extinction and population genetic depletion. **The aim of this report is to investigate the feasibility**

and scope some of the tasks necessary to establish a genome resource bank and to develop the knowledge and skills required to retrievably store amphibian genomes. The methods we propose have global application.

One example of the critical nature of the disappearances among Australian amphibians is illustrated by the situation in the genus *Rheobatrachus,* in which the only two species to have been described are considered to be extinct. The significance of these species, because of their unique habit of gastric-brooding, meant they were the subjects of intense investigation. Consequently their disappearance was examined very closely. *R. silus* was discovered in 1972, its specialised mode of parental care described in 1974, and it was last observed in the wild in 1981. At the time of its discovery it was described as common, though subsequent workers claimed it was never easy to find (Tyler & Davies 1985), the original distribution was confined to the Conondale and Blackall Ranges and occupied less than 100 sq km (Tyler 1991). The second species *R. vitellinus* was discovered in 1984 (Mahony *et al*, 1984), and had disappeared by 1986 (McDonald 1990). Both species were found in relatively pristine environments (Ingram & McDonald 1993; Laurence *et al* 1996; Richards *et al* 1993). Although both species were held in captivity, self-sustaining laboratory populations were not established (Tyler 1991), indeed, mating was not achieved in captivity, although it is true to say that this was not a high priority at the time, since it was assumed that animals could be sourced in appropriate numbers from the wild. With the benefit of hindsight it is incomprehensible that in the later stages of the species decline no frozen tissues were stored to provide a later source for retrieval of genomes. The cryopreservation of tissues would have provided a source of material to re-establish species when appropriate technologies (a subsidiary aim of this project) develop in the future.

1.2   The Concept Of Insurance in Conservation Biology

It is clear that despite the best efforts of conservationists that extinctions are continuing. Extinction is forever, and once it has occurred we have lost the result of millions of years of evolution. What can be done?

There is 'back-up' strategy or Insurance policy that can be applied. It is to preserve the frozen viable cells and DNA from threatened species. Within the frozen cells and nuclei is the store of knowledge about the composition, development, behavior and ecology of a species. We should never underestimate the potential of developing technologies that in the future will allow nuclear DNA to be used routinely for preserving and propagating animals on a large scale. Recent progress in molecular biology has been so fast that we cannot predict what may be possible, using this information, within the next few decades (The Frozen Ark Project, 2005).

We need to take lessons from the past. For most threatened species, the gathering of useful biological information after they are recognized as in danger is often extremely difficult (e.g. the history of the decline and disappearance of the Sharp-snouted day frog, *Taudactylus acutirostris*) and sometimes impossible. We must be pro-active and take out the insurance policy while there is time to sample wild populations to include sufficient genetic diversity.

1.3   Justification For *Ex Situ* Strategies

The ideal strategy for the long-term protection of biological diversity is the preservation of natural communities and populations in the wild. However, *in situ* preservation is not a viable option for the conservation of declining species in the current circumstances. Species diversity is lost forever while investigations proceed into the causes of decline and means of mitigation. The reality is that it is now 20 years since the first disappearance among Australian frogs and no effective strategy to halt declines has emerged. Moreover, some of the factors implicated in the decline, including the action of a virulent pathogen are currently beyond human control, and we must buy time while solutions are investigated. Even if chytridiomycosis due to human related transfer of pathogens is the major cause of declines in frogs, many of the resultant extinctions are already likely to have occurred (Sarkar 2000).

In such a case it is likely that the only way to save susceptible species and populations from extinction is to maintain individuals in artificial conditions under human supervision until the cause is confirmed and mitigated (Clulow *et al* 1999; Mahony *et al* 1999, Holt *et al* 2003). This is a very expensive approach when numerous species are involved and to effectively represent the genetic diversity of a species requires a considerable number of individuals along with special management. We are among the first to recognise that the ideal strategy for the long-term protection of biological diversity is the preservation of natural communities and populations in the wild (Mahony *et al* 1999). However, *in situ* preservation is not a viable option for the conservation of declining species in the current circumstances.

In addition to the total loss of species, for species in small and isolated populations, as well as those in captivity, there is the concern for the loss of genetic variation through genetic drift (Frankham 1995). A number of management approaches can be made to minimize genetic drift but it cannot be removed completely because of the unavoidable random segregation of heterozygotes. Advances in reproductive technologies, particularly sperm cryopreservation (Browne *et al* 1998; 2001; 2002a,b,c,d) allows the effective population size to be increased enormously. Consequently, the amount of drift can be greatly reduced to insure against the loss of genetic diversity in wild and captive populations (Wildt *et al* 1993; Wildt 1997; Carabello *et al* 2001).

1.4 Applications of Cryobiology in the Context of Amphibian Declines: Genome Resource Banking as a special case of the *ex situ* approach.

The approach we propose (cryobiology) is complementary to the classical approach of habitat preservation to prevent species extinction. The maintenance of "seed banks" to preserve genetic variation in rare plants and agriculture is well established. They are also not expensive when combined with modern cryobiology and freezer technology. For example, the Australian Genome Storage Resource Centre, Monash University Melbourne & Taronga Zoo Sydney, holds over 1500 different samples (including semen, oocyte, embryo and reproductive and somatic tissue samples) from 100 endangered species collected over the last 10 years and the Australian Biological Tissue Collection (South Australian Museum) holds over 15,000 frozen (-70^0C) animal tissue samples curated by one manager. Although conservation biologists favour management through habitat conservation, the notion of cryopreservation is gaining acceptance as a legitimate tool to conserve genetic and species diversity under certain circumstances (see papers in Holt *et al* 2003). We have argued in published discussions of this issue, that cryo-storage of amphibian genomes has the potential to be an extremely valuable conservation tool (Clulow *et al* 1999; Mahony *et al* 1999; Watson & Holt 2001).

What must be appreciated is that some technologies relevant to the application of assisted reproduction in the conservation of amphibians already exist, although (with the exception of recent studies by ourselves and some others on sperm cryopreservation) these were not developed with the intention of conserving threatened genomes.

The established procedures include:
- *in vitro* fertilisation (IVF),
- sperm cryopreservation (largely developed in our laboratory at the University of Newcastle),
- techniques for non-invasive induction of sperm and egg release from the gonads,
- nuclear transfer (the capacity to retrieve fully functional individuals by nuclear transfer using early embryonic cells was developed in the early 1950s), and
- androgenesis.

Although few, if any, of these procedures would be regarded as optimised over a range of species, the proof of concept has been demonstrated.

1.5   Reproductive Technologies Associated with Genome Resource Banks

The great majority of amphibians have external fertilization and external embryonic development and thus the reproductive technologies associated with the use and integration of a GBR into a conservation program are much more straightforward than is necessary with mammals.

It should be recognized that the following methods are well developed and currently available.
- Induction of maturation of gametes
- Induction of gamete release
- Collection of gametes
- Short-term storage and maintenance of viability
- In vitro fertilzation (IVF)
- Cryopreservation of gametes, sperm but not oocytes

In theory, it should also be possible to store unfertilized oocytes. However, unfertilized oocytes are much harder to cryopreserve than embryos because the haploid female gamete is highly susceptible to chilling injury and cryoprotectant toxicity. This susceptibility is thought to be associated with the unique characteristics of mature oocytes, namely their large size and the presence of meiotic spindle and cortical granule sin the ooplasm (Candy et al 1994).

1.6   Summary

In summary, the relevant technologies necessary for the application of cryostorage and assisted reproduction in the conservation of amphibians already exist. The established procedures include *in vitro* fertilisation, sperm cryopreservation, and techniques for non-invasive induction of sperm and egg release from the gonads, nuclear transfer and androgenesis. Although few, if any, of these procedures would be regarded as optimised over a range of species, the proof of concept has nevertheless been demonstrated.

1.7   Recommendations

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Establishment of an Amphibian Genome Resource Bank:
A National Amphibian Genome Resource Bank (GRB) be established for threatened Australian amphibians.

The most effective approach to establish the GRB would be to exploit available infrastructure, management systems and governance procedures in existing institutions. These include museums, zoos, and research institutions (universities and CSIRO). Museums should add collections of frozen specimens, thereby adhering to (and indeed ingeniously amplifying) their archival and conservation mission.

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Section 2

Genome Resource Banks

2.1 What is a Genome Resource Bank?

Definition: A Genome Resource Bank (GRB) is a repository of systematically collected germplasm (gametes), embryos, blood products, tissue and DNA for a defined conservation program (Wildt et al 1997).

Most GRBs are specifically applied to wild animals, and there is an emphasis on organized collection of stored germ plasm (mainly cryopreserved spermatozoa from individuals whose contribution to the gene pool is to valuable to loose). Historically, frozen tissue collections from wild animals have been widely used for applied and theoretical research to uncover biodiversity, define species distributions, inform population ecology, and in conservation genetics. Many of these roles are closely allied to the aims of a GRB and we see these two types of collections as being complimentary conservation and research tools. There is also a considerable amount of transfer of expertise learnt with frozen tissue collections to the management and governance of GRBs.

Until recently, frozen tissue collections have provided little practical value in breeding programs for endangered species. Apart from the fact that there was no need to store gametes until the current extinction crisis arose, frozen tissue collections were designed for other purposes, mainly for systematic studies using molecular techniques. The essential difference between these frozen tissue banks and a GRB is the method of freezing. In frozen tissue banks the process of freezing tissues has relied simply on vitrification (immersing the tissue into a freezing environment, usually liquid nitrogen at -170°C) without the use of cryoprotectants. While this method prevents the degradation of enzymes, proteins and DNA, the formation of ice crystals within the cytoplasm damages cellular organelles and membranes. There is little hope that material frozen in such a manner would be useful for nuclear transfer in the future.

The concept of a GRB that we develop here is a focused collection of germplasm, planned in detail, addressing a wide range of scientific and organizational issues from identification of priority species and populations to management and governance. We have derived a considerable amount of the management from policies and practices already adopted by well-established international seed banks.

2.2 Statement of Aims of a Genome Resource Bank for Threatened Australian Amphibians

"To conserve the genetic resource of native Australian amphibians as an insurance against species loss and depletion of genetic diversity in the wild"

Relationship to the overall goal of conservation programs and the Chyrid Threat Abatement Plan.

The objectives of a GRB relate specifically to four areas of the Chytrid TAP:
- Captive husbandry to prevent species extinction.
- Retain and prevent depletion of genetic diversity.

- Reduce the number of individuals of particular species that need to be held in captivity, thus extending resources for a more diverse collection of species.
- Potentially play a vital role in processes to select for resistance to the pathogen.

Species breeding programs typically aim to conserve as much genetic diversity as possible, usually with an aim to maintain evolutionary potential. Achieving this aim by natural reproduction and focused husbandry involves maintaining large numbers of breeding individuals usually dispersed among geographically isolated institutions. Storing germplasm samples from many individuals removes some of the requirements to hold large numbers. Fewer living animals are necessary, and more space becomes available for other rare species. Scientific management of the pedigrees of all animals enables them to be integrated into the planned breeding programs.

2.3   Genome Resource Banks, Do they work?

The GRB concept and strategies have received increasing attention over the past two decades (for reviews see Holt et al 1996, Wildt et al 1997, Holt and Pickard 1999, Watson and Holt 2001, papers in Holt et al 2003). Here we present a brief review of the major international initiatives to establish GRBs and the development of frozen tissue banks for Australian animals.

2.3.1 Existing International Genome Resource Banks

Large-scale organised repositories of germ plasm are more common for plants than for animals, in part because it is technically easier to store plant than animal germ plasm (i.e., seeds of many plants can be dried and stored in refrigerators at 4°C or -20°C, Wildt 1997).

Botanists have already considered the realistic necessities associated with establishing GRBs. Nonetheless, the considerable organizational and governance issues associated with a well planned, prioritised and managed facility should not be overestimated. The Kew Millenium Seed Bank in the UK is one example. Recognizing the impossibility of collecting seeds from every plant species, the program strives to attain modest and pragmatic goals. One target is to collect and store seeds from threatened dryland plant species. To achieve their aims, a dedicated facility has been built and staffed, supported by a long-term funding plan, agreements have been reached with over thirteen international collaborating institutions including the Department of Conservation and Land Management and the Botanic Gardens and Parks Authority of Western Australia (see copy of Kew Seed Bank International Program. Appendix 2).

Similar international GRBs with specific conservation objectives for animal germplasm are less well developed. Although, it should be pointed out that human fertility, animal agriculture and molecular research has benefited greatly from large-scale sperm and embryo storage.

The Conservation Breeding Specialist Group (CBSG) of the IUCN-World Conservation Union's Species Survival Commission has addressed the issue of how GBRs can be implemented for conservation as part of their overall mission (www.cbsg.org). A GRB "Action Plan" concept was formulated with specific guidelines to steer stakeholders through the necessary consideration associated with cryobanking. A prototype GRB has been prepared for the tiger, a species chosen because of its precarious status in nature, with a solid information base on reproductive biology and physiology, and its flagship value.

Announced in 2004 was the "Frozen Ark Project" (ARK) to be based in the United Kingdom with the direct aims of establishing a GRB for endangered animals. Its objectives are similar to the Kew Millenium Seed Bank, and envisages international partnerships and co-operation (see Appendix 1 for draft documents obtained from the ARK project).

The European Center for Animal Cell Culture, Porton Down, England, is another example of a frozen tissue collection. Until recently this national facility did not hold germ plasm samples, but that has now changed. Its role has focused on cultures of agricultural, industrial and medical significance. In some respects this mirrors the role of the Australian Animal Health Laboratory CSIRO at Geelong in Victoria.

The American Museum of Natural History's Ambrose Monell Cryo Collection, launched in 2001, aims to store about one million frozen tissue samples representing DNA of a wide range of species. The objective of the collection are essentially to support a research role and conservation is not the primary objective, *"the Museum's frozen tissue collection will support a broad range of research, and allow scientist, today and in the future, to take full advantage of advance in genomic technology"*.

Of considerable value to this exercise to scope the establishment of a GRB for conservation of critically threatened Australian amphibians is the management procedures developed by these established facilities. These include useful guidelines, policies and practices on the following matters:

1) Collection policy
   - Purpose
   - Acquisition
   - Ensuring that specimens are relevant to and consistent with the purposes and activities of the collection
   - The institution can provide for the storage and preservation of the specimens under conditions and ensure their availability and meet with professionally accepted standards for collection preservation
   - Approval
   - Accessioning specimens
   - Undocumented specimens
   - Collecting in the field
   - Gifts or donations
   - Permanent loans
   - Purchases

2) Care of Collections
   - Documentation
   - Accession record
   - Catalogue of record
   - Specimen database
   - Data, Labelling
   - Physical Map
   - Documents
   - Storage protocols (split between two collections)

3) Use of Collections
- Access, bioresource issues
- Agreement of State Conservation Agencies

2.4 Genetic Resource Banks already established in Australia

There are several frozen tissue collections already established in Australia that achieve major components of the definition of a GRB. The oldest frozen "tissue bank" for native animals (the Australian Biological Tissue Collection, stored and curated by the Evolutionary Biology Unit of the South Australian Museum), is over 20 years old. It stores tissues, blood and DNA for defined research programs (mostly associated with molecular systematics, but it has also been used to identify threatened species, forensic information associated with illegal wildlife collection and trade, parasite identification, population structure analysis for fisheries, and identification of disease organisms). In many respects the collection has contributed greatly to conservation biology by clearly defining the origin and biodiversity of the Australian fauna.

It holds over 15,000 specimens of hundreds of species of native animals. In many instances the frozen tissue sample has a parallel whole animal "voucher" preserved in the fixed material collection of the museum.

To our knowledge the following state and federally funded institutions manage frozen tissue collections, and like the Australian Biological Tissue Collection described above most are designed to support research objectives associated with molecular systematic research.

> Australian National Wildlife Collection: CSIRO Division of Wildlife and Ecology, Canberra.
> Australian Museum, Sydney
> Queensland Museum, Brisbane
> South Australian Museum, Adelaide
> Western Australian Museum, Perth
> Victorian Museum, Melbourne
> Animal Genome Storage Resource Centre (Monash University & Taronga Zoo)

In almost all cases there is inter-institutional agreement for the access and loan of material (this is managed by the Council of Australian Fauna Collections). Management practices appear to have been developed as an extension of long- term procedures used with the curation and management of traditional preserved museum collections. Specialized protocols have been developed to deal with security.

To our knowledge no Australian Museum has a frozen collection that focuses on germ plasm. All collections are of frozen tissues, usually tissues such as heart, liver, kidneys, skin and blood, and occasionally testes or ovaries. In some cases, where the animal is relatively small, whole carcasses may be frozen. In contrast the Animal Genome Storage Resource Centre (Monash University & Taronga Zoo) has a focus on germ plasm.

Three points are common to most of the established frozen animal tissue collections overseas and in Australia.
1) They are research collections and are not directly involved with *ex situ* conservation programs for target taxa.

2) Most collections are held by institutions with established community roles (e.g., State Museums or Zoos) and funding arrangements are included as part of their operating budget. Most have a research related role. Most museums also play an archiving role for natural heritage collections, and genetic heritage is a relatively new concept.

3) Their management protocols have developed from those used for traditional museum research collections.

In several ways they differ from the approach taken by the Millenium Seed Bank and Living Ark projects.

1) These projects have explicit Biodiversity and Conservation goals that are linked to agreements under international conventions on Biodiversity.

2) There is a recognition that ownership of genetic resources belongs to the people of a nation, and the consequences that this brings to issues of loans and use of material.

2.5 Planning to Actively Use a Genome Resource Bank in Genetic Management of Captive Colonies of Threatened Species

2.5.1 Application of GRBs to wildlife species
The following application of GRBs to the conservation of wildlife species is outlined by Wildt et al (1997).

Easy, inexpensive movement of genetic material among living populations.
- Source of new genes that can be infused into small or fragmented populations.
- Saves stress of transport for wild animals.
- Overcomes or reduces the need to remove animals from the wild to support captive populations. Instead "surplus" germ plasm is collected, leaving the entire wild population in nature, where its presence helps to protect native habitat.

Insurance.
Small populations are vulnerable to environmental catastrophes, disease outbreaks, demographic and environmental stochasticity, and genetic drift and inbreeding.

Extending generation interval.
"Genetic diversity is lost only when animals are no longer available to reproduce" (Ballou 1992). As long as viable cryopreserved sperm or embryos remain stored, genes do not die with the animal. Banked germ plasm can be used long into the future. Leibo (1994) reported that cattle sperm cryopreserved for 37 years retained the capacity to fertilize.

Increased efficiency of captive breeding
Populations in different zoos and breeding facilities can be managed to maximizing genetic diversity.
Helps to resolve space problems by reducing the number of living animals needed to meet genetic diversity targets.

Other advantages
Parallel samples of blood and tissues enable biomaterials cryopreserved over time to be screened to identify the onset of a particular epidemic in nature and captive populations.
*"Scientist routinely collect animals and plants as study specimens. Cryobanked germplasm, embryos, tissues blood, and DNA would be another type of collection, but one with considerable*

more conservation potential that provided by the conventional collections of objects, plants and animals found in traditional museums, botanical gardens and zoos." (Wildt et al 1997)

2.5.2 Integrating the technology into practical conservation programs
Being relevant to genetic management and conservation

Bennett (2001) and others have advocated a species-specific approach to GRBs. They argue the need to link such programs to endangered species programs (e.g., directed by actions in a Recovery Plan or Taxon Advisory Group). The point is well made, and we would envisage that an Australian Amphibian GRB would be established with a precise charter, objectives and priorities. However, due to the technical and management requirements we would consider that a facility that deals with all amphibian germ plasm is favourable to separate programs. Restricting the bank to amphibians would provide a useful model by which to judge the value of such an approach to other animal group.

Successful functioning of a Genome Resource Bank involves the combined expertise and collaboration of four rather distinctive partners.
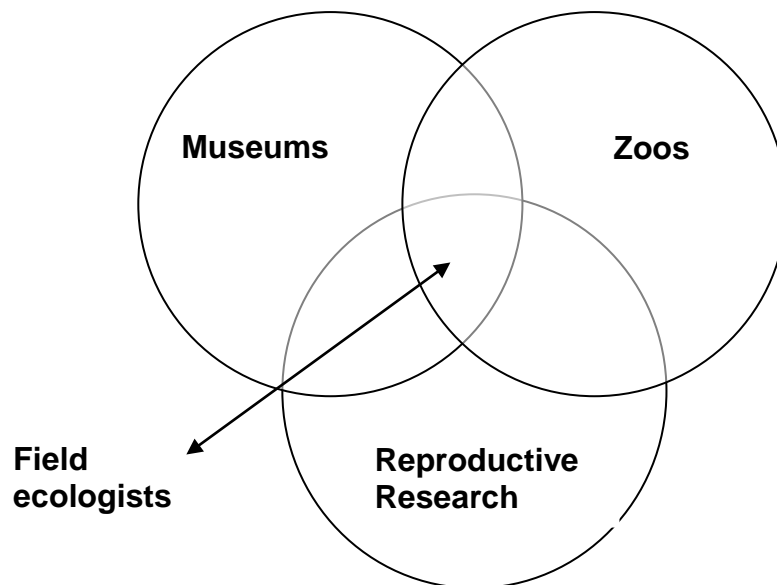
It is possible that all four roles could be within one institution, but this does not appear to likely, at least in Australia.

Museums play the primary role in storing, researching and interpreting natural heritage. While they play a vital role in biodiversity conservation this function is not traditionally associated with *ex situ* conservation and they rarely are involved in captive breeding of endangered species, reintroductions or proactive conservation actions.

Zoos display animals to the public, have expertise in animal husbandry and interpretation, and in recent times have taken a more active role in conservation of endangered species especially with captive breeding programs. Research conducted into animal diseases and in the broad area of reproductive sciences is often association with these programs. Our enquires indicate that it is not common for Australian zoos to house genetic resource collections.

Research in areas dealing with reproductive sciences is generally associated with universities, medical and agricultural institutes. Once again it is not common for genetic resource collections to be housed at these institutions. There are of course exceptions; some of the larger frozen semen and embryo collections are maintained by agriculture and medical research facilities.

One group not traditionally included in the functional GRB model presented above is the field ecologists. They have the knowledge of the animals in the field, their ecology, reproductive biology, and distribution and abundance that are vital for consideration of options for collection of samples and selection of future reintroduction sites. Thus they are a critical link between the museum collections, reproductive sciences and captive breeding groups.

Examination of successful genome resource banks reveals a range of organization structures. The Kew Millenium Seed Bank (see Appendix 1) is an example of a single institution model with storage, research and breeding occurring under one roof. Even field collection is conducted as a routine function, although they do receive and store material from collectors in many parts of the world and have agreements with institutions throughout the world including Australia.

The most effective model from a logistic perspective would be to have all functions under one roof, however this is not likely to be an option for a Amphibian Genome Resource Bank in Australia. This being the case it would seem that the most effective approach is to forge strong links between organizations; museums with a proven capacity to store and manage collections, zoos capable of the animal husbandry, and universities with reproductive research capability to use the material in *ex situ* conservation programs. There is a significant role for field ecologists in accessing and collecting the base constituents of the collections.

Successful models of *ex situ* conservation programs being structured around a 'zoo-centered' focus include examples such as the recovery of the Black Footed Ferret (Howard et al 2003) and a number of programs for large carnivores.

It would seem that for the necessary governance and security required for a genome resource bank the most appropriate institutions are government museums or zoos. These institutions should be brought into a discussion on how their involvement in a Genome Resource Bank may enhance their public role in conservation biology. There is, to our mind, complete synergy between nature conservation and their role in natural heritage interpretation. No doubt this is a changing role, but it is not one that is exactly new to the modern natural history museum or zoo. In the case of museums almost all have embraced modern molecular techniques for investigating biodiversity and have frozen tissue collections that support molecular research laboratories.

2.5 Recommendations

Recommendation 2.1
Develop an Action Plan for an Australian Threatened Amphibian Genome Resource Bank. The Action Plan would be a written document that contains explicit information that justifies the bank on the basis of both *in situ* and *ex situ* conservation.

It should contain:
- Relevant information on species biology (life history and natural reproduction).
- Numbers in the wild and in captivity.

- Accessibility of these animals for donating to the GRB (State agencies are responsible for the conservation of native animals and some consideration would need to be devoted to their role in such a plan).
- Type and amount of materials to be collected, to achieve the genetic management targets.
- A consideration of security issues. We suggest a 3-way division of stored genomes of threatened species; 1) an in perpetuity repository for use only when a species approaches extinction; 2) for routinely managed living animals both *in situ* and *ex situ*, and 3) containing biomaterials for research.
- Technical Aspects: collection, storage, use, research needs, funding, proprietary issues.
- Safety/security
- Database management

Recommendation 2.2
A realistic approach to establishing a GRB is to use facilities that already exist, or alternatively to implement small targeted initiatives dealing with species of special interest.

We recognise that to be effective and useful GRBs are one component in a multi-faceted program to conserve species. Cooperative arrangements need to be forged between wildlife managers, ecologists, museums, zoos, universities, and possibly private industry such as human fertility clinics.

Museums could add collections of frozen germ plasm, thereby adhering to (and indeed ingeniously amplifying) their archival and conservation mission. Government funded institutions such as Museums and public zoos have the advantage of providing a guarantee of safekeeping and security (appropriate stewardship).

Genome Resource Banks could also be developed under the umbrella of the intensive species management strategies that are developed by zoos under established processes such as Species Survival Plans and Taxon Action Groups

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Example of wildlife conservation programs that have relied on GRBs.

Several successful examples of the use of GRBs in conservations programs could be cited (Wyoming Toad, Bighorn sheep, Chimpanzee, Giant Panda, Eld's Deer, Gaur, Bongo, Prezwalski's Horse, Black Footed Ferret, Cheetah), we briefly detail one here.

The Cheetah
The ability to effectively cryopreserve sperm from this species eliminates the need to ever remove another cheetah from the wild to support zoo breeding programs.

Poor record of reproduction in captivity considered being due to low genetic diversity and high levels of inbreeding.

Artificial Insemination achieved in zoos enabled sperm cryopreserved from wild caught males to be used in Artificial Insemination to produce successful litters (Namibia).

The GRB provides a reserve of disease-free gametes that can assist in restoring this species in regions where it has declined.

2.6 Consideration of the Workshop participants

The workshop considered the feasibility of establishing a Genome Resource Bank for threatened Australian amphibians and supporting the Reproductive Sciences that would be necessary to ensure its effectiveness.

Three areas were addressed:
1. Is it scientifically feasible to establish a GRB?
2. Who will manage it?
3. What would it cost in the short and long-term?

(1) Is it scientifically feasible to establish a GRB?

Scientific issues

Question: Which germ plasm (cells/nuclei or tissues) should be cryopreserved?

The ideal situation would be to have a repository composed of cryopreserved mature gametes (sperm and ooocytes) and embryos. The requirement is that the nuclei preserved can be readily retrieved.
- Priority 1. Sperm, Ooocytes and Embryos.
- Priority 2. Diploid totitpotent cells/nuclei. The options are blastomeres and cultured ovarian cells. (Blastomeres are cells obtained from early embyros).
- Priority 3. Somatic tissues (cryoprotectant used prior to freezing) such that the nuclei are not damaged by ice crystal formation. Cell cultures may be preferred over whole tissue or organs.

Current Situation
- It is possible currently only to cryopreserve and retrieve sperm.
- It appears that cryopreservation of oocytes and embryos will not be easily achieved.

Thus sperm would be the first cells that should be considered for cryopreservation

The participants noted that:
- Cryopreservation protocols for sperm have been developed.
- Currently cryopreservation of sperm is conducted in a laboratory setting, however, this process does not rely on extensive equipment and there is no reason to believe that it cannot be conducted successfully in the field.

Question: What associated methods are necessary to collect germ plasm?

Methods to induce sperm release (hormonal induction of wild caught males).
- Methods to achieve sperm release have been developed.
- This can be readily performed in captivity or the field.
- These methods are non-intrusive; they involve injection with a hormone and the subsequent collection of sperm from urine.

Methods to inactivate sperm.
- Protocols to inactivate sperm by altering osmolality have been developed.

Collection Protocols

Many of the species/populations that we consider should be included in a GBR are rare and will therefore be difficult to collect. A significant component of the methods proposed involve techniques such that individuals are not injured or stressed by the process of collection and sampling.

In addition to the non-invasive collection of sperm or other germ line cells we would suggest that the opportunity also be taken to collect a parallel sample of DNA by non-intrusive sampling (eg, by a scraping of skin cells). These biomaterials hold a wealth of information for genetic, disease and as yet unknown future studies. DNA can be amplified and genetic studies completed should they be needed in the future. A number of advantages can be outlined. DNA from non-invasive sampling can be used to identify individuals, determine mating patterns and population structure including effective population size, and measure levels of genetic diversity. It may also provide information on whether the individual was infected with chytrid.

Sampling, collection and handling techniques must be consistent.

We would envisage constructing a GRB by sampling endangered species in nature and storing the material in portable equipment. Minimal numbers of males would be collect to achieve objectives to cover genetic diversity. No transport of animals would be necessary. Sperm would be cryopreserved and frozen in the field. Cryostrored sperm would be transported to the central storage facility.

Other Uses of Genetic Resource Banks

It is well known that animal agriculture has benefited from large-scale sperm and embryo storage for the purpose of improving meat and milk production in domestic livestock (Wildt et al 1997). In addition, a few programs bank biomaterials from animal models used in biomedical research to protect long-term availability for standard genotypes of mice, rats, hamsters, rabbits, cats and dogs, ensuring that researchers are working with uniform animal models that give repeatable results.

Transgenic technology (the ability to incorporate novel genes into injected embryos) has also resulted in the production of thousands of new animal models. Although this technology has exponentially increased research opportunities, the by-product is a proliferation of animal colonies that require expensive housing and care. One solution is to cryopreserve sperm and embryos from genotypes that are not in use currently but are potentially valuable in the future. The Jackson Laboratory in Bar Harbour, Maine and the National Institute of Health in Bethesda, Maryland, collect and cryostore embryos and occasionally, sperm from animal models, usually rodents.

Customized Collections have also been developed for micro-organisms that are used in the environmental, food, and biomedical industries to produce products valued at tens of billions of dollars (Cunningham 1994). The American Type Culture Collection, in Rockville, Maryland, is leader in cold storage research, with more than 80,000 cultures of algae, protozoa, bacteria, bacteriophages, cell lines, hybridomas, fungi, yeast, recombinant DNA materials, and viruses available for worldwide distribution.

Postscript: We are stunned by the apparent misplaced priorities of our scientific effort. That more resources have been directed towards storing the genome of hundreds of transgenic mice lines, that are produced by human laboratory expertise, yet at the same time we have failed to prevent the extinction of up to nine native frog species in the past two decades.

(2) Who will manage it?

This question considered the management of a GRB in the context of whether it is feasible.

Management Issues
Question: What institutions, organizations or individuals would provide the necessary expertise to manage a repository of with high natural heritage significance?

The workshop considered that the institutions capable of managing a GRB are publicly funded and managed facilities whose charter is to research, interpret and protect the natural heritage of Australia. State and National Museums are the most obvious facilities. Zoos also have a role because in this instance there is a link between the stored items and restoration and captive husbandry of endangered species. There was also recognition that research-intensive institutions such a Universities, CSIRO, Government Conservation Agencies and private individuals, should be involved in active collaborative projects.

(3) What would it cost in the short and long-term?

The workshop had some information presented on the costs of basic infrastructure to support a GRB for threatened amphibians. The workshop participants recognised that such an issue is related to the number of specimens that would be stored, space requirements, and security of the collection.

At the very basic level a GRB that covered all the listed Endangered and Vulnerable species on the EPBC Act would mean a total of 27 species being represented by at least one population. If germ plasm from a total of 10 individuals (5 males and 5 females) for each of these 27 species were stored, a total of 270 samples would be collected. For security reasons these would be divided into three independent storage units. Presumable each sample would be represented by multiple replicates. Nonetheless, such a collection could be easily stored in three 80 litre liquid nitrogen dewars.

For more extensive species and population coverage, to increase security and management stewardship a larger facility would be necessary.

In addition to the cost of establishment and maintenance of a GRB is the associated cost of collection of material and associated research in reproductive sciences. It was also recognised that some support would be desirable for education, management and communication functions.

Frequently Asked Questions
1. How long will cryopreserved cells remain viable?
Decades of experience in the cattle industry involving freeze-thawing manipulations of sperm and embryos confirms that, when suitable technology is used, viability is unaltered by cryopreservation.

2. Will diseases be transmitted with frozen samples?

Suitable hygiene techniques can be applied to prevent the accidental transmission of disease contamination from animal to samples or vice versa. A storage cell is available that provides double packaging to prevent leakage during storage.

2.6   What are the challenges?

The workshop participants considered the challenges to a successful GRB.

a.   Making germ plasm readily accessible to breeding programs.

b.   Training those in breeding programs of the significance of germ plasm use.

c.   Infrastructure cost; establishment and continual maintenance.

d.   Convincing government agencies (museums, zoos, research institutions, government conservation agencies and NGOS) that they have a vital role to play. Zoos have, in recent times, recognized the need to be involved in conservation breeding programs as well as providing an opportunity for the public to observe a diverse range of animals. They are continually faced with balancing the ethical considerations of holding animals in captivity with the values of education, enjoyment and conservation.

e.   Once germ plasm is banked, its successful utility is tied to knowledge of the reproductive biology of the target species, and manipulating that biology through technology. In this respect amphibians provide a far simpler target than mammals, because fertilization is external, embryonic development is generally external and there is no need for synchronizing implantation in a surrogate mother.

f.   What is the role of state conservation agencies? Traditionally in Australia they have been responsible for *in situ* conservation programs, although in recent years there has been a move by private industry and investors into this area.

g.   Linking research in Reproductive technologies with the GRB.

h.   Communication.   This is a major challenge, to provide the public, conservation professionals and scientists, many of who are sceptical of the GRB concept with the basic justification for its construction.

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Section 3

Priority Setting for a Genome Resource Bank

3.1 Threatened Species Categories

There are a number of criteria that can be used to determine priorities for acquisitions by a GRB. The first is to use current listings of species of conservation concern and adopt those as priority categories. The simplest approach to achieve this is to consider all amphibian species currently listed on schedules of threatened species in national legislation and legislation of each state and territory. This process can be supplemented by a consideration of other lists based on different priority settings.

Most listings of threatened species in Australia adopt international categories and the criteria on which they are based. However, there has not been a uniform approach to assessment across the various legislatures within Australia. The IUCN (the World Conservation Union) "Red List" classifications of <u>Presumed Extinct</u>, <u>Critically Endangered</u>, <u>Endangered</u>, <u>Vulnerable</u> and <u>Lower Risk</u> reflect degrees of risk of extinction (Mace *et al* 1991, 1993). They are defined largely in terms of the rate of decline in population abundance, area of habitat occupied, and the estimated number of extant populations. While there are numerous other systems used to categorize endangerment, the IUCN "Red List", provides the only international system for comparative purposes, and was the method used in the recently reported Global Amphibian Assessment (Stuart et al 2004). One difficulty with using this approach is that listing is dependent on application to the various government agencies responsible for conservation management. Essentially there is no routine process to assess conservation status against a set of fixed criteria. Another weakness is that the cause of decline is not a consideration in the assessment process.

Other methods of prioritizing conservation risk have been developed and are based on a more refined set of biological criteria than those used in the IUCN ranking. Such a system is that developed by Millsap et al (1992). Recently, this method was used by the FrogsAustralia program (WWF) to investigate the conservation status of Australian frogs with the output a numerical ranking of risk. The scoring system and biological principles behind this approach are outlined by Millsap et al (1984) (see also Gillespie *et al* in preparation). One of its greatest advantages is that as new biological and ecological information are obtained and entered into the database the priority list can be rapidly updated. It also includes the first investigation in Australia of conservation status at a bioregional level. This not only provides a focus on areas where declines have been most apparent but it also indicates where declines have not occurred and where there is a dearth of field survey or information.

A consideration that is implied in each of the prioritizing schemes, but not necessarily recognized in the final lists of species, is the level of population fragmentation. We consider that it is imperative to include population fragmentation in the setting of priorities for a sampling and acquisition strategy for a GRB. This approach is based on the recognition that isolated populations often show levels of genetic differentiation that represent different levels of genetic diversity and the presence of co-adapted gene complexes. It is not appropriate to wait for comparative population genetic studies to be completed before samples should be included in a GRB.

3.2   Planning to actively use a GRB in genetic management of captive colonies of threatened species

Colonies of several threatened species are currently held in captivity in Australia. These colonies provide the opportunity to obtain and store the genetic material of animals without the need to collect animals from nature. Sperm samples could conceivably be collected on a number of occasions from significant individuals to provide sufficient material for use in future genetic management of colonies or for research purposes.

Current examples of native amphibians involved in captive breeding programs:
There are a number of captive breeding programs currently underway for threatened Australian frogs. Objectives differ among the programs but one common objective is the development of knowledge of captive husbandry techniques should they be needed in the near future. Others involve re-introduction projects and population enhancement approaches. Regardless of the project objectives, there is an invaluable opportunity to obtain samples for a GRB from animals in these colonies. This approach maximizes the use of animals already removed from nature. We are aware of the following programs currently underway or proposed.

Species of Conservation significance that are currently held as captive colonies

Corroboree frog	Amphibian Research Centre, Melbourne Zoo and Tidbinbilla NR.
Fleay's barred frog	Lone Pine Sanctuary
Stuttering frog	Melbourne Zoo
Green and golden bell frog	Taronga Zoo, The Wetlands Centre (Newcastle)
Spotted tree frog	Amphibian Research Centre and Melbourne Zoo

There are also proposals to establish a captive colony of the Kroombit tinker frog (*Taudactylus pleioni*). We are also aware of a number species that have been held in research colonies and successfully bred in captivity.

Pseudophryne australis	Newcastle University
Litoria littlejohni	Newcastle University

3.3   Priority setting for wild species and populations

Priorities for inclusion of material from wild populations should be based first on currently threatened species listings (see Section 3.1 above). A list of the amphibians included on threatened species legislation nationally and in each state and territory of Australia is presented below (Table 3.1). Several other approaches to listing the conservation status of Australian frogs have been conducted. Three are notable, the first of which is the conservation assessment provided in the Action Plan for Australian Frogs (Tyler 1995), the second the IUCN workshop listing that applied the IUCN Conservation Categories and provided data to the Global Amphibian Assessment (www.globalamphibians.org), and the third is the FrogsAustralia project (**www.**frogs**.wwf.org.au**) that based conservation status on a broader set of biological variables and extended to a bioregional assessment of Australian frogs.

Under the national Environmental Protection and Biodiversity Conservation (EPBC) Act 1999, there are 4 species listed as presumed extinct, 15 as endangered, and 12 as vulnerable. These

categories could form the primary target list for a GRB for Australian Amphibians at high risk of extinction. We consider that the 27 nationally endangered and vulnerable species should be the primary species targeted for a GRB.

The objective of a GRB would be to cryostore material for all species listed on the endangered and vulnerable categories (Section 3.4).

3.4 Species and Populations with High Conservation Status

Table 3.1. Species listed under the Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Act).

Presumed Extinct: 4 Species

Species	State	Distribution
Rheobatrachus silus,	QLD	Restricted distribution, montane, rainforest, stream breeding
Rheobatrachus vitellinus	QLD	Restricted distribution, montane, rainforest, stream breeding
Taudactylus acutirostris	QLD	Moderate distribution, montane, rainforest, stream breeding
T. diurnus	QLD	Moderate distribution, montane, rainforest, stream breeding

Critically Endangered Species: 0 species are listed under this category

Endangered Species: 15 species listed.

Species	State	Distribution, broad habitat and ecology
Geocrinia alba	WA	Restricted distribution, drainage lines.
Litoria castanea	ACT, NSW	Three disjunct populations, possibly undescribed species, not detected in wild for over 15 years. High altitude pond and swamp breeding.
Litoria lorica	QLD	Restricted distribution, montane, rainforest, stream breeding
Litoria nannotis	QLD	Moderate distribution, rainforest, stream breeding
Litoria nyakalensis	QLD	Restricted distribution, montane, rainforest, stream breeding
Litoria rheocola	QLD	Moderate distribution, montane, rainforest, stream breeding
Litoria spenceri	NSW, VIC	Moderate distribution, stream breeding
Mixophyes fleayi,	QLD	Restricted distribution, montane, rainforest, stream breeding
Mixophyes iteratus	NSW, QLD	Moderate distribution, montane, rainforest, stream breeding
Nyctimystes dayi	QLD	Moderate distribution, montane, rainforest, stream breeding
Philoria frosti	VIC	Restricted distribution, alpine, bog breeding
Pseudophryne corroboree	NSW	Moderate distribution, alpine, bog breeding
Spicospina flammocaerulae	WA	Restricted distribution. Swamp breeding
Taudactylus eungellensis	QLD	Restricted distribution, montane, rainforest, stream breeding
Taudactylus rheophilus	QLD	Restricted distribution, montane, rainforest, stream breeding

Vulnerable Species: 12 species listed.

Species	State	Distribution, broad habitat and ecology
Geocrinia vitellina	WA	Restricted distribution, drainage lines.
Heleioporus australiacus	NSW, VIC	Formerly widely distributed, forest, small creeks and swamps

Litoria aurea	NSW, VIC	Formerly widely distributed, dams and swamps
Litoria littlejohni	NSW, VIC	Wide distribution, forest, small creeks and swamps
Litoria olongburensis	NSW, QLD.	Wide distribution, coastal, small lakes and swamps
Litoria piperata	NSW	Restricted distribution, montane, streams
Llitoria raniformis	ACT, NSW VIC, SA	Formerly widely distributed, dams and swamps
Litoria verreauxi alpina	ACT, NSW, VIC	Moderate distribution, alpine and montane, ponds and soaks
Mixophyes balbus	NSW, VIC	Formerly widely distributed, coastal to montane, stream breeding.
Pseudophryne covaceVIChae	QLD	Restricted distribution, montane, soaks
Pseudophryne pengilleyi	ACT, NSW	Restricted distribution, alpine and montane, soaks
Taudactylus pleioni	QLD	Restricted distribution, montane rainforest, small drainage lines

In addition to the 27 species that are listed nationally there are a further 40 species gazetted on conservation lists among the States and Territories of Australia (Table 3.2). Many of these species are listed in several states. We would propose that this list (Table 3.2) should form the basis for prioritising and selecting species for inclusion into a GRB.

Table 3.2 Threatened species included under Commonwealth, State and Territory legislation. Arranged by family.

Myobatrachidae (ground frogs)

Species	Distribution	EPBC	ACT	NSW	QLD	SA & NT	VIC	WA	TAS
Rheobatrachus silus,	QLD	**PE**			**E**				
Rheobatrachus vitellinus	QLD	**PE**			**E**				
Taudactylus acutirostris	QLD	**PE**			**E**				
Taudactylus diurnus	QLD	**PE**			**E**				
Geocrinia alba	WA	**E**						**R**	
Mixophyes fleayi	QLD	**E**		**E**	**E**				
Mixophyes iteratus	NSW, QLD	**E**		**E**	**E**				
Nyctimystes dayi	QLD	**E**							
Philoria frosti	VIC	**E**					**T**		
Pseudophryne corroboree	NSW	**E**		**E**					
Spicospina flammocaerulae	WA	**E**						**R**	
Taudactylus eungellensisi	QLD	**E**			**E**				
Taudactylus rheophilus	QLD	**E**			**E**				
Geocrinia vitellina	WA	**V**						**R**	
Heleiporus australiacus	NSW, VIC	**V**		**V**			**T**		
Mixophyes balbus.	NSW, VIC	**V**		**E**			**T**		
Pseudophryne covacevichae	QLD.	**V**			**V**				
Pseudophryne pengilleyi	ACT, NSW	**V**	**V**	**V**					
Adelotus brevis	NSW, QLD.				**V**				
Assa darlingtoni	NSW, QLD.				**R**				
Crinia tinnula	NSW, QLD.			**V**	**V**				
Geocrinia laevis	SA, VIC					**R**			
Lechriodus fletcheri	NSW, QLD.				**R**				
Limnodynastes interioris	NSW, VIC						**T**		
Limnodynastes peronii	NSW, QLD, VIC, TAS								**R**
Neobatrachus pictus	NSW, SA, VIC			**E**					
Neobatrachus sutor	SA, WA, NT					**V**			
Philoria kundagungan	NSW, QLD.			**V**	**R**				
Philoria loveridgei	NSW, QLD.			**V**	**R**				
Philoria sphagnicola	NSW			**V**					
Pseudophryne australis	NSW			**V**					
Pseudophryne bibroni	ACT, NSW SA, VIC					**R**			
Pseudophryne occidentalis	SA, WA					**V**			
Pseudophryne semimarmorata	SA, VIC					**V**			
Taudactylus liemi	QLD.				**R**				
Taudactylus pleioni	QLD				**E**				
Uperoleia capitulata	SA, QLD					**R**			

Hylidae (tree frogs)

Species	Distribution	EPBC	ACT	NSW	QLD	SA & NT	VIC	WA	TAS
Litoria aurea	ACT, NSW, VIC	E		E					
Litoria boorolongensis	ACT, NSW	E		E			T		
Litoria castanea	ACT, NSW	E		E					
Litoria lorica	QLD	E			E				
Litoria nannotis	QLD	E			E				
Litoria nyakalensis	QLD	E			E				
Litoria rheocola	QLD	E			E				
Litoria spenceri	NSW, VIC	E		E			T		
Litoria littlejohni	NSW, VIC	V		V			T		
Litoria olongburensis	NSW, QLD.	V		V	V				
Litoria piperata	NSW	V		V					
Litoria verreauxi alpina	ACT, NSW, VIC	V		E			T		
Llitoria raniformis	ACT, NSW, VIC, SA	V		V		V	T		V
Cyclorana cultripes	SA, NSW, NT, QLD					R			
Cyclorana verrucosa	QLD.				R				
Litoria andirrrmalin	QLD				V				
Litoria brevipalmata	NSW, QLD.			V	R				
Litoria cooloolensis	QLD.				R				
Litoria freycineti	NSW, QLD.				V				
Litoria genimaculata	QLD.				R				
Litoria longirostris	QLD.				R				
Litoria pearsoniana	NSW, QLD.				E				
Litoria revelata	NSW, QLD				R				
Litoria subglandulosa	NSW, QLD.			V	V				

Microhylidae and Ranidae (micro- tree frogs and old world frogs)

Species	Distribution	EPBC	ACT	NSW	QLD	SA & NT	VIC	WA	TAS
Cophixalus bombiens	QLD				R				
Cophixalus concinnus	QLD				R				
Cophixalus exiguus	QLD				R				
Cophixalus hosmeri	QLD				R				
Cophixalus infacetus	QLD				R				
Cophixalus mcdonaldi	QLD				R				
Cophixalus monticola	QLD				R				
Cophixalus neglectus	QLD				V				
Cophixalus peninsularis	QLD				R				
Cophixalus saxatalis	QLD				V				
Cophixalus zweifeli	QLD				V				
Sphenophryne fryi	QLD				R				
Sphenophryne robusta	QLD				R				
Rana daemeli	QLD, NT								V

States differ in the use of terms and their meanings when categorizing threatened species. The most common terms and criteria applied are the IUCN threatened species criteria and categories.

Australian Capital Territory. The Nature Conservation Act 1980. E Endangered, V vulnerable

Western Australia Wildlife Conservation Act 1950. Schedule 1. Fauna that are rare or likely to become extinct.

Tasmania Threatened Species Protection Act 1995. V vulnerable, R rare.

Northern Territory Parks and Wildlife Conservation Act 2000. V vulnerable.

South Australian National Parks Act 2003. IUCN categories. V Vulnerable, R Rare.

Victoria Fauna and Flora Guarantee Act 1988. T Threatened fauna.

3.4   Other considerations for the inclusion of material in a GRB

A range of additional considerations should be included in the decision process concerning which species and populations should be collected for a GRB. After focusing on threatened taxa, there are a range of additional priorities and opportunities that can be considered. To some extent, these depend on the financial support that can be obtained for a GRB, or may simply be a matter of available opportunity. If we consider the current situation with museum collections in general and "frozen tissue collections" in particular, there are specific collection targets that may present themselves. These are often driven by a gap analysis of collections, resulting in collecting activity being pursued so that a collection is representative. In other circumstances collections are based on the research interests of staff or on some specific requirement to investigate a taxon, an evolutionary process or perhaps a biogeographic region. An example of this can be observed in the Australian Biological Tissue Collection (ABTC), which has a large collection of tissues from a common species of frog, *Litoria lesueuri,* that occurs on the eastern seaboard and ranges. It is not categorized as threatened, although it has been identified as a species that has declined. The collection contains 433 specimens encompassing 85 locations and largely covers the extensive geographic range of this taxon. This collection was made with research objectives to investigate the systematics and phylogenetics of this widespread taxa, and recent genetic studies have shown that it is in fact composed of three species not one as formerly recognized (Donnellan et al 2004).   Frozen material from this species, collected for research, could also be shared with a GRB.

Museums often become the repositories of collections that are seen to have some value to future generations. Museums and conservation agencies often conduct field surveys to document the biodiversity of sites and regions and in many cases voucher specimens are lodged in collections. Similarly, research conducted by other parties (CSIRO, Universities) often results in the collection and storage of material. These situations also provide an opportunity for frozen tissue to be collected and stored for GRBs. There is a strong ethical imperative to maximize the use of any animal that is removed from nature for research or management purposes.

Below we list some of the priority directions for collections.

1) Clarify the status of tissues in the current "frozen tissue collections" of species that are listed in the Presumed Extinct, Endangered and Vulnerable categories:
For example, the ABTC has specimens of *Taudactylus acutirostris, T. rheophilus, Litoria nyakalensis,* and *Pseudophryne corroboree.* These tissues are of high conservation value, particularly in the case of the two *Taudacytlus* taxa which are now presumed to be extinct in the wild. Management decisions are necessary to decide whether and what portion of the collections should be placed in a "fully secure collection" managed as a last resort collection. It would be unacceptable, in our view, if these tissues were to be used for a range of studies, and eventually

none was available for reconstitution technologies at some time in the future. The ABTC has 47 such critical specimens from three locations and this represents a significant coverage of population genetic diversity and the distributional range of the species.

2) Incorporate samples from isolated populations of otherwise widespread species:
Numerous examples of this situation could be listed. The example of *Litoria revelata* is briefly outlined to illustrate the point. This species has a rather unusual distribution; it has three isolated populations, one in southeast Queensland - northeast NSW, a second in central east Queensland and a third in northeast Queensland. The latter two populations are very small relative to the distance between them. The population at Eungella in central east Queensland is known over an area of less than 10 square kilometer and the population on the Atherton Tablelands in the north occurs over about 100 square kilometers. In such cases it is advisable to store material from each population.

3) Incorporate samples where there is evidence that a species was once widespread and there is current evidence of a loss of populations or a reduction of range:
There are numerous examples of this pattern of decline among Australian frogs. The ideal situation would be to obtain samples from across the geographic distribution of a species and at a range of altitudes where this is pertinent.

4) Incorporate species and populations of Regional Conservation Significance:
There are numerous examples of species that are known from relatively small distributions. In some cases, their conservation status may be related to a small distribution and population size, while in others there is an implicit assumption that the small distribution is an artifact of limited field survey effort in remote areas. An example of a species with a relatively small distribution is *Bryobatrachus nimbus* (=*Crinia nimbus*) from southern Tasmania. There is no indication that this species is threatened, and it occurs in areas of World Heritage wilderness where its habitat is well conserved. However, the same argument was used to forestall *ex situ* conservation efforts for several north Queensland frog that are now presumed extinct.

5) Develop methods to incorporate opportunistic sampling associated with other research:
There are many occasions where specimens are collected for other research work and the cost involved in obtaining approval and collecting involves significant resources. It would be valuable to make connections and build the GRB via multiple institution involvement that ensures access to materials collected in these situations.

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Section 4

Cryopreservation Technologies
for Amphibian Genome Resource Banks

4.1 Current Status

4.1.1 Amphibian Sperm but not Eggs or Embryos can be Cryopreserved

In an ideal world, the manipulation and storage of cells and tissues in an Amphibian GRB would involve simple technical procedures that allowed the retrieval and regeneration of complete amphibians in a few steps from the initial freezing to thawing and embryo regeneration. It would hold only three classes of materials: sperm, (unfertilized) eggs and early embryos. All the desired uses and applications of an amphibian GRB would be achievable if it were possible to successfully retrieve these three types of material from frozen storage. Thawed sperm and thawed unfertilized eggs could be recombined to generate embryos that would develop into tadpoles. Even simpler, the frozen embryos could be simply thawed and allowed to develop into tadpoles in a dish.

Unfortunately, the reality of existing cryopreservation technology as it applies to amphibians is that only one of these three classes of material can be successfully frozen and thawed. **That material is amphibian sperm**. Currently, it is considered that neither unfertilized amphibian eggs or embryos can be successfully retrieved from frozen storage. This pessimistic view of egg and embryo cryopreservation is not based on a scientific literature reporting attempts to freeze eggs and embryos (which doesn't exist for amphibians), but rather on a significant number of reports of attempts to freeze fish eggs and embryos (which have structural and biochemical similarities to amphibian eggs).

Attempts to cryopreserve the embryos of oviparous fish, derived as they are from large, yolky eggs, have not met with success (Hagedorn *et al* 1997a,b, 2004; Liu *et al* 1999). There is evidence that membranes within fish eggs, in particular, the yolk syncytial layer, are poorly permeable to water and cryoprotectants (Hagedorn *et al* 1997a,b; Liu *et al* 1999). The net effect of low permeability to cryoprotectants, and large oocyte size is that damage from either slow or rapid-freezing is severe and usually lethal. Amphibians, like fish, have large eggs (diameters often in the order of 1000 µm) with a significant component of storage substances. The equivalent experiments to those reported for fish have not been reported for amphibians, and there is a need to determine whether similar responses to cryoprotectants and cooling would occur in amphibians (such experiments are currently underway at the University of Newcastle). Nevertheless, personal communications (SP Leibo, Univ of Guelph, Ontario) as well as preliminary experiments at the University of Newcastle with *Bufo marinus* eggs suggest that amphibian eggs may, like fish eggs, be refractory to cryopreservation.

The ability to freeze amphibian sperm, but not eggs or embryos, means that implementation of an amphibian GRB would have to be based around cryopreserving sperm, that would be available for retrieval and use in conservation programmes in the present, and cryopreservation of other cell types (such as body cells from tadpoles and adult frogs, or cells from their testes and ovaries) in anticipation of the development in the future of new technologies to make use of them.

4.1.2 Proven techniques for use in Amphibian GRB programmes.

A number of established reproduction and cryopreservation techniques are available to be used in the operation of an amphibian GRB and its associated programmes. These are listed in Table 4.1 and discussed in more detail in Sections 41.3 to 4.1.5. Sperm and egg collection by hormonal techniques and IVF are well established. Most work on sperm cryopreservation is very recent, but proof of concept has been achieved, and the results are promising across a number of species. Nuclear transfer (cloning) is well established in laboratory science but much work is required to develop it as an applied tool for use in amphibian GRBs. Androgenesis has only been the subject of limited studies in amphibians, with only one report of the generation of viable adults (Gillespie and Armstrong, 1980).

Table 4.1 Existing Procedures in Amphibian Reproductive Technology that may be used within an Amphibian Genome Resource Bank.

Technique	Use	Comments
Hormonal induction of sperm and egg release	Collection of viable sperm and eggs without injuring the adults.	Well established procedures allow the collection of sperm in the urine of males, and ovulation and accumulation of eggs in the oviducts in females; eggs can be collected by manual massage of the abdomen.
In Vitro Fertilisation (IVF)	Fertilisation and generation of a viable embryo that develops into a tadpole in a dish	Amphibian fertilization (in frogs) is external. IVF is a well established laboratory technique for producing amphibian embryos and tadpoles.
Sperm cryopreservation	Storing sperm for an indefinite period	Only developed in recent years, with growing awareness of amphibian crisis. Developed in only a few laboratories, including at Newcastle University. Works with most species tested (although limited research to date).
Nuclear transfer (cloning)	Production of a viable embryo from a single body cell.	Well established laboratory technique for over 50 years (see McKinnell, 1978); requires an inactivated egg to receive the cell to be cloned. Works best when egg and cell are from the same species.
Androgenesis	Production of a viable embryo from only a sperm.	Well established in fish, but only limited reports in amphibians (Gillespie and Armstrong, 1980); requires an inactivated egg (preferably from same species) to receive sperm and generate an embryo.

4.1.3 Significance of the successful cryopreservation of amphibian sperm

Major progress towards retrievably storing the amphibian genome through successful cryopreservation of the haploid (sperm) genome has been achieved in the last ten years. Although the number of papers published in the area of amphibian sperm cryopreservation is relatively small, the available literature indicates that amphibian sperm, like the sperm of many other vertebrate groups, can be successfully cryopreserved (Barton & Guttman 1972; Browne *et al* 1998, 2001, 2002a,b,c,d; Mungnana *et al* 1998). Much of this work has been carried out at the University of Newcastle on *Bufo marinus* and a range of native Australian species from two families (Browne *et al* 1998; 2001, 2002a,b,c,d), and has resulted in the development of a range of techniques for retrieving and cryopreserving sperm from live or recently dead amphibians. The methods developed include procedures for the non-invasive collection of sperm from live males, and the collection of sperm from the testes of recently dead males.

The storage protocols developed at Newcastle University include methods for extended storage of sperm at temperatures above freezing (0°C) as well as frozen storage in liquid nitrogen. The sperm cryopreservation protocol developed at Newcastle University is based on the use of high concentrations of cryoprotectant (DMSO or glycerol), high concentration of osmoticant -sucrose, and a slow freezing rate. The protocol for the non-invasive collection of amphibian sperm by hormonal induction uses chorionic gonadotrophin, and is followed by the addition of cryoprotectants prior to cryopreservation. This protocol allows the collection and cryopreservation of sperm from endangered or other important male amphibians without the necessity of killing them to obtain the testes (the normal method of collecting amphibian sperm in a laboratory situation). It also enables storage of a large amount of population genetic diversity and will allow the removal of the generation effect in captive colonies (Santiago and Cabellero 2000) that are maintained in zoos, or in conservation programmes.

The success in developing freezing protocols for amphibian sperm, from laboratories in Australia and overseas, has made the possibility of establishing an Amphibian GRB based around cryopreserving sperm from endangered species a reality. Although, further work is warranted to refine protocols for target species and broader taxa, the feasibility of the approach has been demonstrated.

4.1.4 Generation of viable amphibian embryos and tadpoles by nuclear transfer

Nuclear transfer of totipotent diploid cells may provide a means to deal with the current difficulties in cryopreserving amphibian eggs because it allows the production of complete organisms from cells that are small enough to be successfully cryopreserved. Nuclear transfer in amphibians using non-cryopreserved material is well established (Briggs & King 1952; McKinnell 1978; Gurdon *et al* 1975; Gurdon 1986), and pre-dates the recent cloning of embryos from mammalian somatic cells (Wilmut *et al* 1997; Wakayama *et al* 1998) by several decades. The generation of viable amphibian offspring from frozen/thawed blastomeres (early embryonic cells) by nuclear transfer techniques would be required if nuclear transfer was to be used in conjunction with a GRB to generate viable frogs from frozen cells. However, this step (nuclear transfer using cryopreserved cells) has not yet been reported, but should be achievable. It should be a key goal of future research.

4.1.5 Androgenesis, a technique for producing viable embryos and tadpoles from only frozen sperm.

The major hurdle to reconstituting individuals using cryopreserved cells in an amphibian GRB is the current inability to freeze mature eggs or early embryos, and since this capacity may be unavailable well into the future, a number of approaches to circumvent this difficulty may need to be employed (nuclear transfer usuing embryonic cells is one that was considered above). Despite the success with sperm cryopreservation, the ability to collect and cryopreserve amphibian sperm has limitations in its usefulness for the regeneration of complete embryos that lead to viable tadpoles and adults. This is obvious, for example, in the case of the application of sperm cryopreservation to the management of endangered species where frozen sperm may be available, but there are no living females. Nevertheless, there is one reproductive manipulation available in lower vertebrates (but not in higher vertebrates because of imprinting effects) that may still enable sperm cryopreservation to generate viable diploid organisms in the absence of female pronuclei from the same species.

This process is ***androgenesis***. Androgenesis, leading to the generation of viable adults, has been widely reported in fish (for a review see Young *et al* 1996), and in at least one amphibian (the axolotl, Gillespie & Armstrong 1980). *Androgenesis involves the fertilization of an oocyte (egg) whose maternal pronucleus has been destroyed (by irradiation or physical removal) before fertilization with a single sperm.* Subsequently, the haploid zygote is subjected to temperature-shock to prevent the first cleavage division after the first nuclear division. As a result, the nucleus is converted to a homozygous, but diploid state, and normal development of the reconstituted diploid embryo proceeds. *In fish and in the one amphibian reported, the resulting diploid animals (androgenotes) are reproductively competent and may be back-crossed, or crossed with other individuals to produce outbred lines.* One problem with this approach in terms of maintaining the genetic integrity of a species is that the mitochondria are derived from the maternal cytoplasm (donor cytoplast). This may be undesirable in the case of regenerating an extinct amphibian species from frozen sperm using the oocyte of a different species. Nevertheless, androgenesis is justified where no live females of the species are available, or where there are no frozen body cells available to use in nuclear transfer.

4.2 Technological Development and Research Objectives for the Short-Medium and Long Term Management of an Amphibian Genome Resource Bank.

Proof of concept with respect to establishing an Amphibian GRB has been achieved. However, research to support the implementation of the GRB is required to optimise its operation, as with the implementation of any new technology to address an issue that has recently emerged. Research objectives can be classified into medium and long term on the basis of priority for optimizing the efficiency of the GRB and likelihood of success within specified time frames. Research issues are summarized in Table 4.2

Table 4.2 Short-Medium and Long Term Research Objectives to improve the effectiveness of an Amphibian Genome Resource Bank. Research Objectives are prioritised according to their significance in establishing and optimizing the GRB at the earliest possible time, and likelihood of successful outcomes.

Research Objective	Priority	Time Scale	Probability of Success	Comments
Sperm Cryopreserva tion protocols for target species and taxa	1	Short	High	Optimise existing cryopreservation protocols for (i) specific target endangered species (ii) broader native taxa such as ground (myobatrachid) and tree (hylid) frogs
Isolation, culture and cryopreservation of early embryonic (stem) cells	1	Short	High	Development of cryopreservation protocols for embryonic stem cells will allow storage of cell lines capable of regenerating whole embryos by nuclear transfer; insurance against species extinction
Cryopreservation of ovarian and testicular tissue and stem cells	1	Short	High	An alternative source of potentially totipotent stem cells for generating embryos
Nuclear Transfer	2	Mediu m	High (same species) Medium (cross-species hybrids)	Technology that needs to be transferred from laboratory species to target GRB species
Androgenesis	2	Mediu	High (same	Demonstrated in only one species; approach needs

			species) Medium (cross-species hybrids)	work in model species, and transfer to target GRB species
Chimeras	3	Long Term	Medium	May be the best way in the long term to produce eggs and sperm from extinct species.
Cryopreservation of eggs and whole embryos	4	Long Term	Low	Success will require development of technologies that do not yet exist; success most likely in fish first

4.2.1 Short-Medium Term, High Priority Research

4.2.1.1 Sperm Cryopreservation protocols for target species and taxa (Priority 1)

Protocols for cryopreserving native amphibian sperm need to be developed for target (endangered and strategic) species that will be maintained in a GRB. This will involve optimizing methods for the non-invasive collection of sperm by hormonal induction, and the adaptation and refinement of existing cryopreservation protocols for target species (especially endangered species, but also species that are being targeted for strategic reasons eg where populations are restricted, or small, or taxa that have few representative species). Protocols will need to be tested in IVF systems to demonstrate the capacity to produce viable offspring from frozen material.

This research is of the highest priority for establishing a viable GRB.

4.2.1.2 Collection, culture and cryopreservation of early embryonic (stem) cells (Priority 1).

Individual cells from early amphibian embryos are diploid and totipotent ie have the potential to be reprogrammed and develop into a complete embryo following their injection into a recipient egg. This process is nuclear transfer (described above in Section X.1.4). The storage of these cells in an amphibian GRB would provide a way around the block to egg and embryo cryopreservation. It would allow the regeneration of whole embryos in one step from frozen material, as long as eggs from the same species were available, or from a compatible sibling species. Consequently, freezing early embryonic cells has the potential to (i) store genetic diversity for re-introduction to a population at a later date (ii) act as an insurance against extinction of living populations and species.

Cryopreservation of early embryonic cells is a procedure that can be implemented in a GRB independently of any capacity to regenerate embryos for particular species by nuclear transfer. Once cryopreserved, the cells can be stored indefinitely, until required procedures for their use are in place.

Although not yet reported in the literature, developing procedures for isolating embryonic cells, culturing them for short periods (if necessary), and cryopreserving them should be relatively simple. Such procedures are in place for similar cells from other vertebrates. This research is of a high priority to achieve the greatest strategic value from an amphibian GRB. It has a high probability of success.

4.2.1.3 Cryopreservation of ovarian and testicular tissue and stem cells (Priority 1)

Both testes and ovaries offer a potential source of cells and tissues for cryopreservation that could yield totipotent stem cells for regenerating whole embryos, as well as immature oocytes for

culturing to a stage where they may be fertilized. Developing methodologies for cryopreserving these cell and tissue types has a high probability of success in amphibians, based on work on similar cell types in other vertebrates (Morris, 1995). Such cells could be cryopreserved indefinitely until nuclear transfer or other procedures are available to make use of them.

The potential of oogonia (gonadal stem cells) in nuclear transfer has not been tested in amphibians but evidence from mammalian studies suggests that these cell types may have the potential for cryopreservation and nuclear transfer (Tesarik *et al* 1999; Ogura *et al* 1998). In mammals, cryopreservation of the most immature oocytes (within primordial follicles) has proved effective for a very wide range of species. This is attributed to their small size, low metabolic requirements and relatively undifferentiated state. The ovary usually contains a heterogenous population of follicles including immature follicles, and can therefore be used as a source of oocytes at any time. Ovarian tissue can therefore be used as a source of oocytes independent of the female's reproductive status or age (Cox et al. 1996). It has also been shown that it is possible to salvage ovarian tissue from mammals up to 48 hours after death (Snow et al 2001). These are important features for a technology designed to aid in the propagation of rare and endangered animals.

In amphibians it would be particularly beneficial to freeze oocytes at a stage prior to vitellogenesis, to avoid the effect of vitellogenesis on the size and freezability of the oocytes. Oocyte development in amphibians is divided into five stages (stage I – V). Each developmental stage has its specific characteristics of membrane composition, protein content, lipid distribution, and organelles organisation. Stage I oocytes are the smallest oocytes (50 – 250 µm), contain few mitochondria and the yolk platelets have not yet formed in the ooplasm. These characteristics make stage I oocytes a good target for cryopreservation. There are no published reports on the cryopreservation of ovarian tissue in frogs, but as in other species it is likely to be significantly easier and more successful than attempts at cryopreserving either mature oocytes or embryos, since in amphibians the mature eggs are so large and lipid rich.

It is suggested that research in this area focus on applying the cryopreservation protocols to the ovaries of amphibians that have been successfully applied to mammalian and insect ovaries. Slow cooling protocols that were first successfully used for the cryopreservation of mouse embryos (Whittingham, 1972), and have proven to be both versatile and effective for ovarian tissue of a wide range of mammalian species (mouse, elephant, human, marsupial) and appear to be associated with little cryoprotectant toxicity (Snow et al. 2004), should be investigated. An advantage of these techniques in relation to the operation of an amphibian GRB would be that they can be used under both laboratory and field conditions (Cleary et al. 2001). Research in this area should evaluate which of the equilibration conditions and cryoprotectants that have proven effective for mammals, are best suited to ovarian tissue and immature oocytes of amphibians.

Testes and testicular cells should also be investigated as a source of pre-meiotic stem cells, along the lines of ovarian tissue, for storage in an amphibian GRB.

4.2.1.4 Adaptation of Nuclear Transfer and Androgenesis procedures to native species stored in an amphibian GRB (Priority 2).

The potential for generating whole embryos from cryopreserved material using nuclear transfer and androgenesis was considered in Sections 4.1.4 and 41.5 respectively. Nuclear transfer and androgenesis are potentially important mechanisms for regenerating whole embryos from material stored in an amphibian GRB, in addition to the standard procedure of IVF. These methodologies are established, to a greater or lesser extent, in laboratory amphibians, with the exception that such

procedures have not been reported using frozen sperm (androgenesis) or frozen cells (using nuclear transfer). Research is needed to: (i) develop protocols that transfer these procedures from laboratory use to amphibian GRB target taxa, including procedures for same and sibling (cross) species embryos from both nuclear transfer and androgenesis (ii) establish protocols for using frozen cells in these procedures.

The priority for this research is not as high as for protocols to store material (cells and tissues) in the bank. This is because stored material can be maintained indefinitely until procedures to regenerate embryos of target taxa are in place.

4.2.2 Long Term, Low Priority Research

4.2.2.1 Chimeras (Priority 3)

Chimeras are organisms that are mixture of cells from two separate organisms (these may be from the same or different species). They have been successfully produced in a number of vertebrate groups including mammals and fish. Although it would not be an objective of an amphibian GRB to produce chimeras that were a mixture of cells from two species for release into the wild, the use of chimeras as a means of producing pure lines of sperm and eggs from populations or species that are extinct in the wild should be considered. Such pure lines of sperm and eggs may be produced where cells (for example, cryopreserved early embryo cells) from the species of interest are injected into a developing embryo of a different species, but are incorporated into the germ line of the host embryo, becoming progenitors of sperm and eggs in testes or ovaries. In this way, sperm and eggs from the target species may be produced (even if it is extinct in the wild) that carry only the genetic material of the species of interest, and not the host species. As such, simple IVF may then be used to re-create the original species, with only nuclear and mitochondrial DNA from the target species.

The production of chimeras using frozen blastomeres (early embryonic cells) injected into eggs has been reported in fish (Lin *et al* 1992; Nilsson & Cloud 1992; Calvi & Maisse 1998; Strussman *et al* 1999). The advantage of small cell size and a normal cytoplasm has facilitated the cryopreservation of fish blastomeres (Calvi & Maisse 1998; Strussman *et al* 1999). The success with freezing fish blastomeres portents well for similar success with amphibian blastomeres.

The generation of amphibian chimeras has not been reported in the literature from either fresh or frozen cells. Thus, the recovery of diploid genomes by production of chimeric amphibian embryos using cryopreserved blastomeres will require research on both the generation of chimeras and the cryopreservation of blastomeres. The development of the capacity to produce inter-species amphibian chimeras using cryopreserved material would be an important technological advance for maximising the utility of an Amphibian GRB. Although it is an important research objective, it is not rated as a high priority at this stage, because of the need to carry out considerable basic research to achieve the objective.

4.2.2.2 Cryopreservation of eggs and whole embryos (Priority 4).

It would be highly desirable for an amphibian GRB to be able to store and retrieve frozen eggs and whole embryos. This should remain a long term research objective. However, research in this area is rated a low priority because of the low probability of success applying the current state of

knowledge in cryobiology. Any major advance that could assist in the development of whole egg and embryo cryopreservation for amphibians is likely to be developed first for fish eggs. There is much interest in this problem in the aquaculture industry, where cryopreservation of fish embryos would have many applications.

4.3 References

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Section 5

A Review of the Collections
of Cryopreserved Tissues of Australian Amphibians

5.1 Australian Collections of Frozen Animal Tissues
The majority of the cryopreserved tissues collections are held by the various state natural history museums. These institutions have formal agreements for the sharing of collections for research purposes and policies and practices are agreed by the <u>Council of Heads of Australian Fauna Collections</u>. Each institute was contacted and a print out of the collection register requested.

Within the frozen tissue collections there are over 9000 individual tissue samples representing 230 species. This represents over 80% of the recognised species of Australian frogs and covers almost all genera (currently 206 species are recognised in Australian). As might be predicted, the collections are limited for many threatened species, but surprisingly, there are tissues stored from several species that are categorised as Presumed Extinct, and numerous from species listed as Endangered (EPBC Act 1999). Some biogeographic regions are well represented while there are significant gaps in others. For example, the east coast has the strongest representation, while Tasmania, the far north and northwest the poorest. Collections of desert species are also minimal.

The great majority of tissues frozen are liver and heart with fewer instances of blood and skin. There are occasional eggs and whole tadpoles and adult frogs frozen. All material is frozen without the use of cryoprotectants. These are chemicals that reduce the damage of ice crystal formation to subcellular structures. In most cases the tissue are dissected from specimens, placed in a labelled plastic tube and frozen directly in liquid nitrogen. Many of the specimens are frozen in the field in portable liquid nitrogen cylinders and transported to the museum or storage facility where long-term storage is in freezers.

Most of the frozen tissues are identified to species level and there is detailed information on location of collection, name of collector and date of collection. For many frozen specimens there is an associated voucher specimen (carcass) stored in the alcohol preserved collection of the museum. There are specimens for which data is absent or deficient, but in general the collections are supported by museum registration databases.

A trend that we detected during this investigation was a move away from frozen collections to the storage of tissues in 80% alcohol. Advances in molecular genetic techniques have made it possible to extract DNA from alcohol preserved material and thereby avoid the need to store fresh material in freezers. Older molecular genetic techniques relied on retrieving cytoplasmic contents such enzymes and proteins for comparative analyses, but more recent advances focus on the nuclear and organelle DNA. In some respects we see this as a retrograde step at a time when frozen material may have important uses in reconstitution technologies in the future.

It seems a small step to expand the role of these important collections to include frozen germ line and somatic cells in a manner that is most effective for reconstitution in the future. Such a collection would compliment and enhance the role of the current collections. It would also provide for an expanded role in conservation biology, which is a natural extension of the charter of these institutions. In addition it would strengthen the connection between museums and research oriented

towards reproductive science, animal breeding and conservation biology.

We have summarised information on the collections in Table 1. There are over 4900 tissue specimens from Australian ground frogs representing 101 species of a total 120 species in this family, and over 3700 tissues from tree frogs representing 70 species of a total 74 species in this family.

Table 2 lists threatened species for which frozen tissues are included in collections. Twenty (20) threatened ground frogs and 11 tree frogs are included in collections.

Tables 3 to 9 provide details on the frozen collections arranged by species, the number of frozen specimen for each species, number of locations represented and the nature of the tissue frozen.

Table 5.1 Summary of the number of species of amphibians for which cryopreserved tissues are stored in Australian institutions. Table 3 to 8 below provide fuller details of the collections in each institution.

Institution	Number of species and specimens stored					
	Myobarachidae (ground frogs)		**Hylidae (tree frogs)**		**Microhylidae, Ranidae, Bufonidae (Micro tree frogs, Ranids)**	**Non-Australian collection**
	No of specimens and species	**No of locations**	**No of specimens and species**	**No of locations**		
Australian National Wildlife Collection. CSIRO Canberra	109 23	47	72 21	41		
Australian Biological Tissue Collection. South Australian Museum	4396 101	730	3154 70	730	92 8	Also includes a significant collection of specimens mostly Papua New Guinea and Pacific Islands
Australian Museum. Sydney.	463 30	91	516 25	56		142 specimens mostly Pacific Islands
Western Australian Museum. Perth	2351 51	674	1046 29	262		

Table 5.2   Threatened species that are included in collections

Institution	*Threatened species represented in tissue collection*
Australian National Wildlife Collection. CSIRO Canberra.	*Myobatrachidae:* *Assa darlingtoni* *Adelotus brevis* *Mixophyes iterates* *Hylidae:* Litoria boorolongensis, L. personiana, L. revelata
Australian Biological Tissue Collection. South Australian Museum	*Myobatrachidae:* **Assa darlingtoni** **Adelotus brevis** **Crinia tinnula** **Heleioporus australiacus** **Mixophyes fleayi, M. balbus, M. iterates** **Pseudophryne australis, P. corroboree, P. occidentalis, P. pengilleyi, P. semimarmorata** **Philoria frosti, P. loveridgei, P. sphagnicolus** *Rheobatrachus silus, R. vitellinus* *Spicospina flammocaerulea* *Taudactylus acutirostris, T. eungellensis, T. rheophilus* *Hylidae:* *Litoria aurea, Litoria boorolongensis, L. nannotis, L. nyakalensis, L. personiana, L. longirostris, L. littlejohni, L. olongburensis, L.rheocola, L. revelata, L. spenceri*
Australian Museum. Sydney.	*Myobatrachidae:* *C. tinnula* *H. asutraliacus* *M. iteratus* *P. australis* *Hylidae:* *L. aurea, L. raniformis, L, subglandulosa, L. spenceri*
Western Australian Museum. Perth	*Myobatrachidae:* *Arenophyrne rotunda, Geocrinia alba, G. leai, G. lutea, G. rosea, Spicospina flammocaerulea*

Table 5.3   Frozen tissues held by the Australian Biological Tissue Collection (ABTC), South Australian Museum.
Family Myobatrachidae (ground frogs)

Genus Species	Number of specimens	No of Locations	Tissues Cryopreserved. L liver; H heart; Tad tadpole; E eggs.
Adelotus brevis	21	10	L, H
Arenophryne rotunda	1	1	L, H
Assa darlingtoni	45	7	L,H, Eggs, some whole animals
Bryobatrachus nimbus (=Crinia nimbus)	6	2	L, H, eggs
Crinia signifera and tinnula + undescribed taxon (specimens in east coast population study)	47	4	L, H
Crina bilingua	28	7	L
Crinia deserticola	25	13	L
Crinia georgiana	40	10	L, H
Crinia glauerti	91	7	L, H
Crinia insignifera	51	5	L, H
Crinia parinsignifera	25	12	L, H, 1 eggs
Crinia pseudinsignifera	90	21	L, H
Crinia remota	44	9	L, H
Crinia riparia	55	13	L, H
Crinia signifera	241	~64	L, H, few eggs
Crinia sloanei	5	5	L
Crinia sp	23	5	L, H
Crinia subinsignfera	5	1	L
Crinia tasmaniensis	2	1	L
Crinis tinnula	42	15	L
Geocrinia victoriana	1	1	L
Heleioporus albopuncatatus	34	5+	L, H
Heleioporus australiacus	67	26	L, H
Heleioporus barycragus	5	3	L, H
Heleioporus eyeri	75	20+	L, H
Heleioporus inornatus	10	2	L, H
Heleioporus psammophilus	19	7	L, H
Lechriodus fletcheri	24	14	L, H, eggs
Limnodynastes convexisculus	25	17	L, H
Limnodynastes depressus	5	2	L, H
Limnodynastes dumerilli	160	50+	L, H, few eggs
Limnodynastes fletcheri	29	10	L, H
Limnodynastes interioris	3	3	L, H
Limnodynastes ornatus	71	37	L, H, few eggs
Limnodynastes peroni	41	23	L, H
Limnodynastes salmini	10	8	L, H
Limnodynastes tasmaniensis	210	55+	L, h, eggs, tads
Limnodynastes terrareginae	17	5	L, H
Limnodynastes spenceri	40	16	L, H, whole
Megistelotis lingarius	5	4	L, H
Metacrinia nichollsi	16	11	L, H
Mixophyes balbus	50	20+	L, H, some eggs
Mixophyes fasciolatus	120	33	L, H
Mixophyes fleayi	18	9	L, H, 1 eggs
Mixophyes iteratus	42	15	L, H, toe, few eggs
Mixophyes schevilli	58	18	L, H, few eggs
Myobatrachus gouldii	21	11	L
Neobatrachus albipes	8	9	L, H, K
Neobatrachus aquilonius	29	8	L, H, 1 egg
Neobatrachus centralis	294	50+	L, H, few eggs

Neobatrachus fulvus	26	15	L, H, few eggs
Neobatrachus kunapalari	118	25+	L, H, few eggs
Neobatrachus pelobatoides	26	7	L, H, few eggs
Neobatrachus pictus	252	30+	L, H, tads, few eggs
Neobatrachus sudelli	155	30+	L, H, tads, few eggs
Neobatrachus sutor	68	21	L, H, few eggs
Neobatrachus wilmorei	14	10	L, H, few eggs
Notaden bennetti	3	2	L
Notaden melanoscaphus	5	2	L. H
Notaden nichollsi	9	6	L, H
Parcrinia haswelli	15	6	L, H
Philoria frosti	9	3	L, H
Philoria (Kyarranus) kundagungan	56	7	L, H, tads, few eggs, ova
Philoria (Kyarranus) loveridgei	9	4	L, H
Philoria (Kyarranus) pughi	27	6	L, H, tad, ova
Philoria (Kyarranus) richmondensis	22	3	L, H, tad, ova
Philoria (Kyarranus) sphagnicola	113	14	L, H, tads, eggs
Pseudophryne australis	28	7	L, H, embyros
Pseudophryne bibroni	154	37	L, H, tads, eggs
Pseudophryne coriacae	100	31	L, H,
Pseudophryne Sp	21	10	L, tad, whole animal, juv
Pseudophryne covacevechi	7	1	L, H
Pseudophryne dendyi	11	4	L, H
Pseudophryne douglasi	4	3	L
Pseudophryne major	30	10	L, H
Pseudophryne guentheri	8	8	L
Pseudophryne occidentalis	69	27+	L. H, eggs
Pseudophryne pengilleyi	9	1	L, H
Pseudophryne raveni	11	4	L, H, toe
Pseudophryne semimarmorata	8	4	L, H, toe
Rheobatrachus silus	12	2?	L, tad, metamorph,
Rheobatrachus vitellinus	1	1	skin
Spicospina flammocaerulea	1	1	L, H, B
Taudactylus actirostris	46	3	L, H, 1 tad
Taudactylus eungellensis	2	1	L, H
Taudactylus leimi	1	1	L
Taudactylus rheophilus	6	1	L, H
Uperoleia altissima	10	2	L, H
Uperoleia aspera	2	1	L
Uperoleia borealis	1	1	L
Uperoleia capitulata	1	1	L
Uperoleia crassa	7	1	L
Uperoleia fusca	64	16	L, H
Uperoleia inundata	11	5	L, H, eggs
Uperoleia laevigata	45	16+	L, H, eggs
Uperoleia lithomoda	17	6	L, H
Uperoleia littlejohni	7	2	L, H
Uperoleia marmorata (X)	3	1	L
Uperoleia micromeles	7	1	L, H
Uperoleia mimula	2	1	L, H
Uperoleia minima	1	1	L, H
Uperoleia mjobergi	7	2?	L,
Uperoleia rugosa	29	11	L, H, eggs
Uperoleia russelli	9	2	L, H, eggs
Uperoleia trachyderma	2	1	L, H
Uperoleia tyleri	9	3	L, H

Table 5.4 Frozen tissues held by Australian Biological Tissue Collection (ABTC), South Australian Museum.

Family Hylidae (tree frogs)

Genus and Species		Number of specimens	No of Locations	Tissues Cryopreserved
Cyclorana "black tail"		1	1	Tad
Cyclorana	*"large"*	1	1	Tad
Cyclorana	*alboguttata*	1	1	LH
Cyclorana	*australis*	30	11	LH, tad, eggs
Cyclorana	*brevipes*	13	2	L
Cyclorana	*cryptotis*	6	1	L
Cyclorana	*cultripes*	17	6	L H
Cyclorana	*longipes*	15	7	L H
Cyclorana	*maculosus*	6	1	L
Cyclorana	*maini*	9	14	L
Cyclorana	*manya*	7	3	L
Cyclorana	*novaehollandiae*	16	8	LB H tad
Cyclorana	*platycephala*	31	16+	LH
Cyclorana	*sp*	1	1	Whole
Cyclorana	*sp*	1	1	L
Cyclorana	*sp*	1	1	Tad
Cyclorana	*sp*	2	1	Tad
Cyclorana	*sp*	2	1	Tad
Cyclorana	*vagita*	2	2	LH eggs
Cyclorana	*verrucosa*	10	2?	LH
Litona	*boorolongensis*	3	1	L, H
Litoria		2	1	Whole
Litoria		1	1	L/HGut
Litoria	*?*	1	1	Tad
Litoria	*?*	1	1	LH
Litoria	*?*	8	1	Tad
Litoria	*adelaidensis*	2	1	L, H
Litoria	*alboguttata*	8	4	L
Litoria	*andiirrmalin*	3	1	L
Litoria	*aurea*	41	1	L/H
Litoria	*barringtonensis*	2	2?	
Litoria	*bicolor*	58	12	LH
Litoria	*booroolongensis*	57	14	L, H, Tad, some eggs
Litoria	*brevipalmata*	112	21	L, H, Tad, toe
Litoria	*burrowsi*	2	2	L
Litoria	*caerulea*	115	35+	L, H, toe
Litoria	*cavernicola*	4	2	L, B, H, K
Litoria	*chloris*	30	8	L/HGut
Litoria	*citropa*	5	3	L
Litoria	*coplandi*	30	11	LH eggs
Litoria	*cyclorhyncha*	3	2	L
Litoria	*dahlii*	12	9	L
Litoria	*dentata*	16	10	L,H,E
Litoria	*dorsalis*	1	1	
Litoria	*electrica*	10	4	LH
Litoria	*ewingii*	44	15+	L
Litoria	*fallax*	64	19	LH eggs
Litoria	*freycineti*	15	6	LH eggs
Litoria	*genimaculata*	51	11	HL
Litoria	*gilleni*	12	3	HRP1 eggs
Litoria	*gracilenta*	65	23	LB

Litoria	inermis	75	30	LH eggs
Litoria	infrafrenata	51	18	LHPR
Litoria	jervisiensis	19	10	LH eggs
Litoria	latopalmata	58	23	LH eggs
Litoria	lesueuri	433	85	LH eggs
Litoria	littlejohni	1	1	LHB
Litoria	longirostrus	2	1	L
Litoria	meiriana	31	11	L
Litoria	microbelos	8	2	L, gut
Litoria	moorei	1	1	L
Litoria	nannotis	11	3	LH
Litoria	nasuta	60	29+	LH
Litoria	nigrofrenata	6	4	L
Litoria	nyakalensis	4	1	L
Litoria	olongburensis	10	7	HL eggs
Litoria	pallida	42	15	HL eggs
Litoria	paraewingi	5	4	HL eggs
Litoria	pearsoniana	31	6	L/HGut
Litoria	peronii	31	18	LHEggs
Litoria	personata	5	1	LH
Litoria	phyllochroa	308	80	LHEGGS, whole animal
Litoria	piperata	33	5	LH
Litoria	raniformis	9	7	L
Litoria	revelata	94	20	LH eggs, tad, toe
Litoria	rheocola	26	4	LH
Litoria	rothii	89	27	LH eggs
Litoria	rubella	215	62+	LH eggs
Litoria	spenceri	234	15	L, H, Whole
Litoria	splendida/caerula	9	?	L
Litoria	subglandulosa	21	11	LH, whole, toe
Litoria	tornieri	35	13	LH
Litoria	tyleri	8	4	LH
Litoria	verreauxii	191	43	LH, eggs
Litoria	wotjulumensis	33	19	LH
Litoria	xanthomera	5	1	LH
Nyctimystes	dayi	10	3	LH

Table 5.5   Frozen tissue held by the Australian Museum.
Family Myobatrachidae (Australian ground frogs)

Genus	Species	Number of specimens	Number of locations	Tissues Cryopreserved
Adelotus	brevis	1	1	L
Crinia	crinia (no id)	8	2	L
Crinia	parinsignifera	1	1	L
Crinia	signifera	23	6	L
Crinia	sloanei	3	2	L
Crinia	tinnula	6	3	L
Heleioporus	australiacus	6	2	L
Limnodynastes	dumerilii	8	6	L
Limnodynastes	fletcheri	10	4	L
Limnodynastes	interioris	8	5	L
Limnodynastes	ornatus	1	1	L
Limnodynastes	peronii	64	9	L

Limnodynastes	*salmini*	10	1	L
Limnodynastes	*tasmaniensis*	54	10	L
Limnodynastes	*terraereginae*	10	3	L
Mixophyes	*iteratus*	2	1	L
Neobatrachus	*centralis*	5	1	L
Neobatrachus	*neobatrachus (no species id)*	20	3	L
Neobatrachus	*sudelli*	47	9	L
Notaden	*bennettii*	6	3	L
Paracrinia	*haswelli*	1	1	L
Pseudophryne	*australis*	124	17+	L
Pseudophryne	*bibronii*	6	3	L
Pseudophryne	*coriacea*	1	1	L
Ranidella	*bilingua*	5	1	L
Uperoleia	*capitulata*	5	1	L
Uperoleia	*inundata*	2	1	L
Uperoleia	*laevigata*	4	3	L
Uperoleia	*rugosa*	5	2	L
Uperoleia	*tyleri/martini*	6	1	L
Uperoleia	*uperoleia (no species id)*	11	4	L

Table 5.6 Frozen tissue held by the Australian Museum.
Family Hylidae (tree frogs)

Genus	Species	Number of specimens	Number of locations	Tissues Cryopreserved
Cyclorana	*brevipes*	20	8	L
Cyclorana	*novaehollandiae*	4	2	L
Cyclorana	*platycephalus*	20	2	L
Litoria	*albogutttata*	2	1	L
Litoria	*aurea*	209	14+	L
Litoria	*caerulea*	19	7	L
Litoria	*coplandi*	7	2	L
Litoria	*dentata*	62	5	L
Litoria	*fallax*	1	1	L
Litoria	*gracilenta*	6	2	L
Litoria	*infrafenata*	10	3+	L
Litoria	*jervisiensis*	1	1	L
Litoria	*lesueuri*	12	4	L
Litoria	*lesueuri/booroolongensis*	8	2	L
Litoria	*litoria (no id)*	15	1	L
Litoria	*moorei*	5	1	L
Litoria	*peronii*	24	6+	L
Litoria	*phyllochroa*	3	1	L
Litoria	*raniformis*	3	1?	L
Litoria	*revelata*	1	1	L
Litoria	*rubella*	51	7	L
Litoria	*spenceri*	2	1	L
Litoria	*subglandulosa*	1	1	L

GENUS	SPECIES	No of specimens	No of locations	Tissues Cryopreserved
Litoria	*tyleri?*	22	3	L
Litoria	*verreauxii*	8	3	L

Table 5.7   Frozen tissue held by the Australian National Wildlife Collection. CSIRO Sustainable Ecosystems, Canberra.
Family Myobatrachidae (ground frogs)

GENUS	SPECIES	No of specimens	No of locations	Tissues Cryopreserved
Adelotus	*brevis*	5	2	L
Assa	*darlingtoni*	1	1	L
Bufo	*marinus*	8	5	L
Crinia	*signifera*	9	3	L
Lechriodus	*fletcheri*	3	2	L
Limnodynastes	*convexiusculus*	2	1	L
Limnodynastes	*dumerilii*	1	1	L
Limnodynastes	*interioris*	1	1	L
Limnodynastes	*ornatus*	3	3	L
Limnodynastes	*peronii*	5	4	L
Limnodynastes	*salmini*	2	2	L
Limnodynastes	*tasmaniensis*	13	3	L
Limnodynastes	*terraereginae*	5	2	L
Mixophyes	*fasciolatus*	2	1	L
Mixophyes	*iteratus*	1	1	L
Neobatrachus	*pictus*	2	1	L
Philoria	*loveridgei*	1	1	L
Pseudophryne	*bibronii*	13	3	L
Pseudophryne	*coriacea*	2	2	L
Pseudophryne	*corroboree*	23	4	L
Pseudophryne	*major*	2	1	L
Uperoleia	*fusca*	2	2	L
Uperoleia	*rugosa*	3	1	L

Table 5.8   Frozen tissue held by the Australian National Wildlife Collection. CSIRO Sustainable Ecosystems, Canberra.
Family Hylidae (tree frogs)

GENUS	SPECIES	No of specimens	No of locations	Tissues Cryopreserved
Cyclorana	*novaehollandiae*	1	1	L
Litoria	*alboguttata*	3	1	L
Litoria	*booroolongensis*	1	1	L
Litoria	*caerulea*	1	1	L
Litoria	*chloris*	1	1	L
Litoria	*dentata*	5	3	L
Litoria	*ewingii*	3	1	L
Litoria	*fallax*	4	4	L
Litoria	*gracilenta*	2	1	L
Litoria	*inermis*	4	1	L

Genus	Species			
Litoria	latopalmata	5	4	L
Litoria	lesueuri	16	7	L
Litoria	nasuta	4	2	L
Litoria	nigrofrenata	1	1	L
Litoria	pearsoniana	2	2	L
Litoria	peronii	5	3	L
Litoria	phyllochroa	9	3	L
Litoria	revelata	1	1	L
Litoria	rothii	2	1	L
Litoria	rubella	1	1	L
Litoria	verreauxii	1	1	L

Table 5.9   Frozen tissues held by the Western Australian Museum.
Family Myobatrachidae (ground frogs)

Genus	Species	Number of specimens	No of Locations	Tissues Cryopreserved. L liver
Arenophryne	rotunda	32	8	L
Crinia	bilingua	10	7	L
Crinia	georgiana	265	40	L
Crinia	glauerti	203	29	L
Crinia	insignifera	104	17	L
Crinia	pseudinsignifera	232	43	L
Crinia	sp.	16	5	L
Crinia	subinsignifera	31	5	L
Geocrinia	alba	2	1	L
Geocrinia	leai	83	24	L
Geocrinia	lutea	1	1	L
Geocrinia	rosea	4	2	L
Geocrinia	sp.	2	1	L
Heleioporus	albopunctatus	15	10	L
Heleioporus	barycragus	1	1	L
Heleioporus	eyrei	171	48	L
Heleioporus	inornatus	8	5	L
Heleioporus	psammophilus	17	12	L
Heleioporus	sp.	12	18	L
Limnodynastes	convexiusculus	10	5	L
Limnodynastes	depressus	5	4	L
Limnodynastes	dorsalis	136	40	L
Limnodynastes	lignarius	5	2	L
Limnodynastes	ornatus	42	16	L
Limnodynastes	spenceri	46	22	L
Metacrinia	nichollsi	15	9	L
Myobatrachus	gouldii	41	20	L
Neobatrachus	albipes	17	7	L
Neobatrachus	aquilonius	54	7	L
Neobatrachus	centralis	4	1	L
Neobatrachus	kunapalari	65	34	L
Neobatrachus	pelobatoides	133	34	L
Neobatrachus	sp.	133	41	L
Neobatrachus	sutor	44	10	L
Neobatrachus	wilsmorei	36	18	L
Notaden	melanoscaphus	1	1	L
Notaden	nichollsi	44	16	L
Notaden	weigeli	2	1	L
Pseudophryne	douglasi	18	7	L

Pseudophryne	*guentheri*	146	41	L
Pseudophryne	*occidentalis*	103	25	L
Spicospina	*flammocaerulea*	1	1	L
Uperoleia	*borealis*	9	6	L
Uperoleia	*glandulosa*	5	2	L
Uperoleia	*lithomoda*	8	2	L
Uperoleia	*micromeles*	1	1	L
Uperoleia	*mjobergi*	3	1	L
Uperoleia	*russelli*	78	17	L
Uperoleia	*sp.*	8	4	L
Uperoleia	*talpa*	4	1	L
Uperoleia	*trachyderma*	2	1	L

**Table 5.10   Frozen tissues held by the Western Australian Museum.
Family Hylidae (tree frogs)**

Genus and Species		Number of specimens	No of Locations	Tissues Cryopreserved
Cyclorana	*alboguttata*	1	1	L
Cyclorana	*australis*	54	14	L
Cyclorana	*cultripes*	1	1	L
Cyclorana	*longipes*	14	5	L
Cyclorana	*maini*	87	22	L
Cyclorana	*platycephala*	23	10	L
Cyclorana	*sp.*	11	4	L
Cyclorana	*vagitus*	7	1	L
Litoria	*adelaidensis*	264	40	L
Litoria	*aurea group*	1	1	L
Litoria	*bicolor*	4	2	L
Litoria	*caerulea*	16	7	L
Litoria	*cavernicola*	5	2	L
Litoria	*coplandi*	30	12	L
Litoria	*cyclorhyncha*	8	3	L
Litoria	*gracilenta*	6	?	L
Litoria	*inermis*	5	5	L
Litoria	*infrafrenata*	1	1	L
Litoria	*meiriana*	4	3	L
Litoria	*moorei*	152	50	L
Litoria	*nasuta*	9	7	L
Litoria	*pallida*	5	2	L
Litoria	*peronii*	1	1	L
Litoria	*rothii*	46	12	L
Litoria	*rubella*	156	45	L
Litoria	*sp*	104		L
Litoria	*splendida*	7	1	L
Litoria	*tornieri*	2	1	L
Litoria	*wotjulumensis*	17	9	L
Litoria	*adelaidensis*	264	40	L
Litoria	*aurea group*	1	1	L
Litoria	*bicolor*	4	2	L
Litoria	*caerulea*	16	7	L
Litoria	*cavernicola*	5	2	L
Litoria	*coplandi*	30	12	L
Litoria	*cyclorhyncha*	8	3	L
Litoria	*gracilenta*	6	?	L

Litoria	*inermis*	5	5	L
Litoria	*infrafrenata*	1	1	L
Litoria	*meiriana*	4	3	L
Litoria	*moorei*	152	50	L

Australian Biological Tissue Collection: The following graphs show the relationship between the number of specimens of a taxon in the collection against the number of geographic locations for ground and tree frogs in the collection of the Australian Biological Tissue Collection
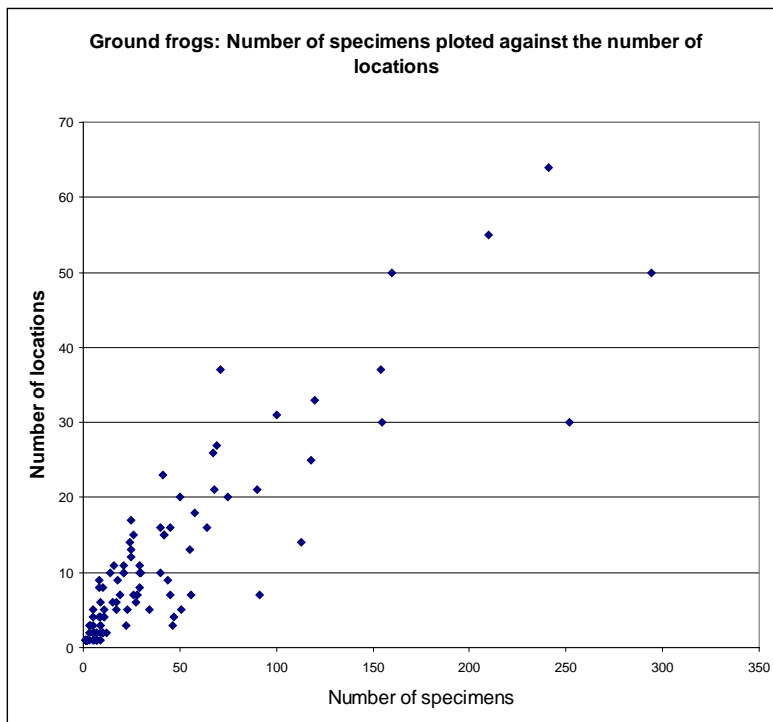
- There are 15 species that are represented by relatively large collections with over 100 specimens all of which are represented by 20 or more locations.
- There are a large number of species, which are represented by less than 50 specimens, the great majority of which come from less than 20 locations.
- There is a strong cluster of species with less than ten specimens from fewer than 5 locations.
- These issues are significant in considerations of the genetic diversity represented for widespread taxa. They also indicate that many threatened species are represented by small samples size and restricted number of locations.
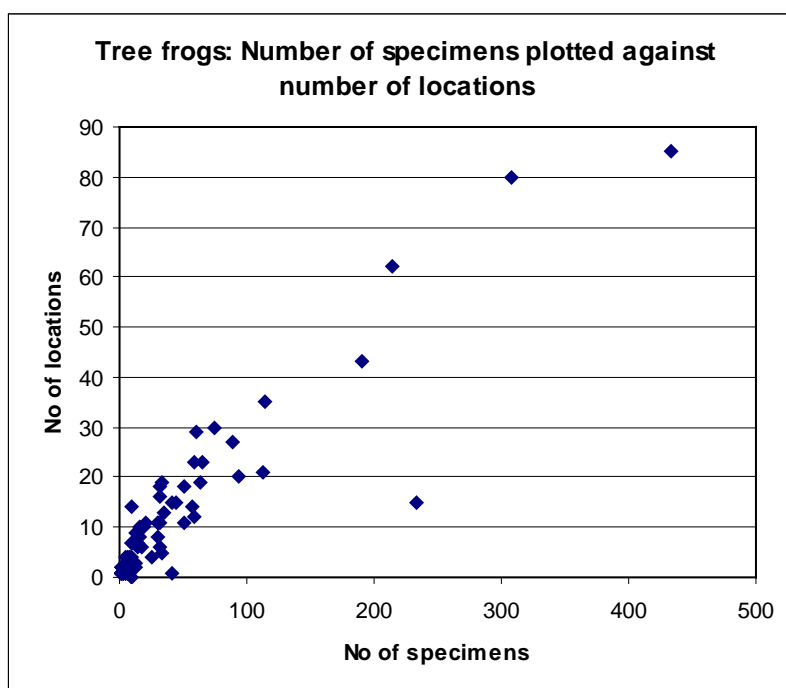
Section 6

Conservation Genetics, Threatened Species
and Genome Banks

6.1   Small Populations and Captive Colonies

Genetic diversity is required for populations to adapt to environmental change. Large populations of naturally outbreeding species usually have extensive genetic diversity, but it is typically reduced in endangered species (Frankham et al 2004). Unfortunately, for many Australian amphibians that have been impacted by chytrid population sizes are now so small that their genetic diversity is compromised. Furthermore, where *ex-situ* colonies have been established to save some of these species the number of founding individuals are relatively small. It is somewhat ironic that infection with a novel pathogen, chytid fungus, has brought several species to extinction despite the fact that they once occurred in large populations. We know nothing of the genetic diversity within these species prior to the impact of chytrid, or whether this was a factor in their demise, the observation is that in the face of a new pathogen these species were unable to respond and no resistant individuals were selected to survive. Our objective is to conserve the genetic record of these species while we are unable to prevent extinction in the wild, with the obvious desire to see the impact of the pathogen mediated and the frogs returned to their natural state.

Genetic considerations in threatened species management arise from the negative effects of small population size and from population fragmentation in threatened species. The objective of Genome Resource Bank (GRB) and genetic management is to preserve threatened species as dynamic entities capable of adapting to environmental change.

6.1.1   Consequences of Small Population Size

6.1.1.1   Genetic Drift
Genetic drift refers to the negative impact of inbreeding which results from the production of offspring from related parents, mutational accumulation and random loss of genetic diversity.

Inbreeding and loss of genetic diversity are inevitable in small closed populations. Threatened species are often composed of small and/or declining populations, and this problem needs to be carefully managed in any threatened species management program. It begins with a judicious selection of animals for the maintenance of genetic diversity, and the necessary management of records.

To achieve acceptable outcomes in short-term survival it is necessary to maintained Effective Population Sizes at greater than 500 individuals. To avoid inbreeding depression and to maintain long-term evolutionary potential effective population sizes between 500-5000 are considered necessary to retain evolutionary potential (Frankham et al 2004).

Genetic deterioration in captive colonies results from inbreeding depression, loss of genetic diversity and genetic adaptation to captivity reduces the probability of successfully reintroducing species back to the wild.

6.1.1.2   Loss of genetic diversity in small natural and captive populations

Random genetic drift occurs in small populations when alleles are lost simply due to chance. When reproduction occurs only a sample of the genetic component present in the parents is transmitted to the offspring. Some alleles, especially rare ones may not be transmitted to the offspring by chance. While it may seem that chance effects would play only a minor role in determining the genetic composition of populations, the situation is magnified in small populations resulting in, loss of genetic diversity. In these situations genetic drift overshadows natural selection.

Evidence that small populations or those founded from a small number of individuals face long term evolutionary consequences can be observed in taxa that have passed through demographic bottlenecks (periods of small population size or with a minimum number of founders contributing to future generations). Many endangered species have suffered bottlenecks, a classic example is the northern elephant seal, which was almost hunted into extinction with less than 100 individuals remaining in the wild prior to protection. Although the population currently numbers many thousands of individuals they show no allelic diversity in allozyme studies. The loss in genetic diversity is considered to be the consequence of the random sampling, or chance, nature of transmission of alleles from one generation to the next.

Loss of genetic diversity arises predominantly from sustained reductions in population size, rather than single generation bottlenecks (Frankham et al 2004). This genetic feature has significant consequences for the planning and use of a GRB. If rapid action is taken when a population is reduced to a small size much of the genetic diversity can be rescued. The rapid action necessary is to ensure that the genetic diversity present is retained by judiciously managing pedigrees to maximise the inheritance of the diversity present. Genome resource banks can play a significant role by reintroducing gene diversity into the lineages.

6.1.1.3   The Problem of Inbreeding Depression

The negative effects of inbreeding on juvenile survival are well known, and its importance in management and conservation of small population has been recognized for over two decades (Soule, 1987, Frankham 1995, Frankham et al 2004). Although the primary threat to amphibians may be a newly emerged pathogen (Daszak et al 1999), inbreeding within the resulting small and isolated populations may increase their susceptibility to extinction. Preventing inbreeding is of profound importance to conservation of endangered species in the wild and in captivity. Inbreeding leads to reductions in genetic diversity (usually measured as heterozygosity), and its consequences are reduced reproductive fitness typically measured by reduced fertility and reproductive output, and increased embryonic failure and juvenile mortality). This in turn increases the risk of extinction to the cause of decline and other stochastic events.

Inbreeding alters genotype frequencies and indirectly alters allele frequencies. The most significant consequence for captive colonies is that homozygotes for deleterious recessives become more frequent. Most large outbreeding populations contain deleterious alleles that are mostly partly recessive, and which occur at low frequencies in mutation-selection balance. Inbreeding increases the risk of exposing these homozygotes.

6.1.1.4 The problem of population fragmentation

The impacts of population isolation and reduction on genetic diversity, evolutionary potential, inbreeding and extinction risk depend on the level of gene flow among fragments. Frankham, Ballou and Briscoe (2004) have warned that all the issues of loss of genetic diversity and inbreeding

depression, with respect to reduced population size, come into play when populations are fragmented.

A great challenge of conservation biology is to manage fragmented populations to minimize the loss of genetic diversity due to inbreeding depression and genetic drift. The tools of translocation and reintroduction are required to minimize extinction risks, and captive breeding programs usually supports these.

One of the characteristics of the declining frog situation in eastern Australia is the fragmentation of formerly widespread and somewhat continuous species into isolated populations. Clear examples occur among representatives of the bell frog group (*Litoria aurea* species group; *L. aurea, L. castanea, L. raniformis*), the stream frog *Litoria boorolongensis*, and the various members of barred river frogs (*Mixophyes*). For example *Litoria boorolongensis* was once distributed in streams at relatively high altitudes on the western slopes of the Great Dividing Range in NSW from the New England Tablelands in the north to the Snowy Mountains in the south. In some regions the frog was also found in eastern flowing streams (Heatwole et al 1996, Hines et al 1999). The northern populations have been reduced to one known population, and the populations on the western slopes of the central and southern tablelands are reduced in distribution and abundance. In a similar fashion the stuttering frog (*Mixophyes balbus*) occurs as a series of isolated populations along the escarpment of the Great Dividing Range with a gap of several hundred kilometres between some populations. The pertinent issue is that the isolated populations are reduced in size to the point where the risk of extinction is high. Unfortunately, we have no idea of the genetic diversity or level of co-adapted gene complexes that are represented by these populations. However, where genetic studies have been conducted on widespread species of amphibians in Australia the results invariably uncover a level of diversity not expected.

In the case of the declining frog situation in eastern Australia, fragmentation has occurred rapidly, and current population sizes vary considerably in the small number of cases examined (see for example studies on the bell frog, *Litoria aurea,* White and Pyke 1996). For many populations that remain the likelihood of migration between populations is extremely low (see example of *Lit boorolongensis* and *Mixophyes balbus* discussed above). It is predicted that since the fragmentation and reduction in population sizes have been relatively rapid (less than 30 years) the remaining populations should not have experienced a dramatic loss of genetic diversity. An investigation of the population genetics of the green and golden bell supports for this position (Burns et al 2004).

6.1.1.5   Non genetic Consequences of Small Population Size
Demographic Stochasticity
Random variation in birth and death rates and sex ratios due to chance alone. Within populations not all members are capable of breeding or contributing to the next generation, some are to old or injured and some to young. The effective population size is generally much less than the population size, often less than 20%.

Environmental Stochasticity
Random unpredictable variation in environmental factors, including catastrophes. The chance of these factors being involved increase with small population size and fragmentation.

6.2 Conservation Genetics and Genome Resource Banks
Genome Resource Banks provide a means to prevent the loss of genetic diversity in small populations, and thus overcome many of the concerns raised by conservation geneticists.

Cyropreserved samples can be regarded as the genetic equivalent to living animals. They achieve this by,

- increasing generation interval, and
- increasing the effective (reproductively viable) population size.

Sperm banks in particular can perform this function. Typically thousands of sperm from an individual can be stored. These can be used for many 'matings' over numerous generations. Thus the contribution of a particular individual with a high level of genetic diversity can be reinstated into a population despite the fact that the animal may have died years earlier. In a similar fashion the storage of germ plasm from as many individuals as possible increases the total population size.

To construct a GRB to maximize genetic diversity requires a combination of genetic knowledge and an understanding of the situation in nature or captivity of the animals of concern. We will concentrate here on the genetic considerations, the field situation requires a collaboration with field ecologists who have knowledge of the particular species or population.

The first question is the sample size that is necessary to represent the genetic diversity present in a population. The level of genetic diversity present determines the answer to this question. Unfortunately, this is a piece of information that is lacking for most endangered species/populations. Conservation biologists recognize that it is desirable to have this information for captive breeding and reintroduction programs (IUCN Guidelines for Reintroductions 1995), but in the current situation this is of secondary priority compared to storing the genome in case of extinction.

In the absence of information on genetic diversity within a population some simple rules can be followed. Obviously, the larger the number of individuals that can be sampled and stored the better, however there are a number of factors which are involved. Attempts should be made to sample unrelated individuals. In a population where breeding is random, estimates based on population genetic theory, indicate that a sample of 5 individuals will contain up to 90% of the genetic diversity within the population. This figure cannot be estimated with certainty because it to depends on the level of genetic diversity within the population. Parallel samples of tissues should be collected, if that is possible, to enable the necessary testing of genetic diversity at a later date should that be necessary.

The second consideration is the number of populations that should be sampled. Once again this will depend on the particular case. If a species is reduced to one population the question is unnecessary (an example would be the Eungella day frog). If a species was once widespread and is now represented by isolated populations, the objective would be to sample from across the former geographic range of the species.

Ideally, it would be valuable to have some information on the genetic make-up of the sample, to ensure that maximum diversity was obtained. In some cases this information may be available to assist sampling decisions, especially where the genetic status of several populations have been investigated (see for example the green and golden bell frog, Burns et al (2004)). However, in most endangered species this information will not be available in the short term. In such cases it will be necessary to operate on broad generalities in the absence of detailed information. Thus it may not be possible to confirm whether the isolated populations of a threatened species are genetically uniform or distinct in the short term but in the absence of such information it would be a cautious approach to obtain germ cell samples from each population. Such a decision would be made with other management and cost factors taken into consideration.

There is good evidence from several genetic studies that the taxonomic status of many amphibians in Australia are poorly resolved. Thus, an apparently widespread and low-risk species may, in reality, consist a complex of distinct species some that are rare or endangered. In the rainforest of mid eastern Australia two species of mountain mist frogs (genus *Philoria*) were considered to be present. Recent genetic comparisons reveal that three previously unrecognized taxa occur, some restricted to isolated mountain areas.

Equally, genetic markers may reveal that populations considered to be threatened, actually belong to common and more widespread species, and are attracting undeserved protection and resources. Recent genetic studies of the genus *Pseudophryne* show that the vulnerable *Pseudophryne covecavichi* is more widely distributed than previously identified (Donnellan and Mahony unpubl data).

6.3 References

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