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Conservation of tropical trees *ex situ* through storage and use

by

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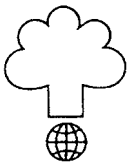

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Cover photo:
Planting seedlings of *Dipterocarpus chartaceus.* Research on seed storage, seedling storage and cryopreservation of indigenous forest trees is carried out at Forest Research Institute Malaysia (FRIM). Such knowledge is important for future conservation efforts. Photo: Ida Theilade.

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Danida Forest Seed Centre (DFSC) is a Danish non-profit institute which has been working with development and transfer of know-how in management of tree genetic resources since 1969. The development objective of DFSC is to contribute to improve the benefits of growing trees for the well-being of people in developing countries. DFSC's programme is financed by the Danish International Development Assistance (Danida).

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1. Introduction

The preferred conservation approach in forestry is to encourage sustainable management and increase the areas of protected forests. Thus, *ex situ* conservation is a last resort, which is used only after it is evident that it is impossible to preserve the ecosystems or habitat. This is mainly due to the long regeneration time required by trees. In contrast, most agricultural crop species are conserved by *ex situ* means using seed banks, field banks and, in certain cases, tissue culture.

Due to the alarming rate of deforestation and loss of species the potential role of *ex situ* in conservation of forest genetic resources, as a complement to the widely used *in situ* strategies, should be further considered. Despite the urgent need, genetic conservation within the framework of forestry has been slow to develop and *ex situ* efforts are generally restricted to species and provenances of proven value (FAO 1993). But when the preservation of ecosystems falter, their fragments may have to be cared for piece by piece. Sometimes this means *ex situ* conservation of forest tree species. This paper deals with *ex situ* storage of seeds, pollen, *in vitro* cultures, and DNA libraries. *Ex situ* conservation of forest trees in living stands, that is arboreta, seedling banks and *ex situ* conservation stands, is discussed in DFSC Technical Note No. 64 (Theilade 2003).

The aim of *ex situ* storage for conserving woody species is to maintain the initial genetic and physiological quality of the germplasm until it is used or regenerated. Achieving this requires consideration of genetic and environmental factors affecting the germplasm in storage. Since seed storage is the most commonly used short to medium term *ex situ* conservation strategy for forest trees, seed biology and its implications for storage are discussed more in detail. Then *ex situ* conservation of forest trees by storage techniques of pollen, *in vitro* cultures, and DNA libraries is reviewed with special emphasis on the relative advantages and disadvantages of each technique.

Finally, the complementary role of *ex situ* with other conservation strategies is discussed and the importance of well-defined objectives highlighted. Do we want to conserve species, alleles or gene complexes? The result is likely a program for conservation of chosen heritable attributes rather than of species. In any case the purpose of *ex situ* conservation must be carefully defined before management planning. Unless *ex situ* material is regenerated and at some stage brought into use, its conservation has no meaning.

2. Practical application of knowledge on biology of forest seeds

Knowledge of seed biology is crucial for proper handling including storage of seeds. In this context, the term 'seed' usually refers to the unit extracted from the fruit and handled as a unit during storage, pre-treatment and sowing. Seed handling encompasses a series of procedures beginning with selection of the best quality of seed source, through collection, processing, storage, pre-treatment to germination. Each link of this chain implies a potential risk of losing seed, and any link in the process is of equal importance (though not necessarily equally sensitive). If a seed dies due to careless handling during collection or processing, even the best storage conditions will not bring it back to life. If a seed dies during storage, the whole preceding effort is wasted. However, some handling procedures become unacceptably expensive if a certain loss is not tolerated during the process. In some cases it is more economical to collect excess seed rather than to try and save everything. This balance must be weighed in each situation (Schmidt 2000).

The whole process of seed storage begins with collection of good quality seeds, both physiologically and genetically. This section focuses on the implications of physiological aspects of *ex situ* storage.

2.1 Seed storage categories

Most agricultural crops have seeds that can be dried and stored at low temperatures for years without losing the ability to germinate. These seeds have been labelled 'orthodox seeds', which reflects that they are considered the most usual and widespread type of seed. However, many tree species, particularly in the tropics, have seeds that do not follow the rules of the orthodox seeds. They can be difficult to store because they do not tolerate drying and have been termed 'recalcitrant seed'. Other seeds do not seem to fit into either of these two categories and are called 'intermediate seed'. It can be argued whether it is possible to categorise all species into these three groups or whether the groups form a continuum from very orthodox seed to very recalcitrant seed. From a practical point of view, it has been established that there are two very important factors concerning seed storage: seed moisture content and storage temperature. The current definitions of the three storage categories are given below (Thomsen 2000).

Once the ovule has been fertilised, a chain of processes starts, which ultimately leads to the formation of a ripe or mature fruit or seed-bearing organ containing mature germinable seeds although germination may be impeded by the development of dormancy. For most species maturity of fruit and seed coincides with dispersal. However, in some species like *Ilex opaca* and *Fraxinus* spp. seeds contain physiologically immature embryos (Nitsch 1971). These seeds are not germinable at fruit maturity but require after-ripening. The phenomenon is often classified as dormancy. On the other hand, seeds may mature well in advance of fruit maturity. Germinable seeds can thus be obtained from green pods of some Leguminosae and unopened cones of some conifers (Sedgley and Griffin 1989). However, seeds picked very early may often have reduced storability and vigour.

Fruit development differs according to fruit type. In animal dispersed fleshy fruits sugar substances are located in the pulp simultaneously with a drastic increase in moisture content (Sedgley and Griffin 1989). The fruit changes colour from green to a usually bright and conspicuous red, orange or yellow, and becomes soft. Simultaneously the pulp usually loosens easily from the seed or stone.

Loss of water and concurrent desiccation occurs late in the development of dry fruits. Thick fruit walls of capsules, samaras and large pods become woody, thin walls papery. Dehydration of the dry fruit implies disintegration of chlorophyll; the fruit changes colour from green to typically yellow, brown or black.

Orthodox seeds tolerate drying (desiccation) to low moisture contents. They also tolerate storage at low temperatures, and can generally be stored for very long periods.

Recalcitrant seeds are damaged by desiccation and tropical species may also be damaged by exposure to low temperatures. They are said to be desiccation and chilling sensitive.

Intermediate seeds are seeds that do not fit into either of the above two categories. They can be desiccated, although not to as low a level as orthodox seeds and they are often chill sensitive.

The late events in seed maturation include formation of storage proteins and hormones, and dehydration in orthodox seeds. In dry fruits dehydration of seeds is concurrent with the general dehydration of the fruits. In fleshy fruits dehydration is the result of the increased osmotic pressure due to sugar formation in the fruit pulp (Schmidt 2000).

Final moisture content in the seeds depends on species and the external environment. It has important implications for the storage properties of the seeds. Orthodox seeds typically dry out to 5-10% during maturation. Recalcitrant seeds maintain relatively high moisture content, typically >40-50%. Intermediate seeds also maintain relatively high moisture content.

Although recalcitrance is predominant in some plant families such as *Dipterocarpaceae* (Box 1), there seems to be no phytogenetic pattern and often two closely related species show very different storage behaviour (Thomsen 2000). Table 1 lists some characteristics that may help to give an idea about storage behaviour.

Chilling injuries occur if recalcitrant seeds at high moisture contents are exposed to temperatures below 15°C. If a species is never exposed to low temperatures in the area of natural distribution, temperature sensitivity should be expected. *Hopea odorata* is a typical recalcitrant species from the humid tropics. It is very desiccation sensitive and does not tolerate temperatures below 15°C while *Quercus robur*, a typical temperate recalcitrant species is desiccation sensitive, but tolerates storage temperatures of -2°C. Thus, a clue to the behaviour of the seed can probably be found in the natural environment of the species (Thomsen 2000).

The use and conservation of particular species is often limited by problems related to seed collection and storage. Seed viability restricts the use of the species to a narrow range from the seed source. *Azadirachta indica* seeds for instance can have a limited viability, which makes immediate sowing necessary, sometimes at times not optimal for seedling survival (Schmidt 2000). Improved seed handling techniques have recently been worked out for this species (Sacandé 2000). Such improved techniques are a key to the increased use and conservation of many species, particularly for the ones with non-orthodox seed storage behaviour.

The recent developments in storage technologies are reviewed below and the possibilities and limitations of storage as a potential long-term conservation measure for tree species is discussed.

2.2 Storage of orthodox seed

Storage of orthodox seeds is the most widely practised method of *ex situ* conservation of plant genetic resources. About 90% of the 6.1 millions accessions stored in genebanks are in fact maintained as seed (FAO, 1996). The techniques involve drying seeds to low moisture content, 3-7% fresh weight basis, depending on the species, and storing them, in hermetically sealed containers, at low temperature, preferably at -18°C or cooler (FAO/IPGRI, 1994). Seeds of many orthodox species may be conserved in this way for several decades and possibly centuries. A series of practical documents are available in literature, covering the main aspects of seed conservation, including:

- design of seed storage facilities for genetic conservation (Cromarty *et al.* 1982);
- principles of seed testing to monitor the viability of seed accessions maintained in genebanks (Ellis *et al.* 1985a);
- methods for removing dormancy and germinating seeds (Ellis *et al.* 1985b);
- methods for processing and handling seeds in genebanks (Hanson 1985).

Table 1. Characteristics of orthodox versus recalcitrant/intermediate seed (Thomsen 2000).

Orthodox seed	**Recalcitrant and intermediate seed**
Low moisture content at shedding	Relatively high moisture content at shedding
Includes dormant and non-dormant species	Usually no dormancy
Perennial, woody or annual, herbaceous species	Mostly perennial, woody species
Found in all ecosystems	Often found in humid ecosystems
Usually small seeds	Often large, fleshy seeds

Box 1

Storage of Dipterocarp seeds

Dipterocarpaceae, which make up the major component of the South East Asian tropical rain forests, is an example of a family where recalcitrant seeds is a major constraint in cultivation and also circumvent *ex situ* storage of seeds.

Early publications mentioning dipterocarp seed storage emphasise their extremely short-lived nature. According to a review of literature (Thompsett 1992) the longevity of seed from 79 tropical recalcitrant species ranged from 14 days for *Shorea dasyphylla* to 365 days for *Hopea hainanensis*. In addition, reduced germination following temperatures below 16° C (chilling damage) occurs in all moist dipterocarp seeds so far examined. Some authors have divided dipterocarp species according to the susceptibility of their seeds to chilling damage: seeds of *Shorea* species in the 'red meranti' and 'balau' groups were said to be especially sensitive whilst all other dipterocarps studied were tolerant to 4°C.

Seeds of *Hopea*, *Cotylelobium* and *Vatica* tend to have greater desiccation tolerance than *Shorea* seeds while most *Dipterocarpus* seeds are relatively intolerant. Several reports indicate optimum storage of recalcitrant dipterocarp seed at temperatures in the range 15-21° C. Other *Dipterocarpus* species, as for example *D. turbinatus* are not recalcitrant.

Associations between desiccation tolerance and seed size, habitat, seed desiccation rate, and longevity have been observed among the species of the *Dipterocarpaceae*. Thus seed of three *Shorea* species from different habitats have different desiccation tolerance. The low-rainfall species *S. roxburghii* has seed which can be dried safely to 35% whereas the monsoon or rain forest species *S. almon* and *S. robusta* cannot be safely dried below 40% moisture content. Smaller seed dry faster and tend to be more desiccation tolerant. The seed with the greatest desiccation tolerance (*S. roxburghii*) is also the seed with the greatest longevity.

Photo 1. *Dipterocarpus alatus* from Thailand. *Dipterocarpaceae*, which make up the major component of the South East Asian tropical rain forests, is an example of a family where recalcitrant seeds is a major constraint in cultivation and also circumvent *ex situ* storage of seeds. Photo: Ida Theilade.

In summary, recalcitrant seeds should be kept at moisture contents above their lowest-safe values. Ventilation is needed to remove toxic gases and to prevent anoxia while the value of fungicides in improving storage life of dipterocarp seeds has yet to be established. More work is needed to assess the optimum moisture contents required for storage of seeds of particular species.

Based on Tompsett's review of the literature on storage of dipterocarp seeds (1992).

The Millennium Seed Bank Project

The Millennium Seed Bank Project aims to conserve seed collections of woody and other species, primarily, from the world's tropical and sub-tropical drylands. The Project has evolved from work on wild species started by the Royal Botanic Gardens, Kew in the 1960s and builds on years of collaboration with botanical, agricultural and forestry organisations across the globe. The impetus of the Convention on Biological Diversity and the threat that some 48,000 plant species may be brought to the edge of extinction within 20 years, has influenced the ambitious aim of conserving seed samples from 24,000 plant species during the years 2000 – 2009. The Project is managed by RBG Kew's Seed Conservation Department (SCD) based at the Wellcome Trust Millennium Building, Wakehurst Place, West Sussex, UK. The Project receives substantial funding from the UK's Millennium Commission, the Wellcome Trust and Orange plc.

The collection numbered over 11,000 population samples of 5,500 plant species prior to the main phase of the Project. All samples are stored long-term at internationally accepted standards. Included within the existing collection are base collections of land stabilisation and agroforestry species held on behalf of FAO / IPGRI and ICRAF.

Samples are collected and, where permitted, distributed, under material transfer agreements. Seed samples are provided in small research quantities only. The key purpose of the collection is to underwrite conservation. This contrasts with many forest seed collections where more immediate use is the aim. Sampling has been directed initially at the species level with the aim of at least one population sample per species. Within each population, the sampling aims to capture a wide representation of the alleles present. These initial samples will be used to learn more about the biology of the species and thereby formulate further sampling strategy.

In order to minimise the need for regeneration with all of the attendant problems, it is important that sample life is maximised. Therefore, a significant research programme, built upon some 25 years of experience underpins the Project. This work feeds into a major training initiative and a database that should facilitate seed conservation in many countries.

Apart from providing advice (recently for example to Mauritius on the conservation of the endangered palm, *Hyophorbe lagenicaulis*), the SCD is involved in methods for determining storage life, elucidating seed storage category and breaking seed dormancy. Work by RBG Kew was the first to produce viability constants for tree seed. This in turn helped demonstrate the potential to keep such seeds alive for periods in excess of a generation time for certain species. SCD plays an important supporting role within the IPGRI / DFSC programme on storage of forest tree seed involving countries such as Tanzania and South Africa. Further projects have looked at the problem of hardseededness in tree seed (supported by IPGRI). Seed desiccation studies have been strengthened by PhD work on soluble sugars, genetic markers and oxidative stress. Recent post-graduate studies on Kenyan *Milletia* and *Euphorbia* and tree seed germination work with Burkina Faso, help illustrate the international flavour of the Research and Technology Programme and its commitment, like that of the MSB Project, to improving tree seed conservation.

Photo 2. The Millennium Seed Bank Project. Scientists from Kenya discuss the processing of *Combretum collinum* seed with a member of RBG Kew staff at the Welcome Trust Millennium Building. Copyright trustees Royal Botanic Gardens, Kew.

An attempt has been made to develop a 'low-input' alternative to the conventional cold storage of seed. The technique is called ultra-dry storage and allows preservation at room temperature. It is considered a useful, low-cost option in those circumstances where no adequate refrigeration can be provided. On the other hand, it has been argued that drying seeds beyond a critical moisture content provided no additional benefit to longevity and may even accelerate seed ageing rates (Ellis *et al.*, 1988; Vertucci and Roos, 1993). Additional research on various aspects of ultra-dry seed storage, including drying techniques such as sun/shade drying (Hay and Probert 2000), or vacuum/freeze drying (Côme, 1983) and their applicability to a broader number of species should therefore be continued.

2.3 Storage of non-orthodox seed

Seeds shed at relatively high moisture content (>40-50% fresh weight) are unable to withstand desiccation and are often sensitive to chilling. These seeds therefore cannot be maintained under conventional seed storage conditions and are described as recalcitrant (Chin, 1988). Even when stored in an optimal manner, their life span is limited to weeks, occasionally months. An IPGRI protocol to determine the precise seed storage behaviour of unknown species is available (Hong and Ellis, 1996).

Of more than 7,000 species, for which information on seed storage behaviour has been published, approximately 3% are recorded as recalcitrant and an additional 4% as possibly recalcitrant (Hong *et al.*, 1996, www. ipgri.cgiar.org). It should be noted that the percentages of intermediate and recalcitrant seed producing species cited are likely to be largely underestimated. These figures are based on scientific and technical publications, which, by default, concern mainly temperate species and crop plants. Many of these plants are of early succession, a habitat in which many species possess seeds that lack dormancy. It can be expected that a large proportion of the species for which no information is available, which are predominantly from tropical or sub-tropical origin will often and even predominantly possess seeds that lack dormancy and will prove recalcitrant to induction of dormancy with current methods. Many long-lived species, often large at maturity, also predominate in these same habitats (Ashton 1988).

More recent investigations have identified species exhibiting 'intermediate' storage behaviour. The term has been used to describe seeds which tolerate a greater degree of drying than recalcitrant seeds but which are less desiccation tolerant than orthodox seeds (Ellis *et al.*, 1990, 1991). Furthermore intermediate seeds are often chilling sensitive. Even though a continuum in desiccation sensitivity is observed from highly desiccation sensitive to relatively, it may not be possible to achieve the long-term conservation at –20°C, which is possible for orthodox seeds tolerant (Berjak and Pammenter, 1994). Included in the intermediate category are some economically important species such as coffee, citrus, rubber, oil palm and many tropical forest tree species tolerant.

Before undertaking any 'high tech' research on methods to improve storage of recalcitrant and intermediate species, it is suggested to examine the development pattern of seeds and perform preliminary experiments to determine their desiccation sensitivity as well as to define storage conditions. IPGRI, in collaboration with DFSC and numerous institutions world-wide have developed a protocol for screening tropical forest tree seeds for their desiccation sensitivity and storage behaviour (Box 2). Even if such experiments do not allow defining long-term storage conditions, increased storage periods may make the whole difference for the use of a species if it allows storage of seeds until the next planting season or transport from the seed source to a prepared planting site.

Box 2

Recalcitrant tropical forest tree seed project

The use of many high-value, tropical forest tree species in tree planting and conservation programmes is hindered by problems associated with seed handling and storage. The seeds of a large proportion of tropical tree species germinate very fast or die soon after they have been collected from the tree. They are called recalcitrant seeds because they are difficult to handle and are difficult to store over long periods of time.

Some of the species that have recalcitrant seeds are highly desirable species but the difficult seed handling often prevent their use. In order to try and overcome the problem, the International Plant Genetic Resources Institute and Danida Forest Seed Centre, together with several national institutes worldwide, initiated a project on handling and storage of such seeds. In the first phase of the project, a protocol for determining the minimum moisture content and optimal storage conditions was developed and tested on about 30 tropical forest species of economical interest.

In the second phase of the project, more than 20 national partners from Africa, Asia and the Americas are working together to screen the desiccation tolerance and storage behaviour of 40 additional tropical forest tree species. In addition, the practical applicability of the results will be tested in large-scale trials of seed handling of *Azadirachta indica*, *Virola koshnyi* and *Vitellaria paradoxa*. All species are screened by two replicating institutions.

Regional training workshops provide practical experience to technicians from national institutions in the application of the screening protocol developed in the first phase. The workshops also provide opportunities for collecting and replicating partners to meet and discuss practical problems on their target species.

Communication between project members is ensured by an informal research network, whose members contribute periodically to a newsletter. This has proven an effective tool for publishing and disseminating the protocol and technical papers as well as other relevant information on trials initiated, progress achieved, practical problems encountered and suggestions to solve them. The results are also made available on the DFSC website: www.dfsc.dk/ipgriworkplan.htm.

Early results show that many of the species are less difficult than expected. Slight modifications of the procedures may lead to longer safe storage periods. For instance, a light drying of the seeds or storage at a certain temperature will in some cases make it possible to keep the seeds for a couple of months instead of a few weeks. These results may make all the difference for the use of the species. In some tropical countries, e.g. Vietnam, there are more than a thousand indigenous species of which some hundreds would be desirable to grow. If proper seed handling methods could be established, some of these species could be included in the tree planting programmes contributing to the national economy and greatly improving the biodiversity of the country.

Photo 3: Seeds of *Neobalanocarpus heimii*. Locally known as Chengal, this species is highly sought after due to its durable and heavy timber properties. Endemic to Peninsular Malaysia it is listed on the IUCN red list as vulnerable. Its highly recalcitrant seed germinate 4-7 days after seed fall, which has hindered seed supplies for conservation and reforestation programmes.
Photo: Ida Theilade.

3. Other techniques for storage of non-orthodox species

Despite decades of research on storage methods for recalcitrant seeds only limited progress has been made. Therefore, it might be necessary to turn to other techniques for storage of non-orthodox species. These are reviewed below:

- **Cryopreservation**: the term refers to seed storage at ultra-low temperature, usually that of liquid nitrogen (-196°C). Coupled with *in vitro* culture, this technique often represents the only safe and cost-effective option for storage of non-orthodox species. For mahogany or neem, seeds are relatively small and tolerant to desiccation, and can thus be cryopreserved directly after partial desiccation (Hu *et al.*, 1994; Marzalina, 1995; Berjak and Dumet, 1996). In cases when seeds are not amenable to cryopreservation, excised embryos or embryonic axes should be used. In this case it must be pointed out that selecting embryos at the right developmental stage is critical for the success of cryopreservation.

  In addition to the desiccation technique preferentially employed for freezing, other methods, including pregrowth-desiccation and encapsulation-dehydration and vitrification should be experimented (Engelmann, 1997).

  With species for which attempts to freeze whole embryos or embryonic axes have proven unsuccessful, various authors have suggested to use shoot apices sampled on the embryos, adventitious buds or somatic embryos induced from embryonic tissues. This might be the only solution for species that lack well-defined embryos. However, in this case, more sophisticated tissue culture procedures have to be developed and mastered. Moreover, using adventitious explants would reduce the range of genetic variability sampled, especially when using somatic embryogenesis, since response to inducing treatments is generally highly genotype-specific and somatic embryo cultures might be obtained from a limited number of genotypes only (Pence 1995; Berjak *et al.* 1996).

  The disadvantage of cryopreservation is the overall difficulties with regeneration of whole plants.

- **Seedling conservation**: conservation of young seedlings arrested in their development by storage at low temperature and/or under low light intensity: this technique has been experimented with *Symphonia globulifera* and *Dryobalanops aromatica* (Corbineau and Côme, 1986; Marzalina *et al.*, 1992).

- ***In vitro* conservation** involves the maintenance of explants in a sterile, pathogen-free environment and is widely used for the conservation of species which produce recalcitrant seeds, no seeds at all, or for material which is propagated vegetatively to maintain particular genotypes (Engelmann, 1997). Although research on *in vitro* techniques only started some 20 years ago this technique has been applied for multiplication, storage and, more recently, for collecting of germplasm material for more than 1,000 species (Bigot, 1987).

  In vitro techniques can be effectively used for collection, multiplication and storage, particularly with problematic species (Engelmann, 1997). Techniques have been developed to introduce recalcitrant seeds and vegetatively propagated material *in vitro*, under aseptic conditions, directly from the field (Withers, 1995). This approach will allow germplasm collections to be made in remote areas (e.g. in the case of highly valuable recalcitrant seeds), or when the transport of the collected fruits would become prohibitively expensive (e.g. because of their size/weight). Also in cases where the target species would not have seeds to be collected, or when budwood would quickly lose viability or is highly contaminated, the establishment of aseptic cultures in the field will facilitate collecting and improve its efficiency (Engelmann, 2000). Plant material with high multiplication rates can be propagated in an aseptic environment through tissue culture systems (e.g. micropropagation and somatic embryogenesis). The miniaturization of explants is an interesting option to reduce space requirements and consequently labouring costs for the maintenance of germplasm collections (Ashmore 1997).

  Different *in vitro* conservation methods are employed, depending on the storage duration required (Engelmann, 1997; Withers and Engelmann 1998). For short- and medium-term storage, various techniques have been devised which allow to reduce growth and to increase the intervals between subcultures. *In vitro* conservation techniques using slow

growth storage have been developed for a wide range of species, including temperate woody plants, fruit trees, horticultural species. Despite the availability of such techniques, the Report on the State of the World's Plant Genetic Resources for Food and Agriculture (FAO, 1996), indicates that only around 38,000 accessions are conserved *in vitro* worldwide. Most conservation programmes are unable to meet requirements for relatively sophisticated equipment, reliable electricity supply and trained staff. In addition, it should be borne in mind that only a limited amount of genetic diversity can be maintained *in vitro*.

- **Pollen storage**: this is comparable to seed storage, since most pollen can be dried to less than 5% moisture content on a dry weight basis and stored below 0^0 C. Some species, however, produce pollen with recalcitrant-type storage characteristics. There is limited experience on the survival and fertilizing capacity of cryopreserved pollen more than five years old (Towill, 1985). However, pollen has a relatively short life compared to seeds (although this varies significantly among species), and viability testing may be time-consuming. For these reasons, despite being a useful technique for species which produce recalcitrant seed (IPGRI, 1996), pollen storage has been used to a limited extent in germplasm conservation (Hoekstra, 1995). Other disadvantages of pollen storage are the small amount produced by many species; the lack of transmission of organelle genomes via pollen; the loss of sex-linked genes in dioecious species; the lack of plant regeneration capacity although first indications exist that pollen can be re-grown into whole plants (Hoekstra, 1995). On the other hand, pollen transfer of pests and diseases is rare with the exception of some virus diseases, thus allowing the safe movement and exchange of germplasm.

- **DNA storage**: this storage method is rapidly increasing in terms of importance. DNA from the nucleus, mitochondrion and chloroplasts are now routinely extracted and immobilised into nitro-cellulose sheets where the DNA can be probed with numerous cloned genes. With the development of PCR (polymerase chain reaction) one can now routinely amplify specific oligonucleotides or genes from the entire mixture of genomic DNA. These advances have led to the formation of an international network of DNA repositories for the storage of genomic DNA (Adams, 1997). The advantage of this technique is that it is efficient and simple and takes up little space. The main disadvantages, besides high-standard requirements in capacity and equipment, lie in problems with subsequent gene isolation, cloning and transfer as well as that it does not allow the regeneration of entire plants (Maxted *et al.*, 1997).

4. Some considerations on *ex situ* storage of trees

The design of any *ex situ* conservation program and which technologies to use must start from considerations on the biological material in question, why do we want to conserve it and how is it going to be used in the future. Thus, purpose must be strictly defined as a prerequisite to management planning. The result will often be a program for conservation of chosen heritable attributes rather than of species. In the following some important limitations and options inherent to *ex situ* conservation is discussed.

4.1 The genetic consequences of *ex situ* conservation

It is generally agreed that most genes do not vary at the population, or even species, level. Most variable alleles are sufficiently abundant to be adequately sampled and conserved without danger of chance extinction through random drift in artificial populations as small as 50 (Marshall and Brown 1975) to 100 (Frankel and Soulé 1981) randomly selected individuals. However, the importance of rare, variable genes in the long-term survival of the species is unknown. It can be expected that a few genes rare in one population, or ecotype, will be common in another. Samples for *ex situ* conservation need to be taken from a range of populations if genetic variability is to be adequately represented (Ashton 1988).

A serious problem is that natural selection cannot be simulated *ex situ*. If the artificial population is established from seed, the progeny has been released from natural selection from the start. Whether *ex situ* conservation takes the form of plants, seed, or tissue culture, genetic rejuvenation through controlled cross-pollination of regenerated plants is periodically obligatory. New unselected gene combinations are inevitably introduced each time. No matter how much care is taken in seed collection following controlled pollination, natural selection cannot be simulated, particularly if it is mediated by interspecific competition (Ashton 1988). Some artificial selection is unavoidable being imposed through the methods adopted for germination, pollination, seed collecting and continuing storage *ex situ*. The extent of the problem with respect to absence of natural selection in storage depends on the generation time of the species and the population structure. Under some circumstances, an absence of natural selection could be an advantage in maintaining potentially useful alleles that might have been lost in the wild.

Ex situ storage of small samples can therefore be expected to lead inevitably to unpredictable genetic change. The proportion of fit genotypes in progeny intended for reintroduction into the wild can thereby be greatly reduced, especially if the original wild donor populations are already small and in decline, and hence already suffering an accumulation of deleterious gene combinations. For all practical intents, therefore, *ex situ* conservation of species leads inevitably, and perhaps irreversibly, to domestication (Ashton 1988**).**

4.2 Limitations of science and technology

When evaluating the effectiveness and limitations of presently available *ex situ* methods for genetic conservation of perennial woody species it becomes clear that, with present knowledge, the storage of recalcitrant seed, the storage of pollen and *in vitro* culture are only viable as short term measures. The only secure, medium to long term *ex situ* conservation method presently available for large-scale use in forestry is the storage of orthodox seeds. In all these cases, however, problems related to regeneration of seed stocks should be kept in mind.

Currently, all methods of *ex situ* preservation of live plant material require periodic regeneration and sexual reproduction of the stock. The latter presupposes knowledge on the breeding system and pattern of genetic variability of all species concerned. This knowledge is often not available for tropical tree species. Thus, storage *per se* may not be the most difficult problem in long term *ex situ* conservation (FAO 1993).

The task of *ex situ* conservation is not made easier by the many cases where mutual interdependence exist. Oaks cannot be conserved without the rich symbiotic fungal flora on their roots on which they depend and which differs between species. Some trees are dependent on bats and flying foxes for pollination and seed dispersal. Large and heavily protected fruits from a large class of valuable timber trees of *Meliaceae* and *Burseraceae* in Southeast Asia appear to be dispersed exclusively by hornbills, as only their strong bills possess the capacity to open the thick husks and extract the large arilate seeds from within (Terbourgh 1990). In some cases, such trees and their symbionts will prove exceptionally difficult to conserve *ex situ*. However, intensive care and biotechnology can preserve some diversity that would otherwise be lost.

Unhappily, high cost is characteristic for high-tech applications, and whereas the capability and the economic resources to apply advanced technologies to preservation is located mostly in wealthy northern countries, the largest problems

of species loss are in poor tropical countries. Funds used for *ex situ* intervention cannot be used to preserve habitat. For less developed countries, habitat preservation is the only realistic strategy, unless help comes from outside (Conway 1988). Even when it does, could not most endangered tree species be conserved *in situ* more cheaply by adequately designed and protected nature reserves and by understanding accommodation outside reserves?

4.3 What is the role for *ex situ* storage of trees?

Species with true orthodox seeds (*Pinus*, *Picea* spp. *Acacia*) have been successfully stored for up to 50 years. With improved protocols for seed collection, handling, processing and storage the longevity of orthodox seeds could be extended to 75-100 years (FAO 1993). Improved storage capabilities clearly facilitate silviculture and use of a species, which may serve as a powerful means to conserve the genetic resource. On the other hand, despite two decades of research towards developing effective storage methods for conserving seeds of recalcitrant tree species, only short-term storage methods have been developed. Storability has been extended from a few days to a week for some of the most difficult species or from a few months to several months for others. Excised embryos of *Hevea brasiliensis* and *Theobroma cacao* have been stored 8-12 months by cryopreservation followed by regeneration using *in vitro* techniques (FAO1993). Such methods are too expensive for operational scale activities, especially in developing countries. However, it is important to note that the potential for cryopreservation has been demonstrated and some institutions like Fort Collins in the USA and NBPGR in India are looking at the longer-term prospects. At present, however, *ex situ* technology is a treatment, which can be used to buy time to preserve options for a few populations and species judged of special value.

In some cases *ex situ* storage may be the only option for conservation over a short interval of time before the species is reintroduced in its natural environment or brought into use in planting programmes. The loss of wild populations is not always the result of irreversible habitat change. It can come about for transient economic or cultural reasons, such as unsustainable harvest, temporarily intensified slash and burn, or correctable environmental reasons, such as certain mining operations, ongoing construction or introduced environmental weeds. Where the threat and destruction may be temporary it makes sense to relocate or store a threatened population for later re-introduction (Ashton 1988). In such case the genetic make-up of the species should as far as possible be maintained as in the example of the Toromiro tree from Easter Island (Box 3).

Ex situ samples can effectively serve as a means to conserve unique or important characteristics rather than species as such. *Ex situ* conservation programs for provenances of tropical pines represent just this (CAMCORE 2000). Unique genes and gene combinations, which may be as important to the survival of endangered species as to the protection of domesticated trees, may be maintained *ex situ*. With respect to most forest tree species, gene combinations can only be conserved clonally. Due to outbreeding, most gene combinations cannot be maintained using seed. Thus, it is imperative for long term conservation that managers of *ex situ* population samples define, as carefully as information allows, the characteristics they are intending to conserve: all alleles in a subsample or only some and, if some, then which? Once a clear set of priorities has been defined, the role of what in effect will become a form of gene library will be considerable (Ashton 1988).

The immediate role of *ex situ* conservation of rare and endangered trees lies more in research and education *per se*. The accessibility of trees in cultivation presents research opportunities not possible with remote and dispersed wild populations as well as opportunities for education and public awareness that would not otherwise exist. This role is absolutely vital if we are to have knowledge about plant populations on the verge of extinction that provides a sufficient basis for their management. This point is brought home most poignantly by consideration of the humid tropics, where species diversity is at its greatest. Long-lived perennials are in the majority, making cultivation easier, but few plants possess seed dormancy and many are of formidable size. Knowledge of the cultural requirements of the minority is non-existent. It is obvious that *ex situ* conservation cannot be an end in itself under such circumstances (Ashton 1988).

Ex situ is a supplement to *in situ* conservation, not an alternative. Of course, the fact remains that tree species, populations and alleles are becoming extinct in the wild, and some can and should be conserved *ex situ*. When extinction in the wild becomes a reality, the means to control artificial selection is greatly reduced and the means to reintroduce alleles lost in culture eliminated. Nonetheless, *ex situ* conservation must often be deemed preferable to loss (Raven 1976).

In these cases a conservation strategy for a particular tree species, population or character requires a holistic approach, combining the different *ex situ* and *in situ* conservation techniques available. Selection of appropriate methods should be based on a range of criteria, including the biology of the species in question (Engels and Wood 1999), practicality and feasibility of the particular method chosen, as well as the cost-effectiveness and security afforded by its application (Maxted *et al.* 1997).

Box 3

Sophora toromiro the lost tree of Easter Island

Easter Island (Rapa Nui) is one of the most remote and smallest inhabited islands. With an area of a little more than 150 km^2 it shares fate with many other small islands of having suffered dramatic environmental degradation and a high level of species extinction. One of the extinct plants of Easter Island is the toromiro tree of the *Leguminosae*. *Sophora toromiro* has, however, survived in cultivation in several botanical gardens.

With the main stage of environmental destruction on Easter Island having passed, the opportunity now exists to restore degraded habitats and reintroduce lost components of the flora. The conservation and re-introduction of *S. toromiro* is being coordinated by the Toromiro Management Group, a collaborative consortium of botanic gardens, geneticists, foresters and archaeologists.

The first scientific collection of *S. toromiro* was made in 1774 during the second of Cook's voyages. Notes at that time suggest that the species survived as scattered thickets on the island and that its valuable timber had many uses, for building materials, household articles, canoes and carvings. The causes of loss of original shrub and forest can be traced to the ecological change following colonisation by Polynesians around 400 AD. Already the first European visitors commented on the island's treeless state and the process of degradation was completed in 1866 with the introduction of rabbits, sheep, pigs, horses and cattle.

The last surviving wild *S. toromiro* specimen was growing on the inner slope of Rano Kau crater and thus protected from predation by livestock. This specimen survived until 1960 when it was chopped down for firewood. It was also the source of the seeds collected by Thor Heyerdahl during the 1955-56 archaeological expedition to Easter Island. The seeds were initially presented to the Naturhistoriska Riksmuseet in Stockholm but in the autumn of 1958 seeds were transferred to the Gothenburg Botanical Garden and six or seven were sown. Stocks currently in cultivation descended from four seedlings germinated at the Gothenburg Botanical Garden in 1959. This initial stock was derived from one tree and was likely to have arisen as a result of self-pollination. In addition, *S. toromiro* is grown at National Botanic Garden, Chile and there are a number of trees growing in private gardens and nurseries where the lack of documentation on origin makes their conservation value debatable.

The Toromiro Management Group has undertaken a global search and located all available toromiro trees in cultivation and a directory of trees is held at Royal Botanic Garden, Kew with associated data from genetic fingerprinting.

It is evident that very little genetic diversity exists within the surviving population. The species apparently persisted with low population numbers over a number of decades; between 1917 and 1960 it probably persisted as one wild individual. It is likely that this bottleneck will have implications for the taxon's long-term survival, which will be dependent upon efficient management of the surviving genotypes. Using genetic data a propagation program is being designed to establish plantations of trees in Europe, Chile and Easter Island. The Toromiro Management Group is working with the Corporatión Nacional Forestal on the repatriation of toromiros to Easter Island. In 1995 an experimental introduction using 150 plants from Bonn and Gothenburg botanic gardens were undertaken but failed.

The future of the toromiro lies in promoting and maintaining collaboration between botanic gardens holding stock and the conservation authorities in both Chile and Easter Island. A single species conservation project like this will only succeed if integrated within broader issues of protected area planning and habitat restoration. This in turn needs to be put into the context of the islands prevailing social and economic requirements. The island has a population of 2800 people, so any planned re-introduction programme must take into account the views and opinions of the islanders. Furthermore, the planned re-introduction and restoration program will work with the constraint that the habitat degradation on Easter Island has been so profound that natural establishment of toromiro trees is no longer feasible. Efforts will focus initially on establishing cultivated field gene banks and assess the ability of restored island habitats to support reintroduced populations.

Toromiro is one of the few examples of *ex situ* storage and cultivation having prevented (or delayed) extinction of a tree species. It is also an example of how difficult it is to reinstate a flora component once species associations and habitat have been altered. Hopefully toromiro will soon again form part of the biological and cultural heritage of Easter Island.

Based on 'Conservation of the extinct toromiro tree' (Maunder 1997) and 'Genetic diversity and pedigree for *Sophora toromiro*: a tree extinct in the wild' (Maunder *et al.* 1999).

Photo 4: *Sophora toromino* - a descendant of the last surviving wild tree of Easter Island grown from seed at the Botanic Garden, Copenhagen. Hopefully the Toromiro tree will soon again form part of the biological and cultural heritage of Easter Island.
Photo: Omar Ingerslev.

5. References

Adams, R.P. 1997. Conservation of DNA: DNA banking. In: Callow, J.A., Ford-Lloyd, B.V. and Newbury, H.J. (eds) Biotechnology and Plant Genetic Resources Conservation and Use. CAB International, Wallingford, pp. 163-174.

Ashmore, S. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. F. Engelmann (Vol. ed.). International Plant Genetic Resources Institute, Rome, Italy.

Ashton, P.S. 1988. Conservation of biological diversity in botanical gardens. In: Biodiversity. E.O. Wilson, ed. National Academy Press, Washington, D.C.

Berjak, P. and D. Dumet, 1996. Cryopreservation of seeds and isolated embryonic axes of neem (Azadirachta indica). Cryo-Letters 17: 99-104.

Berjak, P. and N.W. Pammenter, 1994. Recalcitrance is not an all-or-nothing situation. Seed Sci. Res. 4: 263-264.

Berjak, P., D. Mycock, J. Wesley-Smith, D. Dumet and P. Watt, 1996. Strategies for *in vitro* conservation of hydrated germplasm. Pp. 19-52 in International Workshop on *In vitro* Conservation of Plant Genetic Resources. Universiti Kebangsaan Malaysia and International Plant Genetic Resources Institute, Kuala Lumpur, Malaysia.

Bigot, C. 1987. *In vitro* manipulation of higher plants: some achievements, problems and perspectives. In: Boccon-Gibod, J., Benbadis, A. and Shont, K.C. (eds) Cell Culture Techniques Applied to Plant Production and Plant Breeding. Proceedings of IAPTC French-British Meeting, 8-9 October, Angers, pp. 5-17.

CAMCORE 2000. Conservation and testing of tropical and subtropical forest tree species by the CAMCORE Cooperative. CAMCORE Cooperative, North Carolina State University, Raleigh, USA. 234 pp.

Chin, H.F. 1988. Recalcitrant Seeds: A Status Report. International Plant Genetic Resources Institute, Rome.

Côme D. 1983. Post harvest physiology of seeds as related to quality and germinability. In Post-harvest physiology and crop preservation, M. Lieberman, ed., Plenum Press, New York, pp. 165-190.

Conway, W. 1988. Can technology aid species preservation? In: Biodiversity. E.O. Wilson, ed. National Academy Press, Washington, D.C.

Corbineau, F. and D. Côme, 1986. Experiments on the storage and seedlings of *Symphonia globulifera* L.f. (Guttiferae). Seed Sci. Technol. 14: 585-591.

Cromarty, A.S., R.H. Ellis and E.H. Roberts, 1982. The Design of Seed Storage Facilities for Genetic Conservation. Handbooks for Genebanks No.1. International Board for Plant Genetic Resources, Rome, Italy.

Ellis, R.E., T.D. Hong and E.H. Roberts. 1985a. Handbook of Seed Technology for Genebanks Vol. I: Principles and Methodology. Handbooks for Genebanks No.2. International Board for Plant Genetic Resources, Rome, Italy.

Ellis, R.E., T.D. Hong and E.H. Roberts. 1985b. Handbook of Seed Technology for Genebanks Vol. II: Compendium of Specific Germination Information and Test Recommendations. Handbooks for Genebanks No.3. International Board for Plant Genetic Resources, Rome, Italy.

Ellis, R.E., T.D. Hong and E.H. Roberts. 1988. A low-moisture-content limit to the logarithmic relation between seed moisture and longevity in twelve species. Ann. Bot. 63: 601-611.

Ellis, R.E., T.D. Hong and E.H. Roberts. 1990. An intermediate category of seed storage behaviour? I. coffee. Journal of Experimental Botany 41, 1167-74.

Ellis, R.E., T.D. Hong and E.H. Roberts. 1991. An intermediate category of seed storage behaviour? II. Effects of provenance, immaturity and imbibition on desiccation tolerance in coffee. Journal of Experimental Botany 42, 653-657.

Engelmann, F. 1997. *In vitro* conservation methods. In: Ford-Lloyd, B.V, Newbury, J.H. and Callow, J.A. (eds) Biotechnology and Plant Genetic Resources: Conservation and Use. CAB International, Wallingford, UK, pp. 119-162.

Engelmann, F. 2000. Technologies and strategies for *ex situ* conservation. Proceedings of SAT21 Conference: Science and Technologies for the 21st century. IPGRI, Rome.

Engels, J.M.M. and D. Wood. 1999. Conservation of agrobiodiversity. In Agrobiodiversity: Characterization, Utilization and management. D. Wood and J.M. Lenné (eds.). Pp. 355-386. CAB International, Wellingford.

FAO 1993. *Ex situ* storage of seeds, pollen and *in vitro* cultures of perennial woody plant species. FAO Forestry Paper 113. Rome, Italy.

FAO and IPGRI 1994. Genebank Standards. Food and Agriculture Organization of the United Nations,

Rome, International Plant Genetic Resources Institute, Rome, Italy.
FAO 1996. Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy.
Frankel, O.H. & M.E. Soulé. 1981. Conservation and evolution. Cambridge University Press, New York.
Hanson, J. 1985. Procedures for Handling Seeds in Genebanks. Practical manuals for Genebanks No1. International Board for Plant Genetic Resources, Rome, Italy.
Hay, F. and R. Probert. 2000. Keeping seeds alive. In press in Seed Technology and its Biological Basis, M. Black and J.D. Bewley, Sheffield Academic Press, UK.
Hoekstra, F.A. 1995. Collecting pollen for genetic resources conservation. In: Guarino L., Rao, V.R. and Reid, R. (eds) Collecting Plant Genetic Diversity: Technical Guidelines. IPGRI/FAO/UNEP/IUCN. CAB International, Wallingford, pp. 527-550.
Hong, T.D. and R.H. Ellis. 1996. A protocol to determine seed storage behaviour. In: Engels, J.M.M. and Toll., J. (eds.) IPGRI Technical Bulletin: No.1. IPGRI, Rome.
Hong, T.D., S. Linington and R.H. Ellis. 1996. Seed Storage Behaviour: a Compendium. Handbooks for Genebanks: No. 4. International Plant Genetic Resources Institute, Rome.
Hu, J., C.G. Guo and S.X. Shi. 1994. Partial drying and post-thaw conditioning improve the survival and germination of cryopreserved seeds of tea (*Camellia sinensis*). IPGRI/FAO Plant Genetic Resources Newsletter 98, 25-28.
IPGRI 1996. Programme Activities, Germplasm Maintenance and Use. Annual Report. IPGRI, Rome, pp. 56-65.
Nitsch, J.P. 1971. Perennation through seeds and other structures: Fruit development. In: Plant physiology, a treatise. Steward, F.C. ed. Academic Press.
Marshall, D.R. and A.H.D. Brown. 1975. Optimum sampling strategies in genetic conservation. Pp. 53-80 in: Frankel, O.H. and Hawkes, J.G., eds. Crop Genetic Resources for Today and Tomorrow. Cambridge University Press, Cambridge.
Marzalina, M. 1995. Penyimpanan biji benih mahogani (*Swietenia macrophylla* King). Storage of mahogany seeds (*Swietenia macrophylla* King). PhD thesis, University Kebangsaan Malaysia.
Marzalina, M., S.K. Yap and B. Krishnapillay. 1992. Effect of relative light intensities on *Dryobalanops* aromatica seedlings' growth. Pp. 439-440 in: Ho, H.W., Vidyadran, M.K., Norhani, A., Jainudeen, M.R. and Rani Bahaman (eds.). Proceedings of the National IRPA Seminar - Vol. 11.
Maunder, M. 1997. Conservation of the extinct toromiro tree. Curtis's Botanical Magazine 14: 226-231.
Maunder, M., A. Culham, A. Bordeu, J. Allainguillaumes and M. Wilkinsons. 1999. Genetic diversity and pedigree for *Sophora toromiro* (Leguminosae): a tree extinct in the wild. Molecular Ecology 8: 725-738.
Maxted, N., B.V. Ford-Lloyd and J.G. Hawkes. 1997. Complementary conservation strategies. In: Maxted, N., Ford-Lloyd, B.V. and Hawkes, J.G. (eds.). Plant Genetic Conservation: the *In situ* Approach. Chapman and Hall, London. Pp.15-39.
Pence, V.C. 1995. Cryopreservation of recalcitrant seeds. Pp. 29-52 in Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry Vol. 32 Cryopreservation of plant germplasm I, Springer Verlag, Berlin.
Raven, P.H. 1976. Ethics and attitudes. Pp. 155-179 in Simmons, J.B., Beyer, R.I., Brandham, P.E. Lucas, G.M. and Parry V.T.H. eds. Conservation of threatened plants, Plenum, New York.
Sacandé, M. 2000. Stress, Storage & Survival of Neem Seed. Drukkerij Liesbosch, Nieuwegein.
Schmidt, L. 2000. Guide to handling of tropical and subtropical forest seed. Danida Forest Seed Centre, Denmark.
Sedgley, M. and A.R. Griffin. 1989. Sexual reproduction of seed crops. Academic Press.
Terborgh, J. 1990. Seed and fruit dispersal - commentary. In: Bawa, K.S. and Hadley, M. Edts. Reproductive ecology of tropical forest plants. Man and Biosphere series vol. 7. UNESCO.
Thomsen, K. 2000. Handling of desiccation and temperature sensitive tree seeds. DFSC Series of Technical Notes TN 56. Danida Forest Seed Centre, Humlebaek, Denmark.
Tompsett, P.B. 1992. A review of literature on storage of dipterocarp seeds.
Towill, L.E. 1985. Low temperature and freeze-/vacuum-drying preservation of pollen. In: Kartha, K.K. (ed) Cryopreservation of Plant Cells and Organs. CRC Press, Boca Raton, pp. 171-198.
Vertucci, C. and E.E. Roos. 1993. Theoretical basis of protocols for seed storage. II. The influence of temperature on optimal seed levels. Seed Sci. Res. 3: 201-213.
Withers, L.A. 1995. Collecting *in vitro* for genetic resources conservation. In Guarino, L. and Rao, R. and Reid, R. (eds.) Collecting Plant Genetic Diversity. CAB International, Wallingford, Oxon, UK, pp. 511-515.
Withers, L.A. and F. Engelmann. 1998. *In vitro* conservation of plant genetic resources. Pp. 57-88 in Biotechnology in Agriculture, A. Altman ed., Marcel Dekker Inc., New York.