

## THE CONSERVATION AND UTILIZATION OF GENES FROM ENDANGERED AND EXTINCT PLANTS: DNA BANK-NET

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### SUMMARY

The recent advent of more efficient methods for DNA extraction and amplification (via PCR) now make it both prudent and urgent that we begin to amass significant genomic DNA samples from endangered plants with the greatest priority given to tropical rainforest species. DNA Bank-Net is an association of institutions that have begun to accession DNA and DNA-rich materials for subsequent disbursement of genes and oligonucleotides. The formation and functions of DNA BankNet are presented.

### INTRODUCTION

Since the first plant to plant gene transfer occurred in 1983 (Murai et al., 1983), genes have been transferred to plants from viruses (Nelson et al., 1988), bacteria (Barton et al., 1987; Della-Cioppa et al., 1987; Fischhoff et al., 1987), and even from mammals to plants (Lefebvre et al., 1987; Maiti et al., 1988). Genetic transfers are being performed in order to attain insect, bacteria, viral and fungal resistance, a more nutritionally balanced protein, more efficient photosynthesis, nitrogen fixation, and salt and heavy metal tolerance, to name a few. These kinds of gene transfers from one unrelated organism to another indicate that we must now view the world's genetic resources (genes, DNA) from a horizontal perspective in which gene transfers will cut across species, genera and family boundaries.

For example, a strain of cowpea, *Vigna unguiculata* (L.) Walp., discovered in a market in Ilorin, Nigeria, contains a protein that inhibits trypsin digestion by insects (Redden et al., 1984). This gene has been moved to tobacco (*Nicotiana*) where the trypsin-inhibiting gene is expressed and offers tobacco the same resistance against insects as in cowpea (Newmark, 1987). It is interesting to note that although a very active form of the gene has been found in a Nigerian cowpea, scarcely 100 of the world's 13,000 legume species

have been examined for this gene. Yet, the tropical legumes, one of the most promising groups for the evolution of natural insecticides, will certainly be subject to considerable germplasm loss in the next decade.

The number of novel insecticides, biocides, medicines, etc. that could exist in nature is innumerable. Yet, the principal areas of diversity among plants, the lowland tropical forests, will have been cut or severely damaged within the next 20 years (Raven, 1987, 1988). The Amazon River system, for example, contains eight times as many species as the Mississippi River system (Shulman, 1986). Raven (1987, 1988) estimated that as many as 1.2 million species would become extinct in the next twenty years. The loss of plant species will mean a loss of potential plant derived pharmaceuticals, now estimated at \$2 billion/year in the United States alone (U. S. Congress, 1987).

It also means a loss of genetic diversity present in and available to our current and potential crop species. Cultivated crops are extremely inbred for factors such as yield, uniform flowering and height, and cosmetic features of the products. This narrow genetic base has resulted in several disastrous crop failures. Ireland's potato (*Solanum tuberosum* L.) famine of 1846, which resulted in famine and the emigration of a quarter of its population, was due to the fact (Plucknett et al., 1987) that their potatoes had no resistance to the late blight fungus (*Phytophthora infestans*). This can be traced to the lack of genetic diversity in Irish potatoes, which had been multiplied using clonal materials from just two separate South American introductions to Spain in 1570 and to England in 1590 (Hawkes, 1979).

A more recent example is the southern corn leaf blight fungus (*Helminthosporium maydis*) in 1970 in the United States. Because almost all of the corn (*Zea mays* L.) in the United States was of hybrid origin and contained the Texas cytoplasmic male sterile line, our fields of corn presented an unlimited extremely narrow gene base habitat for the fungus. By the late summer, 1970, plant breeders were scouring corn germplasm collections in Argentina, Hungary, Yugoslavia and the United States for resistant sources (Plucknett et al., 1987). Nurseries and seed fields were used in Hawaii, Florida, the Caribbean, and Central and South America to incorporate the resistance into hybrid corn in time for planting in the spring of 1971 (Ullstrup, 1972). Without these genetic resources this technological feat would not have been possible.

The National Cancer Institute (NCI) is now spending \$8 million over the next 5 years for a massive plant collecting effort in the tropics to find anti-cancer and anti-AIDS virus compounds (Booth, 1987). The plant collectors will gather leaves and/or bark and air-dry the material for shipment to Maryland where it will be extracted and assayed against 100 cancer cell lines and the

AIDS virus. Yet, no genetic resources will be collected! When a promising compound is found, the plants will have to be recollected. For extensive testing (as well as commercial utilization), plantations will have to be established in the tropics to provide material.

Collections of plant specimens have been utilized for the formulation of our understanding of morphological variation among taxa. Indeed, without the great herbaria of the world, our knowledge of plant evolution would be fragmented at the least. As we have moved into the era of utilizing chemical data for systematic and evolutionary studies, methods of preserving plant materials for future (chemical) work have been largely ignored. We are usually content to file a voucher specimen to document our chemical studies. With the present level of support for plant collections, it is unlikely that much of the world's plant species can be preserved by freezing so that scientists might have access to the study of secondary compounds, enzymes, or DNA/RNA in the coming centuries.

#### FORMATION OF DNA BANK-NET

Concurrent with the advancements in gene cloning and transfer, has been the development of technology for the removal and analyses of DNA. DNAs from the nucleus, mitochondrion, and chloroplast are now routinely extracted and immobilized onto nitro-cellulose sheets where the DNA can be probed with numerous cloned genes. Recent advances in the technology for the extraction and immobilization of DNA, coupled with the prospect of the loss of significant plant genetic resources throughout the world, has led to the establishment of DNA Bank-Net, an international network of DNA repositories for the storage of genomic DNA on every continent.

A group of 18 scientists held the organizational meeting at the Royal Botanic Gardens, Kew, London, April, 1991 to share country and institutional experiences using *in vitro* biotechnology and particularly cryostorage of DNA and DNA-rich materials (Adams and Adams, 1991). Relatively few scientists were interested in a 'genetic insurance policy' when the idea of banking genomic DNA from plants was first proposed (Adams, 1988, 1990). However, currently there are 40 institutions (representing 25 nations and every continent, see Appendix I) that have expressed interest in DNA Bank-Net (Figure 1).

The conserved DNA will have numerous uses: molecular phylogenetics and systematics of extant and extinct taxa; production of previously characterized secondary compounds in trans-genic cell cultures; production of trans-genic plants using genes from gene families; *In vitro* expression and study of enzyme structure and function; and genomic probes for research laboratories.

## STRUCTURE AND OPERATION OF DNA BANK-NET

At the organizational meeting of DNA Bank-Net, a task force was convened to define the functions of working (DNA dispensing) and reserve (base) nodes in the DNA Bank network. The group recommended the following functions (Adams and Adams, 1991):

### Working (DNA dispensing) nodes:

- a. Collection of plant material by taxonomists. This may be the primary function of a particular node or be in association with other organizations such as universities, botanic gardens, etc.
- b. DNA extraction by molecular biologists or trained staff.
- c. Long-term preservation of DNA-rich materials and/or extracted DNA in liquid nitrogen.
- d. DNA analysis/gene replication by molecular biologists or trained staff.
- e. Distribution of DNA (genes, gene segments, oligonucleotides, etc.).

### Reserve (base) nodes:

- a. Long term DNA preservation in liquid nitrogen and monitoring of potential DNA degradation.
- b. Act as genetic reserve buffer for working nodes.
- c. Replenishment of DNA if a working node experiences the catastrophic loss of storage parameters and DNA.



Fig. 1. Map of individuals/institutions currently interested in DNA Bank-Net.

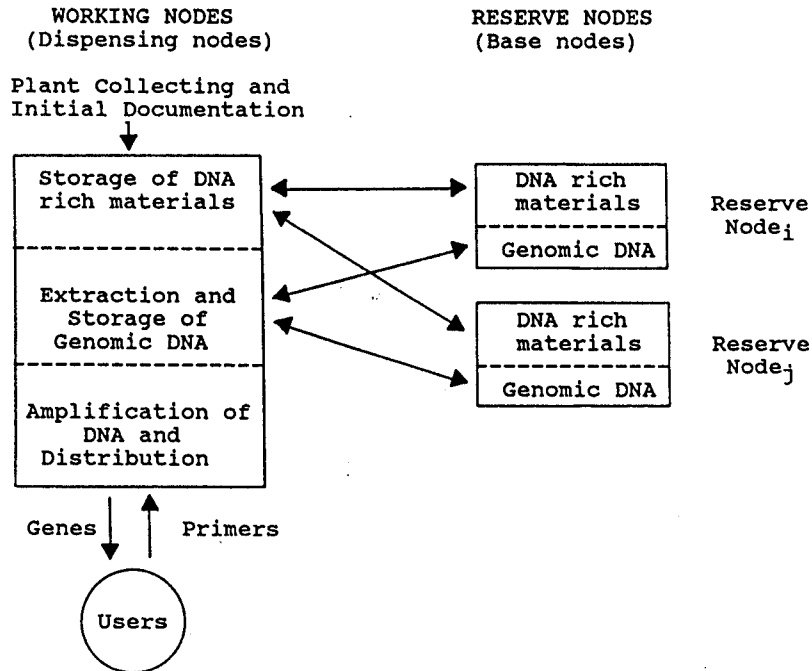


Fig. 2. Schematic representation of the flow of materials and the relationship between working (DNA dispensing), reserve (base) nodes and users.

Figure 2 depicts the relationship between working and reserve nodes. Note the projected flow of plant materials and DNA through the working (DNA dispensing) node. It is likely that some of the working nodes would be actively acquiring and/or dispensing DNA from some geographic area (ex. Africa), yet maintain separate cryovats, functioning as a reserve (base) node for another area (ex. South America).

#### GENERAL REQUIREMENTS FOR NODES IN THE DNA BANK-NET

The task group recommended (Adams and Adams, 1991) that the following were the minimum requirements for nodes:

##### Working (DNA dispensing) nodes:

Personnel: Taxonomists/collectors, biochemists/molecular biologists, technicians for practical work, capable administration

Equipment: Storage facilities (liquid nitrogen, cryovats), extraction facilities (centrifuges, gel electrophoresis, UV spectrophotometer, etc.), DNA Analyses and PCR duplication (PCR thermal cycler, micro-centrifuges, etc.), distribution system (packaging and mailing supplies), computer (database for inventory and correspondence).

**Reserve (base) nodes:**

Personnel: Technicians, capable administration.

Equipment: Storage facilities (liquid nitrogen, cryovats), computer (data base for inventory and correspondence).

Each DNA collection should be split initially into at least 2 or 3 portions. One sample (DNA-rich material or extracted DNA) should be stored at a working (DNA dispensing) node and another portion(s) be stored in at least 1 (one), but desirably 2 (two) back-up reserve (base) nodes. The reserve nodes should be in different countries and if possible on different continents to safeguard the DNA samples against various natural and man-made catastrophes. Figures 3 and 4 show two methods that may be used to distribute DNA-rich materials to working and reserve nodes. In the first example (Fig. 3), materials might be collected in triplicate by a CENARGEN expedition and one of the replicates mailed to the Vavilov Institute (where it would be cryostored as a reserve node), another replicate might be mailed to the Gene Library (where it would be cryostored as the second reserve). CENARGEN would then take the third replicate home to the working node. This method would aid in assuring that the samples actually get sent to the reserve nodes, but it would be critical that considerable identification and label making be done in the field. A second method, shown in figure 4, would be to take all 3 replicates back to CENARGEN and then send the replicates to the Vavilov Institute and the Gene

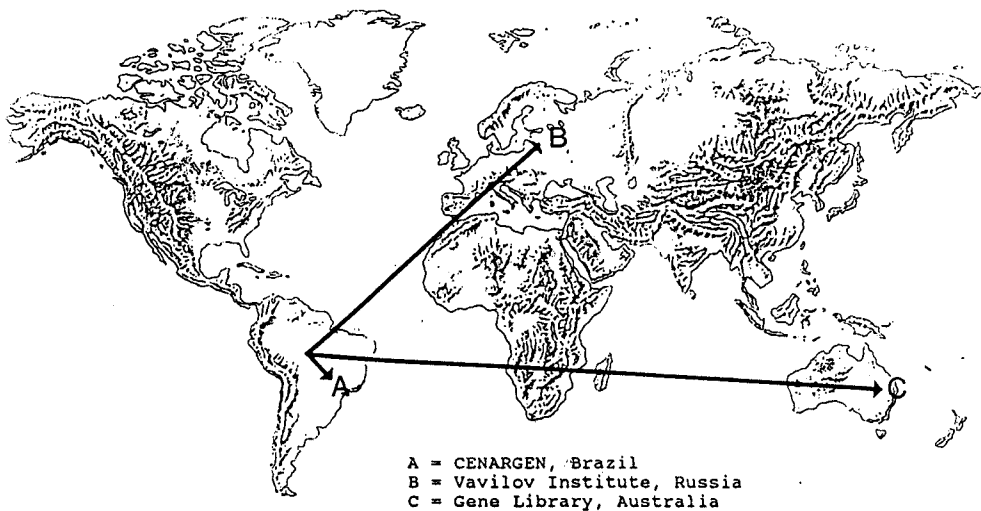


Fig. 3. Hypothetical example of a triplicate collection by CENARGEN in Brazil. The DNA-rich materials are placed in silica gel or drierite for interim preservation and then two replicates sent to reserve nodes at (B) Vavilov Institute and (C) the Gene Library. The third replicate would be taken to the working node (for this accession) at CENARGEN (A).

Library. The plant materials (in silica gel) could be stored in a freezer until the identification and field notes have been accomplished and then shipped in quantity with other samples in off-season periods. No doubt other strategies will be developed with experience. Several general recommendations came from the task groups (Adams and Adams, 1991) and these include:

- a. DNA should be extracted from cryo-preserved DNA-rich materials only when the DNA is needed. Delaying the extraction has the advantage of letting technology catch up, so advanced techniques can be used as they become available.
- b. Working nodes should generally be an existing organization with adequate biochemical expertise and have an associated herbarium. Having an herbarium on site would not be required but a very close, local (in the city) association with a recognized herbarium (Holmgren et al., 1990) is required.
- c. For the working as well as reserve nodes, it is necessary to have a strong institutional commitment, not just a personal commitment, in order that the collection be maintained in perpetuity not just for the lifetime of one person committed to the idea.
- d. Consideration should be made concerning the availability of dependable electricity and liquid nitrogen in determining the feasibility of establishing a node.

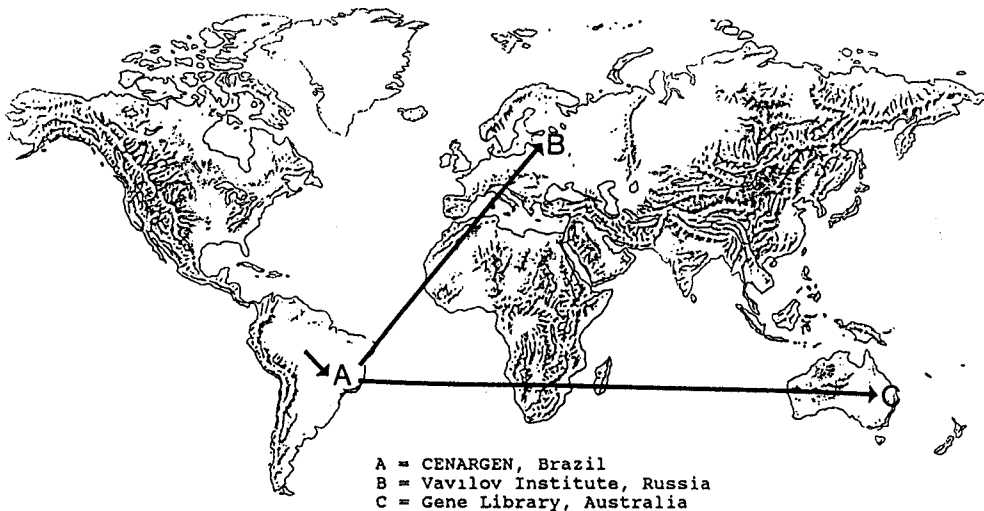


Fig. 4. Hypothetical example of a triplicate collection by CENARGEN in Brazil. The DNA-rich materials are placed in silica gel or drierite for interim preservation and taken to the working node (A, CENARGEN). From CENARGEN (A), two replicates would be sent to reserve nodes at (B) Vavilov Institute and (C) the Gene Library. This procedure would minimize the amount of paperwork needed in the field.

- e. Considerable interest was shown in the concept of storing composite DNA samples (e.g., a composite of DNA from all the legumes in a region, to be used for screening or retrieval of unusual genes).
- f. The need for computer and data base compatibility was expressed. Given the number of flat file and relational data bases that are compatible with dBASE, it would seem that dBASE compatibility would be desirable. No consensus was reached in regards to this nor on the use of a flat file vs. relational data base. It was felt that the critical issue at present was to begin collecting DNA-rich materials.

### SCOPE OF PLANT COLLECTIONS

The task group given this assignment felt that there is a need for an initial focus rather than random collections and that economically useful plants should be given some priority (Adams and Adams, 1991). However, this priority would not include the major crop plants of commercial usage that are widely cultivated (e.g. maize, rice, wheat, etc.), but rather those indigenous species that are tended and/or otherwise used by local people.

One problem with giving a priority to species is that field collecting then becomes 'plant hunting' trips, which tend to be very expensive. It would seem that the cheapest and most practical way to preserve the largest percentage of plant genes would be to utilize the current (and additional) floristic collectors (such as those of the Missouri Botanical Garden, Royal Botanic Gardens, etc.), who are already in the field and familiar with the vegetation in the region. The collections of DNA-rich material (leaves) could be done with little additional effort when specimens are collected.

### DNA COLLECTING PROCEDURES

DNA collectors should be considered the same as all other plant collectors. Consequently they should (Adams and Adams, 1991):

- a. Voucher all collections in recognized herbaria (i.e., listed in Index Herbariorum, ed. 8)
- b. Provide proper label information as to the locality, habitat, etc. for each plant collected.
- c. Follow all procedures concerning permits, convenios, and deposition of duplicate vouchers in the country of origin.
- d. Collect leaf samples and pack them in desiccants (see Adams et al., 1991) immediately (the same day). Leaves are of value as simple long-term storage.
- e. In the case of legumes, samples of root nodules should be taken if possible, but kept as a separate accession.



- f. If a chemical treatment is used in the field, information should be provided concerning the method and some untreated leaves must be stored in desiccant (see d. above).
- g. Fossil plants - When possible, fossil material should be included in DNA Bank-Net. In this case, when destruction of the source material occurs, documentation via photographs and fragments is necessary.
- h. Some material may be accessioned from herbarium specimens under control of local curators using current methods of DNA extraction. Herbarium sheets should be marked if sampled for DNA. Herbarium specimens are limited in supply and their utility appears to be limited to material collected without chemical preservation. Material may be sampled directly from the sheet or the attached specimen envelope if it contains sufficient leaf material (ca. 0.1 - 0.5 g dry wt.) for DNA extraction.

#### INTERIM FIELD STORAGE OF DNA SPECIMENS

The problems associated with bringing back fresh or frozen materials can generally be overcome by specialists (ex. world-wide collections of fresh foliage of *Juniperus* for essential oil analyses and DNA by RPA). However, botanists doing floristic research will likely collect many of the specimens from tropical rare and endangered species. They often collect specimens from scores of different species in a single day. The bulk of the materials that they have to process and ship requires that any protocol for the collection of samples for specialized needs (ex. DNA storage / analyses), must be quick, simple and trouble-free. The generalist collector, working in tropical areas cannot be expected to preserve hundreds or thousands of collections for months under tropical conditions and then arrange transport through customs, all the while keeping the individual specimens frozen.

Fortunately, at least as far as DNA preservation is concerned, interim preservation in silica gel or drierite is an effective way to keep plant materials in the field and/or in transit for several months at ambient temperatures (Adams et al., 1991). Figure 5 shows genomic DNA from fresh spinach stored for up to 6 months still contains high molecular weight DNA (see Adams et al., 1991 for detailed discussion).

#### Protocol for field preservation of foliage

Drierite has a water capacity of 10 to 14 percent, but above %, the capacity varies inversely with temperature (W. A. Hammond Drierite Co.). One would not want to risk possible rehydration of leaves, so storage ratios should be based on the 6.6% capacity. In lab tests, silica gel absorbed 8.85% of its

weight of water after exposure to 100% humidity for 16 h at 22°C. We have found that plant materials contain as much as 92% moisture, so a useful approximation would be to assume the plant is mostly water and use 16 to 20 times the fresh leaf weight for the drierite or silica gel component.

Now that inexpensive (\$100 USD) battery powered, portable balances are available, one could take a supply of jars that hold (for example) 100 g of silica gel and then weigh out 5 g of fresh leaf material and add it to the jar along with silica gel (or drierite). We have found that air dried leaves (suitable for herbarium vouchers) generally contain from 10 to 15% water. Using a robust value of 20% water for air dried leaves, one can weigh out 5 g of air dried leaves (5 g x 20% = 1 g water) or 1 g fresh leaves per 20 g of silica gel. This procedure may seem time consuming, but in practice, we merely do a quick

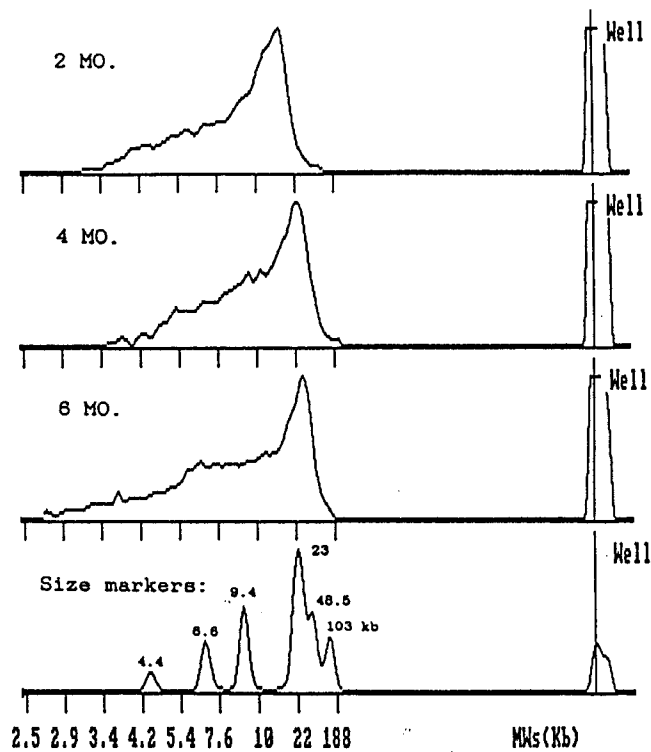


Fig. 5. Video densitometer scans of FIGE of genomic DNA from fresh spinach leaves stored in silica gel at 37°C for 2, 4 and 6 months (from Adams et al., 1991). Notice the gradual increase in lower molecular weight DNA (the peak tailing to the left). Size markers are: lambda DNA cut with HindIII (2.3, 2.0 and 0.5 kbp bands not shown); Lambda DNA (48.5 kbp) and T5 DNA (103 kbp).

check on the leaf area needed to give approximately 1 g (fresh leaves) or 5 g (dried leaves) and then just use that amount of leaf area. For example, for spinach, a 2 cm x 4 cm fresh leaf area weighs about 1 g. So, one can just cut the leaves into roughly 2 cm x 4 cm squares and add one square to 20 g of silica gel. For succulent leaves, a slightly different protocol may be used. Liston et al. (1990) removed succulent leaf material after 24 hours in drierite and placed it in fresh drierite. A note of caution is necessary concerning field drying of specimens for subsequent silica gel/drierite storage. We have experienced difficulty obtaining DNA from leaves dried at temperatures higher than about 55°C. In very rainy conditions where high drying temperatures (from butane stoves, for example) are used to dry specimens, it would seem advisable to merely blot leaves free of surface moisture and then place the fresh leaf material directly into silica gel or drierite. Liston et al., (1990) took 2-5 g of plant tissue and wrapped it in tissue paper to prevent it from fragmenting, then placed it in a 125 ml Nalgene bottle, 1/3 prefilled with drierite (with blue indicator crystals), and then filled the bottle (2/3) with additional drierite.

Plastic bottles are probably to be preferred to glass, to avoid breakage in transit. Using clear jars allows one to check the indicating crystals without opening the jar. The lids should be sealed with vinyl tape to insure against moisture leakage. The use of parafilm to seal containers is not recommended, as we have found it to come loose at 37°C (and of course, at tropical temperatures!).

Silica gel and drierite do differ in one characteristic that may be a consideration. We have found that silica gel can be dried (recharged) at 100°C for 24 h but drierite must be dried at much a higher temperature (200°C). In addition, we could easily dry silica gel in a microwave oven, but were unable to dry (recharge) drierite in a microwave oven. If the desiccant gets wet before use, silica gel appears to be much easier to dry. Silica gel is used in large quantities for flower drying and, thus, may be cheaper, depending on the source. Both drierite and silica gel could be recharged for reuse on subsequent trips, but one should be very careful to remove any leaf fragments. If the materials are to be checked through customs, it is useful to have a small container of silica gel/ drierite that you can open and show the customs agents. A demonstration that the blue indicator crystals will turn pink when you breath on or moisten them is helpful in convincing the customs officials to not open your sealed specimen jars.

#### FUTURE RESEARCH

The vast resources of dried herbarium specimens may hold considerable DNA that would be suitable for PCR. It seems likely that the integrity of DNA

would decrease with the age of specimens. Because there are many types of herbaria storage, preservation and collections, there is a need for systematic investigations of the effect of modes of preparation, collection and storage on the integrity of DNA in the world's major holdings.

One of the major concerns in storing DNA from extinct species is the limited amount of DNA available for distribution. A general process by which the DNA could be immobilized and then specific genes or oligonucleotides amplified is needed. Figure 6 depicts the immobilization of genomic DNA onto nylon as described by Kadokami and Lewis (1990) for cDNA from spiders (*Nephila clavipes*). Amplification would then involve removing the membrane with the bound DNA from cryo-storage (Fig. 7) and amplifying the desired gene, washing away the primers and placing the bound DNA back into cryostorage. Although Kadokami and Lewis (1990) reported successful PCR amplification of membrane bound cDNA, we have not been able to extend their work to genomic plant DNA. Additional research is needed in this area.

Research is needed to amplify the entire genome DNA of a species. Some modification of the GAWTS (Genomic Amplification with Transcript Sequencing, Sommer et al., 1990) type protocol (Fig. 8) needs to be developed for eventual supplementation of DNA reserve stocks and obviate the need for replenishment from outside sources.

#### Concluding remarks

DNA Bank-Net is an association that fills a needs for a professional organization that would function initially as a lead organization and superstructure. The association brings together capabilities and expertise, produces a newsletter, and coordinates DNA banking activities.

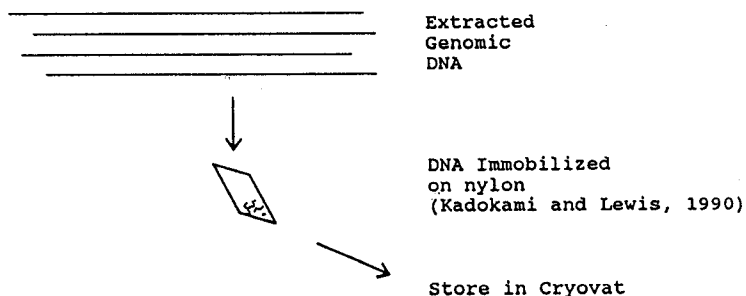


Fig. 6. Scheme for the immobilization of genomic DNA (after Kadokami and Lewis, 1990 and Adams and Adams, 1991).

Technical workshops need to be conducted in order to bring researchers together and develop specific techniques and protocols for DNA extraction, amplification and storage. DNA Bank-Net supports the concept of using license agreements and/or contracts that insure that there is a flow of money back to the countries and/or institutions when commercialization of protected germplasm is achieved. This will guarantee the uninhibited exchange of germplasm for scientific purposes, so that scientific achievements are not obstructed.

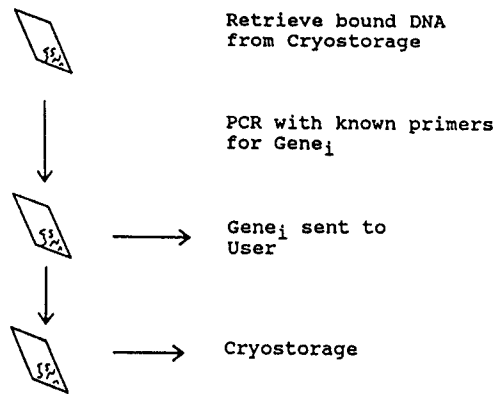


Fig. 7. Conceptual procedure for the amplification of immobilized genomic DNA (after Kadokami and Lewis, 1990 and Adams and Adams, 1991).

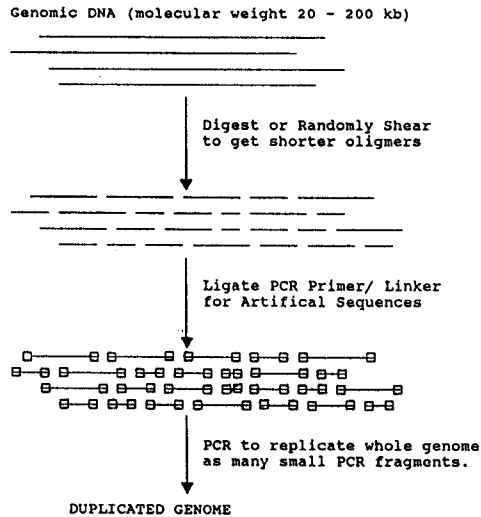


Fig. 8. Possible amplification of genomic DNA by use of a modified genomic amplification with transcript sequencing (GAWTS) method of Somer et al. (1990).

DNA Bank-Net should complement activities already being performed by different institutions, specifically, those working in the area of germplasm collection and conservation.

Technically, all the necessary expertise is available to begin the collection and storage of DNA from endangered species. Now, the vision is needed.

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**Appendix I. Individuals/institutions that have expressed an interest in DNA Bank-Net.**

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