Bioversity International

Conserving coffee genetic resources

F. Engelmann, M.E. Dulloo, C. Astorga, S. Dussert and F. Anthony (editors)

CATIE
Tropical Agricultural Research and Higher Education Center

IRD
Institut de recherche pour le développement

Conserving coffee genetic resources

Complementary strategies for *ex situ* conservation of coffee (*Coffea arabica* L.) genetic resources. A case study in CATIE, Costa Rica

F. Engelmann, M.E. Dulloo, C. Astorga, S. Dussert and F. Anthony (editors)

Bioversity International is an independent international scientific organization that seeks to improve the well-being of present and future generations of people by enhancing conservation and the deployment of agricultural biodiversity on farms and in forests. It is one of 15 centres supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment. Bioversity has its headquarters in Maccarese, near Rome, Italy, with offices in more than 20 other countries worldwide. The Institute operates through four programmes: Diversity for Livelihoods, Understanding and Managing Biodiversity, Global Partnerships, and Commodities for Livelihoods.

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Foreword

It is now well recognized that an appropriate conservation strategy for a particular plant genepool requires a holistic approach, combining in a complementary manner the different *ex situ* and *in situ* conservation techniques available. Selection of the appropriate methods should be based on a range of criteria, including the biological nature of the species in question and the practicality and feasibility of the particular method chosen, as well as the cost-effectiveness and security afforded by its application. Considerations of complementarity with respect to the efficiency and cost-effectiveness of the various conservation methods chosen are also important. Complementarity is a flexible concept, which evolves with the availability of techniques aiming at conserving, propagating and characterizing the genetic resources in question. Research on the development of complementary conservation strategies gained momentum at the beginning of the 1990s, and is coordinated by Bioversity International (Bioversity)[1].

This research area is of particular relevance for species with seeds displaying non-orthodox storage behaviour, whose traditional *ex situ* storage method is the field genebank. In some ways, this method offers a satisfactory approach to conservation. The genetic resources under conservation can be readily accessed and observed, thus permitting detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security. The genetic resources are exposed to pests, diseases and other natural hazards, such as drought, weather damage, human error and vandalism. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance, and even their very survival in times of economic stringency. Even under the best circumstances, field genebanks require considerable inputs in the form of land, labour, management and materials, and, in addition, their capacity to ensure the maintenance of much diversity is limited.

For many years, non-orthodox seed research has been recognized by Bioversity as an area of critical importance for the conservation of plant genetic resources, and numerous projects on this topic have been or are being implemented in collaboration with research institutions and genebanks worldwide. Many of these projects have focused on the development of cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196°C), as cryopreservation is the only method currently available to ensure the safe and cost-effective conservation of germplasm of non-orthodox-seed species.

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[1] Formerly the International Plant Genetic Resources Institute (IPGRI).

Recognizing the tremendous potential interest of the results achieved at IRD with cryopreservation of coffee seeds, Bioversity decided to support a research programme aiming at transferring and testing on a large scale in a genebank located in a developing country the freezing protocol developed in France. This was performed in 1998–2000 in the framework of two successive projects with IRD and CATIE, Costa Rica. CATIE was an ideal partner as it fulfilled the set of criteria required for participation in such a project. Indeed, CATIE holds one of the largest field collections of coffee worldwide, mainly of *Coffea arabica*. CATIE's fully equipped biotechnology laboratory includes all the facilities required for cryopreservation and molecular biology research, as well as highly skilled scientific and technical staff. Moreover, Bioversity and CATIE have a long and successful collaboration history in various areas, including cryopreservation of tropical plant germplasm. At the time of the initiation of this programme, IRD staff were also working in CATIE on a collaborative research project on the characterization and rationalization of the CATIE coffee germplasm collection using molecular tools. We were thus in an ideal situation to study how new technologies (molecular biology and cryopreservation) could be efficiently employed to complement more classical ones to characterize and rationalize an *ex situ* germplasm collection, and to improve its conservation status.

To our knowledge, the work described in this publication represents the first example of the application of these techniques in a genebank located in a developing country, in the framework of the development of an *ex situ* complementary conservation strategy for *C. arabica*, i.e. a crop of commercial importance at the global level. We hope that this publication will help in stimulating research on complementary conservation strategies for other problem crops, as well as on the biotechnological tools required to implement them.

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Partners in this publication

Institut de recherche pour le développement (IRD)

Originally founded in 1944, IRD (the former ORSTOM) is a public science and technology research institute, reporting to the French ministries in charge of research and development cooperation. Working throughout the tropics, IRD conducts its research in close cooperation with its numerous partner countries with a view to assisting the economic, social and cultural development of developing countries. IRD fulfils three main missions: research, training, and consultancy; it also collaborates in the dissemination of scientific and technical information in developing countries.

IRD has research and service units, joint research units with universities and other research bodies, and it collaborates with observatories and technology-specific support networks. The scientific activities of the Institute are interdisciplinary and cover six priority topics, namely environmental hazards; global warming; desertification; water resources; management of marine and continental ecosystems; nutrition, health, emerging diseases, education, migration and poverty reduction policies. Working in Africa, Asia, Indian Ocean nations, Latin America and the Pacific area, IRD operates in 35 countries and in five French overseas territories. IRD participates in major world research programmes conducted in the South through its network of representatives and numerous researchers in the tropical area.

Centro Agronómico Tropical de Investigación y Enseñanza (CATIE)

The Tropical Agricultural Research and Higher Education Center, CATIE, is a regional centre dedicated to research and graduate education in agriculture, and to the management, conservation and sustainable use of natural resources.

Its Regular Members include the Inter-American Institute for Cooperation on Agriculture (IICA); Belize; Bolivia; Colombia; Costa Rica; the Dominican Republic; El Salvador; Guatemala; Honduras; Mexico; Nicaragua; Panama; Paraguay; and Venezuela. CATIE's core budget is strengthened by generous annual contributions from these members.

CATIE's thematic lines of emphasis include agro-ecology, agroforestry systems, organic agriculture, integrated pest management (IPM) and biological control, genetic resources conservation, and plant breeding. Emphasis is also given to natural resource management, including forestry, biodiversity and protected areas, and to socio-economic issues.

CATIE's strategies include networking at the regional and international level for generation, adaptation and validation of new technologies, dissemination of information, and execution of development projects in the field.

The centre works with strategic research, development and education partners from approximately 100 institutions to conduct its activities throughout Latin America.

Bioversity International

Bioversity International is an independent international scientific organization that seeks to improve the well-being of present and future generations of people by enhancing conservation and the deployment of agricultural biodiversity on farms and in forests. It is one of 15 centres supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment. Bioversity has its headquarters in Maccarese, near Rome, Italy, with offices in more than 20 other countries worldwide. The Institute operates through four programmes: Diversity for Livelihoods; Understanding and Managing Biodiversity; Global Partnerships; and Commodities for Livelihoods.

I. Introduction

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Coffee is one of the most important beverages in the world and is consumed by more than a third of the world's population. It is also a very important commodity crop for many developing countries, once contributing over US$ 10–11 billion annually (Bolvenkel et al. 1993) and providing a source of income for thousands of small-scale farmers, as well as being a significant source of employment. However, during the crisis years that began in 2000 and continued to the end of 2004, earnings slumped to just over US$ 5.5 billion annually, while the value of retail sales in industrialized countries continued to remain healthy and to increase steadily, increasing to exceed US$ 70 billion (Osorio 2005).

The commercial coffee comes from two main species, *Coffea arabica* L. and *C. canephora* Pierre ex Froehner, and many varieties of coffee have been developed in response to widespread prevalence of pests and diseases, such as Coffee berry borer, Coffee berry disease, Coffee leaf rust, and, more recently, *Fusarium* wilt and others, all of which undermine coffee production and quality. It is recognized that the cultivated varieties, in particular *C. arabica*, have a very narrow genetic base (Anthony et al. 2002) and their improvement depends on the availability of adequate amounts of genetic diversity. The genus *Coffea* is endemic to the Old World tropics of Africa, particularly Madagascar, and over 100 wild species are found in the Afrotropical-Madagascar region, including the Comoros and the Mascarene Islands (Chevalier 1947; Bridson and Verdcourt 1988; Stoffelen 1998). This region, together with farmers' fields growing old and traditional coffee varieties, represents the ultimate source of coffee genetic diversity, on which the future of coffee improvement depends. However, deforestation and encroachment by agricultural activities, population pressures and economic hardships threaten all these reservoirs of genetic diversity, and with these threats comes the danger of significant erosion of the *Coffea* genepool. Chapter 2 of this publication provides a detailed account of the coffee genetic resources and the threats they are facing. The conservation of coffee genetic resources has not received much attention recently, but efforts to collect and conserve coffee genetic resources were initiated in the 1960s and 1970s by ORSTOM (now IRD), FAO and IBPGR (now Bioversity), and several options for their conservations have been developed.

Conservation options

Two basic conservation strategies, each comprising various techniques, are employed to conserve genetic diversity, namely *in situ* and *ex situ* conservation (Engelmann and Engels 2002). Article 2 of the Convention on Biological Diversity provides the following definitions for these categories (UNCED 1992):

- *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitat.

- *In situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.

There is an obvious fundamental difference between these two strategies: *ex situ* conservation involves the sampling, transfer and storage of target taxa from the collecting area, whereas *in situ* conservation involves the designation, management and monitoring of target taxa where they are encountered (Maxted et al. 1997). Another difference lies with the more dynamic nature of *in situ* conservation compared with the more static *ex situ* conservation. These two basic conservation strategies are further subdivided into specific techniques, including seed storage, *in vitro* storage, DNA storage, pollen storage, field genebank and botanic garden conservation for *ex situ*, and protected area, on-farm and home garden conservation for *in situ*, each technique presenting its own advantages and limitations (Engels and Wood 1999). *Ex situ* conservation techniques are in particular appropriate for the conservation of crops and their wild relatives, while *in situ* conservation is especially appropriate for wild species and for landrace material on-farm.

In situ conservation

In situ conservation offers the possibility of conserving a greater diversity of species and genepools at the same time. It is a dynamic conservation process, as plants continue to evolve with changes in their environment, most importantly pests and diseases (Maxted et al. 1997; Hodgkin and Ramanatha Rao 2002). It is suitable for crop evolution and genetic studies, and represents a viable alternative for conservation of non-orthodox-seed species. However, *in situ* conservation leaves the plant material vulnerable to natural and human-induced disasters, and the plant material is not readily accessible for use. The appropriate management regimes are poorly understood and high levels of supervision and monitoring are required to implement *in situ* conservation. Finally, the amount of genetic diversity that can be conserved in any one reserve is not easily measurable.

On-farm conservation

On-farm conservation is also a dynamic process in which plants continue to evolve (Jarvis et al. 2000; Watson and Eyzaguirre 2002). It ensures the conservation of valuable genetic diversity in traditional landraces, weedy crops and ancestral forms. The material is easily accessible for use by farmers and local communities. However, it is vulnerable to changes in management practices and the appropriate management regimes are poorly understood. On-farm conservation requires the maintenance of traditional cultivation systems. The amount of genetic diversity that can be conserved on farm remains to be evaluated.

Conservation in field genebanks

Conservation in field genebanks is suitable for species with non-orthodox storage behaviour. In some ways, this method offers a satisfactory approach to conservation (Engelmann and Engels 2002). The genetic resources under conservation can be readily accessed and observed, thus permitting detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security (Engelmann 1997a; Withers and Engels 1990). The plants are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism. In addition, they are not in a condition that is readily conducive to germplasm exchange because of the great risks of disease transfer through the exchange of vegetative material. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very

survival in times of economic stringency (Dulloo et al. 2001). Even under the best circumstances, field genebanks require considerable inputs in the form of land (often needing multiple sites to allow for rotation), labour, management and materials, and, in addition, their capacity to ensure the maintenance of much diversity is limited (Engelmann and Engels 2002).

In vitro conservation

In vitro conservation methods represent a relatively easy alternative for medium- to long-term conservation of a large number of non-orthodox, sterile or clonal species (Withers and Engelmann 1998). Cryopreservation (in liquid nitrogen at -196°C) provides long-term safety of the stored material, with limited maintenance and monitoring once the material is in storage. Germplasm exchange is facilitated by *in vitro* methods as they permit the production of virus-free material through meristem culture and their rapid multiplication (Engelmann 1997b). However, there are risks of somaclonal variation in some species when maintained under *in vitro* slow growth. *In vitro* storage is relatively high-tech and maintenance costs of the material are high. Individual slow growth and cryopreservation protocols need to be developed or adapted for most species. Difficulties are encountered in storing non-orthodox-seed species, and only a limited number of accessions can be conserved, especially when using slow growth storage.

Pollen storage

Pollen conservation is another viable alternative for conserving species with non-orthodox seeds. Pollen storage is a relatively easy and low-cost procedure (Towill and Walters 2000). Using pollen facilitates germplasm exchange, as a relatively small quantity of material is required for a single sample. Most importantly, pollen is generally less likely to be infected by pathogens than other propagules. The disadvantages of pollen conservation are that only paternal material is conserved, i.e. less than half of the total genetic make-up of an organism; individual regeneration protocols need to be developed to produce haploid plants; and further research is needed to produce diploid plants.

DNA storage

DNA storage in DNA libraries is yet another alternative for conserving species with non-orthodox seeds (Adams and Adams 1992). It is also a relatively easy and low-cost procedure. It is particularly useful for conserving specific genes responsible for heritable characteristics of particular value (e.g. disease resistance). DNA is easily accessible and is especially convenient for exchange among plant breeders. However, procedures for regenerating entire plants from conserved DNA are not available at present, and numerous problems exist with gene isolation, cloning and transfer.

Seed storage

Seeds are regarded as the most convenient material for *ex situ* conservation, and they make secure medium- to long-term conservation feasible (FAO 1996). Seed storage is both efficient and reproducible, allowing the conservation of a wide range of genetic diversity. Seeds are also a convenient form for germplasm use and exchange. Moreover, they require only limited maintenance and monitoring once the material is placed in storage. However, seed storage does not allow for the conservation of useful genotypes. There are risks of losing genetic diversity with each regeneration cycle and it is a static conservation process, as it 'freezes' the evolutionary development of useful characteristics, especially related to resistance to pests and diseases. Most importantly, the usability of seed for long-term storage depends on its storage behaviour.

A large number of plant species have seeds that are termed 'orthodox', meaning that the seeds are desiccation tolerant and can be dehydrated down to low water contents, and that they are also

cold tolerant and can be stored at low temperature for extended periods (Roberts 1973). There are three main categories of plant species for which seed conservation presents a problem. Firstly, some crops, such as banana and plantain, do not produce seeds and are thus propagated vegetatively. Secondly, some species, such as potato or sugar-cane, include both sterile genotypes and genotypes producing orthodox seeds. However, these seeds are generally highly heterozygous and are thus of limited interest for the conservation of particular genotypes. These species are normally maintained as clones. Thirdly, numerous fruit and forest tree species, especially of tropical origin, produce recalcitrant seeds, i.e. seeds that cannot be dried to sufficiently low moisture level to allow their storage at low temperature (Roberts 1973; Chin and Roberts 1980). There is also a large number of species, termed intermediate (Ellis et al. 1990), for which the seeds can be dried to some extent, but their long-term conservation remains problematic.

Cryopreservation

The traditional *ex situ* conservation method for these difficult-to-store categories of plant species is in the form of field collections, which present both advantages and major drawbacks, as described previously. Cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196°C), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of the genetic resources of the problem species mentioned above. At this temperature, all cellular division and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, protected from contamination, and require very limited maintenance (Engelmann 2000).

Cryopreservation of vegetatively propagated species is becoming a reality and is used routinely for long-term conservation of an increasing number of germplasm collections (Engelmann 2004). As concerns non-orthodox-seed species, a number of review papers have been published in the last decade that present extensive lists of plant species whose embryos or embryonic axes have been successfully cryopreserved (Kartha and Engelmann 1994; Bajaj 1995; Pence 1995; Engelmann et al. 1995; Engelmann 1997a, b; Engelmann and Takagi 2000; Towill and Bajaj 2002). This might lead to the conclusion that freezing of embryos is a routine procedure applicable to numerous species, whatever their storage characteristics. However, careful examination of the species mentioned in these papers reveals that only a limited number of truly recalcitrant seed species are in fact included. This is because research in this area is recent and addressed by very few teams worldwide and because recalcitrance is a dynamic concept that evolves with research on the biology of species and improvement in storage procedures. Some species previously classified as recalcitrant have thus been moved to the intermediate or even sub-orthodox categories, and can now be stored using classical or new storage techniques (Engelmann 2000).

In comparison with the results obtained with vegetatively propagated species, cryopreserved storage of non-orthodox seeds is still at a very preliminary research stage. There are a number of reasons behind this situation, including the huge number of (mainly wild) species falling within this storage category, a lack or insufficient knowledge of their biology, the inexistence or non-operationality of *in vitro* culture protocols for most of these species, and the large heterogeneity in the physical, biochemical and physiological characteristics of their seeds (most importantly concerning their moisture content) within and between seed lots (Engelmann 2000). Fortunately, there are various options to consider for improving storage of non-orthodox seeds, including employing very precisely controlled desiccation and cooling conditions, using other cryopreservation techniques that have so far seldom been employed, and selecting seeds or embryos at the right developmental stage, which is a parameter of critical importance for the success of any cryopreservation experiment (Engelmann 1999).

Development of complementary conservation strategies

It is now well recognized that an appropriate conservation strategy for a particular plant genepool requires a holistic approach, combining in a complementary manner the different *ex situ* and *in situ* conservation techniques available (Engelmann and Engels 2002). *In situ* and *ex situ* methods, including a range of techniques for the latter (storage of germplasm as seeds, plants in the field, pollen, *in vitro* cultures under slow growth, cryopreserved explants, DNA sequences), are options available for the different genepool elements (cultivated species, including landraces and modern varieties; wild relatives; weedy types; etc.). Selection of the appropriate method should be based on a range of criteria, including the biological nature of the species in question; the practicality and feasibility of the particular method chosen (which depends on the availability of the necessary infrastructure); as well as the cost-effectiveness and security afforded by its application (Maxted et al. 1997). Considerations of complementarity with respect to the efficiency and cost-effectiveness of the various conservation methods chosen are also important. In many instances, the development of appropriate complementary conservation strategies requires further research to define the criteria, refine the method and test its application for a range of genepools and situations (Dulloo et al. 1998, 2005; Nissilä et al. 1999; Ramanatha Rao et al. 1999). An important area in this is the linkage between *in situ* and *ex situ* components of the strategy, especially with respect to the dynamic nature of the former and the static, but potentially more secure, approach of the latter (Engelmann and Engels 2002; Reed et al. 2004). Cryopreservation needs to be integrated as a key component in the development of complementary conservation strategies for non-orthodox-seed species since, as mentioned previously, it is the only technique currently available to ensure the safe and cost-efficient long-term conservation for species producing non-orthodox seeds.

Another key component in the development of conservation strategies is the construction of core collections, which can be used either for conservation projects or evaluation purposes. A core collection is a subset of a larger germplasm collection and contains the maximum possible genetic diversity of the species with the minimum of repetitiveness (Frankel 1984). Despite the simple formulation of the core collection concept, construction of core collections appears often to be difficult because of lack of evaluation data for the whole collection. In many cases, a pragmatic approach can be encouraged, with the objective of structuring the germplasm accessions using passport data (see Chapter 5).

Research on the development of complementary conservation strategies and of the relevant storage methods required for their implementation can be effectively carried out through collaborative studies, involving fundamental and applied research organizations within countries, as well as through close cooperation with international institutions concerned with conservation research. Part of the work presented in this publication originates from the occurrence of such a situation with coffee.

Conservation of coffee germplasm

The conservation of coffee germplasm is closely associated with *C. arabica* domestication and has predominantly involved conservation in field genebanks because of the non-orthodox nature of coffee seeds. It first began on farms in the centre of origin, Ethiopia. Subsequently, worldwide extension of coffee cultivation contributed to the establishment of field genebanks in producer countries. The size of the collections increased greatly during the second half of the last century, when coffee germplasm collections were made during explorations across Africa (see Chapter 2). Considering the lifespan of coffee trees (about 30 years) and the inherent problems associated with maintenance of field genebanks (as described above), there is now an urgent need for rejuvenating the ageing coffee trees (Dulloo et al. 2001) and for the development of complementary methods of conservation.

Numerous *in vitro* techniques have thus been developed for medium-term storage of coffee germplasm (Dussert et al. 1997c). The establishment of an *in vitro* coffee core collection was initiated in 1991 at IRD Montpellier (France) but, a few years later, the limits of this technique was recognized with the occurrence of some genotypic selection and intraspecific genetic drift (Dussert et al. 1997a).

Research on the conservation of coffee seeds has also been promoted by Bioversity and IRD. Though *C. arabica* seeds can withstand desiccation down to 0.08-0.10 g $H_2O.g^{-1}$ dw water content (fresh weight basis) (Becwar et al. 1983; Ellis et al. 1990), they cannot be considered orthodox because they remain cold-sensitive (van der Vossen 1977; Couturon 1980; Ellis et al. 1990) and desiccation does not increase their longevity (van der Vossen 1977; Ellis et al. 1990). Fully hydrated seeds stored at 19°C under 100% relative humidity remained viable for 36 months for *C. arabica* and 15 months for *C. canephora* and *C. stenophylla* (Couturon 1980). Because of their intermediate storage behaviour, coffee seeds cannot be used for long-term conservation and coffee genetic resources are conventionally conserved as trees in field genebanks.

This highlights the importance of developing cryopreservation protocols for long-term conservation of coffee germplasm (Dussert et al. 2002). Research for cryopreservation techniques was performed with different explants, including seeds (Normah and Vendagasalam 1992), zygotic embryos (Abdelnour-Esquivel et al. 1992; Normah and Vendagasalam 1992), apices (Mari et al. 1995) and somatic embryos (Bertrand-Desbrunais et al. 1988; Tessereau et al. 1994).

However, seeds were considered the most interesting material for long-term conservation of coffee genetic resources using cryopreservation. Indeed, they are the only explant type for which a cryopreservation protocol could be developed that would not include any *in vitro* step, thereby allowing its implementation under low-tech conditions. In addition, seeds represent the base propagation unit for an autogamous species such as *C. arabica* and can be efficiently used for gene pool conservation in the case of allogamous species. Research was thus actively pursued at IRD Montpellier, leading after several years to the establishment of a simple, robust and efficient cryopreservation protocol based on the determination of very precise conditions for desiccation and freezing of seeds, which was applicable to a range of coffee species (Dussert et al. 1997b, 1998, 1999, 2000, 2002).

Past research has also shown that pollen can also be effectively stored under vacuum at -18°C and remain viable for more than two years and fertile for at least six months. (Walyaro and van der Vossen 1977). Regrettably, to our knowledge, there been no further research on coffee pollen conservation, and this represents a major gap in coffee conservation research.

Research on the *in situ* conservation of coffee genetic resources has lagged behind the efforts made in developing *ex situ* conservation techniques. *In situ* conservation of coffee germplasm has often resulted passively from the establishment and management of protected areas in biodiversity hotspots (Dulloo et al. 1998). The natural habitats of coffee are principally forest ecosystems, and it is widely known that the biological diversity in these habitats is under threat from high rates of deforestation, land clearing and introduced invasive species. Efforts to conserve natural populations of coffee germplasm are very limited, and known examples come from work done in Ethiopia (Gole 2002) and in Mauritius (Dulloo 1998). There is still much to be done within the areas of coffee diversity hotspots in Madagascar and on mainland Africa, particularly in Tanzania, while major areas within the central Africa region, such as Gabon and the Central African Republic, still remain unexplored.

Aims of this publication

For many years, non-orthodox-seed research has been recognized by Bioversity and its predecessors as an area of critical importance for the conservation of plant genetic resources, and

numerous projects on this topic have been or are being implemented in collaboration with research institutions and genebanks worldwide (Engelmann and Engels 2002; Engelmann 2003). Recognizing the tremendous potential implications of the results achieved at IRD for long-term storage of coffee germplasm, and also for other non-orthodox-seed species, Bioversity decided to support a research programme aiming at transferring and testing on a large scale in a genebank located in a developing country the freezing protocol developed in France. This was implemented in 1998–2000 in the framework of two successive projects with IRD and CATIE, Costa Rica. CATIE was an ideal partner as it fulfilled the set of criteria required for participating in such a project. Indeed, CATIE holds one of the largest field collections of coffee worldwide, mainly of *C. arabica*, with 1852 accessions of this species (9760 trees), including wild plants and varieties from the diversity centre (Ethiopia), varieties from the dispersion centre (Yemen), varieties derived from two genetic populations spread worldwide in the 18th century (known as Typica and Bourbon), introgressed lines derived from interspecific hybrids, mutants and other selected genotypes (see Chapter 3). CATIE's fully equipped biotechnology laboratory includes all the facilities required for cryopreservation and molecular biology research, as well as highly skilled scientific and technical staff. Moreover, Bioversity and CATIE have a long and successful collaboration history in various areas, including cryopreservation of tropical plant germplasm (Abdelnour-Esquivel 2000; Engelmann 2003). At the time of the initiation of this programme, CATIE was also implementing collaborative research projects on coffee with IRD and another French research institute, CIRAD (*Centre de coopération internationale en recherche agronomique pour le développement*). Staff from these two institutes were working on a permanent basis in CATIE. These collaborative projects included research on the characterization and rationalization of the CATIE coffee germplasm collection (see Chapters 3 to 5); on the utilization of the material from the coffee collection for improvement purposes (Anthony et al. 1999; Bertrand et al. 1999, 2005); and on the use of biotechnology for large-scale propagation of improved material (Etienne et al. 1999, 2002; Etienne and Bertrand 2001).

The aim of this publication is to illustrate how new technologies (molecular biology and cryopreservation) can be efficiently employed to complement more classical ones for characterizing and rationalizing an *ex situ* germplasm collection, and to improve its conservation status. To our knowledge, the work described in this publication represents the first example of the application of these techniques in a genebank located in a developing country, in the framework of the development of an *ex situ* complementary conservation strategy for *C. arabica*, i.e. a crop of commercial importance at the global level. The approach that was applied to coffee genetic resources might be used with other perennial plants whose seeds also display non-orthodox storage behaviour.

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II. Coffee genetic resources

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Introduction

Coffee has become one of the most popular beverages in the world, but its consumption remained low until the 17th century. Wild plants of *Coffea arabica* L. were discovered in about AD 850 in Ethiopia (Smith 1985), but the centre of genetic diversity also includes the Boma Plateau of Sudan (Thomas 1942) and Mount Marsabit of Kenya (Bridson 1982; Anthony et al. 1987). Coffee spread to Arabia (now Yemen) probably in the 14th century (Chevalier 1929a), then to Mecca, whence it was taken home by pilgrims to other parts of the Islamic world. Spread of coffee consumption to Europe is dated to 1615, when Venetian merchants brought coffee beans from Mocha to Europe (Smith 1985). This started a lucrative trade for the Arabians for 100 years, during which time they were the sole providers of coffee. Several expeditions were then sent by the Dutch, the French and the British to obtain coffee seeds or plants from Arabia, which led to the worldwide dissemination of two genetic populations—Typica and Bourbon—in the 18th century (see Chapter 4).

The interest in other coffee species came later, during the course of Africa's exploration at the end of the 19th century and the beginning of the 20th century. Only four coffee species were known in 1834 (Chevalier 1929b), but they were 36 in 1901 (de Wildeman 1901), about 50 in 1929 (Chevalier 1929b) and up to a hundred species nowadays (Chevalier 1947; Bridson and Verdcourt 1988; Stoffelen 1998). Several new species have been described recently (Stoffelen et al. 1996, 1997a, b, 1999, [2006], 2007; Davis 2001; Davis and Rakotonasolo 2000; 2001a, b, 2003; Cheek et al. 2002; Davis and Mvungi 2004; Sonké and Stoffelen 2004), indicating that the inventory of wild coffee is not yet complete. Based on flowering and flower characters, taxonomists have classified the coffee species into two genera, *Coffea* L. and *Psilanthus* Hook. f. (Leroy 1980; Bridson 1987), but this distinction is not supported by molecular data analysis (Lashermes et al. 1997; Cros et al. 1998). All *Coffea* species are native to the inter-tropical forests of Africa, Madagascar and the Mascarene islands, while the *Psilanthus* species originate from Africa, India, Malaysia, Papua New Guinea and Australia (Bridson 1987; Bridson and Verdcourt 1988). Three groups of species have been identified in the genus *Coffea* on the basis of biogeographical data: in the Madagascar region, in East Africa, and in Central and West Africa (Chevalier 1947; Bridson and Verdcourt 1988; Stoffelen 1998).

Coffee trees differ greatly in morphology, size and ecological adaptation. Particular attention has been paid to the genus *Coffea*, which includes the two cultivated species of economic importance, *C. arabica* (Arabica coffee) and *C. canephora* (Robusta coffee). Of the two, Arabica coffee accounts for 70% of the market, compared with 30% for Robusta. All *Coffea* species are diploid (2n=2x=22) and generally self-incompatible, except for *C. arabica*, which is tetraploid (2n=4x=44) and self-compatible. Nevertheless, the coffee species share a common genome, making possible interspecific hybridizations and hybrid production either within *Coffea* species (Charrier 1978; Louarn 1992; Le Pierrès 1995), or between *Coffea* and *Psilanthus* species (Couturon et al. 1998). This shows the potential value of genetic resources as sources for transfer of new characters from diploid species into the genome of *C. arabica* cultivars.

This chapter discusses the major collecting expeditions of coffee genetic resources made and the genebanks where the collected materials were introduced. The importance of the resources being conserved in these genebanks is then discussed, particularly in regard to some of the traits of agronomic interest to show their importance to coffee breeding programmes and genomic projects.

Coffee genetic resources collecting

Interest in coffee genetic resources increased during the second half of the 20th century, as breeders became aware that deforestation was causing the erosion of coffee habitats, thereby threatening coffee genetic resources. It was estimated that the closed high forest in Ethiopia had declined to only 18% by 1997, which represents a loss of 60% in less than 30 years (Gole et al. 2002). Considering the socio-economic importance of *C. arabica* cultivation, two large surveys were organized in Ethiopia: by FAO in 1964–65 (Fernie et al. 1968) and by ORSTOM (now IRD) in 1966 (Guillaumet and Hallé 1978). Collecting of other species started at the same period in the Madagascar region through a joint initiative of the Paris Museum of Natural History, CIRAD and ORSTOM. In Africa, survey missions were conducted in seven countries between 1975 and 1987 by ORSTOM (Table 2.1). Lastly, a mission was organized by IPGRI in Yemen (Eskes 1989), an area considered to be the first centre of dispersion for *C. arabica* outside Ethiopia (Meyer 1965).

Table 2.1. Main collections of coffee genetic resources (years of collection, countries surveyed, institutions involved, number of accessions collected, and references).

Year	Country	Institutions involved in the collecting missions	No. of accessions	Reference(s)
1964–65	Ethiopia	FAO	620	Fernie et al. 1968
1966	Ethiopia	ORSTOM	70	Guillaumet and Hallé 1978
1960–74	Madagascar	Museum of Natural History, Paris, France; CIRAD; ORSTOM	>3000	Charrier 1978
1975	Central African Republic	ORSTOM	>1200	Berthaud and Guillaumet 1978
1975–87	Côte d'Ivoire	ORSTOM	>2000	Berthaud 1986; Le Pierrès et al. 1989
1977	Kenya	ORSTOM	1511	Berthaud et al. 1980; Anthony et al. 1987
1982	Tanzania	ORSTOM	817	Berthaud et al. 1983; Anthony et al. 1987
1983	Cameroon	ORSTOM; IBPGR	1359	Anthony et al. 1985; Anthony 1992
1985	Congo	ORSTOM; IBPGR	1080	de Namur et al. 1987; Anthony 1992
1987	Guinea	ORSTOM; CIRAD	74	Le Pierrès et al. 1989
1989	Yemen	IBPGR	22	Eskes 1989

Notes: ORSTOM (Office de la recherche scientifique et technique outre-mer) is now Institut de recherche pour le développement (IRD), France.
CIRAD is Centre de coopération internationale en recherche agronomique pour le développement, France.
IBPGR (International Board for Plant Genetic Resources) became the International Plant Genetic Resources Institute (IPGRI), which in turn became Bioversity International.

The collected material generally comprised seed in the case of the autogamous species *C. arabica*, and stem cuttings, seedlings and seeds for the other species. An accession can thus correspond to one genotype when stem cuttings were collected or to several genotypes in the case of seed collecting. At least 11 700 accessions, representing 70 *Coffea* species, were collected and introduced into field genebanks (Table 2.2). Of these species, about 50 taxa were native to the Madagascar region; 8 taxa to eastern Africa, including *C. arabica*; 7 taxa to central Africa; and 4 taxa to West Africa (Figure 2.1). New species from Cameroon and Congo were recently described (Stoffelen *et al.* [2006], 2007) and others remain to be identified (Anthony 1992).

Table 2.2. Major coffee genebanks in the world and distribution of the *C. arabica* accessions collected in Ethiopia by FAO (Fernie et al. 1968) and ORSTOM (Guillaumet and Hallé 1978) surveys.

Area	Country	Institute	FAO collection	ORSTOM collection
Latin America	Costa Rica	Centro Agronómico Tropical de Investigación y Enseñanza (CATIE)	original	
	Colombia	Centro Nacional de Investigaciones de Café (CENICAFE)	duplicate‡	duplicate†
	Brazil	Instituto Agronômico de Campinas (IAC)	original	duplicate†
Africa	Côte d'Ivoire	Centre National de Recherche Agronomique (CNRA)	duplicate§	original
	Cameroon	Institut de Recherche Agronomique et de Développement (IRAD)		original
	Ethiopia	Jimma Agricultural Research Centre (JARC)	original	
	Kenya	Coffee Research Foundation (CRF)	duplicate¶	original
	Tanzania	Tanzanian Agricultural Research Organization (TARO)	original	duplicate††
	Madagascar	Centre National de Recherche Appliquée au Développement (FOFIFA)		original
Asia	India	Central Coffee Research Institute (CCRI)	original	
	Indonesia	Indonesian Coffee and Cocoa Research Institute (ICCRI)		

Notes: † germplasm introduced from Cameroon. ‡ germplasm introduced from Costa Rica. § germplasm introduced from Kenya. ¶ germplasm introduced from Tanzania. †† germplasm introduced from Côte d'Ivoire.

Existing coffee genebanks

The report *The State of the World's Plant Genetic Resources for Food and Agriculture* (FAO 1998) reported 21 087 coffee accessions conserved worldwide. As with other crops of economic importance, exchanges of genetic material have led to an increase in the number of duplicates in many genebanks. Accessions of the cultivated species *C. arabica* and *C. canephora* correspond either to wild plants collected in forest habitats or to cultivated plants selected in plantations and breeding centres. The accessions of the cultivated species *C. arabica* have been widely spread, while the other species have had a more restricted distribution.

Accessions of *C. arabica*

Most coffee genebanks were set up during the first half of the 20th century, the earliest being the Indonesian Coffee and Cocoa Research Institute (ICCRI) in 1900, the Agronomic Institute of

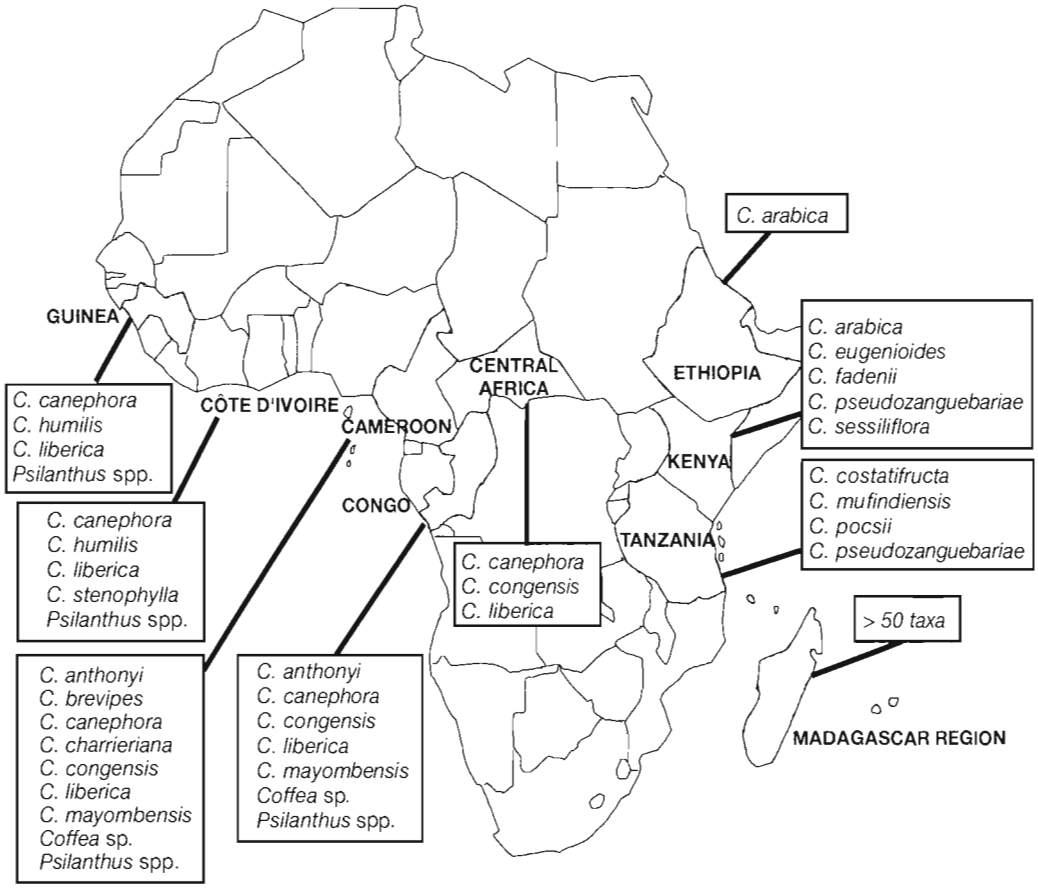

Figure 2.1. Coffee species collected on the African mainland and the Madagascar region, and introduced into field genebanks.

Campinas (IAC) in Brazil in 1924 and the Central Coffee Research Institute (CCRI) in India in 1925 (van der Vossen 2001). Coffee farmers supplied genebanks with materials which displayed good agronomic performance or presented specific traits. Many mutants were thus isolated, as well as numerous varieties selected from the base populations of Typica and Bourbon that were disseminated from Yemen during the 18th century (see Chapter 4). Such selected accessions represent 72% of the genetic resources conserved in the CATIE genebank (see Chapter 3). The accessions collected by FAO and ORSTOM in Ethiopia in the 1960s were introduced in five and four genebanks, respectively (Table 2). Further introductions occurred from these genebanks in Colombia, Côte d'Ivoire and Kenya for the FAO accessions and in Costa Rica, Colombia and Kenya for the ORSTOM accessions. This has contributed to the preservation of corresponding genetic resources, although genetic diversity in duplicated germplasm was often lower than in the original one.

Accessions of other species

Large genebanks of *C. canephora* accessions were set up in several coffee producing countries, including Côte d'Ivoire, Cameroon, Madagascar and India (Charrier and Berthaud 1985). As for *C. arabica* genetic resources, introductions of *C. canephora* originated from plantations and

breeding centres in the first instance, until the collecting of wild plants began in the 1980s. However, only part of the known diversity has been conserved in each genebank, as recently shown in a study using molecular markers (Prakash et al. 2005). Of the five genetic groups identified in this species (Dussert et al. 2003), only one group was well represented in India, two groups were little represented and two other groups were lacking.

Most of the other wild coffee species (i.e. not cultivated) have been introduced into only two genebanks, namely in Madagascar for the Mascarocoffea species and in Côte d'Ivoire for the mainland African species. These unique collections are threatened in the long term because they are nowhere safely duplicated. In the Malagasy genebank, 25% of the accessions and 50% of the genotypes were estimated to have been lost over a period of 20 years (Dulloo et al. 2001). In Côte d'Ivoire, the climate is not optimal for coffee culture and the risk of damage by fire is high (Dulloo et al. 2001). There is therefore an urgent need for duplication of the wild coffee genetic resources conserved in these genebanks. A few genotypes of some species, principally *C. eugenioides* S. Moore, *C. liberica* Hiern, *C. racemosa* Lour. and *C. stenophylla* G. Don, are, however, present in several other genebanks.

Insight into coffee genetic diversity

The genus *Coffea* is characterized by a large number of species whose differentiation has occurred relatively recently, about 5 to 25 million years ago (Anthony and Lashermes 2005). It is thought that the diversity of coffee species and the genetic diversity within them have been the result of a rapid speciation and adaptive radiation process (Cros 1994; Lashermes et al. 1997). Diversity can be analysed at the genetic and morphological levels, the latter often being used as a proxy of the former. Only few studies have been undertaken to examine the level of genetic diversity in wild coffee plants. A good insight into coffee genetic diversity—using phenotypic characteristics such as data on plant morphology, adaptation, biochemical compounds and resistance to pests and diseases as proxies—is given below.

Morphology

There can be wide diversity among morphological traits in coffee plants. Coffee plants can be shrubs or trees, whose height varies from 1 m in *C. humilis* Chev. up to 20 m in *C. liberica* var. *dewevrei* (Chevalier 1947). Leaf size varies considerably, from 2 cm (e.g. *C. eugenioides*) to 48 cm (e.g. *C. magnistipula* Stoff. & Robbr.) in length, and from 0.8 cm (*C. eugenioides*) to 30 cm (e.g. *C. liberica* var. *dewevrei*) in width (Chevalier 1947; Bridson and Verdcourt 1988; Stoffelen 1998). Mature fruits are generally red (e.g. *C. arabica*, *C. canephora*), but also yellow (e.g. *C. liberica*), orange (e.g. *C. congensis* Froehner), violet (e.g. *C. racemosa*) or black (e.g. *C. salvatrix* Swynnerton & Phillipson). Further, white fruits were observed in a *Psilanthus* taxon collected in Congo (Anthony 1992). Bean size is another variable character, which makes necessary the definition of an adequate desiccation time prior to cryopreservation (Dussert et al. 1998). Extreme values of 1000-bean weight were 29 g (e.g. *C. pseudozanguebariae* Bridson) and 198 g (e.g. *C. liberica* var. *liberica*) (Clifford et al. 1989).

Adaptation

Altitude is an important factor structuring forest habitats in Africa (White 1983). The coffee species are distributed from sea level (e.g. *C. liberica* var. *liberica*) to 2100 m (e.g. *C. eugenioides*). Among the cultivated species, *C. canephora* grows in lowland forests, up to 1400 m (Bridson and Verdcourt 1988) while *C. arabica* is found in submontane forests between 1000 and 2000 m (Meyer 1965; Gole et al. 2002). These ecological differences of habitat could explain the variations observed in cold sensitivity among *in vitro* microcuttings of different species stored at various temperatures (Engelmann et al. 1993).

Some species are widely distributed, colonizing diverse environments, e.g. *C. canephora* and *C. liberica* from Guinea to Uganda, but most species have a restricted distribution and display specific adaptations, e.g. *C. congensis* to seasonally flooded areas in the Zaire basin and *C. racemosa* to very dry areas in the coastal region of Mozambique (Charrier and Berthaud 1985). In Kenya, *C. pseudozanguebariae* was found on a coral reef substrate (Anthony et al. 1987). Such ecological data are essential for selecting appropriate growing conditions for living genebanks and for testing of agronomic performance for cultivation (Charrier and Berthaud 1985).

Biochemical components

Our knowledge of the origin of the biochemical diversity in coffee has greatly improved following the analysis of biochemical compounds from wild coffee species collected in Africa in the 1970s and 1980s. Of the numerous compounds found in green coffee beans, attention was firstly focused upon caffeine because of its known pharmacological actions and influence on beverage bitterness. Caffeine content of cultivated species appears moderate in *C. arabica*, varying between 0.76 and 1.82% dry mass basis (% dmb), and high in *C. canephora*, between 1.51 and 3.33% dmb (Anthony et al. 1993; Ky et al. 2001). This constitutes the maximum caffeine content in coffee (Campa et al. 2005). Caffeine-free species were reported in the Madagascar region (d'Ornano et al. 1965), eastern Africa (Hamon et al. 1984) and central Africa (Stoffelen et al. 2007). The other species present a caffeine content ranging from 0.47 to 2.64% dmb (Anthony et al. 1993).

Table 2.3. Variation of biochemical compounds (% dry matter basis) in green coffee (*Coffea* spp.) beans.

Compound	Minimum	Maximum	Reference
Caffeine	0.00	3.19	Clifford et al. 1989
Chlorogenic acids	0.61	14.40	Campa et al. 2005
Sucrose	3.81	10.87	Campa et al. 2004
Trigonelline	0.36	1.99	Campa et al. 2004

Other aroma precursors have been studied, such as chlorogenic acids, which increase bitterness of beverage, and sucrose and trigonelline, which give rise to appreciated flavour products. The data vary greatly for all compounds analysed, especially for chlorogenic acids, where the maximum value is 23 times that of the minimum value (Table 2.3). A relation was found between caffeine and chlorogenic acids, since large contents of dicaffeoylquinic and feruloylquinic acids were only detected in species which contain at least 0.6% dmb caffeine (Anthony et al. 1993). Chlorogenic acids are lower in *C. arabica* than in *C. canephora*, while fat, sucrose and trigonelline are higher (Clifford 1985; Ky et al. 2001).

Pathogen resistance

Resistance to the Coffee leaf rust caused by *Hemileia vastatrix* Berk & Br. was first observed in existing genebanks because of strong attacks in coffee plantations in eastern Africa, India and south Asia in the 1950s, then worldwide. Accessions displaying a high level of resistance were identified in *C. canephora* (Berthaud and Lourd 1982; Kushalappa and Eskes 1989; Montagnon and Leroy 1993), *C. pseudozanguebariae* (Rodrígues Jr. 1980), and with less frequency in *C. liberica*, *C. eugenioides* and *C. salvatrix* (Rodrígues Jr. et al. 1975; Rodrígues Jr. 1980). Accessions highly resistant to *Colletotrichum kahawae* Waller & Bridge (ex *C. coffeanum* Noack) causing the Coffee berry disease (CBD) were detected in *Coffea arabica* and *C. canephora* collections (van der Vossen and Walyaro 1980; Van der Graaf 1981; Rodrígues Jr. et al. 1992).

Resistance to pests has been also sought in genebanks, principally to the root-knot nematodes (*Meloidogyne* spp.) that represent the strongest constraint on *C. arabica* cultivation in many Latin American countries. No accession resistant to *M. exigua* Goeldi was found in *C. arabica*, but some resistance exists in *C. canephora* and *C. racemosa* (Bertrand et al. 2001; Anthony et al. 2003). Resistance to *M. arabicida* López & Salazar and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida was observed in *C. arabica* and *C. canephora* (Anthony et al. 2003). Finally, resistance to the Coffee leaf miner (*Leucoptera coffeella* Guérin-Méneville) was reported in *C. racemosa* (Medina Filho et al. 1977a, b; Guerreiro Filho et al. 1991) and *C. stenophylla* (Cardenas-Murillo and Posada-Ochoa 1984; Guerreiro Filho et al. 1991).

Conclusion

A large amount of coffee genetic diversity has been collected and introduced into field genebanks. Of the 100 species described by taxonomists, more than half have entered conservation, suggesting a large sampling of available genetic resources. However, it has been observed that the coffee genetic resources being conserved in living collections (or field genebanks) are quickly eroding, due to a multitude of reasons, including adaptability problems, vandalism, natural catastrophes and—above all—insufficient funds for maintaining the collections (Dulloo et al. 2001). In Côte d'Ivoire, it was estimated that the cost of germplasm acquisition and genebank establishment represented less than 10% of the total budget allocated to the breeding programme (Charrier et al. 1989). Moreover, except for the cultivated species, the wild species are conserved in a single site: in Madagascar for the species endemic to the region; and in Côte d'Ivoire for the African mainland species. Another problem in coffee genetic resources conservation is the lack of an international structure able to coordinate conservation activities at a global level. There is an urgent need to place the conservation of coffee genetic resources on more secure grounds, and to establish a global strategy for a more efficient and cost-effective rational conservation of these precious resources.

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III. Conservation of coffee genetic resources in the CATIE field genebank

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Introduction

The importance of genebanks in crop breeding has been widely recognized since Vavilov's publications (Vavilov 1935). Genebanks are essential for preserving the genetic diversity of commercial crops and their relatives, and for characterizing the conserved accessions prior to their utilization. For many species that cannot be conserved by seeds, field genebanks became the method of choice for conservation of genetic diversity rather than botanical gardens and introduction centres, which were set up mainly to cultivate wild species, as early as the 16th century. As a part of its institutional mandate, CATIE preserves, multiplies, classifies and promotes the use of its valuable germplasm collections, which include more than 300 plant species with more than 35 000 accessions. The genetic material is available for institutions and organizations involved in plant improvement and production (for details, connect to the CATIE Web site at http://www.catie.ac.cr/research/research.htm).

As for most cultivated plants, conservation of coffee (*Coffea* spp.) genetic resources started with field collections. The sensitivity of coffee seeds to desiccation and cold (Ellis et al. 1990) has long limited the development of other conservation methods. The CATIE genebank is one of the largest and richest worldwide for *C. arabica* coffee, containing 9760 trees of 1852 accessions at the time of writing. It includes wild coffee trees collected in the centre of origin, varieties and mutants selected in various research centres, as well as intra- and interspecific hybrids. The collection is the only genebank available for Latin American and Caribbean countries. An extensive genetic evaluation was carried out in the 1990s with the aim of structuring genetic diversity and of identifying accessions that present interesting characters for the regional improvement programme (see Chapter 4). As a prerequisite to evaluation, an analysis of existing accessions in the genebank was performed in order to classify the accessions according to their genetic origin and to define possible parental linkages.

C. arabica coffee cultivation might have started in the centre of origin of the species, in the south-west of Ethiopia, around the 5th to 8th centuries. It is at that time that coffee trees were introduced to Yemen, possibly by Arabian merchants (see review by Anthony et al. 1999). Two populations, known as Typica and Bourbon, were later disseminated from Yemen to the world during the 18th century. They gave rise to a large number of mutants in Latin America, Africa and Asia (Krug et al. 1939; Chevalier 1947). During the 20th century, the extension of coffee cultivation and the intensification of production revealed that the varieties derived from Typica and Bourbon were sensitive to many pests (e.g. nematodes, Coffee Berry Borer) and diseases (e.g. Coffee leaf rust, Coffee berry disease) (see reviews by Bertrand et al. 1999; Flood et al. 2001). Natural interspecific hybrids between *C. arabica* and *C. canephora* or *C. liberica* constituted the

first sources of resistance to Coffee leaf rust (aka orange rust) caused by *Hemileia vastatrix*. Other interspecific hybrids were later created. The genealogical selection of these descents has led to the diffusion of introgressed lines, resistant to rust and known under the names of Catimor, Sarchimor, Icatu, S.795, etc. Coffee genetic resources have thus varied origins.

This chapter is divided into five sections: (i) a presentation of the accessions conserved in the CATIE genebank; (ii) a description of the conditions of their conservation in the field; (iii) the data management system; (iv) an analysis of the genetic erosion; and (v) the principles of a new conservation strategy for coffee field genebanks.

CATIE field genebank constitution

The introduction of coffee genetic resources started in 1949 at IICA (*Instituto Interamericano de Ciencias Agrícolas*, now *Instituto Interamericano de Cooperación para la Agricultura*) which had available land (1000 ha) close to Turrialba, given by the government of Costa Rica (for details, consult the CATIE Web site at http://www.catie.ac.cr). The field genebank is located in the Cabiria III campus botanical garden, and covers approximately 8.5 ha. The site is situated at 9°38′ N latitude and 83°38′ W longitude, at 602 m above sea level. The average day temperature is 22.5°C and annual rainfall 2600 mm, without any marked dry season. It represents a sub-optimal zone for the culture of *C. arabica* and of the other coffee species (e.g. *C. eugenioides*) usually found at higher altitudes. Coffee produced in the Turrialba region presents normal acidity and good aroma, but small body (for details, see the ICAFE Web site at http://www.icafe.go.cr). Flowerings are multiple and of low intensity; harvest is precocious and is spread over at least four months.

The genebank became CATIE's responsibility after its creation in 1973 by IICA and the government of Costa Rica. Introduction records were maintained by world-renowned coffee researchers, such as J.B.H. Lejeune, P.G. Sylvain and F.L. Wellman. These records were then updated with the support of the German Agency for Technical Cooperation (GTZ) at the beginning of the 1980s. It is on the basis of this information that the analysis of accessions in the genebank was then carried out. Observations were performed in the genebank in order to confirm the taxonomic identification of some introductions.

History of introductions

The accessions introduced in 1949 now represent 1.5% of the total number of conserved accessions. The most massive introductions took place over 20 years, between 1951 and 1970, with an average of 52 accessions introduced annually (Figure 3.1). These introductions constitute 55.6% of the living accessions in the collection. The introduction rate decreased during the following decade (1971–80), with around 20 accessions introduced annually, and then increased between 1981 and 1990, with 43 accessions introduced annually. The 1980s introductions represent almost a quarter of accessions conserved. Since then, additions to the genebank have averaged 17.5 accessions per year.

The chronology of introductions reflects the advances of the breeding programmes which have been developed worldwide. The majority of accessions introduced in the 1950s were Typica- and Bourbon-derived varieties or varieties locally cultivated in the centre of origin (i.e. Ethiopia). These coffee trees were selected at research centres and farms. The following decade (1961–70) was marked by the first large collecting mission in Ethiopia (Fernie et al. 1968). The collected material was distributed to five field genebanks, and CATIE received the most accessions (485). The accessions introduced between 1971 and 1990 were principally introgressed lines, derived from a natural interspecific hybrid *C. arabica* × *C. canephora*, the 'Timor' hybrid (Bettencourt 1973). Finally, new coffee species (*C. brevipes* Hiern, *C. pseudozanguebariae*, *C. sessiliflora* Brid.),

as well as wild *C. arabica*, *C. canephora*, *C. eugenioides* and *C. racemosa*, were introduced in the 1990s. Twenty-one accessions from the IBPGR-funded collecting mission in Yemen were also introduced (Eskes 1989).

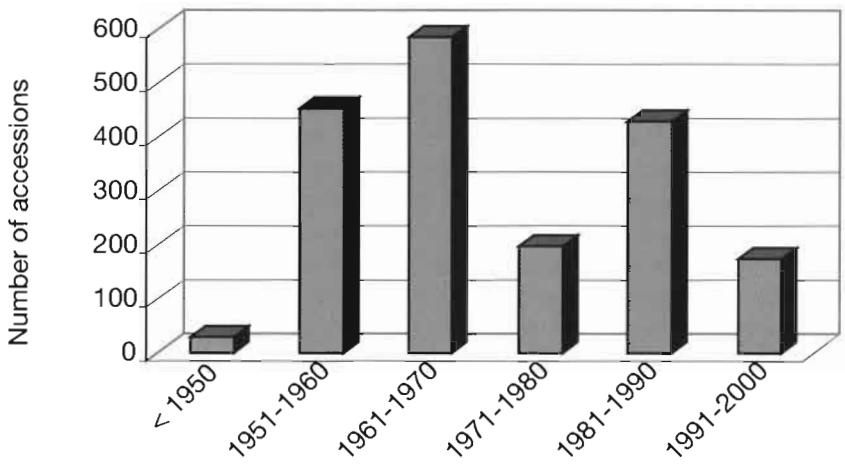

Figure 3.1. Number of coffee accessions introduced to the IICA and subsequently CATIE field genebank per decade, since 1949.

Source of introduced accessions

The coffee accessions introduced to CATIE were received from research centres and plantations located in 31 countries. Latin American (e.g. Brazil, Costa Rica) and Caribbean (e.g. Puerto Rico) countries provided 42% of the accessions (Figure 3.2). Africa provided close to 500 introduced accessions (26%). Two European countries, France and Portugal, made significant contributions, with around 200 accessions each.

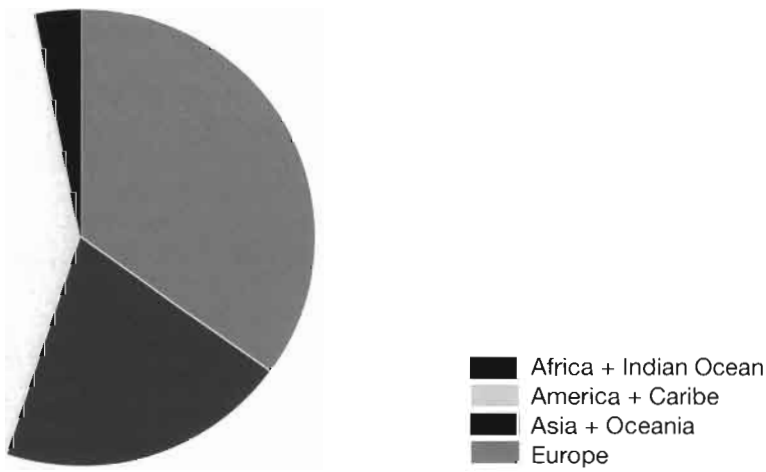

Figure 3.2. Provenance of the coffee accessions introduced to the CATIE field genebank.

Field genebank management

Classification of genetic origins

The large number of accessions in the genebank meant that, before they could be evaluated effectively, it was necessary to classify the accessions according to their genetic origin. The objective was to construct core collections for genotypic and phenotypic analyses of diversity (see Chapter 5). A hierarchical ranking was adopted by choosing the species as first criterion of classification (i.e. *C. arabica* vs. other species and hybrids). The *C. arabica* accessions were then separated into three groups as a function of the selection that had taken place: (i) 'no selection' for wild coffee trees collected in the centre of origin; (ii) 'low' for coffee trees cultivated in the centre of origin and the centre of dispersal (Yemen); and (iii) 'high' for Typica- and Bourbon-derived varieties, mutants and introgressed lines. The accessions classified in this last group often possess parental links due to the selection process.

Various observations can be made from the inventory of the genetic resources of CATIE (Table 3.1). Ninety-one percent of the conserved accessions belong to the species *C. arabica* or to interspecific hybrids involving this species. The other coffee species are under-represented in terms of number and inherent diversity (see Chapter 2). The wild coffee trees collected in Ethiopia by FAO (Fernie et al. 1968) and ORSTOM (now IRD) (Guillaumet and Hallé 1978) constitute 31.5% of the conserved accessions, but only 22.8% of the living trees. The material from the IPGRI collecting expedition in Yemen (Eskes 1989) is only represented by a few (17) trees. In contrast, the accessions originating from selection (group 3) are numerous, representing 45.8% of the total and 58.6% of all trees in the collection. Finally, many intraspecific hybrids are also conserved (15% of all trees).

Table 3.1. Number of coffee (*Coffea* spp.) accessions and corresponding trees conserved in the CATIE field genebank. Within the *C. arabica* cultivated species, three groups were defined on the basis of selection intensity: nil (0), low (+) and high (++).

Identification	Selection	Description	Accessions	Trees
C. arabica	0	Wild plants from the centre of diversity (Ethiopia)	583	2222
	+	Varieties from the centre of diversity (Ethiopia)	191	950
		Varieties from the primary dispersion centre (Yemen)	10	17
	++	Varieties derived from Typica and Bourbon	292	1818
		Introgressed lines from interspecific hybrids	303	1786
		Intraspecific hybrids	169	1467
		Mutants and other selected coffee	84	650
Diploid species		*C. canephora*	83	296
		C. liberica	15	76
		Other species	60	138
Interspecific hybrids		(*C. arabica* × *Coffea* spp.)	19	90
Not classified			43	250
Total			1852	9760

Agronomic practices

Planting

The field genebank is divided into eight sections (A to H), sub-divided into plots. The collection was maintained in a manner similar to that of commercial plantations up to 1998, when the Technical Unit for Support to Research (UTAI) took over its maintenance. Since then, a new strategy of conservation has progressively been implemented (see later in this chapter).

Most of the accessions (91%) have been received in seed form, each seed constituting a genotype. The number of genotypes planted in the genebank varies from four to eight for the majority of accessions. However, 14% of accessions are represented by larger numbers, reaching up to 46 coffee trees.

About 9% of accessions correspond to clones that were introduced as stem cuttings. These accessions are represented by one to ten trees in the genebank, produced by vegetative multiplication (i.e. cutting or grafting).

Coffee trees introduced in seed form are cultivated on their own root system. During the 1990s, grafting on vigorous rootstocks of *C. canephora* var. Nemaya, which is resistant to most root-knot nematodes of Central America (Bertrand et al. 2002), has been used in order to facilitate the adaptation of wild coffee trees (*Coffea* spp.), whose agronomic performances are rather weak.

Spacing between rows of trees and between trees within a row varies according to the section of the genebank. The most classical spacing is 2.5 × 2 m. Extreme spacing distances are 4 × 4 m for the first introductions of *C. canephora* and *C. liberica*, and 2 × 1.5 m for dwarf introgressed lines.

Coffee trees are normally grown under canopy, but shading practice in plantations varies considerably according to ecological conditions, local tradition and the level of management (Mitchell 1988). To provide shade, *Erythrina poeppigiana* trees are planted between the coffee rows, at approximately a 6 × 6 m spacing. Their relatively fast growth requires two pruning treatments annually in order to allow suitable penetration of light at the level of the coffee tree foliage.

Coffee tree maintenance

Coffee trees are maintained with at least three trunks. Pruning is performed once a year, during the dry season, in order to eliminate the oldest stems.

The most common weeds in the coffee collection are grasses, such as *Paspalum paniculatum* and *P. conjugatum*, and forbs, commonly *Bidens pilosa*, *Impatiens walleriana*, *Borreira* spp., *Mitracarpus* spp. and *Richardia scabra*. In the absence of cover plants, weeds are eliminated by applying a herbicide approximately every two months. Rotation of products is respected in order to minimize the development of resistance in the weed flora.

The soil of the coffee genebank is homogeneous for physical structure and chemical composition. However, the presence of a cemented layer makes drainage difficult. The fertility of the soil is medium, and not optimum for coffee, and so requirements are supplied by supplemental fertilizer application. Fertilizers are applied in a uniform manner to all coffee trees, wild or cultivated. Applications consist of 100 g per tree of 20-7-12-3-1.2 (N-P-K-Ca-Mg) in May, 18-5-15-6-2 in September and ammonium nitrate in December. However, variations in the budget allocated to genebank maintenance can affect the fertilization programme, as observed in most coffee field genebanks in the world (Dulloo et al. 2001).

Coffee trees conserved in the genebank are usually comparatively free from the pests and diseases encountered in commercial plantations. The most serious attacks are those provoked by *Hemileia vastatrix*, the pathogenic agent of Coffee leaf rust (orange rust).

Productive trees are more severely affected by defoliation at the time of harvest and immediately afterwards. Treatment against Coffee leaf rust is by application of classical fungicides such as triadimefon, copper hydroxide and ciproconazol. The recent arrival of Coffee Berry Borer (*Hypothenemus hampei* Ferr.) has made necessary the definition of an integrated pest management (IPM) strategy, which reduces insecticide applications. Traps containing a mixture of alcohols, as recommended by the Costa Rica Coffee Institute (ICAFE), have been set up in the genebank.

Harvesting

Harvesting is performed manually, in several passes. The spread flowering pattern implies at least four passes between July and November on each tree. The incidence of the coffee borer has been estimated to be around 5% of harvested fruits. At the last pass, all remaining berries (green, ripe and dry) are picked in order to limit possible refuges for berry borers.

Information system

Accession number

At the time of their introduction, accessions are assigned a unique number (i.e. neither repeated nor re-attributed) in CATIE's introduction records. This number is preceded by the letter 'T', which stands for Turrialba. An accession number corresponds either to several genotypes if the introduction is in the form of seeds, or to a single genotype in the case of a clone. Mixing different genotypes under one unique number is problematic because of preferential autogamy of *C. arabica*, which allows around 10% of allo-pollination at each generation (Carvalho et al. 1991). Presence of illegitimate plants (i.e. not conforming to their genetic origin) constitutes a constant risk with seed samples.

Passport data

Data on the accessions of the field genebank are maintained in a database called 'CaféBase'. This database contains two types of passport information: (i) information on the genetic origin of the accessions; and (ii) information about the provenance (i.e. source) of introduced plant material (Figure 3.3). Information on the origin corresponds to the collecting data (i.e. localization of the forest population, nature of the collected samples) for the wild coffee trees or to the genetic basis for the selection process that has taken place (i.e. Typica, Bourbon, hybrids) for cultivated coffee trees. These data have been extracted from publications and available reports. Information on the provenance of accessions has been found in the introduction records of CATIE. The source of introductions has been international organizations, national coffee research centres or private farms.

Database structure

The passport data of the accessions are stored in two tables (Figure 3.4). One has for its access key the name of the genetic resource and contains information on the genetic origin (wild or cultivated). The other table has for its access key the accession number and contains information on the accession's provenance. These tables can be linked thanks to the presence of a common field: a shortened identifier for each genetic resource (e.g. 'Caturra' instead of the complete identifier '*C. arabica* var. Caturra'). The presence of this link allows collation of data distributed between the two tables and to edit the accession passport using the format presented in Figure 3.4.

T16692

C. arabica origin ET-4 (ORSTOM collection, 1966)

Origin of the genetic resource

Collecting country:	Ethiopia
Collecting site:	Father J. Araya's farm (1720m), 10km W Bonga, Kaffa province
Collecting date:	20/11/1966
Collected material:	seeds of a spontaneous coffee
Collector(s):	J.L. Guillaumet & F. Hallé
Synonym(s):	Ar 4

Source of the accession

Donor name:	IRCC, Paris
Source country:	France
Introduction date:	1985/08
USDA number:	
Other identification:	IRCC 201
Observations:	

T3432

C. arabica var. Maragogipe

Origin of the genetic resource

Selection country:	Brazil
Selection site:	Maragogipe, Bahia
Selection date:	1870
Genealogy:	Mutation of a dominant gen (*MgMg*) in var. Typica
Breeder(s):	C.J. Fernandes
Synonym(s):	Maragogype, Pretoria

Source of the accession

Donor name:	Instituto Agronômico de Campinas
Source country:	Brazil
Introduction date:	1956/02
USDA number:	227711
Other identification:	Brasil X 47127
Observations:	red fruits

Figure 3.3. Examples of passport data of wild (T16692) and cultivated (T3432) coffee accessions.

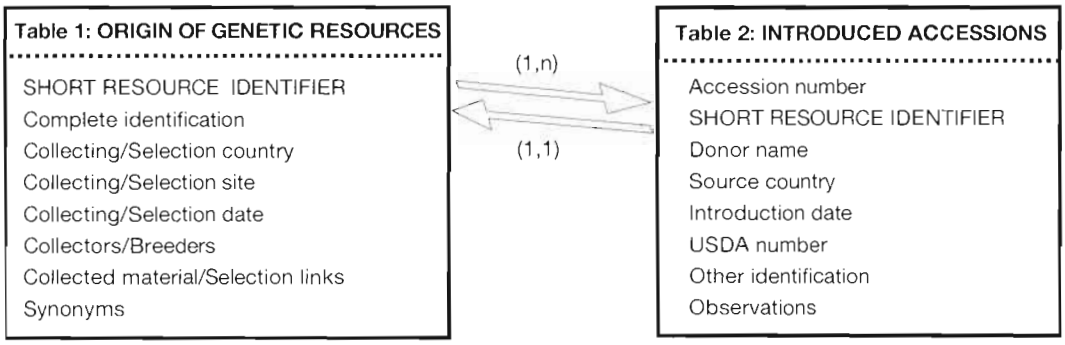

Figure 3.4. Tables of passport data with their links in the database 'CaféBase'. The access keys are indicated using bold letters. The field common to both tables is written in capital letters.

Towards computerized management

Establishment of computerized management of the CATIE genebank came up against the problem of absence of coding of genotypes and of planting sites. The problem of a single number identifying several genotypes has been already noted in this chapter. The absence of a coding system of locations for coffee trees in the field constitutes another obstacle to management computerization. It is fundamental to be able to identify the rows of coffee trees and their position within the row.

Analysis of genetic erosion

Genetic erosion between 1993 and 2002 has been estimated in three areas of the coffee genebank of CATIE:

- Section A, which contains predominantly accessions introduced in the period 1950–60, mainly coffee trees originating from selection.
- Wild coffee trees from the FAO collecting mission in Ethiopia (Fernie et al. 1968), which were planted in section C in 1965.
- Wild coffee accessions from the ORSTOM collecting mission in Ethiopia (Guillaumet and Hallé 1978), which were planted in section F in 1985–86.

Tree mortality, estimated by the number of dead trees, was slightly higher in sections A (14.9%) and F (15.7%) than in section C (11.6%) (Table 3.2). However, in terms of lost accessions, genetic erosion reached an average of 3.6%. This figure concealed significant differences between the three areas of the genebank, as erosion reached 8.2% in section F, but only 2% and 3.6% in sections A and C, respectively. Although coffee trees in section F were planted 20 years after those of section C, the higher mortality in section F cannot be explained by difference in age. The explanation lies more likely in the genetic nature of accessions. As in other large coffee genebanks (e.g. Ethiopia, Kenya, Côte d'Ivoire), cultural practices are close to those employed in commercial plantations and may not be appropriate for the conservation of wild plants collected in the forest (Dulloo et al. 2001). In section F, 45% of introduced trees were lost after eight years in the genebank (Bertrand et al. 1993). This indicates that survival of wild coffee trees was affected soon after their introduction into the field genebank. The greater survival recorded in the oldest plot (section A) can be explained by the higher initial number of trees per accession kept in the collection in this part of the genebank.

Within a given plot, genetic erosion tends to accelerate with the duration of conservation, and thus the age of plants. An analysis, using number of trees per accession as indicators, was

performed for accessions represented by one or two genotypes in the CATIE genebank, i.e. those most threatened in the short term. The erosion rate of these accessions increased regularly during the period 1993–2002 in the three areas of the genebank considered, going from 23% to 29% in section A, from 28% to 33% in section C and from 30% to 51% in section F (Figure 3.5). This analysis shows the seriousness of the situation in section F, where more than one accession out of two is threatened.

Table 3.2. Genetic erosion estimated by the percentage of dead trees and lost accessions in three areas of the CATIE genebank between 1993 and 2002.

Section	Age	Genetic origin	Dead trees	Lost accessions
A	> 45 years	Cultivated	14.9%	2.0%
C	40 years	Wild	11.6%	3.6%
F	20 years	Wild	15.7%	8.2%

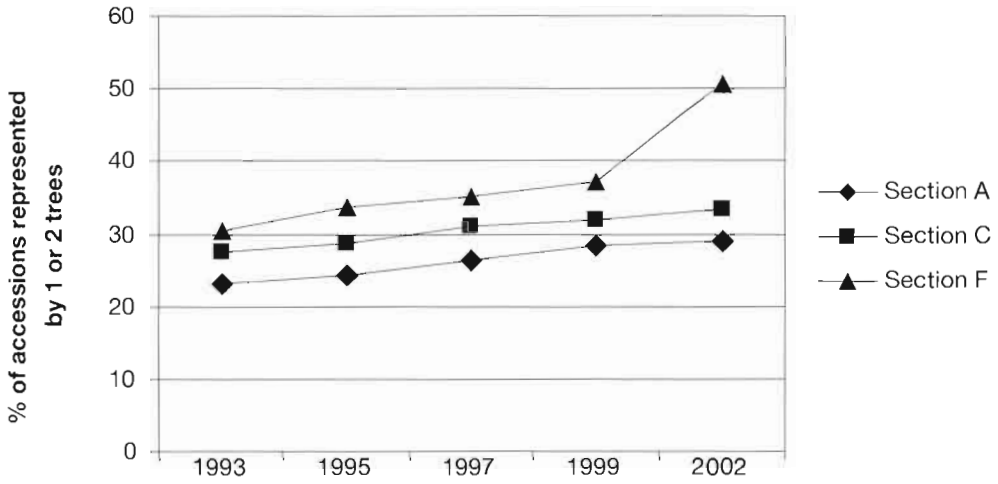

Figure 3.5. Evolution between 1993 and 2002 of the percentage of coffee accessions represented by one or two trees in sections A, C and F of the CATIE field genebank.

New strategy for sustainable field conservation

Given that the rate of genetic erosion is increasing, it has become necessary to develop a new strategy to ensure that the coffee genetic resources are safely conserved in the CATIE field genebank. A renovation project was developed, with two main objectives:

∞ to increase conservation security in the field collection, and

∞ to reduce maintenance costs for the field collection.

These objectives apply to the multiplication of living resources and preparation of fields in the new genebank. The new strategy also identifies priorities for conservation among the accessions currently conserved in the field genebank.

Defining priorities in conservation

Priorities in conservation have been defined based on the conservation cost relative to the 'genetic value' of accessions. This genetic value has been estimated using data from genetic evaluation, or from information on genetic origin for non-evaluated accessions. Three groups of accessions have been identified, corresponding to three levels of decreasing diversity (Table 3.3). The first priority is that group with the highest genetic diversity (i.e. wild coffee and interspecific hybrids). In this group, all the genotypes have to be multiplied and planted in the new genebank. Of second priority are the heterozygous varieties and introgressed lines, where it seems necessary to conserve all accessions, but the number of genotypes can be reduced to four per accession. In the last group, a total of only eight genotypes is considered to be enough to represent accessions of low diversity (i.e. homozygous varieties, mutants and intraspecific hybrids). Applying such a strategy will allow a 30% reduction in the number of coffee trees conserved in the new genebank, without loss of genetic diversity.

Table 3.3. Priority for conservation and renovation, according to the diversity estimated in the accessions.

Priority	Diversity	Genetic origin	Conservation strategy
1	+++	Wild plants (*Coffea* spp.)	All genotypes
		Interspecific hybrids	
2	++	Heterozygous varieties	4 genotypes of each accession
		Introgressed lines	
3	+	Homozygous varieties	8 genotypes maximum, sampled in all accessions
		Mutants	
		Intraspecific hybrids	

Shading to recreate forest conditions

The use of shade in coffee plantations increases tree longevity and reduces pest and disease effects (Somarriba et al. 2004). Flowerings are less intense in plantations under permanent shade than under semi-permanent shade or open sun, which reduces tree production (Mitchell 1988). Moreover, shade trees allow the recreation of a forest niche resembling the natural habitat of wild coffee, which helps the introduced germplasm to adapt to the field genebank conditions. Plantation with native forest species can also contribute to biodiversity preservation, especially for birds, which can be as populous as in forest habitats (Perfecto et al. 1996; Rappole et al. 2003).

Grafting to improve plant growth and development

Root systems poorly adapted to the conservation environment can be compensated for by grafting on vigorous rootstocks. This can also improve resistance to pests and diseases present in the soil. At the low altitude of CATIE, no incompatibility in grafting has been found between *C. arabica* and other coffee species (Couturon 1993). Grafting *C. arabica* varieties on *C. canephora* has affected neither the female fertility (i.e. occurrence of empty fruit or with a single seed) nor the biochemical content of beans (Bertrand and Etienne 2001). Since 1995, all introduced genotypes have been grafted on a *C. canephora* rootstock variety, named 'Nemaya', because of its vigour and resistance to the main root-knot nematodes in Central America (Bertrand et al. 2002). The use of var. Nemaya has proved to be also successful in conserving in the field some *C. eugenioides* and *C. stenophylla* genotypes that had not survived several attempts at growing them on their own roots (J. León, pers. comm.).

Spacing to optimize ground occupation

Coffee species exhibit large diversity in plant habit and morphology: from small shrubs (e.g. *C. brevipes*) to trees exceeding 10 m in height (e.g. *C. liberica*). Within *C. arabica* species, plants with dwarf habit due to gene mutation have to be separated from tall plants, such as wild coffee. Several dwarfism genes have been identified in *C. arabica* (Carvalho et al. 1991). The most famous gene is the *Ct* dominant gene from the variety Caturra, which has been transferred into numerous introgressed lines. Adopting a plantation scheme that allows optimal ground occupation helps to reduce the maintenance cost of the genebank. In practice, the density can vary from 1000 to 3000 trees per hectare, but this number can be greater in the case of dwarf coffee forms.

Conclusions

The management method applied to the coffee genetic resources in CATIE could be used to rationalize other large genebanks of perennial plants. Groups of accessions were defined using available data on the origin of introduced material, and then the genetic groups were submitted to genotypic and phenotypic evaluation (see Chapter 4). Information on the structure of genetic diversity was finally used to define priorities for conservation, giving more weight to genetic groups containing high diversity. Application of such an integrated strategy allows resources (financial, human, technical, spatial) allocated to conservation to be optimized, thus increasing the efficiency of conservation.

Conserving genetic resources in the field is indispensable for evaluating them. However, field genebanks appear to be very vulnerable to local hazards and consequences of global climatic change, as well as from financial resource constraints. As genotypic selection and genetic drift occur in coffee genetic resources maintained *in vitro* (Dussert et al. 1997), research efforts have been focused on the development of a cryopreservation method as a complementary conservation measure, in order to overcome the limitations of field conservation (see Chapter 6). This has been done using a core collection strategy for sampling the accessions (see Chapter 5).

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IV. Characterization and assessment of *Coffea arabica* L. genetic resources conserved in the CATIE field genebank

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Introduction

Awareness of the importance of plant genetic resources increased greatly in the 20th century as it became increasingly evident that this heritage was under threat. The international community responded by initiating a large-scale collection campaign with the aim of preserving as much of the available biodiversity as possible. This led to the construction of many genebanks in the 1960s. The size of these genebanks became so large that they are now very expensive to maintain and new accessions can no longer be systematically characterized. These resources will simply remain 'trite curios' if they are not characterized and assessed to enhance their use in targeted plant improvement programmes. This characterization and evaluation process is also crucial to enable efficient utilization of the genebanks' accessions and to develop core collections, thus making available the most interesting genetic resources.

The history of the CATIE coffee (*Coffea* spp.) genebank resembles that of most genebanks worldwide. Coffee genetic resources began being introduced in 1949, and this initiative continues (see Chapter 3). From 1951 to 1970, this genebank added 50 new accessions annually on average, generating several hundreds of coffee trees to plant every year for 20 years. The collection now includes 9760 coffee trees, representing 1850 accessions, more than 90% of which belong to the cultivated species *C. arabica*. It is one of the largest and most diversified genebanks for this species worldwide.

Coffee germplasm was characterized and assessed to an increasing extent as new constraints to *C. arabica* coffee tree agronomy were identified. The first introduced accessions were assessed chiefly on the basis of the size of the coffee berries and beans produced. These varieties were derived from Typica and Bourbon populations, which were disseminated throughout the world during the 18th century, as well as local varieties cropped in Ethiopia. A new selection criterion was taken into account in the 1970s, i.e. susceptibility to Coffee leaf rust, whose pathogen (*Hemileia vastatrix*) had just been identified in the Americas (Bertrand et al. 1999). Genetic factors that determine coffee resistance to this fungal disease were studied at the Portuguese *Centro de Investigação das Ferrugens do Cafeeiro* (CIFC) (see review by Avelino et al. 1999). Coffee improvement programmes then focused on the selection of the progeny of a natural interspecific hybrid (*C. arabica* × *C. canephora*), called the Timor Hybrid (Bettencourt 1973), which inherited rust resistance from its *C. canephora* parent.

This chapter presents the main characterization and assessment results concerning coffee genetic resources conserved in the CATIE field genebank. The experiments and analyses were undertaken within the framework of a coffee improvement programme that was conducted from 1993 to 2002 in Central America and the Caribbean by the *Programa Cooperativo Regional para el Desarrollo Tecnológico y Modernización de la Caficultura en Centroamérica, República Dominicana y Jamaica* (PROMECAFE) network of the *Instituto Interamericano de Cooperación para la Agricultura* (IICA), with the participation of CATIE, the *Centre de coopération internationale en recherche agronomique pour le développement* (CIRAD, France) and the *Institut de recherche pour le développement* (IRD, France). The programme was designed to broaden the genetic base of cultivated coffee varieties by tapping the diversity of wild coffee accessions collected in the centre of origin of *C. arabica*. Neutral markers (i.e. environment independent) were used for the first time to analyse genetic diversity and polymorphism in *C. arabica*. The results are presented and discussed with respect to the genetic origins of the accessions: (i) coffee accessions from the centre of origin (Ethiopia); (ii) Typica- and Bourbon-derived coffee accessions; and (iii) introgressed lines selected within interspecific hybrid progenies.

Accessions from the diversity centre of *C. arabica*

Two major coffee survey missions were conducted in Ethiopia: by FAO in 1964–65 (Fernie et al. 1968), and by ORSTOM (now IRD) in 1966 (Guillaumet and Hallé 1978). Very few evaluation data concerning these coffee trees are available in the large genebanks hosted in Brazil, Côte d'Ivoire, Ethiopia, Kenya and Tanzania. Since enzymatic markers were found to be relatively inefficient for detecting polymorphism in *C. arabica* (Berthou and Trouslot 1977), the genetic diversity structure in this species was determined after the development of polymerase chain reaction (PCR)-based markers (Lashermes et al. 1996).

Neutral diversity

A hundred and eleven coffee trees, representing 88 accessions collected in Ethiopia, were selected on the basis of their geographical origin and analysed using random amplified polymorphic DNA (RAPD) markers (Anthony et al. 2001). All accessions collected elsewhere than in Kefa and Ilubabor provinces (i.e. Gojjam, Shoa, Sidamo and Harerge provinces) were included in the sampling in order to compensate for their limited populations in the genebank. For Kefa and Ilubabor provinces, at least one accession per collection site was selected. Six accessions of varieties cropped locally in Ethiopia and two accessions of Typica- and Bourbon-derived varieties were also included in the study for comparative purposes.

Wild coffee varieties were classified into four genetic groups that clearly differ from Typica- and Bourbon-derived varieties (Figure 4.1). The Ethiopian 1 group consisted of 78 wild coffee accessions and two Ethiopian varieties. This group included all accessions collected in Gojjam, Ilubabor and Shoa provinces, virtually all of the Kefa accessions, three accessions from Sidamo and one from Harerge (Figure 4.2). This group therefore pooled almost all (apart from a few exceptions) of the accessions from south-western Ethiopia. The other groups were smaller. All accessions classified in the Ethiopian 2, 3 and 4 groups—apart from one accession from Kefa province—were collected in Harerge and Sidamo provinces, which are located east and south of the Great Rift Valley, respectively. The genetic diversity thus seems to be structured into two large complexes separated by the tectonic rift that cuts through Ethiopia from the northeast to the southwest. The Ethiopian varieties were classified in the Ethiopian 1 and 2 groups according to their geographical origins (i.e. south-west vs. south-east).

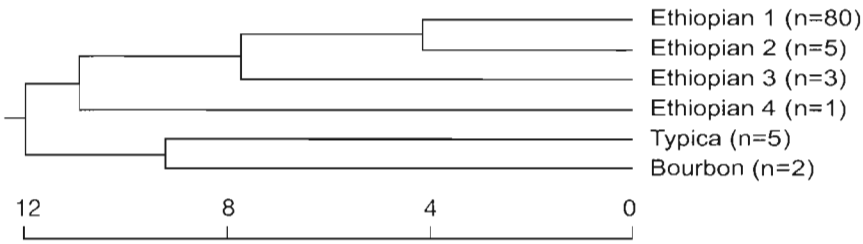

Figure 4.1. Genetic diversity structure in *C. arabica* revealed by RAPD markers (Anthony et al. 2001). The number of accessions per group is indicated in brackets.

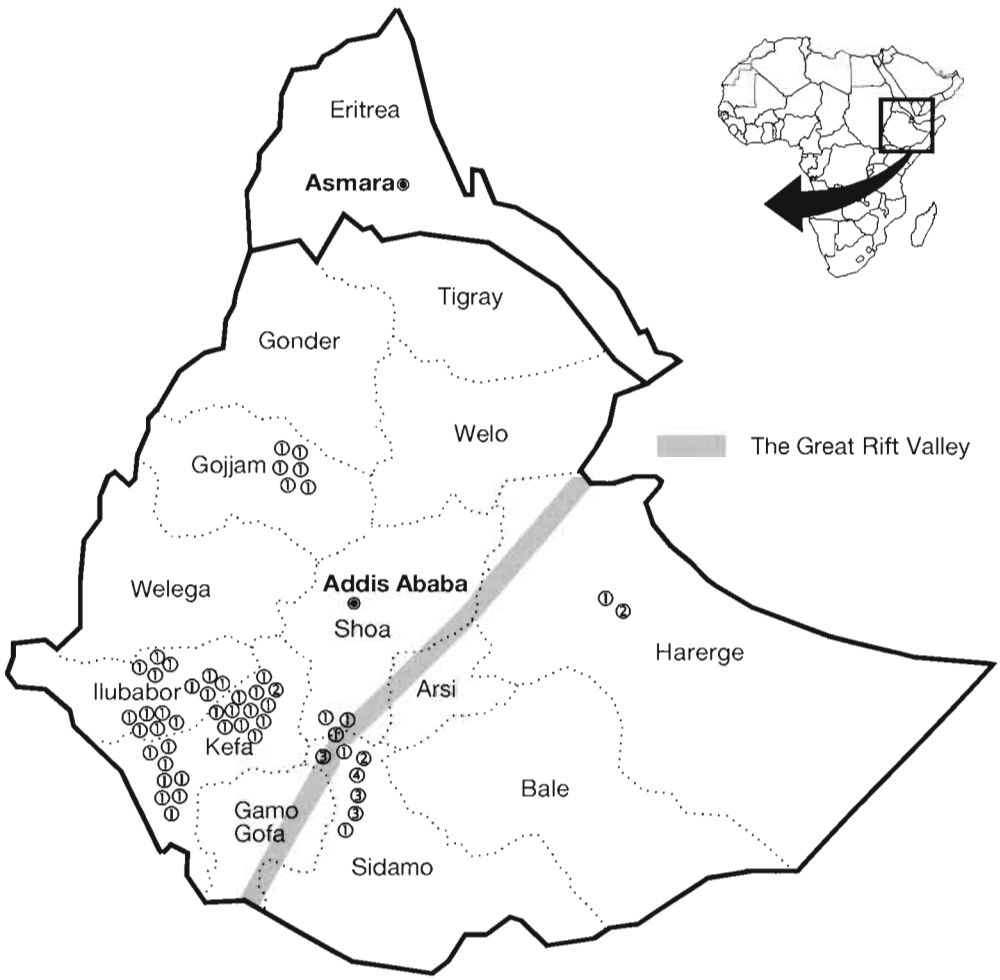

Figure 4.2. Distribution of four Ethiopian genetic groups formed on the basis of RAPD polymorphism (adapted from Anthony et al. 2001). The number of the group in which each accession was classified is circled.

Most of the diversity detected in this study was in the Ethiopian 1 group, i.e. 97% of the markers found in this group were polymorphic, whereas only 45%, 42% and 28% were detected in accessions from the Ethiopian 2, 3 and 4 groups, respectively. Only 59% of the markers produced by all accessions belonging to the three groups from the area south-east of the Great Rift Valley were polymorphic, which is much lower than the percentage noted in the Ethiopian 1 group from the south-western area. Hence, wild coffee accessions collected in Kefa and Ilubabor provinces accounted for most of the observed diversity. It is quite likely that coffee was first cultivated in this region, and this occurred around the 5th century (Lejeune 1958). Moreover, molecular marker analyses highlighted many redundancies within accessions originating from the south-western area.

Phenotypic variability

Phenotypic analyses were carried out to pinpoint wild genotypes with features that could be of interest for the regional coffee genetic improvement programme.

Morphology and fertility

Many agromorphological traits were monitored for several years in the CATIE coffee genebank (Bertrand et al. 1993; Anthony et al. 1999), including internode length, leaf size, fertility, production, and berry and bean defects. The percentage of floating berries (i.e. empty) and berries containing one bean (peaberries), rather than the usual two, were the traits that varied most (Table 4.1). Fertility was almost perfect (with two beans per berry) in some wild coffee accessions, while others produced beans about the same size as those generated by var. Maragogipe, which is famous for its large beans (Krug et al. 1939).

Table 4.1. Fertility and bean weight variability observed in 164 wild coffee trees from Ethiopia, in the CATIE field genebank in 1995 (Anthony et al. 1999).

Character	Min.	Max.	Mean	Variation
Empty fruits (%)	0.0	37.6	5.6	113%
Peaberries (%)	0.3	52.6	10.4	72%
Number of beans per fruit	1.18	2.04	1.75	8%
100-bean weight at 11% moisture (g)	11.8	23.7	17.2	13%

Pest resistance

Wild coffees in the CATIE genebank were evaluated for their resistance to root-knot nematodes (*Meloidogyne* spp.) and to two fungal diseases, Coffee leaf rust and Coffee berry disease (CBD). Resistance to three different nematode species was assessed, including two species from Costa Rica (*M. exigua* and *M. arabicida*) and one from Guatemala (*M. paranaensis*, ex *Meloidogyne* sp. or *M. incognita*). No accessions were found to be resistant to *M. exigua*, but some wild coffee trees from Ethiopia were resistant to *M. arabicida* and *M. paranaensis* (Bertrand et al. 2002; Anthony et al. 2003).

Results of tests in which coffee leaf discs were inoculated with *Hemileia vastatrix* demonstrated that 41% of the wild coffees tested were resistant to strain II, which occurs in Costa Rica (Bertrand et al. 1993). All wild coffees conserved in the genebank (1842 trees) were then screened for disease symptoms (i.e. leaf discoloration and pathogen sporulation). The trees were subsequently assessed several times a year between 1995 and 1998. More than a third of the trees showed no disease symptoms by the end of the assessment period (F. Anthony, unpublished data).

Eighty-two wild coffee accessions were screened by CIRAD for resistance to *Colletotrichum kahawae*, the causal agent of Coffee berry disease (CBD) (Berry and Bieysse, unpub. data). This

led to the identification of genotypes that presented much milder CBD symptoms in comparison with susceptible commercial coffee varieties.

Male sterility

Male sterility is also a very interesting trait for breeding programmes geared towards the selection of heterozygous hybrids (e.g. F_1 hybrids). The transfer of male sterility into a variety will allow dissemination of the heterozygous hybrids as seeds produced by crossing the male-sterile variety and a wild progenitor. Pollen production was checked in more than 7000 coffee accessions hosted in the CATIE genebank (Dufour et al. 1997). Non-pollen-producing genotypes were detected in the wild coffee population, but not in accessions that had been obtained through selection. Studies conducted in Brazil revealed that male sterility is under recessive genetic control in coffee (Mazzafera et al. 1989).

Accessions from selection

The three coffee varieties most cropped worldwide (i.e. vars. Caturra, Catuai and Mundo Novo) are derived from Typica and Bourbon populations that were first disseminated from Yemen in the 18th century. Narrative histories indicate that the Typica population was formed by progeny of a single plant that was cultivated in Amsterdam, and that the Bourbon population was formed by several coffee trees that were introduced to La Réunion (previously called Bourbon Island) (see review by Anthony et al. 1999). Coffee varieties or mutants derived from these populations thus have a narrow genetic base, which limits their breeding potential. This constraint did not, however, prevent growers and breeders from isolating an impressive number of varieties and mutants (Krug et al. 1939; Chevalier 1947). As coffee cropping intensified during the 20th century, their susceptibility to most diseases and pests that occur in coffee plantations (Coffee leaf rust, CBD, nematodes, coffee berry borers, etc.) was revealed (see reviews by Bertrand et al. 1999; Flood et al. 2001).

Neutral diversity

The genetic diversity of coffee trees selected in Typica and Bourbon populations was investigated using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) molecular markers (Anthony et al. 2002). The material assessed consisted of coffee varieties, mutants and hybrids (Typica × Bourbon), in addition to coffee trees cropped in Yemen (Eskes 1989) and a few wild Ethiopian coffee trees. Two distinct groups were revealed, matching their Typica or Bourbon genetic origins, by determining genetic distances in pairs of accessions (Figure 4.3). Catuai coffee trees were classified intermediate between Typica- and Bourbon-derived accessions, in agreement with their hybrid origin. As also noted in a previous study on the genetic diversity of wild coffee (Anthony et al. 2001), the Ethiopian coffee trees differed from Typica- and Bourbon-derived accessions, without forming well-structured groups.

Genetic diversity was found to be low in selected coffee trees. Only 51% and 55% of the markers identified in this study were detected in Typica- and Bourbon-derived accessions, respectively, while the wild accessions had 90%. This diversity was also low in coffee trees from Yemen, which contained 50% of the markers identified. Typica- and Bourbon-derived coffee accessions were distinguished by seven markers specific to each population (i.e. present in all accessions of one population but absent in all accessions of the other population). Polymorphism was substantially reduced in Typica- and Bourbon-derived accessions, with 13% and 24% polymorphic markers, respectively. By comparison, 98% of the markers identified in wild coffee accessions were polymorphic.

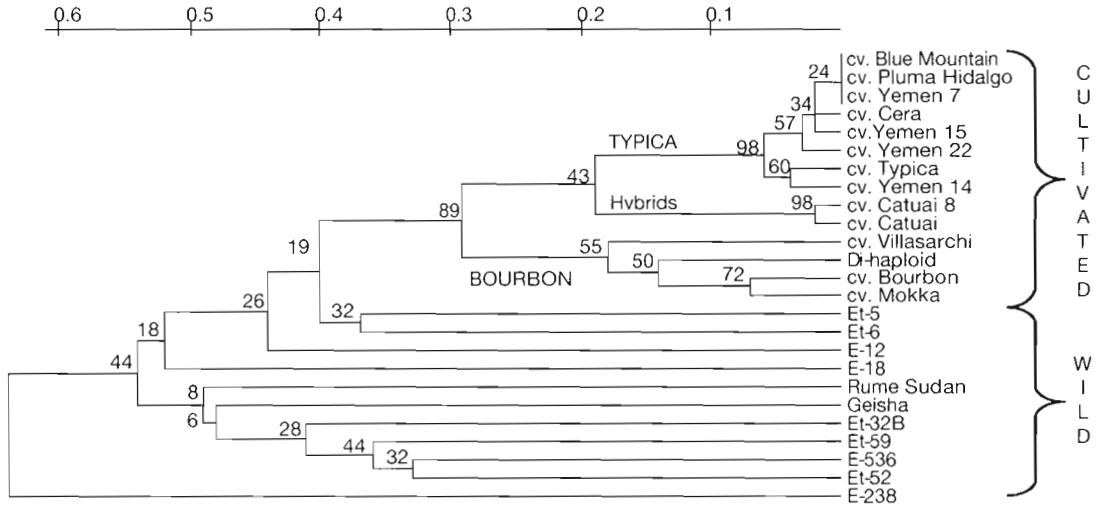

Figure 4.3. Dendrogram of 25 coffee accessions determined according to AFLP-based genetic distance (Anthony et al. 2002). Numbers on the branches are bootstrap values (%) obtained after 200 replicate analyses.

Phenotypic variability

Scientists visiting CATIE's coffee genebank are often surprised by the high variability in phenotypic traits. This feature does not seem to be related to the narrow genetic base of Typica and Bourbon populations that have been used in selection programmes for more than 150 years now. Polymorphism is especially evident in the architecture of coffee trees and in the morphology of their leaves and berries. This apparent diversity is partially due to the presence of many mutants that were isolated in different research centres worldwide and subsequently included in CATIE's coffee genebank. Mutations have had an impact on a wide range of different characters, resulting in stunted growth (e.g. vars. Caturra, San Bernardo and San Ramon), large-sized leaves and beans (e.g. var. Maragogipe), purple leaves (e.g. var. Purpurascens), erect branches (e.g. var. Erecta) and yellow endosperm (e.g. var. Cera). Most of these mutations involved only one gene, sometimes having pleiotropic effects throughout the plant (Carvalho et al. 1991), as noted with the recessive *lr* mutation in var. Laurina, which produces cone-shaped coffee trees with small narrow leaves, narrow beans that are pointed at one end, and whose caffeine content is half that of commercial varieties (Lopes 1971).

Variations observed within Typica- and Bourbon-derived accessions also account for the high apparent variability in CATIE's genebank. Significant differences in agro-morphological traits monitored in leaves, berries and beans were noted between trees of the same accession (Astorga 1999). This polymorphism likely occurred in response to suboptimal conditions when *C. arabica* trees were grown at low elevation (602 m). It was still possible to separate Typica- and Bourbon-derived accessions ($p<0.0001$) on the basis of the colour of young leaves (bronze in Typica *vs.* light green in Bourbon), as delineated by Krug et al. (1939).

Accessions from interspecific hybrid progenies

Rust-resistant introgressed lines currently disseminated worldwide are derived from three interspecific tetraploid hybrids, i.e. Timor Hybrid and Icatu obtained via a *C. arabica* × *C. canephora* cross in Latin America, and S.26 (*C. arabica* × *C. liberica*) in India. At CATIE, Timor Hybrid-derived lines (F_5–F_7) are being assessed, mainly for vigour, production and rust resistance, through

the PROMECAFE network. One advantage of interspecific tetraploid hybrids is that they can undergo recombination, which does not seem to be limited by genetic differentiation of chromosomes from different genomes (Herrera et al. 2002). Genes of these hybrids thus seem to be especially suitable for introgression into the genome of *C. arabica* varieties.

Identification of introgressed DNA

Twenty-one Timor Hybrid-derived accessions were analysed for the introgression of *C. canephora* genetic material using AFLP markers (Lashermes et al. 2000). They were compared with 23 *C. arabica* accessions and 8 *C. canephora* accessions. The Timor Hybrid-derived accessions were distinguished from the *C. arabica* accessions by 178 markers, consisting of 109 additional bands (i.e. present in *C. canephora* and absent in *C. arabica*) and 69 missing bands (i.e. present in *C. arabica* and absent in *C. canephora*). The number of additional and missing bands ranged, respectively, from 18 to 59 and from 0 to 32 among the Timor Hybrid-derived accessions (Figure 4.4). The introgressed fragments were estimated to represent 8% to 27% of the *C. canephora* genome. Assuming that a unique genotype of *C. canephora* was involved in the formation of the Timor Hybrid, the overall 109 introgressed fragments identified in the Timor Hybrid-derived accessions were estimated to represent 51% of the *C. canephora* genome. Most of the introgressed chromosome segments were not eliminated or counter-selected during the selfing and selection process. The introgression was not restricted to chromosome substitution but also involved chromosome recombination, as shown by Herrera et al. (2002).

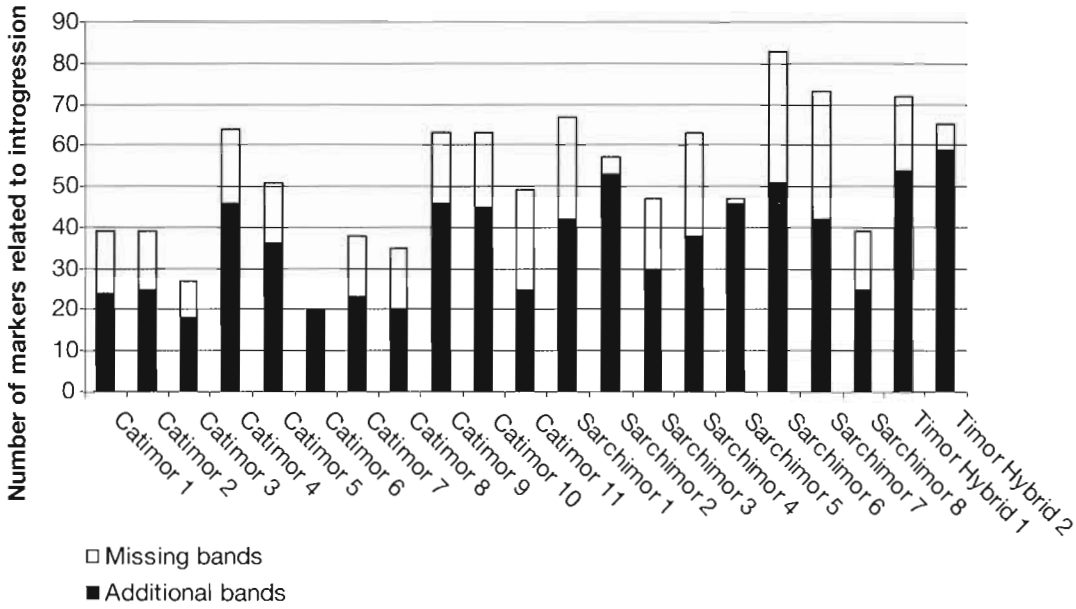

Figure 4.4. Number of AFLP markers attributable to introgression detected in Timor Hybrid-derived genotypes (Lashermes et al. 2000).

Phenotypic variability

The agronomic performances of 27 Timor Hybrid-derived lines were compared with those of two commercial coffee varieties, Caturra and Catuai (Bertrand et al. 1997a), focusing specifically on growth, production and fertility traits, as well as resistance to rust, nematodes and CBD. The results highlighted significant differences between lines for all monitored traits. Lines resistant to

rust or *M. exigua*, or both, were identified. The level of resistance to *M. exigua* was found to be as high in these lines as in *C. canephora* coffee trees (Bertrand et al. 2001). Lines resistant to corky-root, caused by *M. arabicida* and *Fusarium oxysporum* f.sp., were also detected (Bertrand et al. 2002).

Cup quality and chemical composition were studied among 22 introgressed Timor Hybrid-derived lines and compared with data from three non-introgressed varieties (Bertrand et al. 2003). Variability in the analysed characters was found to be rather high in the introgressed lines. There were significant differences between lines for all biochemical compounds analysed, and for acidity and overall standard. Two lines were significantly poorer than the controls with respect to sucrose and beverage acidity. One of them also had a higher chlorogenic acid content and had a poorer overall standard. However, two highly introgressed lines did not differ from the non-introgressed varieties.

Conclusion

C. arabica accessions conserved in the CATIE genebank were classified in three groups on the basis of their genetic origins, as determined by a review of narrative histories on the dissemination of coffee trees worldwide, along with the history of coffee improvement initiatives. The classified groups are as follows: coffee trees from the centre of origin of the species; Typica- and Bourbon-derived varieties and mutants; and introgressed lines selected within interspecific hybrid progeny (*C. arabica* × *Coffea* spp.). The results of the assessment and characterization of these resources highlighted features specific to each of these groups with respect to their innate genetic diversity and extent of polymorphism.

Neutral markers revealed high genetic diversity in coffee trees that were collected in Ethiopia, in contrast with the low diversity detected in Typica- and Bourbon-derived varieties. Polymorphism also seemed to be relatively high in wild Ethiopian coffee trees, but very low in cultivated coffee trees. Phenotypic traits of interest for improvement programmes were found in Ethiopian coffee trees, including male sterility, resistance to Coffee leaf rust and root-knot nematodes. Some of these coffee accessions were found to have almost perfect fertility or an exceptional bean size, or both. Wild coffee trees from the centre of origin thus represent a diversity reservoir that could be tapped to broaden the genetic base of cultivated coffees. Moreover, crosses between wild coffee trees and cultivated varieties generated very productive and vigorous F_1 hybrids in Costa Rica (Bertrand et al. 1997b; Bertrand 2002).

Introgressed lines differ markedly from wild and cultivated coffees. Chromosomes from different genomes can be recombined in first-generation interspecific hybrids, often giving rise to novel polymorphic traits. After five to seven selfed generations, introgressed fragments were found to vary markedly from one line to another, and high variability in the chemical contents of beans and in cup quality were observed. This organoleptic variability could be utilized by selecting lines that produce good quality coffee while also being resistant to coffee diseases and pests.

The genetic resource assessment data discussed in this chapter could now be used to build core collections for long-term germplasm conservation, evaluation and exchange purposes.

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V. Construction of coffee core collections

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Introduction

The concept of a 'core collection' was proposed to enable efficient and cost-effective management and utilization of crop genetic resources (Frankel 1984; Frankel and Brown 1984) and has been interpreted in various ways (van Hintum et al. 2000). Frankel (1984) defined a core collection as a limited set of accessions representing, with a minimum of repetitiveness, the genetic diversity of crop species and its wild relatives. For practical uses, the core collections allow setting up a large representation of the genetic diversity within a reduced set of genotypes, which can be intensively evaluated and widely distributed. However an IPGRI survey of 1346 genebanks and institutions worldwide pointed out considerable confusion and lack of knowledge on what a core collection is (Brown and Spillane 1999). For all intent and purposes, core collections were never intended for conservation purposes, but rather to facilitate use of conserved material. If core collections are to have a meaningful impact on management of germplasm collections, there is a need for greater consensus and knowledge among the curators and users on what is and is not a core collection.

The experience reported here is based on the construction and management of *C. arabica* core collections in CATIE, firstly for evaluation (Anthony et al. 2001), then for long-term conservation (Vasquez et al. 2005).

Coffee core collections

Genetic diversity is not randomly distributed among species and populations, but it can generally be represented by a hierarchical model, a tree (Hamon et al. 1995; Noirot et al. 2003). This model was adopted for constructing coffee core collections, using passport data combined with knowledge of the structure of the gene pools (Noirot et al. 1993).

Evaluation purposes

A representative core collection of the Ethiopian accessions conserved in the CATIE genebank was defined prior to genotypic evaluation (see Chapter 4). Considering the molecular analysis capacity in the CATIE biotechnology laboratory, the core collection was finally composed of 88 Ethiopian accessions (109 genotypes). The sampling was based on geographical data, namely the collecting sites of the FAO (Fernie et al. 1968) and ORSTOM (Guillaumet and Hallé 1978) surveys in Ethiopia. All accessions from the provinces outside south-west Ethiopia were analysed because of their low representation in the genebank (Table 5.1). A selection was necessary within the 482 accessions from south-west Ethiopia. The selection was based on the collecting sites, assuming that the accessions derived from spontaneous trees growing in forest or from subspontaneous plants cultivated on small farms presented a higher diversity than those collected in semi-industrial and industrial farms, where growing coffee trees were not usually derived from spontaneous plants. In fact, visitors to Ethiopia have reported that

it is very difficult to establish from where *C. arabica* is truly native, as it spreads rapidly from cultivation and becomes naturalized in clearings and along trails in the forest (Sylvain 1955; von Strenge 1956; Meyer 1965; Friis 1979). Based on RAPD analysis carried out at CATIE, four groups of accessions were identified (Anthony et al. 2001): one large group in the south-west (Kefa and Ilubabor provinces) and three smaller groups in the south and south-east (Sidamo and Harerge provinces) (see Chapter 4). This result was in accordance with historical data on coffee domestication in south-west Ethiopia (Sylvain 1955; von Strenge 1956; Lejeune 1958). A similar structure of Ethiopian diversity was found using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers (Anthony et al. 2002).

Table 5.1. Geographical origin within Ethiopia of the accessions included in the *C. arabica* core collection for neutral marker analysis of diversity (Anthony et al. 2001).

Region	Province	No. of collection sites	Living accessions in CATIE genebank	No. of accessions selected
South-west	Kefa	337	356	38
South-west	Ilubabor	116	126	31
South	Sidamo	8	8	8
South-east	Harerge	2	2	2
Centre	Gojjam	6	6	6
Centre	Shoa	2	2	2
North	Eritrea[†]	1	1	1

Notes: [†] At the time the accessions were collected, the area was a province of Ethiopia.

Table 5.2. Composition of the *C. arabica* core collection (74 accessions) representative of the genetic diversity present in wild and cultivated accessions from Ethiopia and Yemen, and conserved in the CATIE field genebank.

Origin	Selected accessions
WILD (FAO collection)	T.4472, T.4476, T.4495, T.4497, T.4501, T.4505, T.4579, T.4619, T.4621, T.4661, T.4662, T.4664, T.4665, T.4666, T.4758, T.4759, T.4819, T.4824, T.4837, T.4857, T.4863, T.4864, T.4865, T.4893, T.4900, T.4938, T.4942, T.4945, T.4952, T.4958, T.4960
WILD (ORSTOM collection)	T.16689, T.16690, T.16691, T.16692, T.16694, T.16695, T.16697, T.16700, T.16702, T.16704, T.16705, T.16706, T.16707, T.16709, T.16712, T.16713, T.16714, T.16723, T.16724, T.16726, T.16729, T.16733, T.16737, T.16739, T.17177, T.17205, T.17207, T.17223, T.17232
CULTIVATED (locally in Ethiopia)	T.2710, T.2711, T.2722, T.2724, T.2727, T.2742, T.2748, T.2754, T.2915, T.3097, T.4007
CULTIVATED (locally in Yemen)	T.21233, T.21239, T.21240

Cryopreservation purpose

The classification of Ethiopian accessions into genetic groups was used for constructing a representative core collection of the *C. arabica* gene pool for long-term conservation in liquid nitrogen (see Chapter 6). The sampling also included accessions representing varieties locally cultivated in Yemen, which is considered as the primary centre of *C. arabica* dispersion outside Ethiopia (Meyer 1965). The maximum number of accessions to be included in the core collection was

imposed by financial constraints on storage capacity. It was decided to include 74 accessions in the core (Table 5.2). Because of self-compatibility of *C. arabica*, one genotype per accession was harvested in the CATIE genebank. It was selected on the basis of phenotypic data (not published), principally resistance to Coffee leaf rust (*Hemileia vastatrix*) and root-knot nematodes (*Meloidogyne* spp.). The core collection could thus be considered as representative of the diversity detected by neutral markers, and in addition it was improved in resistance genes.

Conclusion

The construction of core collections provides genebank managers, breeders and research scientists with a manageable number of accessions for their work. The strategy described for coffee could be easily applied to other crops, especially non-orthodox-seed species. Prior to evaluation, a whole collection can be stratified using the data on accession origin, which are commonly recorded in genebanks. After genotypic evaluation, the accessions can be classified according to their genetic group and then sampled within the groups. This contributes to optimize the genetic diversity retained in a subset of the whole collection for long-term preservation.

A pragmatic attitude was adopted for sampling the coffee accessions, taking into account phenotypic traits of interest for breeders as well as technical constraints limiting the core collection size. As frequently mentioned in other crops, the major constraint in constructing core collections is the availability and reliability of data (Ng and Padulosi 1992). It seems important to remember that a core collection formed by simple random sampling of the accessions has surprisingly good retention statistics (Brown 1989) and might actually be better than one biased by poor data (Brown and Spillane 1999).

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VI. Cryopreservation of coffee genetic resources

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Introduction

Because of the non-orthodox storage behaviour of their seeds (van der Vossen 1977; Ellis et al. 1990), coffee (*Coffea* spp.) genetic resources are conserved in field collections. However, since coffee field collections are costly to maintain (Charrier et al. 1989) and leave the material exposed to biotic and abiotic hazards (see Chapter 3), research for alternative methods to field conservation has become a priority (see Chapter 1).

Cryopreservation, i.e. storage at ultra-low temperature of biological material (-196°C, in liquid nitrogen), is the only technique available to ensure safe and cost-effective long-term conservation of coffee germplasm (see Chapter 1). For *C. arabica*, which is autogamous and seed propagated, attention has to be given to seed cryopreservation. Despite early pessimistic reports on the feasibility of coffee seed cryopreservation (Becwar et al. 1983; Stanwood 1985), considerable efforts have been made since 1997 at IRD-Montpellier (France) to investigate the basis of the high sensitivity of coffee seeds to liquid nitrogen (LN) temperature, which research led to the development of a cryopreservation procedure providing satisfactory survival percentages.

Through several years of research using seeds of two well-characterized types (Bourbon and Typica) as models, it was shown that tolerance to LN exposure of whole seeds of *C. arabica* is a complex phenomenon. The interval of water contents allowing seed survival is very narrow, i.e. 0.20-0.23 g $H_2O.g^{-1}$ dw (Dussert et al. 1997, 2003a), and the optimal water status corresponds to the unfreezable water content of seeds (Dussert et al. 2001). After desiccation to the optimal water content and exposure to LN, a high proportion of seeds show hypocotyl and radicle extrusion but a very low percentage of them develop into normal seedlings (Dussert et al. 1997; Eira et al. 1999). If embryos are extracted from frozen seeds after thawing, and then cultivated *in vitro*, a very high proportion of them produce a normal seedling, indicating a different sensitivity to LN exposure between the endosperm and the embryo (Dussert et al. 1997). The percentage of seeds developing into normal seedlings after LN exposure is partly improved by slow cooling of seeds (Dussert et al. 1997). Moreover, controlled rehydration of seeds, through a 6-week osmoconditioning treatment in a –1.25 MPa polyethylene glycol solution, after thawing and before culture under germination conditions, also increases the percentage of seeds developing into normal seedlings (Dussert et al. 2000).

In this way, the percentage of seeds surviving after cryopreservation was improved step by step, reaching values around 40% and 70% in Typica and Bourbon, respectively, in 2000. These percentages of seedling recovery were judged sufficiently high to consider the transfer of the procedure developed at IRD to a coffee genebank and to test whether it could be used as standard protocol for cryopreservation of *C. arabica* genetic resources. It is in this framework that the Bioversity, IRD and CATIE collaborative project on cryopreservation of wild coffee was initiated. The main objective of this study was to test the effects of immersion in LN and post-thaw osmoconditioning on seed and embryo viability within a set of 30 accessions of the CATIE field genebank. This set of 30 accessions is a subset of the 74 accessions of the CATIE coffee core collection (see Chapter 5).

The aim of this chapter is to present the results obtained in the Bioversity-IRD-CATIE collaborative project, whose results have been published recently (Vasquez et al. 2005). Their application to the choice of the standard protocol for the CATIE coffee core collection is also described. The need for optimizing some of the steps is then discussed, taking into account new progress made at IRD subsequent to the project described here.

Choice of the core subset of 30 accessions

The thirty accessions of *C. arabica* used in this study are presented in Table 6.1. They were selected among the 74 accessions of the CATIE coffee core collection (see Chapter 5), which were themselves selected from the CATIE field genebank on the basis of their molecular and agronomic evaluation (see Chapter 4). Twenty-seven accessions were randomly chosen to represent the genetic diversity of the wild material collected by FAO (Fernie et al. 1968) and ORSTOM (Guillaumet and Hallé 1978) in the centre of origin of *C. arabica* (south-west Ethiopia). Two accessions were selected to represent Ethiopian cultivated varieties collected in the same area. The variety Caturra (Bourbon type) was included into the core subset as a comparative cross-reference to the plant material used in previous studies (Dussert et al. 1997, 2000).

Table 6.1. The 30 accessions from the CATIE field genebank analysed for their seed response to cryopreservation.

Type	Origin	Accession number
Wild	Kefa Province	T.4495; T.4621; T.4661; T.4664; T.4665; T.4900; T.16689; T.16697; T.16723; T.16724; T.16726; T.16729; T.16737; T.16733
	Ilubabor Province	T.4824; T.4837; T.4857; T.4863; T.4865; T.16695; T.17177; T.16694; T.16700; T.16702; T.16706; T.16707; T.16712
Cultivated in Ethiopia	Var. Cioiccie	T.2710
	Var. Loulo	T.4007
Cultivated worldwide	Var. Caturra	

Seed preparation, desiccation and cryopreservation

For each accession of the core subset, fresh fruits were harvested manually from the field collection. After wet-process depulping, seeds were partially dehydrated in the shade at ambient temperature for two weeks. Seed water content upon receipt in the laboratory was 0.3-0.4 g $H_2O.g^{-1}$ dw for all accessions. Seeds were desiccated by equilibration for 3 weeks under 78% relative humidity, obtained using an NH_4Cl saturated solution, as described in Dussert et al. (2000, 2001). Seed water content at equilibrium varied between 0.21 and 0.23 g $H_2O.g^{-1}$ dw, depending on the genotype.

The water content of seeds was estimated using 10 replicates of one seed and their dry weight measured after 2 days of desiccation in an oven at 105°C. Before cooling, seeds were hermetically sealed in 15 ml polypropylene tubes (50 seeds per tube). Seeds were precooled to -50°C at 1°C/min using a Cryomed © programmable freezing apparatus, then immersed in LN. Seeds were stored at -196°C for at least one week before thawing. Thawing was carried out by plunging the tubes in a 40°C water-bath for 4 minutes.

After thawing, seeds were either placed directly in germination conditions, or osmoconditioned for 6 weeks before their transfer to germination conditions. Seed culture was carried out according to the method described by Dussert et al. (1997). Osmoconditioning was carried out at 27°C in the dark by placing batches of ten seeds in Petri dishes sealed with Parafilm™ Ribbon on a thin layer of cotton wool imbibed with 20 ml of a -1.25 MPa aqueous PEG 6000 solution, as described in Dussert et al. (2000). Zygotic embryos were extracted from desiccated or desiccated and frozen seeds after disinfection, and cultured *in vitro* for survival assessment. Disinfection, extraction and culture were performed as described in Dussert et al. (1997).

Seed and embryo viability after desiccation

Very high variability was observed within the 30 accessions studied for the viability of seeds after desiccation, since it ranged from 11 to 100%, as estimated by the percentage of seeds developing into normal seedlings when placed in germination conditions (Figure 6.1). In contrast, viability of embryos extracted from desiccated seeds showed little variation and was always very high, since it ranged from 72 to 100%.

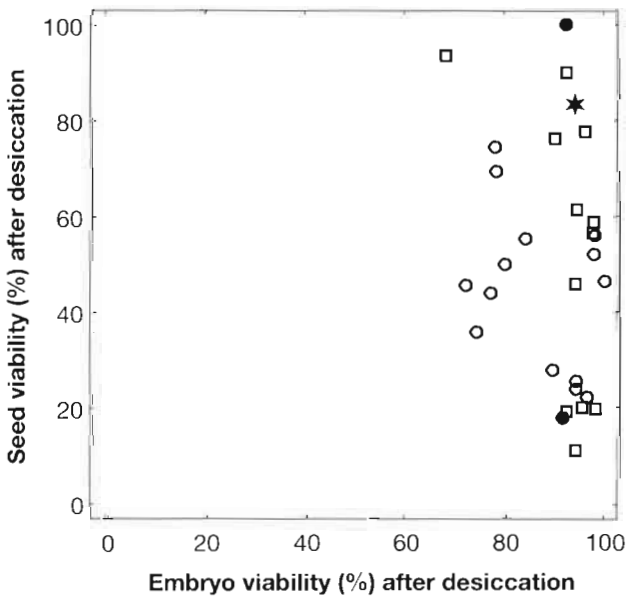

Figure 6.1. Relationship between seed viability (normal seedling development) after desiccation and viability of embryos extracted from desiccated seeds within the core subset studied. The subset included 27 wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

There was no significant correlation between viability of seeds after desiccation and that of zygotic embryos extracted from desiccated seeds. Variability for seed viability after desiccation was not significantly correlated to seed size, nor to seed water content after desiccation. No association was found between the origin of the plant material and seed response to desiccation.

The possibility that this variability is associated with variability for desiccation tolerance cannot be excluded. However, previous studies have shown that the intraspecific variability for seed desiccation tolerance was very low in *C. arabica*, with a loss of seed viability occurring always below 0.12 g $H_2O.g^{-1}$ dw (Ellis et al. 1990; Eira et al. 1999).

From our results, we can rule out that this variability is due to differences in the ability of embryos to develop into normal seedlings, since this was high, with a mean value of 90.1%, independent of seed viability after desiccation. It can thus be speculated that this variability is associated with differences in endosperm quality at harvest. The nature of these differences in endosperm quality are unknown, but they might be related to differences between accessions in the time to achieve complete maturation, or to differences in response of seeds to the post-harvest process, which has been developed for traditional varieties and might be inappropriate for wild forms of *C. arabica*.

Seed viability after cryopreservation and rapid rehydration

Throughout four years of work with Typica and Bourbon seeds (Dussert et al. 1997, 2000, 2001), very low variability was observed among repetitions for the proportion of seeds developing into normal seedlings after cryopreservation under the same conditions, which was always about 15% of the desiccation control. Similarly, with seeds of four other varieties, Eira et al. (1999) observed viability percentages between 10 and 30% after desiccation to the same water content and LN exposure.

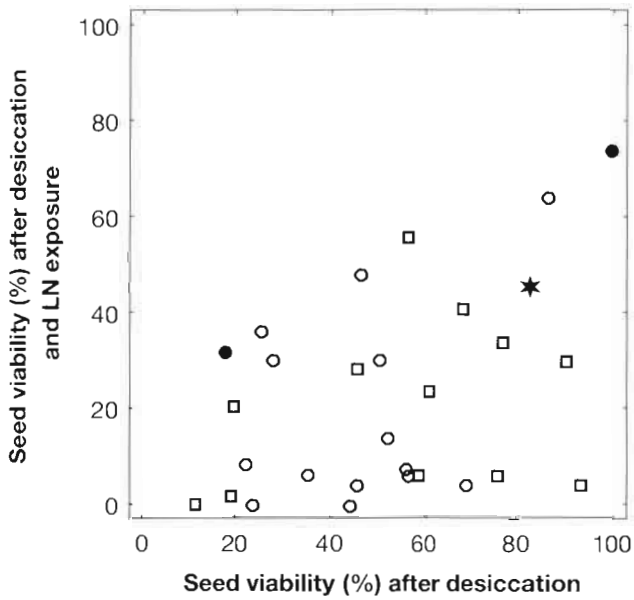

Figure 6.2. Relationship between seed viability (normal seedling development) after desiccation and LN exposure and viability of seeds after desiccation only, within the core subset studied. The subset included 27 wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (★).

In contrast, in the present study, a very high variability for seed sensitivity to LN exposure was observed within the 30 accessions studied. Seed survival after cryopreservation varied from 0 to 74% (0 to 100% when expressed as a percentage of the desiccation control). It is also illustrated in Figure 6.2 by the fact that the points are widely spread between the y = x line (the most LN-tolerant accessions) and the y = 0 line (the most LN-sensitive accessions).

When expressed as the percentage of the desiccation control, seed sensitivity to LN exposure was not correlated to seed size, seed water content after desiccation, or seed and embryo viability after desiccation. There was no apparent association between the origin of the plant material and seed sensitivity to LN exposure. The causes for the variability to LN exposure observed in the present work thus remain to be identified.

This variability could also be expressed through the non-parametric analysis of viability percentages, which showed that, in 8 of the 30 accessions studied, seed viability after desiccation and LN exposure was not significantly different from viability of desiccated seeds, while, in the 22 other accessions, there was a negative effect of LN exposure on seed viability (Table 6.2). The decrease in viability observed in frozen var. Caturra seeds was equivalent to that observed previously in another Bourbon-derived variety (Dussert et al. 2000).

Table 6.2. Multiple comparison of the percentages of seeds developing into normal seedlings after (i) desiccation, (ii) desiccation and LN exposure, and (iii) desiccation, LN exposure and post-thaw osmoconditioning: number of accessions showing, or not, a significant difference for each pair of proportions compared according to the Ryan's test (Ryan 1960).

	Number of accessions
Cooling effect	
Negative effect on seed viability	22
No effect on seed viability	8
Osmoconditioning effect	
Beneficial effect on seed viability	23
No effect on seed viability	7

Seed viability after cryopreservation and controlled rehydration

The post-thaw osmoconditioning treatment resulted in an overall beneficial effect on viability (normal seedling development) of frozen seeds, as illustrated by the fact that all points were located very close to or above the y = x line in Figure 6.3. However, a very high variability for the beneficial effect of seed osmoconditioning was observed within the 30 accessions studied (Figure 6.3). For each accession, this effect could be estimated by the ratio between viability of frozen and osmoconditioned seeds to that of frozen seeds. This variable varied from 1 to 25 among the studied accessions and was not correlated to seed size, seed water content after desiccation, or seed and embryo viability after desiccation. No association was found between the origin of the accessions studied and the viability percentage of osmoconditioned frozen seeds. The beneficial effect of post-thaw seed osmoconditioning observed in Caturra seeds was similar to that observed previously in another Bourbon-derived variety (Dussert et al. 2000). Non-parametric analysis of viability percentages showed that, in 7 of the 30 accessions studied, viability of osmoconditioned frozen seeds was not significantly higher than viability of frozen seeds, while in the 23 other accessions, there was a beneficial effect on seed viability from the post-thaw osmoconditioning treatment (Table 6.2).

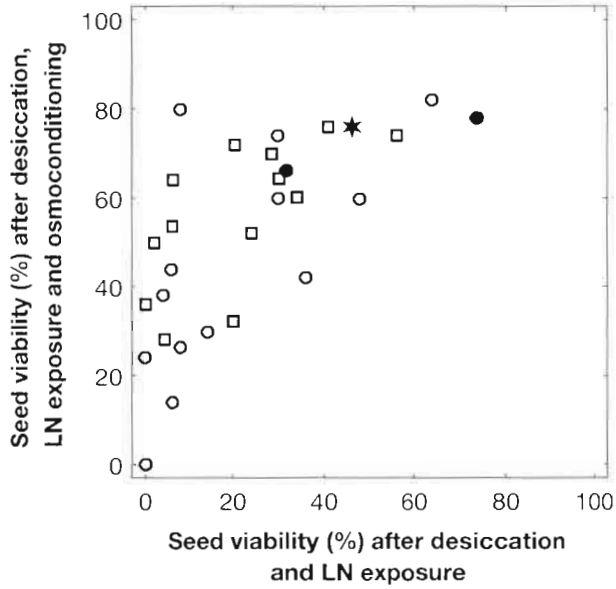

Figure 6.3. Relationship between seed viability (normal seedling development) after desiccation, LN exposure and post-thaw osmoconditioning, and viability of seeds after desiccation and LN exposure only, within the core subset studied. The subset included 27 wild accessions originating from Kefa (O) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

The beneficial effect of post-thaw osmoconditioning on viability of cryopreserved seeds has been shown for the first time with coffee seeds (Dussert et al. 2000). The present study confirms that this treatment improves the proportion of seeds that develop into seedlings after LN exposure, with mean values of 22 and 52% without and with osmoconditioning, respectively. Recent results showing the importance of slowing down the rate of water uptake after cryopreservation suggest that the beneficial effect of post-thaw seed osmoconditioning could be associated with the reduction of imbibitional membrane injury (Dussert et al. 2003a).

Viability of embryos extracted from frozen seeds

Viability (development into normal seedlings) of embryos extracted from frozen seeds was always very high, with a mean value of 76% (Figure 6.4). Moreover, embryo viability after LN exposure was significantly (P=0.034) correlated with that of embryos extracted from desiccated seeds. The slope of the line of regression was close to 1, suggesting that the negative effect of LN exposure on embryo viability was low in comparison with that observed in whole seeds.

In contrast to the results described above with whole seeds, the present results establish clearly that there is no intraspecific variability for the tolerance of embryos to LN exposure. Differences in results obtained with whole seeds and embryos have already been reported in two *C. arabica* varieties (Dussert et al. 1997, 2000) and in other coffee species (Dussert et al. 2001). The very high tolerance of coffee embryos to LN exposure, when seeds are desiccated to their unfreezable water content before cooling, suggests that the decline or the loss of seed viability observed after LN exposure with 22 out of the 30 accessions

studied is due to damage to the endosperm only. In coffee seeds, the transition from the germinated (radicle and hypocotyl emerged) stage to the normal seedling stage appears thus to be strictly dependent on endosperm integrity. The present results suggest thus that the endosperm should be studied to uncover the causes for the intraspecific variability for seed sensitivity to LN exposure.

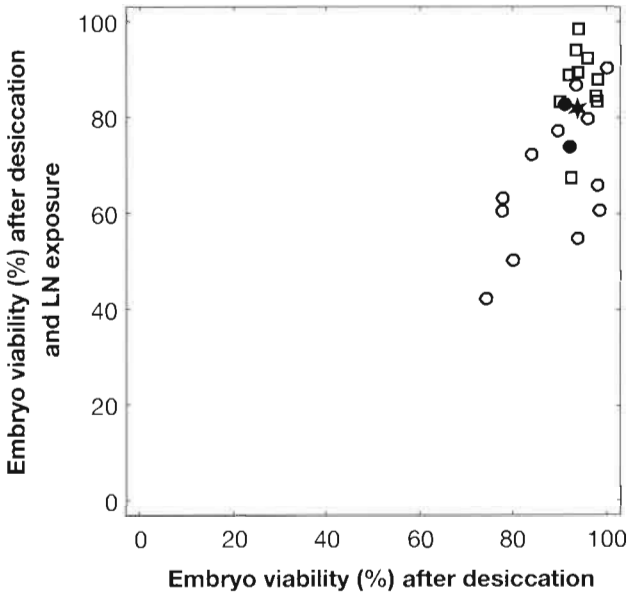

Figure 6.4. Correlation between viability (normal seedling development) of embryos extracted from seeds desiccated and exposed to LN and viability of embryos extracted from desiccated seeds, within the core subset studied. The subset included 27wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

Application to the CATIE cryopreserved coffee core collection

As foreseen in previous reports (Dussert et al. 1997, 2000), two cryopreservation strategies can be employed for routine use in coffee genebanks (for details see Boxes). Each protocol presents its own advantages and drawbacks.

Box 1. Strategy 1

- Desiccation of seeds
- Cooling at 1°C/min to –50°C
- Immersion in liquid nitrogen
- Rapid rewarming (40°C, 4 minutes)
- 6-week osmoconditioning treatment with a –1.25 MPa PEG solution
- Germination in greenhouse

Box 2. Strategy 2

- Desiccation of seeds
- Immersion in liquid nitrogen
- Rapid rewarming (40°C, 4 minutes)
- Seed disinfection
- Extraction of embryos and inoculation in vitro
- 6-week culture period in vitro
- Acclimatization of plantlets
- Transfer to greenhouse

The main advantages of Strategy 1 are that it does not require the use of tissue culture and that seedlings recovered from frozen seeds can be transferred directly to greenhouse conditions. However, it also presents two drawbacks: mean survival is moderate (52%) and a programmable freezer is required. The first drawback is however counterbalanced by the fact that, according to a recent probabilistic study (Dussert et al. 2003b), with a sample size of 50 seeds, the probability to recover at least one plant from the cryopreserved sample is higher than 0.95 for all samples showing a recovery percentage higher than 12%. In the present study, this was the case for 29 of the 30 accessions studied. In order to recover at least five plants, the recovery percentage must be higher than 30%, which was not the case for only five accessions. However, one recovered plant could be considered as being enough to represent the diversity of a given accession because species self-compatibility has contributed to produce homozygous genotypes.

The principal advantages of Strategy 2 are that it enables one to achieve high survival percentages (74% mean) and that it does not require the use of a programmable freezer (direct immersion in LN). However, tissue culture is more time consuming than the standard germination procedure and is associated with additional problems, such as the risk of contamination, which caused the loss of two accessions in the present study, and the acclimatization of *in vitro* plantlets recovered from frozen embryos, which is a second source of plantlet loss. However, these problems should appear less important in the future because the rewarming and the use of a cryobank sample should remain very occasional, allowing samples to be treated very carefully.

Because the second protocol allows the freezing of a higher number of samples simultaneously (direct immersion in liquid nitrogen), it has been chosen by the team of CATIE in charge of this project for the establishment of a cryobank of coffee seeds. In 2002, the 74 accessions of the core collection were cryopreserved according to Strategy 2.

Two very important additional points should also be considered. Firstly, the cryopreserved collection should be duplicated in a secure place other than CATIE, for safety reasons. Secondly, it is essential to cryopreserve a sufficient amount of seeds per accession to ensure their regeneration. The number of seeds to be stored by accession should be calculated as a function of their survival to freezing and of their future utilization. A paper dealing with these issues has already been published (Dussert et al. 2003b) which provides tools for such calculations. In this paper, a simple method, based on the binomial distribution, is proposed to calculate the probability of recovering at least one (or any other fixed number of) plant(s) from a cryobank sample using four given parameters: the percentage of plant recovery observed from a control sample, $p_{obs;}$ the number of propagules used for this control, n_1; the number of propagules in the cryobank sample, n_2; and a chosen risk for the calculation of a confidence interval for the observed plant recovery, α. Using this method, it is possible to assess the number of propagules that should be rewarmed immediately after freezing in order to estimate the plant recovery percentage as a function of the total number of propagules available. It also allows the calculation of the minimum plant recovery percentage to ensure that the probability of recovering at least one (or A, with $A>1$) plant(s) is higher than a fixed probability level, as a function of the control and the cryobank sample sizes. Reciprocally, once the plant recovery percentage has been estimated, it is possible to assess the minimum size of the cryobank sample to obtain a probability to recover at least one (or A, with $A>1$) plant(s) higher than some fixed level.

Prospects

This collaborative project between Bioversity, IRD and CATIE on cryopreservation of coffee germplasm was very fruitful regarding many issues. Not only did it lead to the establishment of the first world coffee cryobank, but also demonstrated the feasibility of transferring proce-

dures set up in a laboratory located out of the coffee growing area to a centre in charge of the conservation of coffee genetic resources located in a developing country.

Secondly, this study highlighted that some of the procedures employed need further optimization. In particular, the unexpected variability observed for seed viability after desiccation clearly showed that the harvest and post-harvest processes have to be re-examined. The fact that the requirement for a programmable freezer disqualified Strategy 1, despite its simplicity, also demonstrated the need for a simpler and low-tech procedure to perform the slow cooling step. Recent trials (unpublished results) have shown that it can achieved by an optimized exposure in a –80°C freezer or in a dry-ice bath.

Finally, considerable progress has been made in the understanding of the mechanisms involved in coffee seed sensitivity to desiccation and LN exposure since the achievement of the project described in the present document (Dussert et al. 2001, 2003a, 2003b, 2004). In particular, the rewarming and rehydration protocols have been significantly improved, allowing achievement of full (100%) survival of frozen seeds. We have indeed shown that pre-heating (soaking seeds in a 40°C water bath for at least 30 minutes) and pre-humidification (placing seeds in water-saturated air at 25 or 37°C (warm pre-humidification) for 24 or 48 h) of seeds after cryopreservation were more efficient procedures than the post-thawing osmoconditioning treatment used in the present study. This improvement will have to be included in future applications of cryopreservation for long-term conservation of coffee germplasm.

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VII. Conclusions and prospects

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Coffee has proven to be an interesting crop for developing complementary strategies and methods for *ex situ* conservation of genetic resources, as an example of a non-orthodox-seed species. As for many crops, coffee field genebanks are today facing many technical, financial and political challenges, which are difficult to resolve (Dulloo et al. 2001). For our review, an integrated action has been applied to the coffee genetic resources conserved in the CATIE field genebank, involving revision of passport data, diversity analysis, core collection construction, and transfer of a cryopreservation protocol from IRD to CATIE. This has contributed to establish, then to cryopreserve, a core collection that can be considered as being representative of *C. arabica* genetic diversity.

The detailed description of the CATIE coffee germplasm collection and its management has highlighted the main problems encountered frequently in large genebank operations. They include the absence of computerization, which can hide the potential presence of off-types, and the difficulties in locating a particular genotype in the collection. Another aspect of crucial importance is the loss of accessions, which might be considered of minor importance due to the relatively low average loss observed. It is in fact extremely serious as some groups, notably the accessions derived from wild coffee, face very drastic losses. This shows that germplasm collections should not be managed uniformly, but that they should be stratified according to the agronomic behaviour of conserved resources in order to adapt the cultural practices and management procedures.

Prior to evaluation, the accessions were classified according to their taxonomy and their geographical or genetic origin. This allowed structuring of the genetic resources and constructing a core collection for genotypic evaluation. The neutral marker analysis led to identification of genetic groups at intraspecific level, which groups were then used for constructing a representative core collection of the diversity conserved in the field genebank. Such an approach has provided genebank managers, breeders and research scientists with a manageable number of accessions for their work. A representative core collection of the Ethiopian accessions was thus constructed and the first world cryobank of *C. arabica* seeds was established at CATIE (Vasquez et al. 2005).

The cryopreservation protocol established in IRD Montpellier has been transferred without any major difficulty to CATIE and applied to a subset of the core collection defined, with plantlet recovery up to 74% of cryopreserved seeds (Vasquez et al. 2005). To our knowledge, this project represents the first example of a cryopreservation protocol being transferred and employed on a large scale in the laboratory of a developing country, in a plant genetic resources conservation context. This active cooperation between developed and developing world institutions has been

a key factor for the success of the project, some experiments being more easily carried out at IRD (e.g. development of cryopreservation protocols) and others at CATIE (e.g. their application to a large number of plants).

One of the significant advantages of this protocol over more classical ones is that no *in vitro* step is necessary at any stage of the protocol for most accessions, i.e. those which show seed survival after freezing, since seeds can be germinated under non-sterile conditions. In cases where survival of whole seeds is nil or very low, then excision and *in vitro* cultivation of zygotic embryos has to be performed, and produces excellent results, as all embryos remain alive inside the seeds, even if they cannot germinate (Dussert et al. 1997). Indeed, cryopreservation damages the endosperm but not the embryo, which conserves its germination and development capacities. The other current drawback of the method is that precooling of seeds to –50°C before their immersion in liquid nitrogen requires the use of a sophisticated programmable freezer. It is hoped that this step can be replaced by a more simple protocol (e.g. using a laboratory deep freezer), which would broaden its applicability.

The availability of the seed cryopreservation protocol as a new complementary technique should have consequences for the management of the coffee genebank. It should be tested on seeds of a broader range of coffee genotypes and species involving notably rare material, material little requested, material with specific characteristics, and material often requested. This should thus have consequences for the number of replicates of a given accession conserved in the field, if it is also stored under cryopreservation, depending on the decisions taken by the curator of the collection. Evaluation data indicated that some accessions present low polymorphism and others probably result from human duplications. Such accessions should no longer be maintained in the field genebank, but only in the form of cryopreserved seeds.

Various additional points should also be considered, such as the necessary safety duplication of the cryopreserved collection at at least one site other than CATIE, and a calculation of the number of seeds that should be stored per accession to ensure their regeneration. A specific field for cryopreserved material should be added to the general collection database. Procedures remain to be established for handling the material (retrieval upon demand for cryopreserved material, replacement of material taken from the cryobank, etc.). All these points could form the subject of a Technical Bulletin for laboratory daily use.

In conclusion, the conservation activities developed for coffee have demonstrated that it is possible to efficiently use cryopreservation for the long-term conservation of germplasm of a species with non-orthodox seeds, in the genebank context of a developing country. There is a huge number of species with non-orthodox seeds for which similar projects would be necessary in order to ensure the safe, long-term and cost-effective conservation of their genetic resources. Such a project is currently being implemented for *Citrus* spp., through collaboration between the Universiti Putra Malaysia (UPM), IRD and Bioversity (Hor et al. 2005). It is our hope that this publication will stimulate research in this area for additional non-orthodox-seed species and pave the way for application of such technologies to other species that have seeds that are difficult to conserve *ex situ*, or species that are at the moment solely dependent on conservation in field genebanks.

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