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BIOTECHNOLOGIES FOR THE MANAGEMENT OF GENETIC RESOURCES FOR FOOD AND AGRICULTURE

by

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This document has been prepared at the request of the Secretariat of the Commission on Genetic Resources for Food and Agriculture by Preetmoninder Lidder and Andrea Sonnino, from the FAO Working Group on Biotechnology as a contribution to the cross-sectoral theme, *Application and integration of biotechnologies in the conservation and utilization of genetic resources*.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABDC-10	FAO international technical conference on Agricultural Biotechnologies in Developing Countries
AbHV	Abalone herpes-like virus
AFLP	Amplified fragment length polymorphism
AFOCEL	Association Forêt-Cellulose
AGeS	Annotation of Microbial Genome Sequences
AI	Artificial insemination
AMF	Arbuscular mycorrhizal fungi
AW-IPM	Area-wide integrated pest management
BecA	Biosciences eastern and central Africa
BiMFG	Bioinformatics tools for Marine and Freshwater Genomics
BOLD	Barcode of Life Data Systems
BWB	Breeding without breeding
CATIE	Centro Agronómico Tropical de Investigación y Enseñanza
CBD	Convention on Biological Diversity
CBOL	Consortium for the Barcode of Life
CBPP	Contagious bovine pleuropneumonia
cDNA	Complementary DNA
C-ELISA	Competitive ELISA
CGIAR	Consultative Group on International Agricultural Research
CGRFA	Commission on Genetic Resources for Food and Agriculture
CIAT	International Centre for Tropical Agriculture
CIBEX	Center for Information Biology Gene Expression Database
CIP	International Potato Center
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
COSAVE	Comité de Sanidad Vegetal del Cono Sur
CSV	Classical swine fever
CWR	Crop wild relatives
DArT	Diversity arrays technology

DDBJ	DNA Databank of Japan
DGGE	Denaturing gradient gel electrophoresis
DH	Doubled haploid
DIVA vaccine	Vaccine capable of differentiating infected and vaccinated animals
DPTF	Database of Poplar Transcription Factors
ECBOL	European Consortium for the Barcode of Life
EHNV	Epizootic haematopoietic necrosis virus
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EMBnet	European Molecular Biology Network
EMS	Ethylmethane sulfonate
EPPO	European and Mediterranean Plant Protection Organization
EST	Expressed sequence tag
ET	Embryo transfer
EU	European Union
EUCAGEN	International Eucalyptus Genome Network
FAO	Food and Agriculture Organization of the United Nations
FGI	Fungal Genome Initiative of The Broad Institute
FMD	Foot and mouth disease
FPPI	*Fusarium graminearum* Protein-Protein Interaction Database
GCP	Generation Challenge Program
GENOLYPTUS	Brazilian Network of Eucalyptus Genome Research
GEO	Gene Expression Omnibus
GLEWS	Global Early Warning and Response System
GM(O)	Genetically modified (organism)
GnRH	Gonadotropin-releasing hormone
GOLD	Genomes OnLine Database
GPMDB	Global Proteome Machine Database
GREP	Global Rinderpest Eradication Programme
GRFA	Genetic resources for food and agriculture
GRIN	Germplasm Resource Information System

GS	Genomic selection
GWAS	Genome-wide association studies
HACCP	Hazard Analysis and Critical Control Point
hCG	Human chorionic gonadotropin
IAEA	International Atomic Energy Agency
iBOL	International Barcode of Life project
ICRAF	World Agroforestry Centre
IFAD	International Fund for Agricultural Development
IHHNV	Infectious hypodermic and haematopoeitic necrosis virus
IHN	Infectious haematopoietic necrosis
IHNV	Infectious haematopoietic necrosis virus
IITA	International Institute of Tropical Agriculture
ILRI	International Livestock Research Institute
IMEx	International Molecular Exchange Consortium
IMTA	Integrated multi-trophic aquaculture
INIBAP	International Network for the Improvement of Banana and Plantain
INSDC	International Nucleotide Sequence Database Collaboration
IPM	Integrated pest management
IPNV	Infectious pancreatic necrosis virus
IPPC	International Plant Protection Convention
IPR	Intellectual property rights
ISAG	International Society of Animal Genetics
ISPM	International Standards for Phytosanitary Measure
IUCN	International Union for Conservation of Nature
IVF	*In vitro* fertilization
KHV	Koi herpesvirus
LAMP PCR	Loop-mediated isothermal amplification PCR
MAS	Marker-assisted selection
MEGAN	MEtaGenome ANalyzer
MNU	N-methyl-N-nitrosourea
MOET	Multiple ovulation and embryo transfer

MPID	*Magnaporthe grisea* Protein-Protein Interaction Database
*Mr*NV	*Macrobrachium rosenbergii* nodavirus
NACA	Network of Aquaculture Centres in Asia-Pacific
NAPPO	North American Plant Protection Organization
NBPGR	National Bureau of Plant Genetic Resources
NCBI	National Centre for Biotechnology Information
N_e	Effective population size
NERICA	New Rice for Africa
NGS	Next generation sequencing
OIE	World Organisation for Animal Health
PATTEC	Pan African Tsetse Eradication Campaign
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PopGenIE	*Populus* Genome Integrative Explorer
PPR	Peste des petits ruminants
PPRV	Peste des petits ruminants virus
PRIDE	PRoteomics IDEntifications database
PSB	P-solubilizing bacteria
PSF	P-solubilizing fungi
QBOL	Quarantine Barcoding of Life
QPM	Quality protein maize
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
RP	Rinderpest
rRNA	Ribosomal RNA
rRT-PCR	Real-time RT-PCR
RT-PCR	Reverse transcriptase PCR
SE	Somatic embryogenesis
SGD	*Saccharomyces* Genome Database

SGRP	System-wide Genetic Resources Programme
SIT	Sterile insect technique
SNP	Single nucleotide polymorphism
TAAPs/TAADs	Transboundary aquatic animal pathogens/diseases
TADs	Transboundary animal diseases
TGGE	Temperature gradient gel electrophoresis
TILLING	Targeting Induced Local Lesions IN Genomes
T-RFLP	Terminal-RFLP
UN	United Nations
UNDP	United Nations Development Programme
UNICEF	United Nations Children's Fund
UNSIC	UN System Influenza Coordinator
WDCM	World Data Center for Micro-organisms
WFCC	World Federation for Culture Collections
WSSV	White spot syndrome virus
WTO	World Trade Organization

EXECUTIVE SUMMARY

At its Twelfth Regular Session, the Commission on Genetic Resources for Food and Agriculture (the Commission) requested FAO to prepare a scoping paper describing the range of biotechnologies being applied to the conservation and utilization of genetic resources for food and agriculture (GRFA), the current status of application of these technologies and matters relevant for their future development, including relevant policy developments in other international forums, for consideration at its next regular session. The scoping paper was reviewed by the Commission's Working Groups on animal, forest and plant genetic resources and subsequently revised in the light of comments received. The revised document[1] will be presented to the Commission for consideration at its Thirteenth Regular Session.

The scoping paper does not present fully comprehensive and detailed information due to length limits. To facilitate discussions and debate on the issue, this more extensive background study paper was commissioned by the Secretariat of the Commission. It provides an overview of biotechnologies applied for the characterization, conservation and utilization of GRFA. It also examines recent developments as well as the current status and potential use(s) of biotechnology applications relevant for the management of crop, forest, animal (livestock), aquatic (fisheries and aquaculture) and microbial GRFA. The document is intended to provide information and insights that can both assist countries to establish, maintain and advance policies in relation to biotechnologies in this area and help to identify gaps which might merit further studies.

In recent years, the land area under agriculture has declined as also has the rate of growth in agricultural productivity while the demand for food continues to escalate. The world pouplation now stands at 6.8 billion and is expected to reach 9 billion in 2045. A broad range of agricultural genetic diversity needs to be available and utilized in order to feed this growing population. Climate change is an added threat to biodiversity that will significantly impact GRFA and food production. There is no simple, all-encompassing solution to the challenges of increasing productivity while conserving genetic diversity. Sustainable management of GRFA requires a multi-pronged approach and as outlined in the paper, biotechnologies can provide powerful tools for the management of GRFA. These tools vary in complexity from those that are relatively simple to those that are more sophisticated. Further, advances in biotechnologies are occurring at a rapid pace and provide novel opportunities for more effective and efficient management of GRFA.

Biotechnology applications must be integrated with ongoing conventional breeding and development programmes in order to succeed. Additionally, the generation, adaptation and adoption of biotechnologies requires a consistent level of financial and human resources and appropriate policies need to be in place. These issues were recognized by Member States at the FAO international technical conference on Agricultural Biotechnologies for Developing Countries (ABDC-10)[2], which took place in March 2010 in Mexico. At the end of the conference, the Member States reached a number of key conclusions, agreeing, *inter alia*, that developing countries should significantly increase sustained investments in capacity building and the development and use of biotechnologies to maintain the natural resource base; that effective and enabling national biotechnology policies and science-based regulatory frameworks can facilitate the development and appropriate use of biotechnologies in developing countries; and that FAO and other relevant international organizations and donors should significantly increase their efforts to support the strengthening of national capacities in the development and appropriate use of pro-poor agricultural biotechnologies.

[1] CGRFA-13/11/3. Status and Trends of Biotechnologies Applied to the Conservation and Utilization of Genetic Resources for Food and Agriculture and Matters Relevant for their Future Development.

[2] CGRFA-13/11/Inf.8. Agricultural Biotechnologies in Developing Countries: Options and Opportunities in Crops, Forestry, Livestock, Fisheries and Agro-Industry to Face the Challenges of Food Insecurity and Climate Change (ABDC-10) – Report.

I. INTRODUCTION

The Convention on Biological Diversity (CBD) defines genetic resources as *"genetic material of actual or potential value"*. Genetic resources for food and agriculture (GRFA) are the raw material for agricultural development and therefore their sustainable use is crucial for global food security, particularly for the rural poor in developing countries. Furthermore, genetic resources are not uniformly distributed on the planet, being richer in the tropical and sub-tropical zones. Consequently, countries and regions are interdependent and benefit-sharing and international cooperation are essential.

The world's food and agriculture production depends upon plant, animal, aquatic, forest, microbial and invertebrate genetic resources. Although many plants are edible and over 7 000 species have been cultivated or collected for food, only 30 crops provide 95 percent of human dietary energy needs with just three of them, i.e. rice, wheat and maize, providing more than 50 percent (FAO, 1997). It should be noted that although the genetic diversity within this small number of major crop species is quite substantial, most crops exhibit less genetic variation than their wild relatives due to domestication bottlenecks (Tanksley and McCouch, 1997). For livestock, of the 50 000 known avian and mammalian species, about 40 have been domesticated, with fewer than 14 species accounting for over 90 percent of global livestock production (FAO, 1999; FAO, 2007a). Within these species, a great variety of breeds have been developed since domestication, and genetic diversity in livestock breeds is usually much greater than that in crop varieties. However, 21 percent of the 8 054 breeds reported globally are at risk of extinction.[3]

For aquatic and forest genetic resources, wild populations play an important role in addition to domesticated populations. More than 300 aquatic species (excluding aquatic plants) are farmed worldwide, while over 1 000 species are harvested from capture fisheries.[4] However, aquaculture is currently the fastest growing animal-food producing sector and improved or domesticated strains are becoming increasingly important (FAO, 2010a). The majority of the world's forest genetic resources are unknown, found in largely unmanaged and undomesticated forests, with only 7 percent being in plantations (FAO, 2010b). It is estimated that the number of tree species varies from 80 000-100 000, of which fewer than 500 have been studied in detail.[5] Finally, micro-organisms (bacteria, fungi and viruses) and invertebrates (insects, spiders and earthworms) include numerous species with great genetic diversity that are invaluable contributors to agro-ecosystems.[6]

Genetic resources are vulnerable to losses and are rapidly dwindling due to habitat change/degradation, overexploitation, pollution, invasive alien species and climate change. According to the 2009 update of the IUCN Red List of Threatened Species™, 36 percent of the 47 677 assessed species are threatened with extinction.[7] The UN General Assembly declared 2010 as the International Year of Biodiversity[8] to coincide with the 2010 Biodiversity Target[9] of achieving a significant reduction in the rate of biodiversity loss. However, this target has not been met. These shortcomings apply equally to agricultural and wild biodiversity. In fact, as confirmed in the third edition of the Global Biodiversity Outlook[10], genetic diversity continues to decline in agricultural systems.

[3] http://www.fao.org/docrep/meeting/021/am131e.pdf

[4] http://www.fao.org/fishery/statistics/software/fishstat/en

[5] http://www.fao.org/nr/cgrfa/cthemes/forest/en/

[6] ftp://ftp.fao.org/docrep/fao/meeting/017/ak534e.pdf

[7] http://iucn.org/about/work/programmes/species/red_list/

[8] http://www.cbd.int/2010/

[9] http://www.cbd.int/2010-target/

[10] http://www.cbd.int/doc/publications/gbo/gbo3-final-en.pdf

As outlined above, the world's food supply is dependent on a small number of species and the continuing reduction in genetic diversity is a major cause for concern. Maintaining genetic diversity is essential since removal of a single species can affect the functioning of global ecosystems. Further, genetically diverse populations have a greater adaptive potential and are more resilient to environmental changes, in addition to being a source of economically and scientifically important traits. Thus conservation of genetic diversity is crucial to ensure its continued availability for adaptation to climate change and future production, market and societal needs. Conservation is also important as it provides insurance against unforeseen catastrophic events and preserves genetic resources of cultural and historical value.

Since GRFA are finite and once lost cannot be regained, proper management is fundamental. Biotechnology applications can provide comparative advantages over, or can increase the effectiveness of, traditional technologies for the characterization, conservation and utilization of GRFA. Indeed, both the *Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture*[11] and the *Global Plan of Action for Animal Genetic Resources*[12] identify a direct or indirect role for agricultural biotechnologies in some of their priority areas.

In this document, the following definition of biotechnology, based on that of the CBD, is used i.e.: "*any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use*".[13] Some of the biotechnologies described, for example the use of molecular markers may be applied to all the agricultural sectors, i.e. they are cross-sectoral, while others are more sector-specific, such as tissue culture (in crops and forest trees), embryo transfer (livestock) and sex-reversal (fish). Issues such as the health or environmental risks of any biotechnology product are beyond the scope of this paper.

The paper aims to provide an updated review of the current state of research, development and application of the many biotechnologies that are currently available and that can be employed for the management of GRFA. Use of biotechnologies, particularly advanced biotechnologies, requires a commitment of financial and human resources. However, detailed discussions on the relative merits of applying the different biotechnologies, compared with each other or with conventional technologies, are not entered into here, as they are often case specific and are influenced by variable factors such as the current costs and technical feasibility of the different technologies.

This document is divided into six main sections. Section 2 presents an overview of biotechnology applications relevant for the characterization, conservation and utilization of GRFA, followed by sector-specific sections (nrs. 3 to 7) that provide details pertinent for the management of crop, forest, animal (livestock), aquatic (fisheries and aquaculture) and microbial GRFA, respectively. Section 8 provides conclusions and outlook.

II. BIOTECHNOLOGIES APPLIED TO THE CHARACTERIZATION, CONSERVATION AND UTILIZATION OF GENETIC RESOURCES FOR FOOD AND AGRICULTURE

For biotechnology applications to succeed, they should complement conventional conservation and breeding activities and build upon existing and active programmes. Conventional breeding has provided enormous benefits in the past and will continue to do so in the future. For example, domestication of aquatic and forest species is relatively recent and they have not been genetically improved to the same extent as crop and livestock species. They may, therefore, derive particular benefits from the use of conventional breeding. Further, relevant components of production and market systems, as well as socio-economic, environmental, and cultural considerations should also be taken into account before the

[11] ftp://ftp.fao.org/docrep/fao/meeting/015/aj631e.pdf

[12] ftp://ftp.fao.org/docrep/fao/010/a1404e/a1404e00.pdf

[13] http://www.cbd.int/convention/articles.shtml?a=cbd-02

decision to apply a particular biotechnology is made. Biotechnologies *per se* are not the solution, but when integrated with ongoing, appropriately designed conservation, breeding and development programmes, they can be of significant assistance in meeting the needs of an expanding and increasingly urbanized population while maintaining the diversity of genetic resources.

2.1 Characterization of GRFA

Characterization is a prerequisite for identifying and prioritizing the genetic resources to be conserved, and is fundamental for optimizing appropriate allocation to conservation programmes when funding resources are limited (Boettcher *et al*., 2010). Characterization also links conservation and utilization as it allows the identification of unique and valuable traits of conserved genetic resources, both *in situ* and *ex situ*, for incorporation into breeding programmes. In addition, characterization of GRFA is essential for ensuring ownership of GRFA (to promote and control bioprospecting, i.e. prospecting for commercially valuable biological or genetic resources and the accompanying traditional knowledge, and to avoid biopiracy, i.e. the illegal appropriation of these resources) as well as access to and fair and equitable sharing of their benefits, especially in conjunction with intellectual property rights (IPR) management.

Genetic resources can be characterized with respect to genotypes, phenotypes, morphological traits, measures of genetic diversity, genetic distance, population size and structure, geographical distribution and degree of endangerment. Biotechnology applications for characterization include molecular markers and the so-called "omic"[14] technologies.

2.1.1 Molecular Markers (Cross-sectoral)

Molecular markers are heritable, identifiable DNA sequences that are found at specific locations within the genome and can be used to detect DNA polymorphism. The first widely used markers were isozymes[15] and they are still being applied today (e.g. to characterize forest trees). However, isozymes show low levels of polymorphism, relatively low abundance and, in many instances, have been replaced by more sensitive techniques.

The assay of molecular markers requires only small amounts of biological material that can be easily transported and stored. Since they are fixed at fertilization, molecular markers are not affected by environmental conditions. They can be used at any growth stage, which is especially advantageous for long-lived species such as forest trees. Additionally, molecular markers can be selectively neutral or associated with functional variation. Neutral markers are particularly useful for understanding population size and structure, estimating relationships between populations and hybrid identification, while functional markers allow specific alleles within pedigrees and populations to be tracked.

Different kinds of molecular marker systems are available such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs) (FAO, 2001). RFLPs are based on the detection of variation in the length of fragments generated when DNA is treated with restriction endonucleases. RAPDs are detected using the polymerase chain reaction (PCR) i.e. the genomic template is amplified with single, short (usually 10-mer) randomly chosen primers. AFLPs are generated by the PCR amplification of restriction endonuclease treated DNA ligated to an adaptor sequence. Microsatellites are segments of DNA, around 5 or fewer bases, that are repeated a variable

[14] Omics is a general term for a broad discipline of science and engineering for analyzing the interactions of biological information objects in various "omes", such as the genome, proteome, etc.

[15] An isozyme is a genetic variant of an enzyme. Isozymes for a given enzyme share the same function, but may differ in level of activity, as a result of minor differences in their amino acid sequence (FAO, 2001).

number of times in tandem and are detected by PCR. SNPs are genetic markers resulting from single base changes at particular positions within a DNA sequence.

The aforementioned markers differ with respect to technical requirements, the amount of time, money and labour needed, reproducibility, level of polymorphism detected, type of expression (dominant or co-dominant) and the number of genetic markers that can be detected throughout the genome (reviewed in FAO, 2003; Okumus and Ciftci, 2003; Budak *et al*., 2004; Schlötterer, 2004; Spooner *et al*., 2005; FAO, 2007b; Duran *et al*., 2009; Teneva, 2009). No single molecular marker system is suitable for all kinds of studies and choice of the marker system used should be based on its appropriateness for the information required, the population/species being studied and the available financial and operational resources. For example, SNPs and microsatellites are excellent choices for parentage analysis while mitochondrial DNA and Y chromosome markers are useful for the identification of maternal and paternal lineages, respectively. Molecular marker information should also be used in conjunction with other information sources (e.g. phenotypic traits, population data and evaluation of the production system) to assist decision-making regarding conservation, especially since populations with low genetic diversity at the molecular level may have unique phenotypes valuable for conservation (Peter *et al.*, 2007).

Irrespective of the kind of molecular marker used, technical infrastructure and know-how, as well as relatively expensive consumables are necessary, although multiplexing for PCR-based markers can significantly improve the speed and efficiency of genotyping leading to reduced costs and labour. However, since marker development costs are higher than running (i.e. typing using known markers) costs, research and development, including in developing countries, may benefit from the large number of markers already available for many species.

Molecular markers can be used to characterize GRFA in a variety of different ways, namely for the:

i) Assessment of intraspecific genetic diversity:

   Molecular markers provide valuable information about intraspecific genetic diversity, i.e. within and between populations of the same species, in all the agricultural sectors. Biological tissue (e.g. blood, leaves, fish fin clips) is sampled from a representative set of individuals from a number of populations of interest, typed for marker loci followed by a statistical analysis of the data to estimate the genetic distance between and genetic variability within the populations being studied (Ruane and Sonnino, 2006). Determination of the amount of genetic diversity present within or between populations as well as the structure and geographic distribution of diversity in populations can aid in the identification of the most suitable strategies for conservation and utilization.

ii) Documentation of domestication events:

   Assessment of genetic distances has contributed to the understanding of the history and timing of domestication by identifying wild populations most closely related to domesticated species as well as the putative centres of origin.

iii) Detection of interspecific variation:

   When species are difficult to identify morphologically, detection of interspecific variation is particularly significant, e.g. for the forestry and fisheries sectors. DNA barcoding is an emerging tool for cataloguing biodiversity that uses a standardized gene region for species identification and discovery. It is being applied on a global scale through an international initiative, the International Barcode of Life project (iBOL)[16] that aims to barcode five million specimens representing 500 000 species. Additional community-based initiatives include the Consortium for

[16] http://www.barcodeoflife.org/content/about/what-ibol

the Barcode of Life (CBOL)[17] that promotes barcoding through information exchange, conferences, outreach and training, and the European Consortium for the Barcode of Life (ECBOL)[18]. Two central DNA barcode databases exist, the Barcode of Life Data Systems (BOLD)[19], which aids collection, management, analysis and use of DNA barcodes, and the International Nucleotide Sequence Database Collaboration (INSDC)[20].

iv) Estimation of effective population size (N_e):

The effective population size (N_e) is a key parameter in the field of population genetics and is defined as the number of individuals in an idealized population that would give rise to the same inbreeding rate or the same amount of genetic drift as in the real population of interest. N_e is generally smaller (often much smaller) than the actual population size and is an important indicator for determining the degree of endangerment of a population. N_e is affected by several factors such as sex ratio, numbers of offspring for individual matings, variable population size, mating systems and selection (Caballero, 1994). N_e is traditionally estimated using pedigree data, censuses and genotype data (Bartley *et al*. 1992) but this information may be difficult to obtain for wild populations (Ruane and Sonnino, 2006).

v) Investigation of gene flow between domesticated populations and their wild relatives:

Gene flow from domesticated populations can influence the genetic variability of fitness traits in wild populations and in some cases lead to outbreeding depression [loss of genotypes important for local adaptation and reduction in reproductive fitness in the first or later generations following attempted crossing of populations (Frankham *et al*., 2011)]. Molecular markers can be used as tools to conserve wild relatives by distinguishing hybrids from non-hybrids, allowing for selection against the hybrids and purging of their genes from the wild population. Markers can also be utilized to preserve cultivated species by detecting introgression of genes from wild relatives and loss of domestication-related traits.

vi) Identification of quantitative trait loci (QTLs)[21]

Molecular markers are routinely used for tracing and mapping alleles of interest in a segregating population. Most economically important traits (e.g. yield, disease resistance) are quantitative, i.e. typically controlled by many genes that have an additive effect and also strongly influenced by the environment. Molecular markers are highly effective tools for the identification of these QTLs.

Markers can be employed to construct genetic linkage maps[22], comprised of markers interspersed at short, regular intervals throughout the genome, with the distances between markers reflecting the degree of linkage. The putative QTLs are then mapped to a small region on the chromosome followed, in some cases, by the identification of the genes responsible for the trait using a positional cloning[23]/candidate gene[24] approach. It should be noted, however, that for QTL

[17] http://www.barcodeoflife.org/content/about/what-cbol

[18] http://www.ecbol.org/

[19] http://www.boldsystems.org/views/login.php

[20] http://www.insdc.org/

[21] A quantitative trait locus is a locus where allelic variation is associated with variation in a quantitative trait, such as yield, tolerance to abiotic stresses, etc. (FAO, 2001).

[22] A linkage map is a linear or circular diagram that shows the relative positions of genes on a chromosome as determined by recombination fraction (FAO, 2001).

[23] A strategy for gene cloning that relies on the identification of closely linked markers to the target trait, and then uses chromosome walking to identify, isolate and characterize the gene(s) responsible for the trait (FAO, 2001).

identification, a major challenge is the technical sophistication associated with creating mapping populations, recording meaningful phenotypes and compiling genetic maps.

vii) Conservation management of genetic resources

Molecular markers are of considerable value in the conservation of plant genetic resources in defining conservation strategies, gap analysis studies and developing sampling strategies for gene banks for prioritizing populations for conservation. Molecular markers can also be used for the management of conserved germplasm by increasing the efficiency of gene bank operations, especially in characterization and regeneration activities.

2.1.2 "Omic" technologies (Cross-sectoral)

Genomics refers to the study of an organism's genome at the DNA level. To date, the genomes of more than 1 000 organisms, including plants, animals, fish[25], forest trees, micro-organisms and invertebrates, have been sequenced. Outputs from genome sequencing can be further enhanced by elucidating patterns of gene expression and gene function through functional genomic technologies such as transcriptomics, proteomics and metabolomics, i.e. mRNA, protein and metabolite profiling respectively, thus providing a thorough gene inventory (Fears, 2007). Additionally, proteomic and metabolomics are not reliant on having a pre-available genome sequence. "Omic" information, analyzed in conjunction with bioinformatics, can be exploited to characterize and utilize GRFA in novel ways, since entire networks of genes (as opposed to single genes) can be analyzed spatially and/or temporally and in a much speedier manner compared to conventional technologies.

Genomic information has the potential to contribute to and solve some key problems in the management of genetic resources (reviewed in Allendorf *et al*., 2010). For example, the accelerated generation of molecular markers throughout the genome can increase the precision and accuracy for estimating and monitoring N_e. The creation of high-density linkage maps can greatly assist QTL mapping and cloning of the corresponding genes due to improved reliability of data and conclusions, making selection for quantitatively inherited characters quicker and more precise.

Specialized fields of genomics are integrating information from numerous sources to benefit from the immense amount of genomic data available and to maximize the characterization benefits of genomic approaches. Comparative genomics takes advantage of synteny[26] and of the conserved functions of genes, regulatory and non-coding sequences, and has facilitated the prediction of candidate genes in close relatives following the availability of the sequences of model/key species (especially important for utilizing the genetic diversity of under-resourced and orphan crop species and less-common livestock species).

Population genomics utilizes genome-wide patterns of sequence variation, at the population level, to detect genes subjected to strong selection pressure, thus contributing to the understanding of adaptive evolution (FAO, 2010c). Genomic scans for detection of "selection signatures" (i.e. increased frequency of nucleotides linked to favourable mutations) can provide insight into the evolutionary history of species and their subdivision into distinct breeds and varieties, with comparisons between species being more informative for the identification of older events, and between and within-populations for revealing more recent episodes of selection.

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[24] A gene known to be located in the same region as a DNA marker that has been shown to be linked to a single-locus trait or to a QTL, and whose deduced function suggests that it could be the source of genetic variation in the trait in question (FAO, 2001).

[25] The term also includes aquatic invertebrates e.g. molluscs, echinoderms and crustaceans.

[26] The occurrence of two or more loci on the same chromosome, without regard to their genetic linkage. The term is increasingly used to describe the conservation of gene order between related species (FAO, 2001).

Landscape genomics combines genomic information with geo-environmental data to determine, for example, which breeds of livestock are best suited for certain production circumstances (FAO, 2007a). Xenogenomics, i.e. functional genomics specifically targeting non-model and non-crop plants with enhanced tolerance to abiotic stresses, is being used to discover novel genes from indigenous and exotic plant species living in extreme conditions (John and Spangenberg, 2005). Metagenomics, the study of the genomes of samples taken directly from the environment, for instance soil samples, is a relatively new field of genetic research that enables studies of unculturable organisms as well as characterization of biodiversity at the ecosystem level (Marco, 2010).

Finally, genomics can be utilized as a tool for bioprospecting indigenous GRFA for economically important traits. Such knowledge is key for developing countries not only to capitalize on the full potential of their genetic resources for economic and conservation benefits but also to prevent the exploitation of their rich biodiversity and avoid unfair sharing of benefits. High-throughput genomic approaches have been utilized by a few developed countries and the private sector, especially for microbial bioprospecting in the Antarctic and Arctic ecosystems (Peck *et al*., 2005; Leary and Walton, 2010).

High levels of financial investments and expertise are required for the set-up and maintenance of laboratories/centres capable of providing "omic" facilities. Further, trained scientists, good internet access and computer facilities are vital to take advantage of the publicly available sequence information and bioinformatic tools. Consequently, these technologies are being used only in some cases across the different agricultural sectors in developing countries. Nevertheless, genomic sequencing is becoming steadily cheaper. High-throughput SNP genotyping costs have decreased by up to 10-fold while data throughput has increased by the same magnitude (Ribaut *et al*., 2010).

2.1.3 Bioinformatics (Cross-sectoral)

The availability of enormous amounts of data generated by the high-throughput "omic" technologies has necessitated the development of methods for processing, analyzing, integrating and interpreting the data. Bioinformatics refers to the research, development and application of computational and statistical tools and information processing methods for the management of biological information. Since bioinformatics is interdisciplinary in nature and blends many fields together, collaboration and synergy between biologists, biostatisticians and bioinformaticists is key for successful data mining, i.e. the extraction of relevant information from large datasets.

Bioinformatic tools can be used for molecular marker discovery/prediction, sequence analyses including prediction of function, genome and chromosome annotation, phylogeny estimation, data mining for genes of interest, high-throughput analysis of gene expression, systems biology and population genetics. Numerous specialized public databases exist. The three major sequence databases are the National Centre for Biotechnology Information (NCBI)[27], the European Molecular Biology Laboratory (EMBL)[28] and the DNA Databank of Japan (DDBJ)[29], which are also the primary sites that provide access to a collection of bioinformatic tools. Microarray and gene expression data can be accessed at the Gene Expression Omnibus (GEO)[30], the ArrayExpress[31], and the Center for Information Biology Gene Expression

[27] http://www.ncbi.nlm.nih.gov/Tools/

[28] http://www.ebi.ac.uk/Tools/

[29] http://www.ddbj.nig.ac.jp

[30] http://www.ncbi.nlm.nih.gov/geo/

[31] http://www.ebi.ac.uk/arrayexpress/

Database (CIBEX)[32]. AgBase[33] provides functional modelling resources for agriculturally important animal, plant, microbe and parasite genomes (McCarthy *et al*., 2011).

At the protein level, annotated protein sequences are provided by UniProt[34], bio-macromolecular structure data can be accessed in the Protein Data Bank (PDB)[35], protein modifications in UniMod[36] and RESID[37], and protein interactions in the International Molecular Exchange Consortium (IMEx) databases[38]. Proteomics data repositories (reviewed in Vizcaíno *et al*., 2010) include the Global Proteome Machine Database (GPMDB)[39], PeptideAtlas[40], the PRoteomics IDEntifications database (PRIDE)[41] and Tranche[42].

Currently, the ability to generate genomic information and the trend at which this capacity is increasing threatens to outpace the corresponding progression in development of hardware and tools for data management, storage and analysis. Bioinformatics may thus be a limiting factor in the full exploitation of genomics for the management of GRFA, and this may be particularly true in developing countries.

2.2 Conservation of GRFA

Two major strategies exist for conservation.[43] *In situ* conservation allows continued evolution and adaptation of a species in response to the environment. Albeit more dynamic, it is exposed to habitat destruction by natural calamities and/or human interference. *Ex situ* conservation can be used to ensure easy and ready accessibility of reproductive material. Gene banks constitute the most significant and widespread means of conserving plant genetic resources. Currently 1 750 gene banks worldwide maintain 7.4 million accessions, with the large majority of the accessions in the form of seeds and the largest collections held by the CGIAR centers in the public domain (FAO, 2010c). The methods described in the preceding section are of utility in monitoring conserved species and/or populations, both *in situ* and *ex situ*.

An effective link between both strategies is important. For example, *ex situ* collections can be used for *in situ* population enhancement (by introducing genetic diversity and thus reducing inbreeding levels), or even to reintroduce rare/extinct species to the wild (Engels *et al*., 2008; Dulloo, 2011). The two strategies are thus complementary rather than alternatives, and effective conservation strategies often incorporate elements of both, to devise the best strategy taking into account the biology of the species to be conserved, technical and financial aspects as well as infrastructural and human resources available (Dulloo *et al*., 2005; Volis and Blecher, 2010; CBSG, 2011).

[32] http://cibex.nig.ac.jp

[33] http://www.agbase.msstate.edu/

[34] http://www.uniprot.org

[35] http://www.wwpdb.org/

[36] http://www.unimod.org/

[37] http://www.ncifcrf.gov/resid/

[38] http://www.imexconsortium.org/

[39] http://gpmdb.thegpm.org/

[40] http://www.peptideatlas.org/

[41] http://www.ebi.ac.uk/pride/

[42] http://www.tranche.proteomecommons.org

[43] According to the CBD and the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), *in situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties. *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitats.

2.2.1 Cryopreservation (Cross-sectoral)

Cryopreservation involves the storage of germplasm at ultra-low temperatures (usually in liquid nitrogen at -196 °C) whereby all metabolic activities are suspended. It is a cost-effective option that allows for long-term storage, reduces the risk of loss (from diseases, disasters, etc.), requires limited space and minimal maintenance, and can offer extinct/selected genetic material for improved breeding in the future, but does not allow for continued genetic adaptation. Its routine use is restricted in developing countries since the availability of economically priced liquid nitrogen is a particular constraint, although it can prove to be a more cost-effective method in the long term provided the initial investment of a cryo facility is made (Dulloo *et al*., 2009).

Cryopreservation is a useful method for long-term storage of germplasm, especially for plant species that are difficult to conserve as seeds due to low dessication tolerance. For aquatic species, cryopreservation has limited application because female gametes and fertilized eggs usually cannot be frozen. For livestock, the term "cryoconservation" is often used to refer to cryopreservation of germplasm for the purpose of genetic conservation, whereas cryopreservation refers to the actual freezing technology and its general application. This distinction is more relevant for livestock than for other sectors, because cryopreservation is more widely applied for uses other than conservation.

2.2.2 In vitro *slow growth storage (Crops and forest trees)*

For crop and forest genetic resources, the majority of the accessions are maintained as seeds in gene banks. A significant number of crop and forest species do not produce orthodox seeds[44] and storage of their germplasm is therefore difficult. Other species, such as root and tuber crops, fruit trees and forest trees are vegetatively propagated because clonal multiplication allows the preservation of their unique genetic setups. In both cases, the germplasm can be preserved in field gene banks or *in vitro*.

Field gene banks are expensive to maintain, require more space and are not very secure. Hence, short to medium term (1-15 years) conservation of vegetatively propagated crops and forest trees is best achieved with *in vitro* slow growth storage, i.e. as sterile tissue/plantlets on nutrient gels. Growth is usually limited by reducing temperature (0-5 ^{o}C for cold-tolerant species and 15-20 ^{o}C for tropical species) and/or light intensity, by modifying the nutrients in the culture medium, reducing oxygen levels (Rao, 2004) and the use of mineral oil overlay (Mathur *et al*., 1991).

Advantages of this method include reduced storage space for maintaining a large number of explants in an aseptic environment, decreased need for frequent subculturing, the potential for high clonal multiplication rates, ease of transfer of germplasm due to the smaller size of cultured material, and reduced need for quarantine during germplasm movement and exchange. However, *in vitro* maintenance is time and labour intensive, requires specialized equipment and has an increased risk of somaclonal variation[45] as well as losses due to contamination of the culture media or mislabelling (Panis and Lambardi, 2006). A prerequisite, of course, is the availability of suitable protocols for *in vitro* culture in the species of interest. This is currently a major limitation for the application of the technology to a large number of forest tree species.

2.2.3 Reproductive biotechnologies (Livestock and fisheries/aquaculture)

A number of reproductive biotechnologies have considerable potential for conserving livestock and fish by facilitating the storage, and eventual multiplication and dissemination, of genetic resources and

[44] Seeds which can be dried to a low moisture content and stored at low temperatures without losing their viability over long periods of time.

[45] The term refers to epigenetic or genetic changes induced during the callus phase of plant cells cultured *in vitro*.

reducing the risk of disease transmission. In livestock, however, the main use of these technologies is not for this purpose but as tools to increase animal production by increasing the number of offspring from elite individuals or by expanding the traditional geographical range of the most productive commercial breeds. This latter application can lead to the loss of indigenous breeds.

Controlled breeding (artificial breeding)
Controlled breeding is widely practised in aquatic species. Adults are spawned under controlled conditions, and often hormones or environmental stimuli are provided to promote spawning. Eggs or larvae are collected for placement alive in contained or open environments. Gametes may also be irradiated or chemically treated to destroy DNA of one sex (androgenesis and gynogenesis), thus allowing manipulation of the embryonic genome.

Artificial insemination
Artificial insemination (AI) is the process of collecting sperm cells from a donor male and manually depositing them into the reproductive tract of an ovulating female to achieve pregnancy. Progesterone monitoring and oestrus synchronization are prerequisites for improving AI efficiency in livestock and ultimately the reproductive potential of animals.

Progesterone monitoring
On their own, traditional methods for recording the reproductive cycle of the animal are often inaccurate. Combined with methods such as radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA), that are used to measure the progesterone level in blood or milk, a more precise assessment of the reproductive status of animals can be made to identify individuals that are anoestrous or non-pregnant.

RIA is based on the use of a radioactively labelled antibody, where the amount of radiation detected indicates the amount of target substance present in the sample (FAO, 2001). ELISA is an antibody-based technique for the diagnosis of the presence and quantity of specific molecules in a mixed sample (FAO, 2001). An enzyme is attached to a reactant (antigen or antibody) in a system that is used to generate a colour signal with an appropriate chromogen and substrate combination.

Oestrus synchronization
Oestrus synchronization is the process of bringing females into oestrous at the desired time by treatment with hormones such as oestradiol, prostaglandins, progesterone, or gonadotropins. Such systems often use a combination of hormones to manipulate either the luteal or follicular phase of the oestrus cycle, while maintaining normal fertility.

Embryo transfer
Embryo transfer (ET) involves the transfer of an embryo from a superior donor female to a less valuable female animal. Embryos for eventual transfer or cryopreservation are normally recovered using multiple ovulation (where the females are hormonally induced to release several oocytes). An alternative is to harvest oocytes from live donor animals (ovum pickup) or ovaries of slaughtered animals, and then fertilize the oocytes *in vitro*, after which the fertilized embryos can be transferred fresh or frozen. On average, the gestation rates of frozen embryos are much lower than those of fresh embryos produced *in vitro*.

In vitro *fertilization*
In vitro fertilization (IVF) involves harvesting the oocytes from the donor's ovaries, culturing them and fertilizing them with sperm *in vitro*, followed either by transfer to recipient females or cryopreservation. IVF is widely practised in aquatic species where gametes are collected from both sexes and physically mixed to induce fertilization. Fertilized eggs or hatched larvae may be placed alive in contained or open environments.

Cloning
Cloning can be accomplished using embryo splitting, somatic cell nuclear transfer or stem cell nuclear transfer. Embryo splitting is performed by surgical bi-section of early tubal stage embryos, while somatic

cell nuclear transfer involves fusing the nucleus of a single diploid cell with an enucleated and unfertilized ovum (Boa-Amponsem and Minozzi, 2006). Embryonic stem cells are derived from the totipotent cells of the early embryo and can give rise to all differentiated cells, including germ line cells.

2.3 Utilization of GRFA

Genetic resources are the raw material for agricultural development and for the continued survival of natural populations. Therefore their sustainable use is crucial for global food security and economic well-being. Biotechnologies are increasingly being applied for the enhancement of GRFA and have had a profound impact on their effective utilization.

2.3.1 Reproductive biotechnologies (Livestock and fisheries/aquaculture)

In addition to the reproductive technologies described earlier, sperm and embryo sexing permits the preferential production of one sex in livestock.

Sperm sexing
Depending upon the species, the X chromosome contains 2-5 percent more DNA than the Y chromosome (Boa-Amponsem and Minozzi, 2006). The DNA contained in the sperm has distinct emission patterns when stained with a fluorescent dye and exposed to light, thus allowing separation by flow cytometry[46].

Embryo sexing
Y chromosome-specific DNA probes, karyotyping, male-antigen specific antibodies and X-linked activity enzymes are used for embryo sexing, with Y chromosome-specific DNA probes being the most reliable.

Hormonal treatment
Similar to livestock, where hormonal treatment is applied for oestrus synchronization (see section 2.2.3), in aquaculture hormonal treatment is used for two main purposes, i.e. controlling the time of reproduction and developing monosex populations. Chemically synthesized hormones are relatively inexpensive and practical to use.

2.3.2 Biotechnologies for disease diagnostics (Cross-sectoral)

Diseases are a major impediment to the sustainable utilization of GRFA. Biotechnologies, based on immunoassays and on nucleic acid detection, for pathogen screening and disease diagnostics are important in all agricultural sectors and can contribute to improved plant and animal disease control and to food safety.

ELISA can be applied either for the detection of an antigen (i.e. pathogen) or for the detection of antibodies produced by the host in response to the pathogen. Both monoclonal[47] and polyclonal[48] antibodies can be employed for the assay. The development of recombinant antigens has further improved the rapidity, specificity, sensitivity and safety of ELISA. Competitive ELISA (C-ELISA) detects the antibody or antigen based on competition between the test serum and the detecting antibody. This method has an added advantage since a relatively crude/impure sample can be used for the assay (OIE, 2009).

PCR is a highly sensitive procedure and the most widely applied molecular technique for disease diagnostics. It allows the amplification of a specific DNA sequence from a complex mixture of heterogeneous sequences. The amplified DNA can then be identified using gel electrophoresis or

[46] Automated measurements on large numbers of individual cells or other small biological materials, made as the cells flow one by one in a fluid stream past optical and/or electronic sensors (FAO, 2001).

[47] An antibody, produced by a hybridoma, directed against a single antigenic determinant of an antigen (FAO, 2001).

[48] A serum sample that contains a mixture of distinct immunoglobulin molecules, each recognizing a different antigenic determinant of a given antigen (FAO, 2001).

hybridization with a labelled DNA probe. Since PCR detects pathogen DNA rather than host antibodies, it provides more rapid results by eliminating the lag period between the initial infection and the appearance of detectable amounts of antibodies.

In the case of certain viruses, whose genomes are made of RNA, reverse transcriptase PCR (RT-PCR) is necessary, i.e. a complementary DNA (cDNA) copy is first synthesized using reverse transcriptase which then acts as the template for amplification. The sensitivity of PCR can be further enhanced by utilizing a technique referred to as nested PCR, i.e. a second set of primers is used to amplify a sub-fragment of the first PCR product. Real-time/quantitative PCR provides quantitative information by measuring the accumulation of the PCR product during the amplification reaction. It is more rapid than conventional PCR techniques, thus reducing the risk of cross-contamination, and can be scaled up for high-throughput applications. The loop-mediated isothermal amplification (LAMP) PCR is a robust cost-effective test that is performed at one temperature (i.e. no need for expensive temperature ramping PCR machines) and the amplified product can be detected visually.

RFLPs can be used to distinguish between isolates of closely related pathogens. When integrated with PCR (PCR-RFLP), it offers improved sensitivity and is particularly valuable when the pathogen is available in small numbers or is difficult to culture. Microarrays may also be used to detect pathogens, especially when more than one pathogen might be present, although more extensive application of this technology for disease diagnosis would require it to become less expensive.

2.3.3 Biotechnologies for disease control (Livestock and fisheries/aquaculture)

Biotechnologies have also been extensively used in the development of vaccines for preventing and thereby managing diseases in livestock and fish. A vaccine is a preparation of dead or attenuated (weakened) pathogens, or of derived antigenic determinants, that can induce the formation of antibodies in a host, and thereby produce host immunity against the pathogen. Molecular techniques can facilitate the identification of potential antigen candidates that may be effective in vaccines (e.g. by using monoclonal antibodies and expression libraries), construction of new candidate vaccines (e.g. by using PCR and cloning) as well as assessment of candidate vaccine efficacy, its mode of action and host response (e.g. by using quantitative RT-PCR) (Kurath, 2008).

The different types of vaccines, produced by molecular methods, are described below (more details available in OIE, 2010a).

Gene deletion vaccines
Gene-deleted pathogens, with deletions in genes associated with virulence or involved in key metabolic pathways, can be used as live vaccines as they retain the immunogenicity of the wild-type organism but have reduced virulence. Live attenuated vaccine strains of bacteria have been created that confer better protection than killed vaccines.

Virus-vectored vaccines
Suitable vectors are essential for the efficient delivery of protective antigens into the animal. Many viruses have been used as vaccine delivery vehicles since their genomes can accommodate large amounts of exogenous DNA. Replication competent as well as replication defective vectors have been commercially developed. Furthermore, a virus may act both as a vector and a self vaccine (e.g. the recombinant capripox virus expressing a peste des petits ruminants virus, i.e. PPRV, antigen).

DNA vaccines
Recombinant DNA technology has made possible the construction of safer and more cost-effective vaccines since only the desired antigen (that is unable to replicate or induce disease) instead of the entire pathogen is used. DNA vaccination involves the direct inoculation, into the animal, of an antigen-encoding bacterial plasmid to elicit the immune response. DNA vaccines can stimulate the induction of both humoral and cell-mediated immune responses, vital for protection against a wide range of pathogens.

They are relatively easy to produce in addition to being very stable with a long shelf life. Additionally, the need for complex vector organisms is obviated.

Subunit vaccines
Subunit vaccines are composed of protein or glycoprotein components of a pathogen that are capable of inducing a protective immune response, and may be produced by conventional biochemical or recombinant DNA technologies. Recombinant subunit vaccines have distinct advantages over live attenuated and inactivated vaccines since they are efficient in inducing humoral and cell-mediated immunological responses and the risks associated with handling the pathogen are eliminated. However, subunit vaccines may be more expensive and may require specific adjuvants[49] to enhance the immune response.

Marker vaccines
In addition to limiting the clinical impact of the disease, marker vaccines optimize the effectiveness of the vaccination strategy. Such vaccines, together with companion diagnostic tests, are of paramount importance for evaluating disease eradication programmes since they are capable of differentiating infected and vaccinated animals (DIVA) in case of an outbreak. DIVA vaccines have at least one less antigenic protein than the corresponding wild-type virus and are based on detecting the serological response either towards a protein whose gene has been deleted in the vaccine strain or against virus non-structural proteins not present in subunit vaccines and highly purified vaccines.

2.3.4 Chromosome set manipulation (Crops, forest trees and fisheries/aquaculture)

Chromosome set manipulation, i.e. alteration of the chromosome set, is used for a range of different purposes in agriculture. In fish and plants, this technique has been used to induce polyploidy (to create sterile individuals) and for uniparental chromosome inheritance (valuable in breeding programmes and for the establishment of monosex populations).

2.3.5 Tissue culture-based techniques (Crops and forest trees)

FAO studies on the development, adoption and application of biotechnologies in developing countries indicate that tissue culture is the most common biotechnology technique for plant genetic resources, being applied in 88 percent of the 25 developing countries surveyed (Sonnino et al., 2007). Similarly, FAO (2004) highlights its importance for the forestry sector, where micropropagation activities were reported in at least 64 countries worldwide, mainly in Asia, Europe and North America.

Wide crossing
Inter-specific hybridization/wide crossing involves crossing plants belonging to two different species that are not normally sexually compatible. It is used to transfer useful characteristics from wild relatives to cultivated species or to combine favourable traits of two different species, but significant amounts of time and scientific expertise need to be invested. Biotechnology approaches such as in vitro embryo rescue and anther culture are crucial to overcome sexual incompatibility and to speed up the process. In vitro embryo rescue utilizes a sequence of tissue culture techniques to enable a fertilized immature embryo, resulting from an interspecific cross, to avoid abortion caused by unbalanced endosperms and to continue growth and development until it can be regenerated into an adult plant. Anther culture is the aseptic culture of immature anthers (within which pollen develops and matures) to generate haploid plants. The chromosome number is then doubled through the application of chemicals such as colchicine or other in vitro techniques. Colchicine is also used to artificially double the chromosome number of sterile wild

[49] An adjuvant is a substance that enhances immune responses when co-administered with the antigen.

hybrid plants in order for functional pollen and eggs to be produced and allopolyploid[50] fertile progenies to be obtained.

Somatic hybridization
Another method to circumvent reproductive barriers and introduce novel genes into a plant's genome from a dissimilar donor species is somatic hybridization. It involves the induced in vitro fusion of protoplasts or cells of two genetically different parents and the subsequent regeneration of adult plants from the resultant hybrid cells. Application of somatic hybridization is limited by the regeneration rate of the hybridized cells, which can be very low for certain hybrid combinations.

Micropropagation
Micropropagation refers to the in vitro multiplication and/or regeneration of plant material under aseptic and controlled environmental conditions to produce thousands or millions of plants for transfer to the field. It is a fast and low-cost method to overcome the accumulation of infectious agents in vegetatively propagated plants and has been used for mass clonal propagation of true-to-type, disease-free material in more than 30 developing and transition countries (Sonnino et al., 2009).

2.3.6 Molecular marker-assisted selection (Cross-sectoral)

An alternative or complement to conventional phenotypic selection is molecular marker-assisted selection (MAS), where the desired trait is selected indirectly by selecting for a marker(s) genetically linked to a gene or genes influencing it. MAS can thus greatly accelerate genetic improvement by enhancing the accuracy of selection and reducing the time needed (particularly when phenotype screening is difficult or when the trait is expressed late in the life of the individual). A successful MAS strategy is dependent upon the availability of a genetic map with sufficient number of regularly spaced markers, tight linkage between the gene or QTL of interest and adjacent markers, adequate recombination between the markers and the rest of the genome as well as the ability to analyze a larger number of individuals in a time- and cost-effective manner. Furthermore, MAS is best utilized when embedded within existing conventional breeding programmes that already have performance and pedigree recording systems in place.

Genomics tools for the simultaneous screening of tens of thousands of genetic markers in the form of SNPs have allowed for the application of so-called genomic selection (GS), an advanced form of MAS. In this procedure, a training set of data consisting of genomic and phenotypic information on a subset of the population is used to establish statistical associations between markers and phenotypes and to develop equations for prediction of breeding values that are then applied to individuals without phenotypic data, i.e. the validation set (Meuwissen *et al*., 2001). GS does not require *a priori* phenotypic information for the validation set and thus can increase the genetic gain by accelerating the breeding cycle.

Even though genotyping is becoming less expensive than phenotyping in applied breeding programmes (due to an increase in the number of markers and reduced cost per data point), GS is not yet applied in developing countries. One reason is that the accuracy of breeding value estimation depends on the number of individuals in the reference population with which to construct the training set (the population should also be genetically and phenotypically characterized), and considerable financial and technical resources are required to establish such reference populations. Moreover, using reference data from developed-country populations for selection is complicated due to differences in breeding objectives between the production systems.

2.3.7 Fermentation (Microbial)

Fermentation is the anaerobic breakdown of complex organic substances into simpler substances by microbes (FAO, 2001). In addition to extending the shelf-life, quality and safety of food, it is widely

[50] A polyploid organism with sets of chromosomes derived from different species (FAO, 2001).

applied to produce a variety of metabolites including vitamins, antimicrobial compounds, enzymes, flavours, fragrances, food additives and a range of other high value-added products.

2.3.8 Biofertilizers (Microbial)

Biofertilizers are preparations containing live or latent cells of agriculturally beneficial strains of micro-organisms that are applied to seed or soil to build-up the numbers of such micro-organisms and accelerate certain microbial processes to augment nutrient acquisition by plants (Motsara and Roy, 2008). Biofertilizers consist of nitrogen fixers (*Rhizobium, Azotobacter*, *Azospirillum,* cyanobacteria/blue-green algae, *Azolla*), phosphate solubilizing micro-organisms and mycorrhizal fungi.

2.3.9 Biopesticides (Microbial)

Biopesticides are living organisms or natural products derived from these organisms that are mass-produced and employed to control pests (Chandler *et al*., 2008; Bailey *et al*., 2010). The organisms employed can be insects, nematodes or micro-organisms while naturally occurring substances include plant extracts and insect pheromones. Micro-organisms are the commonest biopesticides and include protozoa, bacteria, fungi and viruses, and of these, the most dominant is *Bacillus thuringiensis* (Chandler *et al*., 2008).

2.3.10 Bioremediation (Microbial)

Bioremediation refers to the use of living organisms to remove contaminants, pollutants or unwanted substances from soil or water (FAO, 2001). This technology mainly utilizes microbes, although the cultivation of plants can accelerate bioremediation since the rhizosphere[51] provides a favourable environment for microbial proliferation (Wenzel, 2009; Stout and Nüsslein, 2010). Such plant-assisted bioremediation is referred to as phytoremediation and can also be accomplished by exploiting plant-endophyte[52] partnerships (Weyens *et al*., 2009). In aquaculture, integrated multi-trophic aquaculture (IMTA) strategies have been developed for bioremediation, that employ complementary organisms such as bacteria, seaweeds, shellfish, filter feeders and bottom feeders to optimize nutrient utilization and waste treatment (Chávez-Crooker and Obreque-Contreras, 2010).

2.3.11 Probiotics (Microbial)

Probiotics are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Foods containing probiotics are considered to be important in human health and nutrition, with lactic acid bacteria reported to have the most beneficial effects in the human gastrointestinal tract (Burgain *et al*., 2011). Probiotics are also included as feed additives for both livestock and fish, with strains of *Lactobacillus* and *Bifidobacterium* generally used for monogastric animals, and *Aspergillus oryzae* and *Saccharomyces cerevisiae* for ruminants (FAO, 2011c), while *Lactobacillus*, *Carnobacterium*, *Vibrio*, *Bacillus* and *Pseudomonas* are prevalent in finfish and shellfish aquaculture (Balcazar *et al*., 2006). Probiotics are beneficial in multiple ways, such as conferring antagonism against pathogens, strengthening the host's immune system and providing nutritional benefits (Soccol *et al*., 2010). Their efficacy can be increased by supplementation with prebiotics, i.e. non-digestible oligosaccharides (Nayak, 2010a).

[51] The soil region in the immediate vicinity of growing plant roots.

[52] A microorganism that lives inside a plant.

2.3.12 Mutagenesis (Cross-sectoral)

Chemical, radiation or somaclonal mutagenesis can be used to accelerate the process of spontaneous mutation to create novel phenotypes. Mutagenesis is one of the few biotechnologies that is employed more in developing countries than elsewhere, with the FAO/International Atomic Energy Agency (IAEA) partnership being instrumental in technology transfer of mutation breeding approaches.

2.3.13 Transgenesis (cross-sectoral)

Transgenesis/genetic modification refers to the introduction of exogenous DNA or RNA sequences into an organism's genetic material to create a genetically modified organism (GMO). The input gene(s) may be from a different kingdom, a different species within the same kingdom or even from the same species (FAO, 2009a). Genetic modification has been at the centre of a highly polarized debate worldwide in recent years, due to which non-GMO biotechnologies, and their potential benefits for food security and sustainable development in developing countries, have tended to be neglected in most discussions about biotechnology, often hindering their development and application. This is despite the fact that the major breeding and genetic resource management applications to date have come from non-GMO biotechnologies (FAO, 2011a, b, c, d and e). For these reasons, and because of the already very extensive literature regarding the many different issues surrounding their use, GMOs are not discussed in detail in this paper.

III. CURRENT STATUS OF BIOTECHNOLOGIES FOR THE MANAGEMENT OF CROP GENETIC RESOURCES

Estimates indicate that an additional one billion tonnes of cereals will need to be produced annually by 2050 in order to meet the needs of the world's population (FAO, 2009b). It is projected that the majority of this increase in crop production will come from intensification and a small percentage from the expansion of arable land. However, the rate of growth in yields of the major cereal crops has been steadily declining globally; an estimated 75 percent of crop diversity has been lost between 1900 and 2000 (FAO, 2010c), and 16-22 percent of the wild relatives of important food crops of peanut, potato and beans are predicted to disappear by 2055 due to the impact of climate change (Jarvis *et al*., 2008).

Consequently, there is a need for addressing productivity constraints in conjunction with conserving the natural resources base, while addressing the need for improving farming sustainability and facing the potential challenges posed to crops and agrosystems by climate change. Productivity constraints may also be addressed through the focused promotion of non-cereal crops which could benefit the most from future variety improvement because their yields are still far from the theoretical crop productivity potential. Non-cereal crops are also less affected than grains by global price fluctuations, which places them in a favourable position to address food security in the poorest countries. Another critical factor underpinning the future of food security is the conservation and utilization of Crop Wild Relatives (CWR)[53] populations, since they contain far more genetic diversity than the crops themselves. As reviewed in this section, rapid scientific advances in crop biotechnologies, especially in the last two decades, have provided important tools for tapping into the diversity of CWR and for enhancing the management of crop genetic resources, for example, through investigating genetic diversity, developing more effective conservation strategies and obtaining improved crop varieties.

3.1 Molecular markers

Knowledge of the genetic variation within crops and their phylogenetic relationships with wild relatives is important for informed decision-making and designing appropriate conservation and breeding strategies.

[53] The wild ancestors of crop plants and other species closely related to crops.

Molecular markers have been employed for genetic diversity studies in many crop species, including wheat (Hai *et al*., 2007; Zarkti *et al*., 2010), rice (Saker *et al*., 2005; Huang *et al*., 2010a), maize (Lanteri and Barcaccia, 2006; Van Inghelandt *et al*., 2010), barley (Orabi *et al*., 2009; Wang *et al*., 2010a), common bean (Jose *et al*., 2009; Blair *et al*., 2010), sorghum (Ali *et al*., 2008; Pei *et al*., 2010), sugarcane (Kawar *et al*., 2009; Singh *et al*., 2010) and potato (Fu *et al*., 2009; Akkale *et al*., 2010). Markers have provided information on the ecological or geographic patterns of diversity distribution in numerous crops and their wild relatives (Rao, 2004). The use of molecular markers for genetic diversity studies in crop species has increased worldwide in the last decade although it is still limited in developing countries (FAO, 2010c).

Molecular markers have also been used to evaluate the effectiveness of different conservation strategies on the genetic structure of populations (Lanteri and Barcaccia, 2006). Marker analysis of a threatened common bean landrace showed that *in situ* conservation is the most effective way to maintain the diversity (Negri and Tiranti, 2010) while for capsicum, novel genetic variation was found in both the *in situ* population studied and in some *ex situ* accessions, thus supporting conservation of this species via both strategies (Votava *et al*., 2002). The genetic diversity of maize populations maintained *in situ* and *ex situ* was found to be substantially equal (Rice *et al*., 2006).

Determination of the impact of adoption of improved varieties on the genetic diversity of germplasm is essential to assess the need for further incorporation of exotic germplasm into the existing breeding pool. Microsatellite studies with Nordic spring wheat cultivars illustrated that genetic diversity was enhanced by breeders in the first quarter of the 20th century, followed by a decrease and then again an increase during the second quarter of the century (Christiansen *et al*., 2002). Similar results were reported in other studies assessing the diversity of bread wheat in the United Kingdom (Donini *et al*., 2000) and of durum wheat (Maccaferri *et al*., 2003). Molecular investigations with rice cultivars have revealed that while genetic diversity has increased over time in Italy (Mantegazza *et al*., 2008), it has declined to a certain extent in recent decades in China (Wei *et al*., 2009).

Molecular markers have provided insight into the identification of crop progenitors, origins of domestication and the molecular changes underlying domestication traits (Burger *et al*., 2008; Gross and Olsen, 2010). For example, microsatellite data proved that maize is the product of a single domestication event from its wild progenitor in southern Mexico (Matsuoka *et al*., 2002) as is einkorn wheat from its wild relatives in south-east Turkey (Heun *et al*., 1997) and pearl millet in West Africa (Oumar *et al*., 2008). In contrast, multiple domestication events have been uncovered for barley (Morrell and Clegg, 2007), common bean (Chacon *et al*., 2005) and squash (Sanjur *et al*., 2002). To date, nine domestication genes have been identified in plants in addition to 26 other loci that underlie crop diversity associated with human cultural preferences or different agricultural environments (Purugganan and Fuller, 2009).

Introgression between wild populations and cultivated plants is a widespread phenomenon with 12 of the 13 most important food crops of the world hybridizing with wild relatives in some regions of their agricultural distribution (Ellstrand *et al*., 1999). Overwhelming gene flow from crops can deplete the genetic diversity of wild populations, leading in some cases to their genetic extinction, while crop-to-weed gene flow has the potential to promote the evolution of more aggressive weeds (Papa, 2005; Andersson and de Vicente, 2010). Gene flow is particularly difficult to assess when crops and wild species are very closely related. In such cases, molecular markers have proved to be informative tools to monitor gene flow from cultivated crops and ponder over its consequences, for example for rice (Chen *et al*., 2004) and sorghum (Morrell *et al*., 2005) with respect to their wild and weedy relatives. Marker analysis with common bean demonstrated that gene flow was about three- to four-fold higher from domesticated to wild populations than in the reverse direction. Further, the weedy populations had intermediate traits between wild and domesticated bean plants suggesting that they were hybrids, rather than escapes from cultivation (Papa and Gepts, 2003).

Considerable progress has been made in identifying and consequently utilizing QTLs for breeding in crop species. Molecular markers tightly linked with many agronomic, stress tolerance and disease resistance QTLs are available in major crop species (Bernardo, 2008; Collins *et al*., 2008; Yadav *et al*., 2011a) and their wild relatives (Swamy and Sarla, 2008). Genetic linkage maps of cultivated and wild *Vigna* crop species (cowpea, mung bean, rice bean, azuki bean and black gram) have been developed and used to identify QTLs of domestication-related traits (Takeya *et al*., 2011).

QTL mapping experiments are generally heterogeneous and hence comparative QTL mapping is necessary to synthesise all the information for MAS purposes. For a few species (rice, wheat, maize, barley, oat, sorghum, pearl millet, foxtail millet and wild rice), the Gramene QTL database integrates results from independent experiments for 11 624 QTLs for numerous traits.[54] For other species, QTL meta-analysis has been performed for traits of interest to underscore chromosomal regions for use in breeding (Lanaud *et al*., 2009; Danan *et al*., 2011).

Molecular markers are of considerable value in first identifying populations for collecting by developing optimum sampling strategies for gene banks and subsequently, for the management of the accessions held. For example, AFLP marker studies undertaken to evaluate Sri Lankan coconut populations showed that the greatest diversity is found within populations rather than between populations, and hence emphasis should be placed on collecting a large number of plants from a few populations (Perera *et al*., 1998).

Molecular markers assist in the management of conserved germplasm by increasing the efficiency of gene bank operations. They are an effective tool to first characterize accessions in the gene bank and help identify useful traits. Molecular markers can identify both gaps (missing/underrepresented populations) and redundancies (duplicate accessions as opposed to safety duplicates) in collections to guide future acquisition and increase cost-effectiveness.

Currently, less than 30 percent of the 7.4 million plant germplasm accessions held in gene banks worldwide are estimated to be sufficiently distinct (FAO, 2010c). It has been calculated that the additional cost of identifying a duplicate cassava accession (that is to be added to the collection) using molecular characterization, once passport data has been checked, is about 12 times less than the cost of conserving and distributing the material as a different accession in-perpetuity (Horna *et al*., 2010).

The International Potato Center (CIP) in Peru employed molecular markers to compare accessions of sweet potato that appeared identical morphologically, thus identifying duplicates and reducing their clonal collections by approximately two-thirds (Dawson *et al*., 2009). However, in AFLP studies with a wild potato collection, the costs to detect redundancies were estimated to be approximately 2.5 times higher than the savings expected per generation by the reduction of the collection (Van Treuren *et al*., 2004). Thus, the benefits of a reduced collection do not always offset the requisite investments to identify redundancies, which vary depending on the number/type of markers employed, the regeneration costs of the concerned crop, the final level of redundancy detected and the timeframe within which investments returns are expected (Van Treuren *et al*., 2010). Nevertheless, it must be mentioned that progress in marker technologies is proceeding at a fast pace and costs are dropping, albeit not at the same rates witnessed in DNA sequencing technologies. In such a changing landscape, it can be envisioned that markers will play an increasingly important role in the management of gene banks.

Periodic regeneration and multiplication are essential features of gene bank management (to maintain viability and replenish stocks for distribution), following which assessment of genetic integrity is crucial. The size of the seed sample for regeneration should reflect both the reproductive biology of the species under consideration and the degree of homogeneity/heterogeneity of the accession.[55] In this respect,

[54] http://www.gramene.org/qtl/

[55] http://typo3.fao.org/fileadmin/templates/agphome/documents/PGR/ITWG/ITWG5/ITWG5_INF3Upton.pdf

molecular markers can assist in estimating the effective population size (N_e) that will ensure the genetic integrity of the accessions. Molecular markers can be also employed to verify accession identity, detect inadvertent seed mixtures and monitor changes in alleles/allele frequencies as well as gene flow between accessions (Spooner *et al*., 2005; de Vicente *et al*., 2006).

A study investigating the genetic identity of wheat accessions (regenerated 24 times), using microsatellites, did not detect any unintended pollen or seed mixing for any of the accessions, but found that genetic drift had occurred in one case (Börner *et al*., 2000). Another study with AFLPs, comparing wheat accessions (derived from identical sources) duplicated at two different gene banks, concluded that while the overall genetic diversity was conserved at both locations (compared to the original collections preserved without regeneration), there was possible unintentional selection at one gene bank (Hirano *et al*., 2009). Therefore efficient regeneration strategies coupled with continual monitoring of the genetic diversity conserved in gene banks are imperative.

Molecular markers can also be used to make larger collections more accessible and useful for allele mining by developing core collections, i.e. subsets that consist of a small percentage of the entire collection while still representing a broad spectrum of genetic variability. Markers have generated genetic diversity information to assist in the establishment of core collections for many species, including maize (Franco *et al*., 2006; Qi-Lun *et al*., 2008), wheat (Hao *et al*., 2006), rice (Ebana *et al*., 2008), potato (Ghislain *et al*., 2006), chick pea (Upadhyaya *et al*., 2008), grape (Le Cunff *et al*., 2008), cacao and pepper (Marita *et al*., 2000). For creating core collections, emphasis should be placed on methodologies that use data generated by markers in concert with the morphological and agronomical characterization of the accessions (Balfourier *et al*., 2007; Jansen and van Hintum, 2007).

Core collections for certain species, particularly grasses, are still sizeable and therefore of limited use to breeders. Molecular markers have been used to develop thematic core collections that are much smaller in size but exhibit the maximum allelic richness for specific traits of interest (Pessoa-Filho *et al*., 2010). A germplasm collection, with a defined core collection together with various thematic core collections focusing on different traits, thus has the potential to increase the efficiency of germplasm use in breeding programmes.

The establishment and maintenance of gene banks must be coupled with the ability to identify useful genes and utilize the genetic diversity with much greater efficiency. To facilitate the generation and exchange of standardized molecular marker data for plant germplasm held in gene banks, a list of descriptors (de Vicente *et al*., 2004) and guidelines for developing new descriptor lists (Bioversity International, 2007) have been produced although, in general, the amount of characterization data is quite low. The situation is further exacerbated in developing countries, where the percentage of accessions characterized using molecular markers is less than 12 percent, with the exception being 64 percent in the Near East (FAO, 2010c). This lack of adequate characterization is a major impediment to the sustainable use of GRFA (even though the number of accessions deposited is continuously growing), hence characterizing the extensive collections maintained in gene banks must be prioritized.

An additional challenge, especially in developing countries, is the management of the molecular, phenotypic and agricultural data that is being generated across the gene banks. The Germplasm Resource Information System (GRIN)-Global Project[56], a partnership between the Global Crop Diversity Trust, Bioversity International and the Agricultural Research Service of the United States Department of Agriculture, is being developed to provide the world's crop gene banks with a powerful, but easy-to-use plant genetic resource information management system. It will also allow researchers and breeders to access and utilize the information more effectively.

[56] http://www.grin-global.org

3.2 "Omics"

The genomes of several plant species have been sequenced to date, including rice, maize wheat, sorghum, soybean, cassava, potato and tomato (Mochida and Shinozaki, 2010). Whole genome sequences have led to the discovery of thousands of SNPs to create high-density genetic maps (Ganal *et al*., 2009) and together with functional "omic" technologies, have greatly facilitated identification of candidate genes (Langridge and Fleury, 2011). For example, transcriptome profiling in concert with QTL mapping has been used to detect novel candidate genes associated with tuber quality traits in potato (Kloosterman *et al*., 2010) and grain number in rice (Deshmukh *et al*., 2010).

Next generation sequencing (NGS) technologies allow DNA sequencing at a much higher speed and greatly reduced cost, and are being employed for a range of applications (Varshney *et al*., 2009a; Deschamps and Campbell, 2010). Markers can be discovered on a genome-wide scale by aligning short reads of sequence from the genotype of interest to a reference genome (Huang *et al*., 2009a) or through the *de novo* sequencing of species without reference sequences (Bundock *et al*., 2009). In polyploid species with complex genomes, SNPs have been detected after the creation of cDNA libraries to avoid sequencing repetitive regions (Barbazuk *et al*., 2007). Transcriptome sequencing has also been useful for less-characterized species such as chick pea, for the identification of SNPs.[57]

A technology that has recently gained attention for genetic characterization is a hybridization based method, diversity arrays technology (DArT). Diversity panels are created using DNA fragments pooled from various varieties/cultivars which are then hybridized with individual DNA samples. Differential hybridization reveals polymorphisms between the samples (Jaccoud *et al*., 2001). DArT simultaneously assays thousands of markers in parallel across samples, and since it does not require prior DNA sequence information, it is of special interest to orphan crops. It is a low-cost high-throughput system, although a prerequisite is the development and validation of a diagnostic DArT array. This technology has been applied successfully in many species, including wheat (Akbari *et al*., 2006), rice (Xie *et al*., 2006), cassava (Xia *et al*., 2005), barley (Wenzl *et al*., 2004), pigeon pea (Yang *et al*., 2006), sorghum (Mace *et al*., 2009) and oat (Tinker *et al*., 2009).

Genome-wide scans for selection signatures related to domestication have been carried out in only a small number of crop species (Wright *et al*., 2005; Chapman *et al*., 2008). In rice, it has been suggested that selection could leave a genome-wide imprint, rather than a localized signature (Caicedo *et al*., 2007). Genome-wide association mapping studies, correlating phenotype data with genome-wide genotypes, are beginning to be used to detect loci for agronomically important traits in a few crops like wheat (Neumann *et al*., 2011), maize (Tian *et al*., 2011), barley (Cockram *et al*., 2010) and rice (Huang *et al*., 2010b). However, this technique has lower sensitivity for detecting rare alleles and can be ineffective if there is strong genetic differentiation within populations (Nordborg and Weigel, 2008).

Comparative genomics has contributed to understanding genetic diversity, offered insights into evolution and enhanced gene discovery (Duran *et al*., 2008; Flavell, 2008). The CGIAR Generation Challenge Program (GCP)[58] used this approach to identify orthologous genes[59] for improving cereal yields for maize, rice and sorghum in high-aluminium and in low-phosphorous soils.

Transcriptomic approaches have been used to characterize crop species and to improve the understanding of complex responses, for example by evaluating global gene expression changes in response to disease-causing pathogens (Sana *et al*., 2010), symbiotic associations (Hocher *et al*., 2011), abiotic stresses (Narsai *et al*., 2010), low phosphorus (Li *et al*., 2010a) and aluminium phytotoxicity (Mattiello *et al*.,

[57] http://www.intl-pag.org/16/abstracts/PAG16_P05f_385.html

[58] http://www.generationcp.org/

[59] These are genes in different species that can be traced back to the same common ancestor and normally retain the same function in the course of evolution.

2010). Evolving proteomic technologies have facilitated the investigation of developmental processes (Agarwal and Rakwal, 2006), abiotic stress tolerance (Manaa *et al*., 2011; Sobhanian *et al*., 2011), and the detection of plant pathogens as well as study of plant-microbe interactions (Kav *et al*., 2007).

Metabolomics is also emerging as a promising tool for the fundamental biochemical comprehension of plant metabolism, analysis and discovery of a broad range of metabolites, for determining metabolite relationships with specific quality traits and for discriminating taxonomic relationships (Guy *et al*., 2008; Fernie and Schauer, 2009; Summer, 2010; Shepherd *et al*., 2011).

3.3 Bioinformatics

Sequencing is becoming simpler and cheaper and several bioinformatic tools are available for allele mining analysis of these data, such as PLACE[60] and plantCare[61] (Kumar *et al*., 2010). Providers for plant genome sequences and annotations that also facilitate comparative genomic studies include PlantGDB[62] with sequence data for over 70 000 plant species, Phytozome[63] with access to 23 species, Rice Genome Annotation Project[64], MaizeGDB[65], SoyBase[66], Brassica Genome Gateway[67], SOL genomics network[68] for Solanaceae species, GrainGenes[69] for wheat, barley, rye and oat, and Gramene[70] for grasses. Plant-specific proteomic databases include the rice proteome database[71] and the soybean proteome database[72]. Metabolic platforms include the Plant Metabolic Network[73], the Metabolome Tomato Database[74] and the Armec Repository Project[75] for potato. More details on bioinformatic resources for plants can be found in Skuse and Du (2008).

3.4 Cryopreservation

Cryopreservation has been applied to over 200 plant species using diverse materials such as seeds, cell suspensions, callus cultures, meristematic tissue, pollen, and somatic and zygotic embryos (Dulloo *et al*., 2010; Harding, 2010). It is particularly important for the long-term storage of vegetatively propagated crop species such as cassava and banana, as well as species that produce recalcitrant seeds[76] such as coconut and mango. Generally, the plants recovered after cryopreservation maintain their genetic integrity and are true-to-type (Harding, 2004; Liu *et al*., 2008; Zarghami *et al*., 2008), although in some cases variation among genotypes of the same species in DNA structure and methylation patterns have been observed (Kaity *et al*., 2008; Johnston *et al*., 2009).

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[60] http://www.dna.affrc.go.jp/PLACE/

[61] http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

[62] http://www.plantgdb.org/

[63] http://www.phytozome.net/

[64] http://rice.plantbiology.msu.edu/

[65] http://www.maizegdb.org/

[66] http://www.soybase.org/

[67] http://brassica.bbsrc.ac.uk/

[68] http://solgenomics.net/

[69] http://wheat.pw.usda.gov/GG2/index.shtml

[70] http://www.gramene.org/

[71] http://gene64.dna.affrc.go.jp/RPD/

[72] http://proteome.dc.affrc.go.jp/cgi-bin/2d/2d_view_map.cgi

[73] http://www.plantcyc.org/

[74] http://appliedbioinformatics.wur.nl/moto/

[75] http://www.armec.org/MetaboliteLibrary/index.jsp

[76] Recalcitrant seeds are seeds that unable to germinate after cold storage and/or dessication.

Orthodox seeds display natural tolerance to desiccation and cryogenic temperature stresses, and thus can be cryopreserved without any pretreatment. However most hydrated tissues are highly sensitive to freezing injury. Cryopreservation is then carried out employing classical freeze-induced dehydration techniques[77] or newer vitrification techniques[78]. Vitrification is the most extensively used cryopreservation technique since it has higher reproducibility and is more appropriate for complex organs like shoot tips and embryos (Reed, 2008).

Cryopreservation protocols are more advanced for vegetatively propagated species, including for varieties within a given species, and numerous plantation crops, fruit trees as well as roots and tubers have been successfully cryopreserved. The Global Crop Diversity Trust is supporting work on the development and refinement of robust cryopreservation protocols for yam, sweet potato and aroids, and specific genotypes of cassava that are not responding to existing protocols.[79] In contrast, protocols for species that produce recalcitrant seeds are less advanced owing to their seed characteristics, such as high desiccation sensitivity and structural complexity (Engelmann, 2011).

The choice of genetic material for cryopreservation depends on the conservation goal. Cell suspensions and callus cultures are cryopreserved in order to conserve their specific features that might be lost during routine *in vitro* maintenance. For example, rice calli stored in liquid nitrogen exhibit a higher competence for transformation compared to their unfrozen counterparts (Moukadiri *et al*., 1999). Pollen is cryopreserved for use in breeding programmes, preserving nuclear genes and investigating fundamental aspects of pollen biology (Towill and Walters, 2000). Pollen from 600 accessions belonging to 40 species has been cryopreserved in India (Ganeshan and Rajashekaran, 2000), while pollen from more than 700 accessions is cryopreserved in China (Li *et al*., 2009a).

Shoot meristematic tissue is the most commonly used explant for long-term storage of vegetatively propagated species. Over 1 000 old potato varieties are cryopreserved at the Institute of Plant Genetics and Crop Plant Research in Germany (Keller *et al*., 2006), 345 potato accessions at the CIP (Panis and Lambardi, 2006), 540 cassava accessions at the International Center for Tropical Agriculture (CIAT) (Gonzalez-Arnao *et al*., 2008) and 630 banana accessions at the International Network for the Improvement of Banana and Plantain (INIBAP) International Transit Center (Panis *et al*., 2007). In the Republic of Korea, two garlic cryocollections with more than 800 accessions have recently been established (Kim *et al*., 2009).

Cryopreservation is also applied to orthodox seeds, especially of rare and endangered species, to extend seed longevity (Mandal, 2000; Touchell and Dixon, 1994). For example, protocols have been developed to cryopreserve whole seeds of *Coffea arabica* L., which obviate the necessity of germinating seeds or embryos *in vitro* after cryopreservation (Dussert and Engelmann, 2006). Coffee seeds can be rewarmed after cryopreservation and sown directly in vermiculite in the greenhouse. Further, ageing time course studies for lettuce seeds stored at temperatures between 50 and −196 °C have shown that cryopreservation can prolong the shelf-life of orthodox species, compared to storage under conventional optimal conditions of low temperature and low moisture content (Walters *et al*., 2004; Dulloo *et al*., 2010).

Cryopreservation may have practical advantages, even for plant species for which other options are available. A recent study on the comparative costs of maintaining a large coffee field collection with those of establishing a coffee seed cryo-collection at the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) showed that cryopreservation costs less in perpetuity per accession than conservation in field gene banks (especially if the intention is long-term storage), generating economies of scale with the costs further decreasing with an increasing number of cryopreserved accessions (Dulloo *et al*., 2009).

[77] Slow cooling to a defined temperature in the presence of a cryoprotectant, followed by rapid immersion in liquid nitrogen.

[78] Cell dehydration is performed prior to freezing in the presence of cryoprotective media, followed by rapid cooling.

[79] http://www.croptrust.org/

Nonetheless, it should be noted that cryopreservation may not prolong the storage life of germplasm indefinitely. A recent study revealed that while 59 per cent of the strawberry meristems thawed were viable after 28 years of cryopreservation as compared to 56 percent viability after 8 weeks, only 14 per cent of the pea meristems thawed were viable after 28 years, compared to 61 per cent after 26 weeks of cryopreservation (Caswell and Kartha, 2009).

Cryopreservation of plant genetic resources has been reported by both countries in North America, six countries in Asia, four countries in Europe, two in Latin America and the Caribbean, one in the Near East and none in Africa (FAO, 2010c). Among the challenges that restrict wider application of cryopreservation are the complex and time-consuming optimization of efficient protocols for new species, the differential genotypic responses to cryopreservation and lack of knowledge regarding causal factors in cryopreservation sensitivity/tolerance. Studies are ongoing to ascertain genomic responses to cold and desiccation stresses in order to design improved cryopreservation strategies (Volk, 2010).

Cryotherapy, a modification of cryopreservation, is a novel method for pathogen eradication in which the shoot tips are briefly exposed to liquid nitrogen. It has been used mainly to eliminate viruses in banana, grapevine, potato, rasberry, sweet potato and in *Citrus* and *Prunus* species (Wang *et al*., 2009a; Feng *et al*., 2011).

3.5 *In vitro* slow growth storage

In vitro slow growth storage has been successfully applied to a range of species as well as across many genotypes within species. It markedly reduces the frequency of periodic subculturing (ranging from several months to 4 years, depending upon the species) without affecting the viability and regrowth potential of the culture. The efficiency of slow growth protocols depends upon several parameters such as the type of explants and their physiological state. The best results for establishing cultures for storage have been obtained using organized cultures such as apical meristems, axillary buds and embryos since undifferentiated tissues such as calli are more prone to somaclonal variation (Uyoh *et al*., 2003; Rao, 2004).

Variability between accessions in their response to culture conditions has been observed, and culture conditions need to be customized to new material. For instance, an *in vitro* core collection of African coffee germplasm with 21 diversity groups, which was conserved under slow growth, exhibited a great variability in response to the storage conditions (Dussert *et al*., 1997). Bioversity International has developed technical guidelines for the management of *in vitro* crop germplasm collections (Reed *et al*., 2004).

Approximately 8 100 cassava accessions are conserved under slow growth conditions in 13 tissue culture banks worldwide, with 80 percent of these accessions in the CIAT and the International Institute of Tropical Agriculture (IITA) collections.[80] Additionally, collections of potato and sweet potato at the CIP, yam at the IITA, and banana and plantain at the INIBAP International Transit Center are stored using this method. At CIAT, stability of cassava germplasm, after *in vitro* slow growth storage for 10 years, has been ascertained through molecular analysis (Angel *et al*., 1996). Several developing countries, including eight in Africa, have also reported having *in vitro* slow growth storage facilities (FAO, 2010c). The CGIAR System-wide Genetic Resources Programme (SGRP) has developed a Crop Genebank Knowledge Base[81] which provides crop-specific best practices for nine crops and general procedures for different conservation methods including seed banks, field collection, *in vitro* slow growth storage and cryopreservation.

[80] http://cropgenebank.sgrp.cgiar.org/index.php?option=com_content&view=article&id=342&Itemid=487&lang=english

[81] http://cropgenebank.sgrp.cgiar.org/

3.6 Wide crossing

Wide crossing is often employed to obtain a plant that is practically identical to the original crop with the exception of a few desirable genes contributed by the distant relative. The transfer of unwanted characteristics is circumvented through a series of backcrosses (for six or more generations), a time-consuming and laborious process. Introgression libraries, i.e. marker-defined genomic regions taken from the donor parent and introgressed onto the background of elite crop lines, can help accelerate the process (Zamir, 2001).

With biotechnological approaches overcoming inter-specific crossing barriers, there has been a steady increase in the utilization of CWR in plant breeding programmes, with the most widespread use in the development of disease and pest resistance, followed by abiotic stress tolerance, yield increase, cytoplasmic male sterility and fertility restorers, and improved quality (Hajjar and Hodgkin, 2007; Maxted and Kell, 2009). For example, embryo rescue has been successfully used to produce hybrids between Asian rice and all other wild rice species except *O. schlechteri* (Brar, 2005). However, a future concern for wide crossing is the threat to CWR, with many species declining in distribution and abundance (Maxted and Kell, 2009). The technique of embryo rescue has also been utilized in crosses between rye and wheat resulting in the new species triticale which combines the genetic attributes of both species, i.e. the adaptability to environmental conditions of rye with the yield potential and nutritional qualities of wheat (Ammar *et al*., 2004).

In vitro embryo rescue and anther culture have been vital in the development of inter-specific hybrids, including New Rice for Africa (NERICA) varieties produced by cross-breeding African rice (*Oryza glaberrima*) with Asian rice (*O. sativa*). NERICA varieties exhibit higher yields and earlier maturity than Asian rice, increased pest resistance and improved drought tolerance compared to African rice, and a higher protein content than either of the parents (Somado *et al*., 2008; Sonnino *et al*., 2009; FAO, 2011a). These varieties have been released in 30 African countries and have played a key role in enhanced rice harvests (Sonnino *et al*., 2009). Approximately 300 000 hectares are estimated to be under NERICA cultivation in West, Central and East Africa.[82]

Impact assessment studies conducted in West Africa have pointed to significantly positive impacts of NERICA adoption on rice yield in Benin and the Gambia, with higher impacts for women than for men; for example, women rice farmers had a higher additional income gain (Agboh-Noameshie *et al*., 2007; Diagne *et al*., 2009). Analysis in Uganda showed that NERICA has the potential to increase per capita income by USD 16 (10 percent of actual per capita income) and to decrease the poverty incidence with further increases in income possible when combined with effective extension services, seed delivery systems and appropriate market development policies (Kijima *et al*., 2008, 2011).

3.7 Somatic hybridization

Somatic hybridization is a technique that has been used to tap the potential of related or distant species/genera of crops and is especially useful for creating novel combinations of nuclear and/or cytoplasmic genomes. Numerous inter-generic, and intra- and inter-specific somatic hybrids have been reported and genes for quality improvement, resistance against bacterial, fungal, viral and nematode diseases, as well as abiotic stress tolerance have been transferred (Nagata and Bajaj, 2001; Liu *et al*., 2005). Several new commercial varieties of potato and oilseed rape have also been produced through this technology (Murphy, 2007).

Drawbacks of somatic hybridization include genetic instability and low fertility of the hybrids and over the last few years, this technology has been replaced to a large extent by transgenesis. Developing protocols for somatic hybridization is often a long and cumbersome process and poor plantlet

[82] http://go.worldbank.org/OFD841GU60

regeneration efficiency *in vitro* is a limitation in many species. However, somatic hybridization has considerable potential for the future since, unlike transgenesis, it is more efficient for transferring polygenic traits, it does not require the same regulatory approval, and advances in tissue culture and molecular marker techniques have increased the success rate in regenerating genetically stable progeny (FAO, 2011a).

3.8 Micropropagation

Micropropagation is widely used for a range of vegetatively propagated subsistence crops in developing countries such as cassava and banana, commercial plantation crops such as sugarcane and oil palm, niche crops like cardamom and vanilla, and fruit tree crops such as coconut and mango (FAO, 2011a). The most frequent application of micropropagation is for the production of virus-free plantlets through a combination of meristem culture and explant heat treatment, and subsequent mass-scale multiplication of selected plant lines or individuals (Sonnino *et al*., 2009). However, the large-scale use of clonally propagated material can generate completely uniform crops thus further reducing the genetic diversity present in the fields, and therefore care should be taken to include different genotypes as initial sources of plant material.

Socio-economic impact studies, carried out in a few developing countries, indicate that the use of micropropagated material led to increased productivity and enhanced rural livelihoods. In Asia, for example, micropropagated virus-free sweet potato was adopted by 80 percent of the farmers in the Shandong Province of China, within a four year period, leading to, on average, 30 percent increased yields and generating an estimated USD 145 million in net benefits annually (Fuglie *et al*., 1999). In India, integrating micropropagation with disease detection led to the production of virus-free potato stocks that generated a revenue of USD 4 million over a period of 10 years (Naik and Karihaloo, 2007) as well as substantial improvement in sugarcane seedling quality and health, resulting in enhanced yields and economic returns (Jalaja *et al*., 2008). For banana, economic analyses in India have indicated that even though the cost of production per bunch is higher for micropropagated material than for conventional planting material, the gross and net incomes from tissue culture-derived bananas are higher by 35.4 and 42.4 percent respectively (Singh *et al*., 2011a). The introduction of high-yielding and late-blight disease resistant potato cultivars in Viet Nam boosted yields from 10 to 20 tonnes per hectare (Uyen *et al*., 1996).

In Africa, commercially micropropagated crops have been reported in 12 countries (Sonnino *et al*., 2009). In Kenya, over 500 000 farmers have planted micropropagated bananas (Wambugu, 2004) and it has been found that although micropropagated banana production is more capital intensive than traditional banana production, it offers relatively much higher financial returns (Mbogoh *et al*., 2003). Micropropagated sweet potato varieties were taken up by 97 percent of the farmers in the Hwedza District of Zimbabwe leading to greater yields per hectare and net economic returns (Mutandwa, 2008; Sonnino *et al*., 2009). Further, studies carried out in Uganda and Zimbabwe concluded that in order to maximize the socio-economic impact of micropropagated materials, extension and training need to be complemented with service packages such as subsidized planting material, marketing, etc and *ex ante* consideration of adoption patterns (Sonnino *et al*., 2009).

3.9 Chromosome set manipulation

In plants, a rapid and cost-effective approach for inducing sterility (e.g. to produce seedless fruit) is the creation of polyploids, especially triploids. Traditionally, triploidy is induced by first producing tetraploids with colchicine treatment followed by crossing the tetraploids with the diploid counterparts, but this method is tedious and lengthy. A faster method to produce triploids is through the *in vitro* regeneration of plants from the endosperm (a naturally occurring triploid tissue) that can eventually be multiplied by micropropagation (Thomas and Chaturvedi, 2008). Using this technique, triploid varieties

of several fruit crops have been created including most of the citrus fruits, acacias, kiwifruit, loquat, passionflower and pawpaw (FAO, 2011a).

Anther culture offers excellent opportunities for large-scale production of haploids, although problems include the occurrence of albinos and induction of genetic variation. Anther culture techniques have been established for numerous economically important crop species, including vegetables (Juhasz *et al*., 2006) and cereals such as rice, barley and wheat (Germana, 2011). Doubled haploid (DH) plants, produced using *in vitro* anther culture (or alternatively by ovary/ovule culture) and chromosome doubling, are valuable in breeding programmes since they are 100 percent homozygous (i.e. recessive genes are readily apparent) and the need for numerous cycles of inbreeding is thus considerably reduced by shortening the time needed to select desired lines (Dunwell, 2010). However, skilled labour is required to test large populations, leading to increased costs.

Wheat cultivars derived from DHs have been released in Brazil, Canada, China, Europe, Morocco and Tunisia (Jauhar *et al*., 2009; Sonnino *et al*., 2009). Anther culture techniques have also been applied to obtain DH barley (Gomez-Pando *et al*., 2009) and rice (Pauk *et al*., 2009) varieties better suited to the environment. In China, more than 100 new rice cultivars have been developed employing anther culture (Gueye and Ndir, 2010). The Joint FAO/IAEA division has supported and co-ordinated research efforts focusing on the development of reproducible DH protocols, including the publication of a manual with protocols for the production of DHs in 22 plant species (Maluszynski *et al*., 2003).

3.10 Biotechnologies for disease diagnosis

The introduction of monoculture farming has resulted in a decline in genetic diversity, increasing the risk of exposure to pathogens. Diseases in crops, caused by viruses, bacteria, fungi and nematodes, threaten food security in resource-poor countries and cause significant damage and economic losses every year (Vurro *et al*., 2010). Early and accurate detection and diagnosis of plant pathogens is indispensable for predicting disease outbreaks and improving the precision of pesticide and/or fungicide applications as well as other control measures for disease management. Detection of pathogens is also of paramount importance in germplasm collections to enable the storage and exchange of healthy germplasm. Biotechnological diagnostic tools, such as ELISA and PCR-based methods, for pathogen identification can bypass many shortcomings related to culture-based morphological approaches (e.g. for microbial species that are difficult to culture or to identify microscopically).

ELISA is the most widely used diagnostic technique for plant pathogens in developing countries, especially for identifying viruses (FAO, 2005). Numerous ELISA test kits that can detect diseases of root crops, fruits, cereals and vegetables are available commercially. One of the first such ELISA kits was developed by CIP to detect the presence of *Ralstonia solanacearum*, the pathogen that causes bacterial wilt in potato.[83] Recent adaptations in the form of dipstick assays, resulting in increased convenience and portability in concert with lower costs, have further expanded the utility of these technologies in the field.

PCR is routinely employed in the developed world for diagnostic purposes (Vincelli and Tisserat, 2008). PCR-based techniques are also very effective for monitoring the emergence of novel variants of well-known pathogens, such as *Puccinia striiformis* f. sp. *tritici* that causes yellow (stripe) rust of wheat (Milus *et al*., 2009) and *Puccinia graminis* f. sp. *tritici* that causes black (stem) rust of wheat (Visser *et al*., 2009, 2010). Both black and yellow rust are economically damaging diseases affecting wheat production across the world, and there has been a recent escalation in the threat posed by them. The Rust SPORE web portal[84] was launched by FAO and partners in 2010 to mitigate the threat of wheat rust diseases by delivering surveillance information, monitoring pathogens and providing access to information tools.

[83] http://www.isaaa.org/resources/publications/pocketk/22/default.asp; http://www.cipotato.org/potato/pests-and-disease

[84] http://www.fao.org/agriculture/crops/rust/stem/en/

Real-time PCR has high sensitivity and specificity but generally requires expensive laboratory equipment. However, the cost has decreased in recent years and portable thermocyclers have been developed, for example for the on-site diagnosis of bacterial diseases in watermelon (Schaad *et al*., 2001) and grape (Schaad *et al*., 2002). The Cereal Disease Laboratory (United States of America) is currently developing real-time PCR-based assays for the identification of Ug99 and other races of the wheat stem rust pathogen.[85]

The aforementioned assays are restricted in the number of pathogens that can be tested at once, while plants could be infected by several pathogens, some of which may act synergistically to cause a disease complex. Microarrays have been used for discriminating serotypes and subgroups of viruses as well as for species of fungal pathogens (Mumford *et al*., 2006; Nicolaisen *et al*., 2005), although existing microarray methods are complex, expensive and consequently out of reach of many developing countries. An area where microarrays could be applied in the future is in quarantine systems. For example, the European Union (EU) funded project DiagChip aims to develop a diagnostic chip for the simultaneous detection of all the EU-listed quarantine potato pests/pathogens.[86]

Many plant disease diagnostic networks have been established to address issues related to disease diagnosis and pathogen detection, in particular the gap in capacity between developed and developing countries (Miller *et al*., 2009). For example, the Global Plant Clinic[87] has a network of 81 clinics in ten countries across Africa, Asia and Latin America (Boa, 2010). It links stakeholders like diagnostic laboratories and researchers with clinics, and also runs a global diagnostic service in the United Kingdom for further testing to identify new diseases (Wilson, 2010).

The International Plant Protection Convention (IPPC) is a multilateral treaty that aims to increase international cooperation in plant protection by developing International Standards for Phytosanitary Measures (ISPMs).[88] Among the 34 ISPMs that have been adopted so far, one is on "diagnostic protocols for regulated pests", and provides the minimum requirements for reliable and official diagnosis of pests, including guidance on the use of morphological and molecular/biochemical diagnostic techniques.[89] The protocols also offer additional methods to be used in different circumstances such as general surveillance, diagnosis for pests found in imported consignments and the first diagnosis of a pest in an area. In response to the need for regional harmonization, several Regional Plant Protection Organizations (RPPOs) such as the Comité de Sanidad Vegetal del Cono Sur (COSAVE)[90], the European and Mediterranean Plant Protection Organization (EPPO)[91] and the North American Plant Protection Organization (NAPPO)[92], have developed diagnostic protocols of regulated pests in their respective regions (Clover *et al*., 2010).

3.11 Molecular marker-assisted selection

MAS has the potential to increase genetic gain by permitting selection at an earlier stage of development and/or by reducing the generation interval or the number of generations needed prior to releasing a new variety. It is especially advantageous when phenotypic recording is destructive or pyramiding of genes is

[85] http://www.ars.usda.gov/ug99/actionplan.pdf

[86] http://www.diagchip.co.uk/

[87] A consortium of CABI Bioscience, Rothamsted Research, and Central Science Laboratory, United Kingdom.

[88] https://www.ippc.int/

[89] ISPM No. 27 - https://www.ippc.int/file_uploaded/1155903234858_ISPM27_2006_E.pdf

[90] http://www.cosave.org/estandares.php?ver=3

[91] http://archives.eppo.org/EPPOStandards/diagnostics.htm

[92] http://www.nappo.org/Standards/Std-e.html

desired. It is particularly useful for horticultural crops, since most of them are highly heterozygous which makes phenotypic selection difficult (Ibitoye and Akin-Idowu, 2010).

MAS has been used in plant breeding for developing new hybrids and varieties of both annual crops such as cereals and legumes, and perennial crops such as fruit trees, tea and coffee (FAO, 2007b; Collard and Mackill, 2008; FAO, 2011a). For a commercial maize breeding programme in Europe and North America, MAS has been shown to increase the mean performance of progeny for multiple traits such as grain yield and moisture content compared to conventional breeding (Eathington *et al*., 2007).

Sorghum varieties have been developed that are resistant to striga, a weed that infests nearly 100 million hectares of field crops in Africa annually. Marker assisted backcrossing was used to introgress target genes into locally adapted landraces and improved sorghum lines, with broad adaptation and high yield, to produce agronomically superior cultivars that are being grown in several African countries (Ejeta, 2007). Integrated striga management, combining the use of striga resistant cultivars with soil fertility management and moisture conservation, has further increased sorghum productivity (Ejeta and Gressel, 2007).

Quality protein maize (QPM) contains approximately twice as much usable protein as regular maize grown in the tropics, and is of particular use in developing countries to fight malnutrition (Krivanek *et al*., 2007). MAS has been utilized to improve the efficiency of selection to develop QPM hybrids (Babu *et al*., 2005; Danson *et al*., 2006) and commercial cultivars have been released in India (Gupta *et al*., 2009). Other successful examples of hybrids and varieties released using MAS include a pearl millet hybrid resistant to downy mildew in India (Dar *et al*., 2006), drought resistant soybean in the United States of America[93], disease resistant barley varieties in Australia (Eglinton *et al*., 2006), rice varieties with resistance to bacterial blight in China, India, Indonesia and the Philippines (Vogel, 2009), with low amylase in the United States of America (Dwivedi *et al*., 2007), with submergence tolerance in Bangladesh, India and the Philippines[94], and with drought tolerance in India (Vogel, 2009).

In spite of its high potential, MAS is still applied in relatively few breeding programmes in developing countries. This is because an effective MAS strategy requires adequate laboratory capacity and data management, trained personnel and operational resources (Ribaut *et al*., 2010). Another factor has been the scarcity of genetic and genomic resources for orphan crops that play an important role in developing countries, although international collaborations and initiatives are now addressing these issues (Varshney *et al*., 2009b). The CGIAR GCP has also developed a Molecular Marker Toolkit[95] that allows access to current effectively used markers for application in MAS for 12 food security crops (Van Damme *et al*., 2010).

The relative costs of applying MAS are higher than conventional approaches. Studies comparing MAS with conventional selection suggest that the cost-effectiveness of both methods depends on the particular circumstances of the specific application and that the optimal choice of the breeding application should be based on a case-by-case analysis (Dreher *et al*., 2003; Morris *et al*., 2003; William *et al*., 2007). However, MAS is becoming progressively cheaper and more cost-effective.

A recent *ex ante* impact analysis from the CGIAR, taking into account crop yields, farmer adoption rates, market prices, cultivated land area, breeding and dissemination times, input prices and costs of marker development, concluded that MAS in rice and cassava will result in significant incremental benefits over conventional breeding.[96] For example, MAS for tolerance to salinity and phosphorus deficiency in rice is expected to save 3-6 years with projected economic benefits, over 25 years, of USD 50-900 million,

[93] http://www.wisconsinagconnection.com/story-national.php?Id=808&yr=2009

[94] http://www.scidev.net/en/news/india-takes-to-new-flood-tolerant-rice.html

[95] http://s2.generationcp.org/gcp-tmm/web/

[96] http://www.generationcp.org/sp5_impact/exante-norton-conclusions

depending upon the country, stress and time lag (Alpuerto *et al*., 2009). For cassava, MAS for resistance to pests and post-harvest physiological deterioration is estimated to save four years with net benefits over 25 years in the range of USD 34-800 million contingent on various assumptions (Rudi *et al*., 2010).

The incorporation of genomic information is further underpinning crop improvement, and MAS is evolving into genomic selection (GS) in some developed countries (Heffner *et al*., 2009; Jannink *et al*., 2010). Simulation studies have shown the accuracy of estimating the breeding value from GS to be comparable to phenotypic selection, without entailing the time and expense of field evaluation (Zhong *et al*., 2009). A recent study indicated that even at low accuracy, annual gain from GS exceeded that of MAS about three-fold in a high-investment maize breeding programme and two-fold in a low-investment winter wheat breeding programme (Heffner *et al*., 2010). GS, thus, has the potential to lower costs and increase rates of genetic gain, leading to more effective selection strategies. GS models for adult pathogen resistance to wheat stem rust are currently being developed as a promising strategy in the fight against the disease (Rutkoski *et al*., 2011).

3.12 Mutagenesis

Induced mutagenesis has played an important role in the development of superior crop varieties by generating greater genetic diversity in exisiting varieties. It is particularly important for the genetic improvement of vegetatively propagated crops where cross-breeding is not possible or is time-consuming (Mba *et al*., 2009). Induced mutagenesis offers the possibility of introducing desired attributes that have either been lost during evolution or are not present in nature. Another major advantage is the ability to isolate mutants with multiple traits as well as mutant alleles with varying degrees of trait modification. The resultant mutant varieties can be readily commercialized without the regulatory requirements applied to transgenic crops, though the limitation remains that mutagenesis can only be used to manipulate already existing genes, usually by suppressing/deleting their function (Parry *et al*., 2009).

Somaclonal mutagenesis refers to the epigenetic or genetic alterations induced during *in vitro* culture. Typical DNA changes include chromosome number changes, chromosomal rearrangements (e.g. translocations, deletions, insertions and duplications), point mutations and gene methylation or de-methylation. Somaclonal variation is generally considered an undesirable by-product of the stress imposed by tissue culture, but when carefully controlled can provide novel genetic variations to plant breeders. An added benefit is that it can be used to generate variation in vegetatively propagated plants, which are usually less amenable to mutation breeding. This technology has been applied to manipulate various traits for enhancing crop productivity such as disease and pest resistance, drought and salt tolerance, and improved nutritional quality and cultivars have been released in a few crops such as rice, wheat, maize, potato, tomato and sweet potato (Jain, 2001).

As indicated earlier, the Joint FAO/IAEA Division has made irradiation technology more widely available to developing countries through extensive research and development as well as training and capacity development activities (Lagoda, 2009). Almost 3 000 improved crop varieties in about 170 species have been developed through induced mutation and released in an estimated 100 countries generating economic benefits for farmers (FAO/IAEA, 2008). For example, three improved varieties of rice produced a total net profit of USD 348 million for farmers in Viet Nam in 2007 alone while in Peru, the introduction of nine superior barley varieties has resulted in 50 percent increased harvests translating to roughly USD 9 million a year (IAEA, 2008).

The most commonly used chemical mutagens are alkylating agents such as ethylmethane sulfonate (EMS) and N-methyl-N-nitrosourea (MNU), that induce point mutations in DNA. Since point mutations are less detrimental than large chromosomal rearrangements, this method has a higher frequency of achieving a saturated mutant population (Gilchrist and Haughn, 2010). However, point mutations are often recessive and therefore the second or later generations of mutagenized tissues must be screened to identify

homozygous recessive mutations. Chemical mutation-derived varieties have been obtained and commercially released for numerous staple species including rice, wheat, maize, soybean and barley.[97]

A recent approach that combines classical mutagenesis with high-throughput identification of mutations is TILLING (Targeting Induced Local Lesions IN Genomes). DNA from a collection of mutagenized plants is pooled, subjected to PCR amplification and screened for mutations by detecting mismatches in duplexes with non-mutagenized DNA sequences (McCallum *et al*., 2000). TILLING is particularly advantageous as mutations can be detected in pools of small plantlets without the need to screen adult plants for an observable phenotype. It is also amenable to automation, making it especially conducive for crop species that have large and complex polyploid genomes. However, TILLING requires prior DNA sequence information and is a labour-intensive technique. Further, the availability of a mutagenized population is a pre-requisite for TILLING and the development of such populations is expensive and time-consuming for many species.

TILLING platforms and associated large mutagenized populations, valuable resources for screening for traits of interest, have been created for several crops including rice[98], maize[99], durum wheat[100], barley[101], and tomato, rapeseed and pea[102]. The Joint FAO/IAEA Division has developed lower-cost assays and kits for mutation discovery that are affordable for laboratories in developing countries.

TILLING with vegetatively propagated crops, which are less genetically tractable, is still in its infancy. Among the challenges involved in developing a suitable mutagenized population are the choice of mutagen as well as the tissue to be mutagenized. The Joint FAO/IAEA Division is currently establishing TILLING platforms for banana, cassava and yam, for improving food security in developing countries.[103]

A variation on TILLING is EcoTILLING, which looks for polymorphisms in natural populations (Comai *et al*., 2004). It is highly informative for species that are not amenable to mutagenesis and has been applied for allele mining for variation in disease resistance (Barkley and Wang, 2008). It has also been used to detect genetic diversity in inbred lines, cultivars and accessions of agronomically important crop plants (Weil, 2009).

3.13 Transgenesis

Genetically modified crops were first grown commercially in the mid 1990s. In 2010, transgenic crops are estimated to have been cultivated on 148 million hectares in 18 developing and 11 industrialized countries, with 45 percent of the global total being cultivated in the United States of America (James, 2010). Seventeen countries, including 13 developing countries, planted over 50 000 hectares each. The four main transgenic crops grown were soybean, maize, cotton and canola, with herbicide tolerant soybean being the principal crop. Further details on the application of transgenic crops can be found in FAO (2011a).

[97] http://mvgs.iaea.org/

[98] http://tilling.ucdavis.edu/

[99] http://genome.purdue.edu/maizetilling/

[100] http://www.rothamsted.bbsrc.ac.uk/cpi/optiwheat/indexcontent.html

[101] www.gabi-till.de/project/ipk/barley.html; www.distagenomics.unibo.it/TILLMore/

[102] http://www.versailles.inra.fr/urgv/tilling.htm

[103] http://www-naweb.iaea.org/nafa/pbg/public/pbg-nl-25.pdf

IV. CURRENT STATUS OF BIOTECHNOLOGIES FOR THE MANAGEMENT OF FOREST GENETIC RESOURCES

Forests provide a vast array of economic, environmental and social products and services. Thirty percent of the world's forests are primarily used for the production of wood and non-wood forest products, while 8 percent are designated for the protection of soil and water resources (FAO, 2010b). Forests also play a very significant role in carbon sequestration, storing an estimated 289 gigatonnes of carbon in their biomass. The world's total forest area is just over 4 billion hectares, i.e. 31 percent of the total land area, with primary forests accounting for 36 percent and planted forests 7 percent of the total forest area (FAO, 2010b). Approximately 10 million people are employed in forest management and conservation, but millions more rely on forests to a high degree for their livelihoods.

Forests possess much of the world's biodiversity, but this diversity is threatened by a high rate of deforestation due to an expansion in global agricultural and industrial needs. Other threats include diseases, pests and weeds, many of which are introduced from other regions. Forest trees also have certain characteristics that differentiate them from other agricultural sectors, such as crops and livestock - for example, their highly heterozygous nature, long generation intervals, vulnerability to inbreeding depression, narrow regional adaptation and the fact that the majority of the species are undomesticated (FAO, 2011b) - thus generating unique challenges and opportunities for biotechnology applications. As elaborated in this section, biotechnological approaches have advanced considerably in the last decade and have contributed to creating more efficient and effective characterization, conservation and utilization strategies for forest genetic resources. However, in spite of their importance in natural ecosystems, genetic resources of non-commercial species, encompassing also lower plants, have received less attention and therefore there is a pressing need to focus on their sustainable management, including through biotechnological approaches

4.1. Molecular markers

Molecular markers can be used to study the level, structure and origin of genetic variation in both naturally regenerated tropical forests and planted forests. Molecular marker studies, thus far, have suggested that natural populations of most tropical tree species contain higher levels of variation relative to other plants (Kindt *et al*., 2009). However, the extent of genetic diversity in tropical forests is still largely unknown and many economically important species are yet to be identified. Molecular markers have helped to differentiate and inventory species but a thorough cataloguing will require good linkages with field botanists (Dick and Cress, 2009). For tropical trees, a practical protocol guide on molecular marker methods (Muchugi *et al*., 2008a) as well as an accompanying guide on the effective handling and analysis of the datasets generated (Kindt *et al*., 2009) have been produced by the World Agroforestry Centre (ICRAF).

To develop effective conservation strategies, accurate taxonomic identification together with the ability to discriminate hybrids from pure species and to estimate the degree of introgression is essential (Wang and Szmidt, 2001). The phylogenetic relationships of tree species that are difficult to distinguish on the basis of morphological characteristics alone can be resolved in conjunction with the use of molecular markers, for example between species of *Warburgia* (Muchugi *et al*., 2008b), *Populus* (Cervera *et al*., 2005) and *Quercus* (Zeng *et al*., 2010).

Molecular markers have facilitated the identification of natural hybrids across species thus leading to a better understanding of introgression, for instance between *Fraxinus excelsior* and *F. angustifolia* (Fernández-Manjarrés *et al*., 2006), *Populus alba* and *P. tremula* (Lexer *et al*., 2005), *Pinus echinata* and *P. taeda* (Xu *et al*., 2008), and *Quercus suber* and *Q. ilex* (Burgarella *et al*., 2009). Furthermore, natural hybrid zones are valuable for investigating evolutionary processes of speciation (Tovar-Sánchez and Oyama, 2004) as well as for identifying QTLs for adaptive genetic variation in forest trees (Lexer *et al*., 2004).

Determination of the origin of forest reproductive material for planted forests as well as traded wood and wood products is crucial. Molecular markers have been applied to improve traceability through reliable identification of species to control both trade with protected trees and illegal logging (Finkeldey *et al*., 2010). Recently, a standard two locus DNA barcode has been proposed for species discrimination of land plants, including forest trees (CBOL Plant Working Group, 2009).

Molecular markers have offered insights into the domestication of forest trees, such as the origin of olive from the oleaster (Besnard *et al*., 2001; Breton *et al*., 2006). Additionally, based on nuclear and cytoplasmic markers, nine domestication events have been proposed for olive cultivars (Breton *et al*., 2009), while AFLP data have suggested two distinct geographic origins of cultivated *Spondias purpurea* trees in Mesoamerica (Miller and Schaal, 2006). Molecular marker studies have also shed light on the evolutionary history of tree species, for instance the postglacial routes of colonization of *Populus nigra* occurred from two main refugia in Italy and/or the Balkans and Spain (Cottrell *et al*., 2005).

Since most forest trees are outcrossing, they do not generally show evidence of strong genetic differentiation among populations and the highest genetic diversity is found within populations. Molecular markers have been used to measure genetic variation in tropical trees, for example within and between populations of *Calycophyllum spruceanum* in the Peruvian Amazon Basin (Russell *et al*., 1999), *Sesbania sesban* in sub-Saharan Africa (Jamnadass *et al*., 2005), *Tectona grandis* in India (Narayan *et al*., 2007) as well as diverse geographical regions in Indonesia and Thailand (Shrestha *et al*., 2005), and *Guaiacum sanctum* in Costa Rica (Fuchs and Hamrick, 2010). Similar analyses on molecular genetic diversity have been carried out in temperate tree species, for example within and among populations of *Fagus grandifolia* in Mexico (Rowden *et al*., 2004), *Robinia pseudoacacia* in China (Huo *et al*., 2009) and *Sorbus torminalis* in Europe (Rasmussen and Kollmann, 2008). Altitudnal variation has also been observed within populations of tree species, indicating that both vertical and horizontal patterns of genetic diversity must be considered while designing conservation strategies (Ohsawa and Ide, 2008).

Molecular markers have been utilized to evaluate the efficiency of agroforestry systems for conservation of forest genetic resources by comparing the genetic variation across natural forest and proximate planted farm stands. RAPD studies of the timber tree Meru oak in central Kenya showed little differentiation between unmanaged and managed stands (Lengkeek *et al*., 2006). Another study assessing the genetic diversity between planted and natural stands of *Inga edulis* from five sites in the Peruvian Amazon demonstrated lower allelic variation in planted stands, even though on-farm stands contained on average 80 percent of the allelic diversity of natural stands (Hollingsworth *et al*., 2005). An explanation for the difference between the two studies could be that while all on-farm *I. edulis* was of planted origin, the oak trees in Kenya may have been planted or naturally regenerated.

Exploration of the amount and distribution of genetic variation in clonally propagated domesticated stands and sexually reproducing wild populations of *Spondias purpurea* revealed that levels of genetic variation within cultivated stands were significantly lower than in wild populations (Miller and Schaal, 2006). Moreover, within the cultivated populations, trees in orchards harboured less genetic variability than trees in backyard gardens and living fences.

Knowledge of mating systems and gene flow is important for understanding genetic drift, natural selection and population divergence, and to design conservation strategies that maximize connectivity of populations in fragmented forests while minimizing unwanted gene flow. For example, microsatellite data evaluating the mating system and pollen gene flow in oak in northern Thailand showed high outcrossing rates, high levels of gene flow from outside populations and heterogeneity in the pollen composition received by individual trees suggesting that losses of genetic diversity of the species could be prevented at the study site (Pakkad *et al*., 2008). Population genetic diversity in disturbed and undisturbed teak forests within the natural range of the species, the mating system and contemporary gene flow has also been studied using molecular markers (Volkaert *et al*., 2008)

Maternally and paternally inherited markers make it possible to distinguish the separate contributions from pollen and seed in gene flow studies (Jones *et al*., 2006; Sork and Smouse, 2006; Dick *et al*., 2008; Hamza, 2010). Molecular information has demonstrated that gene flow through pollen dispersal is significantly higher (20 to nearly 200 times) than gene flow through seeds, at least among wind-pollinated species (Savolainen *et al*., 2007) and tree species that produce large, immobile seeds (Dow and Ashley, 1998). Molecular markers have been used to study pollen-mediated gene flow in populations of both wind pollinated and animal pollinated trees (Burczyk *et al*., 2004), with long distance pollen dispersal (over 5 to 10 km) not uncommon (Petit and Hampe, 2006), which could have significant implications for the conservation of trees in fragmented stands (White *et al*., 2002; Kamm *et al*., 2009).

Gene flow from planted to natural stands, i.e. anthropogenic hybridization as a result of human activity, can have a profound effect on the diversity and adaptability of wild populations. Most poplar plantations in China, Europe and North America represent a very narrow genetic base (since they are clonally propagated) and could lower the effective population size and alter the evolutionary potential of native poplar populations. Molecular markers have provided evidence for gene flow between cultivated poplars and native black poplar trees, with the frequency of hybridization depending upon the size of the native population compared to the cultivated plantations (Broeck *et al*., 2004; Broeck *et al*., 2005). Extensive hybridization has also been observed between the native North American butternut and the introduced Japanese walnut tree (Hoban *et al*., 2009).

Molecular markers can be used to manage clonally propagated domesticated stands by aiding the selection and identification of clones (Hiraoka *et al*., 2009; Toral Ibañez *et al*., 2009; Aravanopoulos, 2010; Guan *et al*., 2010) and verifying the genetic stability of propagated material (Gangopadhyay *et al*., 2003; Lopes *et al*., 2006; Chandrika and Rai, 2009; Huang *et al*., 2009b). The application of molecular markers for clonal identification and ensuring the genetic fidelity of the mass propagated clones has led to the production of superior teak clones in Malaysia (Goh *et al*., 2007). Molecular markers are routinely used for the correct identification of clones in commercial *Eucalyptus* breeding and production forestry in Australia, Brazil, Chile, Portugal, Spain and South Africa (Grattapaglia, 2008a).

QTL mapping is complicated in forest trees due to growth under conditions of great environmental heterogeneity, long generation intervals, large genome sizes, small segregating populations and lack of multigenerational pedigrees. Nevertheless, QTLs for a variety of traits, such as disease resistance, drought and cold tolerance, wood quality and bud phenology have been mapped in numerous tree species (FAO, 2007b), with growth-related traits being the main targets in tree breeding and plantation forestry (Grattapaglia *et al*., 2009). QTL validation is necessary to identify stable QTLs that are potentially more useful for MAS in forest trees, and is carried out based on repeated QTL detection in additional individuals from the same family or among populations and multiple growing seasons (Brown *et al*., 2003; Devey *et al*., 2004). Alternatively, QTLs can be validated by comparative mapping between species or genera (Chagné *et al*., 2003; Casasoli *et al*., 2006).

4.2 "Omics"

Thus far, *Populus trichocarpa* (Tuskan *et al*., 2006) and *Eucalyptus grandis*[104] are the only forest trees for which the genome sequence has been completed. Currently, genome sequencing of other *Populus* species, Fagaceae and Pinaceae species is also underway (Neale and Kremer, 2011). Based on available DNA sequences, it has been suggested that trees have higher rates of genome-wide recombination (correlated with higher levels of genetic diversity) than short-lived herbs and shrubs, with the exception of conifers which exhibit lower recombination rates than angiosperms (Jaramillo-Correa *et al*., 2010).

[104] http://greenbio.checkbiotech.org/news/eucalyptus_tree_genome_deciphered

SNP frequency in tree species that have been surveyed is high, approximately 1 SNP/100 base pairs, and their discovery has been facilitated by sequencing expressed sequence tags (ESTs) and candidate genes (Novaes *et al*., 2008; Külheim *et al*., 2009; Parchman *et al*., 2010; Ueno *et al*., 2010). High-throughput SNP genotyping coupled with the candidate gene approach has been used for association mapping with phenotypes of interest and aided in the dissection of complex traits such as wood quality, drought or cold tolerance and disease resistance (Eckert *et al*., 2009a; Eckert *et al*., 2009b; Dillon *et al*., 2010). DArT arrays (see section 3.2), with over 8 000 markers, have been developed for population and phylogenetic studies within and between species of *Eucalyptus* (Sansaloni *et al*., 2010; Steane *et al*., 2011).

Comparative mapping studies have uncovered extensive synteny and colinearity in conifers (Krutovsky *et al*., 2004; Pelgas *et al*., 2006), together with small chromosomal disruptions (Shepherd and Williams, 2008) leading to deeper understanding of speciation. The comparative resequencing project[105] is developing resources for Pinaceae comparative genomics. Comparative genomics tools have also become available in *Populus (*Neale and Ingvarsson, 2008; Douglas and DiFazio, 2010) and *Eucalyptus* (Külheim *et al*., 2009; Paiva *et al*., 2011).

Transcriptome profiling is particularly challenging in tree species due to their large genome sizes and lack of reference sequences. In spite of this, it has been utilized to study growth (Park *et al*., 2008; Grönlund *et al*., 2009), adaptation to biotic (Heller *et al*., 2008; Azaiez *et al*., 2009) and abiotic (Holliday *et al*., 2008; Kreuzwieser *et al*., 2009) stress and wood formation (Paiva *et al*., 2008; Wang *et al*., 2009b). Comparative transcriptomics is being employed to study the molecular basis of complex traits such as drought tolerance (Cohen *et al*., 2010) and fungal resistance (Barakat *et al*., 2009). Additionally, transcriptomic data, together with linkage mapping, is being used for the identification of candidate genes (Kirst *et al*., 2004; Sederoff *et al*., 2010).

4.3 Bioinformatics

The principal repository of forest tree genomic data is maintained by the Dendrome Project.[106] The associated TreeGenes database[107] provides information on EST sequences, SNPs, genetic maps, molecular markers, phenotypes and QTLs, as well as tools for their analysis. It focuses primarily on conifers, but resources for *Populus* and *Eucalyptus* are also being integrated (Wegryzn *et al*., 2008). *Populus* specific databases include the RIKEN *Populus* database[108] that contains information on 10 *Populus* species, the Database of Poplar Transcription Factors (DPTF)[109] which currently contains 2 576 putative transcription factors gene models distributed in 64 families, and the *Populus* Genome Integrative Explorer (PopGenIE)[110] with expression tools as well as browser tools for synteny and QTLs (Yang *et al*., 2009a). *Eucalyptus* specific resources include the International Eucalyptus Genome Network (EUCAGEN)[111] and the Brazilian Network of Eucalyptus Genome Research (GENOLYPTUS) (Grattapaglia, 2008b).

4.4 Cryopreservation

The majority of recalcitrant seeds have been identified in trees and shrubs, with approximately 47 percent of the species from evergreen rain forests having seeds that are desiccation sensitive (Tweddle *et al*., 2003). Thus, cryopreservation is especially important for the long-term conservation of forest germplasm.

[105] http://dendrome.ucdavis.edu/crsp

[106] http://dendrome.ucdavis.edu

[107] http://dendrome.ucdavis.edu/treegenes/

[108] http://rpop.psc.riken.jp

[109] http://dptf.cbi.pku.edu.cn/

[110] http://www.popgenie.org/

[111] http://web.up.ac.za/eucagen/

The role of cryopreservation is further highlighted in situations where it may be complicated to find natural stands that are diverse enough for *in situ* conservation. A disadvantage of this technique is the overall difficulty associated with the regeneration of whole trees.

Most forest trees are still undomesticated and cryopreservation protocols have thus been developed and/or optimized for only a limited number of genotypes. Within these, various tissues of softwood and hardwood species have been successfully cryopreserved including embryos, embryogenic cultures, seeds, pollen and shoot tips (Panis and Lambardi, 2006). Cryopreservation (predominantly of shoot tips) is being increasingly applied for hardwood trees such as *Populus*, *Robinia*, *Betula*, *Quercus*, *Fraxinus*, *Morus* and *Eucalyptus* (Haggman *et al*., 2008).

In softwood tree species, cryopreservation has been reported for more than 10 000 genotypes of over 23 conifer species and their hybrids (Tsai and Hubscher, 2004). In conifer clonal forestry, it is used as a suitable and efficient means for the storage of embryogenic cultures awaiting field testing results. Clonal varieties can then be developed by thawing and propagating the desired cryopreserved embryogenic tissue clones that are superior in the field tests (Park, 2002; Sharma, 2005).

So far, there is little evidence of genetic alterations in forest trees caused by cryopreservation. Embryo recovery levels for cryopreserved oak embryogenic lines ranged from 57 to 92 percent, with no genetic instability observed in the regenerated plants (Sanchez *et al*., 2008). Similarly, the genetic fidelity of silver birch meristems and mulberry axillary winter-dormant buds was maintained subsequent to cryopreservation (Ryynanen and Aronen, 2005; Atmakuri *et al*., 2009). The viability of cryopreserved material has also been determined. Dormant European ash seeds, cryopreserved for two years following desiccation to a safe water content, did not exhibit decreased germination after thawing relative to a two year seed storage at -3 ^{o}C (Chmielarz, 2009). Likewise cryopreserved pecan pollen, stored for 1-13 years, did not demonstrate reduced viabilty compared to fresh pollen (Sparks and Yates, 2002).

Implementation of cryopreservation in developing countries has been restricted due to economic constraints. However, cryopreservation of dormant elm buds has been shown to be economically competitive to field clonal archives, with a two-fold cost saving in favour of the cryobank (Harvengt *et al*. 2004). Cryopreservation of forest trees has been initiated in some developing countries, for example for pollen of tree species at the National Bureau of Plant Genetic Resources (NBPGR), India, and for species such as *Dipterocarpus*, *Bambusa* and *Dendrocalamus* in Indonesia (Jalonen *et al*., 2009). Cryogenic repositories for forest tree species include 420 accessions of mulberry at the National Institute of Agrobiological Resources, Japan and 440 elm accessions at Association Forêt-Cellulose (AFOCEL), France (Engelmann, 2011).

4.5 *In vitro* slow growth storage

In vitro slow growth storage techniques require establishment of specific protocols depending on the type of explant and species under consideration. Another issue to be considered with tropical tree species is the presence of endophytes[112] that can cause difficulties for the establishment of sterile cultures (Muralidharan and Kallarackal, 2005).

Successful protocols have been developed for over 30 woody species, including *Pinus radiata, Alnus glutinosa* and species of *Eucalyptus* and *Populus* (FAO, 1994). *In vitro* cultures of *Melia azedarach* apical meristem tips can be maintained for one year without subculture or addition of fresh medium (Scocchi and Mroginski, 2004), of *Eucalyptus grandis* shoot for up to 10 months (Watt *et al*., 2000), 60 months for *Populus* species (Hausman *et al*., 1994) and 6 months for *Cedrus* species (Renau-Morata *et*

[112] An endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease.

al., 2006). In Spain, an *in vitro* collection, with 32 high quality clones, has been established with selected European aspen (Martin *et al*., 2007).

4.6 Micropropagation

Clonal propagation of commercially important tree species is essential in production forestry, for both coniferous and hardwood species, to provide clones of mature and elite genotypes. Additional advantageous aspects of micropropagated trees, compared to trees produced from seedlings, include more uniformity in height and trunk girth, increased biomass production and reduced bark fissuring (Muralidharan and Kallarackal, 2005).

Micropropagation techniques have been applied to over 80 genera of forest trees, with five genera, i.e. *Pinus*, *Picea*, *Eucalyptus*, *Acacia* and *Quercus*, accounting for 50 percent of the documented activities (FAO, 2004). Micropropagation activities have been reported to be most numerous in Asia (38 percent), followed by Europe (33 percent), North America (16 percent), South America (7 percent), Africa (3 percent), Oceania (2 percent) and the Near East (1 percent) (FAO, 2004).

Several endogenous and exogenous factors influence *in vitro* growth and the eventual success of micropropagation. In addition to the seasonal effect, type and age of the explants, genotype is a crucial factor in determining the responsiveness of the material to micropropagation (Yasodha *et al*., 2004; Durkovic and Misalova, 2008; Mashkina *et al*., 2010). *In vitro* multiplication of desired genotypes can be achieved via axillary budding, adventitious budding, or somatic embryogenesis.

Axillary budding refers to the propagation of plants through shoot development from cultured axillary buds[113]. It is the most successful clonal technique for angiosperms and produces the most true-to-type plantlets. Moreover, multiplication rates per subculture cycle can be higher than in adventitious budding. Protocols using this method have been developed for several species (Pijut *et al*., 2011) including *Populus tremula* (Peternel *et al*., 2009), *Tectona grandis* (Shirin *et al*., 2005), *Dalbergia sissoo* (Thirunavoukkarasu *et al*., 2010) as well as species of *Eucalyptus* (Glocke *et al*., 2006; Arya *et al*., 2009), *Acer* (Durkovic and Misalova, 2008) and *Quercus* (Vieitez *et al*., 2009).

Adventitious budding refers to the induction of adventitious[114] buds on non-meristematic tissue and is the preferred method for micropropagation of conifers. Induction rates can be quite high but it is more prone to somaclonal variation. Species of *Pinus* (Alonso *et al*., 2006; Alvarez *et al*., 2009), *Prunus*, *Ulmus* and *Fraxinus* (Durkovic and Misalova, 2008), among others, can be propagated by this technique.

Somatic embryogenesis (SE) is the process of differentiation of somatic embryos from vegetative cells, through the application of exogenous growth regulators to juvenile tissue. SE systems have been developed for both conifer species (Nehra *et al*., 2005) and temperate wood species (Pijut *et al*., 2007, 2011), with conifer species usually being more intractable (Bonga *et al*., 2010). A key advantage of SE is that the embryogenic tissue can be cryopreserved without loss of viability or genetic integrity, while corresponding trees are field-tested (Park, 2002). It has the largest potential multiplication rate and is amenable to handling in automated bioreactors (FAO, 2011b). However, SE is expensive and commercial application of this technique is still limited.

In Malaysia, collaboration between the Sabah Foundation Group and Centre de Coopération Internationale en Recherche Agronomique pour le Developpement (CIRAD, France) led to the development of superior teak clones, with respect to intrinsic wood qualities (Goh *et al*., 2007). These clones are also being exported and preliminary data from trials in Australia, Brazil and the United Republic of Tanzania have indicated that they outperform clones from other sources, displaying a 30

[113] A bud found at the axil of a leaf.

[114] A structure arising at sites other than the usual ones, e.g. shoots from roots or leaves, and embryos from any cell other than a zygote.

percent increase in yield (Goh *et al*., 2010). *Eucalyptus* hybrid clones constitute a considerable portion of existing commercial plantations, particularly in South America. Clonal forestry of selected *E. grandis* hybrid clones has been shown to reduce wood specific consumption[115] by 20 percent while second generation clones derived from hybridization with *E. globulus* have led to a further reduction of 20 percent (Grattapaglia, and Kirst, 2008).

A criticism of clonal forestry has been that it can reduce genetic diversity and make clonal plantations vulnerable to unexpected outbreaks of diseases and pests. Deploying more clonal lines could lower the risk, but also reduce genetic gain. Therefore, an appropriate balance between genetic gain and diversity is essential and it has been proposed that 10 to 30 clones mixed in a plantation should be sufficient to achieve this balance, in concert with a suitable configuration of deployed clones (Park and Klimaszewska, 2003).

4.7 Chromosome set manipulation

Due to their long regeneration time and strong inbreeding depression, forest tree species especially stand to benefit from the production of doubled haploid (DH) plants. In addition to shortening the breeding period, production of DH trees is beneficial for the isolation of recessive traits at sporophytic level. Induced haploid production through anther culture has been reported for about 32 woody species, including *Populus* and *Quercus*, but the success rate has been marginal and efficient anther culture systems are still limited (Andersen, 2005). Forest trees have been shown to be extremely intractable in anther cultures and a major impediment has been the conversion of calli and embryos into plantlets (Srivastava and Chaturvedi, 2008).

Triploids are of economic value in forest trees since they have more vigorous vegetative growth than the corresponding diploids. Triploid *Acaca nilotica* (Garg *et al*., 1996) and mulberry trees (Thomas *et al*., 2000) have been produced by endosperm culture, although this technique has been mostly unutilized in forest trees.

4.8 Molecular marker-assisted selection

Most tree breeding programmes rely on recurrent selection, i.e. implementing cycles of selection with inter-mating, generation after generation. The goal is to improve the overall performance of the population while maintaining genetic diversity, rather than to develop outstanding varieties for immediate use. Thus, advantages of using MAS in tree breeding include reduction of generation time, decreased field-testing costs and increased efficiency of selection for low-heritability traits (Neale and Kremer, 2011).

However, MAS is yet to realize its potential due to a number of reasons, such as the high heterogeneity of breeding populations, the lack of simply inherited traits that could be easily targeted, the difficulty in developing inbred lines to better understand the genetic basis of quantitative traits and the limited number of scientists working in this area (FAO, 2007b; Grattapaglia and Kirst, 2008). Genomic selection has also been evaluated for tree breeding through simulation studies, and initial results have been promising but are contingent upon requirements of effective population size and genotyping density being met (Grattapaglia and Resende, 2011).

A recent innovative strategy in tree breeding has been "breeding without breeding" (BWB), that allows the capture of 75 to 85 percent of the genetic response to selection achieved through conventional breeding programmes, but without performing any controlled crosses or experimental field testing, which are costly and time-consuming (El-Kassaby and Lstibůrek, 2009). BWB combines phenotypic pre-

[115] The amount of wood in cubic metres necessary to produce one ton of pulp.

selection of superior individuals with molecular markers for parentage analysis and pedigree reconstruction to identify elite genotypes retrospectively for establishing seed orchards. It is thus an effective and economic approach that seems to be a viable option for developing countries, especially for tree species that do not have advanced breeding programmes in place (Wang *et al*., 2010b).

4.9 Mutagenesis

Mutation breeding is complicated in forest tree species because recessive mutations are masked in heterozygous plants and it is difficult to obtain homozygous lines. As such, efforts have focused on the creation of dominant mutations, primarily in *Populus* species and hybrids, by activation tagging and enhancer[116] and gene traps (Busov *et al*., 2005). Activation tagging involves insertion of strong enhancers via *Agrobacterium*-mediated transformation[117], followed by screening of the resulting phenotypes in primary transformants and identification of candidate gene(s). Two activation tagged populations have been created in *Populus* – a population of 627 independent lines that have undergone two years of field testing for mutant identification (Busov *et al*., 2011) and a population of 1 800 independent lines (Harrison *et al*., 2007).

Alternative techniques to produce dominant phenotypes include gene and enhancer trapping. Gene trap vectors contain a reporter gene without a functional promoter, while enhancer trap vectors carry a reporter gene preceded by a minimal promoter. The reporter gene is expressed in a fashion that reflects the normal expression pattern of the tagged gene (Groover *et al*., 2004). A collection of poplar gene and enhancer trap lines has been established and is available for screening.[118]

TILLING (see section 3.12) is only just beginning to be applied in forest trees, and in hybrid poplar several induced mutations have been isolated by this method (Mattsson *et al*., 2007). EcoTILLING, that detects natural mutations, has been used to catalogue the level of DNA variation in natural populations of *Populus trichocarpa.* With this technique, 63 novel SNPs were identified in nine target genes for 41 tree accessions (Gilchrist *et al*., 2006). Such data can provide insights into gene function and be informative for association mapping analysis.

4.10 Transgenesis

FAO (2004) reports that forest tree genetic modification research takes place in 35 countries, with 48 percent of the reported activities occuring in North America, 32 percent in Europe, 14 percent in Asia, 5 percent in Oceania and less than 1 percent each in Africa and South America (FAO, 2004). Field trials of GM trees have been restricted largely to four genera, i.e. *Populus*, *Pinus*, *Liquidambar* and *Eucalyptus*. To date, China is the only country where GM trees are reported to be commercially available, involving transgenic poplar trees grown on roughly 300-500 hectares (FAO, 2004, 2011b).

V. CURRENT STATUS OF BIOTECHNOLOGIES FOR THE MANAGEMENT OF ANIMAL GENETIC RESOURCES

Nearly a billion people depend upon livestock for their livelihoods. Livestock provides income, food for human consumption, fibre, leather, fuel, draught power and fertilizer, thus contributing to food security and nutrition. In recent years, there has been a surge in demand for livestock products especially meat and milk, termed the "livestock revolution", driven by continued population growth, rising affluence and

[116] A eukaryotic DNA sequence which increases the transcription of a gene. Located up to several kbp, usually (but not exclusively) upstream of the gene in question. In some cases can activate transcription of a gene with no (known) promoter.

[117] The process of DNA transfer from Agrobacterium tumefaciens to plants, that occurs naturally during crown gall disease, and can be used as a method of transformation.

[118] http://www.fs.fed.us/psw/programs/ifg/genetraps.shtml

urbanization. Livestock is one of the fastest growing sectors of the agricultural economy and contributes 40 percent of the global value of agricultural output. Livestock also contributes 15 percent of total food energy and 25 percent of dietary protein at the global level (FAO, 2009c).

Both conventional technologies and biotechnologies have contributed to increased livestock productivity. However, the challenge is to maintain the diversity in animal genetic resources while simultaneously meeting the increasing demand for animal products. The use of appropriate biotechnologies, as described below, can play an important role in the management of animal GRFA for improved understanding of genetic diversity, enhanced conservation of breeds, increased animal productivity and better disease management.

5.1 Molecular markers

Microsatellites are the most popular markers to estimate genetic diversity in livestock and have been widely used to assess within- and between-breed genetic diversity in cattle (Freeman *et al*., 2006; Egito *et al*., 2007; Amigues *et al*., 2011; Kugonza *et al*., 2011), pigs (Behl *et al*., 2006; Sollero *et al*., 2009), sheep (Peter *et al*., 2007; Tapio *et al*., 2010), goats (Cañon *et al*., 2006; Xu *et al*., 2010), chickens (Hillel *et al*., 2003; Bodzsar *et al*., 2009; Mtileni *et al*., 2011) and horses (Leroy *et al*., 2009; Ling *et al*., 2010). However, information about genetic diversity in many indigenous livestock breeds is still scant.

With the intention of obtaining a global view of animal genetic diversity (by generating reproducible and comparable data and integrating national and regional datasets), panels of 30 microsatellite markers for nine major livestock species[119] have been recommended by FAO and the International Society of Animal Genetics (ISAG). To further standardize results, the development of SNP panels has also been proposed. In order to co-ordinate the various studies undertaken, draft guidelines on molecular genetic characterization have recently been developed by FAO[120] to aid countries in planning, implementing and analyzing genetic diversity of their animal genetic resources.

Country reports prepared for the State of the World's Animal Genetic Resources indicate that in developing countries, genetic distance studies for livestock breeds have been undertaken in four countries in Africa, six in Asia, 11 in Latin America and the Caribbean, and two in the Near and Middle East (FAO, 2007a). Collaborative efforts between FAO, the Joint FAO/IAEA Division and the International Livestock Research Institute (ILRI) are ongoing for molecular characterization of animal genetic resources in Asia and Africa (FAO, 2011c). The Joint FAO/IAEA Division has developed a web-linked database for sharing molecular data for the characterization of several species.[121]

Molecular characterization studies, using mitochondrial DNA, Y chromosomal and autosomal variation, have provided insights into breed history and ancestral populations of many species, including cattle, sheep, goats, horses, pigs and chickens (Groeneveld *et al*., 2010). Molecular markers have also been informative for identifying the geographical site(s) of domestication such as the Near Eastern origin of modern European taurine cattle (Troy *et al*., 2001) and the Indus valley origin of zebu cattle (Ajmone-Marsan *et al*., 2010). Further, molecular marker data in cattle (Loftus *et al*., 1999), goats (Cañón *et al*., 2006) and sheep (Tapio *et al*., 2010) have shown that breeds from near the putative domestication centres have higher levels of genetic diversity, suggesting that these breeds be prioritized for conservation, particularly in the first phase of conservation actions.

A major threat to conservation is introgressive hybridization and therefore early detection is fundamental for effective conservation strategies. Molecular markers have provided evidence for crossing of domestic

[119] http://www.globaldiv.eu/docs/Microsatellite%20markers.pdf

[120] http://www.fao.org/docrep/meeting/021/am135e.pdf

[121] http://www.globalgenomic.com/

pigs with European wild boars and of domestic cattle with wild North American bison (FAO, 2002; Halbert *et al*., 2005). Interestingly, molecular marker data has also demonstrated gene flow from wild populations to domesticated animals, for instance from junglefowl to domesticated populations of Vietnamese chicken (Berthouly *et al*., 2009).

Development of breed-specific brands is a common approach for adding value to the products of local breeds, thus contributing to their continued survival in *in situ* conditions (Tixier-Boichard *et al*., 2006). The price advantage obtained for the branded product can only be maintained if the uniqueness and purity of the breed origin can be demonstrated. Development of panels of molecular markers corresponding to breed-unique alleles has been proposed as a method to monitor the genetic source of such products and assure customers of their origin, thus protecting the market of the local breeds (Dalvit *et al*., 2007).

Parentage determination is essential for accurate pedigree information, genetic evaluation and successful breeding programmes. Parentage analysis with DNA markers is much more precise and reliable than conventional testing with blood groups or biochemical marker systems, and incorrect parentage can be excluded with almost 99 percent accuracy (Glowatzki-Mullis *et al*., 1995). The ISAG has suggested a panel of 12 loci each to be used in cattle (ISAG Conference, 2008a) and horse (ISAG Conference, 2008b) parentage analysis and the recommended loci have been effectively implemented for this purpose (Seyedabadi *et al*., 2006; Ozkan *et al*., 2009; Stevanovic *et al*., 2010). Besides cattle and horses, parentage analysis employing microsatellites has been carried out in other livestock species (Araújo *et al*., 2010; Siwek and Knoll, 2010). SNPs have also been shown to be effective for parentage analysis (Heaton *et al*., 2002; Rohrer *et al*., 2007; Hara *et al*., 2010).

Effective population size (N_e) is extensively used as a criterion for determining the risk status of livestock breeds and molecular markers are commonly employed to estimate N_e, especially of natural populations. Proposed methodologies for estimating N_e from marker data have been reviewed in Wang (2005). Molecular marker-based methods have been used for the estimation of N_e in cattle (Flury *et al*., 2010; Thévenon *et al*., 2007), sheep (Álvarez *et al*., 2008), horses (Goyache *et al*., 2011), chickens (Márquez *et al*., 2010) and pigs (Uimari and Tapio, 2011).

Molecular markers have been used to identify QTLs that affect traits of importance in livestock production, and a large number of studies have been carried out predominantly in developed countries (FAO, 2007b), since in the low-input systems that exist in many developing countries, the necessary phenotypic and pedigree information is often lacking. High resolution linkage maps, to facilitate fine mapping of QTLs, have been created for livestock species such as chickens (Groenen *et al*., 2009), cattle (Arias *et al*., 2009), pigs (Vingborg *et al*., 2009) and sheep (Raadsma *et al*., 2009). Consolidated QTL data on multiple livestock species are publicly available on the Animal QTL database (AnimalQTLdb) for easily locating QTLs and making comparisons within and between species (Hu *et al*., 2007). Currently, this database contains data on 6 344 pig, 4 682 cattle, 2 451 chicken and 454 sheep QTLs, representing 593, 376, 248 and 152 different traits respectively.[122]

5.2 "Omics"

Whole genome sequencing of many domestic animals such as cattle, chickens, horses, pigs, sheep and turkeys has been completed (Fan *et al*., 2010) and has led to the identification of millions of SNPs in the major livestock species[123]. This increased genomic information has, in turn, facilitated candidate gene analysis (Rincón *et al*., 2009; Seichter *et al*., 2011). Comparative genomics studies with the sequences have yielded insights into the biology and evolution of animal species (International Chicken Genome

[122] http://www.animalgenome.org/cgi-bin/QTLdb/index

[123] http://www.ncbi.nlm.nih.gov/SNP/

Sequencing Consortium, 2004; The Bovine Genome Sequencing and Analysis Consortium, 2009; Wade *et al*., 2009).

Subsequent to whole genome sequencing, International HapMap (haplotype mapping) Projects have been developed. These are collaborative efforts for large-scale genotyping of a given species to identify haplotypes, i.e a set of SNPs on a single chromosome that are statistically associated (The Bovine HapMap Consortium, 2009; Cockett *et al*., 2010; Groenen *et al*., 2010). The resulting haplotype maps have facilitated the detection of selection signatures associated with domestication and breed formation (Qanbari *et al*., 2010; Stella *et al*., 2010).

Genome-wide association studies (GWAS), based on high throughput SNP genotyping technologies and combined with phenotypic data, are being used to explore loci associated with complex traits, for example growth rate, milk production and disease related traits, and have the potential to lead to more efficient genomic selection, by contributing to the understanding of biological mechanisms underlying complex traits (Fan *et al*., 2010).

Transcriptomic approaches have provided mechanistic insights into numerous regulatory networks, for example the host response to pathogen exposure (Rinaldi *et al*. 2010; Tuggle *et al*., 2010), biological pathways relevant to traits related to meat performance (Wimmers *et al*., 2010), and ovarian follicle development as well as embryonic development for improving current assisted reproductive technologies (Huang *et al*., 2010c; Grado-Ahuir *et al*., 2011). Genome-wide transcript profiling has also been undertaken for breed comparisons, such as the variation in parasite resistance within and among sheep breeds (MacKinnon *et al*., 2009), differences in pork quality between pig breeds (Gao *et al*., 2011) and hepatic expression of genes in selected lines of the Holstein-Friesian dairy cattle breed (McCarthy *et al*., 2009).

Implementation of proteomic tools has assisted in understanding biological traits, particularly those that affect milk (D'Amato *et al*., 2009; Affolter *et al*., 2010; Wu *et al*., 2010) and meat quality (Chaze *et al*., 2008; Hornshoj *et al*., 2009; Bjarnadottir *et al*., 2010). Proteomic methods have also been applied in animal health to study the pathophysiology of diseases and to identify diagnostic markers for early detection of disease (Bendixen *et al*., 2011). Proteomic strategies for characterizing breeds are just beginning to be exploited (Almeida *et al*., 2010).

5.3 Bioinformatics

In addition to the previously mentioned databases (see section 2.1.3), individual livestock databases also exist: for example, tools for genome annotation, discovery and analysis are available for the bovine genome (Childers *et al*., 2011). Other species-specific databases include the Chicken Variation database (ChickVD)[124], the Pig Genomic Informatics System (PigGIS)[125] and the International Sheep Genomics Consortium[126]. A comprehensive list of databases on livestock genomics is provided in Groeneveld *et al*. (2010).

Advances in bioinformatics are occurring at a very rapid pace and consequently, there is a need for up-to-date training. The Biosciences eastern and central Africa (BecA)[127] Hub Bioinformatics Platform, a specialist node of the European Molecular Biology Network (EMBnet), provides advanced computational

[124] http://chicken.genomics.org.cn/index.jsp

[125] http://pig.genomics.org.cn/

[126] http://www.sheephapmap.org/

[127] BecA is an initiative developed within the framework of Centres of Excellence for Science and Technology in Africa and hosted and managed by the International Livestock Research Institute (ILRI).

capabilities in bioinformatics and is involved in raising awareness and capacity development in the subject in Africa.[128] Training on bioinformatics tools is also provided by the Joint FAO/IAEA Division.[129]

5.4 Cryopreservation

Cryoconservation of animal genetic resources (gametes, embryos, somatic cells) has been carried out in a number of countries, including a few developing countries (FAO, 2007a). Gene banks have also been created for conserving rare livestock breeds (Long *et al*., 2008). However, the costs associated with collecting, cryopreserving and reconstituting animal germplasm are comparatively much higher than those for plants. A recent survey undertaken by FAO in 90 countries illustrates that the number of cryoconservation programmes is approximately half the number of *in situ* programmes for most livestock species, with fully operational gene banks reported in only about 20 percent of the countries.[130]

Semen of most livestock species has been successfully cryopreserved with freezing procedures that are species-specific (FAO, 2007a). Cryoconservation of animal genetic resources in the form of semen is also practical due to its abundant availability and low cost. However, disadvantages include the conservation of only a single complement of chromosomes and the lack of mitochondrial genes. When only stored semen is used to reconstitute breeds by backcrossing with another breed (i.e. when female gametes/live animals of the breed are completely lost), sufficient semen must be available for the required number of backcrosses, which can be several thousand doses for lowly reproductive species. Even with five or more generations of crosses, a non-trivial proportion of the genetic material of the backcrossed breed will remain (Boettcher *et al*., 2005). Moreover, in avian species the W chromosome is absent in males and thus semen cryopreservation cannot be used to conserve the genes on that chromosome (FAO, 2007a).

Due to low permeability to cryoprotectants, cryopreservation of oocytes remains a challenge and is not as well established as for semen and embryos. Nevertheless, significant progress has been made and viable oocytes have been recovered after freezing and thawing in many mammalian species (Crister *et al*., 1997; Dhali *et al*., 2000). Offspring born from embryos produced from cryopreserved oocytes have been reported in cattle, sheep and horses (Prentice and Anzar, 2011). Oocyte cryopreservation of avian species has not been succesful due to the large size and high lipid content. Oocyte storage requires complementary cryoconservation of semen if the genetic material on the Y chromosome of mammalian species is not to be lost.

Embryo cryoconservation allows the conservation of the full genetic complement but is more expensive and requires greater technical capacity than semen cryoconservation (Gandini *et al*., 2007). The success of cryopreservation depends on the origin and developmental stage of the embryos, with *in vivo* derived embryos withstanding cryopreservation better than *in vitro* produced embryos and especially good results being attained with blastocysts. Cryopreservation of embryos has been reported for virtually all of the major mammalian livestock species, though its widespread use is limited to cattle, sheep and goats (Prentice and Anzar, 2011). Cryopreservation of embryos from pigs and equine species has been quite problematic due to their extreme chilling sensitivity, but in recent years, pig embryos have been successfully cryopreserved (Vajta, 2000) and live piglets have been obtained from cryopreserved pig embryos (Nagashima *et al*., 2007). Live offspring, from cryopreserved embryos, have also been obtained in horses (Ulrich and Nowshari, 2002) and other livestock species (Paynter *et al*., 1997; Rodriguez-Dorta *et al*., 2007).

[128] http://hub.africabiosciences.org/Bioinformatics

[129] http://www-naweb.iaea.org/nafa/aph/prospectus-animal-genetics.pdf

[130] http://www.fao.org/docrep/meeting/021/am132e.pdf

Somatic cells can be collected rapidly at favourable costs and easily cryopreserved (Groeneveld *et al*., 2008). However, utilizing the stored material through reproductive somatic cell nuclear transfer is more complex and expensive, and mitochondial genes are lost.

The choice of genetic material for cryoconservation depends on the generation interval and reproductive rate of the species, and costs must also be considered. For example, embryo collection and freezing in livestock is much more expensive than for semen but regeneration using embryos is quicker and cheaper. These differences can vary greatly by species. The use of semen to regenerate a breed of cattle or horse would take much longer and require many more doses of semen than for pigs or rabbits, whereas with embryos the regeneration time could be less than a year and the numbers of embryos required would be similar for all species. Draft guidelines providing technical advice on the cryoconservation of animal genetic resources have been developed by FAO.[131]

5.5 Reproductive biotechnologies

In livestock, both AI and ET can be applied for the future use of cryopreserved GRFA. Once thawed, the semen can be used for backcrossing using AI while the embryos can be transferred to recipient females. AI and ET have also been used to evaluate the viability of cryopreserved germplasm, subsequent to long-term storage. Pure-bred beef cows inseminated with frozen-thawed Angus bull semen, processed during three time periods (from the 1960s through to 2002), resulted in similar pregnancy rates across the different time periods, demonstrating that good quality semen frozen in liquid nitrogen should remain viable indefinitely (Carwell *et al*., 2009). Fogarty *et al*. (2000) showed that cryopreserved sheep embryos, stored for 13 years, could be successfully thawed and transferred to recipient ewes.

AI can have both positive and negative effects on the sustainable use and diversity of animal genetic resources. It has allowed for the tremendous increases in productivity, perhaps increasing the economic sustainability of livestock production, but has contributed to decreased effective population sizes and thus diversity. AI, together with cryoconservation, has facilitated the transboundary gene flow of animal genetic resources, largely in the North-South direction, which has threatened local populations (along with the lack of genetic improvement programmes for local breeds). When AI is applied in the absence of accurate genetic evaluation, it can greatly decrease genetic diversity without yielding gains in productivity. ET can have similar positive or negative effects, but these tend to be less because of the technology's relatively higher cost and lower numbers of offspring per animal.

Artificial insemination

AI is the most widely used reproductive technology in developed and developing countries. It has the best cost-benefit among all reproduction technologies, and has revolutionized the animal breeding industry. It enables a single bull to be used simultaneously in several countries for many inseminations a year and also enhances the efficiency of progeny testing of bulls. AI is applied in cattle, sheep, goats, turkeys, pigs, chickens and rabbits, with its most extensive application being in cattle. It is especially indispensable in the turkey breeding industry since the large size of the males of broad-breasted turkeys, which have been bred for body conformation, precludes natural mating (Flint and Woolliams, 2008).

As per 2002 statistics, more than 100 million AIs in cattle, 40 million in pigs, 3.3 million in sheep and 0.5 million in goats are performed globally every year (Thibier and Wagner, 2002).

In developing countries, most of the AI services are provided by the public sector, though it is still unavailable in many countries in Africa and the Southwest Pacific (FAO, 2007a). When applied, in Africa, Asia, Latin America and the Caribbean, AI is mostly used in cattle (especially in the dairy sector), with semen from exotic breeds predominating for local livestock production (FAO, 2011c).

[131] http://www.fao.org/docrep/meeting/021/am136e.pdf

AI is generally not very expensive and can be carried out by trained livestock keepers. Even so, the effectiveness of AI is often limited by organizational and logistical constraints in developing countries. Moreover, for AI to contribute to increased rural livelihoods, it has to be complemented by other services to maintain the health and fertility of the inseminated animals and provide enhanced market access for the product.

Progesterone monitoring

Progesterone monitoring can be used to verify whether animals have been inseminated at the correct time and detect any animals that later return to oestrus, so that they can be re-inseminated without delay (Dargie, 1990). The Joint FAO/IAEA Division has promoted the development and transfer of progesterone RIA (based on ^{125}I) in about 30 developing countries in Asia, Africa and Latin America for improved livestock productivity (FAO, 2011c).

Oestrus synchronization

The administration of exogenous reproductive hormones to synchronize ovulation is most often used in cattle, sheep and goats, although the use of oestradiol and its related ester derivatives is prohibited in many countries due to concerns about the effects of steroid hormones in the food chain (Lucy *et al*., 2004; Lane *et al*., 2008). In addition to increasing AI efficiency, oestrus synchronization is also of utility for optimizing transfer pregnancy rates when ET is carried out between donor and recipient females. Oestrus synchronization in developing countries is restricted to intensively managed farms or smaller farms with links to farmers' associations that routinely use AI (FAO, 2011c).

Embryo transfer

ET enhances the ability to select superior female genetic material, enables low cost transportation of diploid genetic material and allows for more offspring to be produced from a single female animal. With the intention of ensuring that specific pathogens that could be associated with embryos are controlled and transmission of infection to recipient animals and progeny is avoided, the World Organisation for Animal Health (OIE) has recommended measures on the collection and processing of *in vivo* derived embryos[132] and *in vitro* produced embryo/oocytes[133] from livestock and horses. The OIE has also provided recommendations for the collection and processing of micromanipulated embryos/oocytes (i.e. subjected to biopsy, nuclear transfer, etc.) from livestock and horses.[134]

ET has been applied in many species, including cattle, buffaloes, horses, pigs, sheep and goats. The technology is most widely used in cattle, with 535 164 *in vivo* derived bovine embryos reported to have been transferred in 2009 compared to 352 transfers in goats (down from 20 000 in 2006) and 24 470 in horses worldwide (Stroud, 2010). North America has been the centre of commercial ET activity, with 46 percent of all reported *in vivo* derived bovine embryo transfers in the world, followed by Europe with 18 percent of the total. More frozen *in vivo* derived embryos were transferred in 2009 than fresh embryos except in South America, where four times as many fresh embryos than frozen embryos were transferred. The number of *in vitro* produced bovine embryos transferred increased by 17 percent from 2008 to 2009, with South America responsible for 84 percent of the transfers (Stroud, 2010).

In developing countries, ET is reported to be applied in five countries in Africa, eight in Asia and 12 in Latin America and the Caribbean (FAO, 2007a). ET is expensive and requires highly skilled personnel which explains, in part, its low level of use. Compared with natural breeding, the cost of breeding through multiple ovulation and embryo transfer (MOET) has been estimated to be over 60 times higher (Maxwell and Evans, 2009). A recent study on ET in Mexico showed that the technology is profitable for farmers

[132] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2010/en_chapitre_1.4.7.pdf

[133] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2010/en_chapitre_1.4.8.pdf

[134] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2010/en_chapitre_1.4.9.pdf

only when substantial subsidies are provided (Alarcón and Galina, 2009). Once the subsidized initiative ends, the high costs associated with preparing the donors and recipients, embryo recovery and transfer, and the gestation itself make the ET programme unfeasible for farmers to sustain on their own.

In vitro *fertilization*

In vitro fertilization (IVF) has the potential to accelerate genetic progress by enhancing the accuracy and intensity of selection, reducing the generation interval and improving pregnancy rates in herds with low fertility (Hansen, 2006). IVF may reduce inbreeding since it offers greater flexibility in the mating design of sires to cows. IVF also allows for more efficient utilization of sperm since, compared to AI, a relatively lower number of spermatozoa can produce viable embryos (Boa-Amponsem and Minozzi, 2006). This is particularly valuable when small amounts of sexed sperm are available.

IVF with frozen sperm is mainly applied in commercial cattle embryo transfer in developed countries, and is not yet routinely used in developing countries due to its high cost and requirements for a well-equipped laboratory and skilled technicians. However, this technology has particularly expanded in Brazil and Japan, which account for the majority of *in vitro* fertilized embryos. In 2009, the total number of transferable bovine *in vitro* produced embryos was 379 000, with Brazil responsible for 68 percent, followed by Japan with 20 percent of the world's *in vitro* embryo production (Stroud, 2010). Live offspring from *in vitro* fertilized embryos have also been reported in sheep, goats and pigs.[135]

Recently, attempts have been made to utilize IVF procedures for overcoming reproductive barriers between species and producing hybrids. Owiny *et al*. (2009) demonstrated that although domestic cattle oocytes could be fertilized by African buffalo sperm, the resulting embryo development was slow and low, most likely due to chromosomal disparity.

Cloning

Another reproductive technology that can be used for conservation purposes, particularly when a breed is nearly extinct, is that of cloning. Cloning also offers the opportunity of increasing the uniformity of a given product and dissemination of superior genotypes in commercial populations. Somatic cell nuclear transfer has been successful in cattle, sheep, goats, pigs, rabbits, camels and horses, but the efficiency is low and often cloned offspring show abnormalities (Kues and Niemann, 2004).

Although cloned animals have been produced in a few developing countries (for example buffalo in China and India), it is still at the experimental stage due to the high costs and skills required (FAO, 2011c). According to a survey carried out by OIE in 2005 (with 91 respondent countries), cloning capabilities were reported by 4 percent of the countries in Africa, 23 percent in the Americas (Latin America and the Caribbean and North America), 23 percent in Asia and 18 percent in Europe (MacKenzie, 2005).

Nevertheless, developments in animal cloning have made realistic the conservation of animal genetic resources through cryopreservation of somatic cells rather than germ cells. This strategy can significantly decrease the costs and the level of technical expertise required to collect and bank the genetic material, but relies, for most species, on the assumption that the use of the material for regeneration of new animals will not be necessary until future technological advances have increased the efficiency and reduced the cost and animal welfare implications of creating clones. From a regulatory perspective, the use of cloned animals for food production has been approved by only a few national governments.

[135] http://www.fao.org/docrep/meeting/021/am136e.pdf

Sexing

Sperm and embryo sexing techniques allow for the sex of the progeny to be pre-determined, which is particularly useful for sex-limited and sex-influenced traits (e.g. for cattle, females are desired as dairy animals and males as beef animals).

Sexing of sperm can effectively increase selection intensity within dam136 pathways, enhancing genetic response for traits associated with productivity or other contributors to sustainability. Successful application of sperm sexing has been limited due to the high cost of sexed semen as well as low sperm viability and fertility rates. Consequently, much of the research on sperm sexing has been conducted by the private sector. Sperm sexing in bovine species has been applied commercially in a few countries, including Argentina, Brazil, Canada, Denmark, the Netherlands, the United Kingdom and the United States of America, and approximately two million calves have been produced worldwide from insemination with sexed sperm since 2000 (Rath, 2008). In other livestock species, the technology has not yet reached a stage that allows for commercial application (Rath and Johnson, 2008).

Embryo sexing requires the removal of a small number of cells from the embryo in order for the assay to be performed. Embryo biopsy is thus an invasive technique that calls for a high level of technician skill. The additional costs for embryo biopsy and sexing as well as the increased sensitivity of biopsied embryos to freezing/thawing procedures compared with intact ones, are some of the factors that have limited the application of this technique particularly in developing countries (Heyman, 2010).

5.6 Biotechnologies for disease diagnosis and prevention

Infectious animal diseases cause devastating losses in the livestock industry, limit efficient production and in the case of zoonotic diseases, pose threats to human health. Livestock farmers have suffered severe economic losses due to major outbreaks of transboundary animal diseases (TADs) such as foot and mouth disease (FMD) in Europe, classical swine fever (CSV) in the Caribbean and Europe (1996–2002), rinderpest (RP) in Africa in the 1980s, peste des petits ruminants (PPR) in Bangladesh and India, contagious bovine pleuropneumonia (CBPP) in Eastern and Southern Africa (late 1990s), as well as Rift Valley fever in the Arabian Peninsula (2000) (Domenech *et al*., 2006).

In recent years, FAO has collaborated with various international and regional organizations for preventing, controlling and managing TADs. The Global Framework for Progressive Control of Transboundary Animal Diseases (GF-TADs), a joint FAO/OIE initiative, provides a framework to address endemic and emerging infectious diseases. The FAO-OIE-WHO Global Early Warning and Response System for Major Animal Diseases, including Zoonoses (GLEWS)[137] avoids duplication and co-ordinates the verification processes of the three organizations to improve the early warning and response capacity to animal disease threats. The objective of the multi-agency One World One Health Strategic Framework[138] (FAO-OIE-WHO-UNICEF in collaboration with the World Bank and the UN System Influenza Coordinator - UNSIC) is to diminish the risk and minimize the global impact of epidemics and pandemics due to emerging infectious diseases at the animal-human-ecosystems interface.

Diagnostics and vaccines based on recombinant DNA technology have immensely contributed and are increasingly being used for improving disease control strategies (Balmurugan *et al*., 2010; Barnard, 2010). The OIE has been instrumental in providing updated internationally agreed diagnostic laboratory methods and requirements for the production and control of vaccines for the diseases listed in the OIE *Terrestrial Animal Health Code* (OIE 2010b).

[136] Female parent of an animal, especially domestic livestock.

[137] http://www.glews.net/

[138] http://www.aitoolkit.org/site/DefaultSite/filesystem/documents/OWOH_14Oct08.pdf

Diagnostics

Accurate, rapid and early disease diagnosis allows effective control measures to be implemented. Improved, robust real-time RT-PCR (rRT-PCR) assays have been developed for the detection of viral pathogens that cause FMD, CSV, bluetongue disease, avian influenza and Newcastle disease (Hoffmann *et al*., 2009).

The various ELISA and PCR-based methods that are being used and are recommended to diagnose diseases of global importance are described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE 2010b). The OIE has also adopted a Twinning Programme[139], i.e. a partnership between OIE Reference Laboratories[140] and candidate laboratories to develop laboratory diagnostic methods based on the OIE Standards in developing and transition countries.

Assays may be applied for many purposes (e.g. to document freedom from infection in a defined population, prevent spread of disease through trade, eradicate a disease from a region or country, confirmatory diagnosis of suspect or clinical cases, etc.) and thus need to be validated for their intended purpose. The OIE has developed criteria that must be fulfilled during assay development and validation of all assay types.[141]

The Joint FAO/IAEA division has been at the forefront of developing and delivering early and rapid diagnostic kits to developing countries, as well as providing laboratory networking and on-site training to detect infectious disease agents[142]. Seventy countries use disease diagnostic tests developed or validated by the FAO/IAEA Joint Division to assist their animal disease management programmes (FAO/IAEA, 2008). Currently, efforts are underway to develop penside tests utilizing the loop-mediated isothermal amplification (LAMP) PCR for the diagnosis of avian influenza, CBPP and PPR, that can be applied in rural and difficult to reach remote areas (IAEA, 2010).

The Global Rinderpest Eradication Programme (GREP)[143], spearheaded by FAO and in close association with OIE, was conceived to promote the global eradication of, and verification of freedom from, the disease. No known RP outbreaks have been detected since 2001 and its eradication from all 198 countries of the world was announced in 2011, a milestone in veterinary history.[144] In addition to the development of an improved vaccine against RP, biotechnologies such as C-ELISA and RT-PCR played a vital role in disease diagnosis and surveillance, including for the differential diagnosis between the closely related RP and PPR viruses. Rapid diagnosis, to prevent further spread of disease, was made possible by developing a field-based penside test using eye swab materials.

Vaccines

Vaccination is a cost-effective way to prevent clinical signs of a disease after infection or to help manage and even eradicate an infection. Ensuring vaccine purity, safety, potency and efficacy is indispensable for producing high quality vaccines and the OIE has described the principles of veterinary vaccine production (OIE, 2010b). However, vaccines must complement other aspects of disease management, for instance surveillance and diagnostic proficiency.

Gene-deleted vaccines have been licensed for use against bacterial diseases in horses, chickens, turkeys and sheep (Meeusen *et al*., 2007). Several commercial veterinary vaccines based on viral vectors (e.g.

[139] http://web.oie.int/downld/LABREF/A_Guide.pdf

[140] http://web.oie.int/eng/normes/mmanual/2008/pdf/XX_LIST_LAB.pdf

[141] http://web.oie.int/eng/normes/mmanual/2008/pdf/1.1.04_VALID.pdf

[142] http://www-naweb.iaea.org/nafa/aph/public/aph-nl-52.pdf

[143] http://www.fao.org/ag/againfo/programmes/en/grep/home.html

[144] http://www.fao.org/news/story/en/item/79335/icode/

DNA viruses such as poxviruses, herpesviruses and adenoviruses) have been developed, while many RNA viruses (e.g. Newcastle disease virus and a few retroviruses) are currently being evaluated as vectors (Gerdts *et al*., 2006). Rapid progress is also being made in developing bacteria as vectors (Rogan and Babuik, 2005).

DNA vaccines have been shown to be effective in cattle, sheep and poultry but have been commercially produced only for West Nile virus in horses (Potter *et al*., 2008). Recombinant subunit vaccines against porcine circovirus type 2 (Fachinger *et al*., 2008) as well as atrophic rhinitis (OIE, 2010a) have been commercialized while in recent years, efforts to develop orally delivered plant-based subunit vaccines have emerged (Streatfield and Howard, 2003; Yusibov *et al*., 2011).

Vaccines against economically devastating livestock diseases such as FMD and CSV are not used in disease-free countries as they would interfere with disease surveillance and compromise export interests. In order to address the ethical, welfare and economic aspects of large-scale animal culling in response to outbreaks of these infectious diseases, DIVA vaccines together with their companion diagnostic tests are now available (Beer *et al*., 2007; Grubman, 2005). Furthermore, vaccine banks have been established to guarantee supplies for emergency vaccination in case of outbreaks.

Vaccine banks may either store a ready-to-use formulated vaccine with a short shelf life, or more commonly the antigen component, that can be stored for a long period, for subsequent formulation into vaccine when required. The OIE has published guidelines for international standards for vaccine banks[145].

5.7 Molecular marker-assisted selection

Molecular markers can be used to enhance within-breed selection through MAS. In addition to reduced generation interval and more accurate selection, MAS in livestock can be especially advantageous for traits expressed in one sex, traits that are expressed very late in life as well as traits that usually necessitate slaughtering some of the animals.

MAS has been implemented in large-scale dairy cattle breeding schemes in developed countries (Boichard *et al*., 2002; Bennewitz *et al*., 2004), but its application has been limited in developing countries (FAO, 2007b). Examples of gene or marker tests used in commercial breeding for different species are reviewed in Dekkers (2004). For MAS to succeed and be sustainable within smallholder production systems in developing countries, other system constraints such as animal management, government policies, marketing, etc. need to be addressed (Marshall *et al*., 2011).

The huge variety across breeds can be exploited for genetic improvement through marker-assisted introgression (MAI), i.e. by introgressing individual genes or QTLs from one breed into another through repeated backcrossing. The Booroola gene (*FecB* gene), for enhancing prolificacy, has been introgressed by cross-breeding from the Booroola Merino into Awassi and Assaf breeds in Israel (Gootwine *et al*., 2001; Gootwine *et al*., 2003) and from the small Garole breed into the more productive but lowly fecund Deccani breed in India (Nimbkar *et al*., 2007).

Genomic selection (GS) is fast becoming the new paradigm in animal breeding. Since the genomic breeding value can be predicted at birth, the accuracy of prediction for young animals can be increased. Improved accuracy of predicting breeding values leads to increased genetic gain without increasing the rate of inbreeding (Daetwyler *et al*., 2007). It has also been demonstrated that cross-bred populations can be used as a training data set for GS for predicting breeding values of pure-bred animals without a substantial loss of accuracy compared with training on pure-bred data, thus eliminating the need to track pedigrees (Toosi *et al*., 2010).

[145] http://web.oie.int/eng/normes/mmanual/2008/pdf/1.1.10_VACCINE_BANKS.pdf

GS has the potential to increase response to selection, but phenotype and pedigree recording systems need to be in place. This approach is being used in developed countries, particularly for dairy cattle (Hayes *et al*., 2009) and more recently for chickens (Chen *et al*., 2011), but has high barriers for entry (financial, technical and informational) that have till now precluded its application in developing countries. GS is thus likely to increase the gap in genetic merit for product yield between local and international transboundary breeds, but may also limit the adaptability of these latter breeds to harsh environments, if adaptability is adversely correlated with production.

5.8 Mutagenesis

In the livestock sector, mutagenesis is generally not applied to animal populations. However, the sterile insect technique (SIT), an environment-friendly method for the management of insect pests of agricultural and veterinary importance, has proved to be an effective technique when used as part of an area-wide integrated pest management (AW-IPM) approach (Vreyson and Robinson, 2010). The SIT relies on the introduction of sterility in wild female insects of the pest population when they mate with released radiation-sterilized males, and has been used by 30 countries for the suppression/eradication of key insect pests (FAO/IAEA, 2008). Aspects of the SIT that make it a distinctive IPM tool include species specificity towards the target pest population as well as increased efficiency with decreasing target population density. As the SIT is extremely species-specific, it has limited efficacy for the control of a broader range of vector species.

The most successful SIT campaign eradicated New World screwworm (which causes myiasis in warm-blooded animals, leading to loss of milk, meat or wool production) from Central America, some islands in the Caribbean, Mexico, Panama and southern United States of America, and the area is maintained screwworm-free through the weekly release of 40 million sterile flies in Panama to prevent reinvasion from South America (Robinson *et al*., 2009). The first report of the New World screwworm occurring outside the Americas was in the Libyan Arab Jamahiriya in 1988. FAO, together with IAEA, the International Fund for Agricultural Development (IFAD) and the United Nations Development Programme (UNDP), was successful in containing the disease by dispersing sterile pupae and the country was declared officially screwworm-free in 1992. The annual producer benefits of the eradication programme have been estimated to be USD 796 million, USD 292 million and USD 77.9 million in the United States of America, Mexico and Central America respectively, with an estimated benefit/cost ratio of 5:1 in the infested zone in the Libyan Arab Jamahiriya (Vargas-Terán *et al*., 2005).

Old World screwworm fly has a very wide distribution throughout Asia, tropical and sub-tropical Africa, the Indian subcontinent, parts of the Middle East and Papua New Guinea. Small-scale field trials have shown the technical feasibility of using the SIT in the control of this pest and efforts are underway to use the technique as part of an AW-IPM approach on a much wider scale (Robinson *et al*., 2009).

Trypanosomosis is a severe disease transmitted by tsetse flies in sub-Saharan Africa. When left untreated, it is fatal in livestock and if not lethal, reduces fertility, weight gain, and meat and milk offtake by at least 50 percent (Feldmann *et al*., 2005). The effectiveness of the SIT in creating tsetse-free zones has been demonstrated in Zanzibar (1994-1997), where the tsetse population of *Glossina austeni* was completely eradicated from Unguja Island. Socio-economic assessments after the eradication indicated an increase in the contribution of the livestock sector to agricultural GDP (from 12 percent in 1986 to 34 percent in 1997) and in domestic food production leading to a decrease in food imports. An increase in average income per month of farming households, of 30 percent from 1999 to 2002, was also observed (Feldmann *et al*., 2005). Following this success and the escalating incidence of trypanosomosis, renewed efforts to control tsetse in Africa culminated in the establishment of the Pan African Tsetse Eradication Campaign

(PATTEC)[146], a concerted initiative that uses conventional methods in combination with the SIT to eliminate tsetse and trypanosomosis from Africa.

5.9 Transgenesis

No transgenic livestock have been commercialized as food to date, though a number of transgenic animals with medical or bio-"pharming" applications are at different stages of commercial development (FAO, 2011c). Transgenic production capability has been reported by 8 percent of the countries in Africa, 15 percent in the Americas (Latin America and the Caribbean and North America), 23 percent in Asia and 26 percent in Europe (MacKenzie, 2005).

VI. CURRENT STATUS OF BIOTECHNOLOGIES FOR THE MANAGEMENT OF AQUATIC GENETIC RESOURCES

The fisheries and aquaculture sector provides income and livelihood for almost 45 million people globally and employment in this sector has grown faster than the world's population and employment in traditional agriculture (FAO, 2010a). In 2008, 115 million tonnes of fish was consumed as human food; fish supplied over 3 billion people with at least 15 percent of their average animal protein intake (FAO, 2010a). Aquaculture accounts for 46 percent of the world's food fish and is expected to overtake capture fisheries in this regard. Currently, the most caught species at the global level is the anchoveta, while carps are the most cultured group of species in the world (FAO, 2010d).

In contrast to the crop and livestock sectors, aquatic genetic resources comprise a very large number of species. Most fisheries harvest wild populations and, with a few exceptions, the majority of aquaculture species have short histories of domestication and thus are genetically much closer to their wild counterparts. Structured selective breeding programmes have also been recently established and have been extremely effective for improving a range of characters for enhanced production (Bartley *et al*., 2009). Consequently, the fisheries and aquaculture sector has made less use of biotechnologies compared to the crop and livestock sectors. Nevertheless, as reviewed in this section, the application of biotechnologies for the characterization, conservation and utilization of aquatic genetic resources is steadily increasing, especially in light of the increasing demand for fish and the need for the sustainable management of these resources.

6.1 Molecular markers

Intraspecific characterization studies using isozymes and microsatellites have demonstrated that individual marine species generally exhibit lower levels of inter-population differentiation than freshwater species, since there are fewer barriers to migration and gene flow (Grant, 2006; Primmer, 2006). Molecular markers have been used to document the intraspecific population genetic structure and patterns of diversity in many natural populations, such as neotropical marine and freshwater fish in Latin America (Oliveira *et al*., 2009), coho salmon in North America (Beacham *et al*., 2011), Pacific cod in the Republic of Korea (Kim *et al*., 2010a) and native tilapia in East Africa (Angienda *et al*., 2011), among others. Genetic monitoring of natural populations over time can be applied to design effective conservation and management strategies (Sønstebø *et al*., 2007; Van Doornik *et al*., 2011).

In commercial capture fisheries, the catch often consists of a mixture of distinct populations and stock analysis is important to assess the relative contribution of each population in order to avoid overexploitation of some populations and optimize harvesting of abundant populations. Mixed stock analysis, based on molecular data, has been utilized to monitor stocks of many species including Atlantic salmon (Griffiths *et al*., 2010), Pacific salmon (Flannery *et al*., 2010), Atlantic cod (Wennevik *et al*.,

[146] http://www.africa-union.org/root/au/AUC/SpecialProjects/pattec/pattec.htm

2008), lake sturgeon (Bott *et al*., 2009) and steelhead trout (Winans *et al*., 2004). The ability to link an individual fish product (for example, shark fins and caviar) to the population of origin using molecular data is a robust tool for trade surveillance since it can be used to identify stocks that are major contributors to the market, although it requires prior sampling of the potential source populations (Baker, 2008; Chapman *et al*., 2009).

Molecular markers have also been employed to distinguish the various species that occur together in mixed catches and are difficult to differentiate morphologically (Okumus and Ciftci, 2003; Berntson and Moran, 2009). Molecular markers are valuable as forensic tools, when consumer fraud or illegal possession of species at risk is suspected, especially for identifying early life stages, i.e. eggs and larvae, and morphologically unrecognizable fish products (Ogden, 2008). Applications range from the detection of mislabelled products from threatened or poisonous species (Teletchea, 2009) to the investigation of illegally traded caviar from endangered sturgeon species (Ludwig, 2008) or the discrimination of Atlantic wolffish from the threatened spotted and northern wolffishes, which require legal permits for possession (McCusker *et al*., 2008). Additionally, DNA arrays (based on the mitochondrial gene 16S rRNA) that have the capability to distinguish fish species, including eggs, larvae and pocessed products, have been developed for consumer protection and trade regulation (Kochzius *et al*., 2008, 2010).

Misidentification of cryptic species, i.e. discrete species that are difficult or sometimes impossible to distinguish morphologically but are genetically distinct, can have serious negative consequences in fisheries management (Bickford *et al*., 2007). Molecular markers have helped in cryptic species recognition across a wide variety of marine species, including finfish (Kon *et al*., 2007; Piggott *et al*., 2011), molluscs (Vrijenhoek, 2009) and crustaceans (Belyaeva and Taylor, 2009), suggesting that they are vital for accurately describing species diversity. DNA barcoding, based on a fragment of the mitochondrial gene *cytochrome c oxidase subunit 1*, is being applied for the identification of cryptic species as well as new and invasive species, and for regulatory and enforcement activities (Kim *et al*., 2010b; Radulovici *et al*., 2010; Wong *et al*., 2011). The Fish Barcode of Life Initiative (FISH-BOL)[147] aims to barcode all the world's fishes (Ward *et al*., 2009) and has currently barcoded 8 236 species.

In aquaculture, molecular markers have provided information on genetic variability within and between hatchery stocks (Freitas *et al*., 2007; Blackie *et al*., 2011). It is particularly imperative to maintain high levels of genetic diversity in broodstocks used for replenishing and maintaining the viability of depleted fishery populations, and also to ensure the productivity of genetically improved stocks for fish production. Concerns have been raised about the possible effects of stock enhancement on natural populations as molecular studies comparing hatchery and wild stocks have shown that farmed populations often have reduced genetic diversity and a lower effective population size (Araki and Schmid, 2010).

For an endangered species, *Brycon insignis*, a 28-57 percent reduction in allelic richness was detected in the broodstock used for restocking purposes compared to the wild populations, highlighting the need to maintain genetic variability in captive populations (Matsumoto and Hilsdorf, 2009). Similarly, in contrast to wild populations, lower genetic diversity has been observed in some hatchery populations, for example Atlantic salmon (Blanchet *et al*., 2008), Japanese flounder (Shikano *et al*., 2008) and white seabream (Pereira *et al*., 2010). At the same time, comparable levels of genetic diversity between wild and hatchery populations have also been reported (Pan and Yang, 2010).

Molecular markers have been used to investigate the impact of stock enhancement on wild populations (Okumus and Ciftci, 2003; Povh *et al*., 2008) and to manage broodstocks for stock enhancement and restocking purposes (Congiu *et al*., 2011). Microsatellite data showed that the genetic diversity of Chinook salmon was maintained over multiple generations of supplementation, over a period of 16 years, with increased effective population sizes of both the wild and captive-origin adults (Eldridge and

[147] http://www.fishbol.org/

Killebrew, 2008). Conversely, when a captive broodstock was used for extensive release of red sea bream for 30 years, the disappearance of some rare alleles in the wild populations was detected (Kitada *et al*., 2009).

The most common application of molecular markers (particularly microsatellites) in aquaculture species has been in parentage analysis in order to manage the rate of inbreeding in conventional breeding programmes. Molecular markers are especially useful when physical tagging is not feasible or when individuals are reared communally to minimize environmental variation, as they can retrospectively assign individuals to families after evaluation of individual performance (FAO, 2011d). Parentage analysis with molecular markers has been successfully accomplished in many commercially important species, including Atlantic salmon (Norris *et al*., 2000), Atlantic cod (Herlin *et al*., 2008), gilthead sea bream (Brown *et al*., 2005), Senegal sole (Castro *et al*., 2006), common carp (Vandeputte *et al*., 2004), Japanese flounder (Hara and Sekino, 2003), shrimp (Jerry *et al*., 2006) and mussels (MacAvoy *et al*., 2008), although its potential has not yet been fully exploited in developing countries.

Molecular analysis of parentage has also been utilized in natural populations for increased understanding of mating systems, for example for detecting multiple mating in both sexes in Atlantic salmon (Wilson and Ferguson, 2002), multiple paternity in shrimp, crayfish, crab and lobster (Yue and Chang, 2010; Yue *et al*., 2010), and brood parasitism in cichlids and sunfish (DeWoody and Avise, 2001; Taborsky, 2001). Parentage analysis has been used to evaluate patterns of larval dispersal to determine connectivity among marine populations (Planes *et al*., 2009; Christie *et al*., 2010). Recently, this approach was employed to infer individual movement throughout the life cycle of the stream-dwelling brook charr, especially at very early life stages when individuals are too small and numerous to be tagged by other means (Morrissey and Ferguson, 2011).

Molecular markers are very effective for identifying farmed escapees, the origin of these escapees, as well as for quantifying the interactions between escaped farmed and wild fish. Physically tagging farmed fish is expensive, requires highly developed logistic systems for marking and tracking the fish, and has animal welfare implications. Molecular tagging, on the other hand, can circumvent these issues and has been successfully applied for identifying escaped fish (Glover, 2010). Molecular studies have documented introgression of farmed escapees with wild fish, leading to loss of local adaptation (Bekkevold *et al*., 2006; Skaala *et al*. 2006).

To facilitate molecular characterization, the Network of Aquaculture Centres in Asia-Pacific (NACA) has produced a two-part manual on the application of molecular tools in aquaculture and inland fisheries management, with part 1 focusing on the conceptual basis of population genetic approaches (Nguyen *et al*., 2006a) and part 2 providing laboratory protocols and methodologies for data analysis and project design (Nguyen *et al*., 2006b).

In aquaculture, QTL mapping using commercial populations has been accomplished mainly in developed countries (FAO, 2011d) and, in general, is not as advanced as in the crops and livestock sectors. Genetic linkage maps for QTL identification have been created for various fish and shellfish species such as Atlantic salmon, rainbow trout, channel catfish, tilapia, Japanese flounder, common carp, turbot, sea bass, sea bream, shrimp, oysters and mussels (FAO, 2007b; Liu, 2007; Castaño-Sánchez *et al*., 2010; FAO, 2011d). A number of QTLs for important traits have been mapped in farmed aquatic species, for instance growth, flesh colour, spawning time, sex determination, abiotic stress tolerance and disease resistance (Liu and Cordes, 2004; FAO, 2007b; Presti *et al*., 2009; Baranski *et al*., 2010; Loukovitis *et al*., 2011). Furthermore, aquaculture, while still relatively in its initial stages, can benefit from the large amount of information available for livestock species, especially for the identification of genes homologous to putative candidate genes such as those correlated with growth (De-Santis and Jerry, 2007).

Molecular markers have additional applications in aquaculture. For example, they are routinely applied to assess desired genetic manipulations, such as triploidy and gynogenesis (Jenneckens *et al*., 1999; Presti *et al*., 2009).

6.2 "Omics"

The genomes of five well-known model finfish species, the zebrafish, medaka, spotted green pufferfish, Japanese pufferfish and three-spined stickleback (Oleksiak, 2010) and some marine invertebrates, for example purple sea urchin, have been fully sequenced (Rast and Messier-Solek, 2008). Recently, whole genomic sequencing of three commercial aquaculture species, common carp[148], Nile tilapia[149] and Pacific oyster[150] has been completed while genome projects for Atlantic salmon (Davidson *et al*., 2010), catfish (Lu *et al*., 2011), rainbow trout, flatfish, cod and sea bass are underway (Wenne *et al*., 2007).

For species that lack whole genome sequences, expressed sequence tag (EST) data serve as an important resource for gene discovery as well as for the construction of microarrays (Oleksiak, 2010). Thousands of ESTs are available for some species. For instance, currently there are 498 212 ESTs for Atlantic salmon, 354 466 for channel catfish, 287 967 for rainbow trout and 229 090 for Atlantic cod.[151] Full-length cDNAs, informative tools for functional and structural genome studies, have been obtained for a few aquaculture species, including salmonids (Leong *et al*., 2010) and catfish (Chen *et al*., 2010).

The accelerated ability to sequence has led to the detection of thousands of SNPs as markers, that can contribute to the construction of high-density linkage maps and subsequent QTL identification, in species such as Atlantic salmon (Moen *et al*., 2008), Atlantic cod (Hubert *et al*., 2010), catfish (Liu *et al*., 2011a) and sea bass (Kuhl *et al*., 2011). Diagnostic SNP panels have been assembled to discriminate farmed and wild Atlantic salmon and quantify gene flow (Karlsson *et al*., 2011). Genomic scans with molecular markers have exposed signatures of selection and shed light on adaptive evolution (Nielsen *et al*., 2009; Gomez-Uchida *et al*., 2011).

Functional genomic studies with microarrays (both cDNA and oligonucleotide) can be used to uncover molecular mechanisms underlying productive and adaptive traits in fish (Ferraresso *et al*., 2008, 2010; McAndrew and Napier, 2010; Krasnov *et al*., 2011). Microarrays have been developed for numerous species including Atlantic salmon, rainbow trout, common carp, European flounder, Atlantic halibut, African cichlid and channel catfish. Heterologous microarray analyses, i.e. cross-species hybridization, can be performed for expression profiling of closely related non-model species for which limited sequence information exists (Renn *et al*., 2004; Healy *et al*., 2010). Recently, customized multi-species microarrays have been constructed to assay gene regulation across species (Kassahn, 2008; Baker *et al*., 2009).

Microarrays have been utilized to quantify gene expression in response to altered environmental conditions such as hypoxia and cold (Gracey, 2007; Boswell *et al*., 2009), to infection by pathogens such as bacteria, viruses and fungi (Peatman *et al*., 2007; Roberge *et al*., 2007; Ewart *et al*., 2008; Workenhe *et al*., 2009) as well as to vaccines (Škugor *et al*., 2009) and inflammatory stimulators (Djordjevic *et al*., 2009). Microarray data has yielded insight into diverse biological processes such as nutrition (Taggart *et al*., 2008), reproduction (Cavileer *et al*., 2009), stress physiology (Aluru and Vijayan, 2009), larval development (Douglas *et al*., 2008), life history traits (Aubin-Horth *et al*., 2005) and migration (Miller *et al*., 2011).

Transcriptomics has also been used to study adaptive divergence among natural populations, for example between North Sea and Baltic Sea European flounder populations (Larsen *et al*., 2007) and between dwarf and normal whitefish (St-Cyr *et al*., 2008). In addition, gene expression analyses have been applied in aquatic toxicology to evaluate the impact of anthropogenic contaminants and for the discovery of robust

[148] http://english.big.cas.cn/ns/es/201105/t20110509_69516.html

[149] http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html

[150] http://www.genomics.cn/en/news_show.php?type=show&id=565

[151] http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html

biomarkers[152] (Denslow *et al*., 2007; Hook, 2010). However, to differentiate genetic from environmental effects in natural populations in such experiments, it is essential either that the individuals from different populations are kept under common environmental conditions or that they are subjected to the native environmental conditions experienced by the other populations (Larsen *et al*., 2011).

Transcriptional data from microarrays, together with previously established linkage maps, can be employed to locate candidate genes (Whiteley *et al*., 2008). Next generation sequencing is also proving to be a powerful tool for whole transcriptome characterization and profiling, especially for gene discovery (Jeukens *et al*., 2010; Xiang *et al*., 2010; Johansen *et al*., 2011a). A complementary approach, taking into account both transcriptome and marker data, can be more efficient for defining effective conservation and management strategies (Tymchuk *et al*., 2010).

The potential of comparative genomics has been exploited to map QTLs and identify candidate genes in non-model fish species (Sarropoulou and Fernandes, 2010; Li *et al*., 2011). Proteomic (Forne *et al*., 2010; Sanchez *et al*., 2011) and metabolomic (Flores-Valverde *et al*., 2010; Samuelsson *et al*., 2011) approaches are also being increasingly applied in fish.

6.3 Bioinformatics

Bioinformatics tools for fish are not as comprehensive as for livestock species. In order to co-ordinate and integrate "omic" data for fish, the Bioinformatics tools for Marine and Freshwater Genomics (BiMFG)[153] database has been designed and provides mining tools for analyzing DNA, RNA and protein sequences (Shih *et al*., 2010). Species-specific repositories for storing and integrating genomic data as well as access to bioinformatic tools exist for Atlantic salmon[154], rainbow trout[155], catfish[156], tilapia[157], sea bass[158], sea bream[159] and Atlantic cod[160]. A genome-wide fish metabolic network model (MetaFishNet)[161] is available for analyzing high-throughput gene expression data (Li *et al*., 2010b).

6.4 Cryopreservation

In fish, cryopreservation techniques are mostly applied to sperm and protocols are available for over 200 species of finfish and shellfish, with most studies focused on freshwater species such as salmonids, sturgeons, carps and catfishes (Diwan *et al*., 2010). Cryopreservation of fish ova and embryos still remains a challenge primarily due to their large size and biochemical composition (Yang and Tiersch, 2009), although cryopreservation of oocytes (Hamaragodlu *et al*., 2005; Tervit *et al*. 2005) and isolated embryonic cells (Hiemstra *et al*., 2006; Routray *et al*., 2010) has been demonstrated in some species. Larvae of certain invertebrate species have also been successfully cryopreserved (Paniagua-Chavez and Tiersch, 2001; Kang *et al*., 2009; Wang *et al*., 2011).

Unlike in species with internal fertilization, fish sperm is immotile in the testis and seminal plasma, and generally becomes active and increasingly motile after discharge into the aqueous environment (Alavi and Cosson, 2006). The exception is viviparous fish where the sperm is rendered motile after its release into

[152] A biomarker is a measurable biological quantity that can be linked to either contaminant exposure or effects.

[153] http://bimfg.cs.ntou.edu.tw/

[154] http://www.cgrasp.org/index.html

[155] http://www.irisa.fr/stressgenes/

[156] http://www.catfishgenome.org/cbarbel/

[157] http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html

[158] http://www.bassmap.org/

[159] http://www.bridgemap.tuc.gr/index.htm

[160] http://codgene.ca/index.php

[161] http://metafishnet.appspot.com/

the female genital tract. The activation mode matches the environment where the sperm functions during spawning and post-activation motility is often of short duration. Thus, dilution of sperm in an extender solution that mimics the osmolality of the seminal plasma and inhibits activation of sperm is crucial for cryopreservation (Viveiros, and Godinho, 2009; Yang and Tiersch, 2009).

Thawed sperm is usually less effective than fresh sperm for fertilizing eggs and therefore a higher concentration of cryopreserved sperm is needed to achieve the same level of fertilization (Kurokura and Oo, 2008; Kwantong and Bart, 2009) although increasing the sperm-egg ratio too much decreases the fertilization rate (Magyary *et al*., 2000; Gwo, 2000). Once fertilization has occurred, hatching and survival rates do not seem to differ between fresh and cryopreserved sperm (Chereguini *et al*., 2001; Kwantong and Bart, 2009). Nevertheless, there have been some reports of sperm cryopreservation promoting DNA damage in fish as well as increased embryonic abortion (Cabrita *et al*., 2010).

Recently, spermatogonial transplantation into the gonads of sexually mature fish has been established as a novel method to preserve and propagate germplasm from fish that are still sexually immature or are incapable of providing sperm for cryopreservation (Lacerda *et al*., 2008; Majhi *et al*., 2009). Spermatogonia also exhibit a high level of sexual plasticity and can produce both functional eggs and sperm, which is especially important for species whose eggs cannot be cryopreserved (Okutsu *et al*., 2007). Cryopreserved spermatogonia have been successfully transplanted into triploid recipients to generate pure donor-derived male and female progeny (Yoshizaki *et al*., 2011).

In addition to its advantages described in section 2.2.1, successful cryopreservation of sperm improves the efficiency of hatcheries by providing sperm on demand and permitting out of season breeding. Operational costs can also be significantly reduced by allowing hatcheries to eliminate the need to maintain live males and potentially diverting resources for use with females and larvae (Tiersch *et al*., 2007). On the other hand, the economics of integrating cryopreservation into existing fish hatcheries has been investigated and found to be significant. Compared to public hatcheries, investment and operating costs have been found to be higher for private hatcheries, due to associated taxes and interest (Caffey and Tiersch, 2000).

Cryogenic sperm banks have been established mainly in Europe and North America, but also in some developing countries such as Brazil, India and Malaysia (Harvey, 1998, 2000; Chew *et al*., 2010). Commercial-scale cryopreservation of sperm has also been initiated in a few species such as tetraploid Pacific oysters (Dong *et al*., 2005) and blue catfish (Hu *et al*., 2011).

Compared with livestock germplasm, cryopreservation of fish germplasm has had little commercial application. Barriers to widespread application include variation in sperm characteristics among species and stocks, technical problems, a lack of standardization in practices (for example gamete collection methods, cryoprotectant choice, evaluation of post-thaw sperm quality, etc.) and inconsistencies within the scientific literature (Tiersch, 2008). Further, research has focused mainly on a small number of species with protocols being unrefined or absent for most species.

6.5 Reproductive biotechnologies

Reproductive technologies are widely and easily used in many aquatic species. External fertilization, good response to hormones or environmental spawning cues, and the production of large numbers of gametes that are easily manipulated make reproductive technologies an effective means of increasing the use of aquatic genetic resources.

However, reproductive dysfunctions have been observed in some cases. Some males may produce a reduced amount of milt (a serious issue for hatcheries where fertilization is carried out artificially with selected sperm), low-quality milt or highly viscous milt that cannot fertilize eggs effectively due to diminished dispersal in the water. Captive females often exhibit more severe reproductive problems and fail to undergo oocyte maturation, ovulation or spawning (Mylonas and Zohar, 2007). In such cases,

hormonal treatment is needed for ovulation/spermiation and spawning (Zohar and Mylonas, 2001), and to carry out artificial fertilization, particularly for the creation of hybrids that cannot be produced by natural, spontaneous mating.

Hormonal treatment

Control of reproduction allows for the supply of eggs and fry any time during the year, i.e. independently of the seasons and the natural spawning time of the species. Hormonal treatment is also used to synchronize ovulation for high seasonal fecundity and a predictable seed supply (FAO, 2011d). The timing of spawning can be altered by the manipulation of species-specific environmental factors such as photoperiod and temperature, but these are not well studied for some species or are nearly impossible to simulate in a hatchery. Consequently, the application of exogenous hormones is the only reliable method to induce spawning.

Hormonal therapies are based either on the use of luteinizing hormone preparations such as human chorionic gonadotropin (hCG) and homologous pituitary extracts; gonadotropin-releasing hormone (GnRH) analogues, with the GnRH analogue-delivery system (with controlled release of the hormone for periods from 1 to 5 weeks) being the most extensively employed in aquaculture (Mylonas *et al*., 2010); or serotonin-like hormones in the case of bivalves (Braley, 1985). GnRH analogue treatments offer several advantages such as less species-specificity, i.e. generic use, high potency in small amounts and no risk of disease transmission from the donor fish to recipient broodstocks (Cnaani and Levavi-Sivan, 2009). However, hormonal treatment can affect gamete quality (particularly eggs) depending upon the nature of the treatment, the gonad developmental stage at which it is applied and the method of delivery (Avery *et al*., 2004; Miranda *et al*., 2005). Hormonal treatments have been shown to be effective in a number of cultured fish species, for instance for enhancing spermiation and synchronization of ovulation in salmonid fishes (Mylonas *et al*., 2010), and multiple spawning in carps and flatfish (Agulleiro *et al*., 2006; Routray *et al*., 2007) as well as in molluscs, such as giant clams, through serotonin-induced spawning (Braley, 1985).

Sexual dimorphism for economically important traits such as growth rate, time and age of sexual maturation and carcass composition, is frequently detected in fish species. For example, male tilapia and channel catfish grow faster than females whereas salmonid females are larger with higher flesh quality than males. Development of monosex populations is thus preferred in commercial aquaculture production systems (FAO, 2008). Culture of monosex stocks is also advantageous for reproductive containment and for minimizing competition or territorial behaviour that occurs in mixed sex populations.

Monosex populations can be generated by manual sorting, interspecific hybridization and hormonal treatment. While manual sorting is labour intensive and wasteful with a low success rate, interspecific hybridization is applicable only to specific combinations of species and mating results are inconsistent (Cnaani and Levavi-Sivan, 2009). Another impediment to the use of interspecific hybridization, especially in developing countries, is the necessity of increased management for keeping the parental species separated and avoiding hybrid contamination.

Unlike livestock, fish species display some plasticity in their sexual developmental processes, which can be exploited for manipulation of sex. In many species, even though sex is genetically established at fertilization, phenotypic differentiation occurs at a later stage with the timing dependent upon the species involved (Dunham, 2004). The phenotypic sex can be altered either by the application of androgens for masculinization or estrogens for feminization during the critical period of sexual differentiation, generally during early embryogenesis in salmonids and post-hatching in cichlids and cyprinids (Cnaani and Levavi-Sivan, 2009). Hormones are usually administered through oral or bath treatment, i.e. through the diet or by immersion of eggs and fry in hormone solutions.

The direct induction of sex change by hormones may lead to compromised consumer acceptance even though there is no evidence for the presence of hormonal residues after cessation of the treatment (FAO,

2008). An indirect approach through the combination of sex reversal and breeding is likely to have a wider acceptance, although it requires an understanding of the genetic mechanism of sex determination in the given species and may require progeny testing and multi-generational breeding (FAO, 2011d).

Hormonal sex reversal is widely used on a commercial scale in many countries, for example for the production of all-male Nile tilapia in Israel, the Philippines, Thailand and the United States of America[162], all-female rainbow trout in Europe and the United States of America, and all-female silver barbs in Bangladesh and Thailand (Cnaani and Levavi-Sivan, 2009).

6.6 Chromosome set manipulation

Chromosomal set manipulation in fish includes inducing polyploidy as well as producing gynogens and androgens. Since most fish and shellfish release their eggs into water before fertilization, they can be easily accessed for manipulation of ploidy levels. This technique has been mainly used to create triploids that are advantageous in production and conservation programmes.

Triploids occur naturally in both wild and cultured populations and can be easily induced in many commercial fish and shellfish species. Triploidy is generally induced by physical or chemical shock (with physical treatments being the most successful) and protocols are available for over 30 fish and shellfish species (Dunham, 2004), or alternatively by fertilizing normal haploid eggs with diploid sperm from a tetraploid male (Piferrer *et al*., 2009; Flajshans *et al*., 2010). Triploid production by pressure or temperature shock is not 100 percent effective and species-specific protocols are essential to optimize timing, intensity and duration of treatment in order to obtain the highest triploid yield (Piferrer *et al*., 2006). Crossing between tetraploids and diploids is the most efficient method to produce 100 percent triploids (Dong *et al*., 2005; Zhou *et al*., 2010a). There are some data indicating that sperm diploidy should be confirmed prior to fertilization, since not all tetraploid males produce diploid sperm (Nam and Kim, 2004). Tetraploid production, however, is difficult and viable and fertile tetraploids have been obtained only in a few aquaculture species (Yoshikawa *et al*., 2008).

Triploid fish are usually effectively sterile, which is desired in conservation programmes to prevent introgression of escaped individuals from commercial stocks, including non-native species, into natural populations and hence reduce the genetic impact of farmed escapees. Sterility is also useful to avoid the establishment of introduced exotic species, for example triploid grass carp for aquatic weed control (Pípalová, 2006). The migration behaviour of triploid fish such as salmon has been investigated and these studies have revealed that the return rate of adult triploid fish to the coast and fresh water was substantially diminished compared to the diploids, i.e. triploidization reduces the chances of interbreeding as well as the potential for other negative interactions with wild indigenous salmon in their natal areas (Cotter *et al*., 2000; Wilkins *et al*., 2001). Similarly, restocking of all female triploid brown trout did not noticeably impact wild brown trout and the triploid fish did not participate in spawning activity.[163] Triploidy has also been utilized to increase sterility in fertile hybrids (Na-Nakorn *et al*., 2004), although there is evidence of allotriploid fish producing some viable offspring (Castillo *et al*., 2007).

Since triploid individuals do not devote energy to gamete production, more energy is available for somatic growth. Performance of triploids is species-specific, but they often have similar or less growth than diploids as juveniles and, after maturation, growth is enhanced, especially in shellfish (Piferrer *et al*., 2009). In addition to increased growth (triploids are 17 percent larger than diploids prior to spawning and more than 30 percent larger after spawning), triploid Pacific oysters can be marketed throughout the year due to reduced gonadogenesis (Rasmussen and Morrissey, 2007). Sexual maturity in diploid oysters leads to a decrease in the quality of both taste and texture and thus triploids benefit from higher consumer

[162] http://www.agribusinessweek.com/update-on-tilapia-sex-reversal/

[163] http://www.environment-agency.gov.uk/static/documents/Research/triploid_trout_p3_1882654.pdf

acceptance (Nell, 2002). Triploid rainbow trout females are preferred for commercial production since they have a better carcass yield during the reproductive season and higher body and fillet weights (Werner *et al*., 2008). Triploidy may also skew sex ratios, for example in certain species of fish and shellfish, towards a higher proportion of females, which are preferred due to their larger sizes (Cal *et al*., 2006).

In fish, triploid females are completely sterile and partial to total functional sterility has been observed in males, although offspring sired by artificially produced triploid males are not viable (Manning *et al*., 2004; Feindel *et al*., 2010). Nevertheless, since triploid males can still develop secondary sexual characteristics, the production of all-female triploid populations (by combining triploidy induction with endocrine feminization) is most desirable to reduce any sexual-related disadvantages (Piferrer, 2001). Triploid shellfish exhibit a decrease in gonadal development as opposed to complete sterility. Moreover, in Pacific oysters reversion to diploidy has been observed in a proportion of triploid individuals. Verification of triploidy is therefore crucial to ensure functional sterility in a given species and it has been proposed that at least two full consecutive reproductive cycles should be monitored for this purpose (Piferrer *et al*., 2006).

Triploidy is commercially applied mainly in developed countries for numerous fish species such as trout, salmon, charr and carp, as well as shellfish species such as Pacific oyster, scallops, clams and mussels (Piferrer *et al*., 2009). This technology has not yet been commercialized for shrimp (Sellars *et al*., 2010). Practical implementation of triploidy has not been very successful in developing countries due to high costs and the inability of shock treatment to be 100 percent effective, a serious impediment to large-scale commercial application (FAO, 2011d). Additionally, in many species, triploid induction results in lower early survival rates, reduced performance under adverse environmental conditions and increased deformities (Maxime, 2008; Piferrer *et al*., 2009).

Gynogenesis and androgenesis involve uniparental reproduction, i.e. contribution of chromosomes is from one parent only. The sperm (in gynogenesis) or egg (in androgenesis) is irradiated to destroy the chromosomes, followed by fusion with an untreated egg or sperm, respectively, to form a haploid embryo and then diploidy is restored by inhibiting the first mitotic division using temperature or pressure shock (Dunham, 2004). Androgenetic and gynogenetic doubled haploids (DHs) are thus 100 percent homozygous and have been successfully induced in many fish species, with reported yields of hatched DHs ranging between 1 and 20 percent, although survival rates are often low and fertility is reduced in females (Pandian and Kirankumar, 2003; Komen and Thorgaard, 2007; Zhang *et al*., 2011a). Gynogenesis can also be achieved by the suppression of meiotic divisions (meiosis I in fish and either meiosis I or II in molluscs), but in this case there are regions of heterozygosity due to recombination.

Gynogens and androgens can be used to study the sex determination mechanism in fish. For example, in species with the XX–XY system, all gynogens are expected to be XX and therefore female while androgens should segregate as XX and YY. However, in some cases, deviations from expected progeny sex ratios have been observed due to autosomal and/or environmental factors (Devlin and Nagahama, 2002; Ezaz *et al*., 2004). Gynogenesis and androgenesis can also be employed to create monosex populations but due to high mortality of the manipulated eggs, this has not been feasible on a commercial scale. Combined with sex-reversal technology (for instance crossing XX gynogenetic progeny with sex reversed XX males), large-scale quantities of monosex fish can be produced but this is not yet routine in commercial hatcheries (Beaumont *et al*., 2010).

A second cycle of gynogenesis or androgenesis can give rise to isogenic clonal lines, which are useful for genetic mapping and QTL analysis. Clonal lines have been successfully established in several commercially important species such as rainbow trout, common carp, amago salmon and Nile tilapia (Komen and Thorgaard, 2007). Additionally, androgenesis can be applied to recover extinct or endangered species from stored populations of cryopreserved sperm (Babiak *et al*., 2002; Yasui *et al*., 2010). Gynogenesis has also been suggested as a tool for accelerating the elimination of recessive

deleterious genes and to produce inbred fish with desired traits such as higher growth rates and disease resistance (FAO, 2011d).

6.7 Biotechnologies for disease diagnosis and prevention

Fish are often reared in open systems where they are exposed to a wide variety of environmental pathogens. Additionally, the horizontal spread of pathogens is facilitated through water and when large numbers of fish are reared within ponds/cages/pens, the increased stress levels make them more susceptible to infection (Adams and Thompson, 2011). The potential effect of diseases spreading from cultured to wild fish and vice versa is another major concern (Johansen *et al*., 2011b). As aquaculture production intensifies, the likelihood of diseases occurring also increases, which may pose a serious threat to the sustainability and viability of aquaculture as well as economically important wild fish populations.

Akin to the TADs in the livestock sector, transboundary aquatic animal pathogens/diseases (TAAPs/TAADs) are highly contagious and severely impact aquaculture production and trade. Socio-economic impact studies, carried out in a few countries in Asia and Latin America, have shown that, in addition to a decline in production and sales, TAADs result in export losses, unemployment and lost consumer confidence (Bondad-Reantaso *et al*., 2005). Surveys conducted in 16 Asian countries further demonstrate that annual losses due to disease in the region amount to more than USD 3 billion.[164]

The OIE International Aquatic Animal Health Code currently lists 24 diseases (nine for finfish, seven for molluscs and eight for crustaceans) that require immediate notification in the case of the first occurrence or re-occurrence of a disease in a country or zone of a country that was previously considered to be free of that particular disease, if the disease has occurred in a new host species, if the disease has occurred with a new pathogen strain or in a new disease manifestation, or if the disease has a newly recognized zoonotic potential (OIE, 2010c). In addition to the OIE listed diseases, the NACA/FAO Quarterly Aquatic Animal Disease Report lists four finfish, three mollusc and three crustacean diseases as prevalent or exotic in the Asia-Pacific region (NACA/FAO, 2011).

Diagnostics

Traditional methods for disease diagnosis tend to be slow and labour intensive. Biotechnological (immunological and molecular) methods, on the other hand, provide powerful tools for rapid and accurate detection and identification of pathogens, but can be costly. Often, a combination of traditional and biotechnological methods is needed for definitive disease diagnosis.

Immunological techniques routinely used for the detection of bacterial and viral fish pathogens include direct and indirect fluorescent antibody tests and ELISA, and currently many commercial antibodies and kits are available (Adams and Thompson, 2008). ELISA is utilized to screen and/or confirm the diagnosis of numerous important pathogens causing diseases such as epizootic haematopoietic necrosis and infectious haematopoietic necrosis (IHN) in finfish (OIE, 2010d). However, the low sensitivity of antibody-based methods limits their use in environmental samples.

Another limitation is that molluscs and crustaceans do not produce antibodies in response to infection (FAO, 2011d). Nevertheless, in crustaceans it has been possible to apply ELISA for diagnostics using mouse or rabbit antibodies generated against pathogens purified from infected hosts. For example, diagnostic protocols are available for white spot syndrome virus (WSSV) and *Macrobrachium rosenbergii* nodavirus (*Mr*NV) (OIE, 2010d).

PCR-based diagnostic techniques have been successfully used for the detection of bacteria, viruses, fungi, and parasites in finfish and shellfish (Cunningham, 2002; Poulos *et al*., 2006; FAO, 2011d). PCR and RT-

[164] http://www.oie.int/for-the-media/editorials/detail/article/the-role-of-the-oie-in-aquatic-animal-diseases/

PCR kits are available and commonly used in the crustacean aquaculture industry for pathogens such as WSSV, Taura syndrome virus (TSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) (OIE, 2010d). PCR methodologies have also been extensively used to detect viruses of shrimp broodstock, larvae and postlarvae in developing countries (Peinado-Guevara and López-Meyer, 2006; FAO, 2011d). For example, in India the use of PCR-screened postlarvae, together with improved farm management practices, was incorporated into a project for addressing disease and environmental problems in the shrimp industry. Adoption of better management practices led to improved profits, reduced disease incidence and decreased chemical and antibiotic use (Umesh *et al*., 2010).

In finfish, PCR protocols for confirmatory testing and diagnosis have been developed for viruses such as epizootic haematopoietic necrosis virus (EHNV), infectious haematopoietic necrosis virus (IHNV) and koi herpesvirus (KHV); bacteria such as *Vibrio* spp., *Aeromonas* spp. or *Streptococcus* spp; as well as for parasites such as the ectoparasite *Gyrodactylus salaris* and the oomycete *Aphanomyces invadans* (OIE, 2010d). PCR has also been applied to isolate and identify molluscan disease agents such as *Bonamia ostreae*, *B. exitiosa*, *Marteilia refringens*, *Perkinsus marinus* and *P. olseni*, while real-time PCR is employed for abalone herpes-like virus (AbHV) (OIE, 2010d). Emerging technologies that show promise for diagnostics in fish species include LAMP PCR, which is faster and more sensitive than conventional PCR (Savan *et al*., 2005; Adams and Thompson, 2011) and DNA microarrays that allow multiplexing for different pathogens (Altinok and Kurt, 2003; Kostic *et al*., 2008).

The OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2010d) recommends molecular methods for either direct detection of the pathogen in diseased fish or for the confirmatory identification of the pathogen isolated using the traditional method, but not as screening methods for health certification in international trade. FAO and NACA have also developed regional technical guidelines, including for disease diagnosis, for minimizing the risks of disease due to transboundary movement of live aquatic animals in Asia (FAO/NACA, 2000). These guidelines take into account the technical and financial constraints faced by developing countries and accordingly recommend a three-level process based on the existing national diagnostic capacity (FAO, 2011d).

Vaccines

Since crustaceans and molluscs do not possess an adaptive or specific immune system, vaccines have been used as prophylatics mainly in finfish and have had a significant impact in reducing disease risk. Vaccination has been applied in the commercial aquaculture of many species, including Atlantic salmon, rainbow trout, sea bass, sea bream, tilapia, turbot, channel catfish and yellowtail (Håstein *et al*., 2005). The preponderance of vaccines employed to date have been the traditional inactivated bacterial vaccines. A few inactivated virus vaccines also exist but no vaccines against fish parasites are commercially available. Despite the absence of a true adaptive immune system, vaccination can be used as an intervention strategy in crustaceans (Pereira *et al*., 2009) and recombinant subunit vaccines to control WSSV in shrimp are being developed (Witteveldt *et al*., 2004, 2005; Vaseeharan *et al*., 2006).

So far, only three attenuated live vaccines have been introduced into the market. Of these, two are live bacterial vaccines, *Edwardsiella ictaluri* that has been licensed for use in salmonids in Chile and North America (Sommerset *et al*., 2005) and *Flexibacter columnarae* for channel catfish in the United States of America, while the third is a KHV vaccine in Israel (FAO, 2011d). However, environmental and regulatory concerns have been expressed regarding possible reversion to virulence of the attenuated vaccines and the possibility that they might not be non-pathogenic to other relevant species in the wild.

The only DNA vaccine licensed for use in fish is against IHNV in Canada (Salonius *et al*., 2007). Various DNA vaccines have been tested in experimental challenge trials, with the ones against salmonid rhabdoviruses showing the highest efficacy (Kurath, 2008). DNA vaccines have also been tested for bacterial fish pathogens while vaccines against eukaryotic parasites are still at an early stage of development. A recombinant subunit vaccine against infectious pancreatic necrosis virus (IPNV) is

commercially available (Adams *et al*., 2008) and recently formulations that elicit a strong protective effect against piscirickettsiosis have been developed (Kuzyk *et al*., 2001; Wilhelm *et al*., 2006).

Besides contributing to the enhanced health and welfare of cultured fish, in combination with best management practices, vaccination has additional potential benefits such as the ability to grow vaccinated fish at higher densities for improved efficiency, increased appetite and growth in vaccinated fish and reduced environmental impact due to limited use of antibiotics (Toranzo *et al*., 2009). For example in Norway, the annual use of antimicrobial agents in farmed salmon declined by 98 percent over a period of 17 years, due to the introduction of effective vaccines together with improved health management (FAO/OIE/WHO, 2006). In 2007, the average use of antibiotics in the Chilean salmon industry was 1 400 times greater than in Norway, with 1.17 kg therapeutant/metric tonne produced compared to 0.0008 kg therapeutant/metric tonne in Norway (Burridge *et al*., 2010).

6.8 Molecular marker-assisted selection

The majority of the genetic improvements in aquaculture species to date have been through conventional breeding methods such as selection, cross-breeding and hybridization (Hulata, 2001). For traits that are difficult and/or expensive to measure and that are sex-limited, expressed after a certain age or require killing the fish, for example reproductive traits and flesh quality, MAS can substantially increase the accuracy of selection in breeding programmes. However, it cannot further reduce the generation interval in fish to less than the age at which sexual maturity occurs.

MAS is only beginning to be applied in aquatic species. Documented cases include QTLs for resistance to infectious pancreatic necrosis in Atlantic salmon (Houston *et al*., 2008; Moen *et al*., 2009), for resistance to lymphocystis disease in Japanese flounder (Fuji *et al*., 2007), and for resistance to pasteurellosis in gilthead sea bream (Massault *et al*., 2011) and European seabass[165], that have been incorporated into breeding programmes. Nevertheless, before applying this technology on a larger scale and to increase the likelihood of successful MAS, it is necessary to develop high-density linkage maps to speed up the discovery rate of QTLs, especially in species that have less-developed genomic resources.

For species where dense marker maps and high-throughput genotyping have become increasingly available, genomic selection is an attractive option. Simulation studies, carried out to evaluate the potential of GS in aquaculture, have shown that it can yield high genetic gain, accuracy of selection and very low rates of inbreeding (Sonesson and Meuwissen, 2009). However, it generates extra costs since results have indicated that continuous phenotypic and genotypic testing is fundamental to maintain the accuracy of the genome-wide breeding values over generations.

6.9 Transgenesis

Several transgenic aquatic species have been developed (Dunham, 2003) but have not yet been approved for commercial release as food (FAO, 2011d). Approval is, however, reportedly expected in the United States of America for GM Atlantic salmon that grows twice as fast as its wild counterparts.[166]

VII. CURRENT STATUS OF BIOTECHNOLOGIES FOR THE MANAGEMENT OF MICROBIAL GENETIC RESOURCES

Micro-organisms are highly diverse and range in effect from harmful to beneficial. Some are responsible for plant and animal diseases, while others play an important role in the improvement of agriculture and food production systems and are multi-functional. However, very few micro-organisms associated with

[165] http://www.aquagenomics.es/

[166] http://www.nature.com/news/2010/100914/full/467259a.html

food production ecosystems are domesticated. Micro-organisms are also subject to relatively rapid evolution as they adapt and change in response to the environment quickly, have short generation times and large populations. The World Data Center for Micro-organisms (WDCM) has records of 591 microbial culture collections held in 68 countries, including many developing countries.[167] Yet, this is just a fraction of the world's microbial diversity and the majority of microbes as well as their ecophysiological roles remain unknown. As detailed below, biotechnology applications have added much to the study of microbial diversity as well as facilitated their utilization in the food industry, plant growth promotion, livestock and fish health, and degradation of pollutants.

7.1 Molecular markers

The use of molecular markers is especially important in microbes since an estimated 99 percent of all micro-organisms cannot be isolated and thus cannot be characterized based on physiological or biochemical features (Muyzer, 1999). Markers based on the 16S ribosomal RNA (rRNA) gene are most commonly utilized to explore microbial diversity. However, the 16S rRNA gene may occur in multiple copies per genome; therefore, markers based on single-copy genes such as the one encoding the RNA polymerase β subunit, *rpoB*, provide better resolution, especially at the subspecies level (Case *et al*., 2007).

Following amplification by PCR, polymorphisms can be detected by RFLP (PCR-RFLP) or by other methods such as Terminal-RFLP (T-RFLP), Temperature gradient gel electrophoresis (TGGE) or Denaturing gradient gel electrophoresis (DGGE) (Van Elsas and Boersma, 2011). Alternatively, the PCR product can be cloned and sequenced to inventory microbial diversity and gain insights into the phylogeny of species (Pereira *et al*., 2006; Le Calvez *et al*., 2009; Porwal *et al*., 2009).

PCR-RFLP has been utilized to characterize root-nodule bacteria associated with leguminous plants (Hoque *et al*., 2011) as well as bacteria from the gastrointestinal system of fish (Jensen *et al*., 2002). T-RFLP is based on the detection of restriction digested fluorescently end-labelled amplified PCR fragments and has been used to study the diversity of complex microbial communities, for example to evaluate the diversity of bacterial and fungal communities from manure composts at different stages of composting (Tiquia, 2005), to monitor changes in estuarine bacterial communities (Wu *et al*., 2004) and to identify and discriminate between pseudomonads groups that are involved in the protection of plant roots against soil-borne pathogens (Von Felten *et al*., 2011).

DGGE and TGGE are types of electrophoresis (applying chemical and temperature gradients, respectively, to denature the sample) that separate similar-sized DNA fragments. DGGE is the most widely used technique for microbial community analyses, for example to investigate the spatial distribution of soil microbial communities in desert landscapes (Ben-David *et al*., 2011); compare the phylogenetic diversities of bacteria from aquatic plants (Crump and Koch, 2008); study the dynamics of the bacterial community structure in a wastewater treatment plant (Ding *et al*., 2011); examine the impact of chlorophenols on the soil bacterial and fungal community composition (Caliz *et al*., 2011); and explore the diversity and dynamics of bacterial populations during fermentation processes (Jung *et al*., 2011; Madoroba *et al*., 2011). TGGE has been employed to deduce the microbial community variation in response to soil type, plant type, or plant development (Wieland *et al*., 2001) as well as to analyze the bacterial community composition in stream and river water (Beier *et al*., 2008).

While DGGE and TGGE are useful for characterization at the community level, RFLPs, RAPDs and AFLPs are more informative for intraspecific diversity and strain-level identification (Maukonen *et al*., 2003; Gao and Tao, 2011). For example, RAPDs have been employed to detect genetic diversity in different isolates of the fish pathogen *Aeromonas hydrophila* that are phenotypically homogenous

[167] http://wdcm.nig.ac.jp/statistics.html#1

(Thomas *et al*., 2009) and in marine cyanobacterial strains (Kumari *et al*., 2009). RFLPs have been used to assess rumen microbial diversity (Deng *et al*., 2008), and AFLPs have been utilized to investigate the diversity of rhizobia associated with soybean (Wu *et al*., 2011) and for determining taxonomic and phylogenetic relationships between strains of the nitrogen fixing, filamentous bacteria *Frankia* (Bautista *et al*., 2011). The Joint FAO/IAEA Division has helped in building the capacities of developing countries to use techniques such as RFLP, DGGE and TGGE to evaluate rumen microbial diversity.[168]

Microsatellites have been applied to distinguish between fungal species (Lee and Moorman, 2008) as well as to characterize intraspecific diversity in oomycytes (Vargas *et al*., 2009). Subsequent to whole genome sequencing of many micro-organisms, SNPs have been discovered that are aiding in the phylogenetic characterization of bacterial and viral isolates (Gardner and Slezak, 2010) and in elucidating the population structure in fungi (Abbott *et al*., 2010; Broders *et al*., 2011).

With reference to pathogenic micro-organisms, a multinational consortium, Quarantine Barcoding of Life (QBOL)[169], is developing a DNA barcode identification database for use in plant health diagnostics. The consortium aims to generate barcode sequences for quarantine species and closely related species, to develop generic diagnostic tools based on these barcode sequences, and to develop methodologies to enable the establishment of DNA banks and access to digital specimens (Bonants *et al*., 2010).

7.2 "Omics"

Genomes of microbes are typically small in size and hence easy to sequence. Consequently, over 1 000 microbial genomes have been sequenced and several more are nearing completion (Kumar *et al*., 2011). The availability of genome sequences offers new tools to better characterize candidate starter strains for fermentation processes (Torriani *et al*., 2011; Kleerebezem and de Vos, 2011). Comparative genome hybridization analysis with multi-strain microarrays can be used to investigate intraspecific diversity and to correlate this with industrially-relevant phenotypic diversity (Borneman *et al*., 2010; Siezen *et al*., 2011). Genome mining of bacteria and fungi has unveiled the secondary metabolite product potential of these micro-organisms (Winter *et al*., 2011).

Genome sequencing of rumen microbes is being employed to identify targets for methane mitigation technologies (Buddle *et al*., 2011) as well as to understand the functioning of microbes within the rumen and their impact on ruminant health and performance (Suen *et al*., 2011). Whole genome sequencing of the host together with its symbiont/pathogen, for example *Populus trichocarpa* and its fungal symbiont *Laccaria bicolor*, is a powerful tool to gain new perspectives on the co-evolution of their genomes (Medina and Sachs, 2010).

Advances in microbial transcriptomics have shed additional light on plant-microbe interactions (Bonfante and Genre, 2010), rumen microbial processes (Dodd *et al*., 2010) and livestock host-pathogen relationships (Bannantine and Talaat, 2010). Transcriptomic analyses of food-borne bacterial pathogens in response to specific stresses (associated with food hygiene, processing and preservation measures) have been carried out to enable more effective control strategies to be devised (King *et al*., 2010; Soni *et al*., 2011; Zhang *et al*., 2011b).

A growing number of proteomic approaches are focusing on both symbiotic and pathogenic plant-microbe interactions to unravel the molecular communication between plants and microbes, including the identification of proteins involved in suppression of plant defence responses by symbiotic microbes and of virulence factors responsible for microbial pathogenicity (Mathesius, 2009; González-Fernández *et al*., 2010; Quirino *et al*., 2010). Proteomic analyses of livestock and fish pathogens have been initiated to

[168] http://www-naweb.iaea.org/nafa/aph/crp/aph-molecular-techniques.html

[169] http://www.qbol.wur.nl/UK/about-qbol/

uncover virulence mechanisms and to increase the gamut of antigens available for the formulation of diagnostics and vaccines (Pinto *et al*., 2009; Dumpala *et al*., 2010; Menegatti *et al*., 2010).

Metagenomic approaches are becoming more feasible and cost-effective and are increasingly used to characterize microbial genetic diversity. For example, they are providing insight into bacterial phylogeny (Rosen *et al*., 2009), into the ecology of soil microbes to optimize agricultural production and to identify novel biomolecules (Ghazanfar *et al*., 2010; Moccali and Benedetti, 2010), as well as into rumen microbial function to discover biomass-degrading enzymes (Ghazanfar and Azim, 2009; Hess *et al*., 2011). Nonetheless, efforts should continue to be made to culture micro-organisms, so that the sequence information is used in conjunction with isolation and cultivation to determine the ecophysiological roles of microbes (Yamada and Sekiguchi, 2009).

An integrated strategy, utilizing multiple "omic" technologies, is particularly useful to fully understand the functioning of microbial systems. Several combined transcriptomic and proteomic analyses have been undertaken, to complement each other thus avoiding the detection bias from each technology, for cross-validation purposes and to reveal novel insights into metabolic and regulatory processes (Zhang *et al*., 2010a). Similarly, integrated transcriptomics and metabolomics analysis can be a robust tool to elucidate the molecular machinery underlying physiological processes (Yang *et al*., 2009b).

7.3 Bioinformatics

Fungal genome and proteome databases include the Fungal Genome Initiative of The Broad Institute (FGI)[170], the Sanger Institute fungal sequencing effort[171], the *Saccharomyces* Genome Database (SGD)[172], the *Fusarium graminearum* Protein-Protein Interaction Database (FPPI)[173] and *Magnaporthe grisea* Protein-Protein Interaction Database (MPID)[174] (González-Fernández *et al*., 2010). Several bacterial databases exist, among them the *E. coli* specific EchoBASE[175] and the Annotation of Microbial Genome Sequences (AGeS)[176] system that incorporates tools for integrated high-throughput genome annotation and protein function prediction for completed and draft bacterial genomes (Kumar *et al*., 2011).

The G-InforBIO Database [177] (Tanaka *et al*., 2006) and the Microbial Genome Database (MBGD)[178] facilitate comparative genomic analyses of microbial sequences. The Pathogen-Host Interaction Database catalogues experimentally verified pathogenicity, virulence and effector genes from microbial pathogens that infect plant and animal hosts.[179] Bioinformatic tools and databases for metagenomics include the Genomes OnLine Database (GOLD)[180], the MEtaGenome ANalyzer (MEGAN)[181], StrainInfo[182] and UniFrac[183] (Singh *et al*., 2009; Moccali and Benedetti, 2010).

[170] http://www.broadinstitute.org/annotation/fungi/fgi/index.html

[171] http://www.sanger.ac.uk/Projects/Fungi/

[172] http://www.yeastgenome.org/

[173] http://csb.shu.edu.cn/fppi/

[174] http://bioinformatics.cau.edu.cn/cgi-bin/zzd-cgi/ppi/mpid.pl

[175] http://www.york.ac.uk/res/thomas/howtouse.htm

[176] http://www.bhsai.org/ages.html

[177] http://gbif15.ddbj.nig.ac.jp/inforbio/G-InforBIO/download.html

[178] http://mbgd.genome.ad.jp/

[179] http://www.phi-base.org/

[180] http://www.genomesonline.org/

[181] http://www.megan-db.org/megan-db/home/

[182] http://www.straininfo.net/

[183] http://bmf.colorado.edu/unifrac/

7.4 Cryopreservation

According to the World Federation for Culture Collections (WFCC) guidelines for the establishment and operation of collections of cultures of micro-organisms, in order to minimize the probability of strains being lost, each strain should be maintained by at least two different procedures, and at least one of these should be by lyophilization (freeze-drying) or cryopreservation (WFCC, 2010). Cryopreserving micro-organisms circumvents the need for repeated subculturing, which is time-consuming and prone to contamination. Additionally, it prevents the genetic and physiological changes that could be associated with the long-term culture of actively growing organisms (Day *et al*., 2008). Cryopreservation can be used to preserve microbes that cannot be cultured or those that require growth on their host. It is also the most suitable long-term method for maintaining the stability of secondary metabolite production (Ryan *et al*., 2003).

Numerous factors influence the effectiveness of cryopreservation of micro-organisms, such as species, cell type, age, growth phase, osmolarity and aeration, and composition of the freezing medium. For example, micro-organisms, especially bacteria, that are grown under aerated conditions and are harvested in late log or early stationary phase are more resilient to freezing stress (Kerrigan, 2007). Furthermore, the presence of cryoprotective additives such as sulfoxides, alcohols and derivates, saccharides and polysaccharides, and glycoproteins in the freezing medium can enhance survival rates considerably (Hubalek, 2003). Cooling rates can also vary considerably, with the majority of fungi being successfully preserved at a cooling rate of -1 ^{o}C min^{-1}, while some require a rate of -10 ^{o}C min^{-1} (Smith and Ryan, 2008).

Many micro-organisms have been successfully cryopreserved , including over 4 000 species of fungi, belonging to over 700 genera (Smith *et al*., 2008). Cryopreserved micro-organisms usually exhibit high survival rates (often reaching levels of 100 percent) and good strain stability.[184] For instance, viability of lyophilized yeast is typically between 1 and 30 percent as opposed to greater than 30 percent for cryopreserved yeast (Smith *et al*., 2008). However, selected bacterial species are sensitive to cryopreservation, especially in terms of viability and stability of antigenic, molecular and biochemical properties, and improved protocols need to be developed (Paoli, 2005).

7.5 Pathogen detection in food

Food-borne diseases are a major concern for public health and cause high levels of morbidity and mortality[185], with *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 generally being responsible for the majority of food-borne outbreaks (Velusamy *et al*., 2010). Therefore, the rapid and reliable detection of microbial pathogens in food is essential for ensuring food safety and quality. Microbial analysis also plays a valuable role in assessing the performance of management strategies based on the Hazard Analysis and Critical Control Point (HACCP) (FAO, 1998, 2006).

Conventional methods, such as culture and colony-counting methods, are sensitive and inexpensive but often time-consuming and labour-intensive. To overcome these limitations, a diverse array of biotechnological methods has been developed and is available for the improved detection of food-borne pathogens (Jasson *et al*., 2010; Mandal *et al*., 2011). ELISA is the most prevalent antibody assay for the detection of bacterial pathogens and contaminants in food, and numerous ELISA tests are commercially available as robotized automated systems (Jasson *et al*., 2010). Commercial ELISA kits are also available for detecting mycotoxins in food and animal feed (Schmale and Munkvold, 2009).

[184] http://www.wfcc.nig.ac.jp/tis/info3.html; http://www.wfcc.nig.ac.jp/tis/info4.html

[185] http://www.who.int/foodsafety/foodborne_disease/en/

PCR-based methods are used in the identification of a wide range of pathogens such as *Listeria monocytogenes* (Zunabovic *et al*., 2011), *Staphylococcus aureus* (Riyaz-Ul-Hassan *et al*., 2008), *Salmonella* (Stark and Made, 2007) and *E.coli* O157:H7 (Perry *et al*., 2007) as well as for the simultaneous detection of pathogens (Kim *et al*., 2007; Mukhopadhyay and Mukhopadhyay, 2007; Zhang *et al*., 2009). Quantitative real-time PCR has also been applied for the accurate quantification of pathogens in food samples (Alacorn *et al*., 2006; Malorny *et al*., 2008; Rantsiou *et al*., 2008), and several PCR and real-time PCR based diagnostic kits have been commercialized (Glynn *et al*., 2006).

In recent years, the potential of biosensors in food-borne pathogen detection has been investigated (Gehring, 2011). A biosensor is an analytical device that converts a biological signal into an electrical one. It uses a bioreceptor, i.e. an immobilized biologically-related agent (such as an nucleic acid, enzyme, antibiotic, organelle or whole cell), to detect or measure a chemical compound, and a transducer to convert the recognition event into an electric signal (FAO, 2001; Velusamy *et al*., 2010). Currently, biosensors are being optimized and validated for the routine monitoring and/or identification of bacteria, including field-based pathogen detection (Setterington and Alocilja, 2011; Zordan *et al*., 2011).

7.6 Food preservation and production of food and feed ingredients

Fermentation is a cost-effective food preservation process that can also enhance the flavour, aroma and texture of food, enrich its nutritional quality and digestibility, detoxify contaminated food and decrease cooking time and fuel requirements (Liu *et al*., 2011b). In many developing countries, fermented foods serve as important dietary constituents and are produced primarily at the household and village level. As such, the majority of small-scale fermentations are based on spontaneous processes, resulting from the activities of a variety of micro-organisms associated with the raw food material and the environment. The majority of fermented foods in Africa are produced by spontaneous fermentation, for example Cingwada (fermented cassava) in East and Central Africa, Kenkey (fermented maize) in Ghana and Owoh (fermented cotton seed) in West Africa (FAO, 2011e). However, limitations include enhanced lag phase of microbial growth associated with contamination of competing micro-organisms, i.e. higher probability of spoilage, variable product quality and lower product yield (Holzafpel, 2002).

Starter cultures are preparations of live micro-organisms that are added to initiate and/or accelerate fermentation processes (FAO, 2011e). The starter culture may be obtained through the practice of back-slopping (addition of a sample from a previous successful fermentation batch) or may be a "defined starter culture" consisting of single or multiple strains normally produced by pure culture maintenance and propagation under aseptic conditions (FAO, 2011e). Examples of fermented foods produced using a back-slopping process include fermented cereals and grains in Africa and fermented fish sauces and vegetables in Asia (FAO, 2011e). Strains selected for defined starter cultures should possess several desirable metabolic traits, lack toxicogenic activity and also be suitable for large-scale production (Gänzle, 2009). Defined starter cultures allow process standardization together with lowered health risks, and often incorporate adjunct cultures to inhibit pathogenic or food-spoilage organisms and to improve product quality (Settanni and Moschetti, 2010; Mendoza *et al*., 2011).

Lactic acid bacteria are the predominant micro-organisms in a preponderance of food fermentations. They convert carbohydrates either to lactic acid alone or carbon dioxide and ethanol in addition to lactic acid, and are responsible for many products such as fermented sausage, all fermented milks, pickled vegetables and sour dough bread (Steinkraus, 2002; Flores and Toldra, 2011; Liu *et al*., 2011b). Acetic acid bacteria are important in the food industry due to their ability to oxidize sugars and alcohols into organic acids and are used in the production of vinegar and in cocoa and coffee fermentations (Sengun and Karabiyikli, 2011). A third group of bacteria, belonging to the genus *Bacillus*, hydrolyze proteins to amino acids and peptides and release ammonia. Such alkaline fermentations of plant seeds as well as legumes provide protein-rich condiments especially in Africa and Asia (Parkouda *et al*., 2009). Yeast fermentations, generally involving *Saccharomyces* species, result in the formation of ethanol and carbon dioxide from

sugar, and are widely used for the production of leavened bread and fermented beverages such as wines and beers (Sicard and Legras, 2011).

Fermentation that leads to the nutritional fortification of traditional foods can have a profound impact on the diets of people in developing countries that depend largely on one staple, such as cassava, maize or rice, for subsistence. For example, fermentation of rice to produce tape ketan in Indonesia results in a doubling of protein content and enrichment with lysine, an essential amino acid. Similarly, pulque that is produced by the fermentation of agave juice in Mexico is rich in vitamins like thiamine, riboflavin, niacin, biotin and pantothenic acid (Steinkraus, 2002).

Essential amino acids, produced by microbial fermentation, are also utilized to supplement grain-based livestock feeds, both to increase productivity and to decrease the excretion of nitrogen from the animals into the environment (FAO, 2011c). Currently, the annual global use of L-lysine, the first limiting amino acid for pigs and the second limiting amino acid after methionine for poultry, is estimated to be 900 000 tons followed by 65 000 tons for L-threonine and 1 900 tons for L-tryptophan (Kim, 2010). Feed grade L-valine is marketed in the EU, while L-glutamine, also produced through fermentation processes, is available in South America and selected Asian countries (Kim, 2010). Additionally, exogenous microbial enzymes are increasingly being incorporated in animal feeds. Supplemental phytase, the most extensively used feed enzyme, improves utilization of phosphorus as well as other minerals in pigs and poultry, and can reduce phosphorus excretion by up to 50 percent (Singh *et al*., 2011b). Phytase has recently also been approved for use in salmonid feed in the EU.[186] Other exogenous enzymes included as feed additives to ameliorate digestion are xylanases, glucanases, proteases and amylases (FAO, 2011c).

Microbial enzymes, manufactured by fermentation under controlled conditions, are commonly employed in the food processing industry. For example, α-amylases are applied for converting starch into fructose and glucose syrups (Souza and Magalhães, 2010), proteases such as chymosin are used in cheese-making, pectinases are utilized for extraction, clarification and concentration of fruit juices, and tannases are used for the production of instantaneous tea (Aguilar *et al*., 2008). Micro-organisms are also used to generate volatile flavour chemicals that possess desirable properties such as antimicrobial and antioxidant activities in addition to sensory properties, and more than 100 aroma chemicals are available commercially (Berger, 2009). In recent years, there has been a growing interest in exploiting microbial fermentation processes for the production of bioethanol and biodiesel (Demain, 2009; Cheng and Timilsina, 2010; Ruane *et al*., 2010; Shi *et al*., 2011).

7.7 Biofertilizers

Soils are complex, interactive and dynamic living systems that contain a variety of microbes. There has been increasing worldwide concern regarding the adverse effects of the indiscriminate use of chemical fertilizers on soil productivity and environmental quality. Based on beneficial plant-microbe interactions, it has been possible to develop microbial inoculants or biofertilizers, that are cost effective and environment friendly, for use in agriculture to improve plant performance. In addition to stimulating plant growth and reducing soil pollution, biofertilizers can improve soil structure and fertility, and provide protection against abiotic stresses as well as a broad range of diseases (Yang *et al*., 2009c; Pineda *et al*., 2010).

Nitrogen fixing rhizobial inoculants are the most widely used biofertilizers (Mia and Shamsuddin, 2010) and are applied to leguminous crops as well as other commercially important crops such as rice, maize, sugarcane and wheat, leading to increased grain yield and/or biomass (Bhattacharjee *et al*., 2008). Often,

[186] http://www.fishnewseu.com/latest-news/world/5553-phytase-product-receives-eu-approval-as-feed-additive-for-salmonids.html

inoculating a mixture of bacterial isolates produces a synergistic result (Govindarajan *et al*. 2008), although it is important to determine the compatibility of the strains in the mixture prior to inoculation.

Numerous rhizobial inoculants have been commercialized in developing countries, for example Biofix in Kenya (Odame, 2002) and Rhizofer in Mexico (FAO, 2011a). In Argentina, Bolivia, Paraguay and Uruguay, 70 percent of the soybean crop area is estimated to be inoculated with rhizobia.[187] A study in Thailand showed that the use of rhizobial inoculants, between 1980 and 1993, in soybean, groundnut and mungbean production, led to estimated accumulated benefits of approximately USD 100, USD 17 and USD 4 million, respectively (Boonkerd, 2002). However, another study in Thailand highlighted the varying performance of inoculants in different locations, highlighting the role of farmers' knowledge and experience in the effective application of biofertilizers (Sonnino *et al*., 2009).

A variety of free-living cyanobacteria are found naturally in rice fields, with *Anabaena* and *Nostoc* being the most common nitrogen-fixing organisms. Cyanobacterial application in rice fields can reduce the use of urea fertilizer by 25 to 35 percent (Hashem, 2001) and has resulted in enhanced grain yield in China, Egypt, India, Japan, the Philippines and other rice-growing tropical countries (Vaishampayan *et al*., 2001). Soil-based cyanobacterial biofertilizers can be produced with ease, with the cost of inoculum preparation being one- third that of chemical fertilizers (Sharma *et al*., 2011). However, inoculation of rice fields with selected strains is not a proven technology. Due to problems in the establishment of inoculated strains and their inconsistent performance in increasing yields, there has been localized use of cyanobacterial biofertilizers as inoculants (Prasanna *et al*., 2011).

A few cyanobacterial species also form symbiotic associations, for example *Anabaena azollae* with the water fern *Azolla*. The *Azolla-Anabaena* association has been used as a biofertilizer in Brazil, China, India, Indonesia, Italy, Mexico, the Philippines, Taiwan, Thailand, Viet Nam and several Western African countries (Carrapiço *et al*., 2000; Vaishampayan *et al*., 2001) but in recent years the area devoted to *Azolla* has decreased due to the technology being labour intensive, the availability of cheap sources of urea and potash as well as changing agricultural practices and policies (Roger, 2004). Additional constraints limiting its use include difficulties in maintaining inocula throughout the year, phosphorus limitations in soils, availability and control of water supplies, low tolerance to high temperature and damage by pests (Choudhury and Kennedy, 2004). Regardless, *Azolla* has potential as a multipurpose crop since, in addition to being a nitrogen source for rice crops, it can be employed for reclaiming saline soils, to control weed infestations, to purify wastewater and as an animal feed (Choudhury and Kennedy, 2004; Roger, 2004). For example, a technology called "Azobiofer" has been developed for the production and utilization of *Azolla* for irrigated rice and fish cultivation, which could be profitable for tropical and sub-tropical rice growing countries (Mian, 2002).

Phosphate solubilizing (P-solubilizing) micro-organisms play a key role in the availability of phosphate to plants, and include bacteria such as *Bacillus* and *Pseudomonas* and fungi such as *Aspergillus* and *Penicillium*. Bacteria are more effective in phosphorus solubilization since P-solubilizing bacteria (PSB) constitute 1 to 50 percent of the natural bacterial population in soil, while P-solubilizing fungi (PSF) are only 0.1 to 0.5 percent of the total fungal population (Khan *et al*., 2009a). Commercial PSB biofertilizers include BioP-Plus (*Bacillus coagulans*)[188] and Sukrish-p[189] while JumpStart (*Penicillium bilaiae*) and PR-70 RELEASE (*P. radicum*) are examples of commercially released PSF biofertilizers (Khan *et al*., 2010).

Often, a synergistic effect on plant growth has been observed by co-inoculation of P-solubilizing bacteria and fungi. For example, a microbial preparation termed Indian Agricultural Research Institute (IARI) microphos culture was developed in India that contained two efficient PSB (*Pseudomonas striata* and

[187] http://info.ipni.net/biofertilizers

[188] http://www.soo.co.in/biopplus.htm

[189] http://www.indiamart.com/totalagricare/organic-bio-fertilizer.html

Bacillus polymyxa) and three PSF (*Aspergillus awamori, A. niger* and *Penicillium digitatum*) (Khan *et al*., 2010). Co-inoculation of P-solubilizing micro-organisms with nitrogen fixers such as *Azospirillum* and *Azotobacter* (Yadav *et al*., 2011b) as well as with arbuscular mycorrhizal fungi (Khan *et al*., 2009b) has also been shown to have a positive effect on the growth and nutrient uptake of plants.

Mycorrhiza are fungi that form a symbiotic association with plant roots, and increase plant uptake of phosphorus and zinc in exchange for carbohydrates. The mycorrhizas that are ecologically and economically relevant for agriculture include ectomycorrhizas (that do not penetrate the cells of the plant) and endomycorrhizas such as arbuscular mycorrhizal fungi (AMF) that penetrate the cells forming clusters/arbuscules and vesicles (Habte, 2000). AMF are the most abundant and widely distributed type and are found in about 95 percent of land plant species. Although AMF have a very broad host range, species can vary in abundance in response to different hosts and environmental conditions (Van Diepen *et al*., 2011).

In addition to improved access to nutrients and water, AMF enhance plant productivity through pest and disease suppression, increased drought tolerance and alleviating the detrimental effect of salinity and alkalinity (Cardarelli *et al*., 2010). More than 30 companies worldwide produce commercial mycorrhizal fungal inoculum (Schwartz *et al*., 2006). The application of mycorrhizal fungi has been especially important in forestry where it has contributed to improved tree growth (Ouahmane *et al*., 2007), reafforestation of degraded soils (Duponnois *et al*., 2008) as well as to minimizing the negative influence of exotic tree species on soil microbial communities (Kisa *et al*., 2007). However, there are also some potential concerns regarding their application such as the detrimental effects on host plants and negative impact on the diversity of local fungal and plant communities (Schwartz *et al*., 2006).

Production of organic fertilizer through composting can also be hastened with the addition of cellulose decomposing fungi. For example, in the Philippines, inoculation of substrates such as rice straw with *Trichoderma* reduced composting time to 21-45 days, depending on the type of plant residues used. Moreover, rice and sugarcane farmers that adopted this technology used less chemical fertilizers and had higher yields and net incomes (Sonnino *et al*., 2009).

7.8 Biopesticides

Biopesticides are biological alternatives to chemical pesticides, and as such are environmentally sustainable pest management tools. There has been a rising demand for biopesticides in agriculture due to increased application of integrated pest management (IPM), lower risk to human health and enhanced safety, and minimal negative impact on the environment. Global percentage of biopesticides has thus steadily grown and currently Canada, Mexico and the United States of America dominate the market, employing 44 percent of the products sold worldwide, followed by the EU and Oceania with 20 percent each, Latin America at 10 percent and Asia with six percent (Bailey *et al*., 2010). In contrast, the global market for synthetic pesticides has declined, with a total negative growth of 7.4 percent from 1990 to 2005 (CropLife International, 2006).

Microbial biopesticides offer many advantages, such as high levels of specificity combined with lower developmental and registration costs than those for conventional pesticides (Chandler *et al*., 2008). They are thus particularly attractive and affordable options for developing countries. As of 2010, the number of microbial biopesticides registered/available worldwide were 327 in China, 72 in the United States of America, 42 in Brazil, 40 in Canada, 39 in the EU, 20 in South Africa, 20 in Ukraine, 17 in Cuba, 15 in India, 14 in New Zealand, 13 in the Russian Federation, 11 in Australia, 11 in Kenya, 11 in the Republic of Korea, 9 in Argentina and 8 in the Republic of Moldova (Kabaluk *et al*., 2010).

Microbial biopesticides have had considerable success in controlling plant pests. In China, the fungus *Beauveria bassiana* has been deployed against the Masson's pine caterpillar, a serious forest defoliator. Over a period of 36 years, various applications of this fungus were able to successfully control the forest

pest and dramatically reduce the use of chemical pesticides (Li, 2007). In Brazil, during the growing season of 2007/2008, the fungus *Metarhizium anisopliae* was applied to 250 000 hectares in São Paulo alone for control of the

sugarcane root spittlebug (Kabaluk *et al*., 2010), while in 2004/2005, the *Anticarsia gemmatalis* nucleopolyhedrosis virus was used on 2 million hectares to control the velvet bean caterpillar, a key soybean pest (Sosa-Gómez *et al*., 2008). In India, *Hyblaea puera* nucleoploy hedrovirus (HpNPV) is being commercialized as a biocontrol agent of the teak defoliator *Hyblaea puera*, an important pest of teak plantations causing heavy losses in volume increment (Kumar and Biji, 2009; Wahab, 2009). The proprietary agent Green Muscle®, composed of the spores of the fungus *M. anisopliae* and a mixture of mineral oils, has been instrumental in containing red locust infestations in Africa. An FAO intervention for locust control with Green Muscle® in 2009 prevented an invasion that could have potentially damaged food crops of about 15 million people in Eastern and Southern Africa.[190]

7.9 Bioremediation

A huge amount of pollutants such as heavy metals, mineral oil, polycyclic aromatic hydrocarbons, aromatic hydrocarbons and phenols are expelled into the environment every year. In Europe alone, it is estimated that potentially polluting activities have occurred at approximately three million sites, of which only about 80 000 sites have been remediated in the last 30 years (Guimarães *et al*., 2010). The global intensification of aquaculture has also led to escalating amounts of effluents being discharged that contain feed residues, faeces and antibiotics. The resulting organic enrichment causes environmental deterioration, negatively impacts the ecosystem and may lead to an increased presence of pathogenic bacteria as well as contributing to the spread of viruses (Chávez-Crooker and Obreque-Contreras, 2010).

Treatment of contaminated soil and water can be carried out with traditional physical, chemical and thermal processes, but these are often expensive. Bioremediation is a more efficient, cost-effective and environment-friendly technology for the clean-up and restoration of contaminated sites. Many species of bacteria (e.g. *Bacillus*, *Haemophilus*, *Mycobaterium*, *Pseudomonas* and *Rhodococcus*) and fungi (e.g. *Aspergillus*, *Cladosporium*, *Penicillium*, *Stropharia* and *Trichoderma*) can degrade toxic pollutants, with bacteria being the most active agents of bioremediation (Fernández-Luqueño *et al*., 2011; Tyagi *et al*., 2011).

Bioremediation strategies involve either bioaugmentation, i.e. inoculation of the contaminated site with enriched micro-organisms that degrade the target contaminants, or biostimulation, i.e. the addition of nutrients to favour degradation by a given native microbial population. Bioaugmentation usually works best when micro-organisms are preselected from a contaminated site and a multi-strain consortium is employed (Alisi *et al*., 2009; Li *et al*., 2009b). However, it has been observed that the number of introduced micro-organisms decrease shortly after addition to a site due to abiotic and biotic stresses, competition for limited resources from indigenous microbes as well as antagonistic interactions (Jacques *et al*., 2009).

Adding one or more rate-limiting nutrients, by adding organic matter such as manure, compost, wastewater sludge, etc. accelerates the decontamination rate in biostimulation processes (Fernández-Luqueño *et al*., 2011). A disadvantage of biostimulation compared to bioaugmentation is that its effect is delayed due to the lag between nutrient application and propagation of the microbial population. The decision to implement either or both of these strategies depends upon the circumstances prevailing at the contaminated site and a complementary approach is emerging as promising. Micro-organisms can also be utilized to increase the bioavailability of hydrocarbons for subsequent degradation through the synthesis of surface-active compounds or biosurfactants (Das *et al*., 2008; Banat *et al*., 2010).

[190] http://www.fao.org/news/story/ percent20en/item/21084/icode/en/

A number of commercial bioaugmentation and biostimulation products are available on the market, for example DBC-plus™, Biosolve and S-200, although more evaluation is necessary prior to their implementation in diverse enviornments (Tyagi *et al*., 2011). On-site bioremediation studies have been carried out in a few countries including Canada (Sanscartier *et al*., 2009), China (Liu *et al*., 2010), Japan (Tsutsumi *et al*., 2000) and Spain (Jimenez *et al*., 2006). Bioremediation in aquaculture has also been reported, mainly using bacteria in shrimp hatcheries (Kumar *et al*., 2009; Manju *et al*., 2009).

7.10 Probiotics

Worldwide concern about increased antibiotic administration in livestock and fish and the resulting emergence of antibiotic resistance has led to searches for alternative and effective approaches, such as the use of probiotics. Probiotics have also been reported to play an important role in immunological, digestive and respiratory functions in humans. Once ingested, probiotics produce beneficial physiological effects, although the mechanisms by which these effects are exerted are still poorly understood. Proposed modes of action include competitive exclusion of pathogenic bacteria by competing for nutrients, space and iron; production of antagonistic compounds that inhibit the growth of harmful bacteria; modulation of the host's immune system; and enhanced feed conversion efficiency by providing dietary compounds and enzymes (Farzanfar, 2006; Vine *et al*., 2006; Nayak, 2010b; Soccol *et al*., 2010). A successful probiotic should itself be non-pathogenic and non-toxic, benefit the host, have the capacity to be adherent and colonize the gastrointestinal tract, replicate to high numbers and be stable for long periods under storage as well as in field conditions (Farzanfar, 2006; Soccol *et al*., 2010).

In humans, several studies on potential health benefits of probiotics have been performed. Studies have been carried out on prevention of relapses of inflammatory bowel diseases and ulcerative colitis (Wohlgemuth *et al*., 2010), prevention of antibiotic associated diarrhoea (Fitton and Thomas, 2009), reduction of the duration of acute diarrhoea in children (Henker *et al*., 2008), reduction of the duration of common cold and flu infections, and lowering of the risk of developing eczema if taken by pregnant women and their infants in early life (Weichselbaum, 2009). Many different food products have been formulated with probiotics and are available commercially, such as fermented milks with high or low viscosity in the EU (Soccol *et al*., 2010), yogurt-covered raisins, nutrient bars, chocolate bars and tablets in the United States of America, ice cream in Latin America and yogurt in the Republic of Korea (Burgain *et al*., 2011). With regards to the assessment of the efficacy and safety of probiotics, guidelines have been developed jointly by FAO and WHO that provide a methodology for use in the evaluation of probiotics, and define the criteria and specific levels of scientific evidence needed to make health claims for probiotic foods (FAO/WHO, 2006).

The use of probiotiocs in aquaculture is relatively new, but many studies have been undertaken regarding their application in finfish (Balcazar *et al*., 2006), crustaceans (Farzanfar, 2006), molluscs (Prado *et al*., 2010) as well as larvae (Vine *et al*., 2006). Probiotics are dispensed through feed and/or as a water additive, with supplementation through feed being a better method for successful colonization and establishment in the gut (Nayak, 2010b). Numerous health benefits have been attributed to probiotics in fish, for example improved growth (Lara-Flores *et al*., 2003; Boonthai *et al*., 2011), stimulation of the immune system (Picchietti *et al*., 2009; Zhou *et al*., 2010b), enhanced survival rate (Hjelm *et al*., 2004; Faramarzi *et al*, 2011), facilitation of feed utilization and digestion (Manju *et al*., 2011; Sun *et al*., 2011) as well as control of pathogenic micro-organisms and disease resistance (Vendrell *et al*., 2008; Balcazar *et al*., 2009).

The application of probiotics has been well documented in livestock. In poultry, administration of probiotics has been shown to reduce chick colonization and invasion by *Salmonella* (Higgins *et al*., 2008; Vila *et al*., 2009), decrease mortality (Timmerman *et al*., 2006), increase body weight (Mountzouris *et al*., 2007), and improve egg production and quality (Panda *et al*., 2008). In pigs, probiotic inclusion in feeds has been demonstrated to decrease the pathogen load (Taras *et al*., 2006; Collado *et al*., 2007), ameliorate

gastrointestinal disease symptoms (Zhang *et al*., 2010b) and improve weight gain (Konstantinov *et al*., 2008). In ruminants, supplementation with probiotics has been reported to result in enhanced milk yield (Desnoyers *et al*., 2009), improved growth performance (Frizzo *et al*., 2010), increased dry matter intake (Jouany, 2006), reduced incidence of diarrhoea (Von Buenau *et al*., 2005), increased fibre digestibility (Guedes *et al*., 2008) and lower faecal shedding of the zoonotic pathogen *Escherichia coli* O157:H7 (Schamberger *et al*., 2004).

In spite of their ability to elicit several beneficial effects, the performance of probiotics is often inconsistent and contradictory and depends on several factors such as the choice of microbial strain, diet, dosage level, duration of use, environment and husbandry conditions (Gaggia *et al*., 2010; FAO, 2011c). The situation is further exacerbated by the fact that commercial products for animals often do not meet expected standards since the composition and viability of the strains may differ from those listed on the label (Wannaprasat *et al*., 2009).

Nevertheless, the legislative frameworks in Canada, the EU and the United States of America for the safety evaluation of probiotics have made significant progress (Gaggia *et al*., 2010). In the EU, subsequent to the ban on antimicrobials as growth promoters in 2006, the use of probiotics further expanded to maintain animal productivity (Vila *et al*., 2010). Micro-organisms utilized in animal feed in the EU are mainly bacterial strains of *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and yeast strains belonging to the *Saccharomyces cerevisiae* species and kluyveromyces (Anadón *et al*., 2006). In addition to the aforementioned strains, currently approved micro-organisms for animal feed application in the United States of America include strains of *Aspergillus*, *Bacteroides*, *Bifidobacterium* and *Propionibacterium* (Flint and Garner, 2009).

Probiotics are also prevalent in a number of developing countries. In China, 15 micro-organisms have been approved as feed additives and more than 400 companies are reported to be producing these additives (FAO, 2011c). The use of probiotics in commercial aquaculture in China has led to increased outputs and reduced costs (Qi *et al*., 2009), and in India, the total market value of probiotics in aquaculture is estimated to be USD 109 million (Panigrahi and Azad, 2007).

7.11 Mutagenesis

Mutagenesis has been extensively used for the improvement of micro-organisms of agricultural and industrial importance. Induced mutagenesis has been carried out to create starter strains with enhanced fermentation activity for enzyme (Patel and Goyal, 2010; Awan *et al*., 2011; Ray, 2011; Xu *et al*., 2011) and metabolite production (Demain and Adrio, 2008), or with resistance to viruses (FAO, 2011e). Site-specific mutagenesis allows targeted substitution, insertion or deletion of a single or a few base pairs (Blomqvist *et al*., 2010) and has been applied to optimize properties such as increased thermostability of the enzyme of interest (Singh *et al*., 2011b).

7.12 Transgenesis

Genetic modification, though common in developed countries for microbial strain improvement, is only now beginning to be applied for this purpose in developing countries. A few GM micro-organisms have been approved in the food and beverage industry, for example, GM baker's yeast and brewers' yeast in the United Kingdom as well as two GM yeast strains for wine production in North America (FAO, 2011e). Many food processing enzymes in the United States of America are derived from GM micro-organisms, for example chymosin/rennin, phytase and α-amylase (Olempska-Beer *et al*., 2006). In developing countries, GM micro-organisms are being used to produce enzymes in Argentina, Brazil, China, Cuba and India, amino acids in China and Thailand, as well as nucleic acids and polysaccharides in China (FAO, 2011e). Recombinant bovine somatotropin, to increase milk yield, is produced by transgenic *Escherichia coli* and is banned in Australia, Canada, the EU, Japan and New Zealand, but is

approved in approximately 20 countries including the United States of America and some developing countries such as Brazil, Kenya, Mexico and South Africa (FAO, 2011c).

VIII. CONCLUSIONS AND OUTLOOK

Developing countries are often endowed with a wealth of genetic resources. Agriculture is frequently an integral component of the economy and the harvest of wild populations, as in capture fisheries, forests, and bushmeat, is economically and culturally important. Yet they have not been able to harness this diversity of genetic resources to their full potential for many reasons, among them the lack of appropriate policies, limited human and institutional capacities, low R&D capacity and investment, inadequate infrastructure and low levels of financial investments. The challenge, therefore, remains to manage GRFA effectively so as to conserve and enhance genetic diversity and at the same time sustainably utilize it for increased agricultural productivity as well as to ensure food security for the future.

Agricultural biotechnologies do not represent a "silver bullet" but they are an additional weapon in the arsenal to conserve GRFA and overcome constraints to agricultural production. Developments in biotechnology have been quite substantial in the last two decades and, as reviewed here, biotechnologies have made notable contributions and hold great promise for the management of GRFA. Molecular markers can be used in a variety of ways to characterize genetic resources, to identify priority genetic resources for conservation as well as to effectively manage *ex situ* collections; *in vitro* technologies offer complementary techniques to conventional conservation methods; technologies like tissue culture provide the means to overcome reproductive barriers; diagnostics and vaccines can assist in reducing economic losses due to debilitating diseases; and advances in "omic" technologies are playing an increasingly significant role in understanding the fundamental elements of plant and animal biology. Furthermore, biotechnologies can help to address the impact of emerging issues such as climate change and new diseases, on agriculture (FAO, 2011a, b, c, d and e).

While some biotechnologies such as artificial insemination and micropropagation have been widely adopted and applied in developing countries, uptake of other biotechnologies has been slower. Usually the successful application of a given biotechnology depends on the presence of complementary factors (e.g. training and extension services), rather than on the effectiveness of the biotechnology *per se*. Additionally, non-GMO biotechnologies are often overshadowed by the debate on GMOs and there is a paucity of information/accurate assessments related to the application and potential socio-economic effects of non-GMO biotechnologies (Sonnino *et al*., 2009).

There is no one-size-fits-all solution, since there are substantial differences between sectors, species, regions and countries. Moreover, within developing countries, there is considerable variation in funding and agricultural research capacities. Hence, decisions about what biotechnologies are appropriate and their subsequent development and adoption should be made carefully, based on reliable *ex ante* (e.g. sector-specific requirements and relevance to smallholders' needs) and *ex post* (e.g. adoption rate and genetic impact assessments) analyses, as well as suitability within existing development strategies.

Likewise, an enabling environment, with sound policies in place, is necessary to facilitate the appropriate application of biotechnologies for the management of GRFA. This includes appropriate IPR management, increased investments in the public sector, facilitation of public-private sector partnerships, improved market access, enhanced access to credit, tax subsidies and levies, creation of "biotechnology parks" (to co-locate resources and capabilities), results-based management of research programmes and sharing technologies through collaboration platforms and initiatives, among others (FAO, 2011f, g and h).

The international technical conference on "Agricultural biotechnologies in developing countries: Options and opportunities in crops, forestry, livestock, fisheries and agro-industry to face the challenges of food

insecurity and climate change" (ABDC-10)[191], convened by FAO in March 2010, brought together about 300 policy-makers, scientists and representatives of intergovernmental and international non-governmental organizations, including delegations from 42 FAO Member States. In the conference's keynote address, M.S. Swaminathan succinctly stated "*The bottom line of our national agricultural biotechnology policy should be the economic well being of farm families, food security of the nation, health security of the consumer, biosecurity of agriculture and health, protection of the environment and the security of national and international trade in farm commodities*".

Indeed, at ABDC-10 the Member States reached a number of key conclusions, acknowledging, *inter alia*, that "*Agricultural biotechnologies encompass a wide-range of tools and methodologies that are being applied to an increasing extent in crops, livestock, forestry, fisheries and aquaculture, and agro-industries, to help alleviate hunger and poverty, assist in adaptation to climate change and maintain the natural resource base, in both developing and developed countries"* and agreeing, *inter alia*, that:

"a) Developing countries should significantly increase sustained investments in capacity building and development and safe use of biotechnologies; integrated with other agricultural technologies, including traditional knowledge, and maintain the natural resource base to support in particular, smallholders, producers and small biotechnology based enterprises; employing effective participatory approaches for the robust input from stakeholders in decision-making processes.

b) FAO and other relevant international organizations and donors should significantly increase their efforts to support the strengthening of national capacities in the development and appropriate use of pro-poor agricultural biotechnologies, and that they be directed to the needs of smallholders, consumers, producers and small biotechnology based enterprises in developing countries."

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