Synthesis Report: Microbial and Invertebrate Genetic Resources in the EU

Main topics and outcomes of past research in microbial genetic resources relevant to the EU

Micro-organisms of agricultural interest play a key role in many aspects of food production e.g. soil fertility, crop nutrition, bio-control and bio-fertilizers. It is also important with regard to conservation of food products (toxins and pathogens) as well as in the production of processed foods such as milk, cheese, wine, oil, etc. Therefore their presence and their biodiversity are instrumental for the maintenance of living organisms on earth. Conservation of microbial genetic resources and the safekeeping thereof plays a significant role in maintaining the microbial biodiversity for the further use in agriculture. Microbial culture collections have been around since microbiology began and their basic roles have not changed much over the years (Smith, 2012). They are considered to be a mean to preserve microbial diversity ex situ, they are custodians of microbial diversity and play a key role in the storage and supply of authentic reference material for research and development. Already in 1890 the first public service collection was at the German University of Prague (Smith et al, 2014) and shortly after, several well-known EU collections of today were established. Many of the first deposited strains remain available through the public service collections of today.

The provision of microbial resources for research is now recognised as being an essential component in the advancement of the life sciences. This is clear from the inclusion of micro-organisms in the four domains of life by the Organisation for Economic Cooperation and Development (OECD) where their importance in the development of bio-economy is clear (OECD, 2007). There are different types of collections in Europe: taxonomic collections, patented collections, diverse working collections, taxonomic collections for service, collections that focus on application areas for specific sectors such as plant pathology, food, environmental, health care etc. Smith et al, 2014 Most of these collections have been established during research projects that focused on a specific topic, such as industrial enzymes, antimicrobials or on host crops; they may be linked to a particular sector such as the environment, health care, education, or agriculture. Culture collections vary in size, form and function. They include collections of culturable organisms from algae, bacteria, fungi, yeasts, protozoa to viruses), their replicable parts such as genomes, plasmids and cDNA's, viable but not yet culturable organisms, cell and tissues, databases containing molecular, physiological and structural information relevant to these collections and allied bioinformatics. Over recent years collections have adopted new technologies to characterise and add value to their holdings for example to

preserve mutant strains of fungi and various representatives of other fungal taxa including mating type testers, vegetative compatibility testers, and strains subject to whole genome sequence analyses.

There are several levels at which co-ordination, collaboration and discussion on approaches to microbial resource collection establishment and organization is carried out in Europe. At the regional level the European Culture Collection Organization (ECCO), was established in 1982. ECCO was established to bring managers of the major public service collections in Europe together to discuss common policy, exchange technologies and seek collaborative projects. The organization opened itself to staff and users of microorganisms. A lot of work has already been done by initiatives on microbial sustainability among the ECCO collections (Alexandraki et al, 2013. One of these initiatives is the EMbaRC project. This project has taken the first solid steps towards the development of a coordinated strategy to ensure that authentic high-quality microbial resources are available in Europe to meet the demands of today's research programmes. What they have achieved in this project was to ensure the adequate coverage of microbial resources in Europe, good preservation methods to sustain the holdings and implementation of adequate identification methods for ensuring the preservation of authentic high-quality material (Stackebrandt, 2010).

The total number of microbial species is unknown. But from recent studies it is clear that only a minor part has been discovered and described so far. Identification of organisms by molecular methods was developed in bacteriology in the late 1980s and early 1990s, followed by the mycologists who adapted the methods for their specialized genetics of eukaryotic fungi. As the definition of species is one of the most debated in biology, many researchers over the years studied the taxonomic relationships among different micro-organisms. As it is almost impossible to distinguish among many related species of micro-organisms, these taxonomic studies played and still play a very important role in the microbial research and applied environment. In the past different morphological, physiological and molecular markers have been described that can be used to identify the microbial diversity of agricultural and environmental interest. Nowadays molecular markers play a key role in the study of microbial biodiversity and can be divided into markers for use in identification by identifying species; those used in characterization by describing strains within species on population levels and meta genomic markers that are used for analysis of populations of genomes. Many studies that focused on these aspects gave significant insight on the diversity, identification and genome structure or organisms belonging to the microbial kingdom. As DNA sequencing became easily accessible to most, promotion of a broad-based DNA barcoding initiative was started as a taxon-independent approach for the identification and classification of all groups of organisms. During the last 10 years, DNA sequencing developed further towards a high-throughput technology. Recently, mycology, along with bacteriology, shifted to the opportunity to apply 'meta-omics' approaches, mainly concerning metagenomics, meta-transcriptomics, and meta-metabolomics. In principal, such a transition is a highly welcome situation for the scientific community, as new approaches and methods trigger innovative science (Rambold et al, 2013).

Microorganisms play an important role in the production of bio-ethanol and bio-degrading of material. Many research projects have addressed this issue by studying the relationship and the interaction between fungal isolates (fungal physiology) and the composition of their natural substrates. Important issues that are addressed are the production of extracellular enzymes, metabolic pathways and regulators controlling the fungal response to the substrates present in the environment (bio-ethanol, bio-degradation etc.). Availability of whole genome sequences and the

use of bioinformatics tools also play an important role in these research projects (Khosravi et al, 2015; Rytioja et al, 2014).

Trends in microbial genetic resources research

Preservation of cultures

The primary objective of preserving and storing an organism is to maintain it in a viable state without morphological, physiological, or genetic change until it is required for future use. Ideally, complete viability and stability should be achieved, especially for important research and industrial isolates.
Two of the most popular methods nowadays for conservation of living cultures are freeze-drying (Lyophilisation) and cryopreservation.

- *Freeze-drying* is a highly successful method for preserving bacteria, yeasts and the spores of filamentous fungi. Freeze-drying should be optimized for different organisms and cell types. If this is done it should be successful for the majority of bacteria, sporulating fungi, and yeasts (Santivarangkna et al, 2011). Cultures generally have good viability/stability and can be stored for many years. The process of lyophilisation is relatively complex, can be time-consuming and may be expensive. The preservation of micro-organisms by different drying methodologies has been used for decades. Freeze drying in particular is the preferred method for transporting and storing vast culture collections of micro-organism strain types. The literature on drying and preserving micro-organisms is extensive, but is often specific to one particular strain (Morgan et al, 2006).
- *Cryopreservation* is the best method for maintaining the genomic integrity of microorganisms; in the future, developments and improvements in preservation methodology should allow the method to be applied for microorganisms that at the current time are difficult to maintain in BRC's (Smith and Ryan, 2012). Liquid nitrogen is the preferred cooling agent for cryopreservation, although liquid air or carbon dioxide can be used. Although little metabolic activity takes place below -70°C, recrystallization of ice can occur at temperatures above -139°C and this can cause structural damage during storage. Consequently, the storage of microorganisms at the ultra-low temperature of -190 to -196°C in or above liquid nitrogen is the preferred preservation method for most of the culture collections. Provided adequate care is taken during freezing and thawing, the culture will not undergo change, either phenotypically or genotypically. To reduce the risks of cryo-injury, traditional approaches for cryopreservation have involved controlled cooling at −1°C min-1, typically in the presence of a cryoprotectant such as glycerol, trehalose, or DMSO (Smith and Ryan, 2012; Alexandraki et al, 2013).
- *Sub-cultivation* is a method of periodical cultivation on an agar nutrient medium. It is widely used and it is perhaps the oldest, simplest and most cost effective method for microorganism maintenance and preservation under laboratory and industrial conditions, especially if cultures are required frequently and quickly.
- *Storage under mineral oils* is generally only used for yeasts and filamentous fungi but can be applied successfully to bacteria. Preservation under oil is recommended for storage of organisms in laboratories with limited resources and facilities.
- *Immersion in sterile water* can be used to extend the life of an agar culture.
- *Drying preservation* is performed at room temperature or by heating up at 36 – 40 °C. This preservation method is widely used for brewery and bakery yeasts. (Tsonka and Todor, 2005).

- *Silica gel storage* is relatively simple and involves the inoculation of a suspension of fungal propagules onto cold silica gel. Fungi have been stored for in excess of 25 years using this method. Furthermore, all strains assessed to date, have remained genetically stable.
- *Soil storage* can be applied to a range of microorganisms that can withstand a degree of desiccation for example the spores and resting stages of filamentous fungi and bacteria.

DNA banks

Preservation of whole genomes of many types of micro-organisms in order to send as an alternative to the living cultures become now more common. This is due to the fact that it becomes more difficult to send micro-organisms across the globe because of the strict regulations that exist for organisms on the Dual-use lists, organisms subject to quarantine control and those noted for their exceptional pathogenicity and possible negative impact for food safety or economy. The most common way of preserving DNA is storage at low temperatures (-20°C to -180°C [in liquid nitrogen]). A new procedure for room-temperature storage of DNA was evaluated whereby DNA samples from human tissue, bacteria, and plants were stored under an anoxic and anhydrous atmosphere in small glass vials fitted in stainless-steel, laser-sealed capsules (DNAshells). Based on this study they recommend the use of room temperature storage of precious DNA in DNA shells. This provides an alternative to conventional cold storage and makes it possible to create large-capacity (several million samples) DNA biobanks with numerous advantages for long-term stability, safety, transport, and applications for molecular biology research. Optimal storage of DNA makes possible retrospective (retesting) or prospective (downstream analysis with additional or new genetic markers) testing. Moreover, it is an opportunity for laboratories to store their samples for long periods of time in a highly sustainable environment, with reduced cost, and optimized use of space, lower energy costs, and fewer greenhouse emissions when compared to freezer storage. The protection of the dsDNA molecule for long-term storage is thus much better than that provided by the conventional procedures used for storage at low temperature (Clermont et al, 2014).

Taxonomic issues and name changes

As two or more taxonomic names can be available for a fungal strain, it gives a high instability in fungal names in all sectors, also agriculture. The following declaration effects the desired changes necessary to implement the Declaration on One fungus = One name . In order to advise mycologists as to the most appropriate names to use where the situation is unclear, or to rule on controversial choices that have been made, mechanisms and procedures will need to be developed by the ICTF in consultation with the Committee for Fungi. Major changes in fungal nomenclature will happen in the near future for most of the known taxon described to date. During this name changing process DNA sequencing and phylogenetic studies will play, and already play a very important role (Hawksworth et al, 2011).

DNA sequencing

Hebert (2003) proposed a new technique called DNA barcoding. Hebert and his associates published a paper entitled Biological identifications through DNA barcodes . They proposed a new system of species identification, that is, the discovery of species by using a short segment of DNA from a standardized region of the genome. Nowadays DNA barcoding has become a justifiable tool for the assessment of global biodiversity patterns and it can allow diagnosis of known species to

nontaxonomists. DNA barcoding is a fast, accurate, and standardized method for species level identification, by using short DNA sequences. Barcodes are globally used and it makes it relatively easy to compare sequences as barcodes for most type specimens have to be deposited in the NCBI GenBank and other more specified databases such as Q-bank for Quarantine micro-organisms. The most commonly used barcode gene of bacterial and archaeal communities is the small subunit ribosomal 16S rRNA. However, the tuf barcode performs as well or even better than the 16S rRNA gene and thus provides an easy procedure for strain identification (Lebonah et al, 2014).

The absence of a universally accepted DNA barcode for Fungi, the second largest kingdom of eukaryotic life, (Blackwell, 2011, Mora et al, 2011), is a serious limitation for multitaxon ecological and biodiversity studies. In order to try and standardize the changes in the names and discriminate between fungal species several research groups are working together to try and standardize the molecular method used to discriminate among species and develop a DNA barcode for fungi. Six DNA regions were evaluated as potential DNA barcodes for Fungi by a multinational, multi-laboratory consortium. The internal transcribed spacer (ITS) region was found to have the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. Ideally, the barcode locus would be the same for all kingdoms, but this is unfortunately not the case. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with ITS. For yeasts, the D1/D2 region of the LSU was adopted for characterizing species long before the concept of DNA barcoding was promoted. Currently, ~172,000 full-length fungal ITS sequences are deposited in GenBank, and 56% are associated with a Latin binominal, representing ~15,500 species and 2,500 genera, derived from ~11,500 scientific studies in ~500 journals. Although the ITS sequences have also limitations for identifying species in some groups it is published as the barcode of choice for the fungal kingdom. (Nilsson et al, 2008; Schoch et al, 2012). However, due to the limitations of the ITS region there is now a search for a more suitable barcode. The first steps have been made with this study. The choice of the ideal gene to sequence was based on four major criteria: its presence in all organisms to be studied, ease of PCR amplification and sequencing, it's supposed evolution rate, and the absence of pseudogenes, paralogs or orthologs that could complicate amplification and analysis. The search is on to find new genome regions of high interest that are not located in the 5.8S, 18S, 26S, 28S regions of the rDNA gene cluster or elongation factor 1-alpha. Preliminary data suggest regions of the EF1 alpha as a second barcode region after ITS (Robert et al, 2011).

Additional markers for species identification and population characterization

Several studies that focused on microbes have also used additional markers system for identification purposes, population studies and identifying strains with important traits are Restriction fragment length polymorphisms (RFLPs) based methods, a variation to the hybridization-based method is the polymerase chain reaction/RFLP (PCR/RFLP)-based method also known as Cleaved Amplified Polymorphic Sequence (CAPS), Microsatellites or simple sequence repeats (SSRs), Randomly-amplified polymorphic DNA (RAPDs) and Intersequence simple repeats (ISSRs) and the Amplified fragment length polymorphisms (AFLPs). Many of these markers are used to study natural hybridization processes between closely related species and also to look at the mating type distribution in a population that also give an indication of how diverse the population is and if it is a monoclonal population or not (Groenewald, 2007).

Genome projects

To date, the number of ongoing filamentous fungal genome sequencing projects is almost tenfold fewer than those of bacterial and archaeal genome projects (Baker et al, 2008). The fungi chosen for sequencing represent narrow kingdom diversity; most are pathogens or models. Several ambitious, forward-looking phylogenetic-based genome sequencing programs, designed to capture metabolic diversity within the fungal kingdom, thereby enhancing research into alternative bioenergy sources, bioremediation, and fungal-environment interactions are currently underway. The genome data generated will impact bioenergy research at several levels: biomass crop health and security, genetic and enzymatic robustness for biomass degradation, terrestrial carbon cycling and sequestration, augmentation of the catalog of bioproducts produced by fungal fermentation and single process biomass to ethanol conversion. During the last decade of the 20th century, several model fungi were chosen for genome sequencing, including the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* the filamentous saprobes *Neurospora crassa* and *Aspergillus nidulans* and the filamentous cereal pathogens, *Magnaporthe grisea*, *Ustilago maydis* and *Fusarium graminearum* (Kamper et al. 2006; Cuomo et al, 2007).

With an estimated 1.5 million species, Fungi have an enormous impact on human affairs and ecosystem functioning, owing to their diverse activities as decomposers, pathogens, and mutualistic symbionts. The use of fungi for the continued benefit of human life requires an accurate understanding of how they interact in natural and synthetic communities. The ability to sample environments for complex fungal metagenomes is rapidly becoming a reality and will play an important part in harnessing fungi for industrial, energy and climate management purposes. To bridge the gap in the understanding of fungal diversity, an international research team has embarked on a five-year project to sequence 1000 fungal genomes from across the Fungal Tree of Life (Spatafora, 2011). This project has the core goal of providing reference information to inform research on plant-microbe interactions, microbial emission, capture of greenhouse gasses, and environmental metagenomic sequencing.

Uses and importance of MiGR in food industry

Fermentation was traditionally a process, which served the preservation of perishable food and as such has been used for centuries until now. As new preservation techniques have been developed, the importance of fermentation processes for food preservation has declined (Guizani and Mothershaw, 2007) and nowadays the main purpose of food fermentation is not just to preserve but also to produce a wide variety of fermentation products with specific taste, flavor, aroma and texture. Using various microbial strains, fermentation conditions and chemical engineering achievements, it is now possible to manufacture hundreds of different types of dairy, vegetable, meat products and bread, alcoholic beverages, vinegar and other food acids, as well as oils. It is clear from past research projects that fermentation that involves microbes plays different roles in food processing (Bourdichon et al, 2012).

Food additives generated by microorganisms:

- *Microbial rennets* from various microorganisms are being marketed since the 1970s and have proved satisfactory for the production of different kinds of cheese.
- *Lactase* preparations from *Aspergillus niger*, *Aspergillus oryzae* and *Kluyveromyces lactis*, which are considered safe because these sources already have a history of safe use.

- *Microbial lipases*, which are region-specific and fatty acid specific, are of immense importance and could be exploited for retailoring of vegetable oils. Earlier, lipases of different microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine.
- *Alkaline proteases* have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks.
- *Glucoamylase* and *β-amylase* are used commercially in the production of low calorie beer.
- *Transglutaminase* obtained from microbial fermentation has been applied in the treatment of food of different origins. Food treated with microbial transglutaminase appeared to have an improved flavour, appearance and texture.
- *Flavours* comprise over a quarter of the world market for food additives. Most of the flavouring compounds are produced via chemical synthesis or by extraction from natural materials, but nowadays consumers prefer food related producs that can be labelled as natural. Several microorganisms, including bacteria and fungi, are currently known for their ability to synthesize different aroma compounds (Couto and Sanromán, 2006).

Protective cultures find application in several foods. In fresh fermented products, soft cheeses and smear type cheeses as they are used to control spoilage microorganisms and *Listeria* and in semi-hard and hard cheeses to control spoilage microorganisms.

Probiotics and functional foods constitute worldwide the fastest growing sector in the food industry. At present, probiotic bacteria are mainly incorporated into many dairy products. Fermented dairy products are the most traditional source of probiotic strains of lactobacilli; however, probiotic lactobacilli have been added to cooked pork meat products, snacks, fruit juice, chocolate and chewing gum (Bernardeau et al, 2006; Ranadheera et al, 2010). Further, it is important to develop probiotic products with food and beverages that are part of day-to-day normal diet to maintain minimum therapeutic level easily (Ranadheera et al, 2010). Beverages such as chilled fruit juices, bottled water, and fermented vegetable juices (Prado et al, 2008) are part of big research project.

Fungal physiology linked to bio-processes

Fungal physiology is the basis of biotope and global dispersion of fungal species. It determines the nutrients it can use, how it can survive different and extreme environmental conditions (eg. climate change) and how it compete with other living organisms in its ecosystem. The ability of fungi to survive in every known biotope, both natural and man-made, relies in part on their capacity to use a wide range of carbon sources. In nature, many fungi degrade polymeric carbon sources (e.g. polysaccharides, proteins, lignin) to use the components as carbon source. However, the available carbon sources vary strongly in nature. While some fungi have become specialists that focus on specific carbon sources or specific biotopes, others are more generalists that can grow in many biotopes and use a large variety of carbon sources. Differences in physiology may therefore also reflect species boundaries. Degradation of polymeric carbon sources occurs extracellularly by a broad range of enzymes, of which the production is tightly controlled by a network of regulators within the microbe. This enables fungi to produce an enzyme mixture that is tailored specifically for the available carbon sources at any given time. The released compounds are transported into the cell and metabolized through a variety of metabolic pathways. To study fungal physiology in relation to natural substrates it is therefore necessary to address the production of extracellular enzymes,

metabolic pathways and regulators controlling the fungal response to the substrates present in the environment. The aim of many of the studies that focus on the interaction between the fungi en their environment is to be able to make use of these natural processes by applying it in bio-processes (Rytioja et al, 2014; Khosravi et al, 2015).

Implications for the conservation and use of genetic resources in the EU and globally

Micro-organisms represent a significant percentage of the exiting organisms on earth. Chapman (2009) estimated fungi to represent more than 13% of the Earth's organisms that is considered by many other researchers as a conservative percentage as the estimated number of fungi approaches, or even exceeds that of the insects, which make microbes now the largest group of living organisms on earth. Regarding such mega diversity, it is quite clear that fungi play a dominant role in ecosystems as well as in two- and multi-biont interaction systems and therefor, due to its diversity, play an important part in human live (Chapman, 2009). It is therefore also important to know what you are using, whether it is from the natural population, but also from preserved populations and if it can be applied for further use in agriculture. The provision of microbial resources for research is now recognized as being an essential component in the advancement of the life sciences. The hypothesis is that the full potential of microbial diversity is yet to be harnessed and a coordinated approach to resource provision will accelerate innovation and discovery.

Within the EU several project have been launched that focussed on aspects linked to collections of living microorganisms and how to harmonize microbial conservation within the EU eg. the Global Biological Resource Centre Network (GBRCN) and the Microbial Resources Research Infrastructure (MIRRI). The MIRRI initiative started in 2010 and its preparatory phase began in late 2012 (Schüngel et al, 2013). The aim of this project is to stimulate the use of the known microbial diversity to its full potential and to coordinated approaches to accelerate innovation and discovery of novel products and uses. MIRRI brings together European microbial resource collections with their stakeholders and aim to improve access to enhanced quality microbial resources in an appropriate legal framework, thus under pinning and driving life sciences research. MIRRI will provide coherence in the application of quality standards, homogeneity in data storage and management and sharing of workload to help to release the hidden potential of micro-organisms. It will also enhance existing European microbial collections linking them to non-European country partners globally and will bring added value.

Exchanges of microorganisms between culture collections, laboratories and researchers worldwide have historically occurred in an informal way. These informal exchanges have facilitated research activities, and, as a consequence, our knowledge and exploitation of microbial resources have advanced rapidly. (Kurtzman and Labeda, 2009; WFCC, 2010). Previous studies have shown that informal exchanges among scientists and/or culture collections represent a large percentage of the exchanges (Dedeurwaerdere, 2009). Some collections even distribute material only on an informal basis. This is especially true for the smaller and more specialized collections. As a general trend, however, more and more collections, in both developing and developed countries, are moving toward formal arrangements. The adoption of rather restrictive access measures by several

developing countries, as a reaction to excessive bioprospecting and patenting by developed countries, further threatens the efficacy of an informal regime. Scientists from both developed and developing countries have repeatedly expressed concern about the harm that restrictive access regulations may have on scientific research (Jinnah and Jungcurt, 2009). In February 2009, the European Culture Collection (ECCO) adopted a core Material Transfer Agreement (see www. eccosite.org). The main purpose of the agreement was to make biological material from ECCO collections available under the same core conditions, which were to be implemented by ECCO members either as such, or integrated into their own more extended documents Dedeurwaerdere, 2010; Çaktü and Türkoglu, 2011).

Microbial resource centres have an extremely important role underpinning the conservation of microbial biodiversity and enabling advances in agriculture, food security, biotechnology and education. As microbial resource centres also maintain extensive databases and thus provide access to information on cultures, their characteristics, literature and DNA sequences, for example. Microbial resource centres have played a key role in agricultural and food research over many decades. The Food and Agriculture Organization (FAO) recognises the important role of microbial genetic resources and microbial resource centres for productive agriculture and food security (FAO, 2009) and also in understanding the consequences of climate change (Fujisaka et al, 2009. Not only do these collections provide valuable resources and expertise; the cultures from this research are available for extension of this research. Various reports have called for accelerated research on microbial diversity. Such an initiative would expand knowledge and opportunities for biodiscovery and innovative agriculture and food industries (Sly, 2010).

A few considerations that can strengthen microbial collections and therefore microbial conservation for the future are (Stackebrandt, 2010):

1. Collections that aim to receive Biological Resource Centre (BRC) status will need substantial support for establishing and maintaining quality management and accreditation status, as well as for the expansion of research, training and bioinformatics;

2. Any expansion of collection diversity, both in terms of phylogenetic diversity and in-depth coverage at the level of genus and species, will require increased expertise of curators and technical staff;

3. A strategy needs to be outlined which encourages authors to deposit a higher fraction of strains that are so far under-represented in public collections. The collection community must work with all microbiologists, collection users, editors and research programme funders to ensure that all key strains emanating from research work are preserved for future use and confirmation of results. This is necessary to ensure that investments in delivering outputs from publicly funded research are protected. It is essential that the primary biological materials upon which data in publications or in public databases are based are made available, and preserved as deposited, so that spurious or unusual findings can be further explored or to allow further work as new technologies arise.

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