Visegrad Fund

Next Generation Sequencing: a new approach to deeply discover the microbial contamination of archival documents

Domenico Pangallo

Laboratory of Environmental and Food Microbiology

Institute of molecular biology S A S

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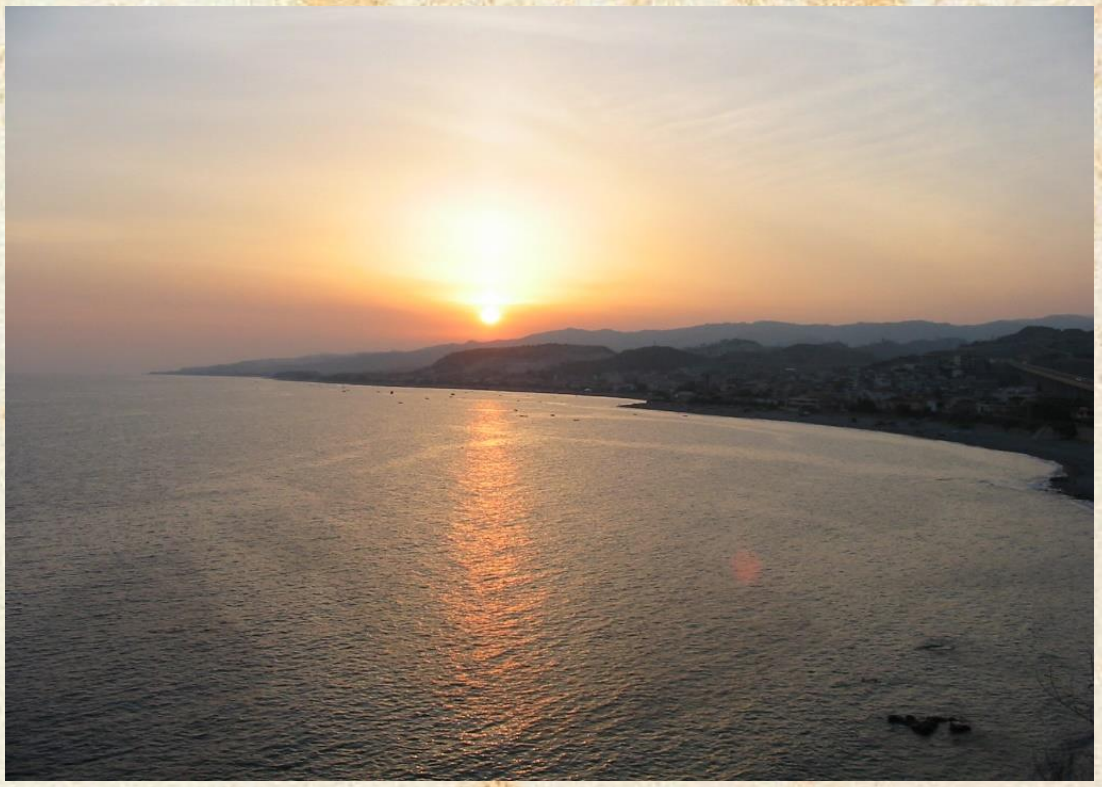
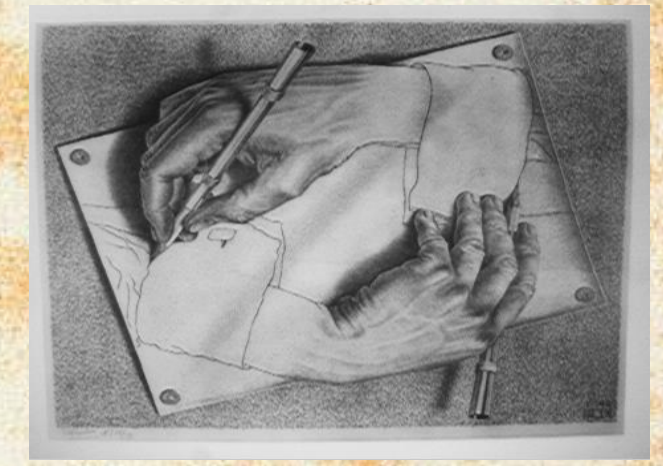


Laboratory of Environmental and Food Microbiology

- Detection of food-borne pathogens by molecular methods
- Detection of non-declared components in food by DNA analysis
- Characterization of the autochthon microflora in traditional food



- Investigation of the biodegradative microflora in art objects
- Study of the biodegradative activities
- Archive documents analysis
- Conservation strategies

It is possible to study the microbial communities by two different strategies:

- **Culture-dependent**

Establish suitable conditions to isolate and cultivate a microbe species.

- **Culture-independent**

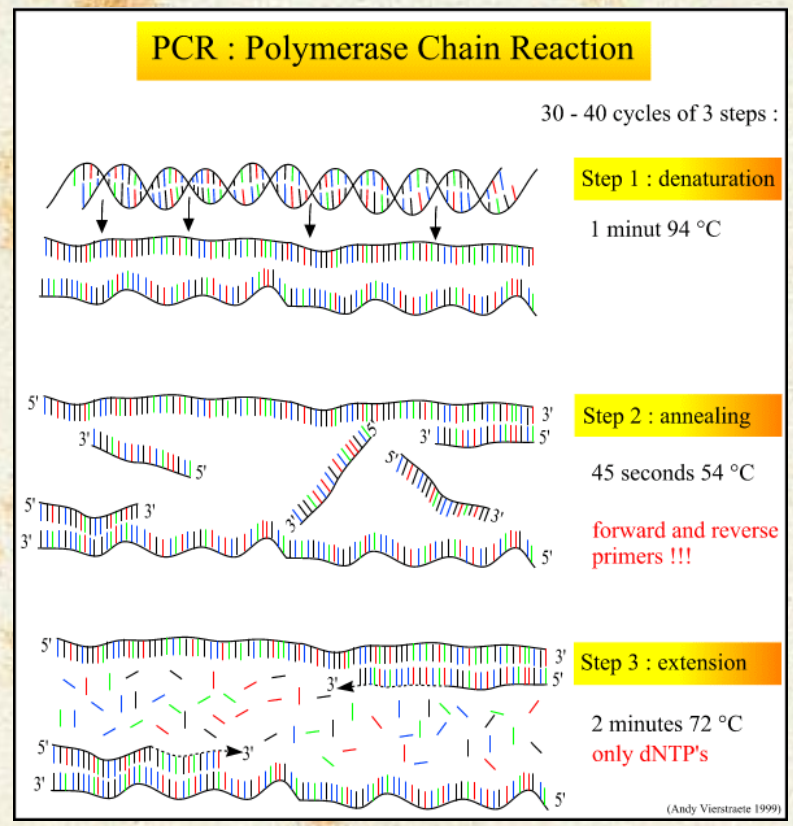
Relies on **molecular methods** to study microbes within their environments.

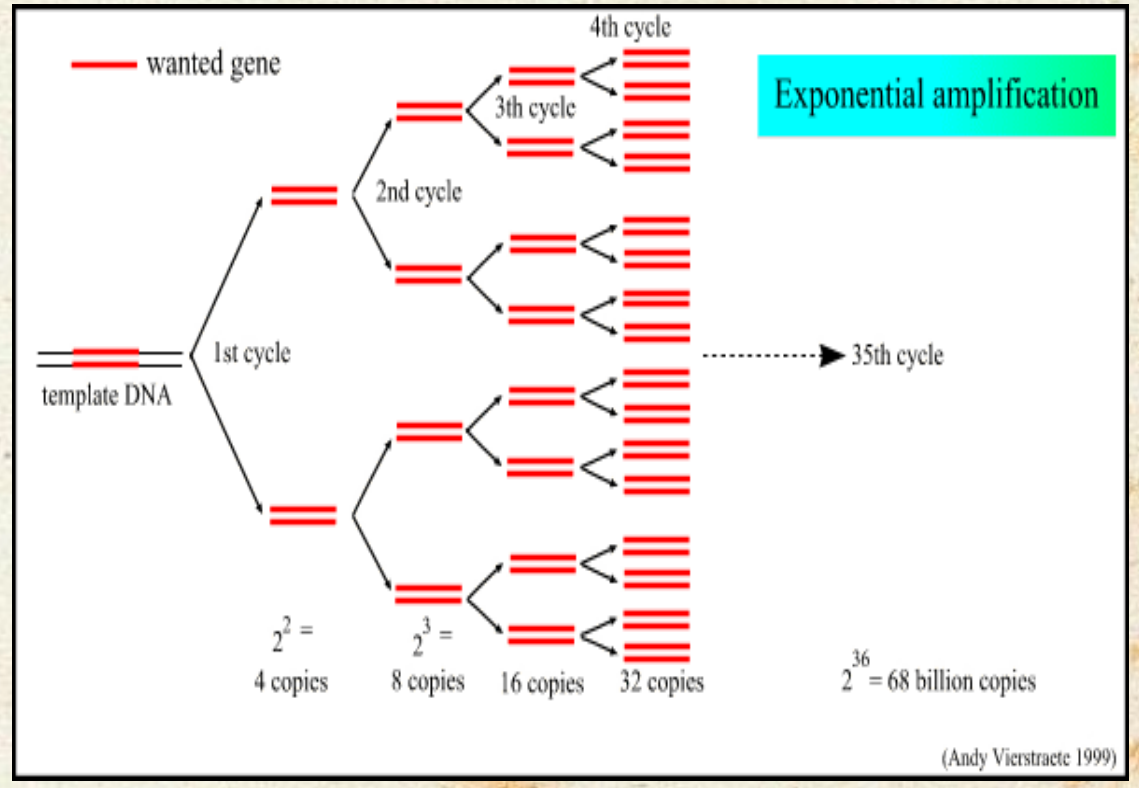
Molecular Biology Approaches

Our laboratory, for **the first time** in the **Slovak scientific scenario**, has used the **culture-independent approaches** in order to analyze the microbial community in cultural heritage items.

In our studies the **molecular biology methods** (nucleotide detection by PCR-based techniques) improved the **culture-dependent strategies** and increased the information of the microbial communities colonizing different environments using a suitable **culture-independent procedure**.

PCR- Polymerase Chain Reaction

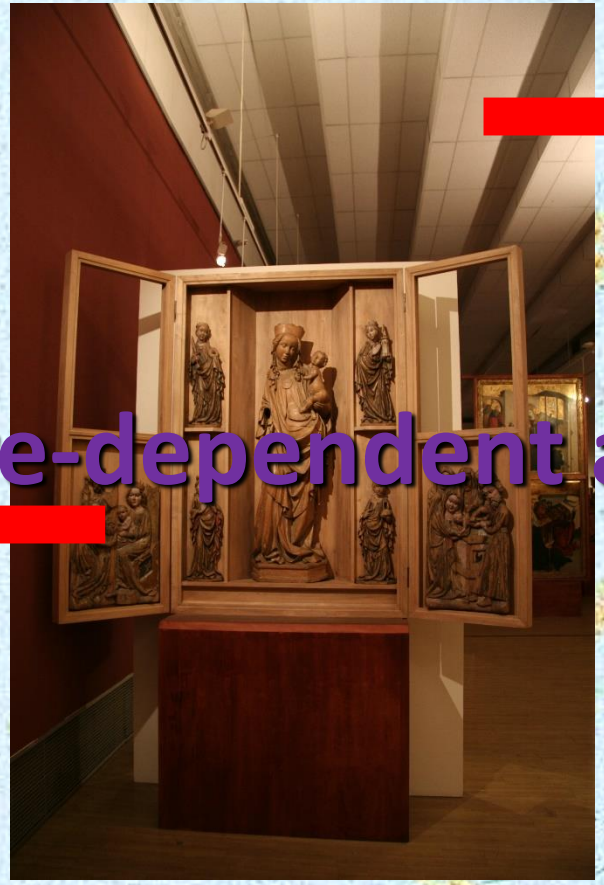




<u>One fragment of DNA</u> is exponential amplified to produce around **<u>68 billion of copies</u>** of the original DNA fragment.

Methods to Study the Microbial Communities Colonizing Cultural and Historical Heritage Items

ENVIRONMENT



Culture-dependent analysis

OBJECT

Typical Microflora present in Indoor Art Objects and Environment

FUNGI

Acremonium sp., Alternaria tenuis, Alternaria solani, Alternaria alternata, Aspergillus niger, Aspergillus flavus, Aspergillus tamari, Aspergillus versicolor, Cladosporium elatum, Cladosporium cladosporoides, Cladosporium herbarum, Cephalosporium sp., Curvularia lunata, Chaetomium globosum, Chaetomium succineum, Fusarium roseum, Fusarium solani, Fusarium oxysporum, Geothrichum sp., Gliocadium sp., Mixotrichum sp., Monilia macrospora, Mucor racemosus, Mycoderma sp., Myrothecium verrucaria, Ophistoma sp., Paecylomyces variabilis, Penicillium bevicompactum, Penicillium frequentans, Penicillium chrysogenum, Pestalotia oxyanthi, Phoma glomerata, Rhizopus nigricans, Trichothecium roseum, Trichothecium sp., Trichoderma viride, Trichoderma longibrachiatum, Trichoderma lignorum, Ulocladium botrytis, Verticillium chlamydosporium, Verticillium albo-atrum, Scopularioupsis brevicaule, Scopulariopsis acremonium, Stachybotrys atra, Spicaria sp.

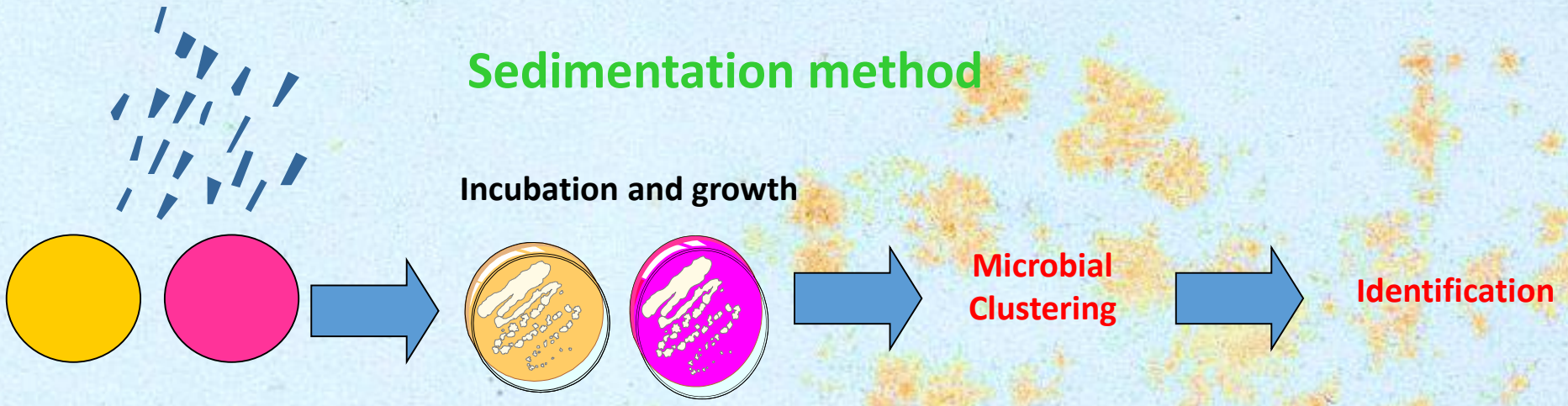
BACTERIA

Aeromonas caviae, Aeromonas sp., Bacillus subtilis, Bacillus cereus, Bacillus circulans, Cellulomonas sp., Cellulomonas cellasea, Cellulomonas cellulans, Cellvibrio mixtus, Chromobacterium sp., Cytophaga aurantiaca, Flavobacterium breve, Micrococcus luteus, Micrococcus roseus, Micrococcus varians, Pseudomanas fluorescens, Pseudomonas elongata, Streptococcus sp., Streptomyces rimosus, Staphylococcus sp., Clostridium sp., Vibrio sp., Xanthomonas sp.

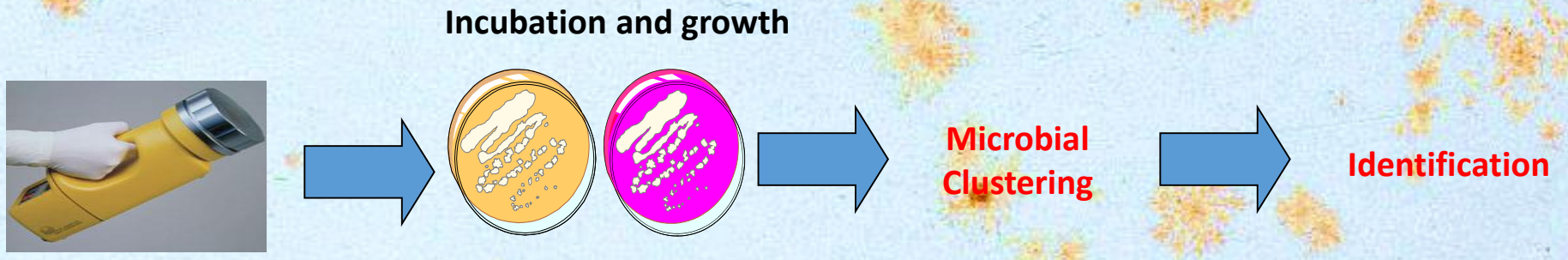
It seams that the fungal community has the biggest responsibility and rule for air contamination and biodegradation of art.

Isolation of Airborne Microorganisms

Sedimentation method



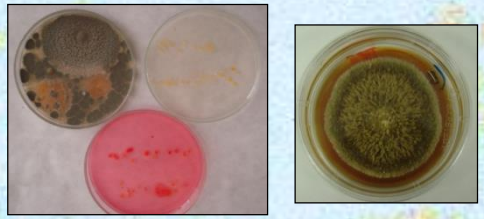
Air-Sampler system



Isolation of Microorganisms from Historical Objects

Historical object

Swabs adhesive tape needles

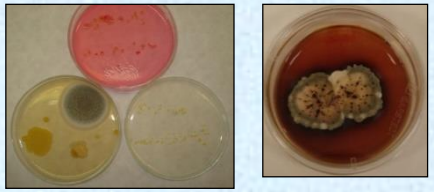


Direct cultivation

Decimal dilution

Microbial Clustering

Identification



Cultivation

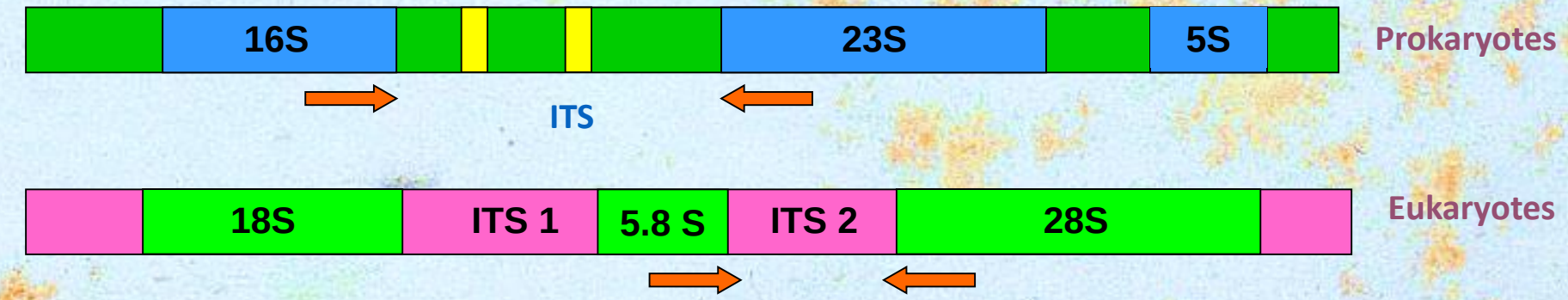
Fungi: MEA, PDA, BRA, DBRC

Bacteria: BHI, LM, R2A, LB, NA

Specific: Cellulose M, Lignin M, Gelatin, $CaCO_3$, with different salt concentrations, etc.

Microbial Clustering by ITS-PCR

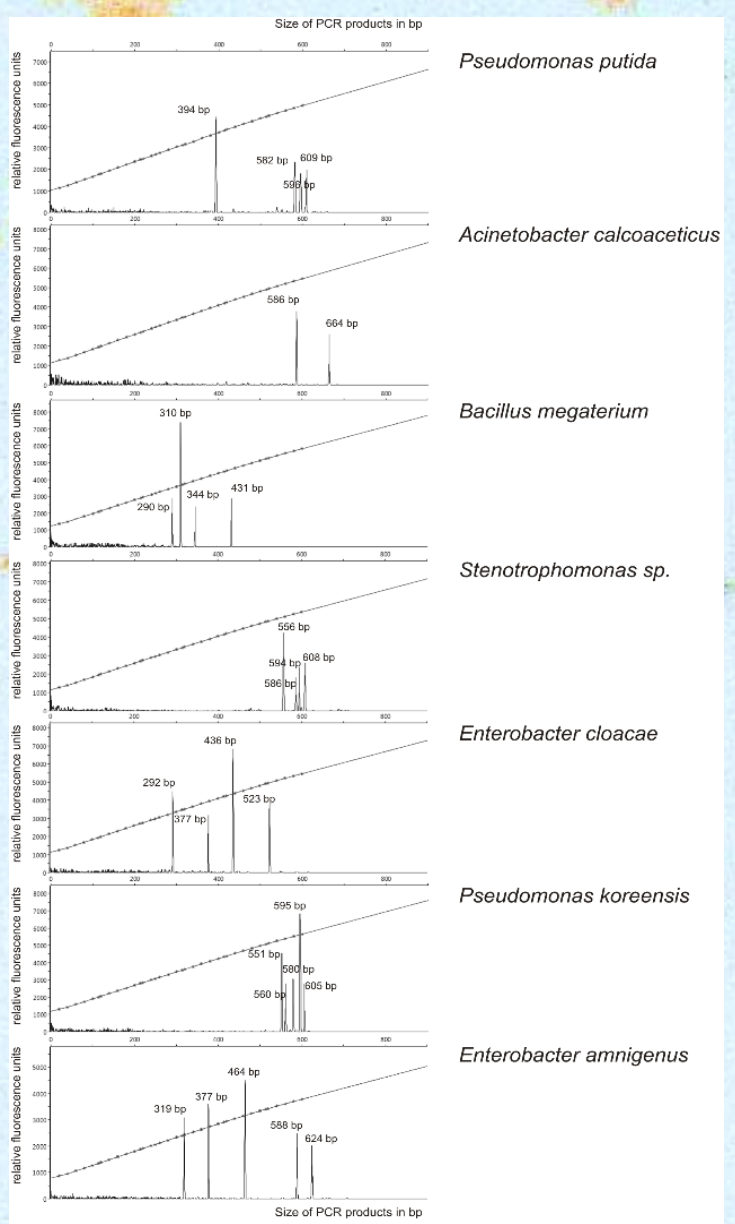
Such strategy is useful in studies of environmental and food microbiology, where a great number of isolates are isolated and it is not possible to establish whether we are going to analyze the same or different strain belonging to the same bacterial group.



The bacterial and fungal **internal transcribed spacers** (ITS) exhibit a large degree of sequence and length variation at the levels of genus and species. ITS are generally found in multiple copies in most bacterial genomes.

By **Fluorescence ITS-PCR (f-ITS)** is possible to create specific bacterial and fungal ITS-PCR fingerprinting patterns in order to cluster the isolates.

BACTERIA



Pseudomonas putida

Acinetobacter calcoaceticus

Bacillus megaterium

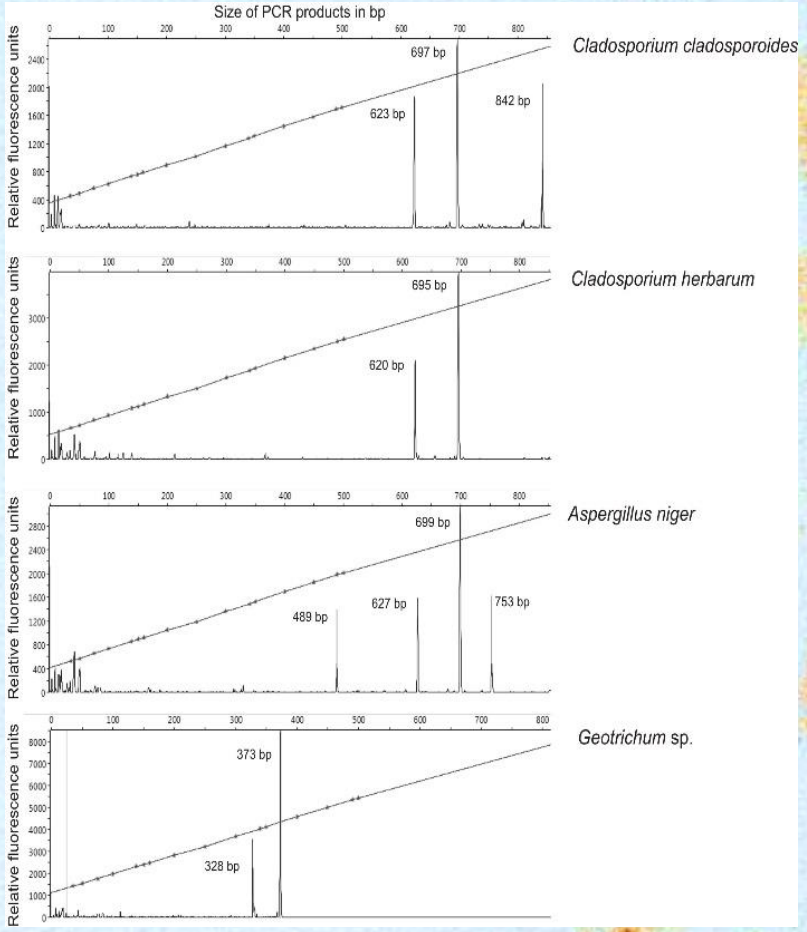
Stenotrophomonas sp.

Enterobacter cloacae

Pseudomonas koreensis

Enterobacter amnigenus

FILAMENTOUS FUNGI



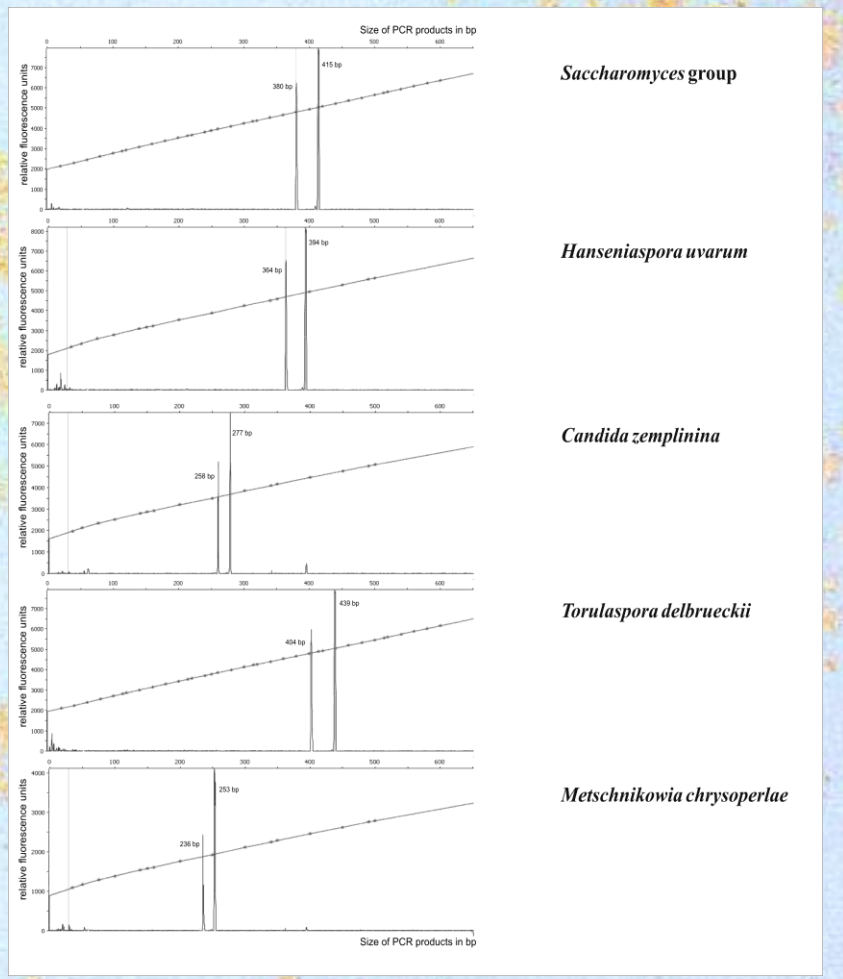
Cladosporium cladosporoides

Cladosporium herbarum

Aspergillus niger

Geotrichum sp.

YEASTS



***Saccharomyces* group**

Hanseniaspora uvarum

Candida zemplinina

Torulaspora delbrueckii

Metschnikowia chrysoperlae

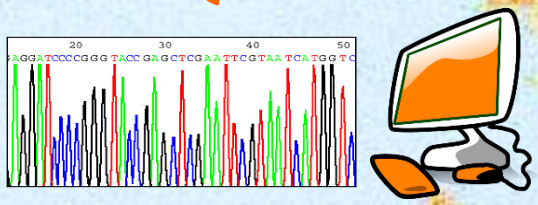
Identification by PCR-based approaches oriented to ribosomal genes

Different ribosomal RNA genes and fragments can be used for microorganisms identification by PCR amplification and sequencing

Prokaryotes

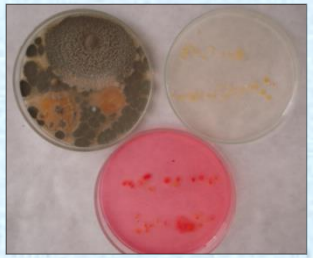
Eukaryotes

SEQUENCING

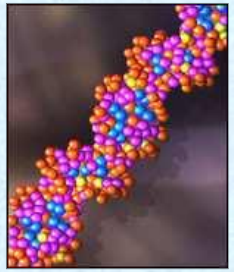


Identification of microorganisms

Microorganisms



Extraction of DNA



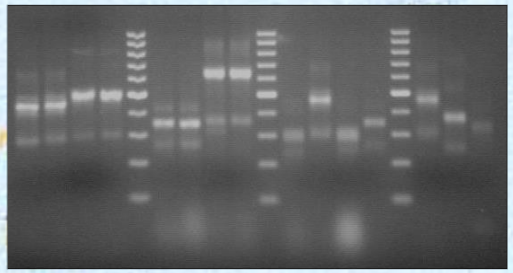
Clustering of isolates

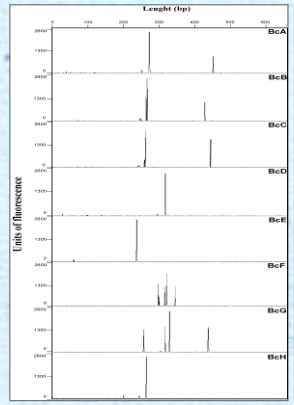
a) prokaryotes - fluorescence ITS PCR

b) eukaryotes - fluorescence ITS PCR

a) Prokaryotic f-ITS

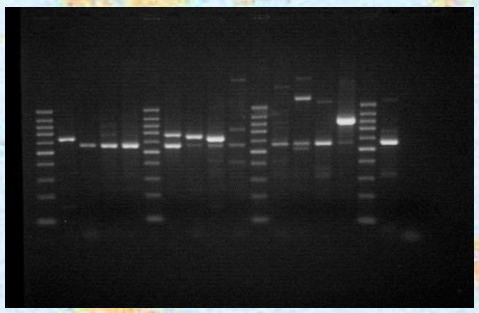
primers FAM G17, L1

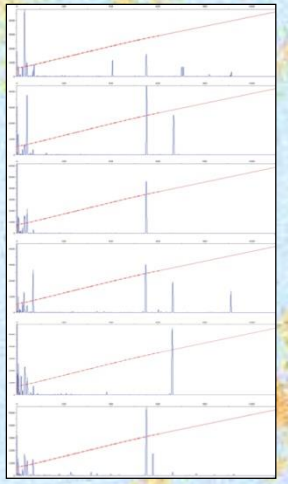




b) Eukaryotic f-ITS

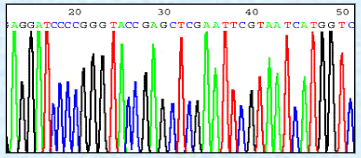
primers ITS3, ITS4-FAM





Identification by Sequencing

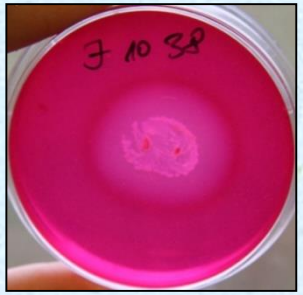
Selected isolates



Prokaryotes – 16S rRNA

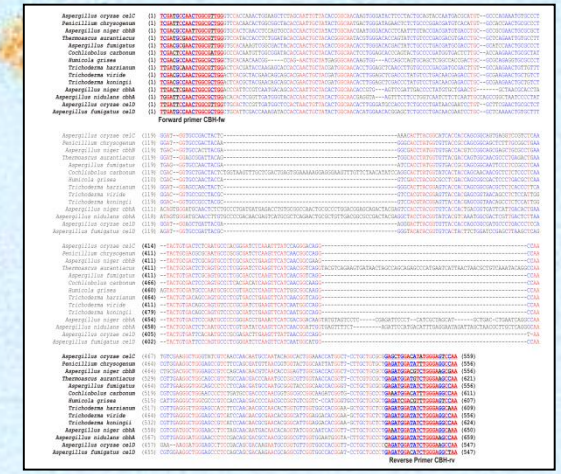
Eukaryotes - ITS fragment or 28S rRNA

A novel PCR-based method oriented on cellobiohydrolase gene for classification of environmental filamentous fungi

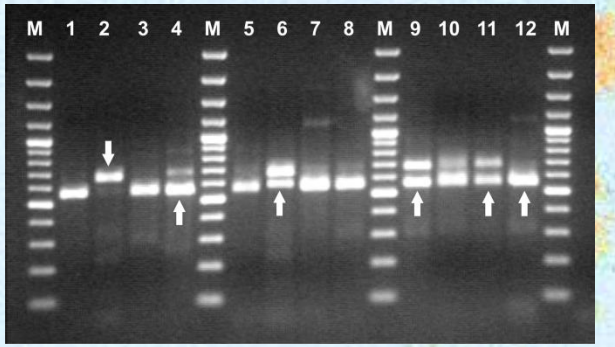


Cellulolytic test

Czapek- Dox agar supplemented with 0.2% of hydroxyethyl cellulose containing covalently linked Ostazin Brilliant Red H-3B; a clear zone around the colonies indicating cellulolytic activity

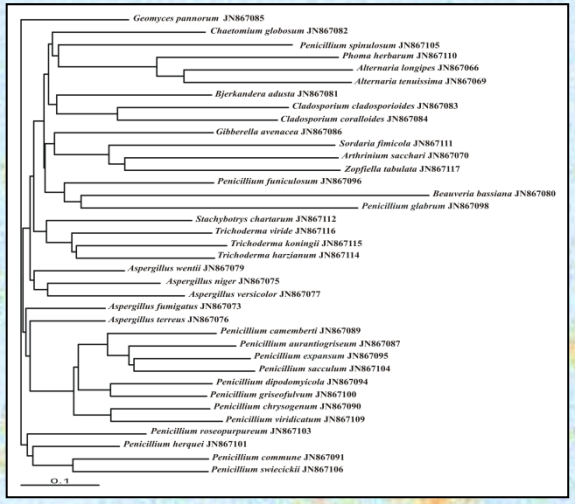


Comparison of 14 sequences obtained from database used for designing the primers CBH-fw and CBH-rev

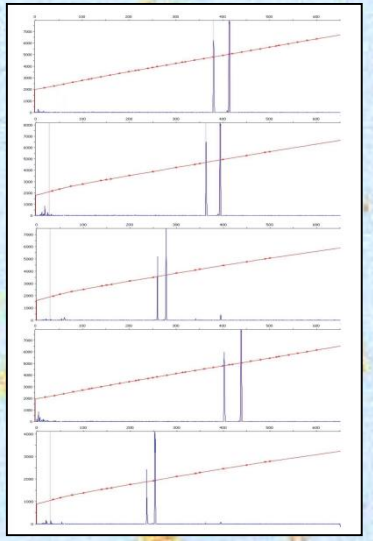


Example of the *cbh* polymorphism visualized on agarose gel. The arrows indicate the bands which were sequenced.1: *Cladosporium herbarum*; 2: *C. cladosporoides*; 3: *Aspergillus niger*; 4: *Penicillium expansum*; 5 and 8: *P. sacculum*; 6: *Alternaria tenuissima*; 7 and 12: *P. chrysogenum*; 9, 10 and 11: *A. fumigatus*

Modification of developed system by adding of 6-carboxy-fluorescein (FAM) label to 5‘ end of CBH-fw primer. Resulting fluorescent products are separated by capillary electrophoresis



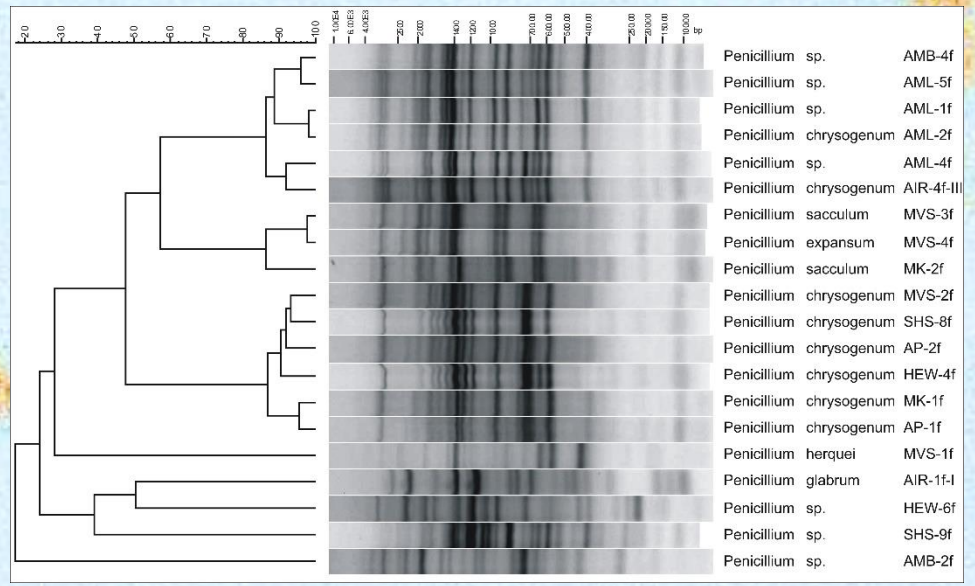
Phylogenetic relationship among the cellobiohydrolase DNA sequences produced by the studied fungal strains.

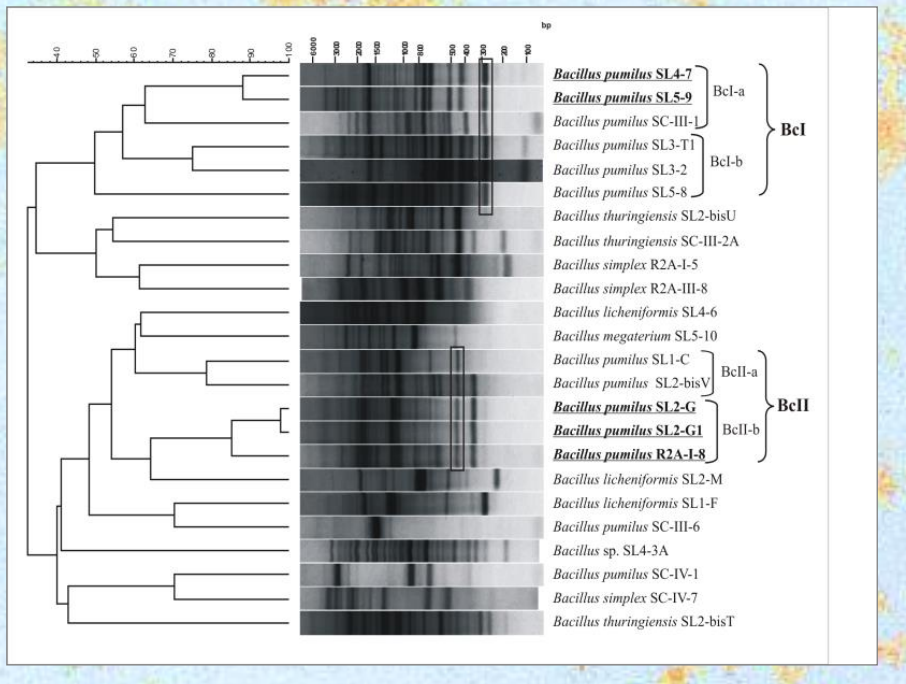


Electrophoretograms of CBH-PCR

RAMP typing

RAMP (**random amplified microsatellite polymorphisms**) - The RAMP method is a PCR assay which includes the combination of one microsatellite primer with a random primer. A special PCR program is used in order to facilitate the annealing of two primers with different Tm.

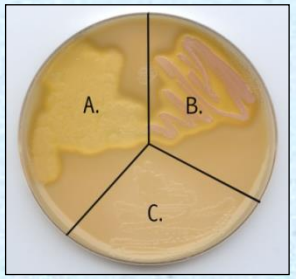




Biodegradation abilities

Characterization of the enzymatic properties of isolated microflora. The presence and the activity of different kinds of enzymes (for example amylases, cellulases, xylanases, depolymerases, pectinases, proteases, lipases, esterases, keratinases) produced by isolated microflora were tested by plate agar assays.

Milk NA



protease

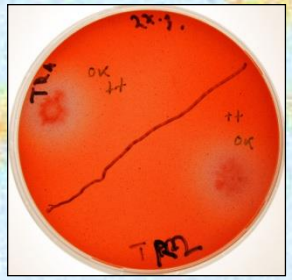
Gelatin medium

protease

Cellulolytic test
hydroxyethyl cellulose

cellulase

Cellulose Congo red medium

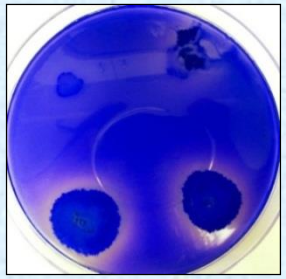


cellulase

Feather Broth

keratinase

Fibroin agar

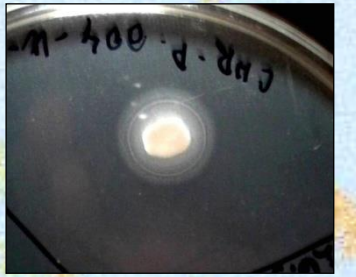


proteinase for fibroin

Spirit Blue medium

lipase

Tween 80 medium



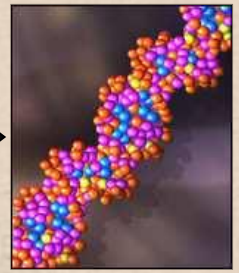
esterase

Culture-independent approaches

Clone Libraries Construction

Air-borne MICRFLORA

DNA / RNA extraction



PCR amplification

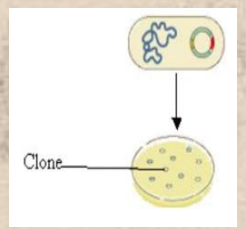
~ 700 bp

Bacterial and cyanobacterial 16S rRNA

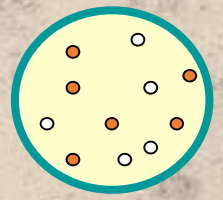
Fungal and Algal ITS

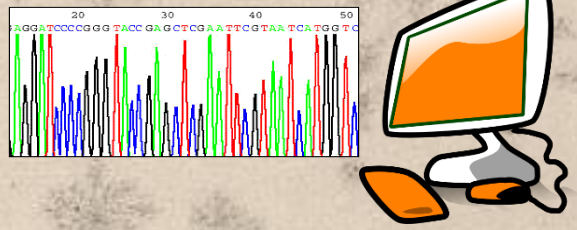
Fungal 28S rRNA

Cloning



Selection of clones



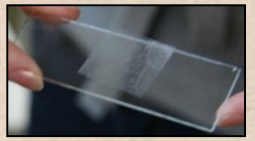


SEQUENCING and IDENTIFICATION

Experimental strategy

sampling

adhesive tape



scraping with scalpel

streaked on R2A plates

wipe of bulk mixed cultures to ependorf

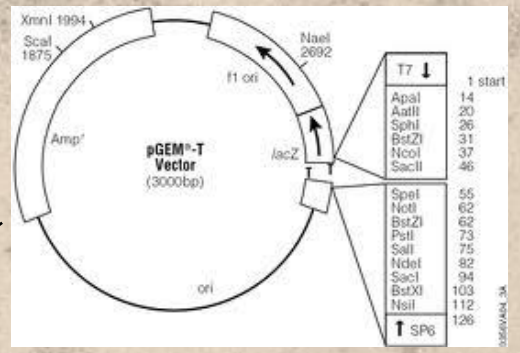
Extraction of DNA from bulk mixed cultures and scrapped material

PCR 16S rDNA

~ 700 bp

Extraction of RNA from scrapped material

Reverse transcription of RNA to cDNA



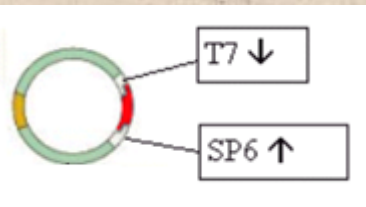
ligation mixture

transformation

E.coli DH5 α

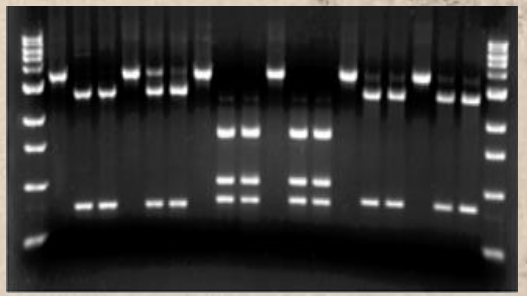
Clone

LB-Agar + Amp + IPTG + X-Gal



PCR amplification of inserts of white colonies

selection of positive clones by restriction digestion by *Hae*III and *Hha*I



sequencing

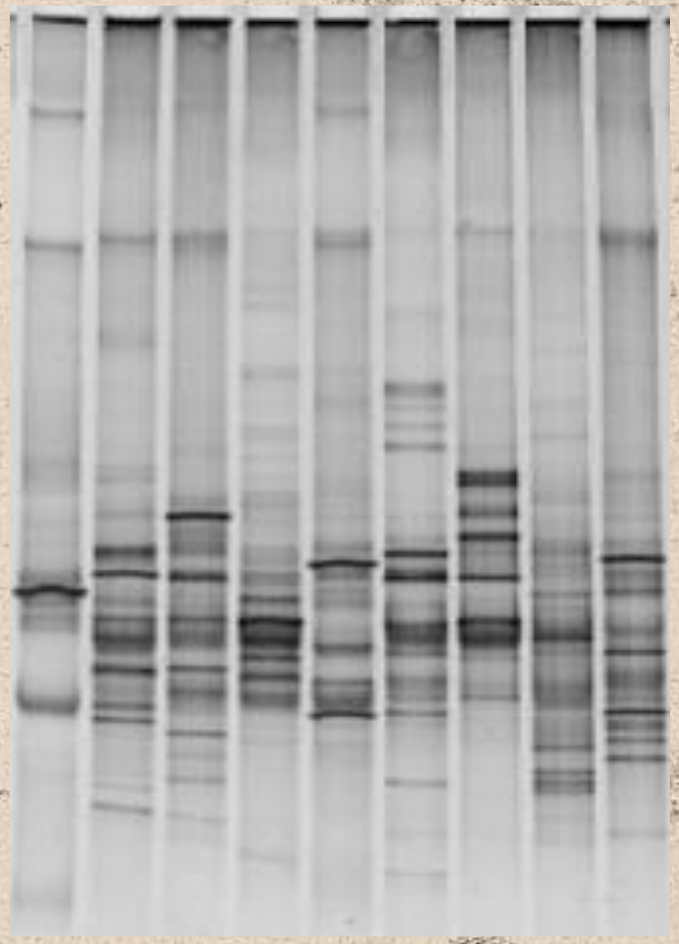
Database Nucleotide Collection	Total RNA / cDNA cloning Sequence similarity (%)	Total DNA cloning Sequence similarity (%)	R2A plate DNA cloning Sequence similarity (%)
	FN297967 Uncultured actinobacterium - (96%)	DQ823212 Uncultured bacterium clone - (94%)	AJ968699 *Phyllobacterium* sp. - (99%)
	GU200958 Uncultured bacterium clone - (90%)	HM186617 Uncultured bacterium clone - (96%)	DQ188524 Uncultured *Lysobacter* sp. clone - (99%)
	NR_025088 *Crossiella* equi - (88%) - ***	FJ937942 *Pseudonocardia* sp. - (97%)	HM186159 Uncultured bacterium clone - (99%)
	HQ385587 Uncultured bacterium clone - (88%)	HM445440 Uncultured bacterium clone - (99%)	JF957297 Uncultured bacterium clone - (97%)
	JF957297 Uncultured bacterium clone - (95%)	HM063022 Uncultured bacterium clone - (99%)	JF957297 Uncultured bacterium clone - (97%)
	JF829480 Uncultured bacterium clone - (89%)	EF188338 Uncultured actinobacterium clone - (95%)	JF957297 Uncultured bacterium clone - (97%)
	HQ132004 Uncultured bacterium clone - (95%),	JN038696 Uncultured bacterium clone - (94%)	DQ188524 Uncultured *Lysobacter* sp. clone - (99%)
	JF266329 Uncultured bacterium clone - (92%)	FJ937942 *Pseudonocardia* sp. - (97%)	HQ682000 Uncultured bacterium clone - (96%)
	HQ864193 Uncultured bacterium clone - (99%)	EU133911 Uncultured bacterium clone - (90%)	DQ188524 Uncultured *Lysobacter* sp. clone - (99%)
	JF266146 Uncultured bacterium clone - (90%)	EU133911 Uncultured bacterium clone - (90%)	GQ183850 *Phyllobacterium* sp. - (99%)
	HM277531 Uncultured bacterium clone - (95%)	JN989287 *Pseudonocardia* sp. - (95%)	HM777012 *Brevundimonas* sp. - (97%)
	HM038053 Uncultured bacterium clone - (93%)	AY921847 Uncultured Acidobacteria bacterium clone - (98%)	HM777012 *Brevundimonas* sp. - (99%)
	JF266146 Uncultured bacterium clone - (91%)	JF957297 Uncultured bacterium clone - (93%)	HQ118734 Uncultured bacterium isolate - (99%)
	DQ823191 Uncultured bacterium clone - (96%)	DQ188529 Uncultured *Lysobacter* sp. clone - (99%)	EF188476 Uncultured alpha proteobacterium clone - (100%)
	FM992788 *Pseudonocardia* sp. - (90%)		AJ968699 *Phyllobacterium* sp. - (99%)
	AB546273 *Pseudonocardia* sp. - (88%)		GQ183850 *Phyllobacterium* sp. - (99%)
	DQ188511 Uncultured *Lysobacter* sp. clone - (95%)		AJ968699 *Phyllobacterium* sp. - (99%)
	FJ478475 Uncultured bacterium clone - (95%)		
	EU800157 Uncultured bacterium clone - (89%)		
	FN567253 Uncultured bacterium clone - (89%)		
	EF188338 Uncultured actinobacterium clone - (94%)		
	AM991227 Uncultured bacterium clone - (99%)		
	HQ864193 Uncultured bacterium clone - (95%)		
	FN659319 Uncultured bacterium clone - (94%)		
	AY694691 Uncultured *Streptomyces* sp. clone - (89%)		
	HM038053 Uncultured bacterium clone - (92%)		
	NR_042003 *Pseudonocardia hydrocarbonoxydans* - (94%) - ***		
	GU200958 Uncultured bacterium clone - (89%)		
	JQ419590 *Kutzneria* sp. - (92%)		
	DQ823212 Uncultured bacterium clone - (94%)		
	HM119284 Uncultured bacterium clone - (87%)		
	FN297971 Uncultured bacterium clone - (91%)		

Database 16S rRNA sequences	Total RNA / cDNA cloning Sequence similarity (%)	Total DNA cloning Sequence similarity (%)	R2A plate DNA cloning Sequence similarity (%)
	NR_042004 *Pseudonocardia halophobica* - (95%)	NR_028867 *Thiorhodospira sibirica* - (89%)	NR_043192 *Phyllobacterium ifriqiyense* - (99%)
	NR_025499 *Alkalilimnicola halodurans* - (87%)	NR_043521 *Desulfohalobium utahense* - (85%)	NR_036925 *Lysobacter enzymogenes* - (99%)
	NR_025088 *Crossiella equi* - (88%) - ***	NR_044562 *Pseudonocardia xinjiangensis* - (97%)	NR_043192 *Phyllobacterium ifriqiyense* - (99%)
	NR_042170 *Cryobacterium psychrophilum* - (88%)	NR_044097 *Thiohalomonas denitrificans* - (92%)	NR_028638 *Chitinophaga japonensis* - (93%)
	NR_028638 *Chitinophaga japonensis* - (91%)	NR_028863 *Thiohalocapsa halophila* - (92%)	NR_028638 *Chitinophaga japonensis* - (93%)
	NR_041803 *Tsukamurella paurometabola* - (87%)	NR_042006 *Pseudonocardia saturnea* - (91%)	NR_028638 *Chitinophaga japonensis* - (93%)
	NR_041965 *Brevundimonas alba* - (94%)	NR_025163 *Desulfonatronum thiodismutans* - (81%)	NR_036925 *Lysobacter enzymogenes* - (98%)
	NR_043717 *Humicoccus flavidus* - (93%)	NR_044562 *Pseudonocardia xinjiangensis* - (96%)	NR_028633 *Brevundimonas nasdae* - (95%)
	NR_041633 *Ilumatobacter fluminis* - (91%)	NR_029324 *Pseudonocardia sulfidoxydans* - (90%)	NR_036925 *Lysobacter enzymogenes* - (98%)
	NR_026064 *Methylocaldum szegediense* - (84%)	NR_042003 *Pseudonocardia hydrocarbonoxydans* - (90%)	NR_043055 *Phyllobacterium catacumbae* - (100%)
	NR_043742 *Pseudonocardia* sp. - (94%)	NR_041993 *Pseudonocardia tetrahydrofuranoxydans* - (95%)	NR_043770 *Brevundimonas aveniformis* - (97%)
	NR_024774 *Actinokineospora terrae* - (89%)	NR_029232 *Clostridium argentinense* - (78%)	NR_041965 *Brevundimonas alba* - (99%)
	NR_042183 *Methylococcus capsulatus* – (84%)	NR_040909 *Chitinophaga filiformis* - (93%)	NR_029046 *Inquilinus limosus* - (99%)
	NR_028638 *Chitinophaga japonensis* - (85%)	NR_036925 *Lysobacter enzymogenes* - (98%)	NR_043192 *Phyllobacterium ifriqiyense* - (99%)
	NR_042005 *Pseudonocardia petroleophila* - (89%)		NR_043192 *Phyllobacterium ifriqiyense* - (99%)
	NR_042006 *Pseudonocardia saturnea* - (87%)		NR_043055 *Phyllobacterium catacumbae* - (100%)
	NR_036925 *Lysobacter enzymogenes* - (94%)		NR_043192 *Phyllobacterium ifriqiyense* - (99%)
	NR_027547 *Anaeromyxobacter dehalogenans* - (83%)		
	NR_041633 *Ilumatobacter fluminis* - (85%)		
	NR_041867 *Nocardia transvalensis* - (89%)		
	NR_042006 *Pseudonocardia saturnea* - (91%)		
	NR_029287 *Nitrospira moscoviensis* - (95%)		
	NR_041633 *Ilumatobacter fluminis* - (88%)		
	NR_043507 *Streptomyces clavifer* - (92%)		
	NR_042004 *Pseudonocardia halophobica* - (90%)		
	NR_042013 *Actinoplanes durhamensis* - (87%)		
	NR_042003 *Pseudonocardia hydrocarbonoxydans* (94%) - ***		
	NR_025088 *Crossiella equi* - (89%) - ***		
	NR_027210 *Lentzea violacea* - (92%)		
	NR_028867 *Thiorhodospira sibirica* - (88%)		
	NR_041633 *Ilumatobacter fluminis* - (84%)		
	NR_029287 *Nitrospira moscoviensis* - (90%)		

DGGE – Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) **is a molecular fingerprinting method** that separates polymerase chain reaction (PCR)-generated DNA products.

The **PCR products of similar size** are **separated** on the basis of their **sequences** in an **denaturing polyacrylamide gel**



DGGE & cloning strategy

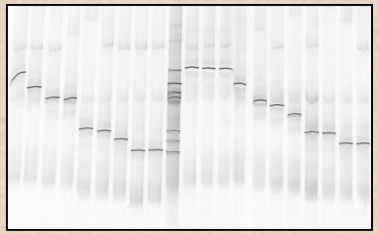
Samples

DNA extraction

PCR amplification of bacterial 16S rRNA or fungal 28S rRNA and ITS

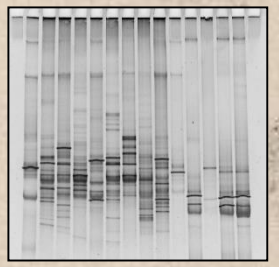
Clone library construction

Screening of clones by semi nested PCR and DGGE.
Comparing them with the corresponding fingerprinting profile



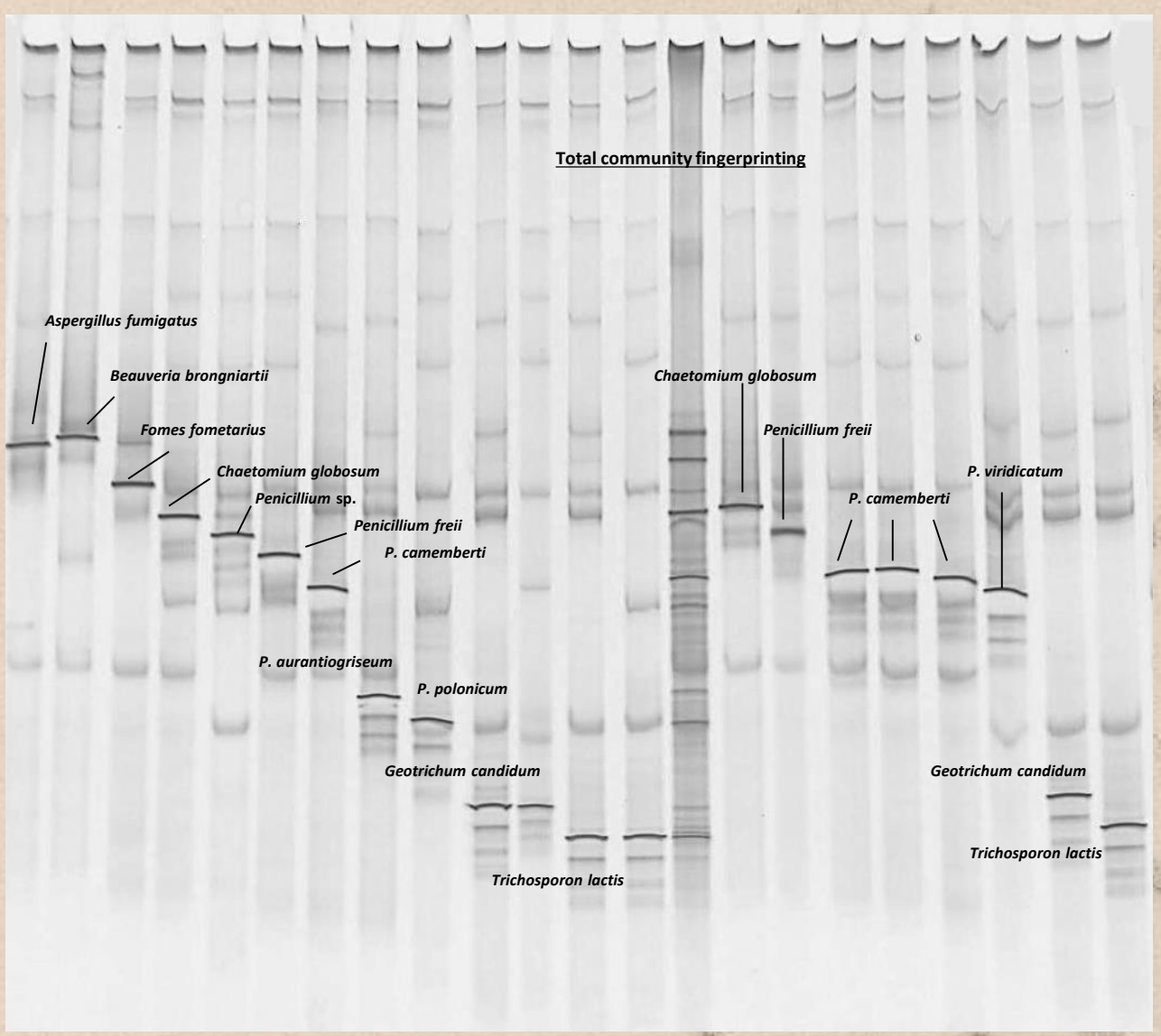
Identification of the different clones by sequencing

Semi nested PCR for DGGE fingerprinting



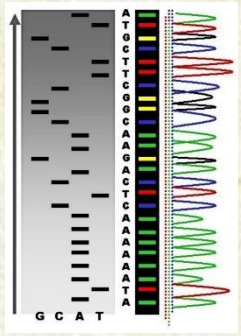
Microbial diversity

Fungi screened by DGGE & cloning approach

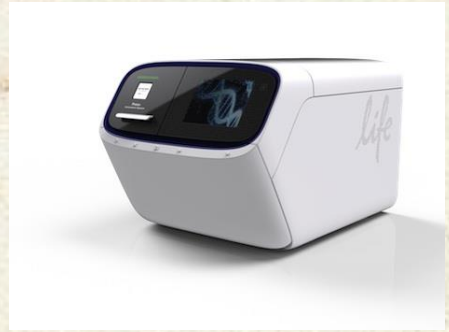


DNA Sequencing

- 1st Generation Sequencing: Sanger method



- 2nd Generation Sequencing (Next Generation Sequencing – NGS): massive parallel sequencing



- 3rd Generation Sequencing: single molecule DNA sequencing in real time

Next Generation Sequencing – NGS: massive parallel sequencing

- **Advantages**

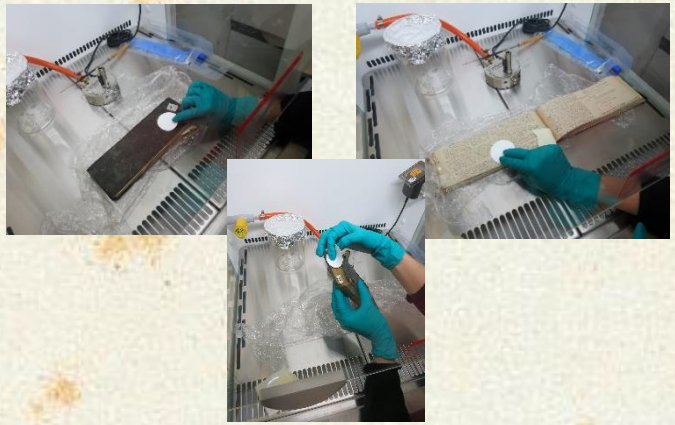
- High-throughput
- Low price
- *de novo* sequencing

- **Disadvantages**

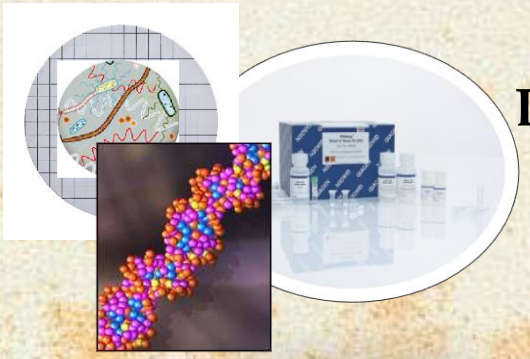
- No accurate sequencing of long homopolymeric fragments
- More challenging data analysis (a good…a **VERY GOOD BIOINFORMATICS TEAM**)


MICROBIAL COMMUNITIES
responsible of
BIODETERIORATION of our
CULTURAL HERITAGE

Microflora detection from book samples



Sampling
with nitrocellulose membrane
4 Samples



DNA extraction

PCR amplification
~ 700 bp

Bacterial 16S rRNA

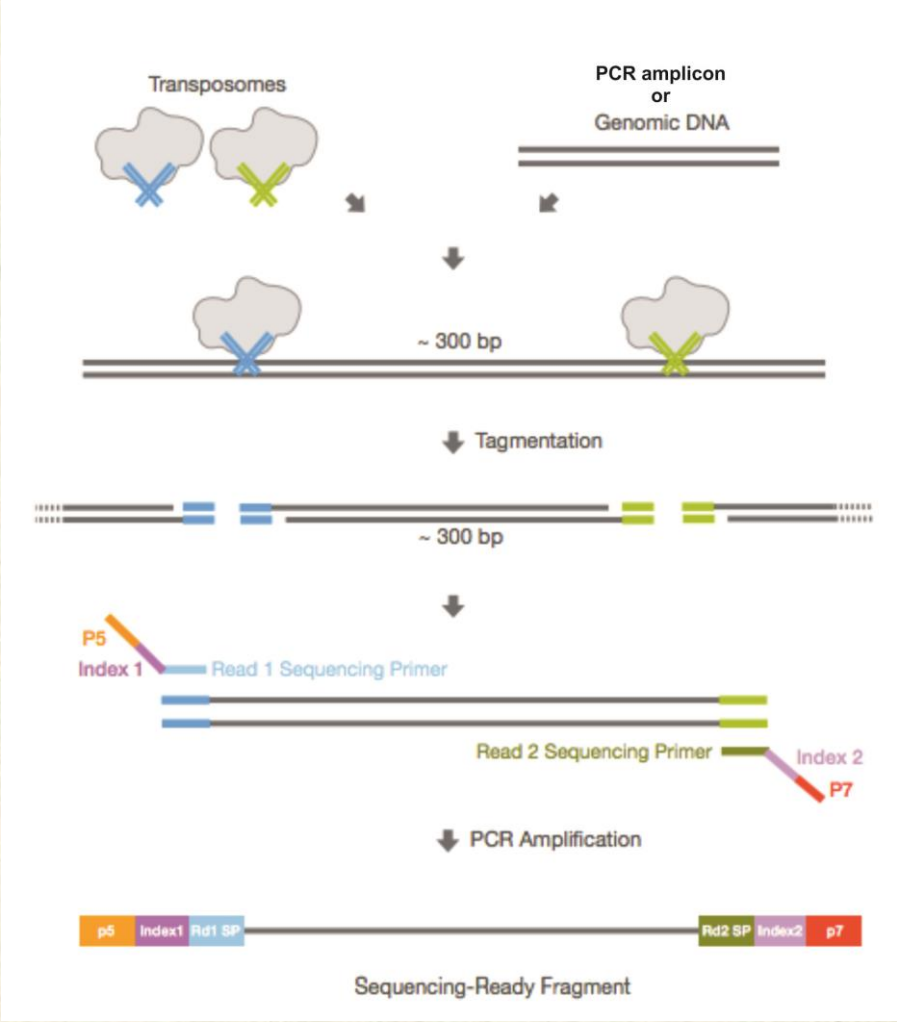
Fungal 28S rRNA

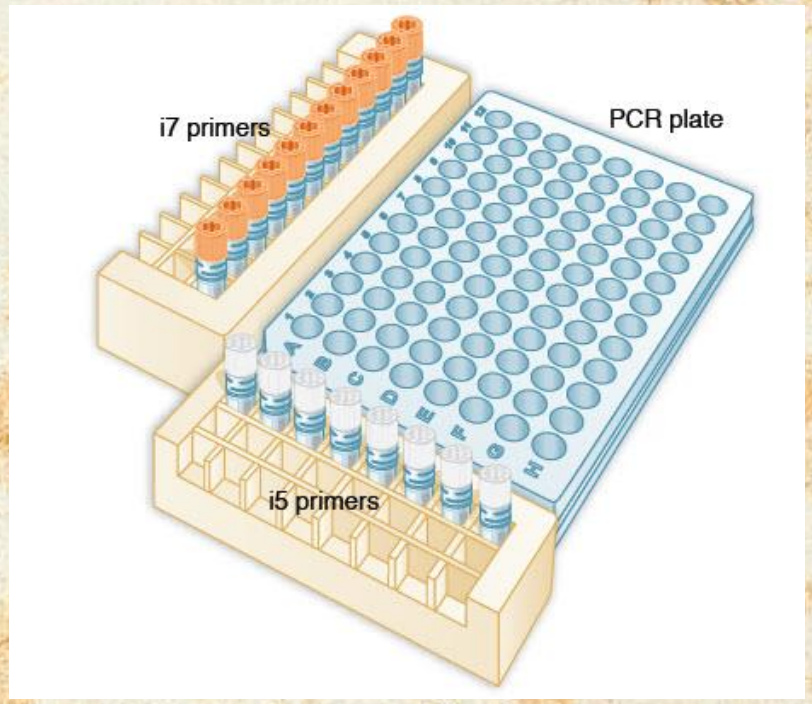
PCR purification

Ready for NGS analysis

Library preparation

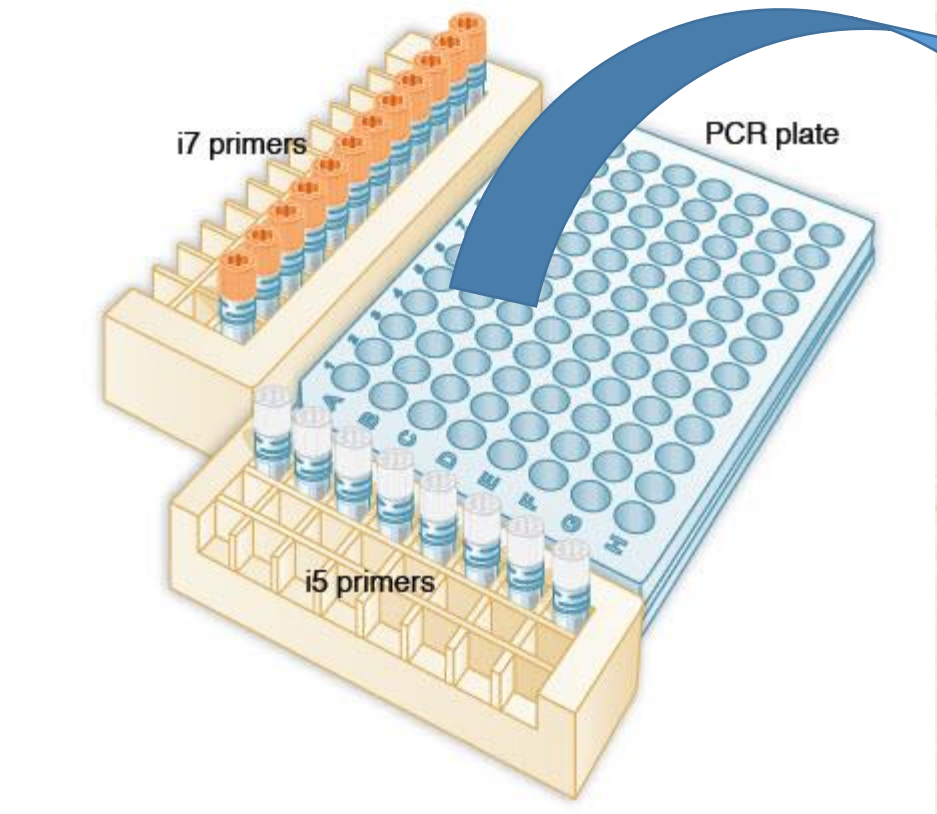
by Nextera XT Index kit

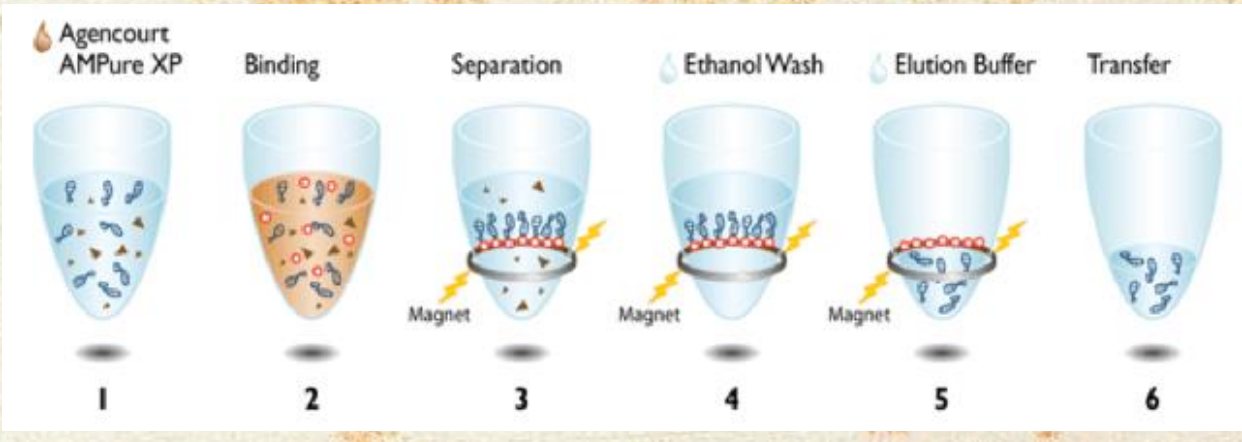




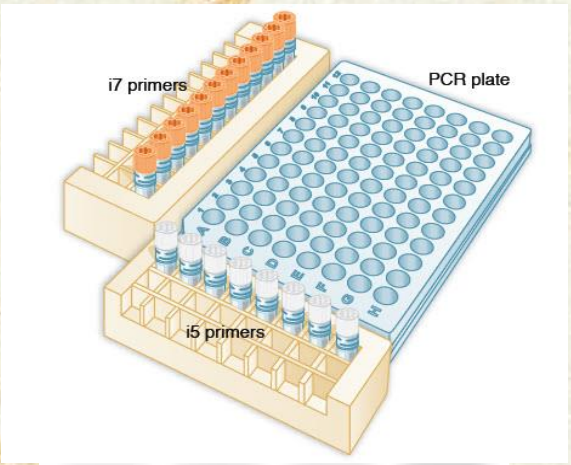
INDEX 1 (I7) SEQUENCE	INDEX 2 (I5) SEQUENCE
N701 TAAGGCGA	S501 TAGATCGC
N702 CGTACTAG	S502 CTCTCTAT
N703 AGGCAGAA	S503 TATCCTCT
N704 TCCTGAGC	S504 AGAGTAGA
N705 GGACTCCT	S505 GTAAGGAG
N706 TAGGCATG	S506 ACTGCATA
N707 CTCTCTAC	S507 AAGGAGTA
N708 CAGAGAGG	S508 CTAAGCCT
N709 GCTACGCT	
N710 CGAGGCTG	
N711 AAGAGGCA	
N712 GTAGAGGA	

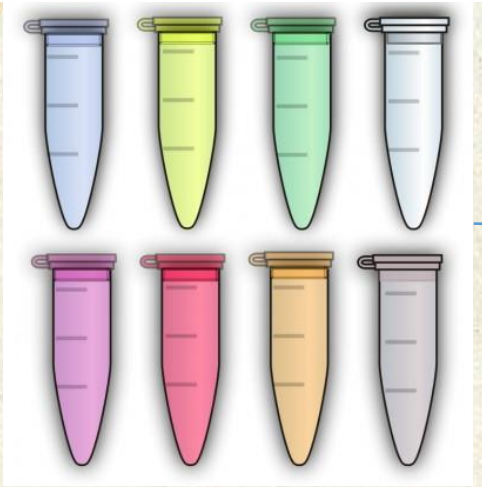
PCR purification



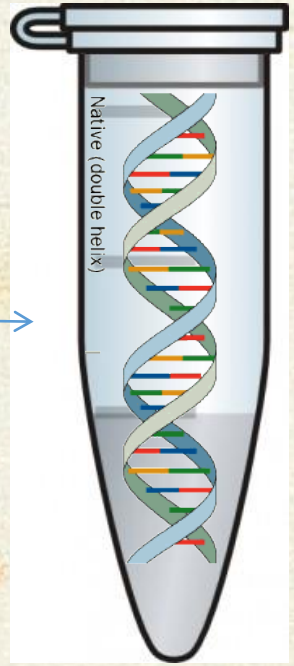


DNA sample pooling, denaturation and dilution





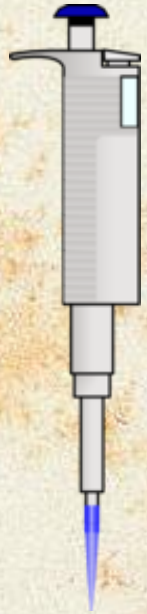
dsDNA libraries (2nM)



dsDNA libraries (2nM)

denaturation (0,2N NaOH) 1st dilution

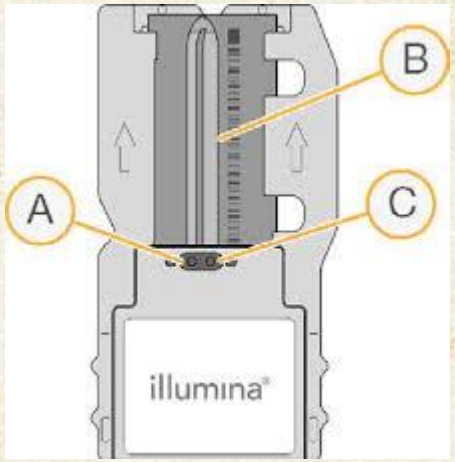
ssDNA (1nM)



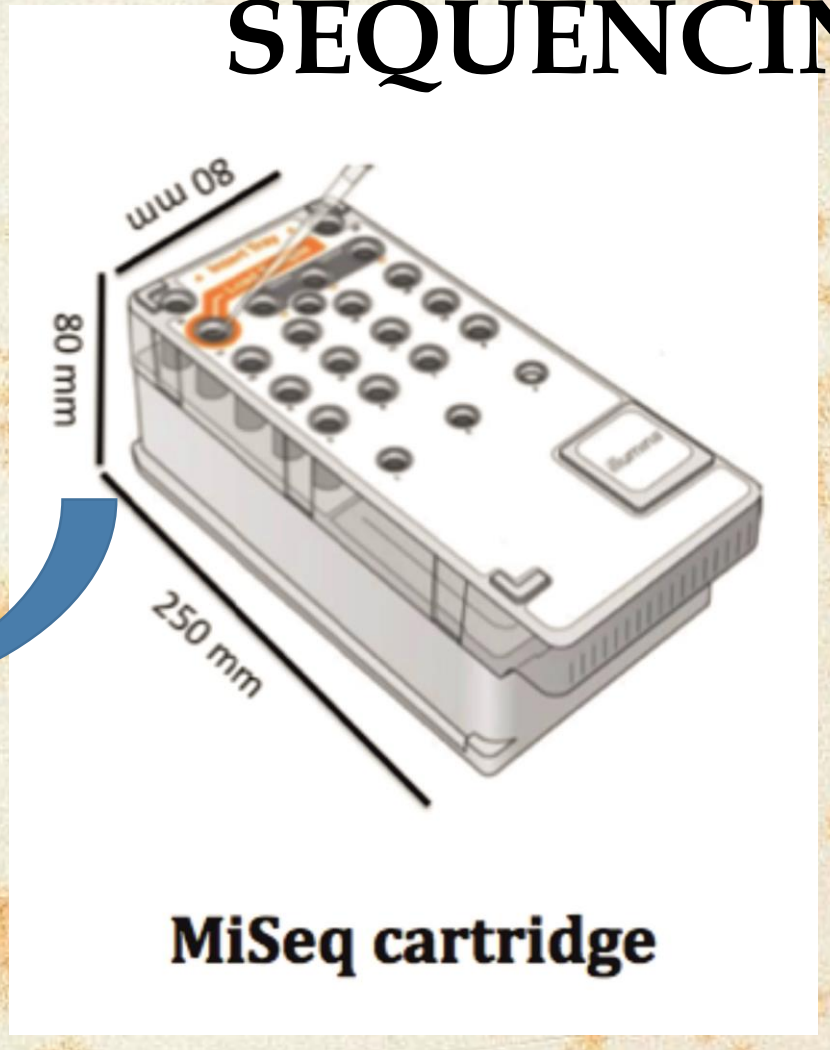
2nd dilution (hybridization buffer)

ssDNA (10-12pM)

SEQUENCING

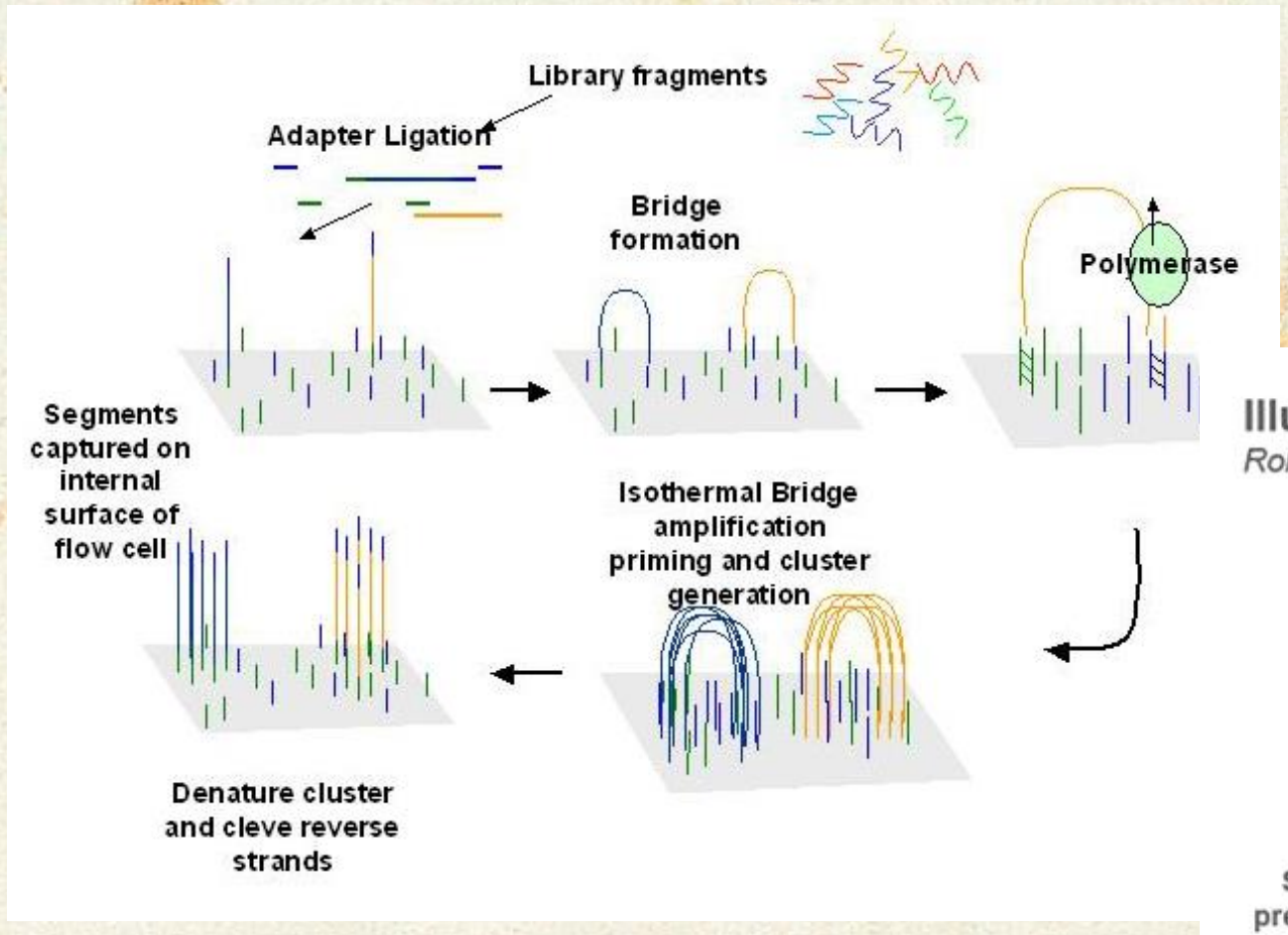


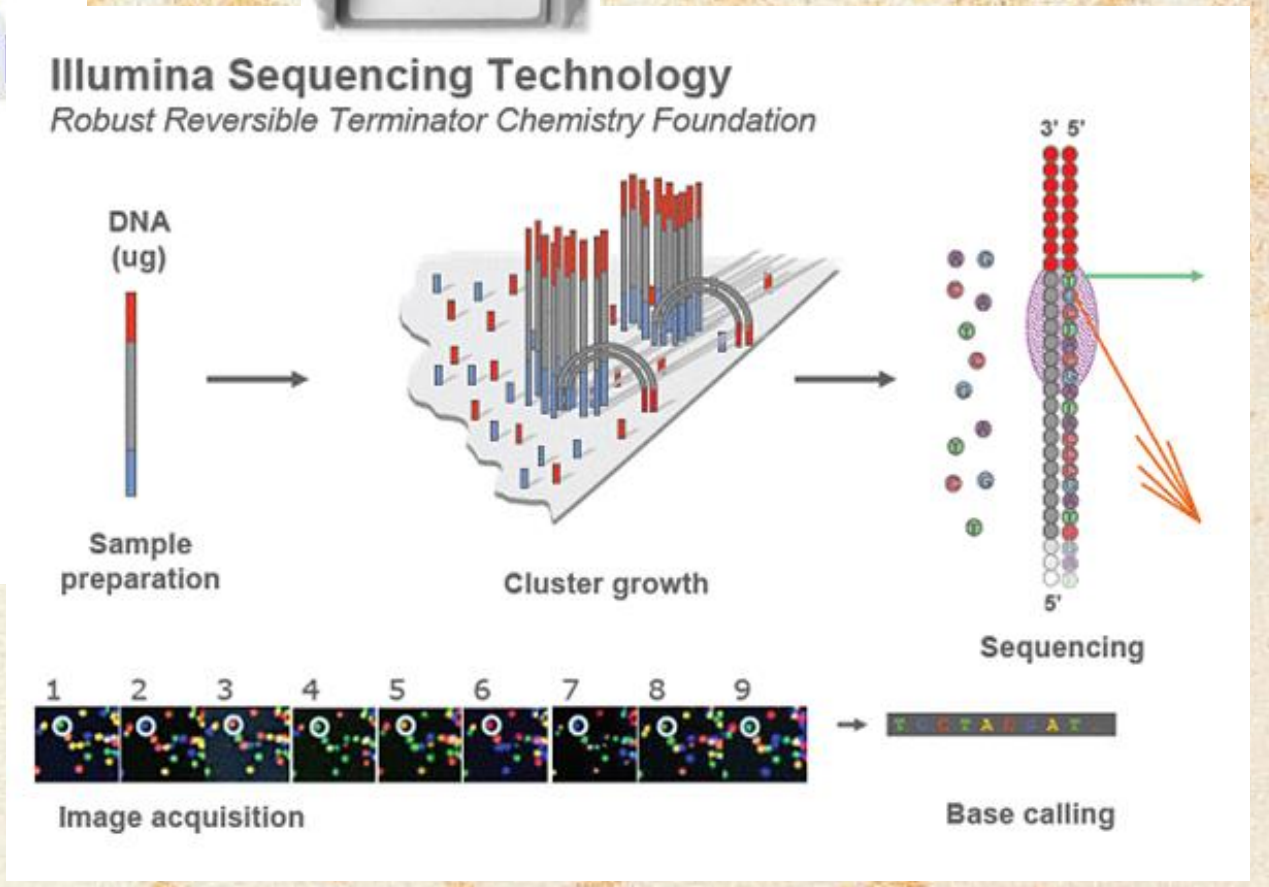
MiSeq chip



MiSeq cartridge

On MiSeq chip





Amazing Bioinformatics Work

BaseSpace®
Genomics Cloud Computing
powered by illumina®

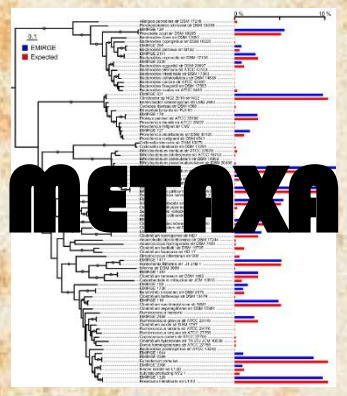
Software

Quantitative Insights Into Microbial Ecology
qiime

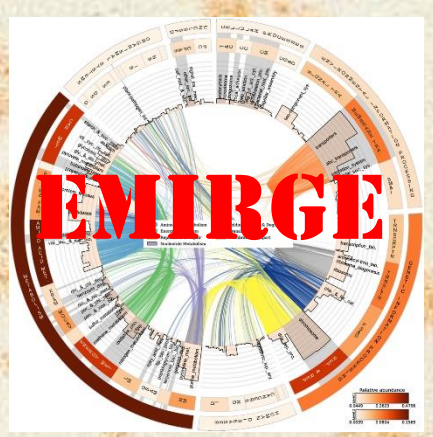
Databases

FunGene
functional gene pipeline & repository

mothur



GREENGENES
The 16S rRNA Gene Database and Tools

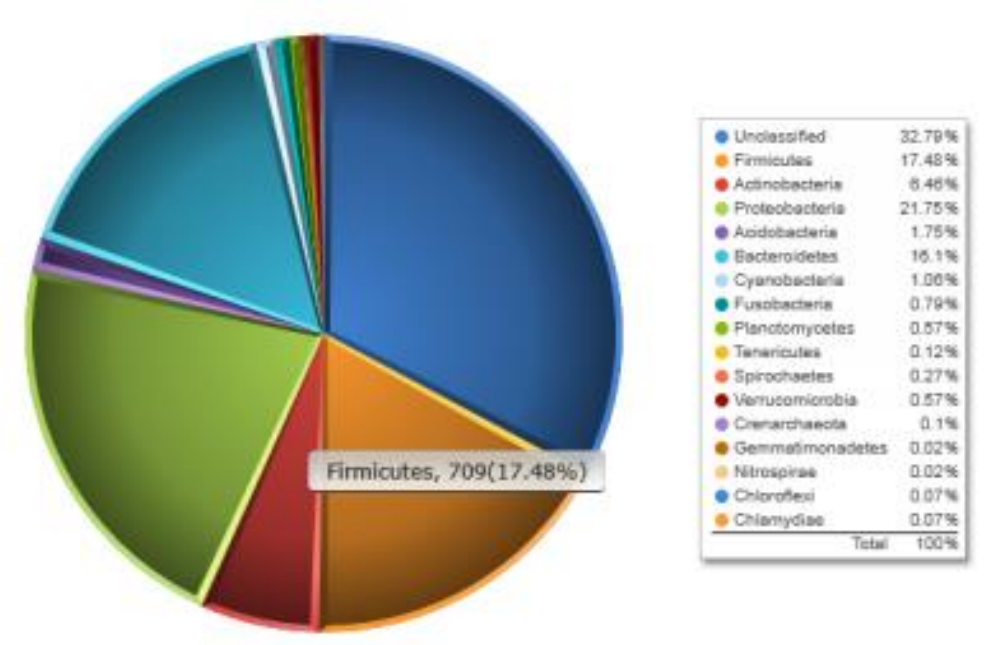


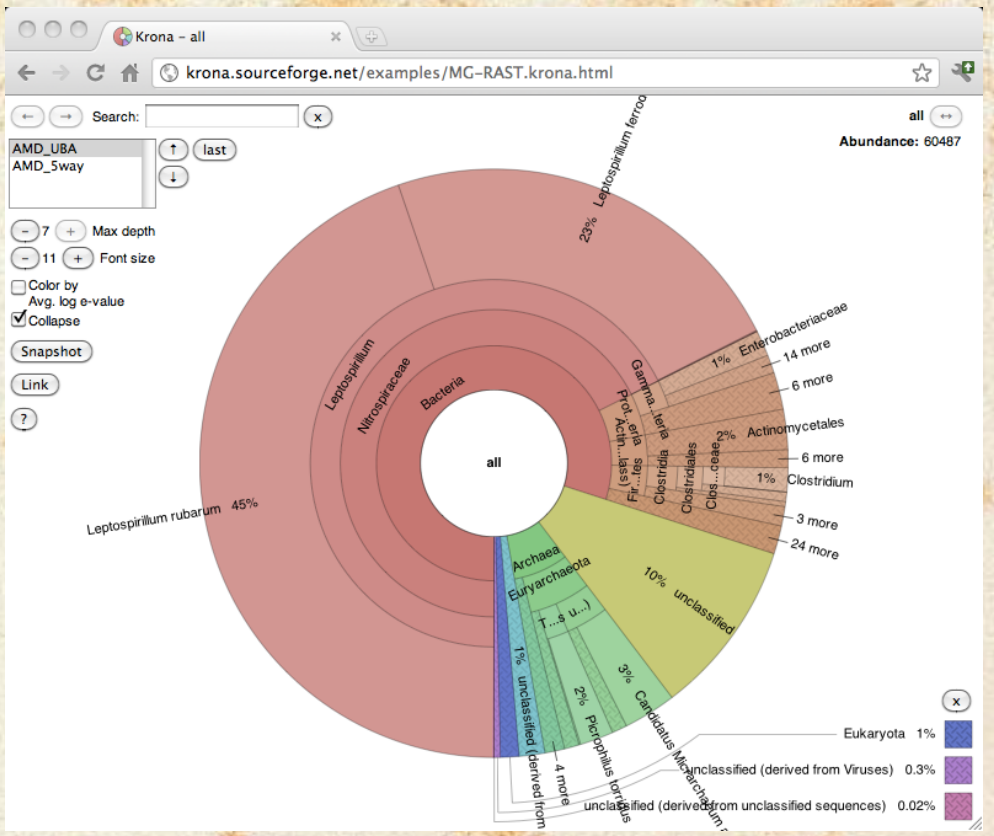
silva
high quality ribosomal RNA databases

rdp

Taxonomic classification

- Assigns taxonomic classification to each read
- 6 taxonomic levels (Domain, Phylum, Class, Order, Family, Genus)

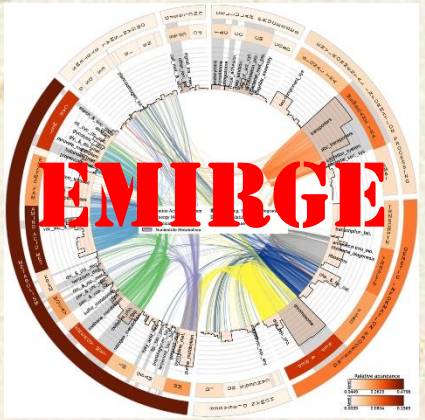


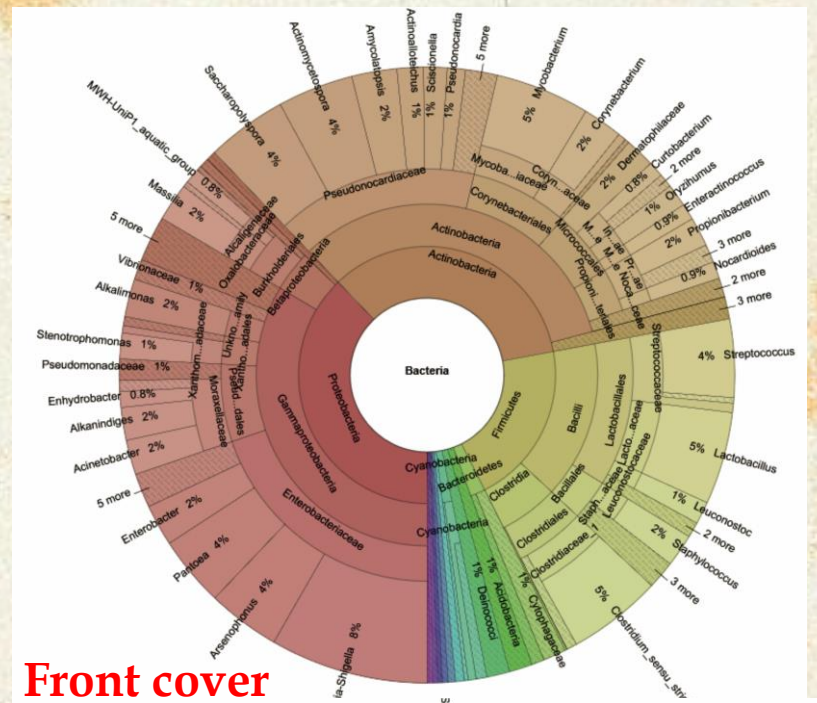


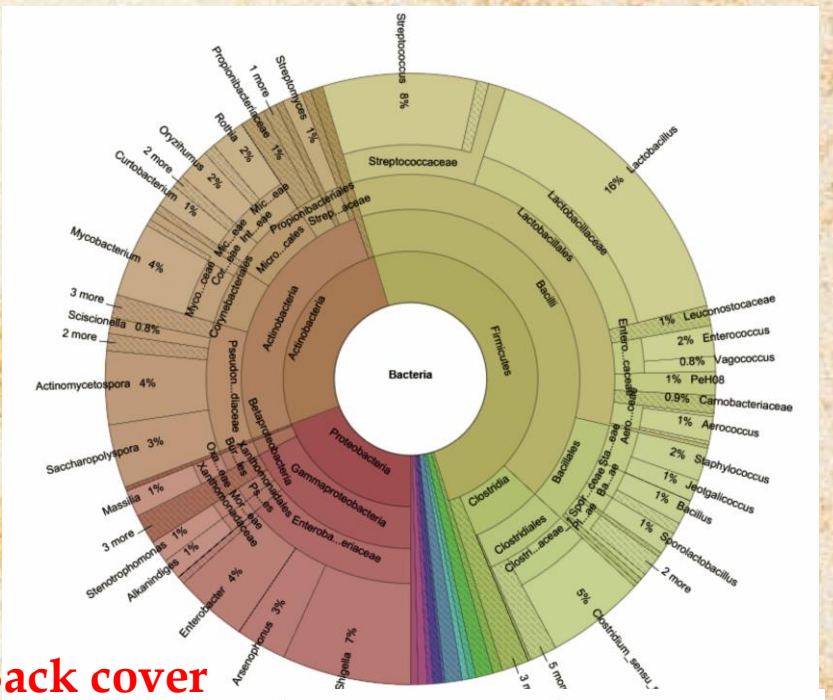
Our results

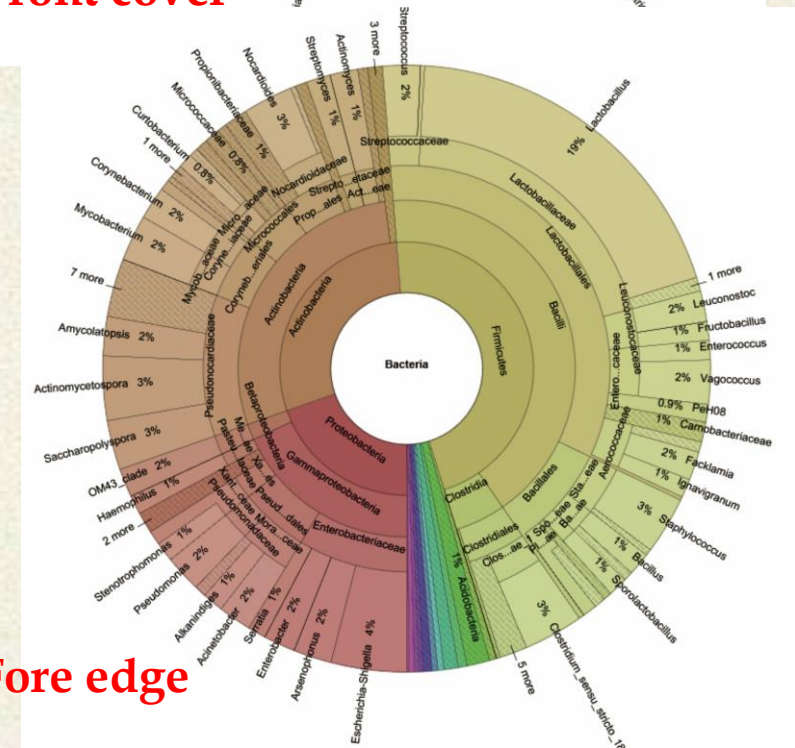
Bacterial 16S rRNA

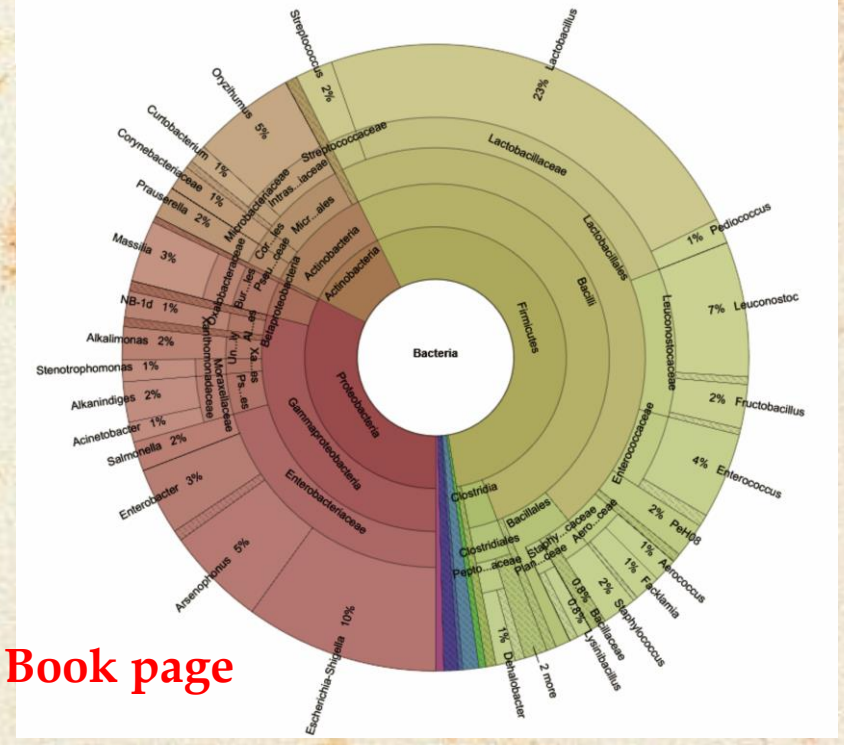
silva
high quality ribosomal RNA databases





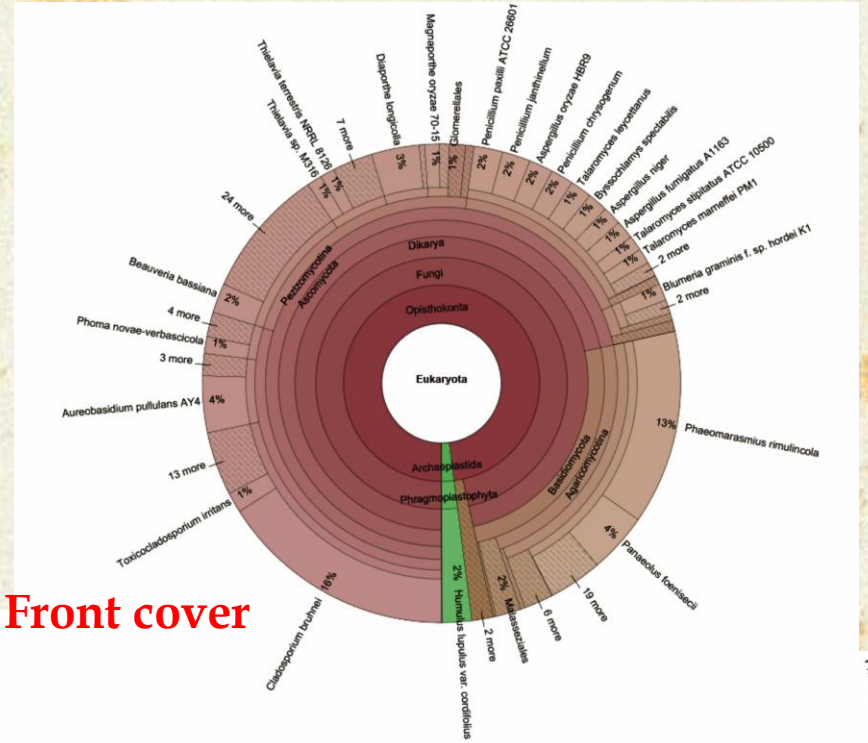




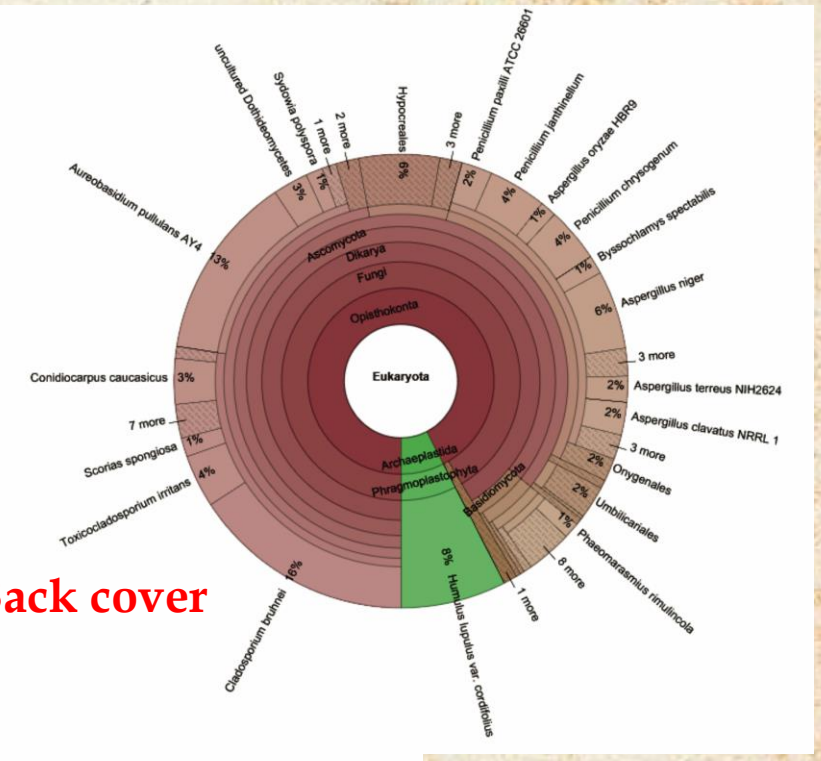


Our results

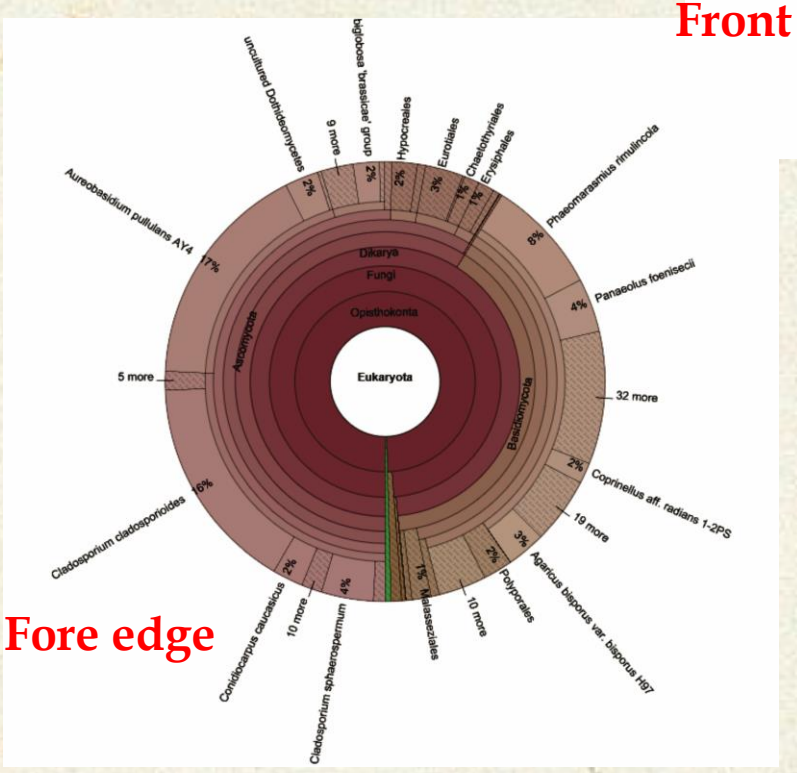
Fungal 28S rRNA



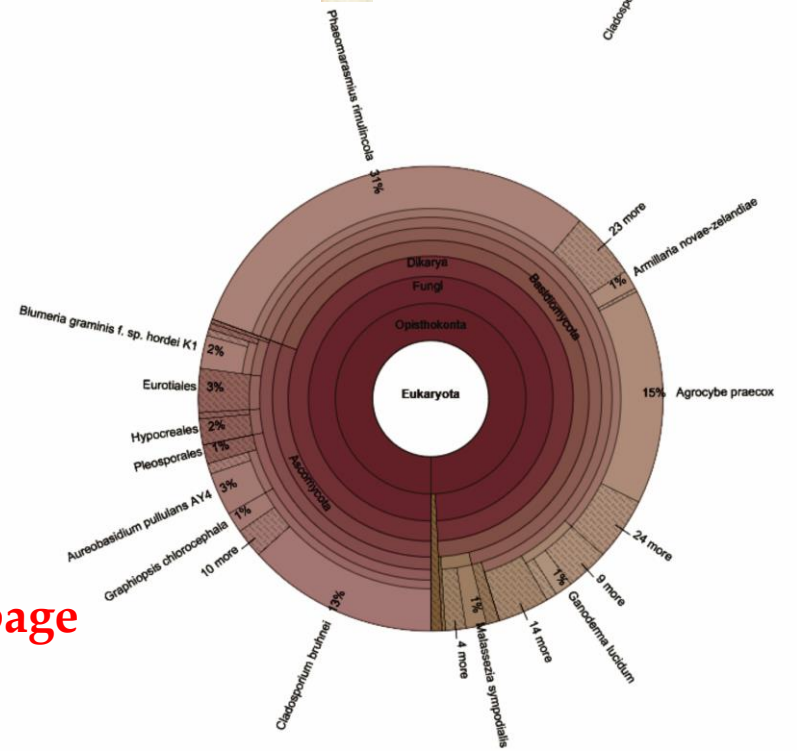
Front cover



Back cover



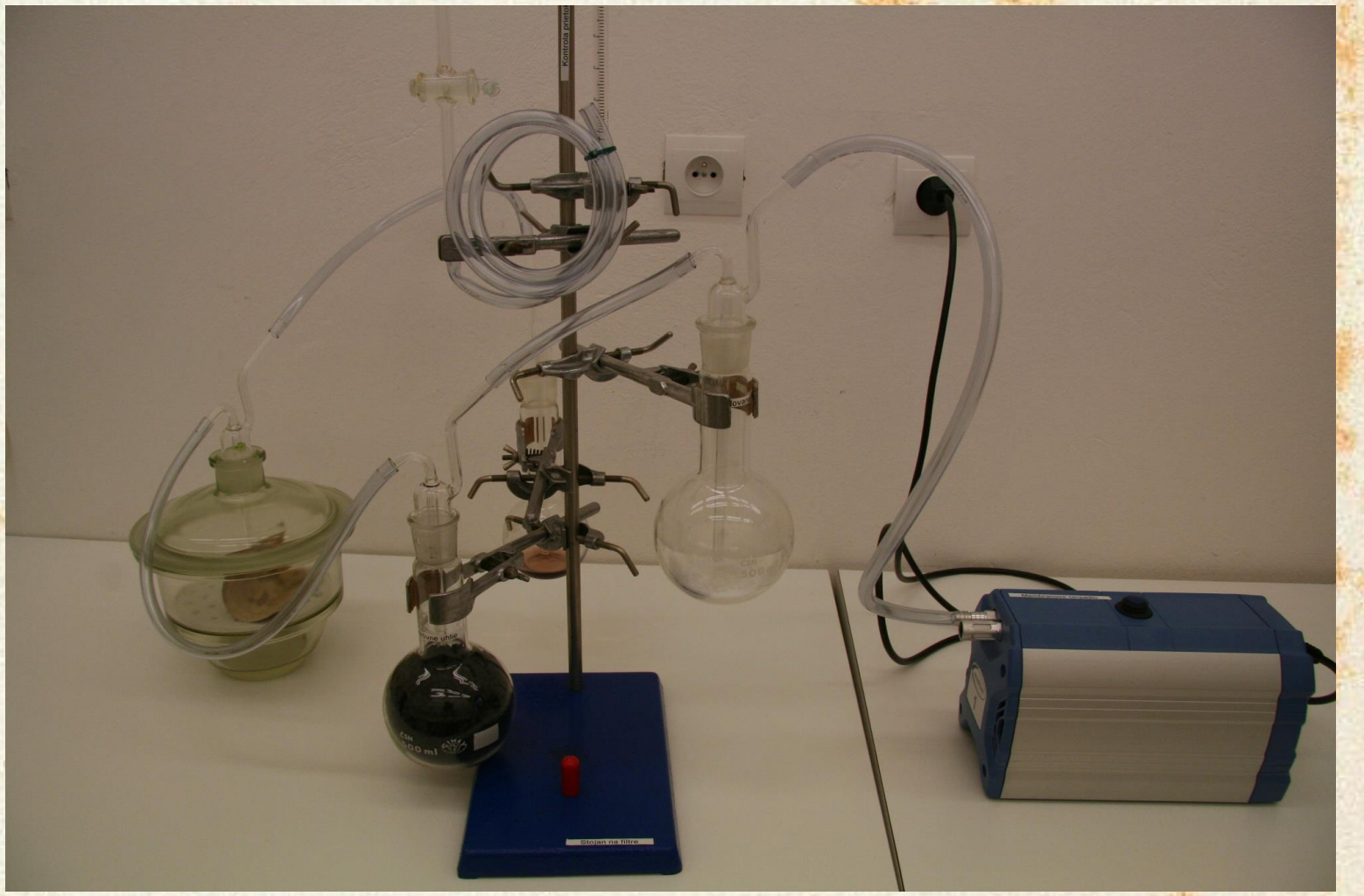
Fore edge



Book page

BACTERIA	FUNGI
Gammaproteobacteria	**Ascomycota**
Pantoea, Shigella, Pseudomonas, Enterbacter.....	*Cladosporium, Aureobasidium, Penicilli, Aspergilli…*
Firmicutes	**Basidiomycota**
Lactobacilli, Streptococci, Bacilli.....	*Agaricus, Phaeomarasmius….*
Actinobacteria	
Streptomyces, Rothia, Mycobacterium.....	
Betaproteobacteria	

Next Step
Disinfection by Essential Oils



Thank you

Der Kuss - G. Klimt

And nice evening…..

Acknowledgements

Visegrad Fund

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Institute of molecular biology S A S

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UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

Department of Chemical Technology of Monument Conservation

Illumina Group

Comenius University, Faculty of Natural Sciences

Department of Molecular Biology

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Tomáš Szemes
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Mária Bučková
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Lenka Jeszeová
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Dominika Svetlíková
Domenico Pangallo