UNIVERSALPLEX:

Multiplex PCR kit for the detection of major taxonomic groups

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MASTER'S THESIS

Master's Degree in Forensic Genetics, Physics and Chemistry

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September 2021

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ACKNOWLEDGEMENTS

First, I would like to acknowledge this project to Santiago Garcia Vallvé for the time he spent to follow and review this project, as to the excellent attention and ideas he suggested me during all the process. I would also like to thank him for believing in me since the beginning and give me a big freedom to design and write this thesis.

Secondly, I would like to thank Filipe Adão Macedo Pereira for bringing me the opportunity to have a wonderful internship in his company from Porto, and for guiding and teaching me that much.

Thirdly, I would like to aknowledge Raúl Beltrán Debón, Cristina Reguant Miranda and Javier Capilla Luque for sending us different samples from bacteria and fungi of big quality and allow us to start with laboratory tests.

Finally, I would like to thank my family and friends for the support and patience I received during this process, but also for the courage they constantly give me in all the decisions I make.

ABSTRACT

Non-human Forensic Genetics is a rapidly growing field from Forensic Sciences that has many applications and involves organisms from the whole tree of life: animals, plants, bacteria and fungi. Depending on the legal case that is studied, professionals might have the aim to identify a specific organism from an unknown sample, starting from zero. This makes them waste money, resources and time as they perform time consuming and sample wasting tests as to have an idea of what does the sample contain. Here, the creation of a new and promising commercial forensic kit is presented. The Universalplex kit has the aim to obtain an early detection of major taxonomic groups, achieving results more quickly and being a useful tool for forensic laboratories, as a preliminary test. It would detect biological material from bacteria, fungi, plants and animals in a single multiplex PCR. The groups specifically analysed in this study are bacteria and fungi. In this work, universal primers for bacteria and fungi have been found and tested both through bioinformatic tools and in the laboratory trough singleplex PCRs. The need to find new universal primers for both groups resulted in the designing of two more optimal pairs of primers, obtaining powerful *in silico* results, despite they have not been tested in the laboratory. Multiplex PCRs have been performed involving every single pair of primers selected for each group, and they have been tested in both groups, as to perform kingdom-crossing tests. A concluding qualitative approach, considering the electropherogram obtained when considering all four groups, has been designed to have a general idea about the usefulness of the Universalplex kit.

1. INTRODUCTION

Forensic Genetics is a branch of Forensic Sciences defined as “the application of genetics to human and non-human material (in the sense of a science with the purpose of studying inherited characteristics for the analysis of inter- and intra-specific variations in populations) for the resolution of legal conflicts” (Carracedo, 2007). Despite this discipline has basically been dedicated to human forensic genetics (HFG), the use of non-human DNA analysis in forensic cases is having a rapid growth, showing successful results (Amorim, 2010). The limited application of non-human forensic genetics (NHFG) is due to many reasons, such as the lack of knowledge of its informative potential, material and theoretical difficulties (Oliveira et al., 2019), the scarce of ethical and legal guidelines and standards, the need of a huge cooperation between scientists of different disciplines (Amorim, 2010, 2019) and the novelty of applying analytical techniques to these kind of samples (Sensabaugh & Kaye, 1998). NHFG has different approaches and can be applied both in a judicial scope or outside a court environment, in order to solve legal conflicts (Oliveira et al., 2019).

1.1. STANDARDIZED APPROACH

The experimental methodologies used in NHFG are similar to those used for human samples, starting with the solicitation of a genetic test and followed with the collection and transportation of samples, correctly labelled and using optimal conditions. It is important to be sure that traceability of samples is well known through the application of chain of custody if the procedure is linked to a legal investigation. In laboratory, extraction and storage steps are performed before the application of a genetic test. In the end, genetic results are compared with other genetic information to finally perform a statistical analysis. Used protocols, kits and techniques have to be correctly validated. However, there are some situations in which biological material correspond to unknown samples or new taxonomic groups never studied before and thus, methods might be not previously available. This happens when dealing with environmental and wildlife materials, where the use of voucher specimens is not possible and identification has to

be done through other strategies; such as sequencing of a gene region and comparison using a database repository like GenBank (Arenas et al., 2017; Linacre et al., 2011; Oliveira et al., 2019).

1.2. MAIN APPLICATIONS

The roles of NHFG are several and are increasing during these last years (Amorim, 2010, 2019; Oliveira et al., 2019):

- Non-human silent witnesses associated with criminalistics and being an associative evidence.
- Frauds, pharmacology and food security, linked to the correct labelling of products.
- Infractions committed upon protected species and their products, in correlation with wildlife crimes.
- Fishing and haunting violations.
- Individual, specific or group identification of organisms or their products in cases of body or property injury and damage, death, robbery, or those involving pathogens, such as in bioterrorism.
- Identity, genealogy and lineages falsification, where kinship tests are performed.
- Consumption products, forbidden businesses or traceability infractions.
- Post-mortem interval estimation through the species identification of cadaveric animals or plants.
- Death cause investigation through the identification of biological toxics production.

1.3. NHFG CLASSIFICATION

The variety of applications derives in the classification of four types of NHFG: 1) zoology, 2) botany, 3) microbiology and 4) food analysis and traceability. Regarding **zoology**, there are already many kits for the identification and parentage testing for animals (Carneiro et al., 2012; Kanthaswamy et al., 2021). These kits are based on the amplification of autosomal STR markers and are performed in a variety of situations,

such as cases of silent witnesses, wildlife law enforcement or animals doping. However, there are more regions used in forensic zoology: barcoding regions such as cytochrome c oxidase I gene (COI) (Linacre et al., 2011; Pentinsaari et al., 2016), cytochrome B gene (CYTB) (de Pancorbo et al., 2004) or rDNA genes. Genotyping of these regions through their amplification with conserved and specific primers and followed with the sequencing, helps to reach species identification by phylogenetic analyses using databases information (Waugh, 2007) (for more information, see point 1.4). What forensic **botany** studies is of relevant importance when talking about cases of illegal drugs trafficking, bioterrorism, transport and commercialization of exotic species or in crime scene investigations (cases where the autopsy and the crime scene evidences are not enough conclusive). Plant evidence can help knowing information about a death, such as the time, the manner, if it was an accident, suicide or homicide, etc. It can also help to know if the crime scene was a primary or a secondary scene (Coyle et al., 2005). There are many barcoding regions studied in plants, such as maturase K (matK), ribulose bisphosphate carboxylase large chain (rbcL) or trnL (UAA) genes (Fazekas et al., 2008; Hollingsworth, 2009). On the other hand, **microbial** forensics is a powerful and promising field that has also many applications. They include: terrorism and use of biological warfare agents, biocrimes, frauds, investigation of infectious disease outbreaks and transmission of pathogens or, with the analysis of the human microbiota, geolocation, body fluid characterization or postmortem interval estimation (Lehman, 2014; Ventura Spagnolo et al., 2019). Despite this, microbial forensics has three main problems: 1) there are insufficient standards and guidelines regarding its use, 2) the lack of big informative reference databases and 3) the poorness value of a found match between reference samples and evidences, due to the asexual character of bacteria and viruses' reproduction (Arenas et al., 2017; Sensabaugh & Kaye, 1998). Last but not least, the **forensics of food analysis and traceability** has the aim to investigate the biological composition of food and other commercial products. Through the study of the geographic origin, the species, variety or cultivar, forensic laboratories can guarantee the exact content of those products when their labels don't provide the right or sufficient information. This is so important to ensure consumer choices, either for health concerns or dietary options (Galimberti et al., 2013; Oliveira et al., 2019).

1.4. TAXONOMIC IDENTIFICATION AND SPECIES ASSIGNMENT

The breakthrough of the polymerase chain reaction (PCR) technique has revolutionized the world of molecular biology and genetics, among others. This technique only requires a small amount of template DNA and it allows the amplification of short fragments, allowing the detection of DNA in degraded samples (Pereira et al., 2008). Its development it's also linked to the invention of many molecular techniques used in forensic genetics. Taxonomic identification of organisms is one of the main applications of NHFG, for example with the identification of wildlife protected species and products, among other cases involved in legal investigations (Amorim et al., 2020). Some of conventional molecular biology techniques used for species identification are: 1) **DNA hybridization** by complementary DNA oligonucleotides based on probes constructed with DNA from target species, 2) **RFLP or STRs analysis** to generate specific band profiles that detect interspecies variation, 3) **SNPs analysis**, to detect single-nucleotide variations between species 4) **AFLP analysis**, consisting in the digestion of purified gDNA with restriction enzymes, the ligation of adapters to the sticky ends of the DNA, and the following amplification of these adapters, 5) the **RAPD profiles analysis**, that is done through the random amplification of DNA fragments using short primers and low annealing temperatures, which generates a specific banding pattern for each specie, 6) **indel variants analysis**, reviewing the length of these hypervariable regions, for example with the SPinDel method 7) **conventional PCR**, producing amplicons using species-specific designed primers, 8) **quantitative PCR (Q-PCR)**, detecting the presence and quantifying the initial amount of a specific DNA fragment, or 9) **DNA sequencing analysis**, comparing the sequence of the PCR-amplified region with reference sequences from a database such as GenBank (Carneiro et al., 2012; Oliveira et al., 2019; Pereira et al., 2008).

The use of the presented genetic techniques usually requires both a previous classification and identification of the taxa to work with, and species-specific molecular toolkits. **DNA barcoding** is a rapid and accurate technique to identify species by the analysis of diversity among a standard target gene's DNA sequence (Hebert et al., 2003; Stoeckle, 2003). If the DNA barcoding is combined with the sequencing tool, it is possible to also solve the problem of complex genomic mixtures (Bell et al., 2016). Sometimes

this taxa filtration and species identification is not achieved due to many reasons such as the unknown origin of the sample, and this can negatively affect the following genetic analysis. To overcome the described problem, it is important to first detect the taxonomic kingdom present in a sample in a more general way and as a first approach. The gold-standard marker usually used to detect organisms from the animal kingdom is COI (Dalton & Kotze, 2011; Dawnay et al., 2007). In bacteria identification it's the 16S rDNA gene (Barghouthi, 2011; Takahashi et al., 2014), while the 18S rDNA gene or the internal transcribed spacer (ITS) region are used in fungi identification (Banos et al., 2018; Stielow et al., 2015; Xu, 2016). There are many genomic, mitochondrial and chloroplast DNA regions used as markers for the identification of plants, such as cyclooxygenase 1 (cox1), matK, rbcL or trnL genes, or the ITS region (Cheng et al., 2016; Demesure et al., 1995).

1.5. THE FUTURE IS BRIGHT

As the importance of this emerging field is notable, the future of NHFG has to face the removing of the limitations mentioned at the beginning of this introduction. NHFG needs a bigger funding, more standards, quality control programs and guidelines, the adaptation and validation of scientific protocols to take them to court, the expansion of databases and phylogenetic studies, a better cooperation between scientists from different fields and the development of powerful probabilistic tools for statistical studies, among others (Amorim, 2019; Arenas et al., 2017; Oliveira et al., 2019; Sensabaugh & Kaye, 1998). Despite gold-standard markers are very good described in literature, the existence of a useful and time-saving tool to correctly identify each taxonomic kingdom at the same time using a single sample is still missing. In this study, we develop a new forensic kit for an early detection of major taxonomic groups in forensic samples of unknown origin. Specifically, we provide the description of methods used to accomplish the detection of biological material from bacteria and fungi kingdoms through a multiplex PCR.

2. HYPOTHESIS AND OBJECTIVES

Forensic laboratories often have to deal with biological samples for which there is no clue about their origin. In consequence, they perform time consuming and sample wasting tests in order to identify the contents of the sample. The main objective of this thesis is to **help designing and developing a new forensic kit** called Universalplex for an early detection of major taxonomic groups in forensic samples of unknown origin. The kit would allow to achieve results more quickly and would be a useful tool for forensic laboratories, as a preliminary test. It would detect biological material from bacteria, fungi, plants and animals in a single reaction: a multiplex PCR. The universalplex method is achieved by the amplification of three hypervariable regions for each taxonomic group labelled with a unique fluorescent dye. The primers are designed in highly conserved genomic regions to guarantee successful amplifications in the highest possible number of species. The groups specifically analysed in this study are bacteria and fungi.

To achieve the main objective, the project has the aim to search universal primers that are specific for each taxonomic group. It intends to design a sequences library for each studied group to test the viability and conservation of the selected primers, as well as design new optimal primers. In addition, a prediction of amplicons length is done for the further design of the multiplex PCR.

Furthermore, a verification of *in silico* results is done in the laboratory. This process includes: DNA extraction from bacteria and fungi samples, DNA quantification, preparation of singleplex PCRs in order to test each pair of primers and finally, the execution of a multiplex PCR to test all the primers in both groups. The conditions of the multiplex PCR are lastly revised so as to be optimal.

3. METHODS

3.1. *IN SILICO* METHODS

3.1.1. Primers search

A preliminary search for universal primers was done for each kingdom using published literature. The aim of this search was to find primers suitable to anneal to all genomes from a specific kingdom, but not capable to anneal to organisms from other kingdoms. As a result from this search, target regions to work with were found. A total of three pairs of primers, and consequently three regions, were selected for each taxa. By picking three different regions from the same group, redundancy is increased and it is easier to be sure the identification is achieved.

3.1.2. Sequences library creation

The development of a library of DNA sequences for each kingdom was done with the software Geneious Prime® 2021.1.1 (Biomatters, New Zealand), using the NCBI Nucleotide database and the NCBI Genome Taxonomy Database, both found inside the mentioned software. The purpose of these libraries was to have a compilation of a coherent number of sequences with which to work having an optimal efficiency and avoiding the collapse of the software, but enough to represent the entire variety of the selected kingdom. The target regions to download coincide with those regions with which the selected primers were supposed to anneal.

3.1.2.1. Bacteria library

Three different bacteria sequences libraries were created: a 16S rDNA gene folder and a 23S rDNA gene folder, both for those species that have these genes available independently, and a cluster (16S-ITR-23S region) folder for those species that have the full sequence of the rDNA cluster available. A total of 499 species representing the major number of bacteria genus were selected (Annex 1).

The species were selected using three different sources of literature and databases. First of all, sequences from species represented on the interactive Tree Of Life (iTOL) source (Letunic & Bork, 2021) were retrieved. Then different scientific publications (Barghouthi, 2011; Hofstadler et al., 2005; Ludwig, 1994; Santos & Ochman, 2004) were checked to ensure the most commonly studied species were represented in the library. Finally, the Alphabetical Table Browser from the Genome Taxonomy Database (Parks et al., 2018, 2020) was consulted to increase the size of libraries and guarantee the representation of a maximum number of bacteria genus.

Lastly, both those sequences having few undetermined bases showed as Ns and other ambiguous positions, plus those sequences starting after or ending before the predicted regions were removed and replaced for new ones.

3.1.2.2. Fungi library

Two different fungi sequences libraries were created: a 18S rDNA gene folder and a 28S rDNA gene folder. A total of 278 species representing the major number of fungi genus were selected (Annex 2).

The species were chosen considering three different sources from literature. Firstly, a list of organisms retrieved from Table S1 from the work "A genome Tree of Life for the Fungi kingdom" (Choi & Kim, 2017) was used to obtain the first set of sequences. Secondly, more sequences were retrieved for those species represented in Figure 1 from the study "Reconstructing the early evolution of Fungi using a six-gene phylogeny" (James et al., 2006). Finally, Figure 3 from the article "High-level classification of the Fungi and a tool for evolutionary ecological analyses" (Tedersoo et al., 2018) was used as the third source to increase the number of selected species and to be sure that the maximum number of fungi genus were represented.

A last step of removing and replacing sequences was performed for those containing ambiguities and undetermined positions, and for those starting after or ending before the predicted regions from genes.

3.1.3. Multiple alignments

Once all sequences libraries were created, a step of alignment was performed for each folder, including all sequences contained inside. Multiple alignments were done with Geneious software using the MAFFT high speed multiple sequence alignment program, with default conditions. A second step of filtering was made to make the alignments cleaner, removing those sequences that were excessively divergent, as it was a clue of inconsistency with NCBI default annotations. Two alignments for each kingdom group were obtained. The 16S bacteria alignment was performed using sequences from the 16S rDNA gene folder and the cluster folder. 23S bacteria alignment was done using sequences from the 23S rDNA gene folder and the cluster folder. On the other hand, 18S fungi alignment was made with sequences from the 18S rDNA gene folder, while 28S alignment was made with sequences from the 28S rDNA genes folder.

3.1.4. Literature primers test

Once alignments were done, consensus sequences were extracted. Sequences from primers were then introduced in the Geneious software. Using the tool “Map primers”, it was possible to anneal primers to their corresponding consensus sequences. Following that, a statistical analysis was done for each primer. First of all, the Nucleotide Statistics from Geneious was used to retrieved some information, such as number of missmatches or pairwise identity. Two sources used to analyse non-specific interactions were: 1) OligoCalc (Kibbe, 2007), to obtain information about possible self-complementarities, as well as to know the melting temperature for each primer, and 2) AutoDimer software (Vallone & Butler, 2004) to check possible cross complementarities between primers.

3.1.5. New primers creation

A part from the selected primers, new ones were manually designed with Geneious software. New primers should keep some parameters: to have a length between 18 and 24 bp, a melting temperature between 52 and 66ºC, avoid secondary structures, GC content range of 30-70%, avoid repetitions of Gs and Cs longer than three bases and,

ideally, place Gs and Cs at the end of primers (Behind The Bench Staff, 2019; Buck et al., 1999).

The aim of this step was to increase the number of *in silico* valid primers with whom to work when designing the final multiplex PCR (for example, in case of facing two amplicons with similar length, to design a shorter/longer amplicon using a different pair of primers).

3.2 EMPIRICAL TESTS

3.2.1 Bacterial and fungal samples

Eleven bacterial samples and eight fungal samples were collected both as colonies in plates or as suspension in eppendorf tubes. Isolated samples were obtained from different groups of research belonging to Rovira i Virgili University (URV) and some other were already available inside IDENTIFICA Genetic Testing laboratory, and are reported in Table 1.

3.2.2 DNA extraction

Bacterial DNA was extracted using the PureLink® Genomic Mini Kit (Life Tecnologies, 2013). 100 mL of each sample was resuspended with 180 µL PureLink® Genomic Digestion Buffer and 20 µL of Proteinase K, and then it was incubated at 55ºC for two hours. After the lysis step, 20 µL of RNase A, 200 µL of PureLink® Genomic Lysis/Bindng Buffer and 200 µL of pure ethanol were added to the lysate, mixing well between all steps. The lysate was then added to the spin column supplied with the kit placed in a collected tube and centrifuged at 10,000 x g 1'. The collection tube was then discarded. To wash the DNA, two steps of adding 500 µL of Washing Buffer, centrifuge at 10,000 x g 1' and discard the collection tube, were performed. Finally, to recover DNA 100 µL of PureLink® Genomic Elution Buffer was used.

Each fungal sample was washed with etanol and then was placed in a mortar in which 500 µL of STE 1X (Sodium Chloride-Tris-EDTA) were added. Fungal cells were then squashed, as quartz particles were added. The lysate was then added to an Eppendorf

Table 1. *Samples used to test in-silico validated primers through PCR amplification: group, assigned code, specie, type of sample and origin of the source*

Group	Code	Phylogeny	Type	Origin
Bacteria	BAC1	*Bacillus sp.*	Suspension	URV
	BAC2	*Klebsiella sp.*	Suspension	URV
	BAC3	*Proteus mirabilis*	Suspension	URV
	BAC4	*Salmonella sp.*	Suspension	URV
	BAC5	*Staphylococcus aureus*	Suspension	URV
	BAC6	*Streptococcus faecalis*	Suspension	URV
	BAC7	*Leunostoc mesenteroides*	Plate	URV
	BAC8	*Lactobacillus brevis*	Plate	URV
	BAC9	*Oenococcus oeni*	Plate	URV
	BAC10	*Pseudomonas aeruginosa*	Plate	URV
	BAC11	*Escherichia coli*	Suspension	Filipe Pereira
Fungi	FUN1	*Alternaria alternata*	Suspension	URV
	FUN2	*Candida albicans*	Suspension	URV
	FUN3	*Candida krusei*	Suspension	URV
	FUN4	*Cladosporium*	Suspension	URV
	FUN5	*Rhizopus oligosporus*	Suspension	URV
	FUN6	*Torulaspora delbrueckii*	Plate	URV
	FUN7	*Saccharomyces cerevisiae*	Plate	Filipe Pereira
	FUN8	*Neurospora crassa*	Suspension	URV

and the classic phenol/chloroform extraction protocol (Al-Samarrai & Schmid, 2000) was followed. Fungal DNA was eluted using 200 µL of TE (Tris-EDTA) tampon.

DNA concentrations were obtained by spectrophotometry using a NanoDrop instrument. The concentration of each DNA sample was adjusted to obtain a final concentation of 10 ng/mL.

3.2.3 PCR amplification

Amplification of target regions using universal primers was performed using the SimpliAmp™ Thermal Cycler from the company Applied Biosystems™, from which primers were also ordered.

Single polymerase chain reactions were conducted in 0,2 mL tubes containing 5 µL of QIAGEN Multiplex PCR Master Mix, 1 µL of the forward primer and 1 µL of the reverse primer (both with a final concentration of 2 µM), 2 µL of RNase-free water and 1 µL of template DNA (or water, in case of negative control). Despite the conditions were later modified to increase the efficiency of the reaction, the initial conditions were: 95ºC for 15 min, 35 cycles of 94ºC for 30', 55ºC for 1 min, and 72ºC for one min, with a final extension of 72ºC for 10 min.

Multiplex polymerase chain reactions were conducted in 0,2 mL tubes containing 5 µL of QIAGEN Multiplex PCR Master Mix, 1 µL of the primers mix[1] (all primers with a final concentration of 2 µM), 3 µL of RNase-free water and 1 µL of template DNA (or water, in case of negative control). Despite the conditions were later modified to increase the efficiency of the reaction, the initial conditions were: 95ºC for 15 min, 30 cycles of 94ºC for 30', 55ºC for 1 min, and 72ºC for one min, with a final extension of 72ºC for 10 min. PCR amplifications were finally assessed on 1% agarose gels in 1X Tris-acetate (TAE).

[1] In order to perform this multiplex PCRs, a mix of all the primers from the same kingdom was performed for each group.

4. RESULTS AND DISCUSSION

4.1. UNIVERSAL BACTERIAL PRIMERS STUDY

4.1.1. *In silico* approach

Three pairs of universal bacteria primers were selected (Decimo et al., 2017; Eshoo et al., 2010; Laloui et al., 2002; Muyzer et al., 1993; Soergel et al., 2012); two pairs mapping the 16S rDNA gene region and one mapping the 23S rDNA gene region. Table 2 lists the characteristics for each pair of primers analyzed. Figure 1 shows the alignment of those primers with the consensus sequence from the multiple alignment of bacteria sequences.

Table 2. *Analysis of the initial three pairs of primers chosen for bacteria. It studies the similarity between primers' sequences and consensus sequences from 16S gene and 23S gene regions obtained after performing multiple alignments.*

Nº	Name	Region	Length	Tm (ºC)	% GC	SC*	MM$^{\nabla}$	PI$^{\blacklozenge}$	3'-end PI $^{\Upsilon}$	Amplicon length
B1	***B16s339F***	16S	18 bp	60.8	66.7	No	No	98.4%	99%	174 bp
	B16s518R		19 bp	61.6	63.2	No	No	98.7%	99.3%	
B2	***B16s1391F***		17 bp	57.3	64.7	No	No	98.5%	99%	121 bp
	B16s1492R		20 bp	56.4	45	No	No	96.5%	98.7%	
B3	***B23s349F***	23S	21 bp	59.5	47.6	No	No	87.2%	95.6%	120 bp
	B23S349R		22 bp	58.4	40.9	No	No	87.5%	96.5%	

** Self complementarity*
∇ Missmatches
$^{\blacklozenge}$ Pairwise identity, which is the average percent identity over the multiple alignment
$^{\Upsilon}$ Pairwise identity of the last 5 bases form the 3' end

Primers were theoretically validated evaluating statistical parameters and stablishing limits and conditions for each of them. The statistical values and conditions considered for primers to be significantly acceptable were the following: 1) ideally, having no self-complementary to avoid the formation of secondary structures such as hairpins, 2) absence of mismatches between the consensus sequence from the studied gene and the sequence of the primer, to make sure the oligo would map the desired region when performing laboratory tests, 3) a global pairwise identity $\geq$ 90%, and 4) the pairwise identity value obtained for the last 5 bases of the 3' end should be $\geq$ 98%. A part from that, the amplicon length resulting from the combination of each pair of primers should

***Figure 1**. Results retrieved from Geneious software. It shows the region from the consensus sequence where each pair of chosen primers map. Bars denote the level of conservation for each position between all bacteria sequences from libraries. Note that those regions where primers align are highly conserved.*

not be very similar, to avoid overlapping signals in the electropherogram obtained from the final multiplex PCR.

Considering the described conditions and having a look to Table 2, it is easy to note that pair B3 was not statistically strong. None of the values for PI reached the stablished limits. At the same time, the length of the resulting amplicon was very similar to the one obtained when considering pair of primers B2. This conduces to the manual design of alternative primers for 23S gene region. After creating different oligonucleotides, a fourth pair of primers (Table 3, Figure 2) was found to be statistically significant and compatible with pairs B1 and B2.

***Table 3**. Analysis of a manually designed pair of primers mapping the 23S rDNA gene region from bacteria.*

Nº	Name	Region	Length	Tm (ºC)	% GC	SC	MM	PI	3'-end PI	Amplicon length
B4	***B23s236F***	23S	18 bp	58.4	64.7	No	No	90.1%	98.4%	239 bp
	B23s470R		20 bp	60.5	55	No	No	94.5%	99.2%	

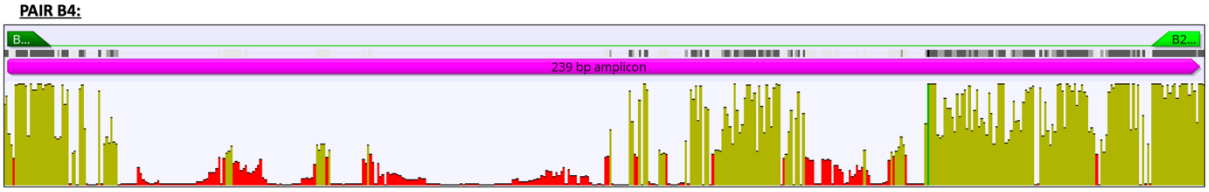

***Figure 2.** Results retrieved from Geneious software. It shows the region from the consensus sequence where the designed pair primers map.*

From a theoretical point of view, the optimal selection of bacterial universal primers to include in the final multiplex PCR would be to choose pairs B1, B2 and B4, and the resulting electropherogram channel would be as shown in Figure 3.

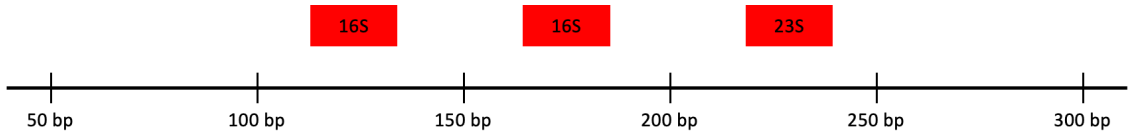

Figure 3. *Representation of the expected bacteria electropherogram channel from the final multiplex PCR. The 16S fragment on the left would correspond to pair B2 (121 bp), the second 16S fragment would be the obtained from pair B1 (174 bp) and the 23S fragment is the one corresponding to pair B4 (239 bp).*

4.1.2. Laboratory approach

Initial selected primers from literature (those from Table 2) were ordered to perform tests in the laboratory. Figure 4 shows the results obtained after testing singleplex PCRs for each pair of primers in five different bacteria species. As it was expected from *in silico* work, all three pairs of primers were annealed in each tested bacteria, and amplicons obtained had the predicted size. No amplicons were found in negative controls.

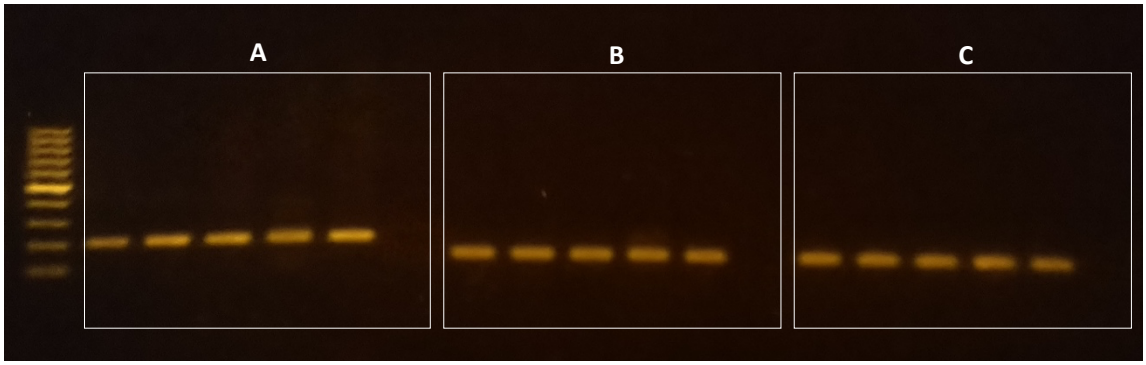

Figure 4. *Agarose gel showing results for three singleplex PCRs tested in five different samples of bacteria to see how each pair of primers amplify. At left, size ladder is found, A) shows results from testing pair of primers B1, B) amplicons obtained testing pair B2, and C) products obtained testing pair B3. In each singleplex the first channel corresponds to Klebsiella sp, the second one to Proteus mirabilis, the third to Salmonella sp., the fourth to Pseudomonas, the fifth to Escherichia coli and the sixth channel stands for negative control.*

4.2. UNIVERSAL FUNGAL PRIMERS STUDY

4.2.1. *In silico* approach

As a result from the literature search, two pair of primers mapping the 18S rDNA fungal gene and one pair of primers annealing to the 28S rDNA fungal region were selected (Borneman & Hartin, 2000; White, T. J., T. D. Bruns, S. B. Lee, 2016; Wu et al., 2003). Table 4 shows the statistics obtained after the analyses of these three pairs. Figure 5

shows the alignment of those primers with the consensus sequence from the multiple alignment of fungi sequences.

Table 4. *Study of the initial three pairs of primers chosen for fungi kingdom. This table shows the similarity between primers' sequences and consensus sequences from 18S gene and 28S gene regions obtained after performing multiple alignments.*

Nº	Name	Region	Length	Tm (ºC)	% GC	SC	MM	PI	PI	Amplicon length
F1	***F18s549F***	18S	18 bp	60.8	66.7	No	No	97.5%	98%	89 bp
	F18s637R		21 bp	53.4	33.3	No	No	91.8%	99.4%	
F2	***F18s1132F***		22 bp	56.4	36.4	No	No	96.2%	97.6%	305 bp
	F18s1436R		21 bp	59.5	47.6	Potential hairpin	No	95.7%	90.1%	
F3	***F28s948F***	28S	19 bp	57.5	52.6	No	No	97.5%	99.2%	194 bp
	F28s1142R		17 bp	54.9	58.8	No	No	96.8%	98.8%	

Figure 5. *Results retrieved from Geneious software. It shows the region from the consensus sequence where each pair of chosen primers map.*

As it happened with bacteria, not all the oligonucleotides achieved the desired values and conditions, thereafter new pairs of primers were created for fungi. In this case, the less acceptable pair was F2: the reverse oligo could potentially present a hairpin formation, besides both oligos were not that statistically strong to be accepted. Furthermore, the length of the resulting amplicon would be among 305 bp, which could be difficult to detect in degraded samples. That is why a manually designed pair of primers for the 18S region was selected (Table 5, Figure 6).

Table 5. *Analysis of a manually designed pair of primers mapping the 18S rDNA gene region from fungi.*

Nº	Name	Region	Length	Tm (ºC)	% GC	SC	MM	PI	3'-end PI	Amplicon length
F4	***F18s986F***	18S	20 bp	55.3	45	No	No	91.9%	98.4%	155 bp
	F18s1006R		19 bp	52.4	42.1	No	No	97.9%	98.3%	

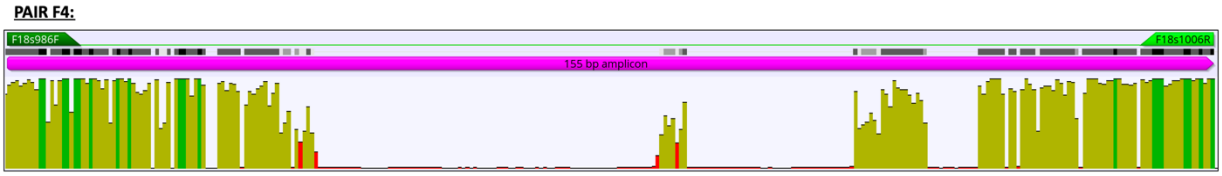

Figure 6. *Results retrieved from Geneious software. It shows the region from the consensus sequence where the designed pair primers map.*

Figure 7 shows what would be expected in the final electropherogram if pairs of primers F1, F3 and F4 were chosen.

Figure 7. *Drawing of the expected fungi channel from the resulting electropherogram of the final multiplex PCR. The first 18S fragment would correspond to pair of oligonucleotides F1 (89 bp), the second 18S fragment would be the obtained from pair F4 (155 bp) and the 28S fragment is the one corresponding to pair F3 (194 bp).*

4.2.2. Laboratory approach

Literature selected pairs of primers (those from Table 4) were tested in the laboratory. Figure 8 shows the obtained results from singleplex PCRs performed in five different species of fungi. No products were found in negative controls and each PCR showed the expected size for its amplicons in each tested sample.

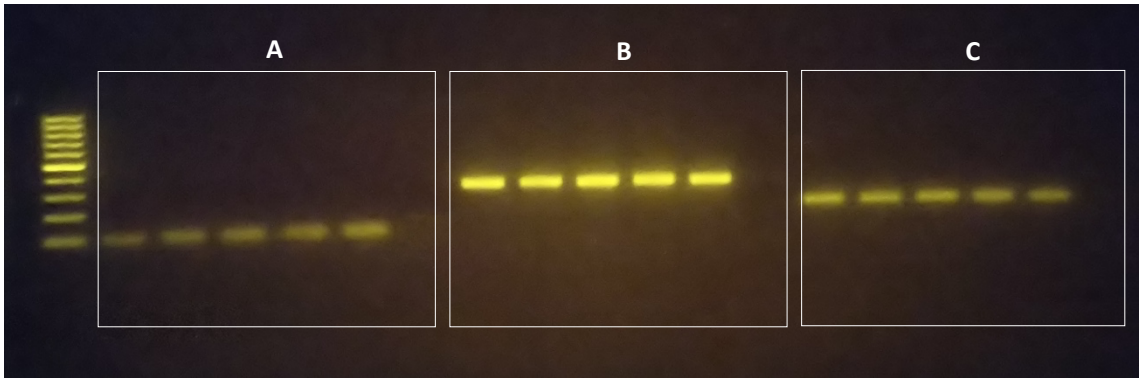

Figure 8. *Agarose gel showing results for the three singleplex PCRs tested in five different species of fungi to see how each pair of primers amplify. At left, size ladder is found, A) shows results from testing pair of primers F1, B) amplicons obtained testing pair F2, and C) products obtained testing pair F3. In each singleplex the first channel corresponds to Alternaria, the second one to Torulaspora delbrueckii, the third to Saccharomyces cerevisiae, the fourth to Neurospora crassa, the fifth to Pleurotus Ostreatus and the sixth channel stands for negative control.*

4.3. MULTIPLEX PCR

A first multiplex PCR approach was performed for both bacteria and fungi. Each primers mix, containing all three pairs from the same kingdom chosen from literature, was tested in different species from the same kingdom. At the same time, the multiplex primers mix was tested in a specie from the other kingdom. Figure 9 shows results from this experiment.

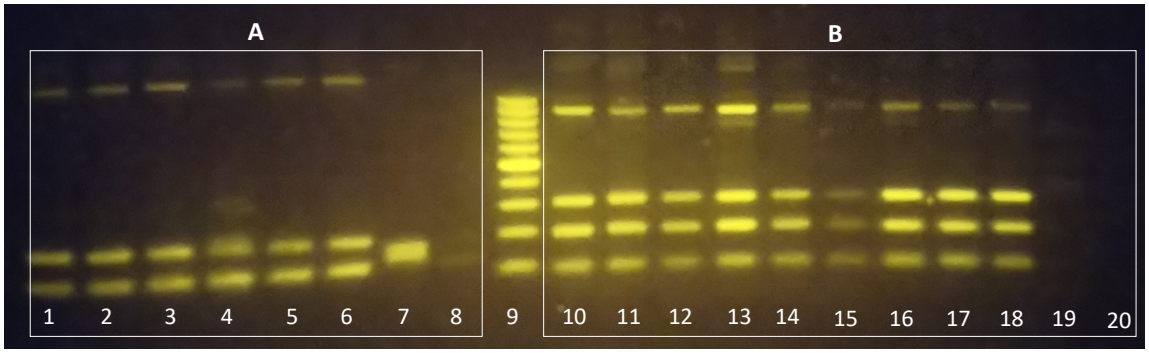

***Figure 9**. Agarose gel that reveals results obtained from the first approach of testing all three pairs of primers for a same kingdom in the same reaction. Region A corresponds to the multiplex PCR performed using pairs 1, 2 and 3 from bacteria. Numbers 1-6 belong to bacteria species (1. Klebsiella sp, 2. Proteus mirabilis, 3. Salmonella sp., 4. Pseudomonas, 5/6. Escherichia coli), number 7 to a fungi specie (Alternaria) and number 8 stands for the negative control. Number 9 corresponds to the size ladder. Region B shows results for the multiplex PCR test done using pairs 1, 2 and 3 from fungi. Numbers 10-18 belong to fungi species (10. Alternaria, 11. Cladosporium, 12. Saccharomyces cerevisiae, 13. Neurospora crassa, 14. Agaricus Bisporus, 15. Agaricus Bisporus var hortensis, 16. Lentinus Edodes, 17. Pleurotus Ostreatus, 18. Lactarius Deliciosus), number 19 shows the result of testing those primers in a bacteria specie (Proteus mirabilis) and number 20 represents the negative control.*

Ignoring top bands, channels number 1 to 6 from Figure 9 are consistent with the *in silico* approach results. Each bacteria specie had two notable bands: the one on the top would be the corresponding to amplicon obtained when considering pair B1, and the one on the bottom would correspond to both other amplicons (those resulting when using B2 and B3). As amplicons B2 and B3 have a very similar theoretical length with just one base of difference, they are not distinguishable when revealing results in an agarose gel and that is why it is just possible to see one big band. Channel number 7 shows a band, which should not appear because the sample analysed corresponds to a fungi specie. It indicates two possible options: 1) the fungi sample could be contaminated with DNA from bacteria, or 2) there are at least two bacterial primers that anneal to fungi genome and it results into the appearance of a notable band. Channels 10 to 18 are in agreement with theoretical results, as they show the appearance of three different bands having the expected size in each fungi specie when testing all three pairs of universal fungal primers (F1, F2 and F3). Channel 19 is also consistent, as it has no bands and it indicates

the absence of amplification when using fungal primers with DNA from bacteria. No notable amplification was found in negative controls (channels 8 and 20). Concerning top bands found in most of the channels, it is suggested that they might correspond to alternative amplicons resulting from the combination of different forward and reverse primers. As these bands are found at the top of the gel, it indicates that they correspond to heavy amplicons and so they have a very large size.

4.4. GLOBAL VIEW AND FURTHER STEPS

As it has been described in above sections, results obtained from computer approaches are mostly congruous to those obtained when testing primers in the laboratory. However, it is necessary to carry out more *in vivo* tests. For example, it is required to increase the number of analyzed species from each kingdom, to have statistically stronger results. It is also necessary to test alternative designed primers for both species (B4 and F4), especially when talking about bacteria to avoid the overlap of those two amplicons found in region A from Figure 9. At the same time, it is fundamental to perform more kingdom-crossing multiplex PCRs in order to see and be sure about the possibility that universal primers from one kingdom anneal to species from another kingdom. In order to avoid the formation of unwanted products, PCR conditions can be adjusted. For example, as the extension time is reduced shorter amplicons are formed. By this it is possible to avoid the formation of big products, such as those heavy bands observed at the top of Figure 9. By changing PCR conditions, it is also possible to avoid the formation of unspecific products: if the annealing temperature is decreased, primers would mainly map strictly similar regions with no mismatches. The reason why this is proposed remains into how similar are oligonucleotides in relation to organisms' genomes. A universal bacterial primer is probable to be more identical to a region from a bacterial genome than to a fungal one. This means primers would easily align to bacterial genomes in comparison to fungal genomes. Decreasing the annealing temperature to a point where primers just anneal to bacterial genomes, might permit to avoid the formation of amplicons in fungi samples using bacteria primers (Channel 7 from Figure 9).

5. CONCLUSIONS

This project reflects the possibility to elaborate a first approach for laboratories in order to achieve the detection of major taxonomic groups within samples of unknown origin. Despite the huge work that is still remaining, good results have been observed. It is possible to find universal primers for each studied kingdom in order to obtain different amplicons when testing them in different species pertaining to that group. However, it is possible to obtain PCR products when those primers are used in another kingdom, due to evident evolutionary reasons and because the chosen regions are highly conserved within the tree of life. Nevertheless, Universalplex still remains as a good idea of a commercial kit for forensic laboratories. It would be a very useful tool for forensic professionals and educational organizations in order to have a fast first approach of identification. With this, they would economize the genetic analyses of biological samples involved in crime scenes.

If chosen primers from all four groups are combined and optimal PCR values (such as annealing temperature, time of each step or number of cycles) are adjusted, good results would be obtained. In order to separate amplicons from each kindom, primers from the final mix would have to be labelled with a specific dye and each group would have a different color. This would permit to elaborate four different channels within the electropherogram and the representation of the multiplex PCR results would be as shown in Figure 10.

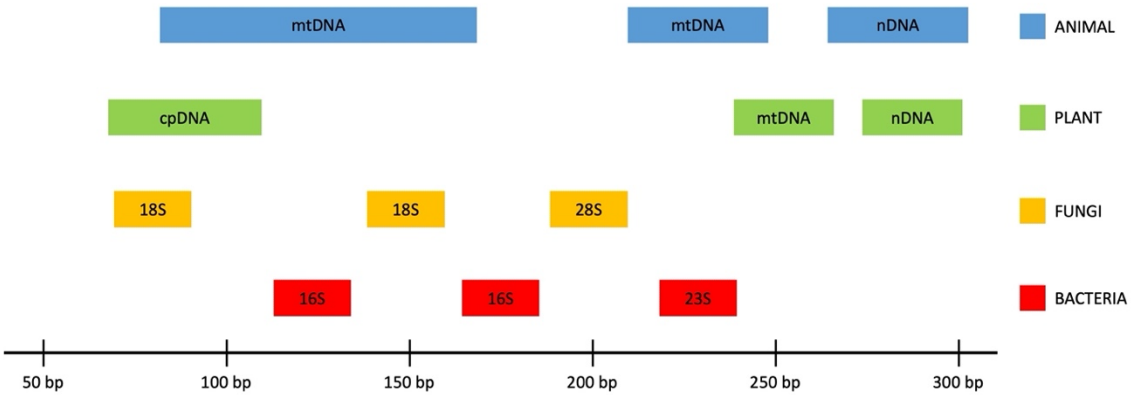

Figure 10. *Universalplex illustration. It shows how would the electropherogram look after testing the three conserved regions from each kingdom. As an approach, bacteria primers would be labelled with red fluorescent dyes, while fungi primers would have orange dyes, plants green dyes and animals blue dyes.*

5.1. USING DNA BARCODING

Apart from the first explained strategy to carry out the Universalplex kit, there is a point that should be reviewed and considered. Conserved regions from all four kingdoms have been considered to develop this method, resulting in the possibility to distinguish an organism at a kingdom level. Nevertheless, it might not be possible to identify them at a species level, due to the high level of conservation of the selected regions. It is suggested to choose other regions where universal kingdom primers are also possible to be found but which results into the amplification of a highly variable region. The importance to include barcoding regions instead of including highly conserved ones would be notable, as researchers and workers from forensic laboratories would be able to firstly find if their sample contains the wanted kingdom (saving money in case they do not find it), and after they would be able to sequence the amplified fragment and determine the specie found in the sample through the application of different bioinformatic tools, such as finding local similarities between sequences using BLAST against GenBank database (Altschul et al., 1990).

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ANNEX 1

List of bacteria species used to create bacteria libraries:

1. *Abditibacterium utsteinense*
2. *Abiotrophia defectiva*
3. *Abyssicoccus albus*
4. *Acaricomes phytoseiuli*
5. *Acaryochloris marina*
6. *Acetanaerobacterium elongatum*
7. *Acetitomaculum ruminis*
8. *Acetivibrio clariflavus*
9. *Acetobacter aceti*
10. *Acetobacterium bakii*
11. *Acetomicrobium flavidum*
12. *Acholeplasma laidlawii*
13. *Achromobacter xylosoxidans*
14. *Acidaminococcus intestini*
15. *Acidianus manzaensis*
16. *Acidibacillus ferrooxidans*
17. *Acidithiobacillus thiooxidans*
18. *Acidobacterium capsulatum*
19. *Acidovorax avenae*
20. *Acinetobacter calcoaceticus*
21. *Actibacterium atlanticum*
22. *Actinoalloteichus cyanogriseus*
23. *Actinobacillus lignieresii*
24. *Actinobaculum suis*
25. *Actinomyces israelii*
26. *Actinoplanes teichomyceticus*
27. *Adlercreutzia equolifaciens*
28. *Aequorivita sp. H23M31*
29. *Aerococcus urinae*
30. *Aeromicrobium erythreum*
31. *Aeromonas hydrophila*
32. *Afipia broomeae*
33. *Agaribacterium haliotis*
34. *Agarilytica rhodophyticola*
35. *Agarivorans gilvus*
36. *Agathobaculum sp. NSJ*
37. *Aggregatibacter actinomycetemcomitans*
38. *Agromyces fucosus*
39. *Akkermansia muciniphila*
40. *Aliarcobacter butzleri*
41. *Aliivibrio fischeri*
42. *Alkalihalobacillus halodurans*
43. *Alteromonas aestuariivivens*
44. *Amycolatopsis orientalis*
45. *Anaerobacillus isosaccharinicus*
46. *Anaerococcus prevotii*
47. *Anaeromyxobacter sp. PSR*
48. *Anaerostipes hadrus*
49. *Anaplasma phagocytophilum*
50. *Apilactobacillus micheneri*
51. *Archaeoglobus sp. QNXS01000036.1*
52. *Arcobacter butzleri*
53. *Arthrobacter agilis*
54. *Azospirillum sp. TSA2s*
55. *Babela massiliensis*

56. *Bacillus anthracis*
57. *Bacteriovorax stolpii*
58. *Bacteroides thetaiotaomicron*
59. *Barnesiella intestinihominis*
60. *Bartonella henselae*
61. *Bdellovibrio bacteriovorus*
62. *Bellilinea caldifistulae*
63. *Bifidobacterium longum*
64. *Bipolaricaulis anaerobius*
65. *Blastococcus saxobsidens*
66. *Blautia hansenii*
67. *Bordetella bronchiseptica*
68. *Borrelia hermsii*
69. *Bosea vestrisii*
70. *Bradyrhizobium japonicum*
71. *Brevibacterium aurantiacum*
72. *Brocadia caroliniensis*
73. *Brucella melitensis*
74. *Buchnera aphidicola*
75. *Burkholderia cepacia*
76. *Butyrivibrio fibrisolvens*
77. *Caballeronia udeis*
78. *Caldanaerobacter subterraneus*
79. *Caldatribacterium californiense*
80. *Caldicellulosiruptor bescii*
81. *Caldithrix abyssi*
82. *Campylobacter jejuni*
83. *Capnocytophaga ochracea*
84. *Carnobacterium maltaromaticum*
85. *Caulobacter vibrioides*
86. *Cellulomonas iranensis*
87. *Cellvibrio japonicus*
88. *Chlamydia pneumoniae*
89. *Chlorobaculum tepidum*
90. *Chromobacterium violaceum*
91. *Chryseobacterium indologenes*
92. *Citrobacter freundii*
93. *Clavibacter michiganensis*
94. *Clostridium acetobutylicum*
95. *Collinsella aerofaciens*
96. *Comamonas thiooxydans*
97. *Companilactobacillus paralimentarius*
98. *Coprococcus sp. OM06*
99. *Coprothermobacter proteolyticus*
100. *Corynebacterium glutamicum*
101. *Coxiella burnetii*
102. *Cryobacterium arcticum*
103. *Cupriavidus metallidurans*
104. *Cutibacterium acnes*
105. *Dehalococcoides mccartyi*
106. *Deinococcus ficus*
107. *Delongbacteria bacterium*
108. *Desulfarculus baarsii*
109. *Desulfitobacterium hafniense*
110. *Desulfobacter hydrogenophilus*
111. *Desulfonatronospira thiodismutans*
112. *Desulfovibrio vulgaris*
113. *Desulfurispirillum indicum*
114. *Desulfurococcus amylolyticus*
115. *Devosia enhydra*
116. *Dialister pneumosintes*
117. *Dickeya solani*
118. *Dictyoglomus turgidum*

119. *Dietzia lutea*
120. *Dolosigranulum pigrum*
121. *Dorea formicigenerans*
122. *Duganella sp. AF9R3*
123. *Duncaniella sp. B8*
124. *Dyella dinghuensis*
125. *Ectothiorhodospira sp. PHS*
126. *Edwardsiella sp. LADL05*
127. *Eggerthella lenta*
128. *Ehrlichia chaffeensis*
129. *Eikenella corrodens*
130. *Eisenbacteria bacterium*
131. *Elizabethkingia meningoseptica*
132. *Elusimicrobium sp. An273*
133. *Empedobacter falsenii*
134. *Endozoicomonas sp. G2_1*
135. *Ensifer adhaerens*
136. *Enterobacter cloacae*
137. *Enterocloster bolteae*
138. *Enterococcus faecalis*
139. *Enterovibrio norvegicus*
140. *Entomoplasma luminosum*
141. *Epibacterium mobile*
142. *Epilithonimonas tenax*
143. *Erwinia amylovora*
144. *Erysipelothrix rhusiopathiae*
145. *Erythrobacter litoralis*
146. *Escherichia coli*
147. *Eubacterium sp. NSJ*
148. *Euzebyella marina*
149. *Exiguobacterium aurantiacum*
150. *Faecalibacterium sp. OF04*
151. *Faecalimonas umbilicata*
152. *Fermentibacter daniensis*
153. *Ferrimicrobium acidiphilum*
154. *Ferrimonas marina*
155. *Ferruginibacter sp. BO*
156. *Fibrobacter succinogenes*
157. *Firestonebacteria bacterium*
158. *Fischerella thermalis*
159. *Flavobacterium hydatis*
160. *Flavonifractor plautii*
161. *Fluviicola riflensis*
162. *Fournierella massiliensis*
163. *Francisella tularensis*
164. *Frankia casuarinae*
165. *Frigoribacterium sp. NBH87*
166. *Fructilactobacillus sanfranciscensis*
167. *Fusicatenibacter saccharivorans*
168. *Fusobacterium necrophorum*
169. *Gallibacterium anatis*
170. *Gallionella capsiferriformans*
171. *Gemella sp. ND 6198*
172. *Gemmata obscuriglobus*
173. *Gemmatimonas aurantiaca*
174. *Geobacillus thermoleovorans*
175. *Geobacter sulfurreducens*
176. *Geodermatophilus aquaeductus*
177. *Geovibrio thiophilus*
178. *Gloeobacter violaceus*
179. *Gluconobacter oxydans*
180. *Glutamicibacter nicotianae*
181. *Glycomyces albidus*
182. *Goldbacteria bacterium*

183. *Gracilibacillus sp. SCU50*
184. *Gramella flava*
185. *Granulibacter bethesdensis*
186. *Haemophilus influenzae*
187. *Hafnia paralvei*
188. *Halanaerobium congolense*
189. *Halarcobacter arenosus*
190. *Haliea salexigens*
191. *Halobacterium hubeiense*
192. *Halococcus hamelinensis*
193. *Helicobacter hepaticus*
194. *Henriciella marina*
195. *Herbaspirillum huttiense*
196. *Holdemanella biformis*
197. *Hungatella hathewayi*
198. *Hydrogenedens sp.*
199. *Hydrogenobaculum sp. SN*
200. *Hydrogenophaga taeniospiralis*
201. *Hydrogenovibrio marinus*
202. *Hymenobacter lapidiphilus*
203. *Hyphomonas atlantica*
204. *Idiomarina loihiensis*
205. *Ilumatobacter fluminis*
206. *Intestinibacter bartlettii*
207. *Intestinimonas butyriciproducens*
208. *Izhakiella australiensis*
209. *Janibacter terrae*
210. *Jannaschia rubra*
211. *Janthinobacterium lividum*
212. *Jeotgalibaca arthritidis*
213. *Jeotgalibacillus proteolyticus*
214. *Jeotgalicoccus psychrophilus*
215. *Jiangella alba*
216. *Kaistella jeonii*
217. *Ketobacter sp. MCCC 1A13808*
218. *Kiloniella majae*
219. *Kingella kingae*
220. *Kitasatospora aureofaciens*
221. *Klebsiella pneumoniae*
222. *Kluyvera cryocrescens*
223. *Kocuria rosea*
224. *Komagataeibacter hansenii*
225. *Kosakonia cowanii*
226. *Kribbella sindirgiensis*
227. *Kushneria konosiri*
228. *Lachnospira pectinoschiza*
229. *Lacticaseibacillus paracasei*
230. *Lactiplantibacillus plantarum*
231. *Lactobacillus johnsonii*
232. *Lactococcus lactis*
233. *Latilactobacillus sakei*
234. *Lawsonia intracellularis*
235. *Leclercia adecarboxylata*
236. *Leeuwenhoekiella aequorea*
237. *Legionella pneumophila*
238. *Leifsonia shinshuensis*
239. *Leisingera aquaemixtae*
240. *Lentilactobacillus buchneri*
241. *Leptospira interrogans*
242. *Leucobacter triazinivorans*
243. *Leuconostoc mesenteroides*
244. *Levilactobacillus brevis*
245. *Ligilactobacillus murinus*
246. *Limnobacter alexandrii*

247. *Limnohabitans sp. 103DPR2*
248. *Limosilactobacillus reuteri*
249. *Lindowbacteria bacterium*
250. *Listeria monocytogenes*
251. *Litoricola lipolytica*
252. *Loigolactobacillus backii*
253. *Longicatena caecimuris*
254. *Luminiphilus syltensis*
255. *Luteimonas wenzhouensis*
256. *Lutibacter oceani*
257. *Lysobacter enzymogenes*
258. *Magnetococcus marinus*
259. *Magnetospirillum gryphiswaldense*
260. *Malaciobacter marinus*
261. *Mannheimia haemolytica*
262. *Maribacter dokdonensis*
263. *Marinobacter flavimaris*
264. *Marinomonas spartinae*
265. *Mariprofundus aestuarium*
266. *Mediterraneibacter glycyrrhizinilyticus*
267. *Megasphaera elsdenii*
268. *Meiothermus taiwanensis*
269. *Mesorhizobium loti*
270. *Methanobacterium sp. A39*
271. *Methylocystis rosea*
272. *Methylomirabilis limnetica*
273. *Methylomonas koyamae*
274. *Methylophaga aminisulfidivorans*
275. *Methylopumilus universalis*
276. *Methylorubrum extorquens*
277. *Methylotenera mobilis*
278. *Micrarchaeum sp. CP060530.1*
279. *Microbacterium ginsengisoli*
280. *Microbulbifer taiwanensis*
281. *Microcystis panniformis*
282. *Micromonospora chalcea*
283. *Microvirga massiliensis*
284. *Moraxella catarrhalis*
285. *Morganella morganii*
286. *Mucilaginibacter kameinonensis*
287. *Muricauda ochracea*
288. *Mycobacterium tuberculosis*
289. *Mycoplasma hyorhinis*
290. *Myroides odoratimimus*
291. *Negativibacillus massiliensis*
292. *Neisseria gonorrhoeae*
293. *Nesterenkonia natronophila*
294. *Nitratireductor aquibiodomus*
295. *Nitrobacter vulgaris*
296. *Nitrosomonas europaea*
297. *Nitrosopumilus maritimus*
298. *Nitrosospira multiformis*
299. *Nitrospina gracilis*
300. *Nocardia nova*
301. *Nocardioides immobilis*
302. *Nocardiopsis gilva*
303. *Nostoc sp. PCC 7120*
304. *Novosphingobium sp. GeG2*
305. *Oceanobacillus iheyensis*
306. *Ochrobactrum intermedium*
307. *Oenococcus oeni*
308. *Olsenella umbonata*
309. *Onion yellows phytoplasma*

310. *Oribacterium oral*
311. *Orientia tsutsugamushi*
312. *Oscillibacter valericigenes*
313. *Ottowia thiooxydans*
314. *Ozemobacter sibiricus*
315. *Pandoraea apista*
316. *Pantoea dispersa*
317. *Parabacteroides distasonis*
318. *Paraburkholderia hospita*
319. *Parachlamydia acanthamoebae*
320. *Paracoccus denitrificans*
321. *Pararhizobium polonicum*
322. *Pasteurella multocida*
323. *Pauljensenia hongkongensis*
324. *Pectobacterium carotovorum*
325. *Pedobacter heparinus*
326. *Peptostreptococcus anaerobius*
327. *Phascolarctobacterium faecium*
328. *Phenylobacterium sp. CCH9*
329. *Phocaeicola plebeius*
330. *Photobacterium profundum*
331. *Photorhabdus luminescens*
332. *Phyllobacterium bourgognense*
333. *Piscirickettsia salmonis*
334. *Planktophila dulcis*
335. *Planktothrix sp. FACHB*
336. *Planococcus faecalis*
337. *Plesiomonas shigelloides*
338. *Polaribacter reichenbachii*
339. *Polaromonas sp. JS666*
340. *Polynucleobacter asymbioticus*
341. *Pontibacter diazotrophicus*
342. *Poribacteria bacterium*
343. *Porphyromonas gingivalis*
344. *Prevotella intermedia*
345. *Prochlorococcus marinus*
346. *Proteiniphilum acetatigenes*
347. *Proteus mirabilis*
348. *Providencia stuartii*
349. *Pseudarthrobacter chlorophenolicus*
350. *Pseudoalteromonas distincta*
351. *Pseudomonas aeruginosa*
352. *Pseudonocardia autotrophica*
353. *Pseudovibrio ascidiaceicola*
354. *Psychrobacter alimentarius*
355. *Psychromonas ingrahamii*
356. *Qipengyuania flava*
357. *Ralstonia pickettii*
358. *Raoultella ornithinolytica*
359. *Rathayibacter rathayi*
360. *Reyranella massiliensis*
361. *Rhizobacter gummiphilus*
362. *Rhizobium leguminosarum*
363. *Rhodanobacter glycinis*
364. *Rhodococcus hoagii*
365. *Rhodoferax antarcticus*
366. *Rhodopirellula sp. JC637*
367. *Rhodopseudomonas palustris*
368. *Rhodovulum sulfidophilum*
369. *Rickettsia conorii*
370. *Rodentibacter pneumotropicus*
371. *Roseburia intestinalis*
372. *Roseibacillus ishigakijimensis*
373. *Roseinatronobacter monicus*

374. *Roseomonas mucosa*
375. *Roseovarius mucosus*
376. *Rothia dentocariosa*
377. *Ruegeria atlantica*
378. *Ruminococcus flavefaciens*
379. *Ruthenibacterium lactatiformans*
380. *Saccharibacillus sp. O16*
381. *Saccharimonas aalborgensis*
382. *Saccharomonospora viridis*
383. *Saccharopolyspora erythraea*
384. *Saccharothrix carnea*
385. *Salinibacter ruber*
386. *Salinicoccus roseus*
387. *Salinisphaera shabanensis*
388. *Salipiger profundus*
389. *Salmonella enterica*
390. *Schaedlerella arabinosiphila*
391. *Secundilactobacillus paracollinoides*
392. *Sediminibacterium sp. C3*
393. *Selenomonas sputigena*
394. *Serinicoccus sp. P2D13*
395. *Serratia marcescens*
396. *Shewanella oneidensis*
397. *Shigella flexneri*
398. *Shimia isoporae*
399. *Sinorhizobium meliloti*
400. *Smithella sp. SC_K08D17*
401. *Snodgrassella alvi*
402. *Sphaerochaeta sp. S2*
403. *Sphingobacterium multivorum*
404. *Sphingobium fuliginis*
405. *Sphingomonas koreensis*
406. *Sphingopyxis granuli*
407. *Spirillospora albida*
408. *Spiroplasma melliferum*
409. *Spirosoma sp. HMF4905*
410. *Spirulina major*
411. *Sporolactobacillus sp. THM7*
412. *Sporosarcina ureae*
413. *Staphylococcus aureus*
414. *Stenotrophomonas maltophilia*
415. *Streptococcus pneumoniae*
416. *Streptomyces avermitilis*
417. *Succiniclasticum ruminis*
418. *Succinivibrio dextrinosolvens*
419. *Sulfitobacter pontiacus*
420. *Sulfuricaulis limicola*
421. *Sulfurimonas sp. H1576*
422. *Sulfurospirillum cavolei*
423. *Sulfurovum sp. NBC37*
424. *Sutterella wadsworthensis*
425. *Synechococcus elongatus*
426. *Synechocystis sp. PCC 6803*
427. *Syntrophosphaera thermopropionivorans*
428. *Syntrophus aciditrophicus*
429. *Tannerella forsythia*
430. *Tatlockia micdadei*
431. *Taylorella equigenitalis*
432. *Tenacibaculum maritimum*
433. *Terrabacter sp. MAHUQ*
434. *Terracidiphilus gabretensis*
435. *Tessaracoccus defluvii*
436. *Tetragenococcus halophilus*

437. *Thalassolituus oleivorans*
438. *Thauera sp. K11*
439. *Thermoanaerobacter mathranii*
440. *Thermoanaerobacterium thermosaccharolyticum*
441. *Thermoclostridium stercorarium*
442. *Thermodesulfobacterium commune*
443. *Thermodesulfobium acidiphilum*
444. *Thermosipho melanesiensis*
445. *Thermotoga maritima*
446. *Thermus thermophilus*
447. *Thioalkalivibrio nitratireducens*
448. *Thiobacillus denitrificans*
449. *Thioclava sediminum*
450. *Thiomonas arsenitoxydans*
451. *Treponema denticola*
452. *Trichormus variabilis*
453. *Trinickia sp. DHG64*
454. *Tropheryma whipplei*
455. *Uliginosibacterium sp. TH139*
456. *Ureaplasma parvum*
457. *Ureibacillus terrenus*
458. *Vagococcus lutrae*
459. *Varibaculum cambriense*
460. *Variovorax boronicumulans*
461. *Veillonella dispar*
462. *Verrucomicrobium spinosum*
463. *Vibrio cholerae*
464. *Virgibacillus necropolis*
465. *Viridibacillus arenosi*
466. *Vogesella indigofera*
467. *Vulcanisaeta distributa*
468. *Vulcanococcus limneticus*
469. *Waddlia chondrophila*
470. *Weeksella virosa*
471. *Weissella paramesenteroides*
472. *Wenxinia marina*
473. *Wenyingzhuangia heitensis*
474. *Wenzhouxiangella sp. AB*
475. *Wigglesworthia glossinidia*
476. *Williamsia sp. 1135*
477. *Winkia neuii*
478. *Winogradskyella sediminis*
479. *Wolbachia pipientis*
480. *Wolinella succinogenes*
481. *Xanthobacter sp. YN2*
482. *Xanthomonas oryzae*
483. *Xenococcus sp. PCC 7305*
484. *Xylanibacterium ulmi*
485. *Xylella fastidiosa*
486. *Xylophilus ampelinus*
487. *Yersinia enterocolitica*
488. *Yokenella regensburgei*
489. *Yonghaparkia alkaliphila*
490. *Yoonia rosea*
491. *Zavarzinia compransoris*
492. *Zeaxanthinibacter enoshimensis*
493. *Zhongshania aliphaticivorans*
494. *Zhouia amylolytica*
495. *Zixibacteria bacterium*
496. *Zobellia galactanivorans*
497. *Zoogloea oleivorans*
498. *Zunongwangia profunda*
499. *Zymomonas mobilis*

ANNEX 2

List of fungi species used to create fungi libraries:

1. Acanthamoeba castellanii
2. Acytostelium digitatum
3. Agaricus bisporus
4. Allomyces arbusculus
5. Amanita bisporigera
6. Amoeboaphelidium protococcarum
7. Ampulloclitocybe clavipes
8. Aphanomyces invadans
9. Aphelidium collabens
10. Archaeospora trappei
11. Armillaria ostoyae
12. Arthrobotrys elegans
13. Arthroderma uncinatum
14. Ascocoryne sarcoides
15. Aspergillus flavus NRRL3357
16. Aureobasidium pullulans
17. Aureococcus anophagefferens
18. Auricularia cornea
19. Babesia ovata
20. Barbatospora ambicaudata
21. Basidiobolus ranarum
22. Batrachochytrium dendrobatidis JEL423
23. Baudoinia antilliensis
24. Beauveria bassiana
25. Bipolaris eleusines
26. Bjerkandera adusta
27. Blastocladiella emersonii
28. Blastocystis sp. AFJ96
29. Blastomyces gilchristii SLH14081
30. Blumeria graminis
31. Boletellus shichianus
32. Bondarzewia berkeleyi
33. Botryobasidium botryosum
34. Botrytis cinerea
35. Brettanomyces naardenensis
36. Calcarisporiella sp. NBRC 105922
37. Calocera cornea
38. Calostoma cinnabarinum
39. Candida albicans 19F
40. Candida auris
41. Capronia pilosella
42. Cercospora zebrina
43. Chaetomium pilosum
44. Chytriomyces hyalinus
45. Cintractia limitata
46. Cladochytrium replicatum
47. Cladophialophora parmeliae
48. Clavaria australiana
49. Clavispora lusitaniae
50. Climacodon septentrionalis
51. Coccidioides immitis RS
52. Coelomomyces stegomyiae
53. Colacogloea peniophorae

54. Colletotrichum tamarilloi
55. Collybia tuberosa
56. Coltricia macropora
57. Conidiobolus coronatus
58. Conidiobolus sp.
59. Coniophora olivacea
60. Coprinopsis atramentaria
61. Coprinus comatus
62. Cordyceps farinosa
63. Cortinarius bolaris
64. Cotylidia sp. MB5
65. Cryptococcus neoformans
66. Cryptosporidium meleagridis
67. Cylindrobasidium laeve
68. Cyllamyces aberensis
69. Dacryopinax spathularia
70. Dactylellina haptotyla
71. Daedalea africana
72. Debaryomyces hansenii
73. Dichomitus sp.
74. Dictyostelium sp. stellarum
75. Dimargaris bacillispora
76. Dothistroma septosporum
77. Echinodontium tinctorium
78. Eimeria meleagrimitis
79. Encephalitozoon hellem
80. Endocarpon petrolepideum
81. Endocronartium harknessii
82. Endogone pisiformis
83. Entamoeba histolytica
84. Enterocytozoon bieneusi H348
85. Entomophthora muscae
86. Entorrhiza citriformis
87. Eremothecium sinecaudum
88. Eutypa lata
89. Exidia uvapassa
90. Fibroporia vaillantii
91. Fibularhizoctonia sp. TMB
92. Fistulina pallida
93. Fistulina subhepatica
94. Flammulina velutipes
95. Fomitiporia mediterranea
96. Fomitiporia rhamnoides
97. Fomitopsis pinicola
98. Fusarium proliferatum
99. Galerina laevis
100. Galerina marginata
101. Ganoderma australe
102. Ganoderma tsugae
103. Gautieria otthii
104. Gelatoporia subvermispora
105. Geosiphon pyriformis
106. Giardia intestinalis
107. Gloeophyllum abietinum
108. Glomus sp. MUCL 43206
109. Gonapodya polymorpha
110. Gregarina sp.
111. Grifola sordulenta
112. Gromochytrium mamkaevae
113. Grosmannia davidsonii
114. Guillardia theta
115. Gymnopus contrarius
116. Hammondia hammondi
117. Hebeloma mesophaeum

118. Henningsomyces sp. FP
119. Heterobasidion annosum
120. Histoplasma capsulatum var. farciminosum
121. Hyaloraphidium curvatum
122. Hydnomerulius pinastri
123. Hydnum rufescens
124. Hygrocybe conica
125. Hygrophoropsis aurantiaca
126. Hyphoderma orphanellum
127. Hypholoma sublateritium
128. Hysterium pulicare
129. Ichthyophthirius multifiliis
130. Jaapia argillacea
131. Kazachstania taianensis
132. Kluyveromyces marxianus
133. Komagataella phaffii GS115
134. Kuzuhaea moniliformis
135. Laccaria ochropurpurea
136. Lachancea thermotolerans
137. Lactarius lignyotus
138. Laetiporus sulphureus
139. Leishmania aethiopica
140. Leptosphaeria sclerotioides
141. Lodderomyces sp. Y
142. Lycoperdon marginatum
143. Macrophomina phaseolina
144. Malassezia pachydermatis
145. Marasmius rotula
146. Melampsora euphorbiae
147. Metarhizium robertsii
148. Meyerozyma guilliermondii
149. Microsporum distortum
150. Millerozyma farinosa
151. Mixia osmundae
152. Moesziomyces antarcticus
153. Moniliophthora roreri
154. Monoblepharella mexicana
155. Monoblepharis micrandra
156. Mortierella sp. MS
157. Mucor racemosus
158. Naegleria gruberi
159. Naumovozyma dairenensis
160. Nectria curta
161. Nematocida parisii ERTm1
162. Neocallimastix frontalis
163. Neofusicoccum ribis
164. Neolentinus lepideus
165. Neospora caninum
166. Neurospora crassa OR74A
167. Nosema adaliae
168. Oedogoniomyces sp. CR84
169. Oidiodendron tenuissimum
170. Olpidium brassicae
171. Omphalotus olearius
172. Ophiostoma piliferum
173. Orphella haysii
174. Orpinomyces sp. OUS1
175. Paracoccidioides brasiliensis
176. Paraglomus occultum
177. Paramecium multimicronucleatum
178. Parastagonospora nodorum SN15
179. Passalora dodonaeae
180. Paxillus filamentosus

181. Penicillium digitatum
182. Peniophora cinerea
183. Perkinsus olseni
184. Pestalotiopsis microspora
185. Phaeoacremonium prunicola
186. Phaeodactylum tricornutum
187. Phanerochaete carnosa
188. Phlebia floridensis
189. Phlebia radiata
190. Phlebiopsis crassa
191. Phycomyces blakesleeanus
192. Physoderma maydis
193. Phytophthora parasitica INRA
194. Piloderma fallax
195. Piptocephalis corymbifera
196. Piromyces sp. PGL01
197. Pisolithus albus
198. Plasmodium falciparum HB3
199. Platygloea disciformis
200. Pleurotus pulmonarius
201. Plicaturopsis crispa
202. Pluteus cervinus
203. Pneumocystis jirovecii
204. Podospora anserina S mat+
205. Polychytrium aggregatum
206. Postia caesia
207. Pseudozyma hubeiensis SY62
208. Puccinia sorghi
209. Punctularia strigosozonata
210. Pyrenophora tritici
211. Pyronema omphalodes CBS 100304
212. Ramaria stricta
213. Ramicandelaber longisporus
214. Rhizoclosmatium sp. JEL347
215. Rhizoctonia solani AG IA
216. Rhizophagus clarus
217. Rhizophlyctis rosea
218. Rhizophydium sp. JEL221
219. Rhizopus stolonifer
220. Rhodotorula babjevae
221. Rhopalomyces elegans
222. Rhytidhysteron rufulum
223. Rozella allomycis
224. Saccharomyces cerevisiae
225. Saprolegnia ferax
226. Scheffersomyces insectosa
227. Schizophyllum commune
228. Schizopora paradoxa
229. Schizosaccharomyces japonicus
230. Scleroderma laeve
231. Sclerotinia sclerotiorum 1980 UF
232. Scutellospora sp. 1 ML
233. Serendipita indica
234. Serpula himantioides
235. Smittium culicis
236. Spathaspora arborariae
237. Sphaerobolus stellatus
238. Sphaerocreas pubescens
239. Sphaerulina quercicola
240. Spiromyces minutus
241. Spizellomyces punctatus DAOM BR117
242. Sporisorium scitamineum
243. Stereum hirsutum

244. Stereum ostrea
245. Sterigmatomyces hyphaenes
246. Suillus spraguei
247. Synchytrium macrosporum
248. Talaromyces rugulosus
249. Taphrina communis
250. Tetrahymena rostrata
251. Tetrapisispora blattae CBS 6284
252. Thalassiosira eccentrica
253. Thecamonas trahens ATCC 50062
254. Theileria annulata
255. Thielavia australiensis
256. Tilletiaria anomala
257. Tilletiopsis washingtonensis
258. Torulaspora globosa
259. Toxoplasma gondii
260. Trametes versicolor
261. Tremella aurantia
262. Trichoderma deliquescens
263. Trichomonas vaginalis
264. Trichophyton rubrum
265. Trypanosoma cruzi
266. Tuber melosporum
267. Tulasnella violea
268. Umbelopsis ramanniana
269. Uncinocarpus reesii
270. Ustilago hordei (reversed)
271. Vanderwaltozyma polyspora
272. Verticillium dahliae VDG2
273. Volvariella volvacea
274. Wallemia muriae
275. Wolfiporia cocos
276. Xylona heveae
277. Yarrowia lipolytica
278. Zymoseptoria tritici