6 MATERIALS AND METHODS

6.1 MYCELIAL CULTURES

Colonies were established by subculturing hyphal fragments on a suitable medium (SUB-CULTURE). *Lentinula edodes* strains were cultivated on both liquid (CYM) and solid medium (PDA). Both media were prepared with distilled water several components (Table 2), and sterilized in an autoclave (120°C for 20min) as shown in Fig. 6.

CYM (1000ml)	**PDA (1000ml)**
Dextrose: 20g	**Potato infusion: 4g**
Peptone: 2g	**Dextrose: 20g**
Yeast extract: 2g	**Agar: 15g**
$Mg.SO_4.7H_20$: 0.5g	
KH_2PO_4: 0.46g	
$K_2H\ PO_4$: 1g	

Table 2. Components of different media used for mycelial cultures

At the end, the strains were subcultured on Petri dishes using inoculating tools, in a sterile laminar flow chamber to prevent any kind of contamination (Fig. 7).

Fig. 6. Autoclaves: steam pressure sterilizers

Fig. 7. Laminar flow chamber with sterilized needles, medium and EtOH

The entire process was conducted under aseptic conditions. The Petri dishes were secured with perforated Parafilm to allow air exchange. The strains grew at different rates and needed to be checked constantly to supervise if any contamination had occurred. Fig. 8 shows the growth of several strains after a couple of weeks.

Fig. 8: Different *L. edodes* strains grown on agar plates for 2 weeks

6.2 FREEZE DRYING

Freeze drying (also known as lyophilization) is a process in which the mycelium is frozen and water is removed by sublimation (change from a solid state – frozen mycelium – to a gaseous state without going through a liquid state). This drying process is accomplished by freezing under reduced pressure in a vacuum condition. The first step in this process is to extract the mycelium from a liquid culture on Petri dish, with the help of a spatula. Afterwards, the mycelium is placed on filter paper so that it absorbs all the excess liquid medium and compressed to minimize its volume (Fig. 9).

Fig. 9. Drying and compressing of the mycelium

The compressed mycelium is placed in properly labelled eppendorf tubes on racks that will be submerged in liquid nitrogen (-170°) for about 2 minutes so that all cellular activities are arrested (Fig. 10).

Fig. 10. Liquid nitrogen handling for freeze-drying procedure

The samples are then placed in a freezer (-20°C) while the Freeze Dry System is set up to -44°C. After placing samples in this apparatus, they remain there for approximately 24 hours so that the sublimation process is completed (Fig. 11).

Fig. 11. Freeze Dry System (Dryzone 4.5)

After the freeze dried samples were ready ten strains were selected (shown on blue in Table 3) on the basis of differences in productivity, mating type and origin.

STRAIN	SPECIES	ORIGIN	COLLECTOR
CP-5	*Lentinula boryana*	Veracruz	García s/n
CP-7	*Lentinula edodes*	Hong Kong	
CP-8	*Lentinula edodes*	USA	B. Harris
CP-9	*Lentinula edodes*	Japan	R. Kurtzman Mori

CP-10	*Lentinula edodes*	Commercial	L. Villarreal
CP-13	*Lentinula* spp.	Japan	Carlos Ortega
CP-95	*Lentinula edodes*	Commercial	Michoacán RL3-105
CP-96	*Lentinula edodes*	Japan	Michoacán, Mori 30-3
CP-97	*Lentinula edodes*	Commercial	
CP-163	*Lentinula edodes*	Commercial	R. Leben
CP-164	*Lentinula* spp.	Cholula, Puebla	A. Largo
CP-172	*Lentinula* spp. (variety Mori)	Japan	Carlos Ortega
CP-173	*Lentinula* spp.	USA	Carlos Ortega
CP-174	*Lentinula* spp.	USA	Carlos Ortega
CP-188	*Lentinula edodes*	Commercial	H. Leal
CP-189	*Lentinula edodes*	Commercial	H. Leal

Table 3. Strains deposited at the COLPOS culture collection. Those strains highlighted (blue) were selected for this study

6.3 DNA EXTRACTION

The Qiagen Plasmid MiniKit 100 (Qiagen, Germany) was used for DNA extraction (Fig. 12), following the "Fungal genomic DNA extraction protocol for filamentous fungi" [19] (All buffer components are found in the APPENDIX 11.4).

1. Grind freeze dried mycelia to a fine powder.
2. Add 650 µl of buffer A and continue to mix till homogeneous.
3. Add 5 units of RNase A (3 µl), mix by inversion and incubate at 70°C for 30 min.
4. Spin for 10 min in microcentrifuge (Brinkmann Instruments, Inc., USA) at room temperature (13,000 rpm).
5. Equilibrate QIAGEN column using QBT buffer (1ml).
6. Add supernatant to column using a blue tip and allow to run through.
7. Wash column 4x 1ml of QC buffer.
8. Elute off with 700 µl of QF buffer into an eppendorf tube.

9. Add 700 µl of propan-2-ol to precipitate DNA, mix by inversion and wait for 2 min. Spin for 20 min (13,000 rpm).
10. Wash the pellet by inversion with 500 µl 70% EtOH.
11. Spin for 10 min (13,000 rpm).
12. Dry in oven for 30 min.
13. Resuspend in 50 µl of TE in water bath at 55°C for 30 min.
14. Assess DNA quality by the method of Sambrook *et al* (1989).

Fig. 12. Gilson pipets, microcentrifuge and QIAGEN columns used for DNA extraction

6.4 DNA QUANTIFICATION (ELECTROPHORESIS)

Gel electrophoresis protocol (Fig. 13):

1. Add 1g of Agarose to 100ml 1x TAE buffer.
2. Heat and dissolve solution till homogeneous (aprox. 2 min), stirring every 20 sec.
3. Add 1 µl EtBr to the agarose solution and pour into electrophoresis tray. Leave for 15-20 min until solidified. Remove comb and dams.
4. Place gel inside electrophoresis chamber.
5. Add 1x TAE buffer until the gel is completely submerged.
6. Mix DNA samples (1 µl) with 3 µl 6x Loading buffer dye and 6 µl 1x TE buffer pH 8.0, achieving a 10 µl volume.

7. Load ladder (3 μl) on gel, followed by the DNA samples and the molecular weight markers (1 μl and 5 μl), respectively.
8. Connect leads to power supply and allow to run at 70-80 Volts.
9. After 1-2 hours, disconnect, place gel on DNA UV-table BIORAD Gel Doc 2000™ (Bio-Rad Laboratories, Inc., USA) and obtain a photograph of migrating DNA bands, using Bio-Rad Quantity One Software.
10. Quantify DNA concentrations using Bio-Rad Quantity One Software, naming samples, background and known quantities (10 Lambda and 50 Lambda respectively).

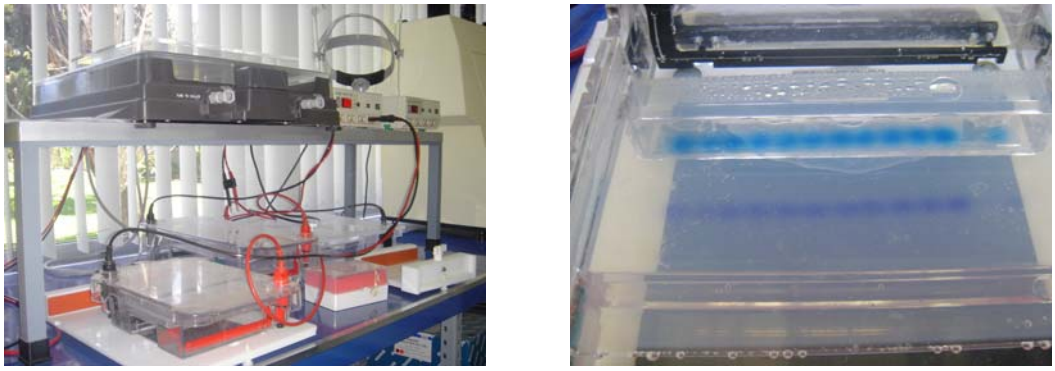

Fig. 13. Electrophoresis equipment (left) and running samples in agarose gel (right)

6.5 PCR AMPLIFICATION OF ITS REGION

Protocol for ITS region, Ampli Taq (Biosystems, USA):

1. Quick spin PCR mastermix, PCR water (nuclease-free) and primers (ITS1 and ITS4 primers were used; their sequences are found in the APPENDIX 11.3).
2. Adjust quantities of PCR water (13-15 μl) and DNA (0.5-2.0 μl) so that the reaction volume equals 50 μl. The amount of DNA used is selected according to its concentration (Table 4).

STRAIN	DNA Concentration (ng/µl)	DNA volume (µl)	PCR water volume (µl)
5	16.6	2	13
7	12.4	2	13
8	62.6	1	14
9	62.1	1	14
13	112	0.5	14.5
95	188.7	0.5	14.5
163	35.3	1.5	13.5
172	191.3	0.5	14.5
188	116.2	0.5	14.5
189	1.6	2	13

Table 4. DNA and water volumes used for PCR reaction

3. Add 25 µl mastermix, pre-calculated PCR water quantity, 5 µl ITS-1 primer and 5 µl ITS-4 primer to each DNA sample.
4. Run a blank sample that contains 15 µl PCR water without DNA.
5. Quick spin samples before introducing them to the thermocycler GeneAmp PCR System 2400 (Perkin Elmer, USA).
6. PCR cycling:

95°C	**1min**	**1 cycle**
94°C	**30sec**	**25 cycles**
50°C	**45sec**	
72°C	**5min**	
72°C	**5min**	**1 cycle**

After this procedure was done, the samples were runned in a 1% Agarose Electrophoresis Gel not to quantify but to ensure the success of the amplification process.

6.6 PCR PURIFICATION (ITS REGION) AND SEQUENCING

The QIAquick PCR Purification Kit (Qiagen, Germany) Protocol was followed. This protocol is designed to purify single or double-stranded DNA fragments from PCR reactions. The fragments are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns (Fig. 14) in a microcentrifuge.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix (e.g. 250 µl of Buffer PB to 50 µl PCR sample).
2. Place a QIAquick spin column in a provided 2ml collection tube.
3. To bind DNA, apply the sample to the column and centrifuge for 1min.
4. Discard flow-through. Place the column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the column and centrifuge for 1min.
6. Discard flow-through and place the column back in the same tube. Centrifuge the column for an additional 1min.
7. Place column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 30 µl nuclease-free water (pH between 7.0 and 8.5) to the center of the column membrane. Let the column stand for 1min and then centrifuge.

Fig. 14. QIAquick PCR Purification Kit

After this procedure was done, the samples were runned in a 1% Agarose Electrophoresis Gel to quantify the purified ITS region DNA, using 2 μl of EtBr instead of 1 μl, followed by illumination with short wave UV.

The purified PCR samples were sent to be sequenced in Houston, Texas (SeqWright DNA Technology Services). Original and edited sequences, as well as their chromatographs, are found in the APPENDIX 11.5 / 11.6.

6.7 PHYLOGENETIC ANALYSIS

Data from the aligned sequences of the different strains was then compared. A dendrogram was created from the similarity matrix. The PHYLOGENETIC TREE generated was based on data resulting from ITS analysis using the computer program DS Gene (Accelrys Inq., USA). The trees were constructed with the NEIGHBOR-JOINING method and Best-tree option. Evolutionary distances are measured as the presence/absence of bases (TAJIMA-NEI's distance). The degree of genetic divergence between two DNA sequences is correlated with the proportion of DNA fragments with which they share.

Molecular data from different electronic databases such as NCBI and EBI (APPENDIX 11.7) were directly compared with the strains studied to facilitate the confirmation of results and their application to other strains without the need to obtain them or repeat experiments. In this manner, the strains were clustered in major groups throughout the world and their relationships with other species (e.g. *L. lateritia*) could be observed.