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7. Molecular Identification of Fungal Isolates/Fungal Pathogens

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7.1 Introduction:

Fungi are morphologically, ecologically and metabolically diverse. By using morphological features alone in the identification of fungi to the species level is difficult. It is unable to solve the confusion of anamorphs (asexual reproductive stage) and telomorphs (sexual reproductive stage) of fungi. Now molecular identification is considered for species level identification of fungus which was done through the three nuclear ribosomal genes and ITS region which is the official DNA barcoding marker for species-level identification of fungi using NCBI-BLAST search for DNA barcoding with numerous curated molecular databases containing fungal sequences to augment or supplant ITS in species-level identification of fungal groups and construct phylogenetic trees from DNA sequences to facilitate fungal species identification. It is further recommended that, whenever possible, both morphology and molecular data may be used for fungal identification.

7.2 Isolation of Genomic DNA:

Isolation of fungal genomic DNA is done by growing the fungi for 3-4 days. Liquid nitrogen is used for crushing the cell mass for both cases.

a. Preparation of Genomic DNA Extraction Buffer:

The following buffers for DNA extraction are prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

Lysis Buffer:

- 50 mM Tris, pH 8.0
- 100 mM EDTA
- 100mM NaCl
- 1% SDS

Genomic DNA Buffer:

- 10 mM Tris, pH 8.0
- mM EDTA

b. Genomic DNA Extraction:

Isolation of fungal genomic DNA is done by growing the fungi for 3-4 days. The mycelia are incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60^0C followed by centrifugation at 12,000 rpm for 15 min.

The supernatant is then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase is further extracted with equal volume of phenol:

Chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min; the aqueous phase is then transferred in a fresh tube and the DNA is precipitated with chilled ethanol (100%).

DNA is pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried.

c. Purification of Genomic DNA:

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure is followed by RNAase treatment. Genomic DNA is resuspended in 100 µl 1 X TE buffer and incubated at 37oC for 30 min with RNAse (60µg). After incubation the sample is re-extracted with PCI (Phenol: Chloroform:

Isoamylalcohol 25:24:1) solution and RNA free DNA is precipitated with chilled ethanol as described earlier.

The quality and quantity of DNA are analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produce clear sharp bands, indicating good quality of DNA.

d. Measurement of DNA Concentration using Spectrophotometry:

The pure sample is (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), used to quantify DNA.

For quantitating DNA absorbance at wavelengths of 260 nm and 280 nm are taken. Quantification is done as follows:

- 1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA
- 1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

Pure preparations of DNA have OD260/OD280 value 1.8.

If there is contamination with protein or phenol, this ratio will be significantly less than the value given above and accurate quantitation of the amount of nucleic acid will not be possible.

e. Agarose Gel Electrophoresis to Check DNA Quality:

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA.

It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample**.**

i. Preparation of DNA Samples for Electrophoresis:

Agarose (0.8%) in 1X TBE buffer is melted, cooled and poured into the gel casting tray with ethidium bromide.

Gels solidify in 15-20 min.

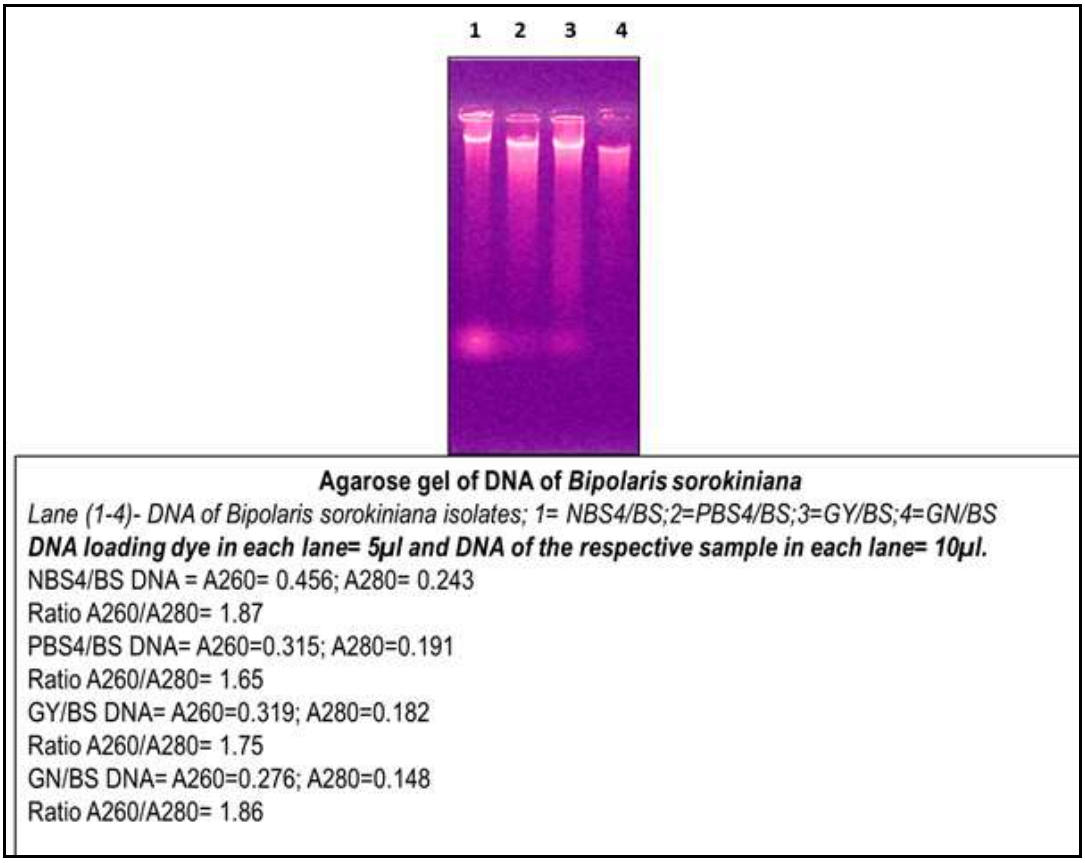
ii. Run Gel Electrophoresis for DNA Fraction:

15µl of sample and 5µl of DNA loading dye mixed properly is loaded in each well of agarose gel (1%).

The electrical head of the gel tank is attached firmly and electric supply is applied at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 min.

The DNA migrated from cathode to anode. Run is continued until the bromophenol blue had migrated an appropriate distance through the gel.

Then electric current is turned off and gel is removed from the tank and examined on UV Tran illuminator and photographed for analysis.



Agarose gel of DNA of *Bipolaris sorokiniana*

Lane (1-4)- DNA of Bipolaris sorokiniana isolates; 1= NBS4/BS;2=PBS4/BS;3=GY/BS;4=GN/BS

DNA loading dye in each lane= 5µl and DNA of the respective sample in each lane= 10µl.

NBS4/BS DNA = A260= 0.456; A280= 0.243
Ratio A260/A280= 1.87
PBS4/BS DNA= A260=0.315; A280=0.191
Ratio A260/A280= 1.65
GY/BS DNA= A260=0.319; A280=0.182
Ratio A260/A280= 1.75
GN/BS DNA= A260=0.276; A280=0.148
Ratio A260/A280= 1.86

7.3 Polymerase Chain Reaction (PCR):

The polymerase chain reaction (PCR) is a laboratory technique for DNA replication that allows a "target" DNA sequence to be selectively amplified. PCR can use the smallest sample of the DNA to be cloned and amplify it to millions of copies in just a few hours. Discovered in 1985 by Kerry Mullis, PCR has become both and essential and routine tool in most biological laboratories.

Principle of PCR: The PCR reaction requires the following components:

- **DNA Template**: It is a double stranded DNA (dsDNA) of interest, separated from the sample.
- **DNA polymerase:** It is usually a thermostable Taq polymerase that does not rapidly denature at high temperatures (98°), and can function at a temperature optimum of about 70°C.
- **Oligonucleotide primers**: It is a short piece of single stranded DNA (often 20-30 base pairs) which is complementary to the 3' ends of the sense and anti-sense strands of the target sequence.
- **Deoxynucleotide Triphosphates**: These are single units of the bases A, T, G, and C (dATP, dTTP, dGTP, dCTP) provide the energy for polymerization and the building blocks for DNA synthesis.

- **Buffer System:** It includes magnesium and potassium to provide the optimal conditions for DNA denaturation and renaturation; also important for polymerase activity, stability and fidelity.

Procedure of PCR: All the PCR components are mixed together and are taken through series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine.

- **Denaturation**: This step involves heating the reaction mixture to 94°C for 15-30 seconds. During this, the double stranded DNA is denatured to single strands due to breakage in weak hydrogen bonds.
- **Annealing**: The reaction temperature is rapidly lowered to 54-60°C for 20-40 seconds. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.
- **Elongation**: Also known at extension, this step usually occurs at 72-80°C (most commonly 72°C). In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction. Under optimal conditions, DNA polymerase will add about 1,000 base pairs (bp) per minute.

With one cycle, a single segment of double-stranded DNA template is amplified into two separate pieces of double-stranded DNA. These two pieces are then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template is increased exponentially.

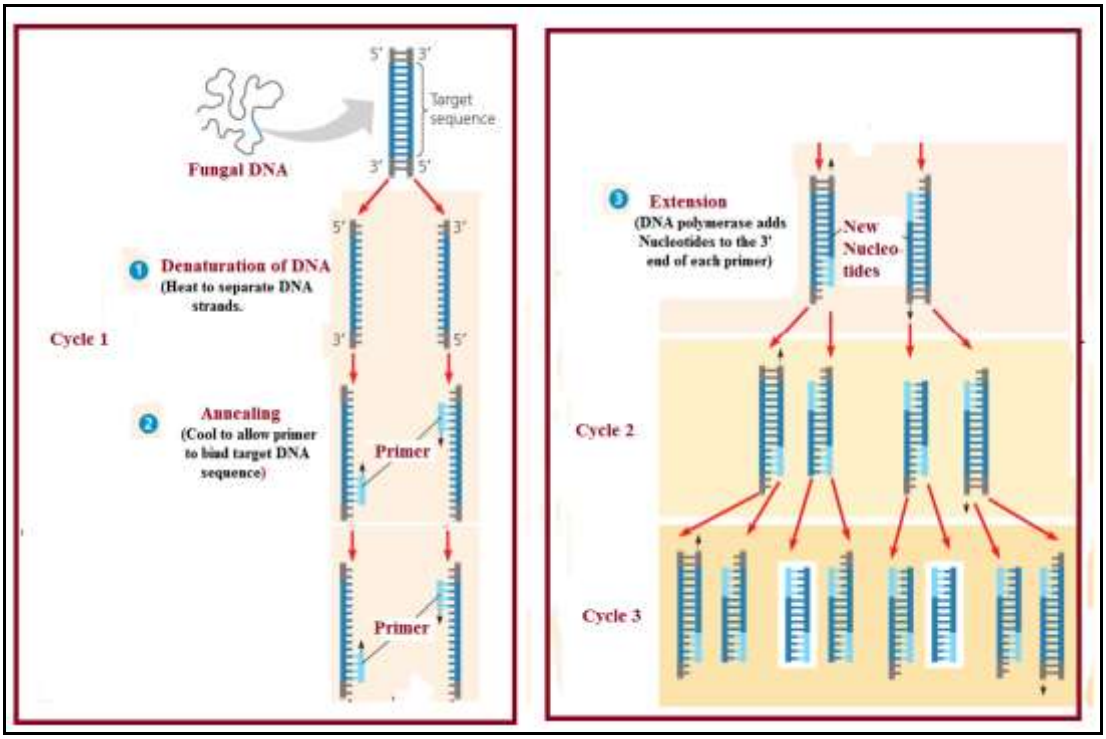


Figure 7.2: Polymerase Chain Reaction (PCR)

7.4 Restriction Endonuclease Digestion of Fungal / Plant DNA:

7.4.1 Restriction Endonucleases:

Restriction endonucleases (REs) are bacterial enzymes that cleave double-stranded DNA.

Type I REs are important in bacterial function but do not cleave DNA at specific sequences.

Type II REs, use to cleave DNA and require highly specific sites for DNA cleavage and are thus extremely useful tools in molecular biology.

These enzymes allow the cloning and purification of defined DNA fragments. REs are present in bacteria use to destroy DNA from foreign sources by cleaving the foreign DNA at specific recognition sites.

The bacteria bearing REs is protected from cleavage because the specific recognition sites in their own DAN are modified, usually by **methylatio**n at one of the bases in the site, making the site no longer a substrate for RE cleavage.

Practically, REs with different recognition site specificities have been purified from various bacterial strains and are used by molecular biologists under defined conditions to cleave purified DNA from eukaryotic sources into defined fragments in an *in vitro* reaction.

Many of the REs are palindromic, meaning the base sequence reads the same backwards and forwards.

The cleavage DNA may be sticky or blunt based on the REs which cut the DNA. The cleavage site specificities of some REs have been listed (Table 7.1).

Table 7.1: Restriction endonucleases (RSs) with their specific recognition sites and nature of cutting end

Microorganisms	REs	Cleavage site	Cleavage product	Nature of cutting
Bacillus amyloliquefaciens H	Bam HI	5'-GlGATCC-3 3-CCTAGlG-3	5-G GATCC-3 3-CCTAG G-5	Sticky end
Bacillus globigii	Bgl	5-AlGATCT-3 3-TCTAGlA-5	5-A GATCT-3 3-TCTAG A-5	Sticky end
Escherichia coli	Eco RI	5-GlAATTC-3 3-CTTAAlG-5	5-G AATTC-3 3-CTTAA G-5	Sticky end
Haemophilus parainfluenzae Rd	Hpa I	5-GTTlAAC-3 3-CAAlTTG-5	5-GTT AAC-3 3-CAA TTG-5	Blunt end
Serratia marcescens	Sma I	5-CCClGGG-3 3-GGGlCCC-5	5-CCC GGG-3 3-GGG CCC-5	Blunt end

7.5 Restriction Fragment Length Polymorphism (RFLP):

Principle:

RFLP is an enzymatic procedure for separation and identification of desired fragments of DNA. Using restriction endonuclease enzymes fragments of DNA is obtained and the desired fragment is detected by using restriction probes. Southern hybridization using restriction endonuclease enzymes for isolation of desired length of DNA fragments is an example of RFLP.

Procedures

A. Step I: Restriction Digestion:

Extraction of desired fragments of DNA was done using suitable restriction endonuclease (RE). The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments. Different sizes of fragments are generated along with the specific desired fragments.

B. Step II: Gel Electrophoresis:

The digested fragments are run in polyacrylamide gel electrophoresis or Agarose gel electrophoresis to separate the fragments on the basis of length or size or molecular weight.

Different sizes of fragments generate different bands.

C. Step III: Denaturation:

The gel is placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

D. Step IV: Blotting:

The single stranded DNA obtained are transferred into charge membrane ie. Nitrocellulose paper by the process called capillary blotting or electro-blotting.

E. Step V: Baking and Blocking:

The nitrocellulose paper transferred with DNA is fixed by autoclaving. Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labelled probe non-specifically to the charged membrane.

F. Step VI: Hybridization and Visualization:

The labelled RFLP probe is hybridized with DNA on the nitrocellulose paper. The RFLP probes are complimentary as well as labelled with radioactive isotopes so they form colour band under visualization by autoradiography.

7.6 Application of RFLP Test:

7.6.1 Genome Mapping: helps in analysis of unique pattern in genome for organism identification and differentiation. It also helps in determining recombination rate in the loci between restriction sites.

7.6.2 Genetic Disease Analysis: After identification of gene for particular genetic or hereditary disease, that gene can be analyzed among other family members.

7.6.3 DNA Finger Printing (Forensic Test): It is the basis of DNA finger printing for paternity test, criminal identification etc.

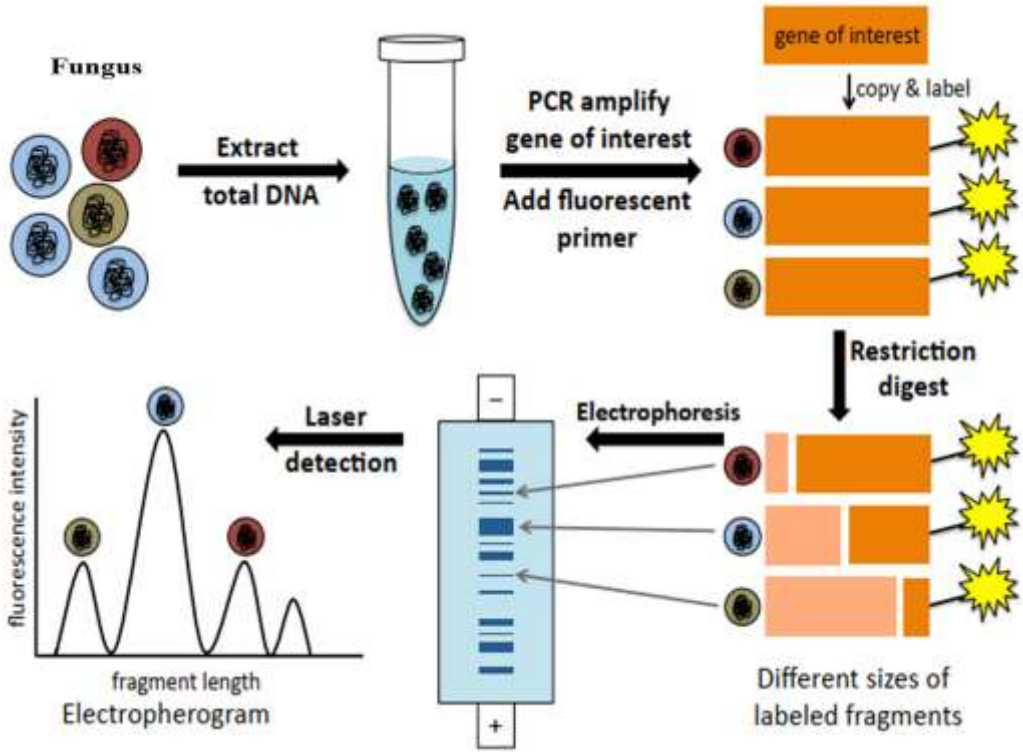


Figure 7.3: Restriction fragment length polymorphism (RFLP)]

7.7 RAPD PCR Analysis:

For RAPD, random primers are selected. PCR is programmed with an initial denaturing at 94ºC for 4 min. followed by 35cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min and extension at 70ºC for 90 s and the final extension at 72ºC for 7 min. in a Primus 96 advanced gradient Thermocycler.

PCR product (20 µl) is mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

a. RAPD Primers: The following primers can be used for RAPD analysis:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
OPA1	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%
A-11	AGGGGTCTTG	10	31.8	76%
A-5	AGGGGTCTTG	10	36.8	73%
OPD6	GGGGTCTTGA	10	32.8	83%
AA-04	CAGGCCCTTC	10	38.2	70%

b. Amplification Conditions: Temperature profile, 94ºC for 4 min followed by 35cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min and extension at 70ºC for 90 s and the final extension at 72ºC for 7 min in a Primus 96 advanced gradient Thermocycler.

c. Analysis of RAPD Bands: RAPD band patterns are initially assessed by eye and isolates are grouped according to their shared band patterns.

d. Scoring of Individual Bands: Two methods of scoring bands are assessed. The first method involved scoring bands using the ***computer programme NTSYSPc*** and the second method is to score the number of shared bands (i.e. bands of equal size) on a gel by eye.

For both methods, photographs of the gels have to be scanned into a computer and saved as graphics files.

e. Reconstruction of the Phylogenetic Tree: As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer. More complex analyses involve cladistic analysis of data and reconstruction of the phylogenetic tree.

f. UPGMA Method:

The image of the gel electrophoresis is documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands are scored and analysed following UPGMA cluster analysis protocol (Sneath and Sokal, 1973) and computed ***In Silico into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W)***. The SIMQUAL program is used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate is evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix is created which is used to calculate the Jaccard similarity coefficient for each pair wise comparison.

Jaccard coefficients are clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with ***arithmetic average (UPGMA) algorithm*** in NTSYSpc.

7.7.1 ITS PCR Analysis:

For ITS-PCR amplification, genomic DNA is amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR is programmed with an initial denaturing at 94ºC for 5 min. followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 59ºC for 30 sec and extension at 70ºC for 2 min and the final extension at 72ºC for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) is mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

a. ITS- PCR Primers: The following ITS primers can be used as example:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplicon size (bp)
***ITS-PCR primers: For example:- Fcg17F & Fcg17R for Fusarium* sp. *and T/ITS 1 & T/ITS4 for Trichoderma* sp.**					
Fcg17F	TCGATATACCGTGCGATTTCC	21	65.0	47%	~570
Fcg17R	TACAGACACCGTCAGGGGG	19	66.0	63%	
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%	~600
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%	

b. Amplification Conditions: Temperature profile, 94ºC for 5 min. followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 59ºC for 30 sec and extension at 70ºC for 2 min and the final extension at 72ºC for 7 min in a Primus 96 advanced gradient Thermocycler.

c. Sequencing of rDNA Gene: The rDNA is used for sequencing purpose. DNA sequencing can be done bidirectionally using the ITS primer pairs by Genei Bangalore.

d. Sequence Analysis: DNA sequence information is analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4 (Tamura et al. 2004; 2007), NTSYSpc as well as the few online softwares.

e. Chomatogram of Sequence: The chromatogram of the DNA sequence is analysed by the software Chromus.

f. Editing and Alignment of Sequence Data: All the DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms (Thomson et al. 1994).

g. BLAST Analysis of the Sequences: The DNA sequences are analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism is done on the basis of homology of sequence.


Molecular Identification of BS-29 isolate
100bp ladder
T1
T4
Contig sequence : BS-29
>BS-29
TTTCCGTAGGTGAACCTGCGGAGGGATCATTACACAACAAA
ATATGAAGGCCTGGCTTCGCGGCCGGCTGAAATATTTTTTT
CACCCATGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGT
TCGCCCGCCACCAGGACCAAACCATAAACCTTTTTTTTATG
CAGTTGCAATCAGCGTCAGTAAAAACAATGTAATTATTACAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
CGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTA
TTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCA
AGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTCCCTCTTTCT
GGGAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGG
TTTCGGAGCGCAGCACATATTTTGCGCTTTGTATCAGGAGA
AAAGGACGGTAATCCATCAAGACTCTACATTTTTAACTTTTG
ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGA
Identification of BS-29 isolate (Acc.No.-KM 066949- Bipolaris sorokiniana) by 18S rDNA sequencing and chromatogram of sequence
Source:- J. Mycopathol. Res. 54(1) : 117-125, 2016; (ISSN 0971-3719)

Figure 7.4:

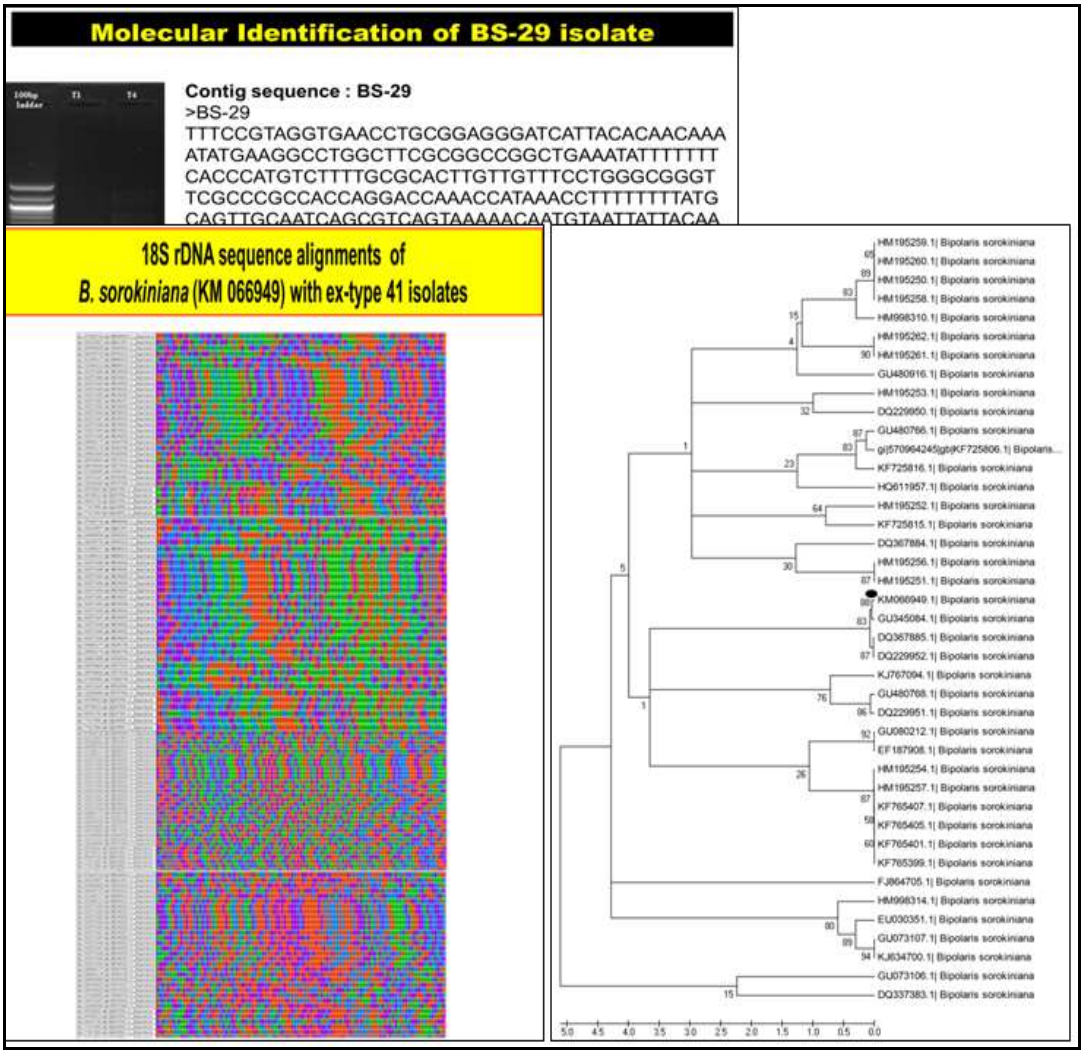


Figure 7.5:

7.8 Submission of rDNA Gene to NCBI Genbank:

The DNA sequences are deposited to ***NCBI GenBank*** through ***BankIt procedure*** and approved as the IT'S sequence after complete annotation and given accession numbers.

7.8.1 Submitting Sequences using Specific NCBI Submission Tool- BankIt:

7.8.2 Submission using BankIt:

How do I create a submission to GenBank using BankIt?

a. Review the Requirements for GenBank Submissions through BankIt and make sure you can provide the required information for your submission.

b. If you have never submitted to GenBank, scan the GenBank Sample Record to familiarize yourself with GenBank record field definitions.
c. For examples of specific types of GenBank submissions, see the GenBank Annotation Example page.

7.8.3. Login to My NCBI:

a. Go to the BankIt home page and click on "***Sign in to use BankIt***" located in a yellow box on the right at the top of the page. You will go to the My NCBI login page.
b. If you do not already have an NCBI account, click on "***Register for an NCBI account***" located below the login text boxes.
   The boxes marked with an asterisk (*) indicate the minimum amount of information we need to create an account for you. Enter the required information and click the "Create ***account***" button.
c. c. ***Login to BankIt and begin your submission***. The submission process has well marked steps where you will be prompted to provide contact information and your data.
d. d. The BankIt home page contains links to sequence annotation examples.

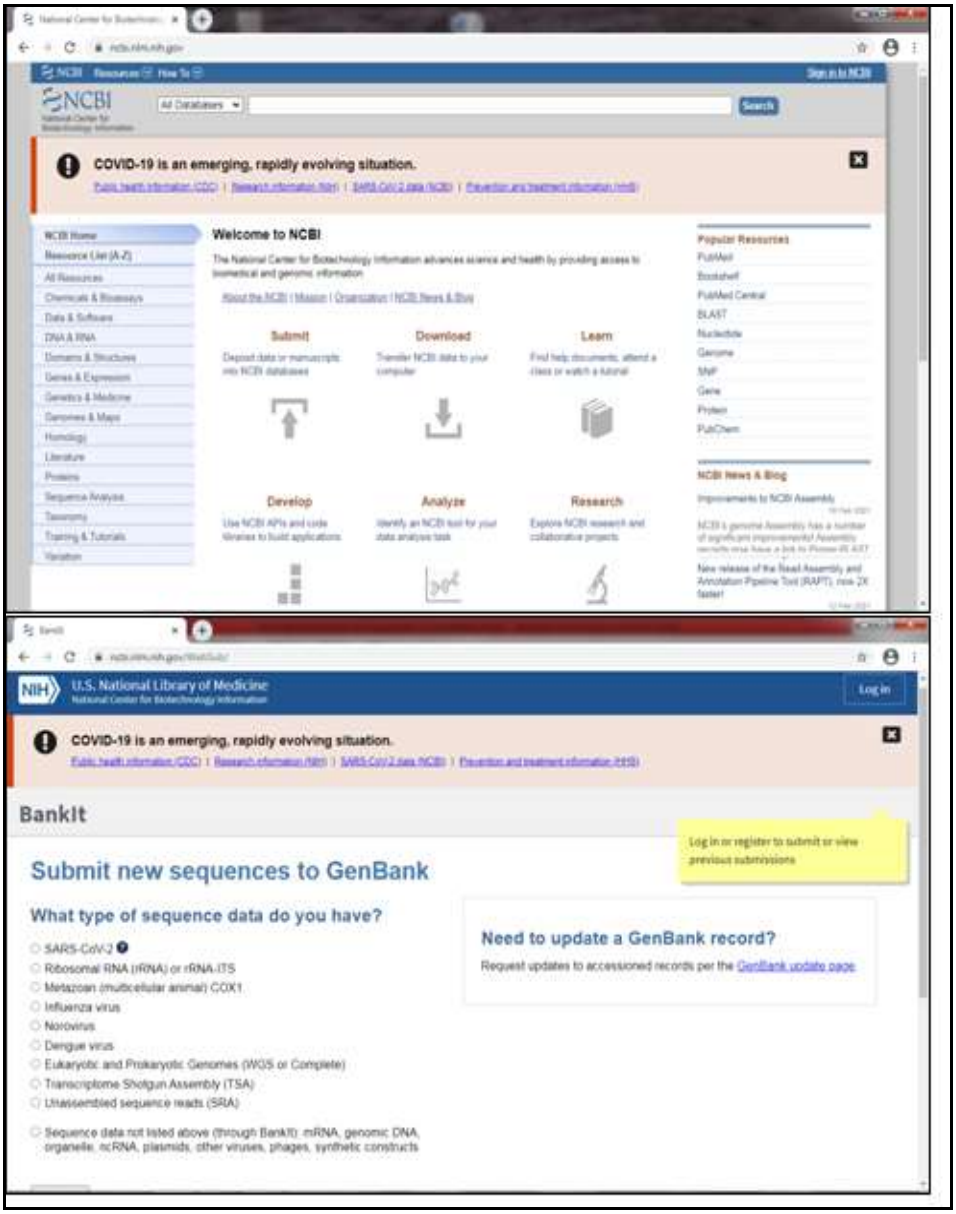


Figure 7.6

NCBI and Banklt websites

List of few private companies in India exhibiting facilities of Molecular Identification of fungal isolates/pathogens through 18S rDNA partial sequencing method

A. Xcelris Labs Limited: Sangeeta Complex, Near Parimal Crossing, Ellisbridge, Ahmedabad - 380006, Gujarat, India. http://www.xcelrisgenomics.com/

B. Chromous Biotech Pvt. Ltd.: 236, Pushpagiri Bhavan, 'F' Block, Opp. Cauvery School Sahakaranagar, Bengaluru-560092 https://www.chromous.com

C. Sci Genom: Plot no: 43A, SDF, 3rd floor, A Block, CSEZ, Kakkanad, Cochin, Kerala – 682037 https://www.scigenom.com

D. Credora Life Science: 176/1, Horamavu Outer Ring Road, Horamavu Village, Bengaluru 560043 https://www.credora.in

E. C-SIX LABS: C - SIX LABS PRIVATE LTD No.6/858-M, 2nd Floor, Suite 345, Valamkootil Towers, Judgemukku, Thrikkakara P.O, Kakkanadu, Kochi, Ernakulam, Kerala – 682021 https://www.csixlabs.in

F. Eminent Biosciences: 91, Sector a, Mahalakshmi Nagar, Indore - 452010, Madhya Pradesh, India https://www.eminentbio.com

G. Yaazh Xenomics: No.326, 1st Floor, Thadagam Road, Opp. Mayflower Westmount Apartment, Above Covai Deepan Restaurant, Near GCT College, Coimbatore – 641013

https://www.yaazhxenomics.in

H. geneOmbio: VEDANT, S.No. 39/3, H.No.1043, Yogi Park, Off Mumbai-Bangalore Expressway, Baner, Pune, Maharashtra 411045 https://www.geneombiotechnologies.com

7.9 References:

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2. Tamura K, Dudley J, Nei M, Kumar S, MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol Biol Evol, 24: 1596-1599, 2007.
3. Tamura K, Nei M, Kumar S, Prospects for inferring very large phylogenies by using the neighbor-joining method, Proc Natl Acad Sci USA, 101: 11030-11035, 2004.
4. Thompson J, Higgins D, Gibson T, and CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res, 22:4673-4680, 1994.