Chapter 2

Morphological and molecular identification of pathogenic clinical *Fusarium* isolates

2.1 Introduction:

In developing countries, corneal blindness because of keratitis is very common and caused by several pathogens like *Fusarium, Aspergillus, Candida* and dematiaceous species (Gajjar *et al*., 2013; Nielsen *et al*., 2015). *Fusarium* species emerged as very important opportunistic pathogen causing localised to life threatening infections (Zapater, 1986). Keratitis due to members of FSSC and FOSC are most common (Dallé da Rosa *et al*., 2018; Guarro, 2013; Salah *et al*., 2015). However, keratitis because of *F. dimerum* (Vismer *et al*., 2002), *F. vericilliodes* (Sun *et al*., 2015), *F. sacchari* (Bansal *et al*., 2016) and *F. polyphialidicum* (Guarro *et al*., 2003) is very rare. Keratitis caused by fungus is known as mycotic keratitis and reported worldwide. The infection is most common in tropical and subtropical regions of the world like China (An *et al*., 2016; Sun *et al*., 2015), Brazil (Oechsler *et al*., 2013) and India (Tupaki-Sreepurna *et al*., 2017). Also the incidences are increasing in United States (Chang *et al*., 2006) and Europe (Van Diepeningen *et al*., 2015). Antifungal susceptibility revealed that the members of FSSC are highly resistant to antifungal therapy (Al-Hatmi *et al*., 2017; Azor *et al*., 2007; O'Donnell *et al*., 2008). Also there were reports of keratitis outbreaks in U.S. because of contact lens associated keratitis caused by FSSC (O'Donnell *et al*., 2007).

2.2 Materials and Methods:

2.2.1 Collection of fungal isolates:

Fungal isolates (n=22) were provided by Iladevi cataract and IOL research center, Ahmedabad (n=11) and L.V. Prasad eye institute, Hyderabad (n=11).

2.2.2 Maintenance of fungal isolates:

All the isolates were grown on PDA and SDA plates for 7-8 days and then sealed with parafilm and stored at 4ºC for 3 months. Fungal spores were collected from 7-8 days actively growing fungus by pouring sterile PBS (Phosphate buffered saline) on SDA/ PDA plates and supernatant was collected in to sterile falcon tube and stored at -20ºC. Fungal isolates were also stored for long period by using sterile filter paper discs. 10 mm disc of 7-8 days old actively grown culture was inoculated on sterile PDA plates and 10mm sterile whatman no 1 filter paper discs were placed on PDA plates. One

drop of sterile PDB was added on each disc. Fungus was allowed to grow completely on plate for 8-10 days. After 10 days, filter paper discs which were covered completely with fungi were removed with the help of sterile forceps and taken in to sterile petriplate and allowed to dry in desiccator. Completely dried discs were transferred in to sterile envelope and sealed and stored at -80ºC.

2.2.3 Identification of fungal strain:

2.2.3.1 Morphological examination:

The fungus was grown on SDA and PDA plate at 30ºC for 7 days. Identification on the basis of morphological characteristics included whether the fungus is yeast or mold, colony diameter, colour or pigmentation, type of hyphae (cottony, velvety or powdery), slow growing or fast growing and some other characteristics.

2.2.3.2 Microscopic examination:

For microscopic examination the fungus was mounted with lactophenol cotton blue (Himedia, India) and examined under light microscope at different magnification (10X, 40X and 100X). The microscopic identification was done using specific characters like spore morphology like size and shape and presence of septate or aseptate hyphae.

2.2.3.3 Molecular identification:

For molecular identification the DNA was isolated from fungi and Polymerase chain reaction (PCR) using internally transcribed spacer (ITS) and translation elongation factor 1 alpha (TEF-1-apha) primers was done. The amplified product was sequenced and subjected to BLAST (Basic Local Alignment Search Tool) for identification.

2.2.3.3.1 DNA isolation from fungi:

Fungal isolates were grown in PDB at 30ºC for 7 days and mycelial mat was collected by filtration using whatman no.1 Filter paper (Merck, USA). Fungal mat was washed with distilled water (D/W) and dried by decanting the water. 60-90 mg of dried mat was taken in to sterile mortar pestle and 500 µl of warm TES (Tris-EDTA-Sodium dodecyl sulphate) lysis buffer was added to it. Fungal mat was grinded in mortar pestle and collected in sterile

microcentrifuge tube (MCT). 50 µg of proteinase K (Merck, USA) was added to it and kept at room temperature (RT) for overnight and next day incubated at 60ºC for 60 minutes. After incubation, 140 µl of 5M NaCl (Merck, USA) and 64 µl of 10% CTAB (Merck, USA) was added to MCT. Tubes were rotated slowly and incubated at 65ºC for 10 minutes. After incubation, equal volume of mixture and freshly prepared chloroform (Merck, USA): isoamyl alcohol (Merck, USA) (24:1) (V/V) was taken and mixed well followed by centrifugation at 14,000xg for 10 minutes. After centrifugation, 3 layers were formed in tube and upper layer was taken in to new autoclaved MCT and to that 0.6 volume of cold isopropanol (Merck, USA) and 0.1 volume of 3M sodium acetate (Merck, USA) (pH 5.2) was added. Centrifugation was carried out at 14,000xg for 10 minutes. After centrifugation, white pellet was formed at the bottom of tube. Supernatant was discarded and pellet was washed twice with 70% ethanol and centrifuged at 10,000xg for 5 minutes. The obtained pellet was dissolved in TE buffer and stored at -20ºC till further use. The quality of DNA was checked by agarose gel electrophoresis using 0.8% agarose gel and by taking ratio of 260/280 nm and quantification was done by measuring absorbance at 260 nm using spectrophotometer (Multiscan Go, Thermoscientific, USA).

2.2.3.3.2 Polymerase chain reaction of fungal DNA:

After DNA extraction, ITS and TEF1-alpha regions were amplified using respective primers which are universal for the amplification of fungal DNA. The universal primers used for fungal amplification were ITS1 (F): 5'TCCGTAGGTGAACCTGCGG 3' which hybridizes at the end of 18S rDNA and ITS4 (R): 5'TCCTCCGCTTATTGATATGC3' which hybridizes at the beginning of 28S rDNA, Ef-1 (F): 5'ATGGGTAAGGAAGACAAGAC3' which hybridizes at start of exon 1 and Ef-2 (R): 5'GGAAGTACCAGTGATCATGTT3' which hybridizes at start of exon 4. 100 ng of genomic DNA was used as a template for a PCR reaction. For ITS amplification, 25 µl PCR mixture was prepared which contained 100 ng of genomic DNA template, 1.5 µl of 10 pmol forward primers (ITS 1- Eurofins scientific, India), 1.5 µl of 10 pmol reverse primer (ITS 4- Eurofins scientific, India), 12.5 µl of 2X PCR mastermix (Takara, Japan) and final volume was

adjusted to 25 µl using dH_2O. PCR reaction involved 1 cycle at 95ºC for 5 minutes, followed by 35 cycles with a denaturation step at 95ºC for 30 seconds, an annealing step at 55ºC for 1 minute, an extension step at 72ºC for 1 minute and final extension at 72ºC for 6 minutes. For TEF amplification, 25 µl PCR mixture was prepared which contained 100 ng of genomic DNA template, 2.0 µl of 10 pmol forward primers (Ef 1- Eurofins scientific, India), 2.0 µl of 10 pmol reverse primer (Ef 2- Eurofins scientific, India), 12.5 µl of 2X PCR Mastermix (Takara, Japan) and final volume was adjusted to 25 µl using dH_2O. PCR reaction involved 1 cycle at 94ºC for 3 minutes, followed by 35 cycles with a denaturation step at 94ºC for 30 seconds, an annealing step at 53ºC for 30 seconds, an extension step at 72ºC for 45 seconds and final extension at 72ºC for 5 minutes. The amplified PCR products were stored at 4ºC till further use. The amplified products were electrophoresed on 2% agarose gel to check the amplification. Sequencing was outsourced at Labreq Bioscientific. Sequence analysis was carried out by using BLASTN (Basic Local Alignment Search Tool) similarity search (http://www.ncbi.nlm.nih.gov/BLAST) and *Fusarium* MLST database similarity search.

2.2.3.3.3 Agarose gel electrophoresis:

The PCR amplified products of ITS and TEF were subjected to electrophoresis to confirm the purity and size. The presumed size of ITS amplified products was approximately 600 base pairs and TEF amplified product was 700 base pairs. The casting tray was wiped with 95% ethanol and taped on both the ends. For 2% agarose gel, 1 g of agarose powder was added to 50 ml 1X TBE (Tris-Boric acid-EDTA) buffer and kept in a water bath (70-80ºC) until agarose was dissolved. After complete dissolution of agarose, ethidium bromide (Himedia, India) at a final concentration of 5 µg/ml was added. This solution was added to the taped casting tray with a comb placed in it. The gel was allowed to solidify at RT for 15-20 minutes. After solidification of agarose gel, combs and tapes were removed and tray was kept in electrophoresis chamber. 1X TBE was poured in the chamber up to the gel was completely immersed in buffer. The amplified products (10 µl) were mixed with gel loading dye (6X) and added into wells; in one well 100 bp

molecular weight marker (TAKARA, Japan) was added. The current was passed through the buffer in black (negative) to red (positive) direction and voltage was set to 100V. After the run was complete ($3/4^{th}$ of the gel), power was switched off and gel as immediately visualized using Gel Doc (BIO-RAD, USA). Fluorescent bands were observed against dark background. The size of amplicons were compared with marker.

2.2.3.3.4 Phylogenetic analysis:

All sequences were aligned using ClustalW. The Phylogenetic tree was generated using maximum parsimony analysis (MEGA X).

2.3 Results:

2.3.1 Morphological identification:

Morphological identification of *Fusarium* was done on the basis of the growth characters on PDA which is commonly used for the growth of fungi. Mainly 4 types of *Fusarium* species were identified.

The first type of isolates were fast growing, highly filamentous, fluffy and cottony on PDA and some of them released red to orange coloured pigment in media. The fungus was fully grown within 7 days. Spore production was seen after maturation of fungus. The colour of mycelia visibly was whitish. Margin of colony vary from smooth to irregular but mostly the margin observed was smooth. All these characters were typical of *Fusarium solani* species and the isolates having these characters were tentatively identified as *F. solani* (CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50 and Cc240) (Figure 2.1 to Figure 2.6). Majority of isolates were *F. solani*.

The second group of fungus was with slow and flat growth. The colony was fully grown within 12-15 days. The surface appeared slimy and slightly yellowish, peach to orange with irregular margins. The aerial mycelium was floccose and whitish sometimes. These isolates did not secrete any pigment. This group was identified morphologically as *Fusarium dimerum* (Cc26, Cc119 and CSH4) (Figure 2.7).

The third type of isolates were rapidly growing, highly filamentous and cottony on PDA but they did not release pigments in to the media but the mycelia itself was pigmented from bottom which was red to purple in colour. The fungus was fully grown within 5-7 days. The colony was having smooth as well as rough margin. After maturation, thread like structure was observed in the centre of colony (Cc52, Cc61, Cc167 and Cc215) (Figure 2.8 and Figure 2.9). These isolates were tentatively grouped as *Fusarium sacchari*.

The fourth type of isolate was also similar to *F. solani* and *F. sacchari* in morphology but did not produce any pigment in media. The growth of fungus was rapid. It appeared white cottony fluffy and elevated. The pigmentation was not observed initially but with age slight yellow pigmentation was observed. The margin was smooth and was grouped as *F. equiseti* (Cc172) (Figure 2.10).

In the present study three control strains were used which were *F. solani* (MTCC 2935) and *F. sacchari* (MTCC 3004 and MTCC 9671).

2.3.2 Microscopic identification:

2.3.2.1: *Fusarium solani*:

Microscopic examination of isolates having morphological characters of *F. solani* (CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50 and Cc240) showed presence of conidiophore which was arising laterally from aerial hyphae. The hyphae was long, septate and branched. Conidiogenic cells were long and narrow towards the apex which generate one celled microspores. Monophialides were produced on a distinct collarette. The number of septa in microcondia was from 0-1 and in macroconidia was from 2-4. The shape of macroconidia was sickle shaped and elongated with blunt ends. Macroconidia were producing on shorter branches, usually slightly curved with short blunt apical and indistinctly pedicellate basal cells, mostly 3-septate, occasionally septate, Microspore was oval as well as clavate and curved. Microconidia were found abundantly, produced on elongate conidiophores. (Figure 2.1 to Figure 2.6).

2.3.2.2 *F. dimerum*:

The isolates having colony characteristics of *F. dimerum* (Cc26, Cc119 and CSH4) had following microscopic features. The macrospore was curved with pedicellate cells. The spores were fusiform in shape. Spores were found in group and attached to hyphae sometimes. Both macrospores and microspores were present. Macrospores were long, boat shaped and sometime curved; microspores were small curved or oval (Figure 2.7)

2.3.2.3 *F. sacchari*:

Characteristics of *F. sacchari* is formation of microcondia from monophialids and polyphialids. Cc52, Cc61, Cc167 and Cc215 showed formation of spores from short conidiogenic cells present in polyphialids. The hyphae was long, branched and septate. Both macrospore and microspore were found. Macrospores were elongated and 2-4 septate straight basal cells. Microspores were oval to clavate in shape and had 0-1 septa. Conidiophore was polyphiadilic. The spores were smaller in size. (Figure 2.8 and Figure 2.9).

2.3.2.4 *F. incarnatum-equiseti*:

The main feature of *F. equiseti* is presence of chlamydospores. In microscopic examination of Cc172, the chlamydospores were present and they were found in chains as well as solitary. The surface of chlamydospore was smooth to rough. Microcondia were found which were having oval as well as elongated apical cells and had 0-1 septa. Formation of microconidia was from polyphialids. However, in stressed condition of 3-8 septate macrospore was observed (Figure 2.10).

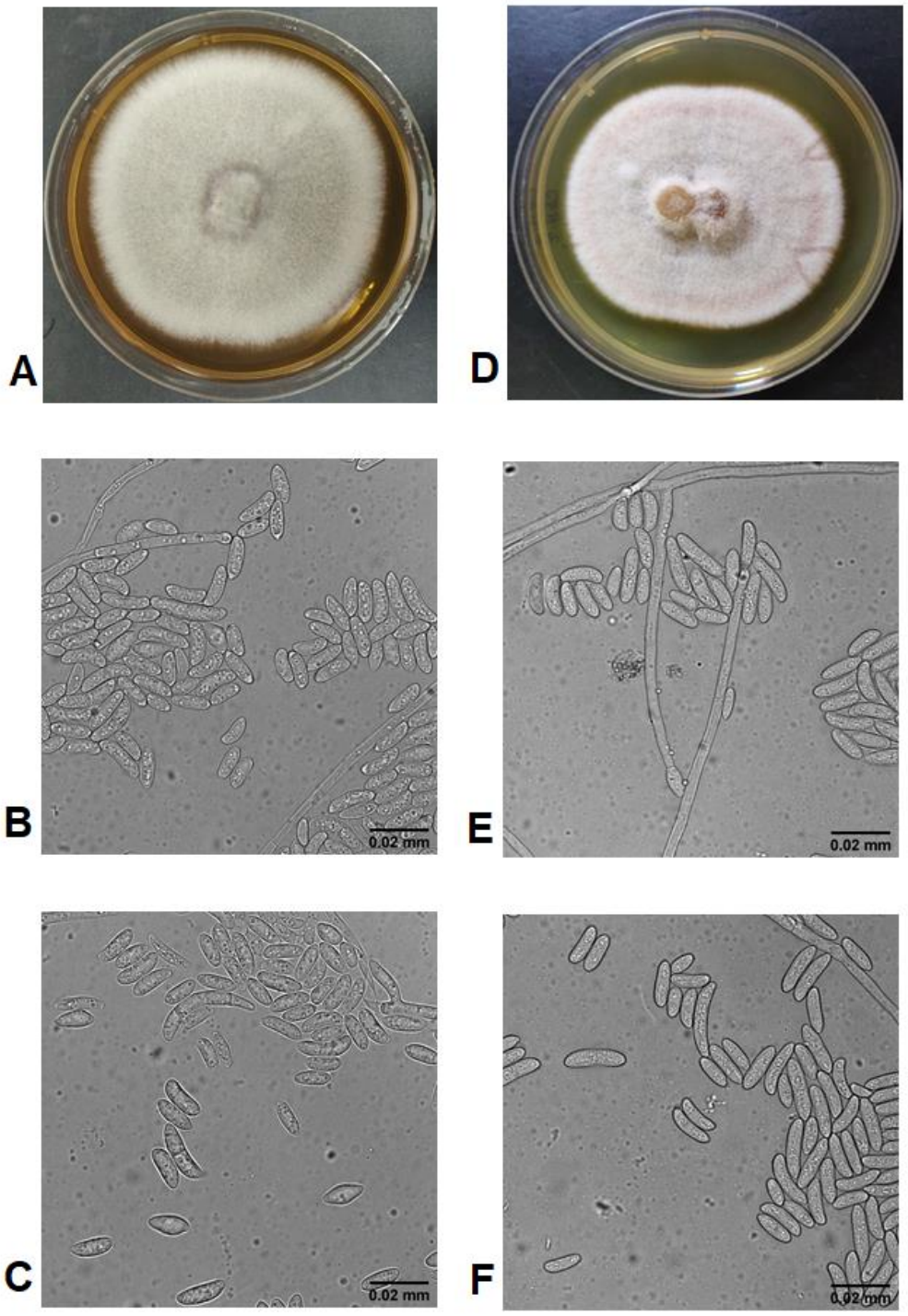

Figure 2.1: Morphology and microscopy of CSH1 and CSH2. (A) & (D): Colony morphology of CSH1 (white, fluffy and cottony) and CSH2 (white and cottony), respectively. Presence of flaccose was detected in CSH2. **(B) & (E):** Spore generation from conidiogenic cells of CSH1 and CSH2, respectively. **(C) & (F):** Presence of macrospore in CSH1 and CSH2, respectively.

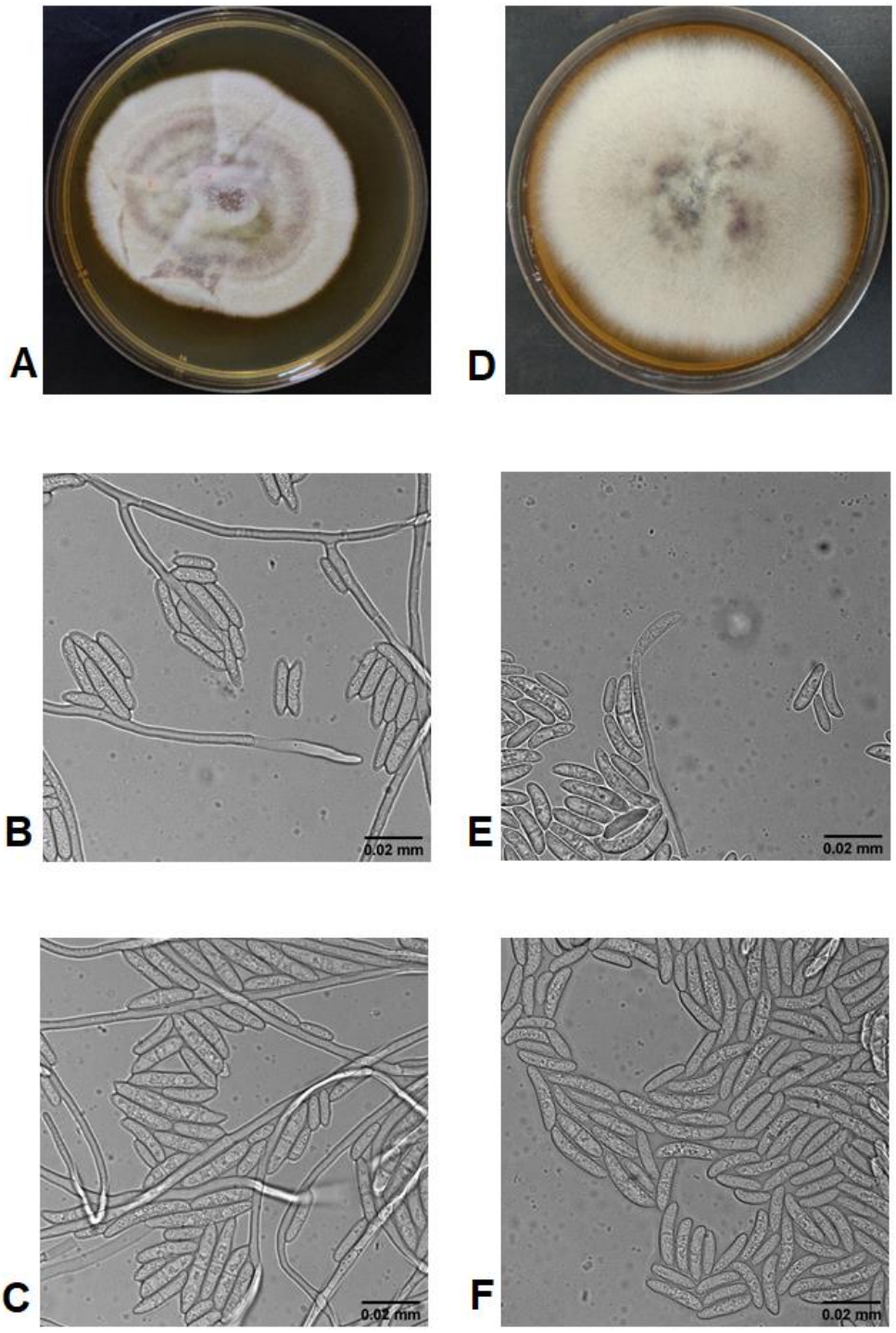

Figure 2.2: Morphology and microscopy of CSH3 and CSH5. (A) & (D): Colony morphology of CSH3 (white and cottony) and CSH5 (white, fluffy and cottony), respectively. **(B) & (E):** Spore generation from conidiogenic cells of CSH3 and CSH5, respectively. **(C) & (F):** Presence of macrospore in CSH3 and CSH5, respectively.

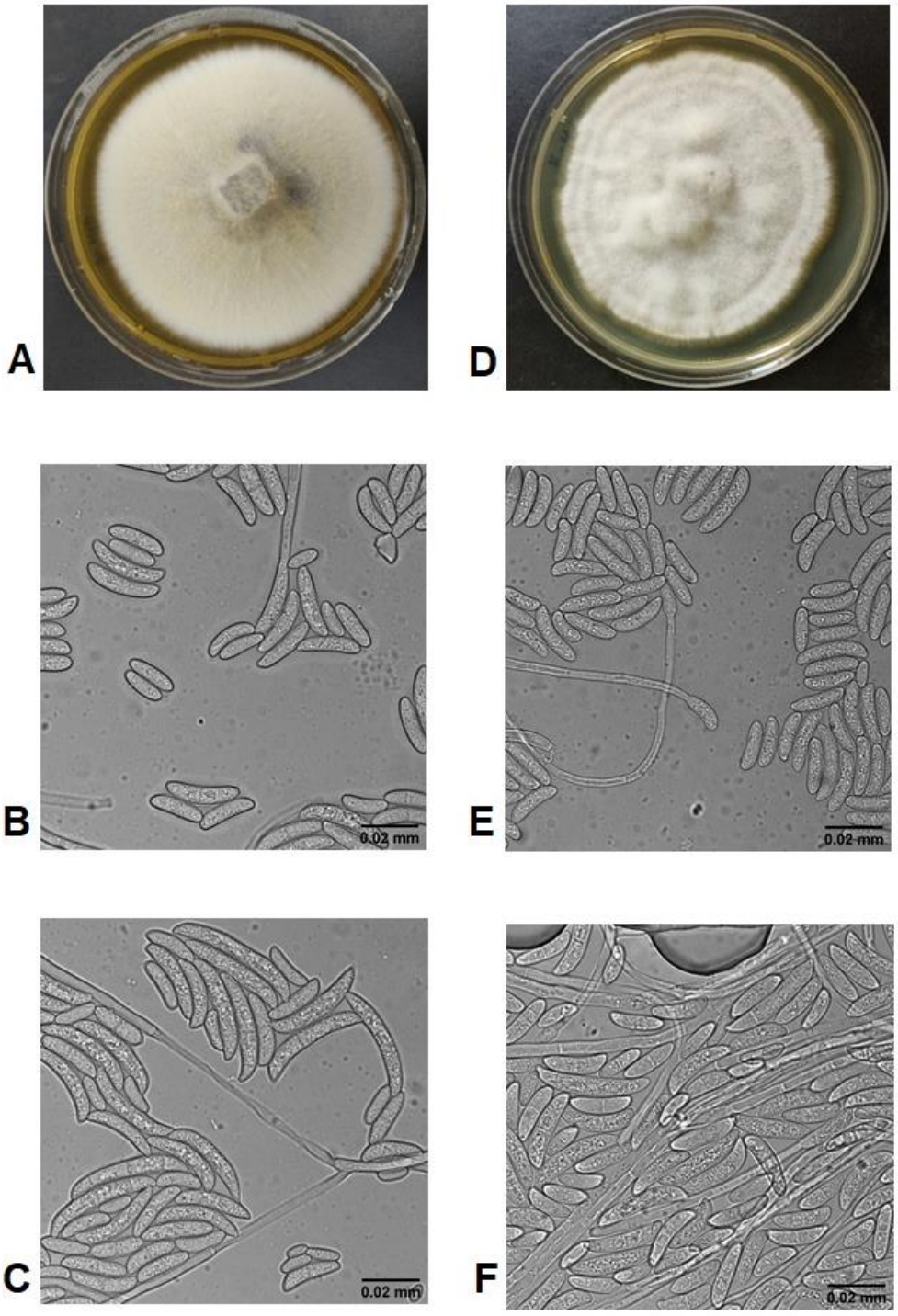

Figure 2.3: Morphology and microscopy of CSH6 and CSH7. (A) & (D): Colony morphology of CSH6 (white, cottony and fluffy) and CSH7 (white and cottony), respectively. Flaccose like structure was detected in CSH7. **(B) & (E):** Spore generation from conidiogenic cells of CSH6 and CSH7, respectively. **(C) & (F):** Presence of macrospore in CSH6 and CSH7, respectively.

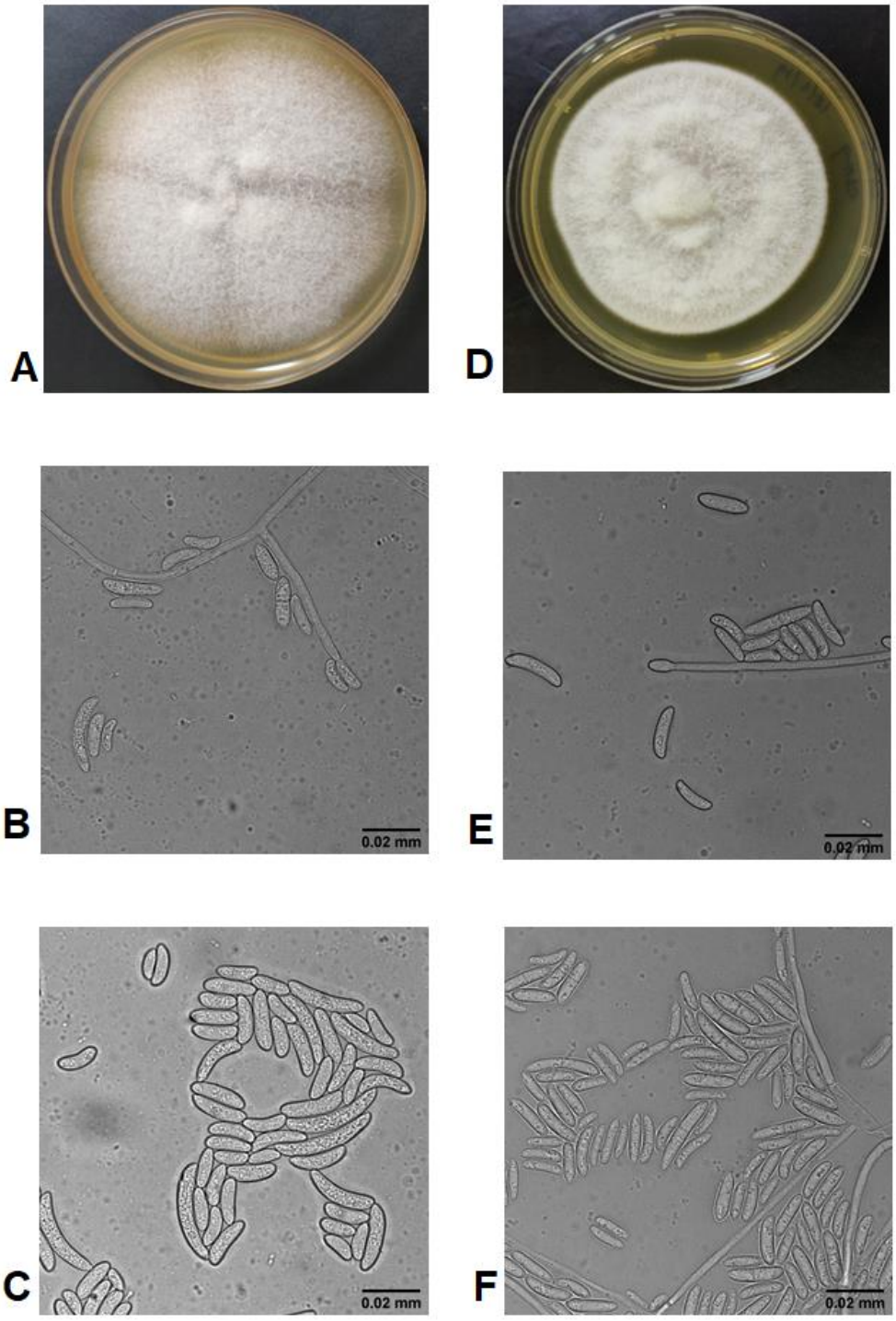

Figure 2.4: Morphology and microscopy of CSH8 and CSH9. (A) & (D): Colony morphology of CSH8 and CSH9, respectively. White and cottony colony. Flaccose like structure was detected in CSH9. **(B) & (E):** Spore generation from conidiogenic cells of CSH8 and CSH9, respectively. **(C) & (F):** Presence of macrospore in CSH8 and CSH9, respectively.

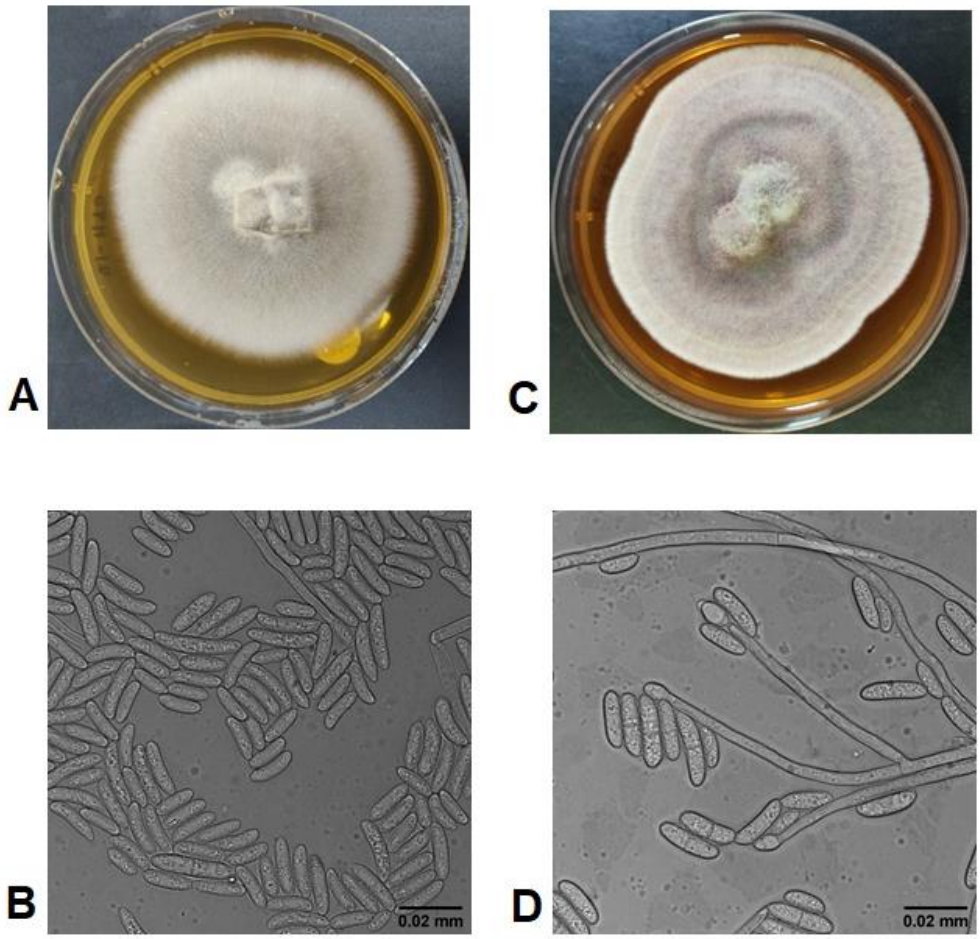

Figure 2.5: Morphology and microscopy of CSH10 and CSH11. (A) & (C): Colony morphology of CSH10 and CSH11, respectively. White and cottony colony. **(B) & (D):** Spore generation from conidiogenic cells of CSH10 and CSH11, respectively. Macrospores are present in both CSH10 and CSH11.

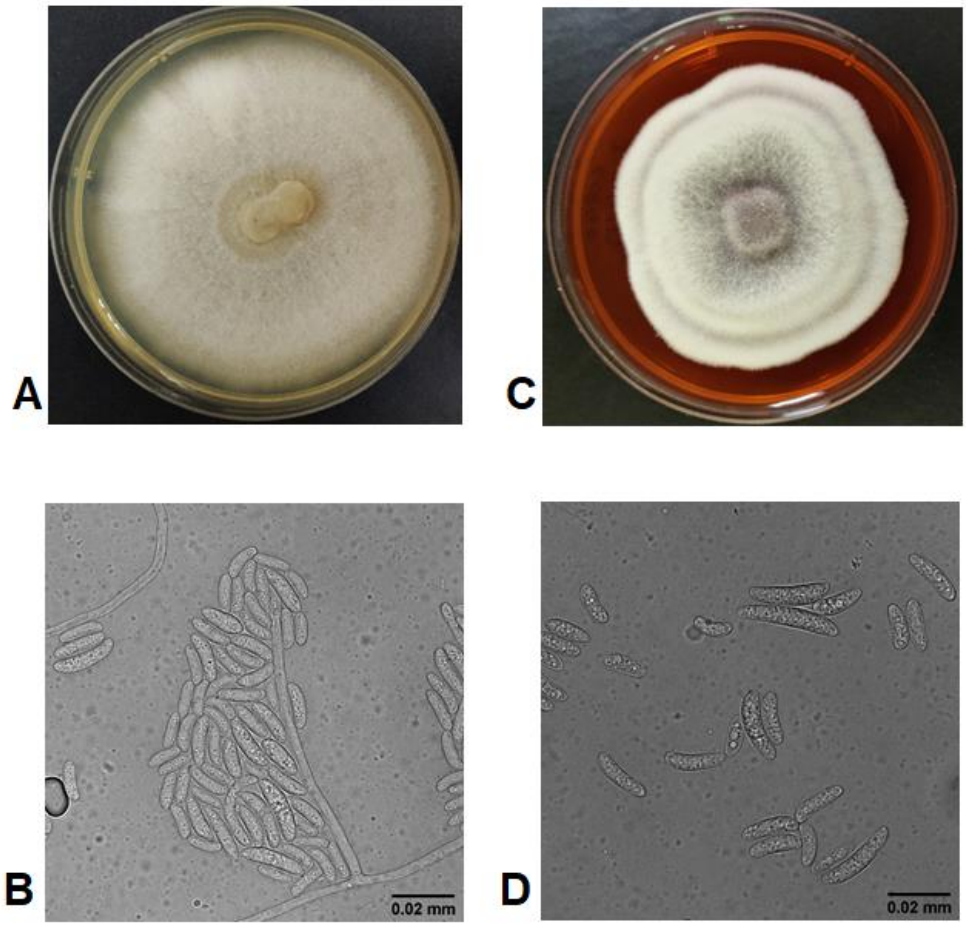

Figure 2.6: Morphology and microscopy of Cc50 and Cc240. (A) & (C): Colony morphology of Cc50 (White and cottony) and Cc240 (White, fluffy and cottony), respectively. **(B):** Spore generation from conidiogenic cells of Cc50 and 3-celled macrospore was detected. **(D):** 2-3-celled macrospore was detected in Cc240.

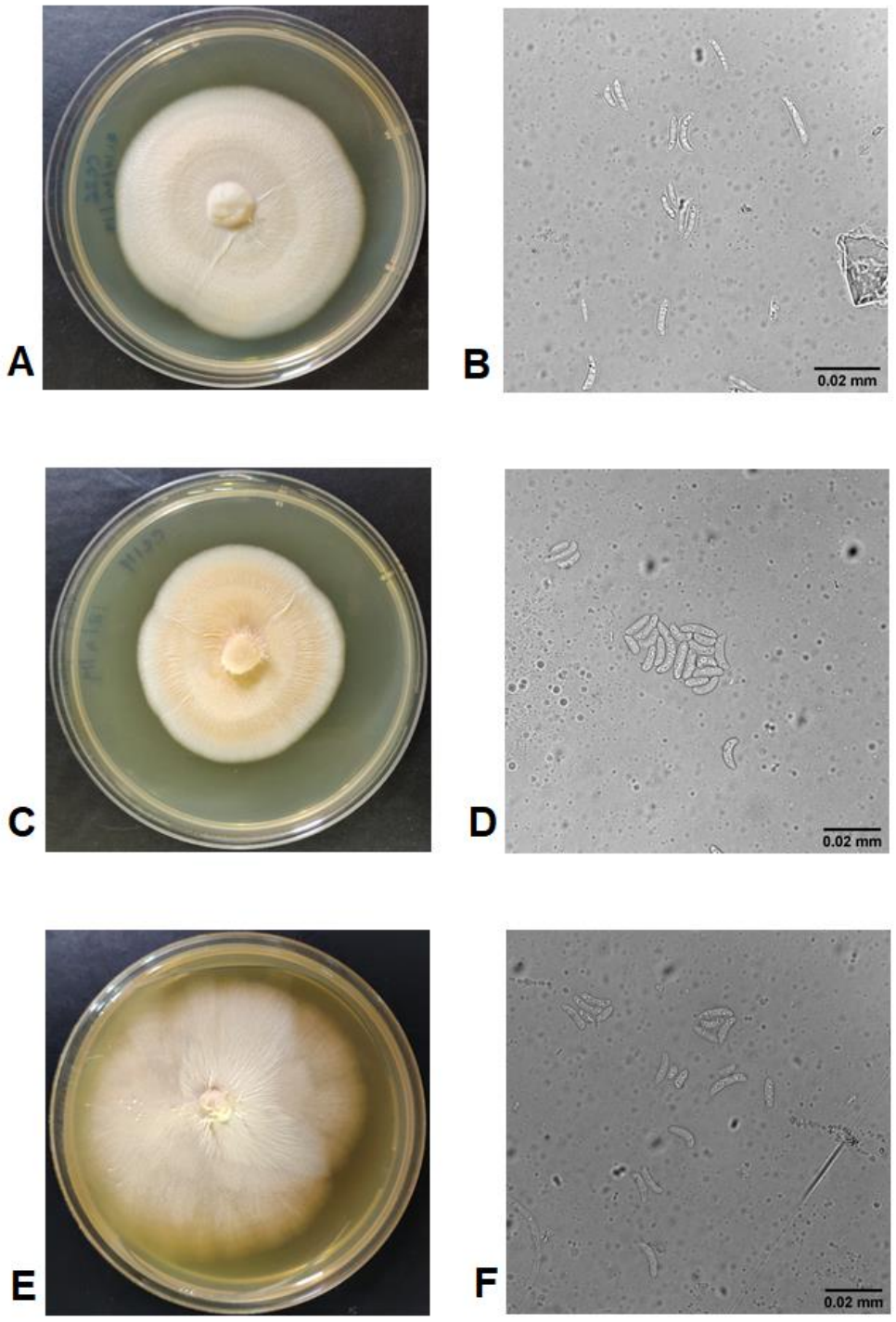

Figure 2.7: Morphology and microscopy of Cc26, Cc119 and CSH4. (A), (C) & (E): Colony morphology of Cc26, Cc119 and CSH4, respectively. Flat colony with slightly creamish white to apricot pigmentation. **(B), (D) & (F):** Curved spores of Cc26, Cc119 and CSH4 isolates, respectively.

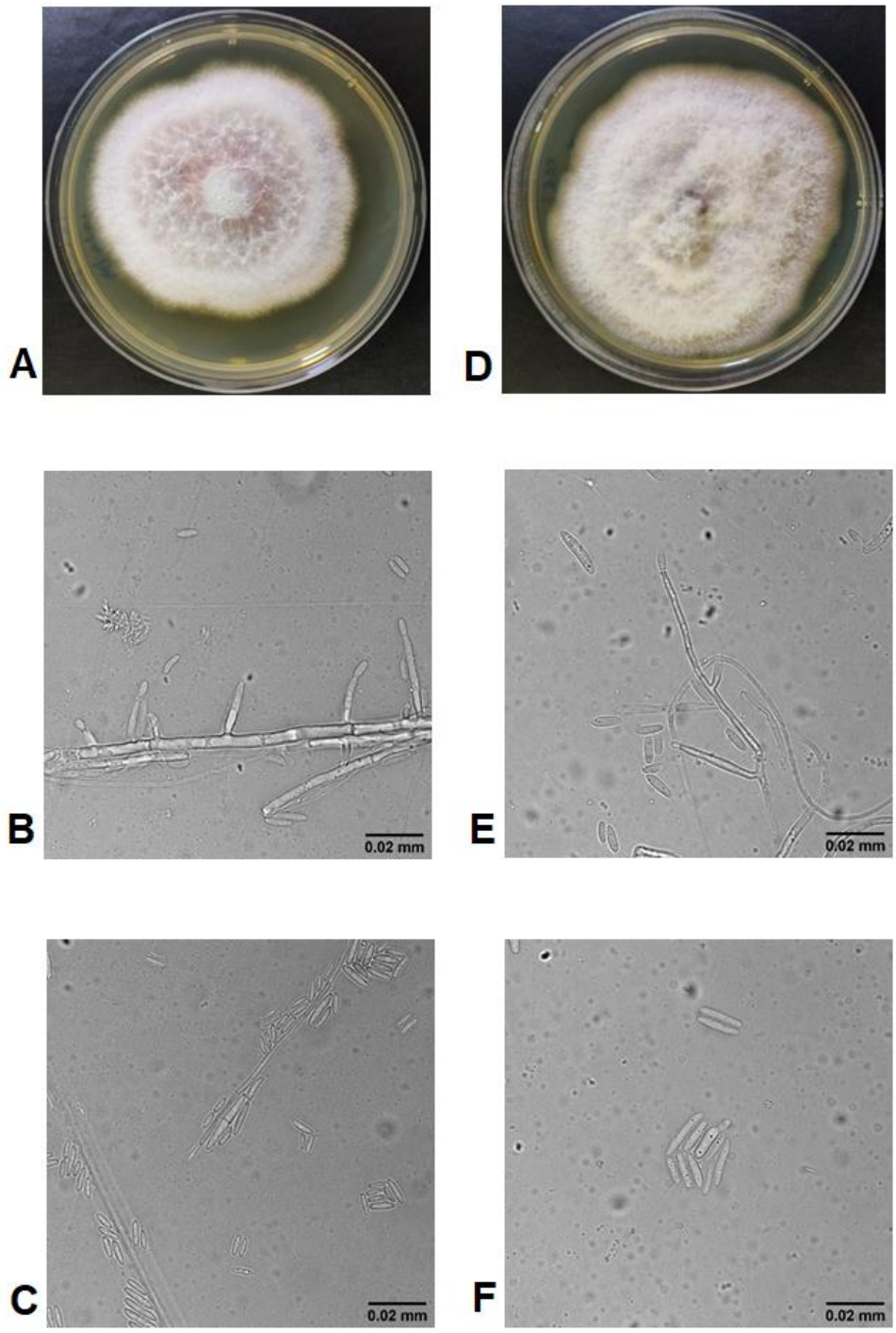

Figure 2.8: Morphology and microscopy of Cc52 and Cc61. (A) & (D): Colony morphology of Cc52 and Cc61, respectively. White to slightly purple and fluffy colony. **(B) & (E):** Spore generation from conidiogenic cells of Cc52 and Cc61, respectively. Presence of polyphialids detected in both Cc52 and Cc61. **(C) & (F):** Presence of macrospore in Cc52 and Cc61, respectively.

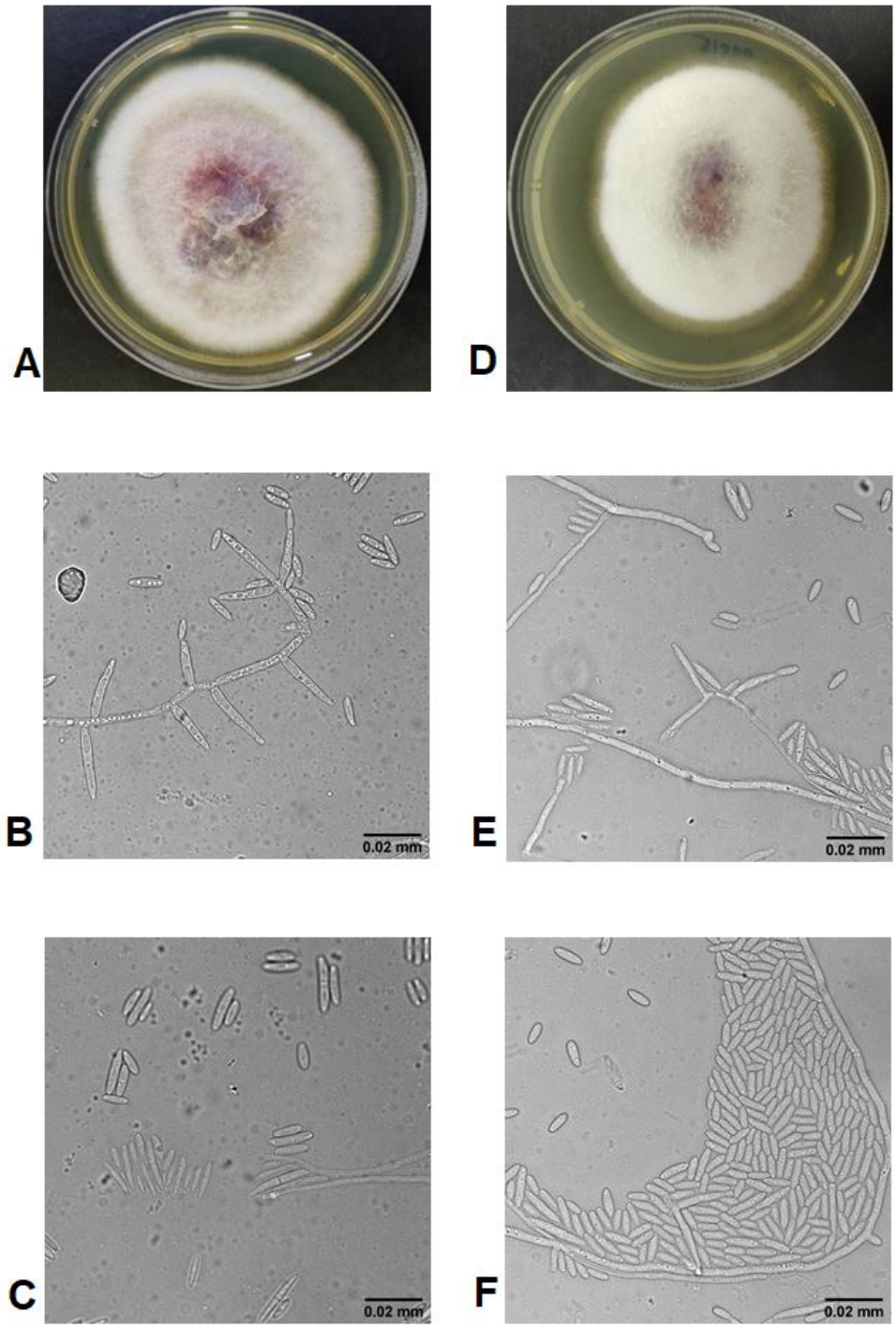

Figure 2.9: Morphology and microscopy of Cc167 and Cc215. (A) & (D): Colony morphology of Cc167 and Cc215, respectively. White to slightly purple and fluffy colony. **(B) & (E):** Spore generation from conidiogenic cells of Cc167 and Cc215, respectively. Presence of polyphialids detected in both Cc167 and Cc215. **(C) & (F):** Presence of macrospore in Cc167 and Cc215, respectively.

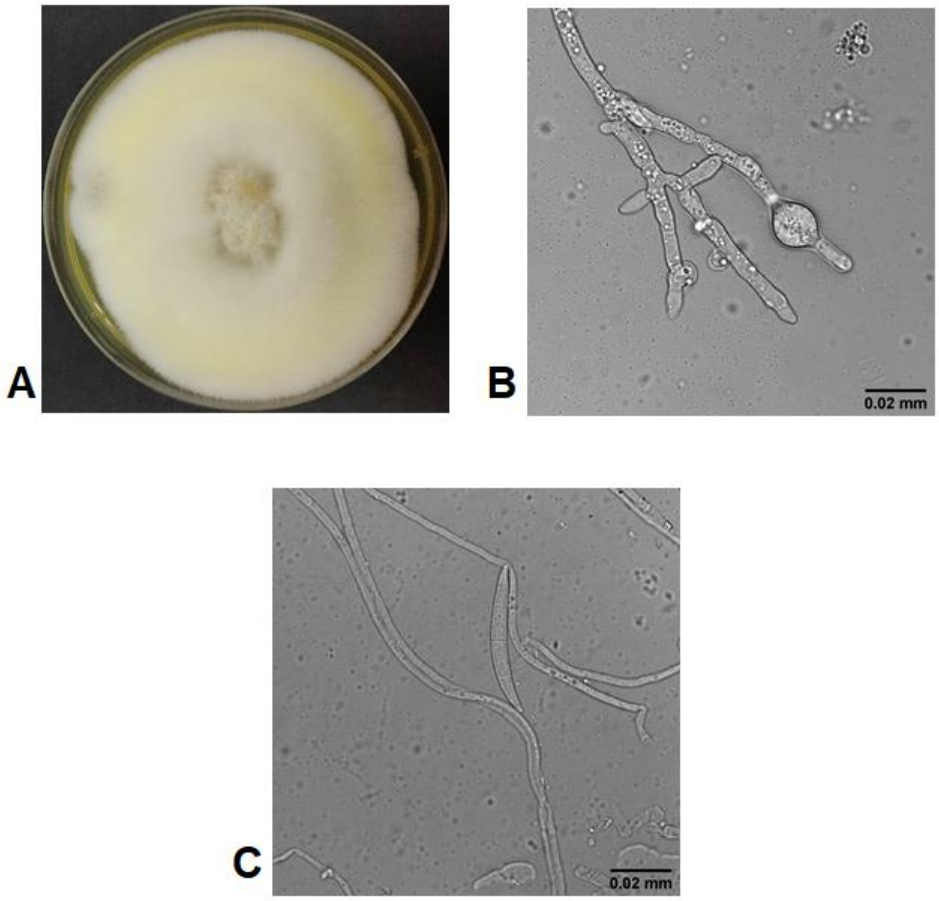

Figure 2.10: Morphology and microscopy of Cc172. (A): Colony morphology of Cc172 (White, fluffy and cottony). **(B):** Presence of chlamydospores. **(C):** Presence of macrospore when grown in stress media.

2.3.3 Molecular identification:

All the *Fusarium* isolates after morphological and microscopic examination were further identified at molecular level. The molecular identification was carried out by amplifying two conserved regions of fungal kingdom, ITS and TEF. Amplification of ITS region was carried out using universal ITS1 primer (Forward) and ITS4 primer (Reverse). The amplification of TEF region carried out using EF1 primer (Forward) and EF2 primer (Reverse). The extracted fungal DNA after quality check was used for PCR amplification of ITS and TEF region. After amplification of ITS and TEF region the obtained products were analysed on 2% agarose gel electrophoresis (Figure 2.11).

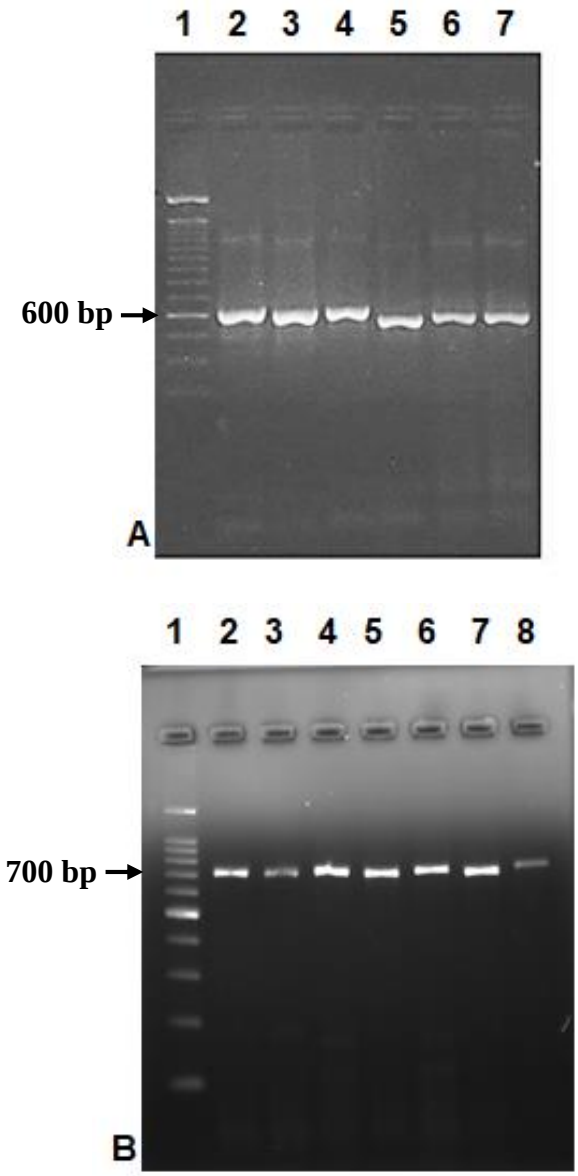

Figure 2.11: Representative image of 2% Agarose gel of amplified DNA isolated from different *Fusarium* isolates. **(A) PCR amplified ITS product (600bp):** Lane 1: 100 bp ladder, lane 2: Cc215, lane 3: Cs2, lane 4: Cc50, lane 5: CSH1, lane 6: CSH4, lane 7: Cc26. **(B) PCR amplified TEF product (700bp):** Lane 1: 100 bp ladder, lane 2: Cc215, lane 3: Cs2, lane 4: Cc50, lane 5: CSH1, lane 6: CSH4, lane 7: Cc26 and lane 7: Cs1.

The sequencing was carried out at facility of Eurofins, India. The obtained ITS and TEF sequences were subjected for identification to NCBI BLAST and *Fusarium* MLST database. The sequence which showed 95-100% similarity with expect value of zero were only taken in to consideration. The ITS sequences were submitted to genebank and accession number was provided by

NCBI. Table 2.1 shows the isolate and Genebank accession number and similarity with existing sequences in NCBI and *Fusarium* MLST database. Table 2.2 shows similarity of TEF sequences of *Fusarium* isolates with existing sequences in NCBI and *Fusarium* MLST database.

All the isolates used in the present study were identified up to species level using the ITS and TEF sequencing. Isolates identified as *F. solani* using ITS sequences were CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50, Cc240, Cc61, Cc167, Cc215 and Cc172. Isolates identified as *F. delphinoides* were Cc26, Cc119 and Cc52. CSH4 was identified as *F. dimerum*. Both *F. delphinoides* and *F. dimerum* belongs to *F. dimerum* species complex (FDSC).

Isolates identified as *F. solani* using TEF sequences were CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50 and Cc240. Isolates identified as *F. delphinoides* were Cc26 and Cc119. CSH4 was identified as *F. dimerum*. Isolates identified as *F. sacchari* were Cc52, Cc61, Cc167 and Cc215. Cc172 was identified as *F. incarnatum-equiseti*.

The MLST database is considered more accurate for *Fusarium* identification and when the ITS and TEF sequences of *Fusarium solani* isolates were evaluated using *Fusarium* MLST database, the match of ITS sequences was to *F. solani* and *H. haematococca,* and match of TEF sequences was to *F. solani* and FSSC in maximum number of isolates. Both *F. solani* and *H. haematococca* belongs to FSSC. Hence all *Fusarium solani* isolates were considered as FSSC. Both *F. delphinoides* and *F. dimerum* belongs to FDSC. Hence, Cc26, Cc119 and CSH4 were considered as FDSC. The phylogenetic relationships among all isolates were examined with distance analyses using maximum parsimony method by ClstalW in MegaX software. Figure 2.12 shows the tree generated for ITS sequences and Figure 2.13 shows the tree generated for TEF sequences.

Table 2.1 Name of the fungi and accession number of ITS sequences submitted to genebank, used in the present study. Similarity with sequences in NCBI and *Fusarium* MLST database.

Sr. No.	Isolate No.	Name	Accession No. of submitted sequence	Sequence similarity with existing sequence on NCBI database	Sequence similarity with existing sequence on *Fusarium* MLST database
1	**CSH1**	*Fusarium cf. solani*	MG136903.1	*F. solani* Ac. No.- KU528858.1 % Identity- 99%	*F. solani* Ac. No.- HM214445 % Identity- 100%
2	**CSH2**	*Fusarium cf. solani*	MG775565.1	*F. solani* Ac. No.- MH879587.1 % Identity- 98.89%	*Haematonectria haematococca* Ac. No.- HQ439152 % Identity- 100%
3	**CSH3**	*Fusarium cf. solani*	MG775557.1	*F. solani* Ac. No.- KR527142.1 % Identity- 99.25%	FSSC Ac. No.- AM412642 % Identity- 100%
4	**CSH5**	*Fusarium solani*	MN855526	*F. solani* Ac. No.- KX583231.1 % Identity- 98.34%	*F. solani* Ac. No.- AY310442 % Identity- 99.60%
5	**CSH6**	*Fusarium cf. solani*	MG775558.1	*F. solani* Ac. No.- HM214445.1 % Identity- 99.62%	*H. haematococca* Ac. No.- HM214445 % Identity- 100%
6	**CSH7**	*Fusarium cf. solani*	MG775559.1	*F. solani* Ac.No: MF800959.1 % Identity: 100%	*H. haematococca* Ac. No.- HQ384397 % Identity- 100%
7	**CSH8**	*Fusarium cf. solani*	MG775560.1	*F. solani* Ac.No.-KX965602.1 % Identity: 99.08%	*H. haematococca* Ac. No.- EU314970 % Identity- 100%

8	**CSH9**	*Fusarium cf. solani*	MG775561.1	*F. solani* Ac. No.-MF685335.1 % Identity: 99%	*H. haematococca* Ac. No.- JQ277276 % Identity- 100%
9	**CSH10**	*Fusarium cf. solani*	MG775562.1	*F. solani* Ac. No.-KX965602.1 % Identity: 99.40%	*F. solani* Ac. No.-KX385047 % Identity: 99.60%
10	**CSH11**	*Fusarium cf. solani*	MG775563.1	*F. solani* Ac. No.- MG775565.1 % Identity: 99.40%	*F. solani* Ac. No.-CBS 318.73 % Identity: 100%
11	**Cs1**	*Fusarium cf. solani*	MN698739	*Nectria haematococca* Ac. No.- KJ620369.1 % Identity: 98.50%	*F. solani* Ac. No.-FR691777 % Identity: 99.58%
12	**Cs2**	*Fusarium solani*	MN855523	*F. solani* Ac. No.- LC318412.1 % Identity: 99.62%	*F. solani* Ac. No.- CBS224.34 % Identity: 100%
13	**Cc50**	*Fusarium solani*	KM017134	*F. solani* Ac. No.- KC156597.1 % Identity: 99%	*F. solani* Ac. No.- KY484971 % Identity: 99.80%
14	**Cc240**	*Fusarium solani*	MN855566	*F. solani* Ac. No.- LC318412.1 % Identity: 99.44%	*F. solani* Ac. No.- LC318412.1 % Identity: 99.80%
15	**Cc26**	*Fusarium delphinoides*	KM017139	*F. delphinoides* Ac. No.- MN583389.1 % Identity: 100%	*F. delphinoides* Ac. No.- KY436171 % Identity: 100%
16	**Cc119**	*Fusarium delphinoides*	KM017141	*F. delphinoides* Ac. No.- MN396227.1 % Identity: 100%	*F. delphinoides* Ac. No.- KY436171 % Identity: 100%

17	**CSH4**	*Fusarium cf. dimerum*	MG775564.1	*F. delphenoides* Ac. No.- MG020724.1 % Identity- 99.61%	*BisiFusarium dimerum* Ac. No.- CBS 254.50 % Identity- 98.04%
18	**Cc52**	*Fusarium delphinoides*	JQ910153	*F. delphinoides* Ac. No.- MG020724.1 % Identity: 99.60%	*F. delphinoides* Ac. No.- EU721707 % Identity: 100%
19	**Cc61**	*Fusarium solani*	MN855402	*F. solani* Ac. No.-MK680186.1 % Identity: 99.35%	*F. solani* Ac.No.-KC478614 % Identity: 100%
20	**Cc167**	*Fusarium solani*	KM017143	*F. solani* Ac. No.-MN583474.1 % Identity: 100%	*F. solani* Ac. No.-CBS 318.73 % Identity: 100%
21	**Cc215**	*Fusarium solani*	KM017137	*F. solani* Ac. No.-MN653259.1 % Identity: 100%	*F. solani* Ac. No.-HQ265420 % Identity: 100%
22	**Cc172**	*Fusarium solani*	KM017144	*F. solani* Ac. No.-MN602620.1 % Identity: 100%	*F. solani* Ac. No.-KF717534 % Identity: 100%

Table 2.2 Similarity of TEF sequences of isolates used in present study with existing sequences in NCBI and *Fusarium* MLST database.

Sr. No.	Isolate No.	Sequence similarity with existing sequence on NCBI database	Sequence similarity with existing sequence on *Fusarium* MLST database
1	**CSH1**	*F. solani* Ac. No.- KY486652.1 % Identity- 97.63%	*F. solani* species complex Ac. No.- DQ247011 % Identity- 98.23%
2	**CSH2**	*F. solani* Ac. No.- HE647943.1 % Identity- 99%	FSSC Ac. No.- DQ246855 % Identity- 99.54%
3	**CSH3**	*F. solani* Ac. No.- HE647943.1 % Identity- 99%	FSSC Ac. No.- DQ246855 % Identity- 99.69%
4	**CSH5**	*F. solani* Ac. No.- HE647953.1 % Identity- 99%	FSSC Ac. No.- DQ247034 % Identity- 99.69%
5	**CSH6**	*F. solani* Ac. No.- HE647911.1 % Identity- 99.27%	FSSC Ac. No.- CNRMA12.929 % Identity- 99.67%
6	**CSH7**	*F. solani* Ac. No.- HE647908.1 % Identity- 99%	FSSC Ac. No.- DQ246889 % Identity- 99.84%
7	**CSH8**	*F. solani* Ac. No.- HE647936.1 % Identity- 99%	FSSC Ac. No.- DQ246855 % Identity- 99.84%
8	**CSH9**	*F. solani* Ac. No.- HE647935.1 % Identity- 99.01%	*F. solani* Ac. No.- DQ246834 % Identity- 99.40%
9	**CSH10**	*F. solani* Ac. No.- HE647943.1 % Identity- 99%	*F. solani* Ac. No.- DQ246834 % Identity- 98.31%
10	**CSH11**	*F. solani* Ac. No.- HE647911.1 % Identity- 98.07%	*F. solani* Ac. No.- DQ247095 % Identity- 96.79%
11	**Cs1**	*F. solani* Ac. No.- HE647946.1 % Identity- 99%	*F. solani* Ac. No.- DQ247642 % Identity- 99.09%
12	**Cs2**	*F. solani* Ac. No.- HE647943.1 % Identity- 99%	FSSC Ac. No.- CNRMA12.929 % Identity- 99.37%

13	**Cc50**	*F. solani* Ac. No.- KR816154.1 % Identity- 99%	*F. solani* Ac. No.- DQ247642 % Identity- 99.36%
14	**Cc240**	*F. solani* Ac. No.- HQ731056.1 % Identity- 97.59%	FSSC Ac. No.- CNRMA14.808 % Identity- 98.28%
15	**Cc26**	*F. delphinoides* Ac. No.- MF411137.1 % Identity- 95.14%	*F. delphinoides* Ac. No.- EU926313 % Identity- 94.89%
16	**Cc119**	*F. delphinoides* Ac. No.- EU926292.1 % Identity- 95.10%	*Fusarium* sp. 3 (*cf. F. delphenoides*) Ac. No.- EU926313 % Identity- 94.89%
17	**CSH4**	*F. dimerum* Ac. No.- JN235524.1 % Identity- 99%	*BiFusarium dimerum* Ac. No.- CNRMA14.624 % Identity- 98.79%
18	**Cc52**	*F. sacchari* Ac. No.- JF740708.1 % Identity- 99%	*F. sacchari* Ac. No.- AF160278 % Identity- 99.21%
19	**Cc61**	*F. sacchari* Ac. No.- FJ603491.1 % Identity- 99%	*F. sacchari* Ac. No.- AF160278 % Identity- 99.34%
20	**Cc167**	*F. sacchari* Ac. No.- HE647912.1 % Identity- 99%	*F. sacchari* Ac. No.- AF160278 % Identity- 99.68%
21	**Cc215**	*F. sacchari* Ac. No.- FJ603497.1 % Identity- 99%	*F. sacchari* Ac. No.- AF160278 % Identity- 98.27%
22	**Cc172**	*F.cf. incarnatum/equiseti* Ac. No.- KF962949.1 % Identity- 98.21%	*F.incarnatum-equiseti* species complex Ac. No.- GQ505617 % Identity- 98.15%

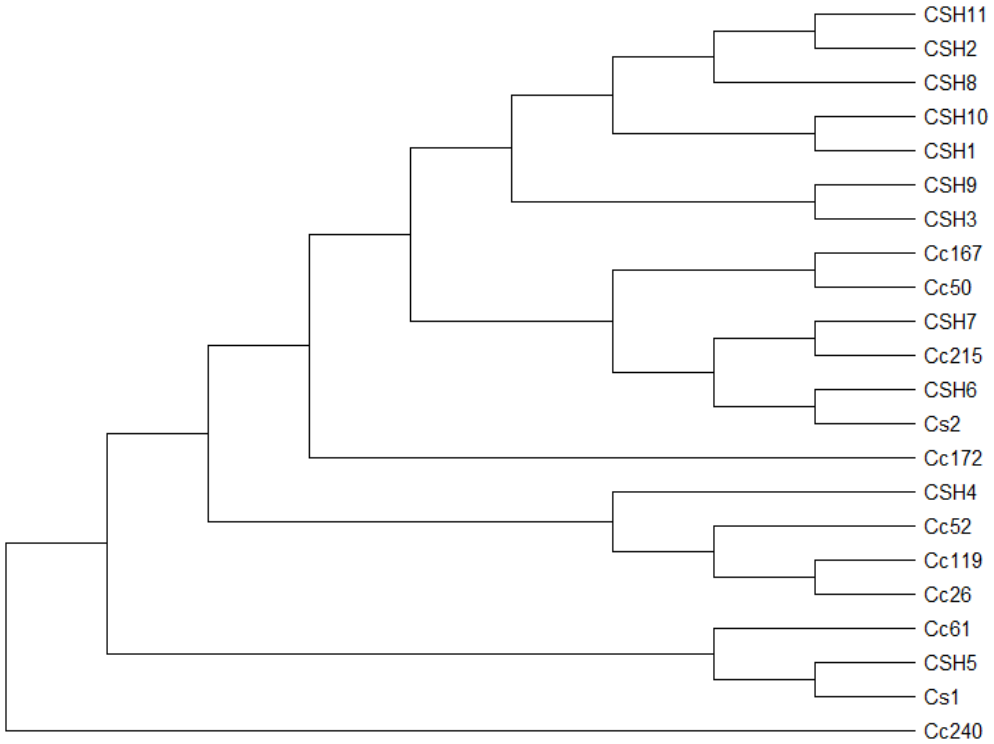

Figure 2.12: Phylogeny tree of *Fusarium* isolates based on ITS gene sequence analysis.

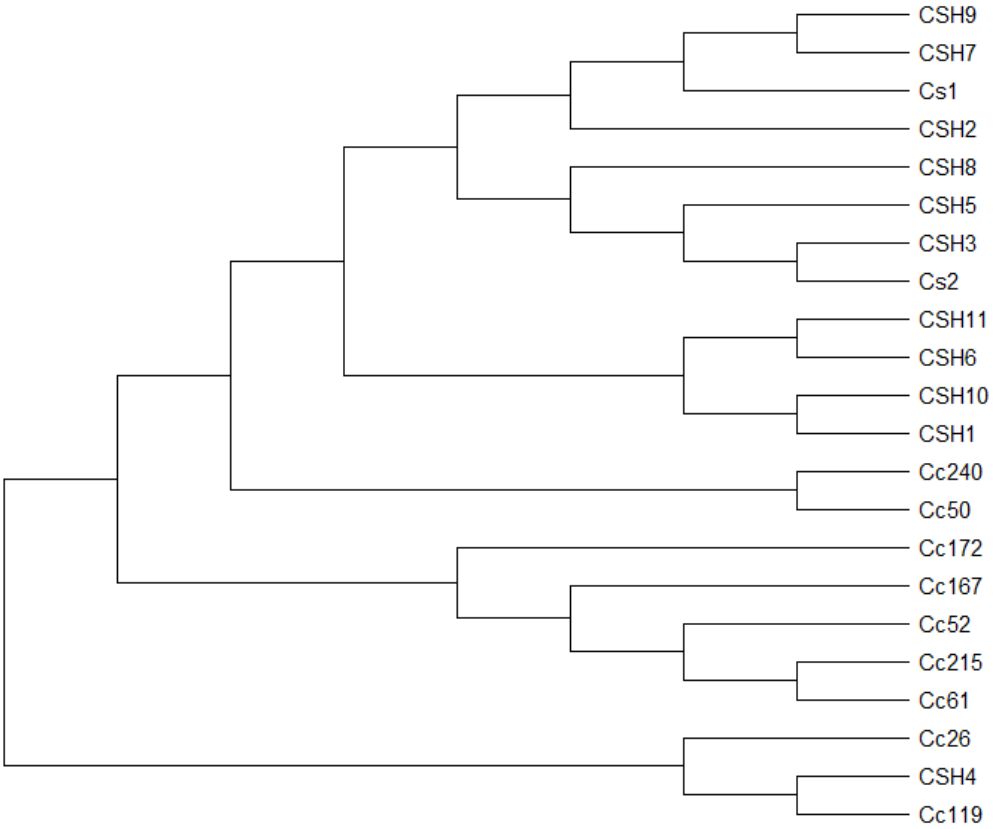

Figure 2.13: Phylogeny tree of *Fusarium* isolates based on TEF gene sequence analysis.

2.4 Discussion:

In present study, 22 clinical isolates were used. The isolates were provided from Iladevi cataract and IOL research centre, Ahmedabad and L.V. Prasad eye institute, Hyderabad. The isolates were collected at hospitals from corneal scrapping of keratitis patients. The isolates were grown in PDA and PDB medium. Preliminary identification was done morphologically with the help of "Atlas of Clinical fungi" (De Hoog *et al.*, 2009) and microscopically using lactophenol cotton blue staining as well as without staining. The main morphological feature of *Fusarium* is white colony to slightly pigmented colony. The pigmentation can be found in mycelia and/or in media. The pigmentations are usually yellow, red, orange and purple.

In present study, on the basis of morphological and microscopical features, 14 isolates were groped as FSSC, three isolates were groped as FDSC, four isolates were grouped as *F. sacchari* and one isolate was grouped as *F. incarnatum-equiseti*. Further characterization of isolates was done using molecular techniques.

Spores produced by *Fusarium* spp. are of three types, microconidia, macroconidia and chlamydospores (Nelson *et al*., 1994). The macroconidia are long, pointed at both ends, septate, sickle-shaped, curved and has basal foot cell (Smith, 2007). Macroconidia are produced in phialides which are small openings at the tip of the conidiophore from which the spores are emerging one by one. Besides the macroconidia, there are other type of spores produced by *Fusarium* which help in distinguishing the species in genus *Fusarium*. Microconidia are also produced which are present on aerial mycelium. They are usually one celled, and are oval-shaped, pear-shaped and spherical in structure (Smith, 2007). Sometimes microconidia from phialides remain attached to each other in sequence to form chains (Leslie and Summerell, 2006; Nelson *et al*., 1994). A monophialide is a condiophore which has one opening or pore through which spores are released, while a polyphialide has several openings trough which spores are released (Nelson *et al*., 1994). Resting spores, chlamydospores, found in some, but not in all *Fusarium* spp. Chlamydospores are sphaerical, approximately 7-16 μm in diameter. They

found singly but sometimes they are doubles, in chains and in clumps (Smith, 2007). They have thick and rough cell walls. Their cytoplasm contains big pool of nutrients which is seen as oily globules microscopically (Leslie and Summerell, 2006; Nelson *et al*., 1994).

The macroconidia of *F. solani* are cylindrical in the central area and the spore wall is heavy and strong. Macroconidia are curved as well as straight. They are produced in sporodochia on long phialides and sometimes they merge and form mat on the surface. The spores are blunted. Microconidia are oval as well as spherical in shape. Sclerotia are also sometimes present. Members of *F. solani* sometimes are air-borne, they produce ascospores which borne in asci of perithecia. Perithecia are reddish to red-brown in colour. Many isolates of this species are homothallic but there are some heterothallic members also. Pathogenic isolates are known to produce perithecia (Smith, 2007).

The species belongs to *F. dimerum* species complex are comparatively slow growing. After 7 days of growth, the colonies become orange to apricot coloured with white flaccose aerial mycelium sometimes. Presence of loosely branched conidiophores with short, often swollen phialides observed. During initial development, the macroconidia protrude from phialides in cylindrical form and later become curved or falcate. The macroconidia have pedicillate basal cells (Do Carmo *et al*., 2016).

Morphological and microscopic features of *F. sacchari* involves, fast growing colony with whitish fluffy mycelia. The colony becomes purple with age. Microconidia are produced in false head form monophialids and polyphialids. Macroconidia are oval, slender, spetate and slightly flacate. The key feature for *F. sacchari* is absence of chlamydospores and microconidial chain. Presence of simple and prostrate conidiophores can be observed (Nordahliawate *et al*., 2008).

Species belonging to the *F. incarnatum-equiseti*, produce straight to slightly curved macroconidia on sporodochia. The species also produced abundant mesoconidia, while chlamydospores were produced by some strains and were generally solitary and not abundant. Presence of a prominent foot-shaped basal

cell was found. Elongated apical cell of macroconidia and chlamydospores were produced in chains (Avila *et al*., 2019).

FSSC: FSSC contains 60 or more distinct species in 3 major clades. It is most commonly encountered species causing human infection globally (Van Diepeningen *et al*., 2014; Zhang *et al*., 2006). Biogeographically located and plant associated species belongs to clade 1 and 2 (O'Donnell *et al*., 2008) while species from clinical or veterinary source belongs to clade 3 and are capable of causing life threatening opportunistic infections to humans (Zhang *et al*., 2006). FSSC has homothallic and heterothallic species (O'Donnell, 2000). It has several telomorphic names, which are *N. haematococca* (O'Donnell, 2000), *Hypomyces solani* (Reichle *et al*., 1964) and *H. haematococca* (Rossman *et al*., 1999). Members of FSSC can cause systemic as well as localised (cutenous and superficial) infections (Van Diepeningen *et al*., 2015). It is known to cause onchomycoses (Ghannoum *et al*., 2000), keratitis and disseminated fusariosis in immunocompromised host (Homa *et al*., 2018). The main species belongs to FSSC includes *F. solani sensu latu*, *F. falciformae*, *F. keratoplasticum*, *F. lichenicola*, *F. petroliphilum*, *F. solani sensu stricto*, *Neocosmospora gamsii* and *N. suttoniana* (Herkert *et al*., 2019; Homa *et al*., 2018; Van Diepeningen *et al*., 2014).

F. dimerum species complex (FDSC): (FDSC) contains at least 12 lineages, of which *F. dimerum sensu stricto*, *F. delphinoides*, and *F. penzigii* have been involved in human infection. Before recognition and identification of FDSC, several case reports were published. FDSC is known to cause onychomycoses, keratitis and other localized infections to disseminated infections in haemato-oncological patients (Letscher-Bru *et al*., 2002; Schroers *et al*., 2009; Van Diepeningen *et al*., 2014).

F. incarnatum-equiseti species complex (FIESC): Within the FIESC, 28 species can be recognized, which are organized into two main clades which are *F. equiseti* and *F. incarnatum* or *F. semitectum*. These two groups are known to cause onychomycoses, skin, eye, and deep localized and disseminated infections, especially in leukemic patients (O'Donnell *et al*., 2009).

F. fujikuroi species complex (FFSC): Members of *F. fujikuroi* species complex (FFSC) mainly involve *F. proliferatum, F. verticillioides, F. acutatum, F. anthophilum, F. andiyazi, F. nygamai, F. sacchari, F. andiyazi, F. subglutinans, F. napiforme and F. thapsinum.* The two members of the FFSC are commonly observed in human infections are *F. proliferatum* and *F. verticillioides.* Members of FFSC are increasingly identified in especially invasive and disseminated infections in haematooncological patients. Some species of FFSC species, e.g., *F. acutatum, F. anthophilum, F. andiyazi, F. nygamai,* and *F. sacchari* have a limited geographic distribution and/or are associated with specific climatic conditions (Krulder *et al*., 1996; Van Diepeningen *et al*., 2014).

For molecular identification of fungi, several genes are reported which are conserved for particular genus and species. These genes involve ribosomal RNA (rRNA), translation elongation factor 1- α, β-tubulin (tub2), calmodulin, intergenic spacer region (IGS), the second largest subunit of RNA polymerase II (RPB2) and domains D1/D2 of the nuclear large subunit (LSU). Initially majority of fungal isolates were identified using ITS region sequences using universal primers ITS1 and ITS4. The ITS region became marker of choice for fungal identification as it is intron rich region of protein coding gene and they evolve at higher rate (Geiser, 2004; O'Donnell *et al*., 1998). The transcriptional unit is composed of 18S, 5.8S, and 28S rDNA genes. Between the 18S and 5.8S, and between 5.8S and 28S rDNA gene subunits are intergenic transcribed spacer regions (ITS 1 and 2) that are not translated into rRNA (Figure 2.14). Although rDNA genes are highly conserved, the ITS regions are variable and rich in informative sites. Hence, considered as good candidates for phylogenetic classification of fungal species (Alfonso, 2008; Oechsler *et al.*, 2009). However, some fungi contain non-orthologous copies of ITS2 which contribute to incorrect identification and phylogenetic interference (O'Donnell *et al*., 1998, 1997). It has been reported that for identification of *Fusarium* species, ITS region is too conserved and one would not be able to discriminate between species complex as well as not able to identify isolate at species level. Also, it has been reported that for identification of *Ascomycota* (*Fusarium* belongs to *Ascomycota* family), there

is need for longer barcode than ITS region which helps for identification of fungi at species level.

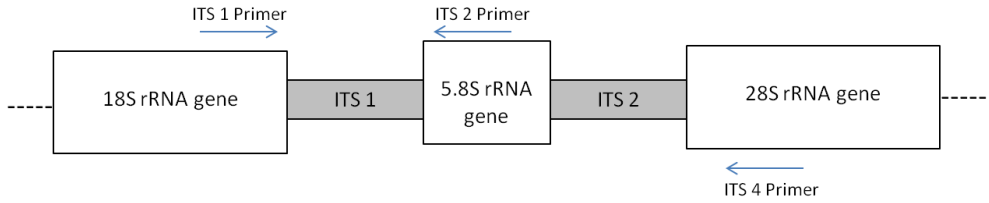

Figure 2.14: Structure of the rRNA gene cluster and positions of fungal PCR primers. The cluster is split into coding (18S, 5.8S and 28S genes) and non-coding (Internally Transcribed Spacer or ITS) regions. The positions of the PCR primers and their direction of synthesis are indicated by arrows.

For identification of molecular sibling and species identification at species level in *Fusarium* species, translation elongation factor-1α (TEF-1α) region or are recommended as molecular marker (Lewis *et al*., 2011; O'Donnell *et al*., 2010, 1998; Seifert and Lévesque, 2004; Van Diepeningen *et al*., 2014; Robert *et al*., 2011). TEF gene encodes for essential part of protein and it is highly informative and no non-orthologous copies of this gene were found. The universal primers have been designed which work across vast phylogenetic genus. This gene was first used as marker in *Lepidoptera* to understand generic level and species level relationship (Cho *et al*., 1995; Mitchell *et al*., 1997). They were developed for understanding of lineage in *F. oxysporum* species complex (O'Donnell *et al*., 1998). The ef1 and ef2 primer amplifies around 700 bp region which flanks 3 introns which is half of the length of total amplicon in all fusaria (Figure 2.15). TEF gene appears as single copy in *Fusarium* and is consistent in nature. It shows high level of sequence polymorphism among closely related species. Compared to other intron rich protein coding genes like calmodulin, histone H3 and beta-tubulin, TEF shows high polymorphism. Hence TEF became marker of choice in *Fusarium* species (Geiser *et al*., 2004).

In present study, 22 pathogenic isolates were identified using ITS and TEF 1 alpha genes as molecular markers. We were able to identify isolates belongs to FSSC, FDSC, FFSC and FIESC groups. Majority of isolates belongs to FSSC group. Using ITS as molecular marker 19 isolates were identified as FSSC and

four isolates were identified as FDSC. Using TEF as molecular marker, 14 isolates were identified as FSSC, four isolates were identified as *F. sacchari* which belongs to FFSC group. Two isolates were identified as *F. delphenoides* and one isolate was identified as *F. dimerum*. However all 3 belongs to FDSC group. One isolate belonged to FIESC group.

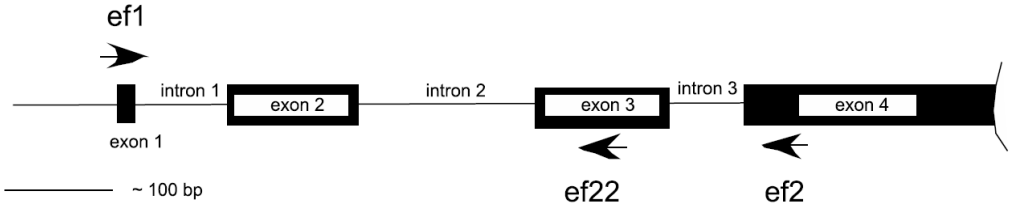

Figure 2.15: Map of TEF gene region in *Fusarium* with primer location. The positions of the PCR primers and their direction of synthesis are indicated by arrows. (Geiser *et al*., 2004).

There are several reports of identification of *Fusarium* species using ITS and TEF genes as a molecular marker. Oechsler and group has identified 41 *Fusarium* isolates from keratitis patients using ITS region as molecular marker. They reported 36 FSSC isolates, 2 FOSC isolates, 1 FDSC isolate and 2 isolates were not grouped in any species complex (Oechsler *et al*., 2013). Rosa and group have reported identification of keratitis causing *Fusarium* using TEF and RPB2 gene regions and they identified 6 FSSC isolates, 2 FOSC isolates, 1 FIESC isolate and 1 FFSC isolate (Dallé da Rosa *et al*., 2018). In one study, the *Fusarium* was identified from HIV patients using TEF gene as molecular marker using loop mediated isothermal amplification (LAMP) technique (Ferdousi *et al*., 2014). Identification of 148 isolates of *Fusarium* spp. using TEF alpha gene sequencing has been reported and identified species were *F. avenaceum, F. culmorum, F. flocciferum, F. graminearum, F. equiseti, F. lunulosporum, F. torulosum, F. cerealis, F. sambucinum, F. tricinctum* and *F. venenatum* (Kristensen *et al*., 2005). Species of FIESC complex has been also identified using TEF alpha region and RPB2 region sequencing (Avila *et al*., 2019). Identification of *F. keratoplasticum* and *F. pseudensiforme* were carried out using TEF alpha sequencing (Al-Hatmi *et al*., 2017). Van Diepeningen and group has reported identification of *Fusarium* spp. from dermatological ward in Thailand. They

used ITS, TEF alpha and RPB2 as markers. They identified 37 FSSC isolates of which *F. faliformae* and *F. keratoplasticum* were more predominant. One FOSC isolate and six FIESC isolates were also identified (Van Diepeningen *et al*., 2015). A study on *Fusarium* causing keratitis was carried out in Chennai, India, where researchers had identified 9 FSSC isolates from which *F. faliformae* and *F. keratoplasticum* were more predominant. The authors also identified one isolate as *F. sporotrichoides*. All the isolates were identified using TEF alpha and RPB2 as molecular marker (Tupaki-Sreepurna *et al*., 2017). Salah and group have reported identification of *Fusarium* species causing systemic and local infection in humans using RPB2 and TEF 1 alpha gene sequencing. They identified 75% FSSC isolates, 13.6% FFSC isolates, 6.5% FDSC isolates and 4.5% FOSC isolates (Salah *et al*., 2015). *Fusarium riograndense* sp. *nov*. is a new species identified in FSSC causing fungal rhinosinusitis using RPB2, ITS and TEF 1 alpha phylogenetic markers (Dallé da Rosa *et al*., 2018). In Germany, a study on *Fusarium* keratitis was carried out where ITS, RPB2 and TEF 1 alpha were selected as molecular marker. They identified 13 FSSC, 6 FOSC and FFSC isolates (Walther *et al*., 2017).

Looking at morphological characterization, in the colony morphology of FSSC, *F. sacchari* and *F. equiseti*, there was slight difference. We could not strongly discriminate between FSSC, FIESC and *F. sacchari* based on colony morphology. But we were able to discriminate them on the basis of microscopic features and molecular markers. The characteristic microscopic feature for species to become FSSC is long hyphae with monophilides which has long apex while for species to become *F. sacchari*, presence of polyphialides is important. We were able to see long monophialides but were not able to detect polyphialids in following isolates. Cc50, Cc240, CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2. In further studied these isolates were termed as FSSC. We have detected presence of polyphialids in isolates Cc52, Cc61, Cc167 and Cc215. Also, the molecular identification from TEF 1 alpha sequencing of above isolates indicated that Cc52, Cc61, Cc167 and Cc215 belongs to *F. sacchari* group. Colony morphology of isolates Cc26, Cc119 and CSH4 is similar to isolates of FDSC group and all three isolates were identified as FDSC using both ITS

region and TEF 1 alpha region sequencing. In further studies, these isolates were termed as FDSC. In case of isolate Cc172, the colony morphology strongly could not discriminate that isolate belonged to between FSSC or FIESC group. The microscopic examination showed the presence of chlamydospores which were present in chains as well as solitary. This is key feature for isolate to be identified as FIESC. The other microscopic features were also different from features of FSSC. Also TEF 1 alpha sequencing revealed that Cc172 belongs to FIESC group. While Cc172 ITS region sequencing alone was not able to discriminate between FSSC and FIESC group.

These results are in accordance with literature where it has been reported that ITS region as molecular marker is not able to discriminate between isolates at species level while TEF 1 alpha is the marker of choice for identification of *Fusarium* species because of its presence as single copy in *Fusarium* genus.