MULTILOCUS SEQUENCE TYPING (MLST) BASED GENETIC VARIATION ANALYSIS OF SHISHAM DIEBACK ASSOCIATED STRAINS OF *CERATOCYSTIS FIMBRIATA* SENSU LATO SPECIES COMPLEX IN PAKISTAN

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Abstract. *Ceratocystis fimbriata* sensu lato species complex is a group of highly diverse cryptic species known to be associated with different diseases in a range of host plants. For the accurate identification of fungal pathogens belonging to this species complex, molecular approaches have been employed in juxtaposition with morphological characterization. Different DNA based methods have become the routine practice of mycologist community throughout the world. Among them, multilocus sequence typing (MLST) approach is gaining popularity. Four fungal taxonomy groups have been established MLST scheme for few fungi, to date. However, in *Ceratocystis fimbriata* species complex, no report on MLST based genotyping is available yet. Therefore, by considering this perspective, we attempted MLST method for genotyping and diversity analysis of this species complex. We investigated ITS and TEF1-α loci, for two multilocus sequence typing scheme. Allelic profile of each locus gave 23 sequence types (STs) and 22 Allele types (ATs). However, the typing efficiency of ITS region was high as compared to TEF1-α. The phylogenetic network parallelograms based on neighbor net algorithm also showed genetic diversity in studied isolates. This relationship study was further supported by generating phylogram, which resolved these isolates in three different clades showing unequivocal phylogenetic diversity and relatedness.
Keywords: *Ceratocystis fimbriata, Dalbergia sissoo, genotyping variation, sequence types (STs), allele types (ATs)*

Introduction

Ceratocystis fimbriata complex represents a group of cryptic species of sever plant pathogen(s) and saprobe(s) as well (Wyk, 2005). *C. fimbriata* complex parasitizes a wide range of plant families from herbaceous to woody plants. The *C. fimbriata* was first time reported in Sweet potato in 1890 (Halsted, 1890). Since 1980, several fungi of species were isolated and identified from a range of host plants from around the globe (Seifert et al., 1993; Wyk et al., 2005). *C. fimbriata* is widely accepted as a complex that comprised of Latin American, North American, Asian and the African clades that include many fungal species (Johnson et al., 2005; Heath et al., 2009). In past few decades, new taxa have been evolved extensively in this complex with special reference to advancement in DNA based identification methods (Fourie et al., 2015). Different *Ceratocystis* species in the *C. fimbriata* sensu lato species complex have been reported to date that are associated with different plant diseases (Harrington, 2000). The fungi of this species complex causes root rot, cankers and vascular wilts and staining in woody hosts (Wingfield et al., 1996; Marin et al., 2003; Al Adawi et al., 2013).

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The pathogens belong to Ceratocystis fimbriata sensu lato species complex are soil borne with fruity odor characteristics. This feature facilitates their dispersal by insects and beetles (Wyk et al., 2005). With wide host rang, this species complex has been recognized as of diverse taxonomic entity. However, with the aid of molecular based studies, different taxa have been recognized and diverged as distinct species from this species complex. It has been documented by DNA based studies that Ceratocystis fimbriata species complex represents diverse and discrete grouping based on geography and host (Harrington, 2000; Baker et al., 2003). Previously, in this complex, species delineation was made based on single gene genealogy of ITS (internal transcribed spacer) region. Nevertheless, multiple gene genealogies and analyzing phylogenetic diversity had been attempted to specify species boundaries.

However, there are diverse molecular tools employing for genotypic diversity analysis. Whereas, multilocus sequence typing (MLST) tool has become more popular scheme for genetic diversity studies. In fungal molecular biology, so far established MLST schemes are for Fusarium species (Westerdijk Fungal Biodiversity Institute), Candida species (Imperial College), Aspergillus (Oxford University), Bipolaris species and Scedosporium apiospermum and S. boydii species (International Society of Human and Animal Mycology, ISHAM). However, in Ceratocystis species complex, MLST scheme has not been established yet. Although this complex is a cryptic species complex and there is a great room of genetic diversity. By considering this perspective, we have attempted two multilocus sequence typing (MLST) method for genotyping and analyzing genetic variation in Ceratocystis species complex. This may be used for further studies based on other loci. Hence, the data generated by MLST scheme could be used for epidemiological studies for which accurate recognition of different species, subspecies and strains are required.

Material and methods

Fungal isolates

We used 23 isolates of *Ceratocystis fimbriata* sensu lato from our collection of fungal isolates associated to dieback affected shisham (*Dalbergia sissoo*) plants. Each isolate was characterized based on its morphocultural characteristics and was labelled as FMB (Fungal Molecular Biology Laboratory-Culture Collection) with a unique identification number as given in *Table 1*.

DNA extraction

Fungal DNA was extracted with GeneJET Genomic DNA Purification Kit (Thermoscientific, USA). The DNA samples were quantified with a NanoDrop 8000 spectrophotometer (Thermofisher). However, DNA quality was checked on agarose gel electrophoresis and stored at -20 °C until further use.

Molecular biology and computational biology

We used two genes, internal transcribed spacer (ITS) and translation elongation factor 1 α (TEF1-α), for polymerase chain reaction (PCR), sequencing and phylogenetic analysis. PCR amplifications of these two genetic regions was carried out in Veriti^{TM} 96 wells thermalcycler (Applied Biosystems) using 25 µl reaction volume with the ITS (White et al., 1990) and TEF1-α (Oliveira et al., 2015; Li et al., 2017) sequence regions

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based primer pairs. PCR products were purified using a FavorPrep PCR purification kit (Favorgen Biotech Corporation, Taiwan) and were sequenced in both directions (Eurofins Genomics DNA sequencing services, USA). High quality trimmed sequences were generated using BioEdit software version 7.2.6.1. For getting consensus sequences of sequences obtained using forward and reverse primers DNASTAR Lasergene v. 7.1.0 SeqMan pro (SeqManTMII) software package was used. These generated sequences were compared with the sequences available in NCBI database using BLASTn and were deposited in GenBank under accession numbers, given in the *Table 1*.

Table 1. *A list of isolates name, strain accession number and GenBank accession numbers of sequences of ITS and TEF1-α*

Sr #	Isolate name	Strain accession number	Location	GenBank accession numbers	
				ITS	TEF1-α
1	FMB-GHP1E-SD	FMB 0146	Punjab, Pakistan	MK816923	MK890310
2	FMB-GHP2E-SD	FMB 0147	Punjab, Pakistan	MK816925	MK890311
3	FMB-GHP3-SD	FMB 0148	Punjab, Pakistan	MK816928	MK890312
4	FMB-GHP4-SD	FMB 0149	Punjab, Pakistan	MK816929	MK890313
5	FMB-GHP5-SD	FMB 0150	Punjab, Pakistan	MK816932	MK890314
6	FMB-GHP6-SD	FMB 0160	Punjab, Pakistan	MK816933	MK890315
7	FMB-GHP7-SD	FMB 0161	Punjab, Pakistan	MK816934	MK890316
8	FMB-GHP8-SD	FMB 0162	Punjab, Pakistan	MK816957	MK890317
9	FMB-M12-SD	FMB 0033	Punjab, Pakistan	MF767443	MK890321
10	FMB-BWP-SD	FMB 0163	Punjab, Pakistan	MK811131	MK820654
11	FMB-VR-SD	FMB 0164	Punjab, Pakistan	MK813838	MK890320
12	FMB-ST-SD	FMB 0165	Punjab, Pakistan	MK813839	MK890322
13	FMB-BR-SD	FMB 0166	Punjab, Pakistan	MK811133	MK820656
14	FMB-FSD-SD	FMB 0167	Punjab, Pakistan	MK811134	MK850855
15	FMB-TTS-SD	FMB 0168	Punjab, Pakistan	MK811125	MK890319
16	FMB-BLCN-01	FMB 0169	Baluchistan, Pakistan	MK811095	MK850853
17	FMB-BLCN-02	FMB 0170	Baluchistan, Pakistan	MK811097	MK820655
18	FMB-Thatta-SD	FMB 0171	Sindh, Pakistan	MK811092	MK850852
19	FMB-Hyderabad-SD	FMB 0172	Sindh, Pakistan	MK811093	MK850851
20	FMB-Nosheroferoz-SD	FMB 0173	Sindh, Pakistan	MK811094	MK850850
21	FMB-GL-SD	FMB 0174	KPK, Pakistan	MK811103	MK890318
22	FMB-BZ1-SD	FMB 0175	KPK, Pakistan	MK811102	MK910747
23	FMB-PZ1-SD	FMB 0176	KPK, Pakistan	MK811101	MK850854

Genotyping variation analysis of ITS and TEF1-α genes

Nucleotide sequences of ITS and TEF1-α genes were used for genetic distance analysis. Genotypic variation of each locus was assessed using MLSTest v1.0 software. The allele types (ATs) number, sequence types (STs) number, number of polymorphism, typing efficiency and discrimination power at 95% confidence interval were computed using this software. Neighbor net algorithm was used to perform phylogenetic network of each loci by splitsTree4 V4.14.8 software.

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Nucleotide sequences of both genes were concatenated using Geneious software v. 4.8.5 in the order as ITS and TEF1-α to generate concatenated dataset file. The best evolutionary model for concatenated dataset was selected from Bayesian Information Criterion (BIC) using MEGA 7.0 software. However, evolutionary model with lowest BIC value was selected to construct a maximum likelihood phylogram. Sequences were aligned with ClustalW for phylogenetic tree construction. A phylogenetic tree of the 23 aligned sequences based on best evolutionary model was obtained for the heuristic search in bootstrap analysis with 1000 replications by applying the Neighbor-Joining method to a matrix of pairwise distances estimated through Maximum Composite Likelihood (MCL) approach using MEGA7 (Molecular Evolutionary Genetics Analysis version 7.0) software.

Results

Twenty-three fungal isolates collected from dieback disease affected tissues of *Dalbergia sissoo* (Shisham) were characterized based on morphological and genomic investigations. These characterizations has set them under the *Ceratocystis* species complex. Molecular characterization was based on the direct sequencing of ITS and TEF1-α regions. The generated sequences of these isolates were subjected to BLASTn tool to search their homology with already available sequences of the type materials in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence similarity using BLASTn showed significant similarity with Ceratocystis species of *Ceratocystis fimbriata* sensu lato complex.

The Genotypic variation profiling of 23 isolates belongs to *Ceratocystis fimbriata* sensu lato complex was generated using MLSTest software. The analysis showed 23 best different sequence types (STs) numbers and 22 allele types (ATs) numbers of both loci (*Table 2*). However, the frequency of each allele types (ATs) numbers in each isolate is given in *Table 3*.

The allelic profile of both genes including number of alleles, discrimination power (95% confidential interval), typing efficacy and number of polymorphisms were calculated, given in the *Table 4*. A diverse genetic relationship was found among the genotyped variants of 23 *Ceratocystis fimbriata* sensu lato after analyzing phylogenetic network of each of the both loci (ITS and TEF1-α) using neighbor net algorithm (*Fig. 1*).

Among 24 calculated models of concatenated dataset, the best evolutionary model of concatenated dataset (ITS and TEF1-α) for maximum likelihood (ML) phylogenetic tree was K2 (Kimura 2-parameter) with BIC (Bayesian Information Criterion) score 2721.116. This model was selected based on the theory that evolutionary models with the lowest BIC values are considered to describe best substitution pattern. The phylogenetic tree (*Fig. 2*) with highest log likelihood (-1145.9648) was drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were 749 positions in the final dataset.

The evolutionary hierarchy was inferred using ML method based on the K2 model, which categorized these fungal isolates into three discrete clades. The phylogram showed that the clade III was outer group and comprised of FMB-BZ1-SD, FMB-M12-SD, FMB-Thatta-SD, FMB-ST-SD, FMB-GHP2E-SD, FMB-BLCN-01, and FMB-GHP1E-SD with 96% bootstrap support. However, Clade I and Clade II were shown to be evolutionary diverged from Clade III with 96% and 86% bootstrap support respectively (*Fig. 2*).
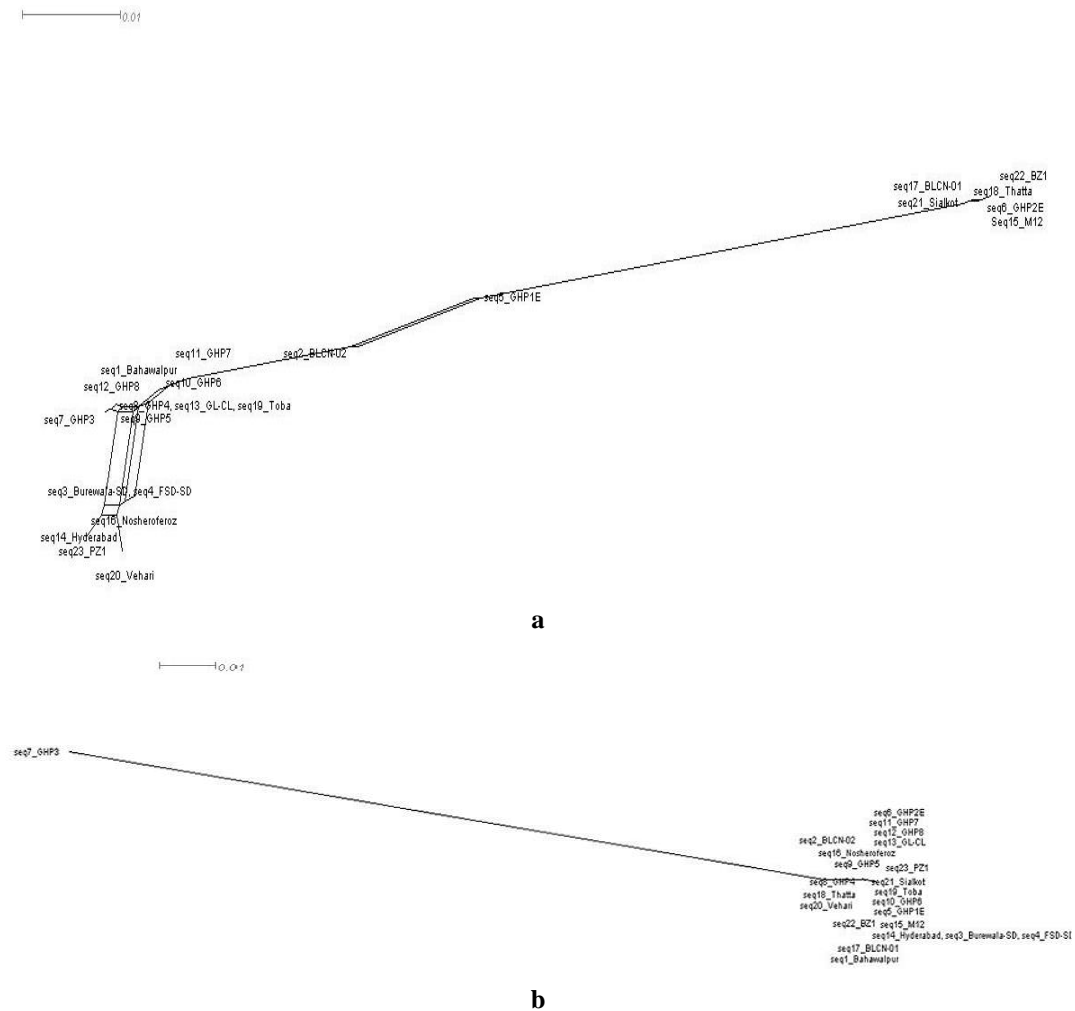
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Figure 1. *Phylogenetic network of two loci, (a) ITS, (b) TEF1- α with neighbor net algorithm by split tree software*

Clade I majorly comprised of fungal isolates from Punjab (a province of Pakistan), as FMB-GHP3-SD, FMB-GHP4-SD, FMB-GHP5-SD, FMB-GHP6-SD, FMB-GHP7-SD, FMB-BWP-SD, and FMB-TTS-SD except FMB-BLCN-02, which was collected from Baluchistan (a province of Pakistan). These fungal isolates made cladding with 96% bootstrap support. However, in clade II fungal isolates from KPK, Sindh and Punjab (provinces of Pakistan) made grouping. In clade II, the isolate FMB-PZ1-SD (KPK) was evolutionary diverged from clade III with 86% bootstrap support. However, fungal isolates of this group, FMB-Nosheroferoz-SD, FMB-Hyderabad-SD (Sindh), FMB-BR-SD, FMB-FSD-SD and FMB-VR-SD (Punjab) were shown to diverged from FMB-PZ1-SD and grouped together in this clade with 67% bootstrap support (*Fig. 2*). Based on the phylogenetic networks of both loci and phylogenetic tree of concatenated dataset, among these 23 studied isolates, 9 isolates (FMB-BLCN-02, FMB-GHP3-SD FMB-GHP7-SD, FMB-PZ1-SD, FMB-FSD-SD, FMB-Nosheroferoz-SD, FMB-BZ1-SD, FMB-M12-SD and FMB-GHP1E-SD) were selected for phylogenetic analyses to delimit the species boundaries of Ceratocystis species associated to dieback disease of Dalbergia sissoo. These selected isolates showed more genotyping variation among all studied isolates by multilocus sequence typing (MLST) approach.

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Table 2. *A list of sequence types (STs) numbers and allele types (ATs) numbers of ITS and TEF1-α loci in isolates of Ceratocystis fimbriata sensu lato complex*

Isolate name	Strain number	Allele types (ATs)		Sequence types (STs)
		TEF1-α	ITS	
FMB-BR-SD	FMB 0166	1	1	1
FMB-PZ1-SD	FMB 0176	2	2	2
FMB-FSD-SD	FMB 0167	3	3	3
FMB-Nosheroferoz-SD	FMB 0173	4	4	4
FMB-Hyderabad-SD	FMB 0172	5	5	5
FMB-VR-SD	FMB 0164	6	5	6
FMB-BLCN-02	FMB 0170	7	6	7
FMB-GHP8-SD	FMB 0162	8	7	8
FMB-GHP5-SD	FMB 0150	9	8	9
FMB-GHP4-SD	FMB 0149	10	9	10
FMB-GL-SD	FMB 0174	7	10	11
FMB-BWP-SD	FMB 0163	11	11	12
FMB-GHP7-SD	FMB 0161	12	12	13
FMB-GHP3-SD	FMB 0148	13	13	14
FMB-GHP6-SD	FMB 0160	14	14	15
FMB-TTS-SD	FMB 0168	15	15	16
FMB-GHP2E-SD	FMB 0147	16	16	17
FMB-BLCN-01	FMB 0169	17	17	18
FMB-M12-SD	FMB 0033	18	18	19
FMB-BZ1-SD	FMB 0175	19	19	20
FMB-Thatta-SD	FMB 0171	20	20	21
FMB-ST-SD	FMB 0165	21	21	22
FMB-GHP1E-SD	FMB 0146	22	22	23

Pertaining to allelic profile for multilocus sequence typing (MLST), typing efficiency of ITS locus was more (0.124) than TEF1-α (0.021). This genotyping supports the phylogram generated through concatenated dataset of both loci. This genotyping results have deduced that, the ITS region has more resolving and typing power than TEF1-α region to species identification and discrimination in case of Ceratocystis species of *Ceratocystis fimbriata* sensu lato complex. This is the first report of using two multilocus sequence typing (MLST) scheme for studying and typing genetic variation in Ceratocystis species complex.

Discussion

There are different approaches that have been employing for genotypic diversity studies. Among of them, multilocus sequence typing (MLST) has become more robust scheme. In the field of fungal molecular biology, MLST scheme Fusarium species, Candida species, Aspergillus, Bipolaris species and Scedosporium apiospermum and S. boydii species have been established by four working groups of Fungal taxonomy. However, in Ceratocystis species complex, MLST scheme has not been established yet. Although this complex is a cryptic species complex and there is a great room of genetic diversity. By considering this perspective, we have attempted two multilocus sequence typing (MLST) method in Ceratocystis species complex.

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Table 3. *A list of allele types (ATs) numbers of ITS and TEF1-α with their frequency in each isolate*

Gene	ATs	Frequency	Isolate name
TEF1-α	1	1	FMB-BR-SD
	2	1	FMB-PZ1-SD
	3	1	FMB-FSD-SD
	4	1	FMB-Nosheroferoz-SD
	5	1	FMB-Hyderabad-SD
	6	1	FMB-VR-SD
	7	2	FMB-GL-SD, FMB-BLCN-02
	8	1	FMB-GHP8-SD
	9	1	FMB- GHP5-SD
	10	1	FMB-GHP4-SD
	11	1	FMB-BWP-SD
	12	1	FMB-GHP7-SD
	13	1	FMB-GHP3-SD
	14	1	FMB-GHP6-SD
	15	1	FMB-TTS-SD
	16	1	FMB-GHP2E-SD
	17	1	FMB-BLCN-01
	18	1	FMB-M12-SD
	19	1	FMB-BZ1-SD
	20	1	FMB-Thatta-SD
	21	1	FMB-ST-SD
	22	1	FMB-GHP1E-SD
ITS	1	1	FMB-BR-SD
	2	1	FMB-PZ1-SD
	3	1	FMB-FSD-SD
	4	1	FMB-Nosheroferoz-SD
	5	2	FMB-Hyderabad-SD, FMB-VR-SD
	6	1	FMB-BLCN-02
	7	1	FMB-GHP8-SD
	8	1	FMB- GHP5-SD
	9	1	FMB-GHP4-SD
	10	1	FMB-GL-SD
	11	1	FMB-BWP-SD
	12	1	FMB-GHP7-SD
	13	1	FMB-GHP3-SD
	14	1	FMB-GHP6-SD
	15	1	FMB-TTS-SD
	16	1	FMB-GHP2E-SD
	17	1	FMB-BLCN-01
	18	1	FMB-M12-SD
	19	1	FMB-BZ1-SD
	20	1	FMB-Thatta-SD
	21	1	FMB-ST-SD
	22	1	FMB-GHP1E-SD

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Table 4. *Allelic profile including number of polymorphism, number of alleles, typing efficiency, discriminatory power of ITS and TEF1- α genes*

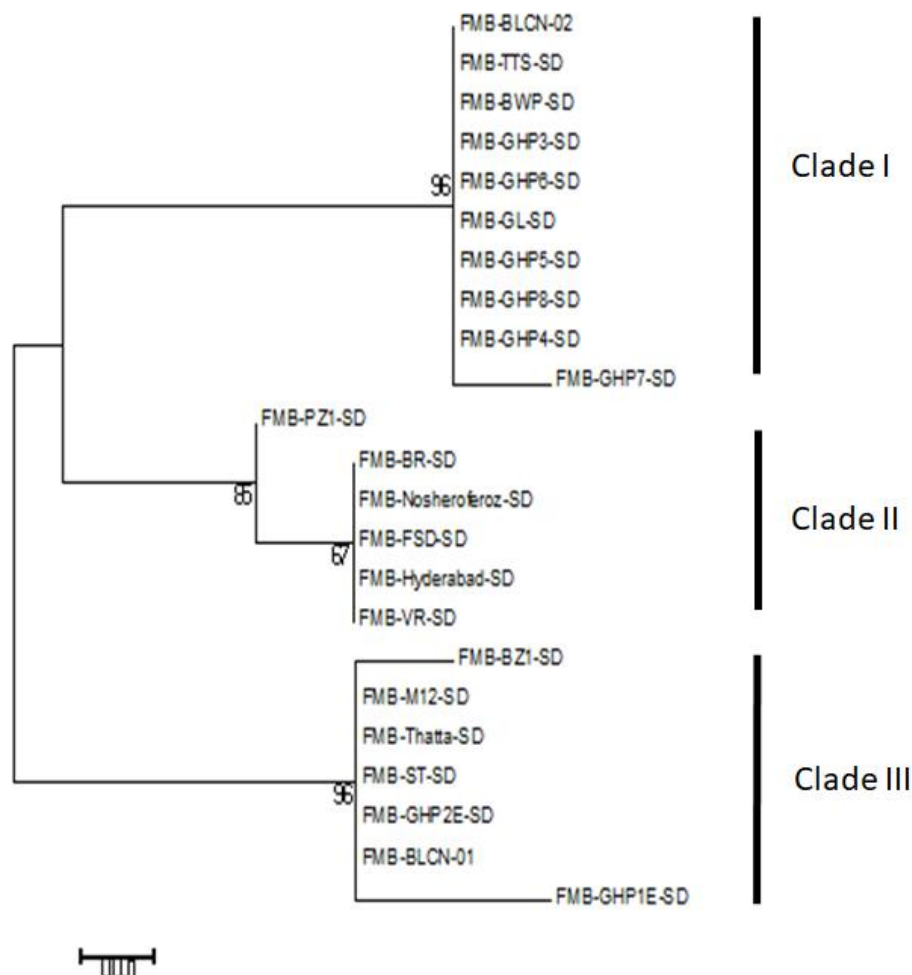| Locus	No. of alleles	No. of polymorphisms	Typing efficiency	Discrimination power (95% confidence interval)
ITS	22	177	0.124	0.996(0.985-1)
TEF1- α	22	1049	0.021	0.996(0.985-1)

Figure 2. *A maximum likelihood (ML) phylogenetic tree of concatenated dataset of two loci (ITS and TEF1-α) based on evolutionary model K2 (Kimura 2-parameter)*

We have attempted to study the genetic variation and typing analysis among the isolates of Ceratocystis species. We have 23 isolates of this fungus collected from dieback tissues of Dalbergia sissoo. Initially we have used 2 loci, internal transcribed spacer (ITS) and translation elongation factor 1 α (TEF1-α), for MLST based genotyping analysis of *Ceratocystis fimbriata* sensu lato species complex. Successful sequencing of both loci in all isolates was attempted. We identified 23 STs (sequence types) in both loci. The number of polymorphism for each locus was varied which was 177 for ITS and 1049 for TEF1-α.

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The sequences of 23 isolates of *Ceratocystis fimbriata* sensu lato species complex were analyzed by applying neighbor net algorithm using Splits-Tree software. The graph of ITS region-based sequences generated by Splits-Tree was shown as parallelogram, which implied all could be diverse but characters are shared by a set of species. However, the graph of TEF1-α showed the clustering of species at the one end of graphical string except FMB-GHP3-SD on its other end. The genetic network not shown to be able to describe genetic variation. This phylogenetic network results supported the allelic profiles generated by MLST tool which showed typing efficiency of ITS is more (0.124) as compared to TEF1-α (0.021), however, their discriminatory power was same (0.996). The sequence types (STs) number were 23 in case of both loci, which depicted that each isolates had one ST type. However, the allele types (ATs) number was 22 in both loci. Each isolates has one type of allele except FMB-GL-SD, FMB-BLCN-02 that have same type of allele of TEF1-α. Similarly, in case of ITS loci, each isolate has one type of allele except FMB-Hyderabad-SD, FMB-VR-SD that possess same type of allele.

In nutshell, here we have presented first two multilocus sequence typing (MLST) scheme based relationship analysis among *Ceratocystis fimbriata* sensu lato species complex associated to dieback disease in Dalbergia sissoo in Pakistan to studying and typing genetic variation in Ceratocystis species complex. In future, more loci may be explored for allelic profiling of these fungal isolates. Allele types (ATs) of those loci could be appraised to apprise sequence types (STs) numbers of these fungal isolates in way that is more precise.

Conclusion

Ceratocystis fimbriata sensu lato species complex is a group of cryptic species. Several species have been evolved and identified as discrete taxa. In Ceratocystis fimbriata species complex, no report on MLST based genotyping is available yet. We investigated two loci (ITS and TEF1-α) of 23 fungal isolates from dieback disease affected tissues of Dalbergia sissoo for MLST scheme. In allelic profile, the typing efficiency of ITS region was shown to be high by scoring the value of 0.124 as compared to TEF1-α with 0.021 typing score. This allelic profile result was supported by phylogenetic network parallelograms based on neighbor net algorithm, which showed genetic diversity in studied isolates though some were sharing characters. This relationship study was further supported by generating phylogram of concatenated dataset by applying K2 evolutionary model in maximum likelihood phylogenetic analysis, which resolved phylogenetic diversity and relatedness among these fungal isolates with maximum bootstrap support.

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