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GENETIC DIVERSITY OF *Alternaria* ISOLATES IN PAKISTAN

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ABSTRACT

Total genomic DNA of all isolates of *Alternaria* was isolated by CTAB method and molecular characterization by ITS sequence. The genetic identification of different isolates of *Alternaria* was confirmed by the DNA sequence analysis, a rapid mean of identification. Sequence similarities were determined by molecular method using DNA and ITS region. DNA analysis not only enables the species identification but also permits phylogenetic analysis. Internally transcribed spacer (ITS) region including 5.8S rRNA coding region in ribosomal DNA is one of the favorite targets for this purpose. The ITS region was amplified using the polymerase chain reaction (PCR) and the universal primers ITSI and ITS4. The focus of present study was on the genetic diversity analysis of the ITS regions of rRNA gene complex of local isolates of *Alternaria* in Pakistan. The genomic DNA of these isolates KI1, KI4, KI5, KI6, KI, KI8, KI9, and KI10 was amplified using primers ITS1 and ITS4 designed at the end, and start of conserved 18S and 28S region and between ITS1 and ITS4 respectively. By the comparison of isolates of all species have maximum genetic diversity only KI7 and KI8 have maximum homology with each other that showed that both are may be originated from same ancestor. Their phylogenetic relationships in GenBank were analyzed. Morphological and molecular data obtained might be useful in determining the taxonomy and diversity of *Alternaria* species.

Keywords: ITS region, Genetic diversity, *Alternaria,* BLAST, CLC BIO, CTAB.

INTRODUCTION

The Genus *Alternaria* comprises of unique, ubiquitous diverse group of pathogenic and saprophytic fungal species [1]. *Alternaria* has wide spread distribution in nature and belongs to the *Dematiaceae* of the hyphomycetes in the fungi imperfecti. It is opportunistic pathogen that acts as plant pathogens, weak facultative parasites, endophytes, saprobes, found on plants and other substrata worldwide. They are serious well known pathogens that cause diseases of agricultural and economic crops such as Blights, Leaf spots. Blotches results annually billion dollars loss [2]. This pathogen population shows diversity in many citrus species. This pathogen specially attacks to the aerial parts of plants such as fruits in favorable environmental weather conditions causing considerable economic losses per year. *Alternaria* metabolites have variety of biological activity designated as phytotoxins, mycotoxins, cytotoxic and antimicrobial activities that took the attention of pharmacologist and plant pathologist to

research in this field and on its application for healthy purposes [3,4]. In recent years, there has been substantial progress in the development of innovative methods to analyze fungi (and other organisms) at the molecular level. For example, the genetic variation of pathogenic and saprobic *A. alternata* isolates was assessed using RAPD, RFLPs, DNA hybridization, AFLP and DNA sequences [5,4]. *A. alternata*, endophytic isolates however, have also been investigated using random amplified microsatellite (RAMS) markers which was originally used to measure genetic diversity of plants and animals [6,7], it has also been applied in studies of endophytic fungi. With several molecular methods being developed, many species-specific primers targeting several genomic regions such as internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) [8,9] or by using nonspecific primers for polymerase chain reaction (PCR) amplification of the ITS regions and subsequent analysis with restriction fragment length polymorphism (RFLP) [10]. The ITS regions are located between the repeating array of nuclear 18S, 5.8S and and 28S ribosomal RNA genes and have 100-200 genome. These regions are rapidly evolving and thus have been routinely used in species-level phylogeny in a wide range of organisms [11]. In *B. xylophilus*, the intraspecific variation of the ITS regions have been used to study the genetic structure of isolates from various regions of the world through sequencing [12] and RFLP analysis [10]. Studies about genetic diversity of *Alternaria* species are focused worldwide. But yet there were not enough studies about genetic diversity and fungal *Alternaria* distribution with more study about diseases damages and its importance. Thus genetical study helps in determining genetic diversity of pathogens and adaptation to various methods of the pathogen controls and management. Metabolic rate slows down at the time of preservation. Some species are sensitive to the temperature specially mastigomycotina. A non-sensitive fungus doubles their shelf life by this preservation.

MATERIALS AND METHODS

Procurement of Fungal Cultures

Following isolates of Genera *Alternaria* were obtained from Fungal Culture Bank of Pakistan (FCBP) Institute of Agricultural Sciences (IAGS) University of the Punjab Lahore Pakistan. Different isolates of *Alternaria* were maintained on potato dextrose agar (PDA) and Malt extract agar (MEA) at 28 ± 2°C for 7 days. All these cultures were preserved at 4°C until needed.

DNA Extraction

Revived Fungal cultures was cultured in ME medium (Malt Extract) for 5-7 days and incubated at 28 °C± 2°C on shaker at 110-120 rpm. The mycelial mat was collected by filtering through a No.1 Whatman filter paper and washed with sterile distilled water. The fresh mycelia mat was further used in DNA extraction. The dried or lyophilized mycelia mat was ground into fine powder by using liquid Nitrogen with autoclaved sterilized pestle and mortar. Then DNA was extracted using hexadecyl trimethyl -ammonium bromide (CTAB) extraction method with some modification as described by Guo et al. [13].

Polymerase Chain Reaction

Amplification of ITS region

PCR was performed for the amplification of nuclear rDNA region containing the Internal Transcribed Spacer (ITS) regions (ITS1 and ITS4) in the *Alternaria* sp. Following primers were used for amplification of ITS region (Table 2). For

Table 1. Revived isolates from FCBP

Accession no of FCBP	Culture name	Source of isolation	Date of isolation
117	*Alternaria alternata*	Decaying wood, Chashma, Khanaspur.	09.09.03
963	*Alternaria pluriseptata*	Rose leaf, Lahore.	06.02.09
988	*Alternaria pluriseptata*	Brassica, Sargodha.	23.05.09
1092	*Alternaria citri*	Citrus fruit, Lahore.	29.12.09
1107	*Alternaria senecionicola*	*Solanum melongena* (Eggplant) leaf, Lahore.	20.01.11
1129	*Alternaria alternata*	*Psidium guajava* L. (Guava fruit), Lahore.	25.02.11
1131	*Alternaria pluriseptata*	Textile industry effluent, Sheikhupora.	28.02.11
1169	*Alternaria alternata*	Gram pod spot, Lahore.	23.01.12

Table 2. Primers were used in the amplification of ITS region

Serial	Primer name	Sequences (5' -3')
1	ITS1	TCCGTAGGTGAACCTGCGG
2	ITS4	TCCTCCGCTTATTGATATGC

PCR a reaction mixture of 25 µL containing 5 uL DNA extracted from *Fusarium*, 2.5 µL of 2 mM dNTPs, 2.5 µL of 10X Taq polymerase buffer (Fermentas), 1.5 µL of 25 mM $MgCl_2$, 0.25 µL of *Taq* DNA polymerase (Fermentas), 0.5 µL of each primers and 12.25 µL of SDDW will be prepared. PCR amplified product will be tested on 1% agarose gel.

Gel Electrophoresis

Agarose gel (1% w/v) containing 0.5µg/mL ethidium bromide will be prepared in 1X Tris-acetate EDTA (TAE) buffer (20 mM Tris-acetate and 0.5 mM EDTA [pH 8.0] DNA will be mixed with 10X loading dye, loaded on the gel alongwith 1kb DNA ladder (Vivantis) for size comparison and will be electorphoresed at an electric potential of 60 volts. The concentration and quality of extracted genomic DNA was checked by running the samples on 1% gel. A molecular marker of 100 bp and 1kbp (Fermentas) were used to compare the resultant bands with the molecular marker. Result of gel was observed under ultra violet light in the gel documentation system. Image of the gel was observed and captured by using the JPG IT program.

Sequencing and BLAST Analysis

Purified PCR products in microfuge tubes were labelled properly and sent to Macrogen (South Korea) for sequencing using the primers ITS1 and ITS4. The sequence data were assembled and analysed with the aid of the Lasergene package of sequence analysis software (DNAStar Inc., Madison, WI, USA). Sequence similarity searches (Blast) were performed by comparing the sequence to other fusarium sequences in the database

(http://www.ncbi.nlm.nih.gov/BLAST/). Final sequences were submitted to the EMBL database (http://www.ebi.ac.uk/embl). Multiple sequence alignments were performed using Clustal X [14] and the Meg Align program of Lasergene. Phylogenetic trees were constructed using the Neighbour Joining algorithm of Clustal X and displayed, manipulated and printed using Tree view (Page, 1996).

RESULTS AND DISCUSSION

Molecular Phylogenetic Tree Constructed by CLCBIO by Muscle Alignment

Molecular neighbor joining tree was formed by CLCBIO, software used for the phylogenetic tree construction by align muscle, with 1000 boot steps in which minimum JC+G value and minimum BCI value was used. Evolutionary analyses were conducted in CLCBIO, the software supports read mapping and high throughput sequencing data, detection of structural variations on whole genomes of any size, RNA sequence analysis, Small RNA analysis and BLAST.

Discussion

In this study, genetic diversity among 8 *Alternaria* isolates was evaluated through sequence analysis of the ITS region. The ITS region proved to be a useful tool for assessing genetic variability within this pathogen. Because the ITS regions have important biological meaning in rRNA processing. The ITS sequences of *Alternaria* sp. were deposited in GenBank under different accession No. Different *Alternaria* species that were taken from (FCBP) show considerable levels of genetic variation. Intraspecific genetic divergence based on the percentage of homology. So sequence variations found in all these species that were much higher in *Alternaria* species. The high intraspecific genetic variation in *Alternaria species* is mainly due to the existence of deep divergent clades as revealed by the phylogenetic analysis. Genetic distance was different among all *Alternaria* species and genetic identity ranged from the maximum value of divergence and minimum value of homology. The phylogenetic tree was constructed by MEGA06 and CLCBIO software. Evolutionary analyses were conducted in CLCBIO and MEGA06 the software supports read mapping and high throughput sequencing data, detection of structural variations on whole genomes of any size, RNA sequence analysis, Small RNA analysis and BLAST. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The analysis involved 32 nucleotide sequences. There were a total of 467 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. According to the data collected from the molecular phylogenetic tree the sequence of KI1 (FCBP Accession NO 117; PK2003) was *Alternaria alternata* isolated from the decaying wood of chashma, khanaspur from Pakistan has 100 percent homology with sequence isolated from India (Accession No NCBI the KJ173524). Both Pakistan and India showed same climatic conditions and favorable environmental conditions, so both are same species of the genera *Alternaria*. Due to same geographical zone these were similar to each other, and it has 99.8 percent homology with the KC337039 and JF835808 while KC337039 that was *Alternaria tenuissima* isolated from china in 2013 and JF835808 was *Alternaria alternata* isolated from China in 2011.

KI4 (FCBP Accession NO 963) was *Alternaria pluriseptata* isolated from Rose leaf , Lahore from Pakistan in 2009 showed 99 percent homology with KC337039 and the 98.6 percent homology with the JN108906. KI4 also show homology with KI5

due to the sequence KC337039. While the KC337039 which showed 99 percent homology was the *Alternaria tenuissima* isolated from China in 2013. JN108906 has 98.6 percent homology with the Ki4 that was *Alternaria brassicae* isolated from India in 2013. KI5 (FCBP Accession NO 988) was *Alternaria pluriseptata* isolated from Brassica, Sargodha from Pakistan in 2009 has 99.6, 99.4 and 98.4 percent homology with the HG974561, KC337039 and KJ735924 respectively. HG974561 that showed 99.6 percent resemblance with Ki5 was the *Alternaria alternata* isolated from Sweden in 2014. KC337039 showed 99.4 percent homology with the Ki5 was the *Alternaria tenuissima* isolated from China in 2013. KJ735924 showed 98.4 percent homology with the KI5 that was *Alternaria alternata* isolated from India in 2014. Ki5 also showed homology with KI4.

Table 3. Nucleotide sequences that were used in blast from the NCBI site

Serial No	Accession No.	Country
1	KC337039	China
2	HG974561	Sweden
3	JF835808	China
4	KJ173524	India
5	KJ735924	India
6	JN108906	India
7	JN108903	India
8	KJ592052	India
9	JX406564	China
10	JX406499	China
11	HG798746	Saudi Arab
12	KJ547594	India
13	JX241640	China
14	KF644352	China
15	JX406564	China
16	KF998988	China
17	KF941273	USA

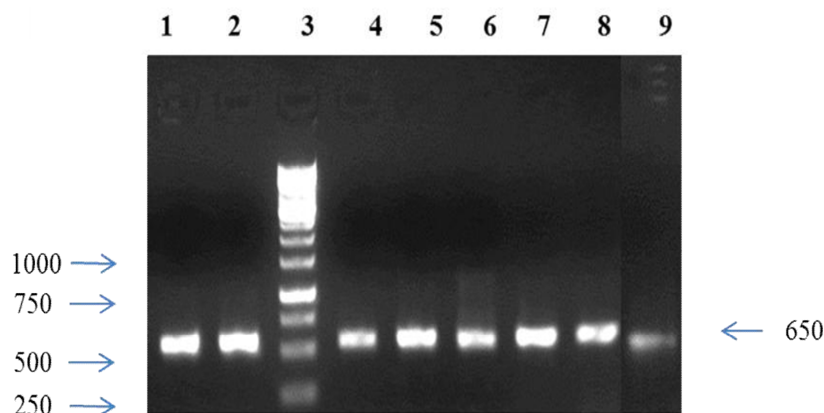

Fig: 1 Gel electrophoresis showing DNA of different isolates of Alternaria.

Lane 1: 117 *Alternaria alternata*
Lane 2: 963 *Alternaria pluriseptata*
Lane 3: DNA molecular marker of 1kb
Lane 4: 988 *Alternaria pluriseptata*
Lane 5: 1092 *Alternaria citri*
Lane 6: 1107 *Alternaria senecionicola*
Lane 7: 1129 *Alternaria alternata*
Lane 8: 1131 *Alternaria pluriseptata*
Lane 9: 1169 *Alternaria alternata*

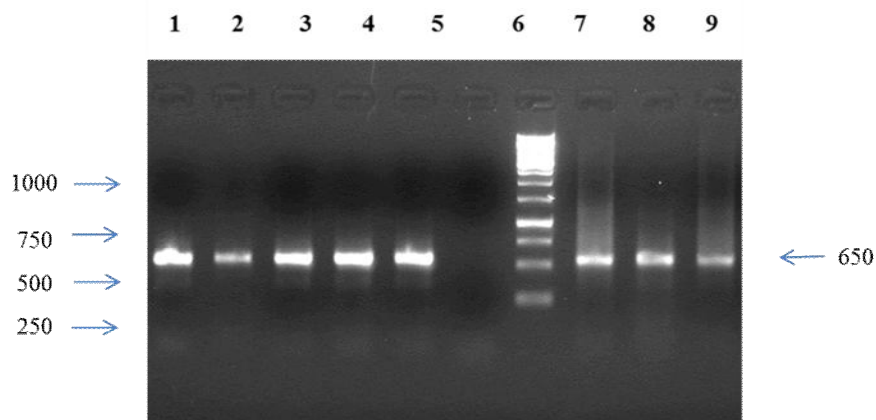

Fig: 2 Gel electrophoresis for purified PCR product

Lane 1: 117 *Alternaria alternata*
Lane 2: 963 *Alternaria pluriseptata*
Lane 3: 988 *Alternaria pluriseptata*
Lane 4: 1092 *Alternaria citri*
Lane 5: 1107 *Alternaria senecionicola*

Lane 6: Lane 6: DNA molecular marker of 1kb
Lane 7: 1129 *Alternaria alternata*
Lane 8: 1131 *Alternaria pluriseptata*
Lane 9: 1169 *Alternaria alternata*

Table 4. NCBI Nucleotides with maximum homology with sequenced FCBP Nucleotides

Serial no.	Accession no.	Code	Sequence	Similarity	Country
1	117	KI1	KJ173524	100	India
2	963	KI4	KC337039	99	China
3	988	KI5	HG974561	99.6	Sweden
4	1092	KI6	KC337039	98.8	China
5	1107	KI7	JF835808	98.6	China
6	1129	KI8	JF835808	95	China
7	1131	KI9	HG974561	94	Sweden
8	1169	KI10	JX406513	90	China

Table 5. NCBI nucleotides with maximum homology with sequenced FCBP nucleotide

Samples	NCBI nucleotides and percentage of homology		
	1	**2**	**3**
KI1	KJ173524	KC337039	JF835808
	1oo	99.8	99.8
KI4	KC337039	JN108906	KJ735924
	99	98.6	96
KI5	HG974561	KC337039	KJ735924
	99.6	99.4	98.4

Samples	NCBI nucleotides and percentage of homology		
	1	2	3
KI6	KC337039 98.8	KJ173524 97.7	HG97456 95.1
KI7	JF835808 98.6	KC337039 94.9	HG974561 92.4
KI8	JF835808 95.3	HG974561 91.2	KI6 90
KI9	HG974561 94.4	JF835808 94.4	KI5 85
KI10	KJ173524 96	JX406513 95	JX406564 92

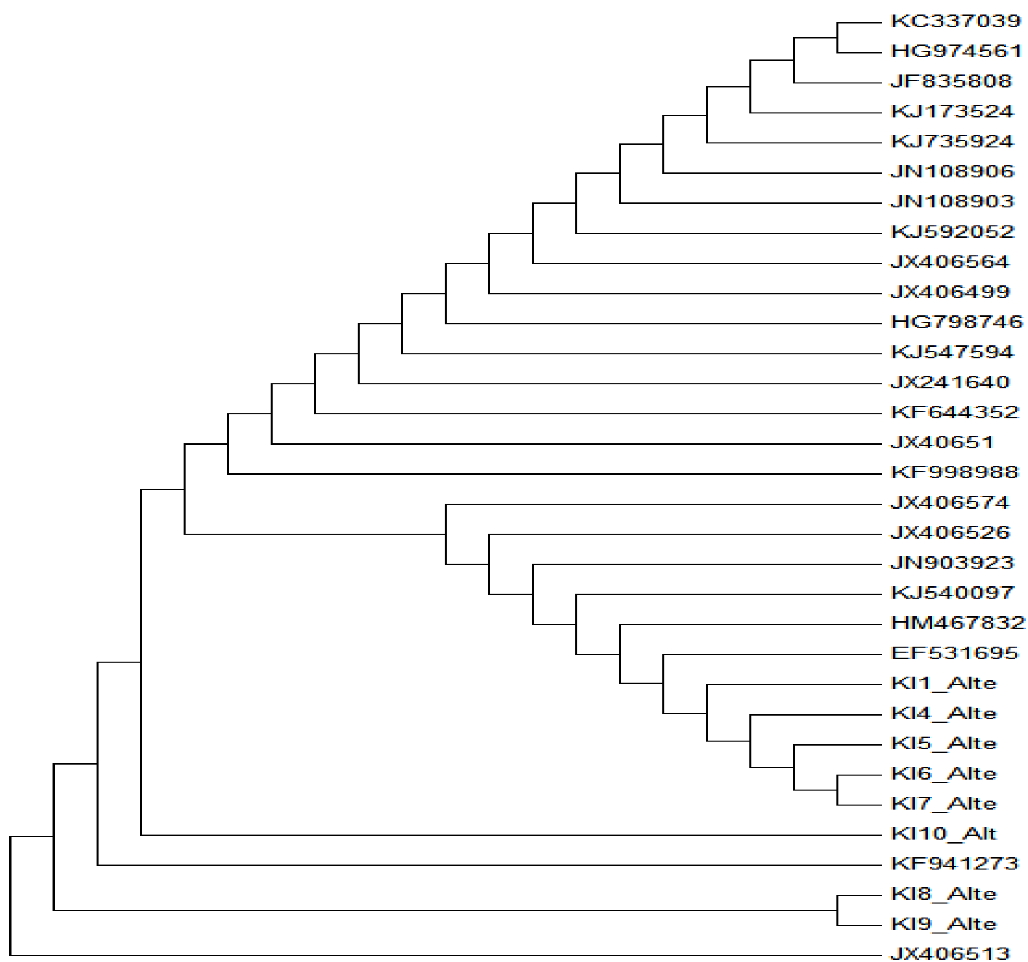

Fig. 3. Molecular Phylogenetic tree constructed by MEGA06

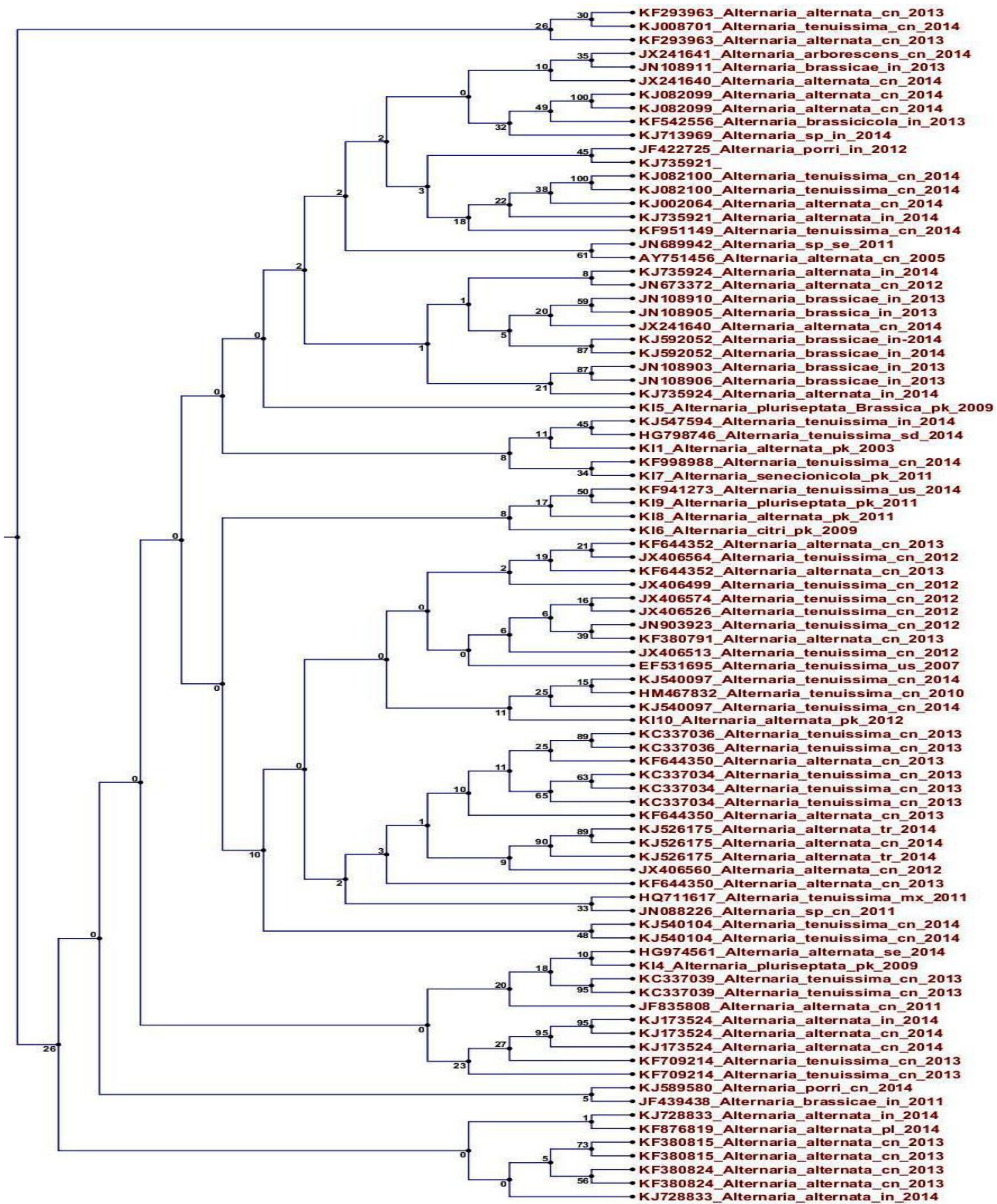

Fig. 4. Molecular phylogenetic tree constructed by CLCBIO

KI6 (FCBP Accession NO 1092) was *Alternaria citri* isolated from citrus fruits, Lahore from Pakistan in 2009 has showed 98.8 and 97.7 percent homology with KC337039 and KJ173524. KC337039 showed 98.8 percent homology was the specie *Alternaria tenuissima* isolated from China in 2013. KI6 somehow showed homology with KI7.

KI7 (FCBP Accession NO 1107) was *Alternaria senecionicola* isolated from *Solanum melongena* leaf, Lahore from Pakistan in 2011has 98.6 and 94.9 percent homology with the JF835808 and KC337039 respectively. JF835808 was the *Alternaria alternata* isolated from China in 2011. KC337039 was the *Alternaria tenuissima* isolated from China in 2013. KI8 (FCBP Accession NO 1129) was *Alternaria alternata* isolated from *Psidium guajava* L. (Guava fruit), Lahore, from Pakistan in 2011. It showed 95.3 and 91.2 percent homology with the JF835808 and HG974561 respectively. JF835808 was *Alternaria alternata* isolated from China in 2011showed 95.3 percent homology. HG974561 was *Alternaria alternata* isolated from Sweden in 2014 showed 91.2 percent homology. This sequence showed diversity. KI9 was the *Alternaria pluriseptata* isolated from Textile industry effluent, Sheikhupora from Pakistan in 2011. It showed homology 94.4 percent with the HG974561 that was *Alternaria alternata* isolated from Sweden in 2014 and JF835808 that was *Alternaria alternata* isolated from China in 2011.This sequence showed diversity in genetics. KI10 was the *Alternaria alternata* isolated from Gram pod spot, Lahore, Pakistan in 2012. It showed homology with the KJ173524 that was *Alternaria alternata* isolated from India in 2014. JX406513 that was *Alternaria tenuissima* isolated from China in 2012, and the JX406564 was *Alternaria tenuissima* isolated from China in 2012.This sequence also showed great diversity in the genome. These isolates have highest homology as in KI1 isolated from Pakistan with the isolates of India because the climatic conditions, the environment, the agricultural soil conditions and the geographical zone were the same. So these showed most homology with India and China. The variability developed because of different climatic conditions, soil conditions and the geographical zones. As in the KI9 that was *Alternaria pluriseptata* KI10 that was *Alternaria alternate,* the variation noted and these have no homology. Great level of diversity was noted. In this study it was concluded that dependence on environmental factors, such as temperature and soil moisture affected the *Alternaria species*. By the comparisons of all isolates it was concluded that they were not similar with each other at species level but show similarity at genus level. So it has been concluded that *Alternaria* species may have changed their genetic material with passage of time for their survival. It can be concluded that morphologically similar strains of the same fungal species may show genetic variations so by this study species genotypes were identified which add knowledge about *Alternaria* species genotypes in Pakistan and this work helps to understand the genetic mechanisms for the efficient breeding programs to breed the resistant cultivars of different crops.

CONCLUSION

The genomic DNA of isolates KI1, KI4, KI5, KI6, KI, KI8, KI9, and KI10 was amplified using primers ITS1 and ITS4 designed at the end, and start of conserved 18S and 28S region and between ITS1 and ITS4 respectively. By the comparison of

isolates of all species have maximum genetic diversity while KI7 and KI8 have maximum homology with each other that showed that both are may be originated from same ancestor. Their phylogenetic relationships in GenBank were analyzed. Morphological and molecular data obtained might be useful in determining the taxonomy and diversity of *Alternaria* species.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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