RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume 58 Issue 3: 532-538 (June 2024)

Molecular Identification and Genetic Diversity of Alternaria Isolates Causing Leaf Spot Disease in Cotton from Major Cotton Growing Areas of South Zone of India

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10.18805/IJARe.A-6104

ABSTRACT

Background: Alternaria leaf spot caused by two species namely *Alternaria macrospora* and *Alternaria alternata* is an important foliar disease of cotton. Conidial morphology showed that most of the isolates in the study belonged to *A. macrospora.* Molecular confirmation is necessary to strengthen the identification of species in Alternaria. Genetic diversity study of Alternaria isolates using ISSR and hyper variable SSR primers will provide variation and grouping among the isolates collected from major areas of South Zone of India. Present study was conducted to identify Alternaria isolates at species level using molecular methods (species specific primers) and genetic diversity analysis using ITS, SSR and ISSR primers.

Methods: Reported species-specific primers such as AmF and AmR as well as AaF2 and AaF3 were used for Alternaria species identification. ITS region was amplified through ITS1 and ITS4 and sequences were used for identification and clustering of isolates. Thirteen hyper variable SSR primers specific to Alternaria were designed based on the sequences retrieved from NCBI and used for diversity study. Six different ISSR primers were also used for genetic diversity study.

Result: Reported species-specific primers found not suitable to identify *A. macrospora* and *A. alternata* at species level. Two SSR primers were found to be effective in showing variability among the isolates. Six clusters were formed at 71 percent genetic dissimilarity among 15 isolates of Alternaria through ISSR primers. Five clusters were formed in ITS sequence's diversity analysis. Blasting of ITS sequences of 15 selected isolates at NCBI showed that all belong to *A. alternata*. This was due to absence or presence of very few sequences of *A. macrospora* in NCBI database itself. Further house-keeping genes like Alt a1, Plasma membrane ATPase, GAPDH and TEF -1 α sequence analysis will be useful for confirmation of *A. macrospora* at species level.

Key words: Alternaria species, Cotton, ISSR, ITS, Molecular identification and characterization, SSR.

INTRODUCTION

Cotton the "King of fibre" is infected by different pathogens during various crop growth stages. Fungi, bacteria and viruses are the pathogenic organisms, which cause number of diseases in different parts of cotton plant. Among leaf spot diseases, Alternaria leaf spot caused by *Alternaria macrospora* and *Alternaria alternata* is an important disease responsible for considerable yield loss in cotton during boll maturity and bursting stage. Under favourable weather conditions, Alternaria disease can cause yield reduction up to 26.59% (Monga *et al*., 2013) and 38.23% (Bhattiprolu and Prasada Rao, 2009). Alternaria blight was the major disease observed in farmer fields of central zone and the disease intensity ranged between 2.16 to 24.12 per cent irrespective of variety/hybrids and locations (AICRP on Cotton, Annual report 2016-17). Alternaria leaf spot disease incidence varied from 0.0 to 12.0 PDI in different cultivars/ hybrids tested in Research farm, Surat, Gujarat (Sandipan *et al.,* 2019). Alternaria leaf spot of cotton recorded as 5.03 PDI in protected condition and 11.85 PDI under unprotected conditions in Jaadoo BG II in the experiment conducted in Lam farm, Guntur, Andhra Pradesh (Baba *et al.,* 2022). Average disease severity index of Alternaria leaf spot of cotton ranged from 21.5 to 87.0% in New Mexico (Zhu *et al.,* 2019). Two species of Alternaria namely *A. macrospora* and *A. alternata* are reported to cause the leaf spot disease. Traditionally these two species are identified and differentiated based on long or short beak length of the muriform conidia. Sometimes the identification of Alternaria is difficult due to mixed infection of other similar looking fungus. The confusion is compounded by the presence of other morphologically similar and closely related pathogens such as *Ulocladium* and *Stemphylium*, which produced morphologically similar conidia (Lawrence *et al*., 2012).

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How to cite this article: Sampathkumar, A. and Raghavendra, K.P. (2024). Molecular Identification and Genetic Diversity of Alternaria Isolates Causing Leaf Spot Disease in Cotton from Major Cotton Growing Areas of South Zone of India. Indian Journal of Agricultural Research. 58(3): 532-538. doi: 10.18805/IJARe.A-6104.

Submitted: 23-04-2023 **Accepted:** 02-05-2023 **Online:** 09-06-2023

For precise identification of Alternaria, ITS region amplification and sequencing were followed. Some species-specific primers were developed by different workers for species identification.

Molecular approaches, mainly the polymerase chain reaction have been used widely as the tool for detection of fungal pathogens (Schaad and Frederick, 2002). Molecular characterization and phylogenetic genetic studies have demonstrated a clear distinction between large and small spored Alternaria species (Woudenberg *et al*., 2013). Alternaria species identification which cause the black spot disease of tomato was carried out according to morphological description and by ITS gene sequencing (Saleem and El-Shahir, 2022). Present study was conducted with the following objectives. 1. Alternaria species identification using molecular methods and 2. Genetic diversity analysis of Alternaria using ITS region, SSR and ISSR primers.

MATERIALS AND METHODS

Collection of leaf spot samples, isolation, morphology and pathogenicity

The Alternaria leaf spot samples were collected from major cotton growing areas south India comprising the states of Tamil Nadu, Andhra Pradesh, Telangana and Karnataka. The pathogen was isolated from the samples by tissue segment method using potato dextrose agar (PDA) medium. The pathogenicity and virulence of the isolates were tested on 30 days old susceptible cotton cultivar LRA5166 using spray inoculation method under glass house conditions. Cultural characters like colour, texture and growth pattern were studied. Morphological characters like colony shape, conidial shape and size, sporulation capacity of isolates was studied on PDA. The conidial size was measured using compound microscope.

Genomic DNA isolation

Genomic DNA was isolated as per procedure given by Van Burik *et al.* (1998) using CTAB method. Ten days old PD broth multiplied Alternaria fungal mat was harvested for each isolate and the moisture was removed using filter paper. Dried mat was ground in pestle and mortar using liquid nitrogen. DNA was extracted using series of steps according to the method mentioned above.

Molecular identification of alternaria isolates

Reported species-specific primers in earlier studies for *A. macrospora* namely AmF and AmR as well as *A. alternata* specific primers namely Aa F2 and Aa F3 (Table 1) have been used to identify the pathogen at species level (Sangeetha, 2014). Alternaria genus specific primers from previous study also used to identify the pathogen at genus level. Primers namely forward primer Alt-for and Alt-rev (Sangeetha, 2014) were used. ITS primers namely ITS 1 and ITS 4 were used for amplification of ITS region followed by sequencing for further analysis. Genetic diversity analysis

Table 1: Alternaria species specific primers, hyper variable SSR primers and ISSR primers used in the study.

Gene	Primer sequences					Amplicon size (bp)
	Forward	no. of bases	gene	Reverse	no. of bases	
Alternaria gene specific primers						
ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'	19	ITS4	5'- TCC TCC GCT TAT TGA TAT GC-3'	20	560
AmF	5'-CGG TAC TAC TGT CAT CTT CG-3'	20	AmR	5'- CTT ACG GTA CCT GAG TTG AC-3'	20	442
Aa F2	5'-TGC AAT CAG CGT CAG TAA CAA AT-3'	23	Aa F3	5'- ATG GAT GCT AGA CCT TTG CTG AT-3'	23	320
Alt-for	5-ATG CAG TTC ACC ACC ATC GC-3	20	Alt-rev	5-ACG AGG GTG AYG TAG GCG TC-3'	20	
Hyper variable SSR primers						
SSR1	5'-ACG TAC AGC TCG AAG TGT GG-3'	20		5'-GTT TTG CTC AAG CGA CCG G-3'	19	179
SSR5	5'-CGT CTT CCG GGA GAA AGA CC-3'	20		5'-TTC CAC GGA GGG ATA TTC GC-3'	20	224
ISSR Primers						
UBC 807	5'-AG AG AG AG AG AG AG AG T-3'					
UBC 809	5'-AG AG AG AG AG AG AG G-3'					
UBC 834	5'-AG AG AG AG AG AG AG CT-3'					
UBC 835	5'-AG AG AG AG AG AG AG CC-3'					
UBC 842	5'-GA GA GA GA GA GA GA TG-3'					
UBC 856	5'-AC AC AC AC AC AC AC CA-3'					

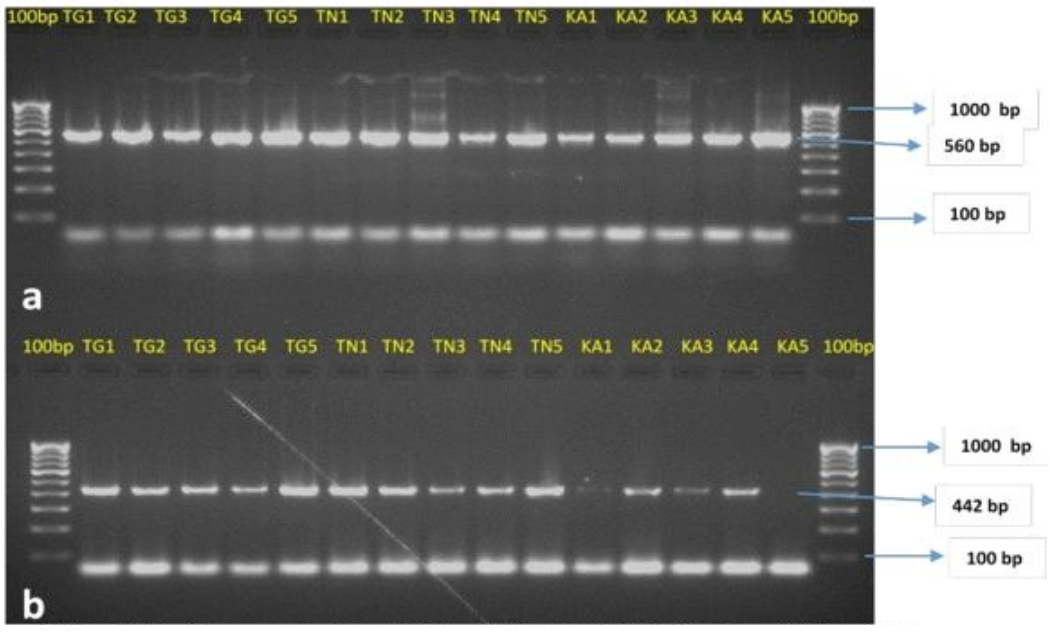
of Alternaria isolates was done using hyper variable SSR primers and ISSR primers. Thirteen hyper variable SSR Primers (Table 1) were designed from *Alternaria alternata* sequences retrieved from NCBI website. ISSR primers (Table 1) were selected from literature and used for genetic diversity analysis.

The 10 µl PCR reaction mixture contained 2 µl of template DNA (50 ng concentration), 1 µl each of forward and reverse primers, 5 µl of PCR master mix (Smart prime 2X master mix-Red) and 1 µl of sterile water. PCR cycling conditions include initial denaturation of 95°C for 4 minutes followed by 35 cycles of 94°C for 30 seconds (denaturation), 55-59°C (based on primer) for 30 seconds (annealing) and 72°C for 30 seconds (extension). The final extension was at 72°C for 10 minutes. PCR amplification was performed with 10 ml reaction in Himedia Prima 96 plus PCR machine (Himedia laboratories Pvt Ltd, Mumbai, India). The PCR products were resolved on 1.5% agarose gel electrophoresis stained with ethidium bromide (0.5 mg/ml), photographed and analysed using gel documentation system (GelONE gel documentation system, Clever Scientific, UK). The ITS sequence alignment was performed using Clustal W and phylogenetic tree was constructed with MEGA 6 as mentioned by Tamura *et al.* (2013). The phylogeny was tested through neighbour joining method by 1,000 bootstraps with specified cut off value.

RESULTS AND DISCUSSION

The present study was aimed to find major species of Alternaria in cotton growing areas of south zone. Alternaria survives in infected plant debris during off season. The pathogen infects all the four cultivated species of Gossypium (Srinivasan, 1994). One-hundred and forty-one Alternaria leaf spot samples have been collected from different cotton growing regions of India and 96 isolates were isolated using PDA medium. Colony characters varied from grey to ashy grey, whitish grey, blackish grey with smooth or irregular margin. All the isolates were pathogenic to cotton and pathogenicity was proved on susceptible cultivar LRA 5166.

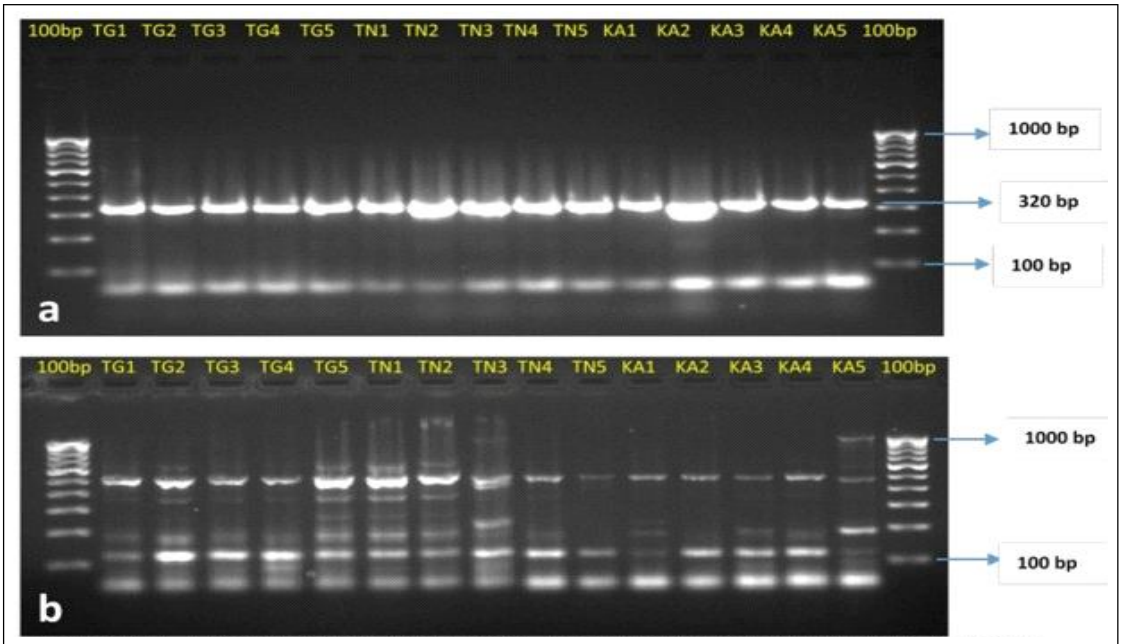
Fifteen representative isolates, each five from Karnataka (KA1 to KA5), Tamil Nadu (TN1 to TN5) and Telangana (TG1 to TG5) were selected for molecular characterization and identification. Among five isolates, four *A. macrospora* and one *A. alternata* were selected through conidial characters. Long beak of conidia was the characteristic feature of *A. macrospora* (Vasudeva, 1960). Short beak length was the characteristic feature of *A. alternata* (Srinivasan, 1994). ITS primers generated 560 bp bands for all the isolates (Fig 1). Alternaria genus specific primers amplified multiple bands for all the isolates (Fig 2). *A. macrospora* specific primers (Am) amplified 442 bp for all isolates (Fig 1). *A. alternata* species specific primers (Aa) amplified 320 bp for all the isolates (Fig 2). Both of these primers could not identify or differentiate the two species of Alternaria. Sequences of ITS region showed that all the fifteen isolates were belonged to *A. alternata*. Dendrogram was constructed based on ITS sequences and isolates formed five different clusters among them (Fig 3). Blasting of ITS sequences of 15 selected isolates at NCBI showed that all were belonging to *A. alternata* (Table 2). This was



TG1 to TG5 -Telangana isolates; TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates; M (Marker): 100 bp ladder. 560 bp amplification for ITS region and 442 bp for Am primers for all isolates

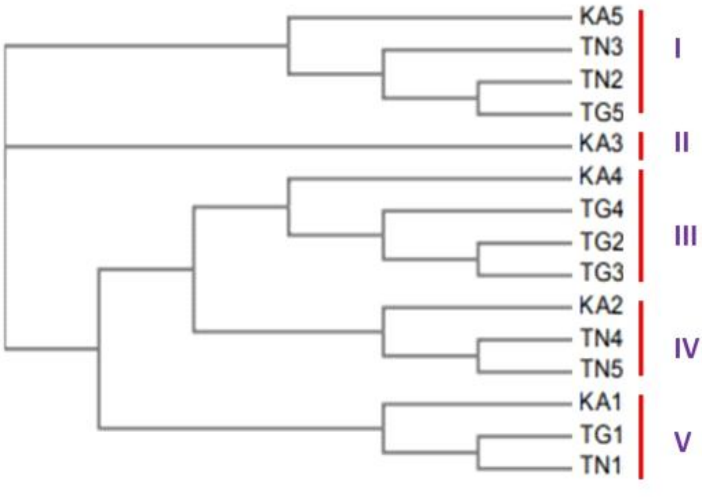
Fig 1: Molecular identification of Alternaria isolates through a) ITS region and b) *A. macrospora* specific primers using ITS (1 and 4) and Am (F and R) primers.

due to absence/very few sequences availability of *A. macrospora* in NCBI itself. Further GAPDH and TEF-1 α gene sequence analysis will be useful for confirmation of *A. macrospora* at species level. Many workers reported same kind of results. They identified the species based on ITS region in combination with housekeeping genes. Alternaria was divided into 24 sections on the basis of morphological and multiloci molecular phylogeny (Lawrence *et al.,* 2013). Molecular phylogenetic analyses of Alternaria sp. from rubber based on ITS rDNA, GAPDH and TEF1-alpha sequences demonstrate that the phytopathogen falls in the clade of the section Porri (Cai *et al.,* 2019). Zhu *et al*. (2019)



TG1 to TG5 -Telangana isolates; TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates;
M (Marker): 100 bp ladder. 320 bp amplification for Aa primers and multiple bands for Alt primers for all isolates.

Fig 2: Molecular identification of Alternaria isolates through a) *A. alternata* specific Aa (F2 and F3) primers and b) Alternaria genus specific Alt (for and rev) primers.



Clusters formed by the respective isolates are shown (I, II, III, IV and V). TG1 to TG5 -Telangana isolates;
TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates.

Fig 3: Phylogenetic tree generated from the ITS gene sequences of 15 Alternaria isolates.

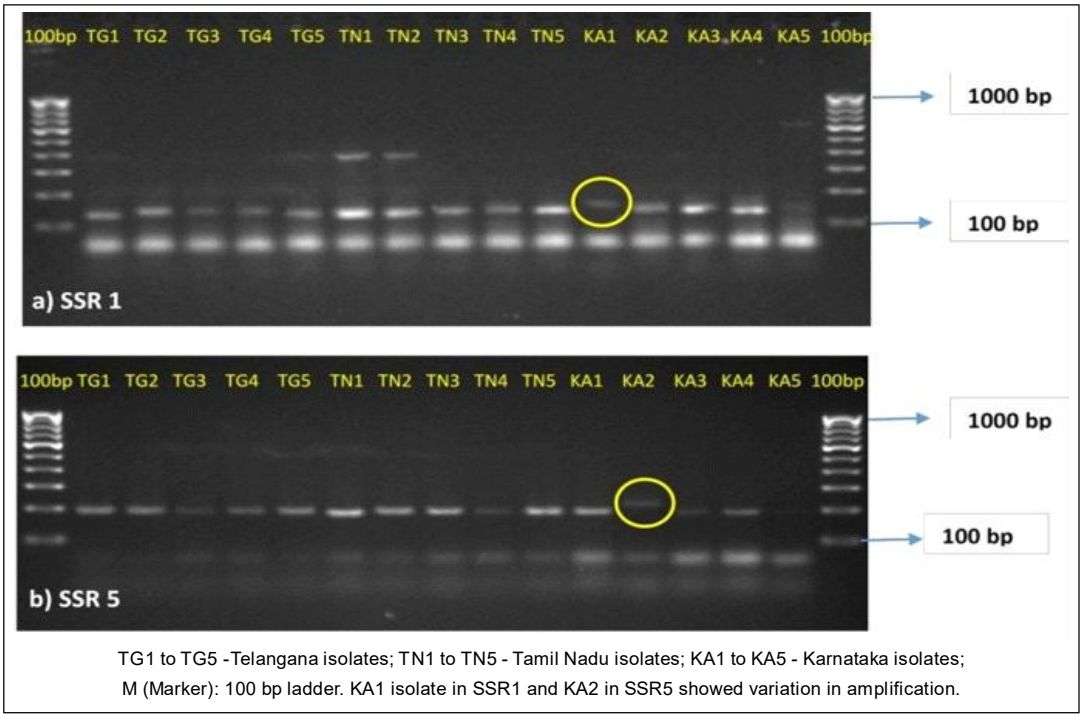
identified the 14 Alternaria isolates collected from Cotton in Southern New Mexico in USA as *A. alternata* based on ITS and plasma membrane ATPase gene sequences. Zhang *et al*. (2021) identified and differentiated two Alternaria species such as *A. solani* and *A. tomatophila* using Alt a1 and Calmodulin gene sequences.

Genetic diversity of Alternaria was studied using hyper variable SSR and ISSR primers. Thirteen SSR primers designed from sequences of *A. alternata* retrieved from NCBI were not useful for generating polymorphism among isolates except two primers. SSR1 and SSR5 primers showed variation among KA1 and KA2 isolates in PCR amplification (Fig 4). This necessitates that the SSR primers to be designed using the sequences of *A. macrospora* rather than *A. alternata.* As SSR primers are Co-dominant in nature they are highly useful for study of genetic diversity among isolates. ISSR primers generated lot of polymorphisms among the isolates and found suitable for variability study. ISSR primers such as UBC 807 and 809 (Fig 5) and UBC 834, 835 842 and 856 generated number of amplicons with various sizes.

Six different ISSR primers generated six clusters among 15 Alternaria isolates at 71 per cent genetic dissimilarity

Table 2: Sequencing of ITS region of 15 Alternaria isolates and identification through NCBI nucleotide alignment.

Isolate code	Identity through NCBI	Query coverage (%)	Per cent similarity
TG 1	*A. alternata*	90	91.81
TG 2	*A. alternata*	90	95.03
TG 3	*A. alternata*	93	93.08
TG 4	*A. alternata*	92	92.48
TG 5	*A. alternata*	79	86.65
TN 1	*A. alternata*	88	88.37
TN 2	*A. alternata*	81	83.41
TN 3	*A. alternata*	92	80.31
TN 4	*A. alternata*	82	92.19
TN 5	*A. alternata*	65	96.34
KA 1	*A. alternata*	69	92.14
KA 2	*A. alternata*	69	97.02
KA 3	*A. alternata*	68	84.33
KA 4	*A. alternata*	92	94.05
KA 5	*Sarocladium* sp.	60	92.93

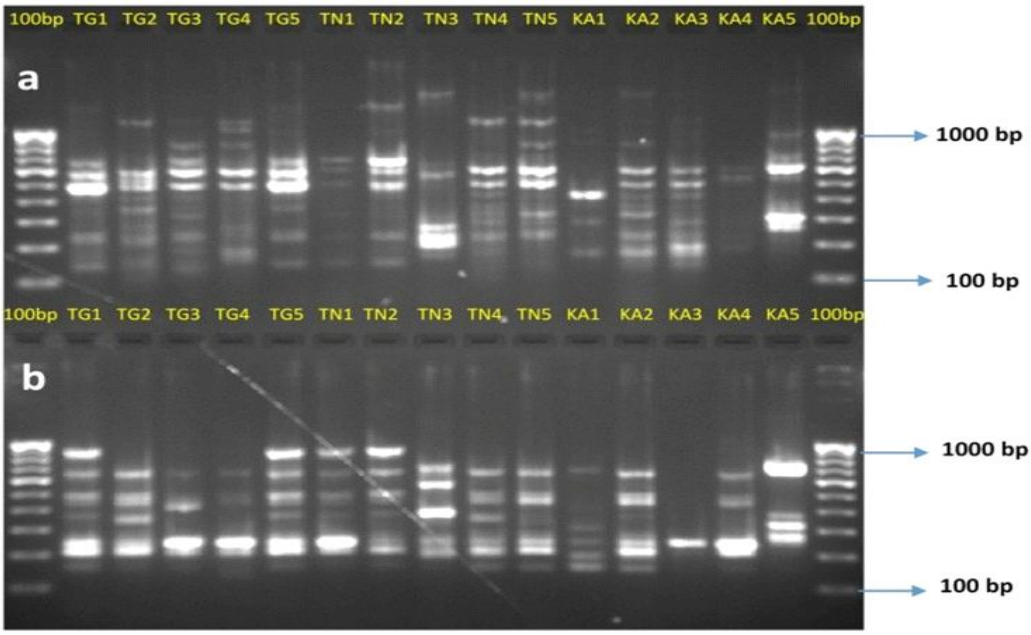


TG1 to TG5 -Telangana isolates; TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates; M (Marker): 100 bp ladder. KA1 isolate in SSR1 and KA2 in SSR5 showed variation in amplification.

Fig 4: Amplification of Alternaria isolates through a) SSR1 and b) SSR5 primers.

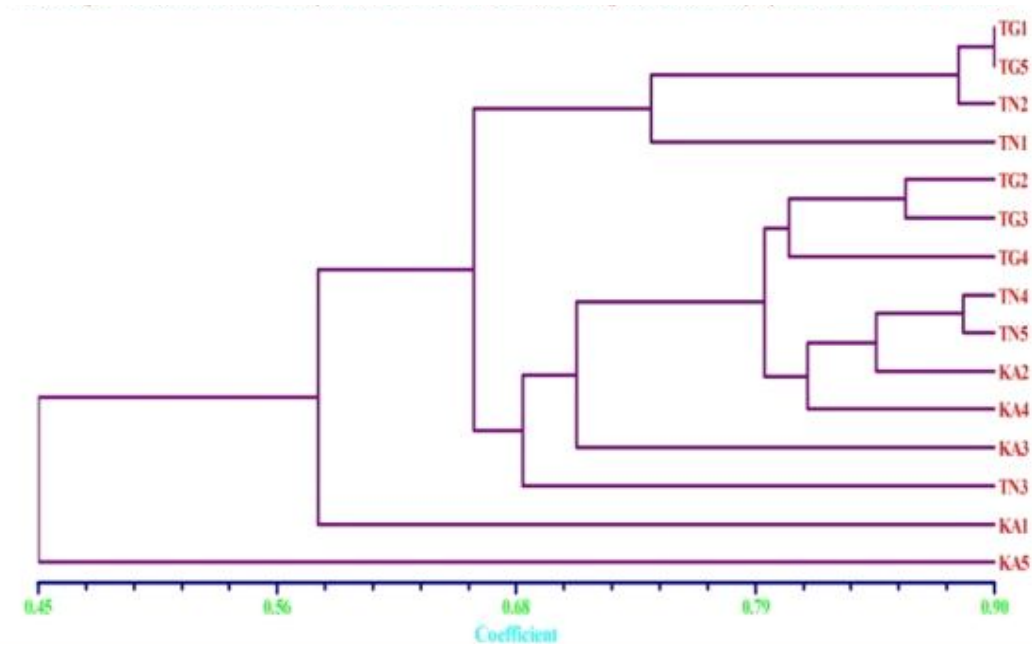
(Fig 6). Five clusters were formed in ITS sequence's diversity analysis (Fig 3). These clustering of isolates were not uniform neither on geographical location nor virulence of the isolates. Clustering was random between isolates selected in this study.

From this study, it is confirmed that ITS region is not suitable to identify and delineate the species of Alternaria. Small-spored *Alternaria* spp. identification was difficult due to lack of variation in nuclear ribosomal ITS regions (Pryor and Michailides, 2002). Multilocus phylogeny using GAPDH



TG1 to TG5 -Telangana isolates; TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates; M (Marker): 100 bp ladder.

Fig 5: Amplification of Alternaria isolates through ISSR primers a) UBC 807 and b) UBC 809 primers.



Clustering of isolates are shown. TG1 to TG5 -Telangana isolates; TN1 to TN5 - Tamil Nadu isolates; KA1 to KA5 - Karnataka isolates.

Fig 6: Dendrogram based on cluster analysis (UPGMA) of the estimate of genetic similarity by ISSR-PCR of Alternaria isolates.

and TEF1 gene regions was able to identify Alternaria isolates from potato as *A. alternata* (Dube, 2014).

CONCLUSION

Previously reported primers were found not suitable for species identification of Alternaria. Out of 13, two SSR primers found to be effective for variability among pathogen isolates. Six clusters were formed at 71 per cent genetic dissimilarity among the 15 isolates using six different ISSR primers. Five clusters were formed in ITS sequence's diversity analysis. Blasting of 15 ITS sequences at NCBI showed that all belonged to *A. alternata.* This was due to absence or presence of very few sequences of *A. macrospora* in NCBI itself. Further GAPDH (Glyceraldehyde 3- Phosphate dehydrogenase) and TEF -1 α (Translation elongation factor -1 α) and other housekeeping gene sequence analysis will be useful for confirmation of *A. macrospora* at species level.

Conflict of interest

All authors declare that they have no conflict of interest.

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