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Molecular Characterization of *Ampelomyces* spp. Using RAPD and ISSR Markers Against Okra (*Abelmoschus esculentus* (L.) Moench) Powdery Mildew

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ABSTRACT

A survey was conducted in major okra growing districts of Tamil Nadu during June 2021 to assess the incidence of powdery mildew and to collect different isolates of *A. quisqualis*. Ten isolates of *Ampelomyces* were collected and these were tentatively identified as *A. quisqualis* using specific primer pair for *A. quisqualis*. We have used 5 random amplified polymorphic DNA (RAPD) markers to estimate the genetic variation among the 10 isolates of *Ampelomyces* spp. A dendrogram was constructed based on Jaccard's similarity coefficient using the marker data from the *A. quisqualis* isolates with unweighted pair group method (UPGMA). Analysis of these polymorphisms revealed 4 distinct groups, in agreement with previous studies. The minimum similarity index was recorded at 20 per cent between TNAU-AQ104 and TNAU-AQ107 even though they were from same cluster group, thus confirming that the *Ampelomyces* isolates from entirely different geographical areas can also share some genetic relationship. The ISSR analysis based on the dendrogram results also revealed that the ten isolates showed 20 to 85 percent similarity coefficient. This result indicated the identification patterns of *Ampelomyces* spp.

Key words: *Powdery mildew,* A. quisqualis, *RAPD markers, Polymorphism, ISSR*

Introduction

Okra (*Abelmoschus esculentus* (L.) Moench) belonging to the family Malvaceae popularly known as Bhendi or Ladies finger is one of the important vegetables grown in India (Elkhalifa *et al*., 2021). It is grown for its tender green pods in the tropical, sub-tropical region and also in the warmer parts of temperate region (Dhankhar and Mishra, 2004).

Some of the major factors limiting okra production, amongst several others, include the use of locally unimproved varieties, high incidence of pest

and disease burden, a narrow genetic base of existing varieties and sub-optimal planting densities. Among these, powdery mildew caused by *Erysiphe cichoracearum* DC is a destructive disease worldwide (Eshiet and Brisibe, 2014) which causes an yield loss of 20 to 40 per cent or 17 to 86.6 per cent (Sharma *et al*., 2017; Kaur *et al*., 2019; Athira *et al*., 2017). The disease affects all stages of growth causing premature defoliation. *Erysiphe cichoracearum*, the powdery mildew pathogen of okra is causing the most widespread disease generally favoured by dry atmospheric and soil conditions (Younes *et al*., 2014).

Control of powdery mildews relies mainly on the use of chemical fungicides, yet intensive use of these can result in the accumulation of residual chemicals that are potentially hazardous to humans and the environment (Athira *et al*., 2017). In order to reduce the use of chemical pesticides, alternative methods for controlling powdery mildews have been studied, including the use of microbial biocontrol agents (Ons *et al*., 2020).

Pycnidial fungi belonging to the genus *Ampelomyces* are common intracellular mycoparasites of powdery mildews worldwide. Some strains have already been developed as commercial biocontrol agents (BCAs) of *Erysiphe necator* and other powdery mildew species infecting important crops (Pintye *et al*., 2012a; Liang *et al*., 2007).

To understand the phylogenetic relationship between the different isolates, the nuclear ribosomal DNA internal transcribed spacer (ITS) can be used. Today, DNA methods are commonly used for identification and phylogenetic classification and different PCR-based strategies have been used to characterise species and strains of different antagonistic fungi. Data produced with randomly amplified polymorphic DNA (RAPD) have been used to differentiate numerous fungi (Hermosa *et al*., 2001). Highly sensitive diagnostic assays, based on the polymerase chain reaction (PCR), have also been implemented successfully for the identification of important fungi (Bohuski *et al*., 2015). With this background, the present study was carried out envisaging the molecular variability of *Ampelomyces quisqualis* using RAPD and ISSR markers.

Materials and Methods

Survey for the incidence of powdery mildew and collection of isolates of *A. quisqualis* collected from major bhendi growing districts of Tamil Nadu. (Table 1). The method of DNA isolation, primer used and RAPD-PCR were followed as per procedure given by Thakur and Sandhu (2002). The cultures of *A. quisqualis* collected from various places maintained in potato dextrose agar slants were transferred to potato dextrose agar medium plates and incubated at room temperature for two to four days. *A. quisqualis* isolates were then transferred into 250 ml Erlenmeyer flasks containing 150 ml potato dextrose broth and were incubated at room temperature for 14 days. Mycelium was harvested by filtration through sterile filter and stored at -70 ^{0}C until used for DNA extraction. To extract the DNA,

Table 1. Survey for the incidence of powdery mildew and collection of isolates of *A. quisqualis* in major bhendi growing districts of Tamil Nadu

S. No	Place of collection	Village/Taluk	Stage of the crop	Variety/ Hybrid cultivated	Mean PDI of powdery mildew	*A. quisqualis* isolates no. assigned
1	Coimbatore	Thondamputhur	Vegetative	COBH h 1	63.45^{a}	TNAU-AQ101
2	Madurai	Devankuruchi	Pod harvest	Ganga	24.72^{f}	TNAU-AQ102
3	Dharmapuri	Papparapatty	Fruit formation	NS 801	18.53^{g}	TNAU-AQ103
4	Dindigul	Ambilikkai	Flowering	COBH h 1	54.27^{b}	TNAU-AQ104
5	Trichy	Thimampatty	Fruit formation	Sakthi	37.51^{d}	TNAU-AQ105
6	Erode	Muthampalayam	Flowering	RASI 5	43.23^{cd}	TNAU-AQ106
7	Salem	Idappadi	Vegetative	NS 531	15.54^{h}	TNAU-AQ107
8	Tirupur	Nallur	Fruit formation	COBH h 1	47.13^{c}	TNAU-AQ108
9	Theni	Kovilpatti	Flowering	OH-016	35.47^{de}	TNAU-AQ109
10	Cuddalore	Ramapuram	Fruit formation	Sakthi	30.50^{e}	TNAU-AQ110

* PDI- Percent Disease Index

* Values are means of three replications.

Means followed by a common letter are not significantly different at 5% level by DMRT

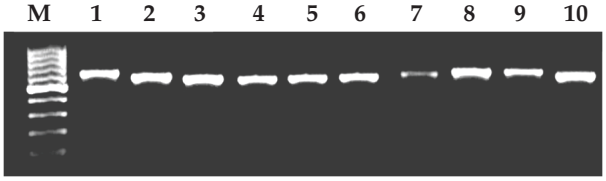
one gram of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2 per cent CTAB extraction buffer (10 mM trisbase (pH-8.0), 20mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2 per cent), mercapto ethanol (0.1 per cent) and PVP (0.2 per cent) at 65 ^{0}C for 1 h. The suspension was added with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by a centrifuge at 12,000 rpm for 5 min. The supernatant was transferred to clean tube and mixed with equal volume of ice-cold isopropanol. It was incubated at 25 ^{0}C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol. Again, incubation was given for 15 min. The pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the DNA concentration was estimated spectrophotometrically.

RAPD profiles were produced by the method of Baysal *et al*., (2008). Primers and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. Amplification reaction mixture contained total volume of 20 ml and standardized as follows for taxonomic analysis of RAPDs: 0.5 to 5 ng of template DNA (in 10 ml of H_2O), 0.6 mM primer, 50 mM each dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.5 U of Taq DNA polymerase. In each amplification reaction, a control sample without DNA was included. Samples were quickly transferred in Master cycler gradient (Eppendorf, Germany). Thermal cycling conditions were: initial denaturation at 92^{0}C for 4 min, followed by 40 denaturation cycles at 92 ^{0}C for 1 min, annealing at 37 ^{0}C for 2 min, and extension at 72 ^{0}C for 3 min. A final extension step at 72^{0}C for 3 min was also performed to ensure complete extension of the amplified products. The amplified fragments were analyzed by electrophoresis of 10 ml of the amplification reaction mixture in 1.5 per cent agarose gels run in 1 x TAE buffer.

The banding patterns were scored for RAPD and ISSR primers in each *A. quisqualis* isolate starting from the small size fragment to large sized one. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix to analyse genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) (Akshitha *et al*., 2022). A dendrogram was constructed based on Jaccard's similarity coefficient using the marker data from the *A. quisqualis* isolates with unweighted pair group method (UPGMA) (Pintye *et al*., 2012b)

Results and Discussion

PCR amplification of ITS region in the ten isolates of *Ampleomyces* spp. was performed using the universal primers of forward ITS1 and reverse ITS4. (Kanipriya *et al*., 2019). The *Ampelomyces* genus was amplified as a fragment of 560 bp corresponding to the region of the 18s-28s rDNA intervening sequence for *Ampleomyces* spp (Marvaldi *et al*., 2009). All the ten isolates were tentatively identified as *Ampelomyces* using molecular characterization have amplified an amplicon of 560 bp in PCR analysis and they were identified as *Ampelomyces* spp (Fig.1). The results are in accordance with (Narayanasamy and narayanasamy, 2013) who reported that the size of the PCR product of *A. quisqualis* varied from 500-600 bp. (Badotti *et al*., 2017) reported that ITS regions have been used successfully to identify and differentiate closely related fungal species. The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi (Ghosh *et al*., 2007).



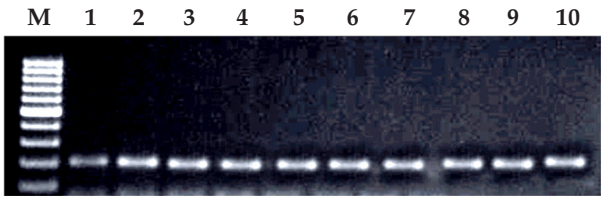
M. 100 bp Marker

1.	TNAU-AQ101 (Coimbatore)	6.	TNAU-AQ106 (Erode)
2.	TNAU-AQ102 (Madurai)	7.	TNAU-AQ107 (Salem)
3.	TNAU-AQ103 (Dharmapuri)	8.	TNAU-AQ108 (Tirupur)
4.	TNAU-AQ104 (Dindigul)	9.	TNAU-AQ109 (Theni)
5.	TNAU-AQ105 (Trichy)	10.	TNAU-AQ110 (Cuddalore)

Fig. 1. PCR amplification of ITS region

Further, with specific primer of *A. quisqualis,* the isolates were subjected to PCR and it was amplified at 200 bp respectively. Finally, all the ten isolates were tentatively identified as *A. quisqualis* using specific primer pair for *A. quisqualis* (Fig. 2). The results are in accordance with the previous study conducted by (Kanipriya *et al*., 2019).

In the RAPD analysis the DNA fragments of different isolates of *A. quisqualis* were amplified using RAPD primers (Avis *et al*., 2001). Each RAPD pattern was compared with other patterns and Euclidean distance matrix was calculated for *A. quisqualis*.



M. 100 bp Marker
1. TNAU-AQ101 (Coimbatore)
2. TNAU-AQ102 (Madurai)
3. TNAU-AQ103 (Dharmapuri)
4. TNAU-AQ104 (Dindigul)
5. TNAU-AQ105 (Trichy)
6. TNAU-AQ106 (Erode)
7. TNAU-AQ107 (Salem)
8. TNAU-AQ108 (Tirupur)
9. TNAU-AQ109 (Theni)
10. TNAU-AQ110 (Cuddalore)

Fig. 2. PCR amplification using specific primer pair for *A. quisqualis*

The relationship between the isolates examined was represented by a dendrogram by using UPGMA. (Fig. 3). The isolates TNAU-AQ106 and TNAU-AQ108 showed similar RAPD pattern (85 per cent) and also isolate TNAU-AQ110 shared greater similarity (69.5 per cent) with the isolate TNAU-AQ108 in RAPD pattern even they were from different locations. While the isolate TNAU-AQ104 and TNAU-AQ108 having the 65 per cent similarity index and the minimum similarity index was recorded at 20 per cent between TNAU-AQ104 and TNAU-AQ107 even though they were from same cluster group, thus confirming that the *Ampelomyces* isolates from entirely different geographical areas can also share some genetic relationship (Table 2).

In the ISSR analysis the DNA fragments of different isolates of *A. quisqualis* were amplified using ISSR primers. Each ISSR pattern was compared with other patterns and Euclidean distance matrix was calculated for *A. quisqualis*. The relationship between the isolates examined was represented by a dendrogram by using UPGMA. (Fig. 4). The isolates TNAU-AQ108 and TNAU-AQ110 showed similar ISSR pattern (85 per cent) and also isolate TNAU-

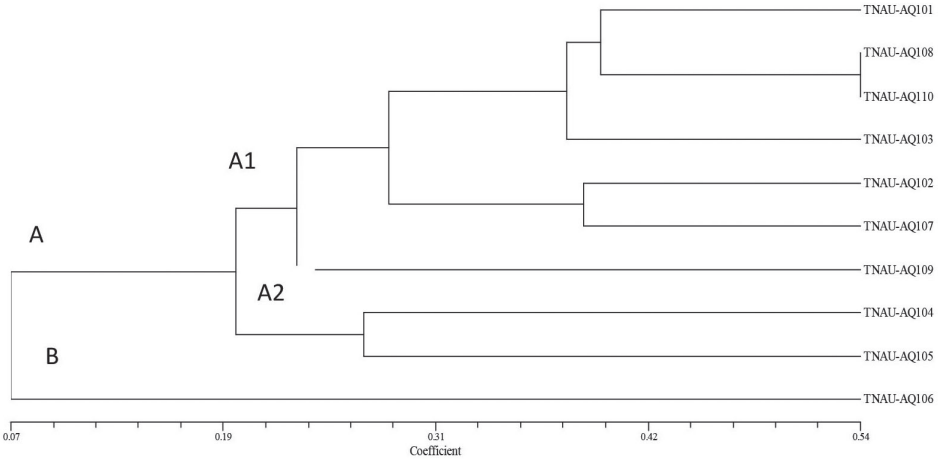


Fig. 3. Dendrogram constructed with UPGMA clustering method among 10 isolates of *A. quisqualis* isolated from different regions of bhendi growing areas in Tamil Nadu using RAPD primers

Table 2. Similarity matrix of A. quisqualis isolated from different ecosystems of Tamil Nadu generated through RAPD primers

Isolates	TNAU-AQ101	TNAU-AQ102	TNAU-AQ103	TNAU-AQ104	TNAU-AQ105	TNAU-AQ106	TNAU-AQ107	TNAU-AQ107	TNAU-AQ108	TNAU-AQ109	TNAU-AQ110
TNAU-AQ101	1.00										
TNAU-AQ102	0.22	1.00									
TNAU-AQ103	0.25	0.18	1.00								
TNAU-AQ104	0.27	0.20	0.29	1.00							
TNAU-AQ105	0.25	0.37	0.26	0.28	1.00						
TNAU-AQ106	0.31	0.20	0.33	0.36	0.17	1.00					
TNAU-AQ107	0.23	0.21	0.25	0.12	0.25	0.14	1.00				
TNAU-AQ108	0.31	0.24	0.21	0.30	0.26	0.52	0.26	1.00			
TNAU-AQ109	0.33	0.24	0.21	0.31	0.33	0.28	0.18	0.42	1.00		
TNAU-AQ110	0.39	0.344	0.19	0.45	0.33	0.40	0.12	0.34	0.30	1.00	000

Table 3. Similarity matrix of *A. quisqualis* isolated from different ecosystems of Tamil Nadu generated through ISSR primers

Isolates	TNAU-AQ101	TNAU-AQ102	TNAU-AQ103	TNAU-AQ104	TNAU-AQ105	TNAU-AQ106	TNAU-AQ107	TNAU-AQ107	TNAU-AQ108	TNAU-AQ109	TNAU-AQ110
TNAU-AQ101	1.00										
TNAU-AQ102	0.26	1.00									
TNAU-AQ103	0.39	0.25	1.00								
TNAU-AQ104	0.20	0.19	0.25	1.00							
TNAU-AQ105	0.17	0.17	0.18	0.28	1.00						
TNAU-AQ106	0.10	0.03	0.16	0.05	0.06	1.00					
TNAU-AQ107	0.34	0.46	0.27	0.31	0.13	0.08	1.00				
TNAU-AQ108	0.42	0.23	0.38	0.27	0.20	0.04	0.29	1.00			
TNAU-AQ109	0.20	0.29	0.30	0.22	0.20	0.05	0.26	0.21	1.00		
TNAU-AQ110	0.39	0.21	0.35	0.25	0.13	0.12	0.37	0.56	0.25	1.00	000

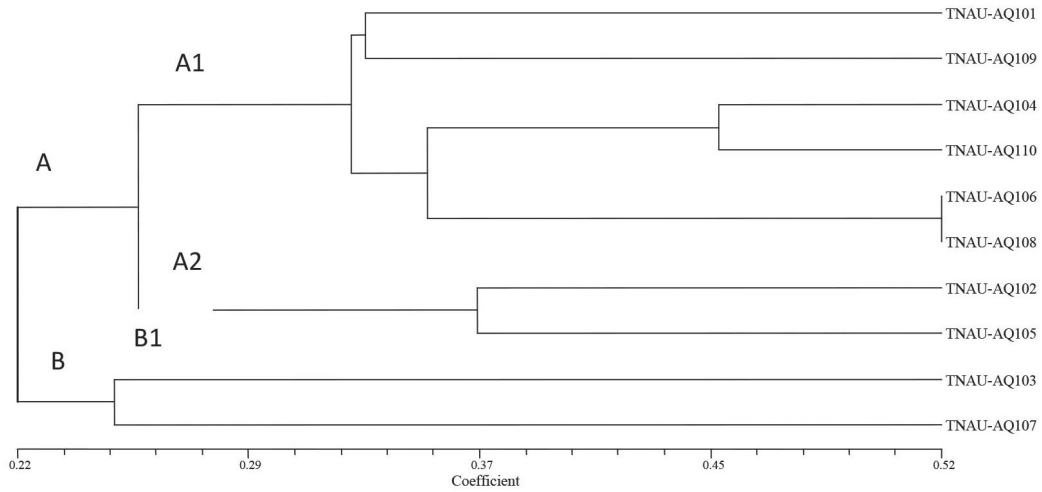


Fig. 4. Dendrogram constructed with UPGMA clustering method among 10 isolates of *A. quisqualis* isolated from different regions of bhendi growing areas in Tamil Nadu using ISSR primers

AQ101 shared greater similarity (69.5 per cent) with the isolate TNAU-AQ110 in ISSR pattern even they were from different locations. While the isolate TNAU-AQ101 and TNAU-AQ110 having the 65 per cent similarity index and the minimum similarity index was recorded at 20 percent between TNAU-AQ101 and TNAU-AQ106 even though they were from same cluster group, thus confirming that the *A. quisqualis* isolates from entirely different geographical areas can also share some genetic relationship (Table 3).

Conclusion

In the present study, the isolation of DNA from ten isolates of *A. quisqualis* were done from major okra growing districts in Tamil Nadu. PCR based detection was done using the universal primers of forward ITS1 (5′CTTGGTCA TTTAGGAAGTAA-3′) and reverse ITS 4 (5′TCCTCCGTTATTG ATATGC-3′). The *Ampelomyces* genus was amplified as a fragment of 560 bp. So, the PCR amplification has confirmed that all the isolates were *Ampelomyces* spp. ITS regions have been used successfully to identify and differentiate closely related fungal species. The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi. Further, with specific primer of *A. quisqualis*, the isolates were subjected to PCR and it was amplified at 200 bp respectively. This work was supplemented with the selection of nine inter-simple sequence re-

peat (ISSR) markers for strain-specific identification of *Ampelomyces* mycoparasites to monitor the environmental fate of strains applied as BCAs. Since molecular study may reveal any variation that exists among the isolates in support of the variation in physiological properties. The genetic distances among strains calculated based on ISSR patterns have also highlighted the genetic diversity of *Ampelomyces* mycoparasites naturally occurring in okra powdery mildew.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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