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Molecular variability in *Pythium aphanidermatum* causing rhizome rot of ginger

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Abstract
Ginger crop is one of the most important spice crops cultivated in India. Which suffers from several diseases, among which rhizome rot complex caused by *P. aphanidermatum, F. oxysporum* f. sp. *zingiberi, S. rolfsii, R. solanacearum* and *M. arenaria* is one of the serious diseases observed regularly in ginger growing areas. As major pathogen for rhizome rot is *P. aphanidermatum*, on that basis molecular studies were conducted. Molecular variability of eight isolates of *P. aphanidermatum* was studied applying STR PCR, using 14 primers *viz.* P18CCA1-41, P18TGA2-9, P18TTC3-29, P18CA2-23, P18GAA1-71, P18TCC3-25, P18GAA1-72, P18GAA3-34, P18GT4-20, P18CAT1-74, P18TTG1-79, P18ACA2-81, P18AC2-18, and P18TTC1-42. The dendrogram analysis revealed phylogenetic relationship of pathogen in two clusters. In the Cluster I, four isolates of *P. aphanidermatum viz.*, Iso-2, Iso-29, Iso-7 and Iso-36 found. Cluster II comprised of four isolates *viz.*, Iso-14, Iso-17, Iso-32 Iso-33.

Keywords: Rhizome rot, *Pythium aphanidermatum*, PCR, STR (SSR), dendrogram, primers

Introduction
Ginger is one of the most important and most widely used spice crop grown in the world. It has spread in most of the tropical and subtropical parts of China-India region, due to its universal appeal. Ginger botanically known as *Zingiber officinale* belongs to the family Zingiberaceae. It is a tropical horticultural plant, valued all over the world as an unparallel spice in culinary preparations and for its medicinal properties. It is an herbaceous perennial, rhizomatous spice erect, with many fibrous roots, aerial shoots (pseudostem) with leaves, and underground stem (rhizome). India is considered as “home of spices” as out of 80 types of spices grown in world about 50 types are grown in India (Rathore and Shekhawat, 2008) [12].
Ancient Indians considered ginger as the "mahashoudha" (the great medicine). One of the most important value-added products to emerge is "Ginger Tea". Brought out by the Tata group (Zachariah *et al*., 1993; Nair, 2013) [18, 11].
India is the largest producer of ginger in the world accounting for about one-third of the total world output, followed by Thailand and Japan (Kadam *et al*., 2015) [7]. India, China, Nepal, and Thailand are the major producers of ginger in the world. India and Indonesia have the largest area under cultivation. The area under this crop in India was 174 thousand ha. with 1846.0 thousand MT production and productivity of 10.60 MT/ha, where the total area under ginger crop in Maharashtra state was 9.45 thousand ha with 140.60 thousand MT production and productivity of 14.87 MT/ha (Anonymous, 2019) [1].
Major ginger-growing states in India are Kerala, Sikkim, Meghalaya, West Bengal, Orissa, Tamil Nadu, Karnataka, Andhra Pradesh. Maharashtra and Himachal Pradesh (Shukla and Gupta 2015) [15].
In some parts of Maharashtra due to favourable environmental condition and soil this crop has become backbone of farmers income. Particularly in Satara, Aurangabad districts and some pockets of other districts. In Marathwada, the major area under ginger cultivation is mainly concentrated in the Aurangabad district and its adjacent parts (Anonymous, 2020) [1].
Ginger crop suffers from a wide variety of diseases caused by fungi, bacteria and nematode. They are Soft rot (*Pythium aphanidermatum, Pythium gracile, Pythium myriotylum, Pythium ultimum*), Yellows (*Fusarium oxysporum* f. sp. *zingiberi. Fusarium moniliforme*), Leaf spots (*Phyllosticta zingiberi*), Leaf blight/Dry rot (*Rhizoctonia solani*, *Rhizoctonia bataticola*), Basal rot (*Sclerotium rolfsii*), Thread blight (*Pellicularia filamentosa*), Sheath rot (*Fusarium* spp.), Bacterial wilt *(Ralstonia solanacearum*), Viral diseases like Cucumber mosaic virus (CMV) and Nematodes (*Meloidogyne* spp.).

Amongst the diseases the rhizome rot is a serious disease of ginger causing considerable economic losses to growers in different countries. In India, the rhizome rot is also considered as important disease and is prevalent in most of the ginger-growing areas. This disease may cause losses to the extent of 50-90 per cent and it is a major constraint in ginger cultivation. The term rhizome rot is generally used for all disease affecting the rhizome in respective of pathogens involved, since the ultimate result is partial or total loss of rhizome. The pathogens involved decide the nature of damage and exhibition of symptoms. The major disease and pathogens responsible for rhizome rot are soft rot caused by *Pythium* spp. yellows caused by *Fusarium* spp. sclerotium rot caused by *Sclerotiton rolfsii*, bacterial will caused by *Ralstonia solanacearum* and nematode caused by *Meloidogyne* spp. The individual pathogen infection may also lead to cause rhizome rot and in combination also (Kulkarni, 2011; Debnath *et al*., 2010; Dohroo, 2005, Sagar *et al*., 2008) [8, 3, 5, 14].

Considering the importance of crop in region the current studies to explore the exact cause, to formulate the strategy to manage the disease the current studies was undertaken.

Material and Methods

Molecular variability among eight isolates of *P. aphanidermatum* was analysed by using STR (Short Tandem Repeats) the detailed protocols of isolation of DNA and gel electrophoresis are given below.

Isolation genomic DNA

The genomic DNA of eight test isolates of *P. aphanidermatum* were isolated, separately by using standard 2% Cetyltrimethyl ammonium bromide (CTAB) extraction method. The broth cultures of isolates grown on potato dextrose were used for isolation of genomic DNA, 250 ml capacity conical flasks filled with Potato dextrose broth (100 ml/flask) were inoculated with isolates to generate the mycelium. Mycelial mat of the test isolate was ground (0.5 g mycelium) with white glass wool, using sterile mortar and pestle.

About 500 mg of lyophilized fungal material was ground in liquid nitrogen, dispersed in 800 µl of 2% CTAB extraction buffer at 65 ºC in water bath for 45 min. An equal volume of Phenol: Chloroform: Iso-amyl alcohol (25:24:1 v/v) was added, mixed well and centrifuged (7000 rpm, 10 min). The upper aqueous phase was transferred to a fresh tube and added equal volume of Chloroform: Iso-amyl alcohol (24: 1), mixed well and centrifuged (1000 rpm, 10 min.). Further, nucleic acids were precipitated by adding 0.6 volume of ice-cold isopropanol and collected by centrifugation (12000 rpm for 30 min.). Pellet was washed twice with 70% ethanol, air dried and solubilised in 200 µl TE buffer (10 mm Tris-HCL, 1 mm EDTA, pH 8.0). Further RNA contamination was removed by RNase treatment (Fermentas U.K.) for one hour at 37 ºC. The extracted DNA was resolved on 0.8% agarose gel, quantified by spectrophotometer and stored at -20 ºC for further use.

Quantification of DNA

Spectrophotometer was used for quantitative and qualitative analysis of the DNA of the test isolates. Five µl of DNA sample was added in Cuvette carrying 0.995 µl of sterile H_2O and absorbance was measured at 280 nm wavelengths. Similarly, the purity of DNA was checked by measuring the ratio of OD at A260/A280 nm. The quantification of DNA was calculated by using following formula.

$$\text{DNA } (\mu g/\mu l) = \frac{\text{OD at 260 nm X dilution factor X 50}}{1000}$$

STR analysis of *P. aphanidermatum* isolates

The PCR protocol for STR reaction was optimized with various PCR components and thermal cycler program as detailed below.

Table A: PCR Components used for molecular characterization study of *P. aphanidermatum* isolates

Sr. No.	PCR Components	Required Concentration	Volume/ reaction
1.	PCR Buffer	1 X	2.5 µl
2.	$Mgcl_2$	1.5 mM	1.5 µl
3.	dNTP mix	200 µm	0.2 µl
4.	Random Primers (Kit-Aoperontech)	0.4 pm	1 ml
5.	*Taq* DNA Polymerase	1.5 U	0.3 µl
6.	Template DNA	25 ng	1 µl
7.	Nuclease free water	-	18 µl
-	Total	-	25.0 µl

Master mix (25 µl) containing all of the above reactants, except template DNA were dispensed in autoclaved PCR tubes (0.2 ml). Genomic DNA of each isolate of *P. aphanidermatum* was added to the individual tubes containing the master mix. The content of each tube was mixed by tapping with fingers, followed by a brief spun to collect the content at bottom of the tube. These tubes were placed in Thermocycler (Bio Rad, USA) and subjected to PCR according to the standardized protocol given below.

Standardized PCR protocols for amplification of DNA

A PCR master mix in sterile distilled water with all of the above-mentioned compounds in required quantities were prepared and amplifications were done through thermal cycler, using following PCR conditions.

Table B: PCR protocols

Sr. No.	Steps	Temperature		Time
1.	Initial Denaturation	94 ºC		4min
2.	Denaturation	94 ºC	40 cycles	1min
3.	Annealing	37 ºC		1min
4.	Primer Extension	72 ºC		2min
5.	Final Extension	72 ºC		10min
6.	Final hold	4 ºC		Forever

The amplified STR product was separated by electrophoresis in 3% agarose gel with 1 X TAE buffer, stained with ethidium bromide (0.5 µg/ml) at 90 V for 1.0 to 1.5 hrs. and photographed using gel documentation system (Alpha Innotech, USA). The sizes of the amplification product were estimated using 100 bp to 1 kb ladder (Fermentas, UK). The polymorphism was detected by comparing STR product of the test isolates of *P. aphanidermatum*.

The amplified products generated from STR-PCR reaction were resolved on 3% agarose gel. The STR amplicons showing monomorphic and polymorphic pattern were scored and amplicon size was determined by comparison with 1 kb DNA ladder (Fermentas, U.K.).

STR fingerprint data was scored in larger allele (A), smaller

allele (B), mixed one (AB) and no allele (0) forms, data matrices were generated and used to plot dendrogram exploited for phylogenetic analysis, by using Jacquards' similarity coefficient, using the software NTSYS pc2.02i.

Results and Discussion

It was difficult to distinguish these species using traditional morphological differences. The suitability of Simple Tandem Repeats (STR) was used to detect the variations among the isolates of *P. aphanidermatum* (Table 1, Plate 1, 2, 3, 4, 5, 6, 7 and 8 and Fig. 1).

The PCR amplification

The PCR amplification reaction was optimized by varying concentration of PCR components. Amplification reaction was carried out in 25 µl reaction mixtures containing 25 ng of fungal genomic DNA, 1X PCR buffer, 1.5 mm $MgCl_2$, 0.25 mm dNTPs, 10 pmol primers and 1.50U of *Taq* DNA polymerase. PCR amplification was performed in master cycler gradient, Eppendorf PCR thermocycler and the program consisted of an initial denaturing at 94 ºC for 4 min, followed by 39 cycles comprising denaturation at 94 ºC, 1 min, annealing at 37 ºC and extension of 2 min. at 72 ºC. The final extension was set at 72 ºC for 10 min. PCR amplified product was separated by electrophoresis on 3% agarose gel in 1X TAE buffer, stained with Ethidium bromide and visualized under gel documentation system. dNTPs, 10 pmol primers and 1.50 U of *Taq* DNA polymerase. PCR amplification was performed in master cycler gradient, Eppendorf PCR thermocycler, and the program consisted of an initial denaturing at 94 ºC for 4 min, followed by 39 cycles comprising denaturation at 94 ºC, 1 min, annealing at 37 ºC and extension of 2 min. at 72 ºC. The final extension was set at 72 ºC for 10 min. PCR amplified product was separated by electrophoresis on 3% agarose gel in 1X TAE buffer, stained with Ethidium bromide and visualized under gel documentation system.

Table 1: List of STRs primers and alleleic data from gel images of *Pythium aphanidermatum*

Primers	Iso-2	Iso-7	Iso-14	Iso-17	Iso-29	Iso-32	Iso-33	Iso-36
P18CCA1-41	O	O	O	A	O	O	O	O
P18TGA2-9	A	B	B	A	O	O	O	B
P18TTC3-29	A	O	A	O	O	O	O	A
P18CA2-23	O	O	O	O	O	O	O	O
P18GAA1-71	O	A	A	O	O	O	O	A
P18TCC3-25	A	A	A	O	O	O	O	B
P18GAA1-72	O	O	O	O	O	O	O	O
P18GAA3-34	A	A	B	O	A	O	O	A
P18GT4-20	B	A	A	O	O	A	B	O
P18CAT1-74	O	O	O	O	O	O	O	O
P18TTG1-79	O	O	O	O	O	O	O	O
P18ACA2-81	A	AB	AB	AB	O	AB	O	AB
P18AC2-18	A	O	B	A	O	A	O	O
P18TTC1-42	A	B	AB	AB	O	AB	A	AB

*Larger allele (A), Smaller allele (B), Mixed one (AB) and No allele (0)

Alleleic data generated from gel images of *P. aphanidermatum* could be grouped as under. In Iso-2 larger allele (A) were found with P18TGA2-9, P18TTC3-29, P18TCC3-25, P18GAA3-34, P18ACA2-81, P18AC2-18 and P18TTC1-42 markers, smaller allele (B) were found with only P18GT4-20 and remaining markers showed no amplification (O), none of marker showed mixed allele product (AB). Iso-7 showed larger alleles (A) with P18GAA1-71, P18TCC3-25, P18GAA3-34 and P18GT4-20 markers, smaller alleles (B) with P18TGA2-9 and P18TTC1-42, mixed allele showed (AB) with P18ACA2-81 marker, remaining marker showed no amplification. In Iso-14, larger alleles were found with P18TTC3-29, P18GAA1-71 and P18TCC3-25 markers, smaller alleles (B) with P18TGA2-9, P18GAA3-34 and P18AC2-18 markers. Only two markers showed mixed allele product *i.e.* P18ACA2-81 and P18TTC1-42, remaining marker showed no amplification.

Similarly, in Iso-17 all markers showed no amplification except P18AC2-18, P18CCA1-41 and P18TGA2-9 (larger alleles), P18ACA2-81 and P18TTC1-42 (mixed alleles). None of the markers showed amplification except P18GAA3-34 (larger allele) in Iso-29. Marker P18GT4-20 and P18AC2-18 showed larger alleles (A) and P18ACA2-81 and P18TTC1-42 showed smaller alleles (B), remaining marker was not amplified in Iso-32. Similarly, only two markers showed amplification *i.e.* P18GT4-20 (smaller allele) and P18TTC1-42 (larger allele) in Iso-33. In Iso-36 markers P18TTC3-29, P18GAA1-71 and P18GAA3-34 showed larger alleles (A), whereas P18TGA2-9 and P18TCC3-25 showed smaller alleles (B) and P18ACA2-81 and P18TTC1-42 showed mixed allele product (AB), remaining markers showed no amplification (O).

Diversity analysis using STR marker

The genomic DNA of 8 isolates of *P. aphanidermatum* isolated from rhizome rot diseased specimens of ginger was subjected for PCR amplification by using STR primers. Initially 14 STR primer *viz.*, P18CCA1-41, P18TGA2-9, P18TTC3-29, P18CA2-23, P18GAA1-71, P18TCC3-25, P18GAA1-72, P18GAA3-34, P18GT4-20, P18CAT1-74, P18TTG1-79, P18ACA2-81, P18AC2-18 and P18TTC1-42. Among these, 9.

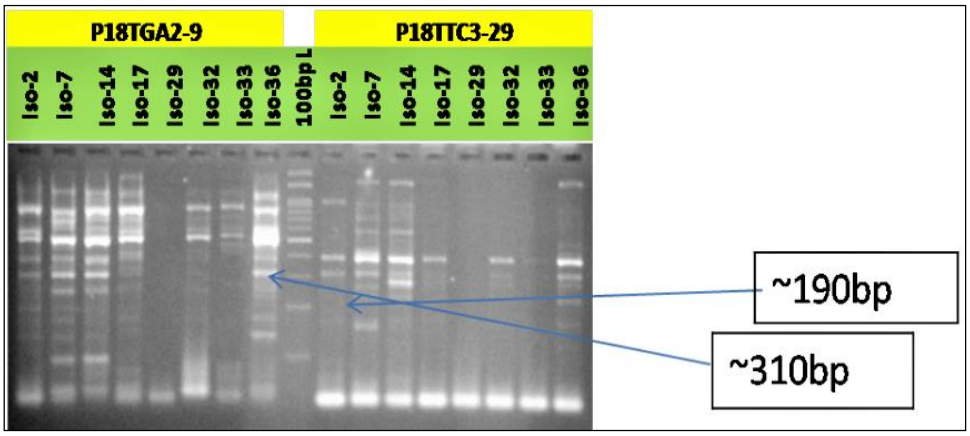

Plate 1: Primer P18TGA2-9 and P18TTC3-29

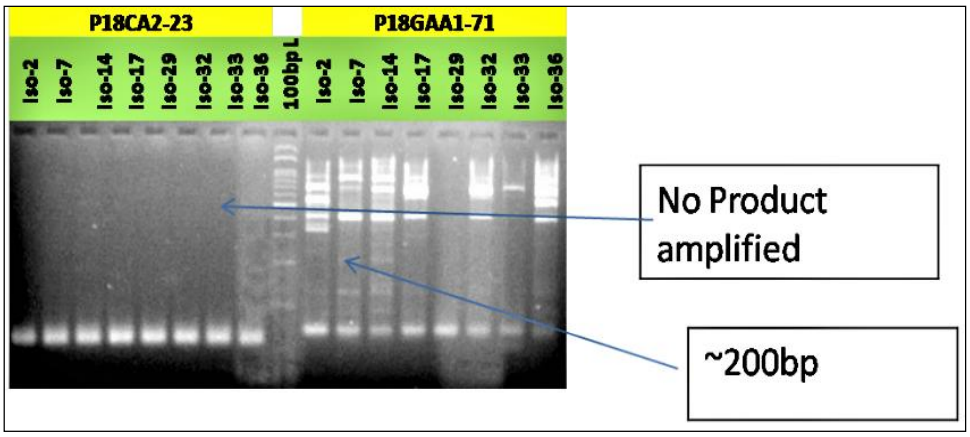

Plate 2: Primer P18CA2-23 and P18GAA1-71

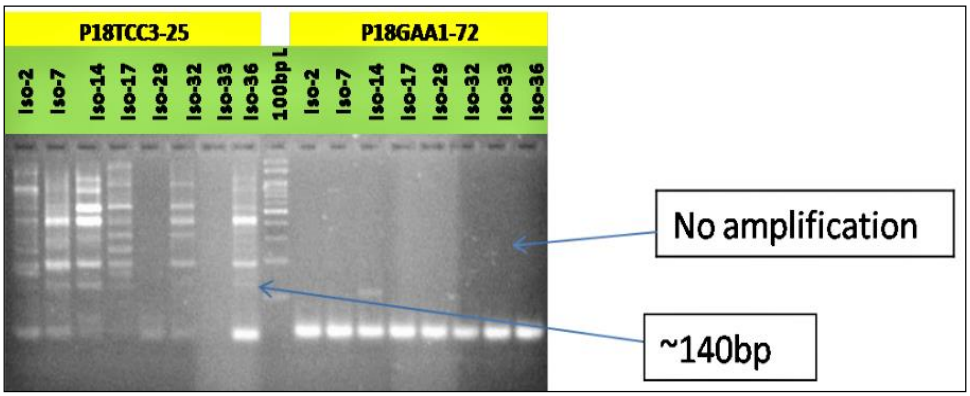

Plate 3: Primer P18TCC3-25 and P18GAA1-72

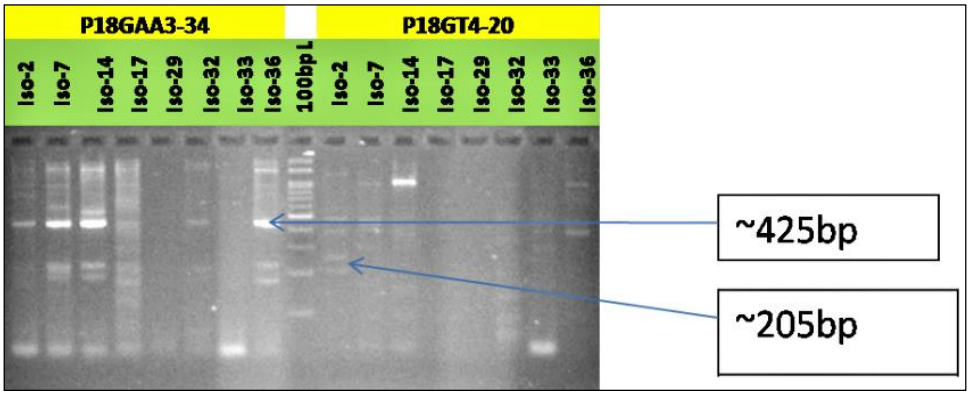

Plate 4: Primer P18GAA3-34 and P18GT4-20

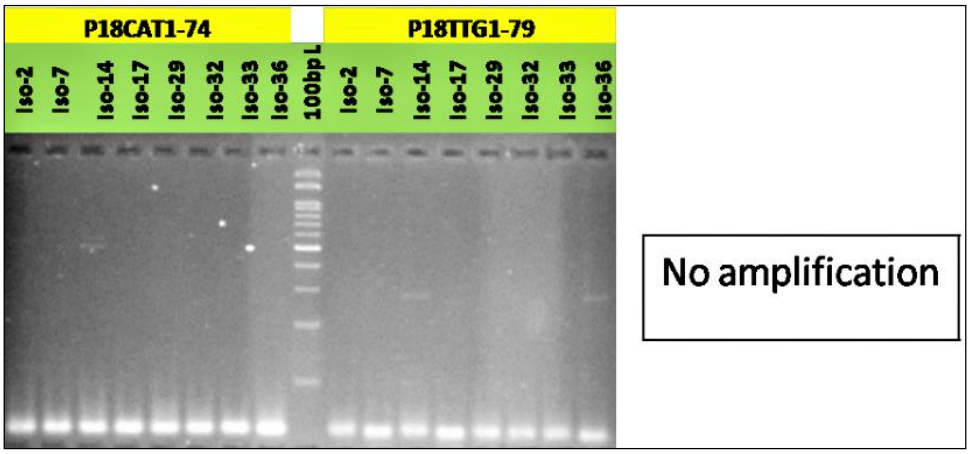

Plate 5: Primer P18CAT1-74 and P18TTG1-79

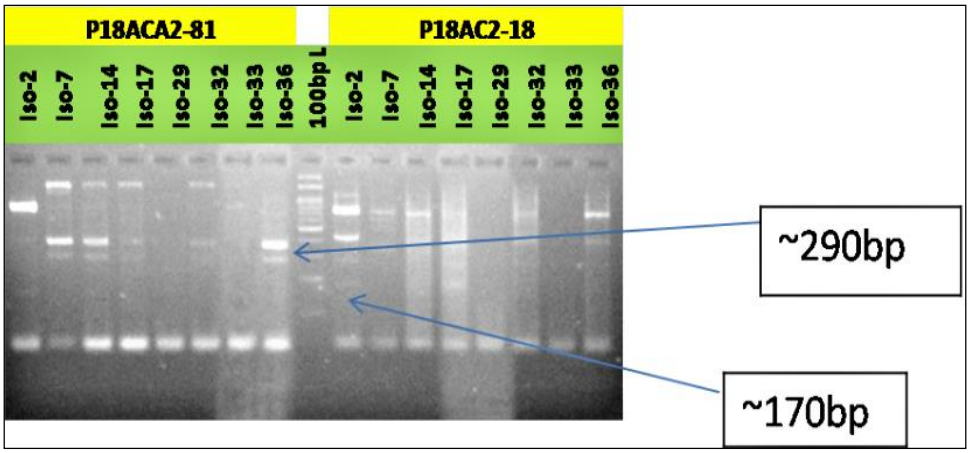

Plate 6: Primer P18ACA2-81 and P18AC2-18

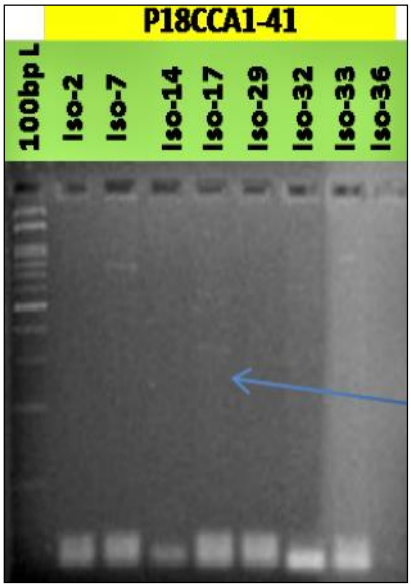

Plate 7: Primer P18CCA1-41

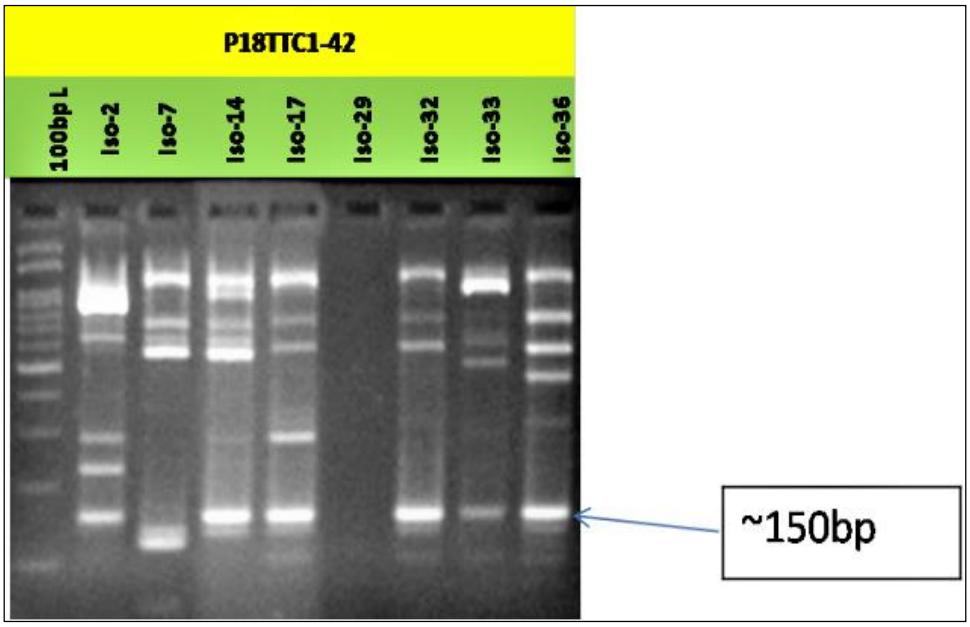

Plate 8: Primer P18TTC1-42

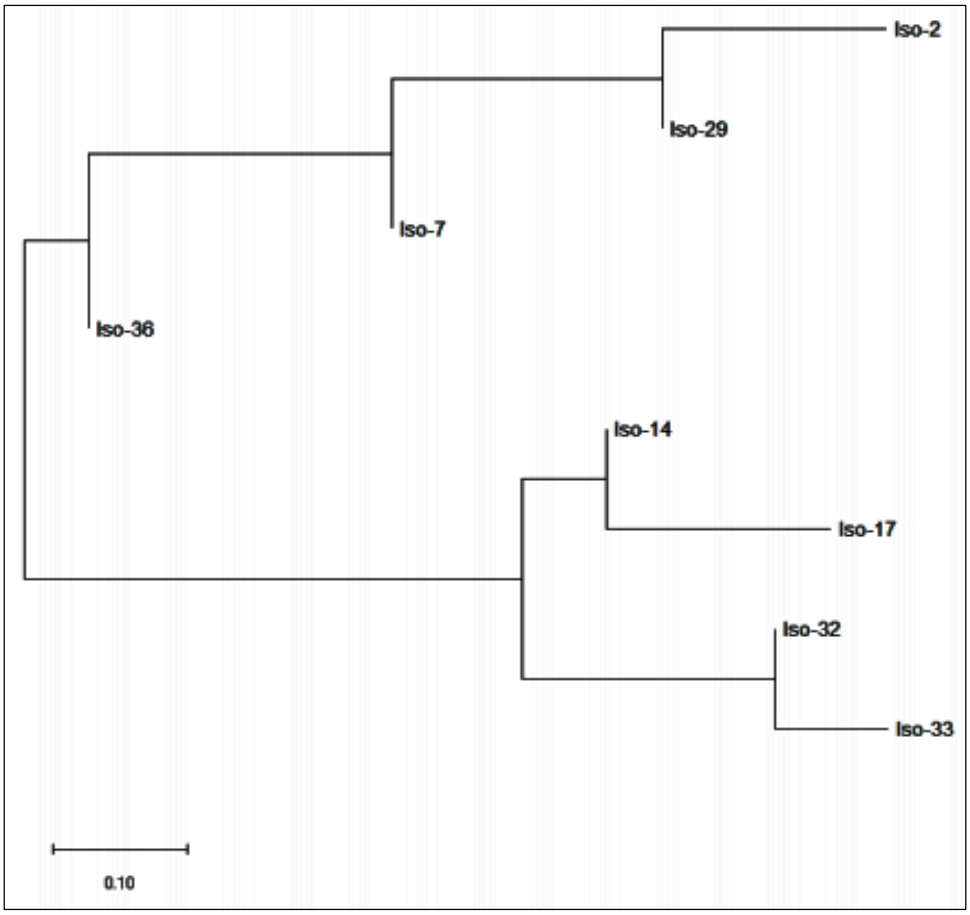

Fig 1: Phylogenetic tree image for *P. aphanidermatum*

primers produced large number of reproducible amplicons, which were employed for molecular characterization of the 8 test isolates.

Randomly selected 8 isolates of *P. aphanidermatum* obtained from the districts *viz.*, Sorjan (Parbhani) (Iso-2), Hiwara (Hingoli) (Iso-7), Pankanergaon (Nanded) (Iso-14), Tapargaon (Aurangabad) (Iso-17), Dhasla (Jalna) (Iso-29), Satephal (Beed) (Iso-32), Bavi (Osmanabad) (Iso-33) and Chakur (Latur) (Iso-36) characterized at molecular level by using 14 primers. Results (PLATE X) indicated that among the primers tested, *viz.*, P18TGA2-9, P18TTC3-29, P18GAA1-71, P18TCC3-25, P18GAA3-34, P18GT4-20, P18ACA2-81 and P18AC2-18 were found polymorphic bands, which generated significant data for discrimination of the test isolates. The average size of amplicons generated by the test primers was ranged between 140 bp to 425 bp.

Cluster analysis of STR DNA fingerprint

Dendrogram generated based on analysis of STR data grouped all the eight test isolates of *P. aphanidermatum* into two major clusters (Fig. 1.). These clusters were formed on the basis of genus as well as regional level. In the Cluster I, maximum four isolates of *P. aphanidermatum* from Sorjan (Parbhani) (Iso-2), Dhasla (Jalna) (Iso-29), Hiwara (Hingoli) (Iso-7) and Chakur (Latur) (Iso-36) districts of Maharashtra state. Cluster II comprised of four isolates from Pankanergaon (Nanded) (Iso-14), Tapargaon (Aurangabad) (Iso-17), Satephal (Beed) (Iso-32) and Bavi (Osmanabad) (Iso-33) districts of Maharashtra state.

Genetic/molecular diversity and polymorphism among *Pythium* spp. by employing SSR (STR) markers was reported earlier by many workers. Vasseur *et al.* (2005) [16] obtained polymorphism by using Inter Simple Sequence Repeat (ISSR)-PCR method in which two primers (CAC) 5 and (CCA) 5 detected polymorphism, and isolates were classified among 11 molecular clusters. Lee and Moorman (2008) [9] studied six simple sequence repeat (SSR)-enriched genome libraries from *Pythium aphanidermatum*, *P. irregulare* and *P. cryptoirregulare* constructed to develop SSR markers. They reported Analysis of isolates from each *Pythium* species using SSR markers showed the high degree of gene diversity and polymorphic information content (PIC) value in the three species.

Similar results of genetic variability among isolates of *Pythium* spp. were reported by several workers (Rosso *et al.*, 2008; Lee *et al.*, 2010; Weiland *et al.*, 2015; Huzar-Novakowiski and Dorrance, 2018 and Del Castillo Munera *et al.*, 2019) [8, 10, 15, 6, 4].

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