rDNA Sequence and Phylogenetic Analysis of *Macrophomina phaseolina,* Root Rot Pathogen of *Citrus reticulata* (Blanco)

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Abstract : The loose-skinned mandarin orange (*Citrus reticulata* Blanco) is one of the most economically important and popular fruits in the world, constituting about 41% of the total citrus fruits produced in India. Recently there have been severe signs of citrus decline caused by *Macrophomina phaseolina* resulting in decreased fruit production in orchards of Darjeeling. In the present study, initially, a fungus was isolated from diseased roots of *Citrus reticulata* which was morphologically identified as *Macrophomina phaseolina.* Genomic DNA of *M. phaseolina* isolated from mandarin rhizosphere was purified and PCR amplification of 18S rDNA was done using genus specific ITS-1 and ITS4 primers. Amplified product (550bp) was sequenced and aligned against ex-type strain sequences of *M. phaseolina* from NCBI GenBank using BLAST and phylogenetic analysis was obtained using MEGA4 software. The evolutionary history was inferred using the UPGMA method. Amplification of ITS1 region of the rDNA can be considered as a rapid technique for identifying pathogens successfully in all cases.

Key words: rDNA · ITS · *Macrophomina phaseolina* · *Citrus reticulata*

INTRODUCTION

Citrus reticulata (Blanco) is the principal fruit crop of Darjeeling hills known as 'Darjeeling mandarin'. It is considered as the most important cash crop of the marginal families of the hills. But recently its decline is posing a threat to the sustainability of citriculture and the stakeholders respectively [1]. Citriculture in the hilly terrain is undertaken as secondary and tertiary source of income. However, extensive cultivation is seldom undertaken owing to the topography of the terrain. This has resulted in establishment of accountable smaller orange grooves or orchards. Moreover, in the recent past the area under cultivation as well as the production has declined. This can be attributed to various parameters ranging from on-field cultural practices, quality of planting materials, occurrence of diseases that may be due to viruses, fungi, insects or bacteria and soil quality. The evident symptoms that can be observed are defoliation of young shoots and twigs and ultimate drying up of the whole plant which is an indicative of the orange orchards being affected by "citrus dieback" disease. A multidisciplinary attempt has been made to

assess the conditions of the orchards and analyse the relative abundance of the contributing factors for the citrus decline. Lack of proper knowledge on agro-techniques of orchard management of citrus cultivation in terms of nursery management, proper seedling selection, soil health status, plant protection measures, amount of manuring and fertilizer required for the plant, irrigation in winter, mulching practice, pruning practice, intercropping (suitable and unsuitable crop) etc. after the establishment of orchard singly or in combination has lead to citrus decline in Darjeeling hills. It has been reported that chronic infection of foot and root rot alone or coupled with the infection of *Fusarium* and *Macrophomina* were associated to large number of dieback plants in combination with nutrient deficiencies.

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions with the genome [2]. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [3]. Phylogenetic species concepts

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between five or more gene trees has been proposed by Taylor et al [4]. These molecular techniques indicating interrelations among species combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relationships. ITS sequences of rDNA analysis and universally primed polymerase chain reaction have been used to categorize the isolates of *Talaromyces flavus* and *Trichoderma* species [5].

The purpose of the present study was rapid identification of *Macrophomina phaseolina*, a root rot pathogen of *Citrus reticulata* (Blanco) in Darjeeling hills based on the sequence analysis of ITS regions of the rDNA gene and development of rDNA markers for analysis of genetic variability.

MATERIALS AND METHODS

Fungal Culture: Fungal pathogen (*Macrophomina phaseolina*) was isolated from samples of diseased roots of *Citrus reticulata* grown in Mirik busty by culturing pieces of internal tissues. Healthy mandarin seedlings (1-year-old) were further inoculated with this isolated organism for completion of Koch's postulate. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Macrophomina phaseolina.* Mycelia-septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelial mat even and appressed. Sclerotia – black, moderate size (34-78 µ in diameter), round or irregular uniformly reticulate with no difference in internal structure. The culture has been deposited (NAIMCC-F-02902) in the National agriculturally important microbial culture collection (NBAIM), Mau, India.

Genomic DNA Extraction: Genomic DNA was isolated from 4 day old fungal mycelia of *M. phaseolina* by a modified Raeder and Broda method [6]. Fungal mycelia from 3-4 days old cultures grown on potato dextrose broths was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 4 h at 65°C followed by centrifugation at 12,000 rpm for 15 min.

The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min and further extracted with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (in the ratio of 1:4 v/v) was added followed by 0.5M Na-acetate (in the ratio of 1:10 v/v). Next, isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Qualitative and Quantitative Estimation of DNA: The extraction of total genomic DNA as per the above procedure was followed by RNAase treatment. Genomic DNA was re-suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region and sequencing: Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers (Table 1).

For amplification of the ITS regions of the ribosomal DNA primer pairs, ITS1 and ITS4 were used. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

Table 1 : The nucleotide sequence used for ITS PCR of root pathogens of *C. reticulata*

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplicaon size (bp)
Macrophomina sp.					
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56%	~550
ITS4	TCCTCCGCTTATTTGATATGC	21	63	59%	

PCR product (10 µl) was mixed with loading buffer (5µ1) containing 0.25% bromophenol blue, 40 % w/v sucrose in water and then loaded in 1.5% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. Nucleotide base pairs of the amplicons were determined on the basis of its migration and conformation relative to the molecular size marker (1000 base pair, wide range DNA ladder, Genie,Bangalore) PCR products were sent for sequencing to Chromous biotech, Bangalore, India.

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc.) Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pair wise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA) [7]. Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained [8]. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura [7].

RESULTS

Genomic DNA of *Macrophomina phaseolina,* the isolate obtained from mandarin root tissue collected from Mirik orchard, which was established as the causal organism of root rot of *Citrus reticulata* was amplified. Main focus was on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *M. phaseolina.* ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 for sequencing of its

18S rDNA region. Amplified products of size in the range of 550bp was produced by the primer pairs (Fig 1). PCR products produced sequences and chromatogram and 18S rDNA sequence of *M. phaseolina* that could be aligned and showed satisfactory homology with ex-type strain of *M. phaseolina* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 1 region. ITS1 showed the highest number of nucleotide substitutions and it was used for the phylogenetic study.

Studies involving isolates of *M. phaseolina* revealed that the partial sequence of ITS1- 5.8S-ITS4 rRNA gene is as variable as rDNA regions. The sequence information was then analysed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *M. phaseolina* with 100% similarity. This sequence has been deposited to NCBI genebank to get accession number.

Identified *M. phaseolina* rDNA gene sequences obtained from NCBI genebank (Table 2) of various host plants were selected for comparison with the rDNA gene sequence of *M. phaseolina* isolate of mandarin plant. The sequence alignment of the isolate of *M. phaseolina* shows variation in this gene. These available sequences of *M. phaseolina* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software for determining the conserved regions of rDNA gene. This partial sequence was deposited to NCBI database (Acc. No.JN241996). Multiple
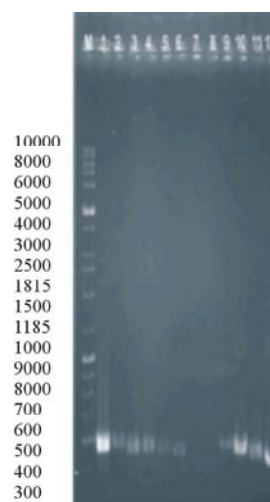

Fig. 1: ITS-PCR amplified products of Macrophomina phaseolina (RHS/S565). Lane M-high range DNA ladder, lanes 1&2 - M. phaseolina

Table 2 : NCBI GenBank rDNA sequences *of Macrophoina phaseolina*

Acc. No.	Sequences(bp)	Host	Country	Authors
JN241996	310	*Citrus reticulata*	India	Chakraborty et al 2011
DQ314733	527	*Glycine hispida*	India	Chaudhury et al 2005
DQ233666	495	*Glycine max*	India	Chaudhury et al 2005
DQ233664	441	*Abelmoschus esculentus*	India	Chaudhury et al 2005
DQ233663	519	*Cyamopsis tetragonoloba*	India	Chaudhury et al 2005
DQ233662	432	*Cyamopsis tetragonoloba*	India	Chaudhury et al 2005
HQ713771	511	*Pinus sylvestris*	Switzerland	Grunig and Sieber 2011
HQ380051	685	*Helianthus annuus*	Turkey	Mahmoud and Budak 2011
EU754070	998	*Glycine max*	Denmark	de Gruyter et al 2010
EU754169	1326	*Glycine max*	Denmark	de Gruyter et al 2010
GU251105	500	*Pinus dulcis*	USA	Inderbitzin et al 2010
HQ625638	496	*Phaseolus vulgaris*	Nicaragua	Marcenaro et al 2011
HQ625641	478	*Phaseolus vulgaris*	Nicaragua	Marcenaro and Valkonen 2011
HQ649831	558	*Inula crithmoides*	Spain	Macia-Vicente et al. 2011
HQ649832	560	*Inula crithmoides*	Spain	Macia-Vicente et al 2011
HQ649833	558	*Inula crithmoides*	Spain	Macia-Vicente et al 2011
JN672592	917	*Ipomoea batatas*	Spain	Hipol,2011
DQ377905	842	*Eucalyptus sp*	Uganda	Crous et al 2008
HM990163	534	*Morus alba*	India	Gangwar et al 2011
EF446288	562	*Morus alba*	India	Therese et al 2009

Table 3 : Similarity of rDNA sequences within the groups of *M. phaseolina*

	JN 241996	EU 754070	EU 754169	GU 251105	HQ 625638	HQ 625641	HQ 649831	HQ 649832	HQ 649833	JN 672592	DQ 377905	DQ 233663	HM 990163	DQ 233662	EF 446288	HQ 713771	DQ 314733	DQ 233666	DQ 233664
JN241996																			
EU754070	1.3																		
EU754169	1.7	0.1																	
GU251105	0.4	0.2	0.4																
HQ625638	0.4	0.2	0.4	0.0															
HQ625641	0.5	0.2	0.4	0.0	0.0														
HQ649831	0.4	0.2	0.4	0.0	0.0	0.0													
HQ649832	0.4	0.2	0.4	0.0	0.0	0.0	0.0												
HQ649833	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0											
JN672592	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0										
DQ377905	1.7	0.1	0.0	0.4	0.4	0.4	0.4	0.4	0.4	0.4									
DQ233663	0.4	0.9	0.9	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.9								
HM990163	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3							
DQ233662	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.0						
EF446288	0.4	0.3	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.2	0.0	0.0					
HQ713771	0.5	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.0				
DQ314733	0.9	1.3	1.2	0.7	0.7	0.6	0.7	0.7	0.7	0.7	1.2	0.3	0.7	0.7	0.5	0.7			
DQ233666	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.0	0.0	0.0	0.0	0.7		
DQ233664	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.0	0.0	0.0	0.0	0.7	0.0	
HQ380051	0.4	0.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.0	0.0	0.0	0.0	0.7	0.0	0.0

Table 4 : Nucleotides status of rDNA sequence of *M. phaseolina*

Nucleotide (s)	Times found	Percentage	Nucleotide (s)	Times found	Percentage
G	67	21.61	tg	19	6.15
A	78	25.16	ta	12	3.88
T	80	25.81	tt	32	10.36
C	85	27.42	tc	16	5.18
Gg	29	9.39	cg	12	3.88
Ga	11	3.56	ca	13	4.21
Gt	11	3.56	ct	20	6.47
Gc	16	5.18	cc	40	12.94
Ag	6	1.94	g,c	152	49.03
Aa	42	13.59	a,t	158	50.97
At	17	5.50	ac	13	4.21

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#JN241996 C CTC CG CGG CCG CCC CCC TTT TGG GGG GTG GCT AGT GCC  [ 246]
#EU754070 . T.. G. G.C T.C TTG GTG CA. GAT AAC T.A A.G .A. CG.  [ 246]
#EU754169 G TCT A. A.. GT. AGA AT. CCG .AT .C. ... .G. T.C CTT  [ 246]
#GU251105 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ625638 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ625641 T T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ649831 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ649832 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ649833 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#JN672592 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#DQ377905 G TCT A. A.. GT. AGA AT. CCG .AT .C. ... .G. T.C CTT  [ 246]
#DQ233663 T T.G .. .AC TT. TTG TTT C.G G.C ... T.C ..C C.C CA.  [ 246]
#HM990163 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#DQ233662 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#EF446288 . T.. .. ... ... ... ... ... ... ... ... ... ... TT.  [ 246]
#HQ713771 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#DQ314733 T T.G .. TAC TTC TTG TTT C.. G.T ... T.C ..C CAC CA.  [ 246]
#DQ233666 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#DQ233664 . T.S .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]
#HQ380051 . T.. .. ... ... ... ... ... ... ... ... ... ... ...  [ 246]

#JN241996 CCC CCG AAG T ATC CAC CT CCA GT AAA CGT TTG AG TCT GA  [ 296]
#EU754070 ATG G.C TT. C CGG .GA GG TTC AA .TT TC. GCC CA A.. TT  [ 296]
#EU754169 AG. .AT GT. . C.. .TT GA .G. AG TTG TT. GG. CA G.. CT  [ 296]
#GU251105 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ625638 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ625641 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ649831 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ649832 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ649833 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#JN672592 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#DQ377905 AG. .AT GT. . C.. .TT GA .G. AG TTG TT. GG. CA G.. CT  [ 296]
#DQ233663 .AG GAC C.C C ..A A.. .. TTT .C .GT T.C AAT C. ..A .T  [ 296]
#HM990163 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#DQ233662 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#EF446288 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ713771 .G. .A. .G. . ... A.A .. ... .G ... ... .GC .. ... ..  [ 296]
#DQ314733 TAG GAC ..A C ..A A.. .. TTT .. ..T T.C AAT C. ..A .T  [ 296]
#DQ233666 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#DQ233664 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]
#HQ380051 .G. .A. .G. . ... A.A .. ... .. ... ... .GC .. ... ..  [ 296]

#JN241996 AA ATA TAA A AAC TAA AAC TTT CA AAA CGG TT TGG TTT TG  [ 347]
#EU754070 .T GAG AT. G GC. ..C C.T GG. AT C.. ... CG G.. AA. .A  [ 347]
#EU754169 .T GAG ... T TCT .CT ..A GC. A. T.C ... CC A.A GAC C.  [ 347]
#GU251105 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ625638 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ625641 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ649831 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ649832 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ649833 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#JN672592 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#DQ377905 .T GAG ... T TCT .CT ..A GC. A. T.C ... CC A.A GAC C.  [ 347]
#DQ233663 .. CAT ... C .TT ..C ... ... A. C.. ... C. ... ..C ..  [ 347]
#HM990163 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#DQ233662 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#EF446288 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ713771 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#DQ314733 .. CAT ... T ..T ..C ... ... A. C.. ... C. ... ..C ..  [ 347]
#DQ233666 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#DQ233664 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
#HQ380051 .. ... ... . ... ... ... ... A. C.. ... C. ... ..C ..  [ 347]
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Fig. 2: Continue

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#JN241996 G C ATC AAG AAA AAA CCA CC GAA AGG CAA AAA TTA TGG GG [ 395]
#EU754070 . T ..T CC. G.G ..G ..G AG A.. C.. .T. CC. CAT CCA A. [ 395]
#EU754169 A T .G. GCA C.. G.G TG. T. ... ..A TG. ... GC. CTT T. [ 395]
#GU251105 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ625638 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ625641 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ649831 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ649832 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ649833 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#JN672592 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#DQ377905 A T .G. GCA C.. G.G TG. T. ... ..A TG. ... GC. CTT T. [ 395]
#DQ233663 . . ... G.T G.. G.. .G. G. ... .T. .G. T.C G.. GT. T. [ 395]
#HM990163 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#DQ233662 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#EF446288 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ713771 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#DQ314733 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. GT. T. [ 395]
#DQ233666 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#DQ233664 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]
#HQ380051 . . ... G.T G.. G.. .G. G. ... .T. .G. T.. G.. AT. T. [ 395]


#JN241996 A ATT GT A ATT CAT GGA ACC ATC TAA TCT TTG AAC C CCC C [ 439]
#EU754070 G .AG .A . GGC GCG CA. .TT .C. C.. ..C CGA C.. G GAG G [ 439]
#EU754169 G .AA .A . G.. A.A AAG TA. G.G A.. .TG ... ..A G GGA A [ 439]
#GU251105 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ625638 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ625641 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ649831 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ649832 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ649833 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#JN672592 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#DQ377905 G .AA .A . G.. A.A AAG TA. G.G A.. .TG ... ..A G GGA A [ 439]
#DQ233663 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HM990163 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#DQ233662 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#EF446288 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ713771 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#DQ314733 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#DQ233666 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#DQ233664 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]
#HQ380051 . ... .A . ... ..G T.. .T. ... G.. ... ... ... G .A. A [ 439]


#JN241996 TT GCC CCC CTT GGG ATT CG GGC ATG CCT GTT CAA ACT TC [ 482]
#EU754070 .A .TG A.A A.A AAT .C. GA ..G C.C TT. TGG GTC TTG .A [ 482]
#EU754169 GC ..T TG. AGC CA. .C. T. TC. GCA GT. .C. ... C.G GT [ 482]
#GU251105 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ625638 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ625641 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ649831 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ649832 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ649833 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#JN672592 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#DQ377905 GC ..T TG. AGC CA. .C. T. TC. GCA GT. .C. ... C.G GT [ 482]
#DQ233663 .. ..G ... T.. ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HM990163 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#DQ233662 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#EF446288 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ713771 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#DQ314733 .. ..G ... T.. ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#DQ233666 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#DQ233664 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
#HQ380051 .. ..G ... ... ..T ... .. ... ... ... ... .G. G.G .. [ 482]
```

Fig. 2: Continue

```
#JN241996 C TTT CCA CCC TTT GCT  [ 498]
#EU754070 A ..G GA. TGA G.A CAA  [ 498]
#EU754169 . .CC TG. ..G G.G TAC  [ 498]
#GU251105 A ... .A. ... .CA AGC  [ 498]
#HQ625638 A ... .A. ... .CA AGC  [ 498]
#HQ625641 A ... .A. ... .CA AGC  [ 498]
#HQ649831 A ... .A. ... .CA AGC  [ 498]
#HQ649832 A ... .A. ... .CA AGC  [ 498]
#HQ649833 A ... .A. ... .CA AGC  [ 498]
#JN672592 A ... .A. ... .CA AGC  [ 498]
#DQ377905 . .CC TG. ..G G.G TAC  [ 498]
#DQ233663 A ... GT. ... .CA AGC  [ 498]
#HM990163 A ... .A. ... .CA AGC  [ 498]
#DQ233662 A ... .A. ... .CA AGC  [ 498]
#EF446288 A ... .A. ... .CA AGC  [ 498]
#HQ713771 A ... .A. ... .CA AGC  [ 498]
#DQ314733 A ... GT. ... .CA AGC  [ 498]
#DQ233666 A ... .A. ... .CA AGC  [ 498]
#DQ233664 A ... .A. ... .CA AGC  [ 498]
#HQ380051 A ... .A. ... .CA AGC  [ 498]
```

```
DataType=Nucleotide CodeTable=Standard;  NSeqs=20 NSites=296, Identical=.
Missing=? Indel=-;!Domain=Data property=Coding CodonStart=1;
```

Fig. 2: Nucleotide sequence alignments of rDNA repeats (partial) encoding ITS region of different isolates of Macrophomina phaseolina used for analysis
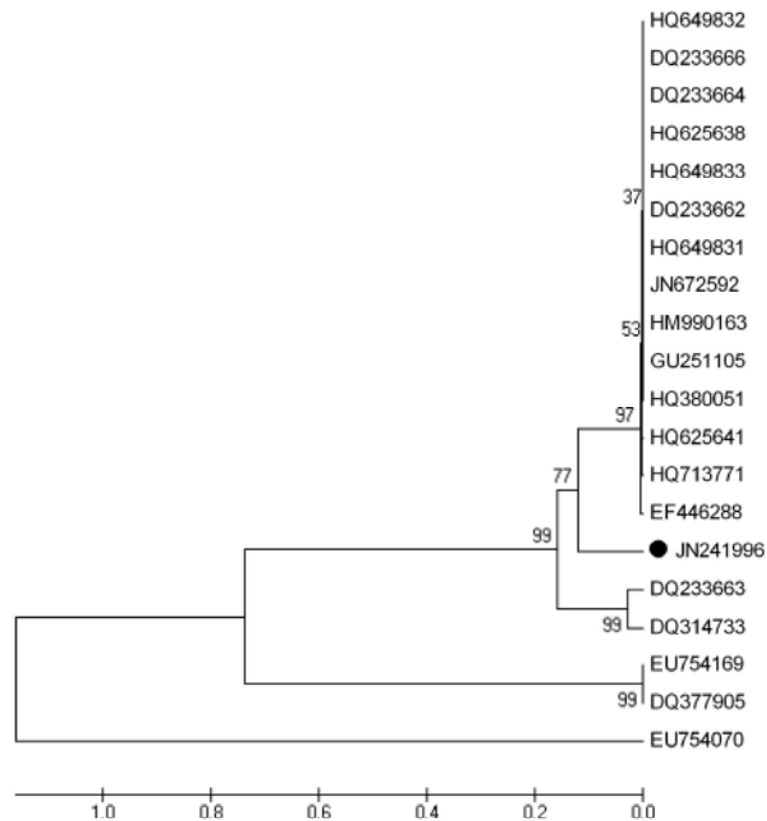
Fig. 3: Phylogenetic placement of Macrophomina phaseolina (JN241996) with extype strains from NCBI genebank

sequence alignment revealed that there were quite a number of gaps introduced in the alignment within the ITS region which were closely related. Similar sequence indicated that the isolates were closely related. From the sequence alignment, variations were observed between other *M. phaseolina* isolates in species level (Fig 2). Multiple and pair wise sequence alignment were generated and used to calculate evolutionary distances and percent of sequence similarity values (Table 3) and to construct a phylogenetic tree.

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.56368608 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 294 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Fig 3).

Next, combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html (Table 4).

The of rDNA fragments sequenced with 310 bases of ITS region starting with "GTATACCTAC" presented in the table following this protocol revealed that the 'C' content of the sequence is maximum (27.42 %) with highest repetition of 85. Combinations like GC were also maximum at the level 5.18 % which occurred at least 16 times in the entire sequence.

The DNA Molecular Weight rDNA sequence of ITS region is 95420.76 Da (http:// www.ualberta.ca/ ~stothard/javascript/dna_mw.html). A total of 1 open reading frames for the designated sequence was calculated with the help of ORF finder available from http://www.ualberta.ca/~stothard/javascript/orf_find.html for residue sequence of *M. phaseolina* starting at " GTATACCTAC ".

ORF number 1 in reading frame 1 on the direct strand extends from base 142 to base 237.

AACTTTCCAAAACGGGTTTTTTGGTTTTGGGCATC AAGAAAAAACCCACCGAAAGGCAAAAATTATGG GGAATTGCTAATTCATGGAACCATCTAA

Translation of ORF number 1 in reading frame 1 on the direct strand.
NFPKRVFWFWASRKNPPKGKNYGELLIHGTI*

DISCUSSION

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. Differences in the nucleotide composition of the variable ITS region have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens [8-12]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [3]. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [2]. They also occur in multiple copies with up to 200 copies per haploid genome [13] arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes.

In the present study, ITS regions of ribosomal genes for the construction of primers were used to identify *M. phaseolina.* ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers for *M. phaseolina.* Amplified products of size in the range of 550bp was produced by the primer pairs.

Amplification of DNA fragments of *M. phaseolina* with specific primers indicate the usefulness of molecular technique for their detection and identification. Using the specific primers ITS 1 and ITS 4, only a single band of 550 bp was generated in the amplification pattern of all the isolates. *M. phaseolina* as first described by [14] suggested that isolates from one specific host are more suited to colonize it. Later, differences in pathogenicity among the isolates of soybean and sorghum have been observed [15]. This has been further confirmed with isolates from soybean, sorghum and cotton [16].

Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region. The overall study was established the rDNA gene sequence of *Macrophomina paseolina* a root rot pathogen of *Citrus reticulata* (Blanco) based on the bioinformatics tools with sequence analysis of ITS regions of the rDNA gene for rapid identification and development of rDNA markers for analysis of genetic variability within the outgroups. We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database.

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