Pak. J. Bot., 52(3): 1073-1082, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-3(9)

MOLECULAR CHARACTERIZATION OF NOVEL ISOLATES OF *RHIZOCTONIA SOLANI, TRICHODERMA ATROVIRIDE* AND *FUSARIUM* SPP. ISOLATED FROM DIFFERENT PLANTS AND CUTTING WOODS IN IRAQ

AQEEL N. AL-ABEDY[1*], R.G. AL-JANABI[1], Z.A. AL-TMEME[1], ALAA T.SALIM[1] AND MUHAMMAD ASHFAQ[2*]

[1]*Plant Protection Department, Agriculture College, University of Karbala, Iraq*
[2]*Department of Plant Pathology, MNS-University of Agriculture Multan, Pakistan*
[*]*Corresponding author's email: alabedyaqeel0@gmail.com; mashfaq1642@gmail.com*

Abstract

Three fungal (*Fusarium, Rhizoctonia* and *Trichoderma*) species isolated from diseased tomato, maize and wood decayed plants from Iraq were morphologically identified. ITS1 and ITS4 based genetic regions were used for molecular identification and phylogenetic relationship revealed that that *Rhizoctonia solani* isolates had 99% similarities to Indian, USA and Mexicans isolates. *Fusarium solani* showed 99% nucleotide identity to Indian, Chinese, Mexicans and Brazilian isolates. *Fusarium proliferatum* showed >99% nucleotide identity to Asian isolates whereas *Trichoderma atroviride* showed 99% similarity to Chinese isolates. These identified fungal isolates of *F. solani*, *F. proliferatum* and *T. atroviride* were not previously reported from Iraq and therefore sequences of these fungi were deposited in the GenBank database (NCBI) under the accession numbers KY283953(*R. solani*), KX000895 (*F. solani)*, KX350051 (*F. proliferatum)* and KU985151 (*T. atroviride*) for the first time.

Key words: *Fusarium proliferatum, Rhizoctonia solani, Trichoderma atroviride*.

Introduction

Fungi are diverse in nature and have been discovered from remote, desert, polar-regions, tropical and subtropical regions in extreme environments worldwide (Siddiquee, 2017). Soil of Iraq is diverse and good for tomato production throughout the whole year. In the winter, tomatoes are produced in the desert area of al-Zubayr and Samawa and are produced in spring in Najaf and Karbala area. In the summer, tomatoes are produced in the Kut areas in Nu'maniyah and Al khales. Tomatoes have significant nutritional value and are an important source of lycopene, which is a powerful antioxidant that acts as an anti-carcinogen. They are also a good source of vitamins A, B and C, potassium, iron and calcium.

Soil is a major reservoir for microorganisms such as *Rhizoctonia solani* and *Fusarium* spp. are considered to be the most severe and dangerous fungi affecting many vegetables and field crops, their symptoms usually appear on the plants ((Hsuan *et al.,* 2011). The genus *Fusarium* is one of the most diverse and pathologically important fungi and usual identification of species of this fungus is based on their micro and macroscopic features and morphological characters alone may lead to incorrect identification. *Trichoderma* is commonly used as biological control agents in crop protection and their products as alternatives to synthetic agro-chemicals. They are presently marketed as bio-pesticides, biofertilizers, growth enhancers and stimulants of natural resistance. This fungus has broad spectrum efficacy due to their ability of protecting plants, enhancing vegetative growth and managing pathogen populations under numerous agricultural conditions, as well as to act as soil amendments/inoculants for improvement of nutrient ability, decomposition and biodegradation.The quick and accurate identification of fungi is one of the efficient approaches to disease management. Morphological identification of fungi sometimes gives an accurate result (Hsuan *et al.,* 2011) but is not a reliable because it needs a lot of experience for taxonomic classification of fungi of closely related species. There are many constraints in the proper morphological identification. Environmental factors such as pH, temperature and humidity are more important which affect the size, shape and colors of spores and fungal colonies (Wang *et al*., 2008; Zhang *et al*., 2012; Huang *et al*., 2016).). It still also plays an important role in sorting isolates into smaller groups before other methods of identification can be applied (Leslie & Summerell, 2006; Hsuan *et al.,* 2011). Genome level identification by polymerase chain reaction (PCR) contributed to its accuracy, sensitivity and ability to detect genetic differences and to eliminate the disadvantages of conventional methods in diagnosing many organisms (Giantsis *et al.,* 2016; Stanis *et al.,* 2016). PCR technique has been used to diagnose many microorganisms, including fungi such as *Fusarium* spp., *Cladosporium* spp. and *Aspergillus* spp., (Romberg & Davis, 2007; Alaei *et al.,* 2012; Arif *et al.,* 2012; Alhussaini *et al.,* 2016). Keeping in view the importance of fungal classification, this research work was conducted to isolate and identify five fungal isolates by using PCR technique and determining the nucleotide sequence to investigate the genetic identities and dissimilarities among these isolated fungi as well as previously reported fungi elsewhere.

Material and Methods

Survey and sample collection: Fields were selected from Babylon, Najaf, Karbala, and Mosul provinces at various locations in Iraq. Non random sampling was done on the basis of symptomology. Plants showing yellowing, stunting, wilting, rotting, dead leaves, and decayed woods were collected from tomato, maize and wooden tree plants of farmer fields. Samples were wrapped in brown bag, properly labeled and stored in icebox. Samples were then brought to the Laboratory of Plant Pathology, Department of Plant Protection at College of Agriculture, University of Karbala, Iraq.

Isolation, identification and preservation: Samples were properly washed in running tap water and dried. Potato sucrose agar (PSA) media was prepared and autoclaved at 121ºC, 15psi for 20 mins, after autoclaving antibiotic chloramphenicol was added at 20mg/ml. PSA media was poured into Petri plates and allowed it to solidify. Infected samples were cut into small pieces and surface sterilized with 10% sodium hypochlorite solution (Clorox) for 2 minutes and rinse with distilled water twice to remove leftover of Clorox. The treated infected samples were dried and transferred to already prepared PSA media in petri plates. Petri plates were then incubated at 25±2°C for 3-5days. The fungal colonies were picked and transferred to new Petri plates. Repeated the same process by using a single spore technique to obtain a pure culture as shown in Fig. 1. All fungal isolates were morphologically identified by using a compound microscope. Pure cultures of all fungal isolates were maintained on slant and also preserved in 50% glycerol at 70ºC for further use.

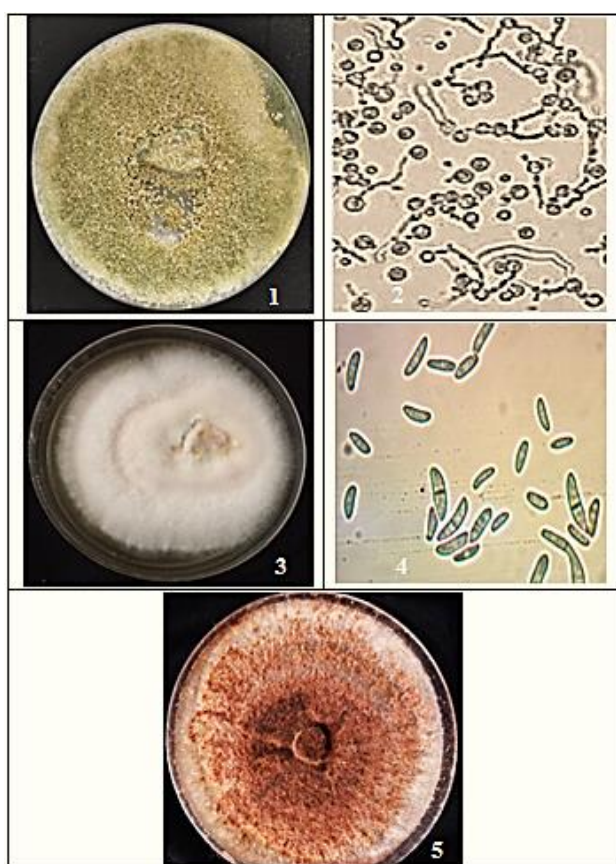

Fig. 1. *T. artoviride* (1) spores and mycelium (2) *Fusarium culture* (3) *Fusarium* spores (4) *R. solani* (5).

DNA extraction, PCR amplification and DNA sequencing: Hyphae and spores of all pure fungus cultures were scraped from the surface of culture media using heated sterilized needle and 50-100 mg of fresh fungal culture were transferred to an Eppendorf tube. These fungal cultures were stored at -80ºC. Total DNA extraction was followed by using genomic DNA extraction kit (Favrogen, Taiwan, Cat. No. FATGK001) as per manufacturer's instructions. The quality and quantity of DNA extracted from each isolate was measured by a UV spectrophotometer (Thermo Scientific, Germany) and subsequently DNA was stored at -20ºC until use.

ITS region of fungal isolates was amplified by using universal primers set of ITS1 (TCCGTTGGTGAA CCAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) (White *et al.,* 1990). Master mixture was prepared by adding 1 µl each primer (10 pmol), 2 µ1 10X PCR buffer, 2 µl dNTPs (2 mM), 3 µl template DNA (30 ng/µ1), 1 unit *Taq* polymerase enzyme and adjust the volume with nucleases free water. PCR amplification was done in the thermal cycler by providing a specific program of denaturation at 94°C for 1 minute followed 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72ºC for 1 minute and post extension at 72°C for 5 minutes. Amplified PCR products were analyzed on 1% (w/v) agarose gel containing 4µl of ethidium bromide (100µg/ml) in 0.5X Tris-borate EDTA (TBE) buffer. Amplified samples were mixed with 6X loading dye (bromophenol blue) and electrophoresis at 120V until the bromophenol migrated approximately two third the length of the gel. After electrophoresis, DNA bands were observed on gel documentation apparatus and photographs were taken by using VilberLourmat, Taiwan gel documentation system.

PCR-amplified products were gel-purified using the FavorPrep PCR purification kit (Cat. No. FAGCK 001, Favorgen, Taiwan) and sent along with the primer pairs (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions. Nucleotide sequences were aligned and compared with the sequences of the other fungal isolates available at the NCBI database using the Basic Local Alignment Search Tool (BLAST) (Zhang *et al.,* 2000). Phylogenetic analysis of all fungal nucleotide sequences were compared by using MEGA 6.

Results and Discussion

All the fungal isolates from maize, tomato and wood cuttings were cultured on PDA and PSA media. Pure cultures were morphologically identified under compound microscope as shown in Fig. 1.

PCR amplification and nucleotide sequencing of the ITS region of fungal isolates: DNA from all fungal cultures was successfully extracted and amplified using ITS primer pair. Fungal PCR product of 500bp was amplified from each sample using the universal primer pair ITS1 and ITS4 as shown in Fig. 2(a). Quality and quantity of purified DNA was checked by running gel as shown in Fig. 2(b). The main objective of this study was to determine the effectiveness of using PCR for the purpose of sequencing and eventual species identification. Partial ITS region of fungal isolates was sequenced and BLAST in the NCBI database. The results revealed that all these sequences were 99 %t identical to the isolate of *Rhizoctinia solani, Trichoderma atroviride, Fusarium proliferatum* and *Fusarium solani*.

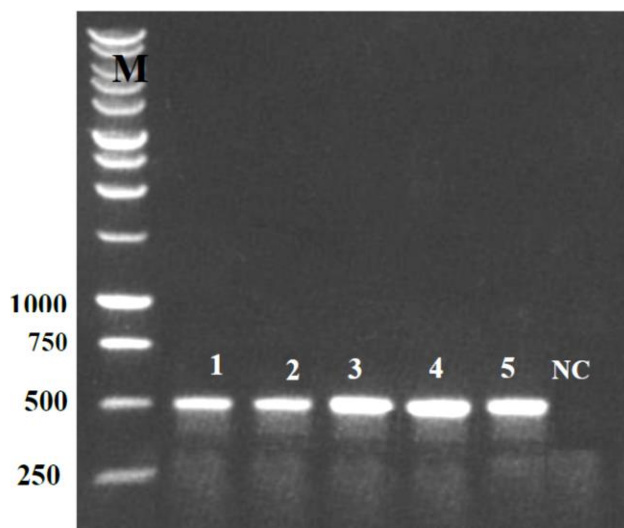

Fig. 2(a). Sharp and clear bands of 500bp amplified PCR products of internal transcribed spacer (ITS) region of *F. proliferatum* (1), *F. solani* (2) and *R. solani* (3 and 4); *T. atroviridae* (5) **M**= 1Kbp DNA ladder marker. NC: Negative control (no template DNA added).

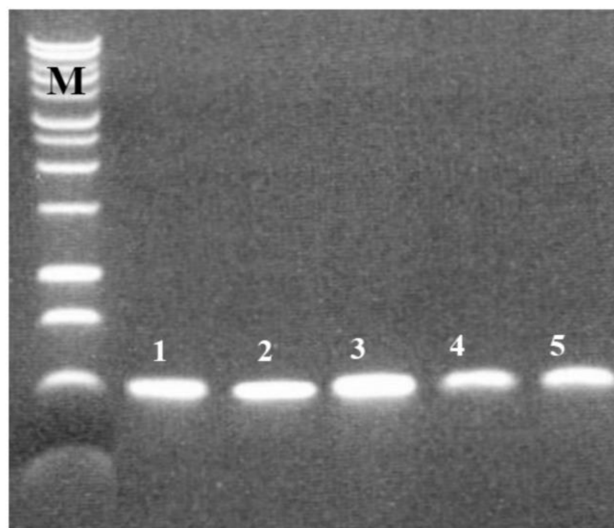

Fig. 2(b). Quality and Quantity of purified DNA by using a Favrogen PCR purification kit were observed in 1% gel. (1) *F. proliferatum*; (2) *F. solani* and (3 and 4)*R. solani*; and *T. atroviridae*. **M**, 1kbp DNA ladder marker.

By comparing partial sequence of ITS region (ITS1, 5.8S rDNA and ITS4) of the *R. solani* isolate to other *R. solani* isolates already available at NCBI GenBank database, results revealed that the genetic similarity percentage of nucleotide sequence ranged between 92-99% as shown in Table 1. Genetic similarity of Iraqi isolate of *Rhizoctonia solani* have 99% to Indian isolates (KJ466117, JX535004 and JX535004), Iraq isolates (KF372653, KF372646, KF372645 and KF372646) and Mexico isolates (KX592586) whereas the lowest homology percentage of 93.2% was found with Malaysia (KX674533) and Iraq (KF372657 and KF372662) isolates. The identity of difference/similarities between the sequences from different isolates with their IDs are shown in Table 1.

Genetic relationship analysis among the *R. solani* isolates and other isolates from GenBank are used to construct a phylogenetic tree. For the phylogenetic tree, the neighbor joining methods was used to compare the sequences. The phylogenetic tree was constructed using the ITS region consists of two main clades identified among the isolates of *R. solani* analyzed as shown in Fig. 3.

PCR amplified product of *Fusarium solani* BLAST in NCBI database, the nucleotide sequence shows 99% similarity with the other *F. solani* isolates identified in different geographic regions of the world and registered in NCBI (Table 2). Whereas phylogenetic tree was also constructed by using MEGA 6 software for neighbor joining method as shown in Fig. 4. Results revealed that our *F. solani* isolate resembles more to China, India and Mexico isolates. A species of *Fusarium* was isolated and identified as *F. proliferatum*, the sequence and blast analysis showed 98-99% similarities to other isolates already present in gene bank data base as shown in the Table 3. Phylogenetic tree was made by the neighbor joining method as shown in the Fig. 5.

As given in Table 4, results also revealed that the genetic similarity of the ITS-generated sequence of *R. solani* isolated in the study was entirely identical to the Iraqi *R. solani* isolate (Accession No.: FJ746917.1), followed by *R. solani* isolates isolated from USA (Accession No.: FJ746906.1) and China (Accession No.: FJ746917.1) that had a genetic similarity of 88%. It was also found that the genetic differences based on the ITS-sequenced region of the *R. solani* used in this study ranged between 90-95% with those previously identified *R. solani* isolates and published in NCBI. Phylogenetic tree was made by the neighbor joining method as shown in Fig. 6. The PCR-amplified fragment of the *T. atroviridae* isolate was also sequenced, and the nucleotide sequences had 99% identity with all other *T. atroviridae* isolates previously registered in the GenBank data base. Comparison and list of all their IDs with percentage of similarity is shown in Table 5. Whereas phylogenetic tree is shown in Fig. 7 by Neighbor Joining Method using MEGA 6 software. In the present study *R. solani*, *F. solani*, *F. proliferatum, T. atroviride* were isolated from tomato, corn plant, wood cutting using the conserve region of ITS1 and ITS4 of rDNA. By comparing the nucleotide sequence, it was found that the sequences of Iraq isolates have 99% identity to other isolates of fungi *R. solani*, *F. solani*, *F. proliferatum, T. atroviride*; therefore, the identified fungal sequences have been aligned and registered in Genbank under the accession numbers KY283953 (*R. solani*), KX000895 (*F. solani*), KX350051 (*F. proliferatum*) and KU985151 (*T. atroviride*).

Fungal diagnostics have increased dramatically with the introduction of molecular tools i.e., PCR. PCR and sequencing has widely used as a rapid and accurate techniques to identify many pathogenic plant fungi, bacteria viruses and nematodes. Molecular markers help to eliminate the limitations in identification on the basis of morphological characters (Henry *et al.,* 2000, Zakiah *et al.,* 2016). Despite the morphological characters are best in sorting fungal isolates into smaller groups, but it needs time, efforts and expertise to be an taxonomist for proper identification, especially up to species level identification (Leslie & Summerell, 2006; Yang *et al.,* 2007; Wang *et al.*, 2008; Zhang *et al.,* 2012; and Huang *et al.*, 2016). Morphological character also depends on many other factors such as moisture, light, pH and composition of the growth medium can change the color, shapes and sizes of spores and fungal colonies growing pattern (Zhang *et al.,* 2012; Huang *et al.,* 2016).

Table 1. Comparison of the whole region (ITS1, 5.8S rDNA and ITS4) of *R. solani*, isolated in this study, with those of *R. solani* isolates available at NCBI.

Fungus	Isolate/ strain name	Origin	Sequences similarities in GenBank database	
			GenBank accession number	Sequence similarity (%)
R. solani	Babylon*	Iraq	KY283953(registered now)	100
R. solani	RsolTeaIN1	India	KJ466117	99
R. solani	MML4001	India	JX535004	99
R. solani	IQ49	Iraq	KF372653	99
R. solani	IQ23	Iraq	KF372645	99
R. solani	IQ35	Iraq	KF372646	99
R. solani	AYSDIN 18S	Mexico	KX592586	99
R. solani	IQ30	Iraq	KF372657	98
R. solani	IQ40	Iraq	KF372662	98
R. solani	SPM1	Malaysia	KX674533	98
R. solani	Rae354	Taiwan	AY684921	97
R. solani	RUPP93	India	JF701784	95
R. solani	BPRhi 01	India	KM434130	95
R. solani	RKLC1	India	JF701742	95
R. solani	IQ34	Iraq	KF372660	95
R. solani	RKNG9	India	JF701745	94
R. solani	RKNM3	India	KC997793	94
R. solani	RKNM8	India	JF701744	93
R. solani	R43	Canada	EU730814	92
R. solani	RDLM6	India	JF701717	92
R. solani	AQNOAH	Iraq	KY055374	92
R. solani	F14	USA	FJ492073	92

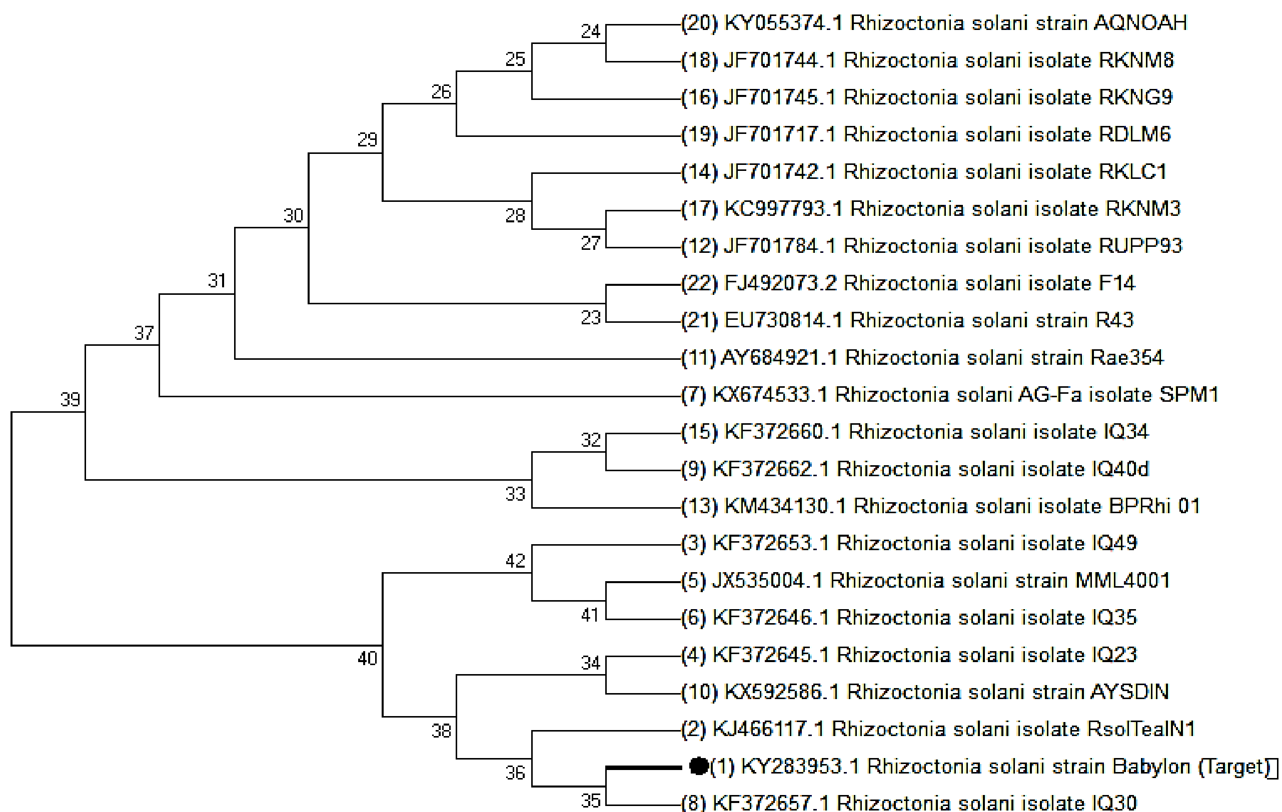

Fig. 3. A phylogenetic tree generated using the Neighbor-Joining Method based on a comparison of the partial ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *R. solani* isolate, indicated by black dote (●), with those of other *R. solani* isolates available in GenBank (NCBI).

Table 2. Comparison of partial region (ITS1, 5.8S rDNA and ITS4) of *F. solani*, other *F. solani* IDs available at NCBI.

Fungus	Isolate/ strain name	Origin	The most similar sequences in GenBank database	
			GenBank accession number	Sequence similarity (%)
*F. solani**	AQRAJAA	Iraq	KX000895(register now	100
F. solani	NBAIM: 350	India	EU214559	99
F. solani	Zbf-R5	China	KX079483	99
F. solani	G6D1	China	KT375695	99
F. solani	G8A1	China	KT375684	99
F. solani	87	Mexico	KP137446	99
F. solani	86	Mexico	KP137444	99
F. solani	59	Mexico	KP137443	99
F. solani	84	Mexico	KP137441	99
F. solani	TVD	Canada	KF494125	99
F. solani	M5_1H	Hungary	KJ584550	99
F. solani	1A44	China	KF572456	99
F. solani	Fs1	India	KC156593	99
F. solani	CS11723	China	JX406551	99
F. solani	Fusa22	India	JX135091	99
F. solani	bxq33104	China	EF534185	99
F. solani	JM6201508003	China	KT366735	99
F. solani	ABL1	India	KJ729475	99
F. solani	UOM AE	India	KF923870	99
F. solani	TUFs8	Saudi Arabia	HG798753	99
F. solani	MML4006	India	JX535009	99
F. solani	BK-CB20	India	JQ954888	99
F. solani	LCPANCF01	India	JN786598	99
F. solani	UENFCF251	Brazil	JN006813	99
F. solani	GIFUUHFA10	India	GQ121291	99
F. solani	XBH4	China	AB369465	99
F. solani	MML4007	India	JX535010	99
F. solani	P1	India	GQ451337	99
F. solani	MML4012	India	JX535014	99
F. solani	MML4011	India	JX535013	99
F. solani	CIIDIRC-2	Mexico	JQ956460	99
F. solani	FS5	Ireland	HQ265423	99
F. solani	MHE 49 MC	South Africa	KY617066	99
F. solani	51	South Africa	KY587307	99
F. solani	DET-59	Brazil	KX385047	99
F. solani	SQU14015	Oman	KY684277	99
F. solani	Fs-150P	Spain	KY484958	99
F. solani	18 FS	Iran	KX929305	99

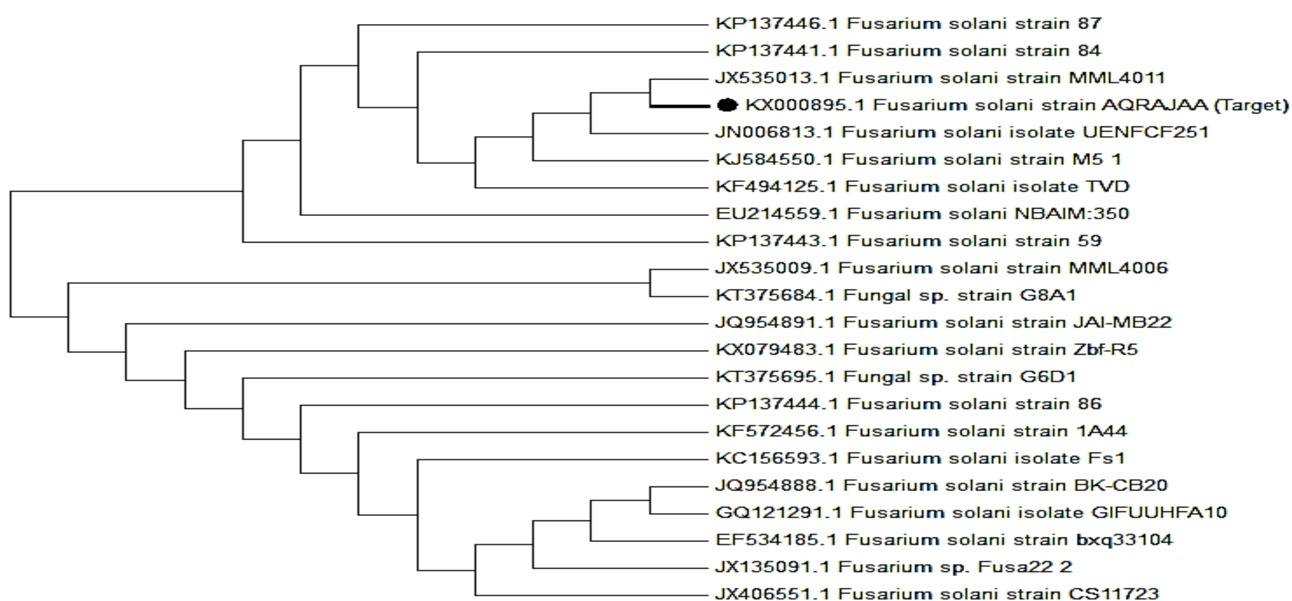

Fig. 4. A phylogenetic tree generated using the Neighbor-Joining Method based on a comparison of the partial ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *F. solani* isolate, indicated by black dote (●), with those of other *F. solani* isolates available in GenBank (NCBI).

In Peninsular Malaysia, studies on *Fusarium* spp. are often based on morphological characters which could lead to the incorrect species identification (Hsuan *et al*., 2011). In one of those studies, it was found that there are some limitations on the use of morphological characters for the identification of some fungi such as species in the *G. fujikuroi* species complex as some species, i.e., *F. proliferatum, F. fujikuroi*, *F. sacchari*, *F. subglutinans, F. verticillioides* and *F. andiyazi*, have very close morphological characters (Hsuan *et al.,* 2011). It was found through the re-diagnosis using the PCR technique that there was an error in the morphological identification of many fungi identified in previous studies such as species belonged to *Fusarium* spp., e.g., *Fusarium subglutinans* and *F. verticillioides* (Wulff *et al.,* 2010 and Hsuan *et al.,* 2011).

Differences in the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA), repeat units are well-investigated sequences that are found in multiple copies and can be isolated and selected by PCR amplification (Alhussaini *et al.,* 2016). PCR amplification of ITS region has been provided a high efficiency in diagnosing many fungi such as *Pythium* spp., *R. solani* and *F. verticillioides* (Hsuan *et al.,* 2011; Arif *et al.,* 2012; Walch *et al.,* 2016 and Alhussaini *et al.,* 2016).

The rapid and accurate identification of plant pathogenic fungi is one of the most important needs because of its importance for the development of effective disease control management, quarantine purposes and as a basis for making correct decisions to achieve a complete protection for crops and other natural resources from fungal pathogens (Rossman & Palm-Hernández, 2018).

Table 3. Comparison of partial region (ITS1, 5.8S rDNA and ITS4) of *F. proliferatum* with the other isolates of *F. proliferatum* available at NCBI.

Fungus	Isolate/ strain name	Origin	The most similar sequences in GenBank database	
			GenBank Accession Number	Sequence similarity (%)
*F. proliferatum**	Iraq	Iraq	KU98515	100
F. proliferatum	CDR1P1F2	India	KF986684	99
F. proliferatum	-	China	FJ040179	99
F. proliferatum	E26F	India	KY425734	99
F. proliferatum	A2S1-D96	Malaysia	KJ767073	99
F. proliferatum	A1S1-D30	Malaysia	KJ767072	99
F. proliferatum	CE1	China	KJ576800	99
F. proliferatum	D1	Italy	EU151484	99
F. proliferatum	GSLZA-F-8	China	KX029333	99
F. proliferatum	3	Italy	KJ608094	99
F. proliferatum	870066	France	GU594758	99
F. proliferatum	SAPB4	India	KX343956	99
F. proliferatum	A1S1-D12	Malaysia	KJ767071	99
F. proliferatum	MPT-7	China	KJ634671	99
F. proliferatum	UOA/HCPF	Greece	KC254041	99
F. proliferatum	CanR-8	China	JF817300	99
F. proliferatum	8	China	HQ380789	99
F. proliferatum	47PC	Colombia	HQ248202	99
F. proliferatum	D2	Italy	EU151485	99
F. proliferatum	A545	China	KX463001	99
F. proliferatum	F8	China	KU133370	99
F. proliferatum	FP15	Algeria	KR856363	99
F. proliferatum	P9-94	Brazil	KJ439117	99
F. proliferatum	NY-XX-A-08-I-	China	HM590497	99
F. proliferatum	MW10	China	KT803067	99
F. proliferatum	CBS	Netherlands	KM231816	99
F. proliferatum	-	China	KC466547	99
F. proliferatum	189MC/F	Malaysia	GU066729	99
F. proliferatum	165PG/F	Malaysia	GU066714	99
F. proliferatum	38MC/F	Malaysia	GU066624	99
F. proliferatum	PO1	Italy	EU151487	99

Table 4. Comparison of IDs downloaded from the NCBI database to my own isolate of *rhizoctonia solani* isolate of whole region (ITS1, 5.8S rDNA and ITS4) of *R. solani* isolated in this study, with that of other *R. solani* isolates/ strains available at NCBI.

Fungus	Isolate/ strain name	Origin	The most similar sequences in GenBank database	
			GenBank accession number	Sequence similarity (%)
*R. solani** (my isolate)	-	Iraq	Registration is in progress	100
R. solani	RKNG9	India	JF701745	100
R. solani	RKNM8	India	JF701744	98
R. solani	AQNOAH	Iraq	KY055374	98
R. solani	RKLC1	India	JF701742	97
R. solani	RUPP93	India	JF701784	96
R. solani	Rae354	Taiwan	AY684921	96
R. solani	AG-Fa	Malaysia	KX674533	96
R. solani	IQ49	Iraq	KF372653	96
R. solani	RKNM3	India	KC997793	96
R. solani	MML4001	India	JX535004	96
R. solani	AYSDIN	Mexico	KX592586	96
R. solani	IQ23	Iraq	KF372645	95
R. solani	IQ40	Iraq	KF372662	95
R. solani	RsolTeaIN1	India	KJ466117	95
R. solani	RDLM6	India	JF701717	94
R. solani	IQ30	Iraq	KF372657	94
R. solani	Babylon	Iraq	KY283953	94
R. solani	RT 5-3	USA	FJ746908	92
R. solani	CHR09-19	China	HQ270173	92
R. solani	RT 8-2	China	FJ746916	92
R. solani	EV_7	USA	KX118354	92
R. solani	KARS02_2_5	USA	KX118362	92
R. solani	RT 8-3	USA	FJ746917	92
R. solani	KARS02_1_9	USA	KX118361	92
R. solani	KARS02_1_8	USA	KX118360	92
R. solani	CR 8	Brazil	KT362074	92
R. solani	BVT_16	USA	KX118335	92
R. solani	RT 8-1	USA	FJ746915	92
R. solani	BVT_20	USA	KX118337	92
R. solani	RUPC95	India	JF701771	92
R. solani	KARS02_1_6	USA	KX118359	92
R. solani	EV_19	USA	KX118351	92
R. solani	RT 5-2	USA	FJ746907	92
R. solani	G14	Costa Rica	JX294349	91

Table 5. Comparison of whole region (ITS1, 5.8S rDNA and ITS4) of *T. atroviride* with other isolates of *T. atroviride* available at NCBI.

Fungus	Isolate/ strain name	Origin	The most similar sequences in GenBank database	
			GenBank accession number	Sequence similarity (%)
*T. atroviride** (my isolate)	AL-Abedy	Iraq (now register)	KX350051	100
T. atroviride	S54	Brazil	MF076590	99
T. atroviride	HG38	China	KX099656	99
T. atroviride	PCW3	South Africa	KY073424	99
T. atroviride	CTCCSJ	China	KU896322	99
T. atroviride	CCTCC: AV4	China	KT588284	99
T. atroviride	CCTCC: AO**6**	China	KT588268	99
T. atroviride	CCTCC: AO**5**	China	KT588268	99
T. atroviride	CCTCC: AO**4**	China	KT588241	99
T. atroviride	CCTCC: AV2	China	KT588236	99
T. atroviride	ZNAF13	China	KR868399	99
T. atroviride	ZNAF5	China	KR868397	99
T. atroviride	ZNBW2	China	KR868393	99
T. atroviride	ZNWPL4	China	KR868391	99
T. atroviride	ZNWPL7	China	KR868388	99
T. atroviride	ZNWPL4	China	KR868391	99
T. atroviride	ZNWPL7	China	KR868388	99
T. atroviride	ZNWPL10	China	KR868387	99
T. atroviride	ZNAF9	China	KR868385	99
T. atroviride	ZNH6	China	KR868382	99
T. atroviride	ZNH2	China	KR868380	99
T. atroviride	ZNAF16	China	KR868378	99
T. atroviride	ZNAF14	China	KR868377	99
T. atroviride	ZNAF12	China	KR868352	99
T. atroviride	ZNWPL5	China	KR868351	99
T. atroviride	ZNR2	China	KR86834	99
T. atroviride	239	China	KX357830	99
T. atroviride	265	China	KX357837	99
T. atroviride	282	China	KX357839	99
T. atroviride	292	China	KX357841	99
T. atroviride	QT21970	China	KY209916	99
T. atroviride	JCM 9410	China	LC228651	99
T. atroviride	10261	South Africa	KX379158	99
T. atroviride	ZNR9	China	KR868356	99
T. atroviride	ZNAF19	China	KR868350	99
T. atroviride	wxm144	China	HM047764	99
T. atroviride	SA2I1F2	Brazil	KX421474	99
T. atroviride	2015005	China	KY484995	99
T. atroviride	ZNAF18	China	KR868392	99

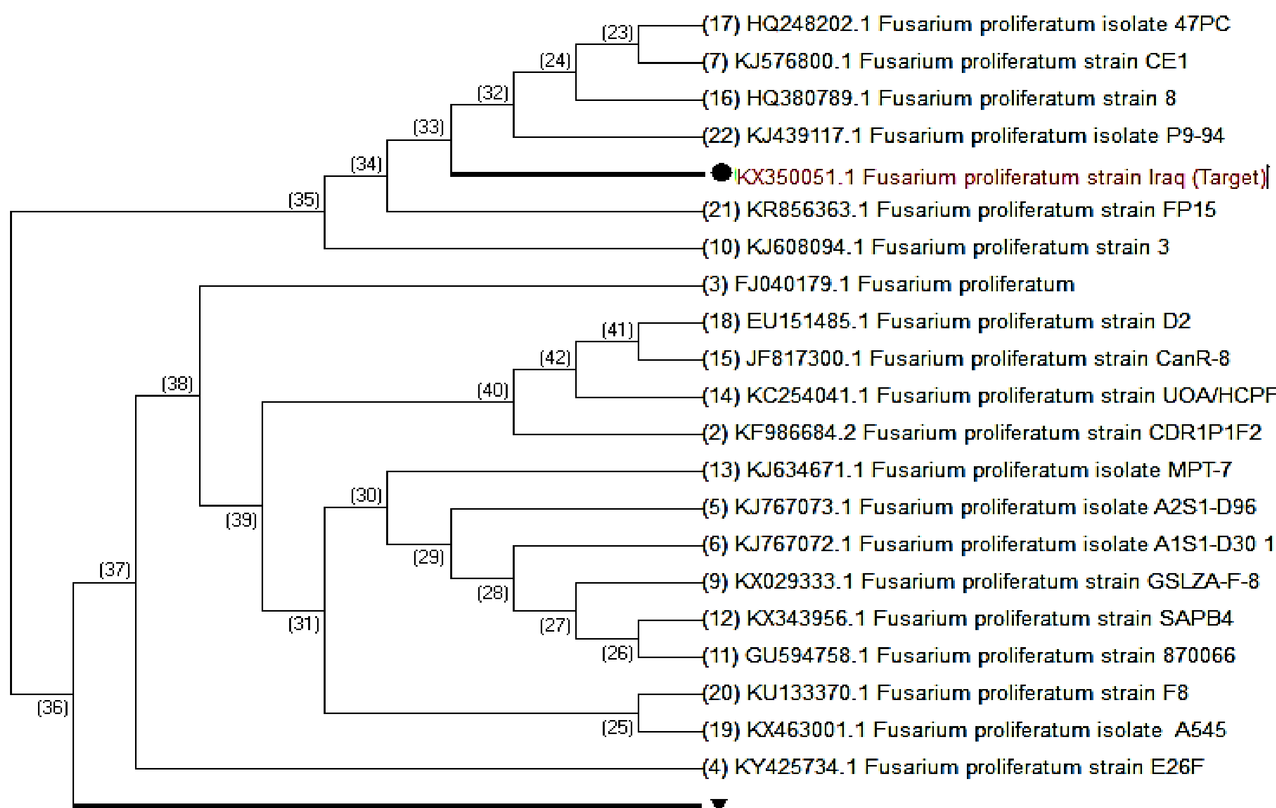

Fig. 5. A phylogenetic tree generated using the Neighbor-Joining Method based on a comparison of the whole ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *F. proliferatum* isolate used in this study, indicated by black dote (●), with those of other *F. proliferatum* isolates available in GenBank (NCBI).

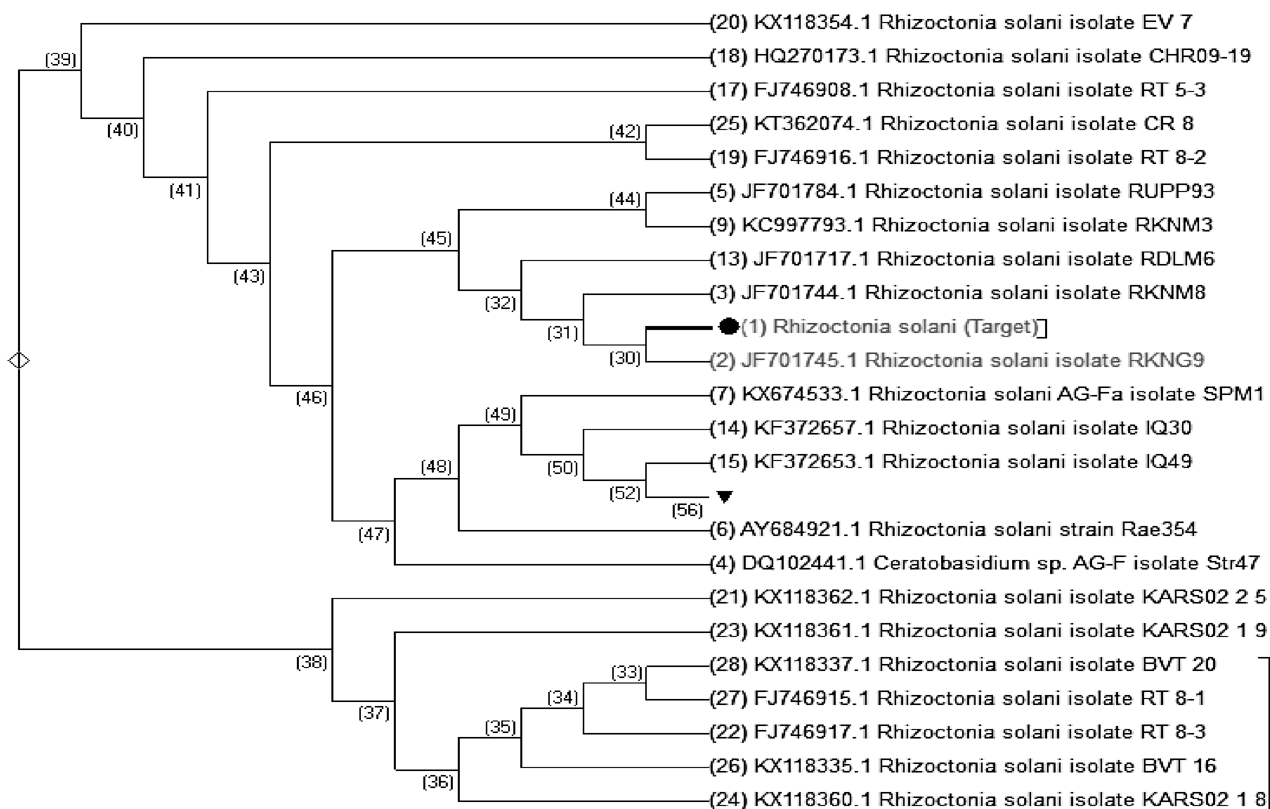

Fig. 6. A phylogenetic tree generated using the Neighbor-Joining Method based on a comparison of the whole ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *R. solani* isolate used in this study, indicated by black dote (●), with those of other *R. solani* isolates available in GenBank (NCBI).

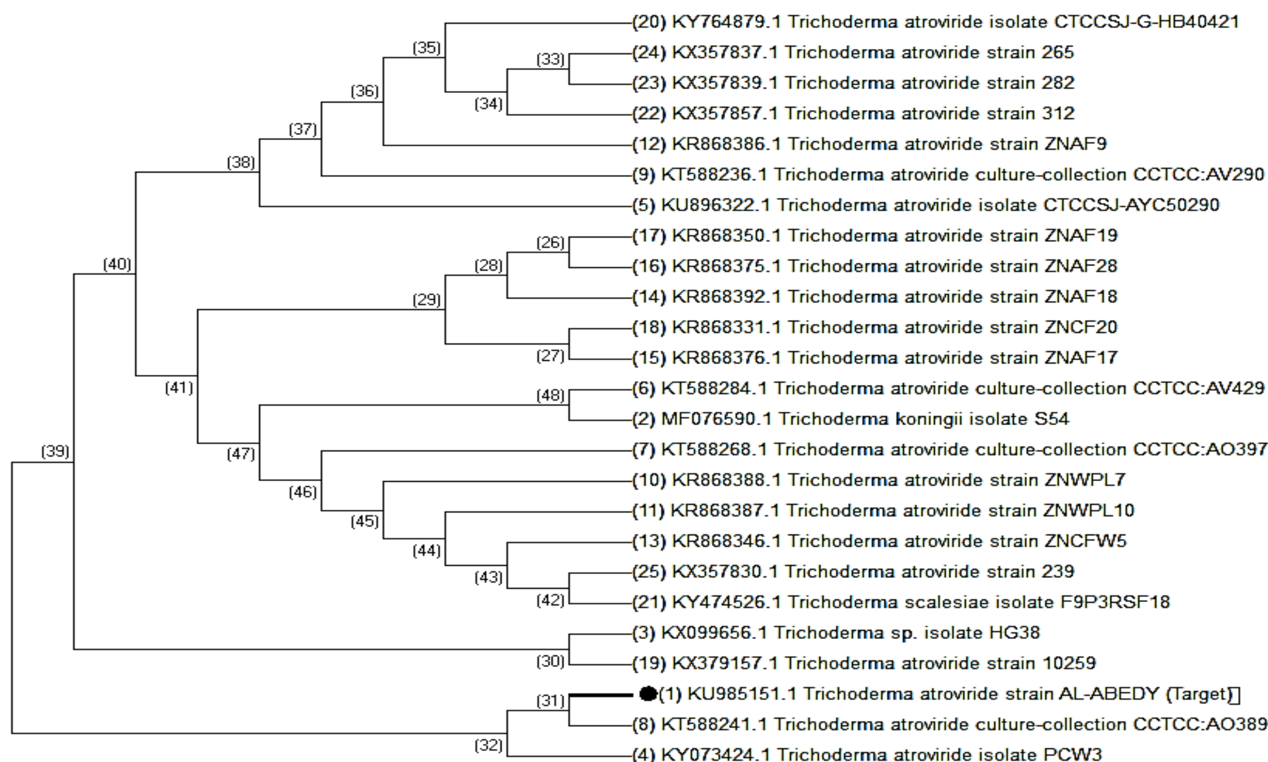

Fig. 7. A phylogenetic tree generated using the Neighbor-Joining Method based on a comparison of the whole ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *T. atroviride* isolate used in this study, indicated by black dote (●), with those of other *T. atroviride* isolates available in GenBank (NCBI).

References

Alaei, H., A.H. Mohammadi and A. Dehghani. 2012. Molecular characterization of the rDNA-ITS sequence and a PCR diagnostic technique for *Pileolaria terebinthi*, the cause of pistachio rust. *Phytopathologia Mediterranea*, 488-495.

Alhussaini, M.S., M.A. Moslem, M.I. Alghonaim, A.A. Al-Ghanayem, A.A.I. AL-Yahya, H.M. Hefny and A.M. Saadabi. 2016. Characterization of *Cladosporium* species by internal transcribed spacer-PCR and microsatellites-PCR. *Pak. J. Biol. Sci*., 7: 143-157.

Arif, M., S. Chawla, N.W. Zaidi, J.K. Rayar, M. Variar and U.S. Singh. 2012. Development of specific primers for genus *Fusarium* and *F. solani* using rDNA sub-unit and transcription elongation factor (TEF-1α) gene. *Afr. J. Biotech*., 11(2): 444-447.

Farr, D.F., G.F. Bills, G.P. Chamuris and A.Y. Rossman. 1989. Fungi on plants and plant products in the United States. APS PRESS: St. Paul, USA. pp. 1-1252.

Giantsis, I.A., A. Chaskopoulou and M.C. Bon. 2017. Direct multiplex PCR (dmPCR) for the identification of six phlebotomine sand fly species (Diptera: Psychodidae), including major leishmania vectors of the mediterranean. *J. Econ. Entmol*., 110(1): 172-182.

Henry, T., P.C. Iwen and S.H. Hinrichs. 2000. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J. Clin. Microb*., 38(4): 1510-151.

Hsuan, H.M., S. Baharuddin and Z. Latiffah. 2011. Molecular identification of *Fusarium* species in *Gibberella fujikuroi* species complex from rice, sugarcane and maize from Peninsular Malaysia. *Int. J. Mol. Sci*., 12: 6722-6732.

Huang, A., J.W. Li, Z.Q. Shen, X.W. Wang and M. Jin. 2016. High-throughput identification of clinical pathogenic fungi by hybridization to an oligonucleotide microarray. *J. Clinic Microbiol*., 44: 3299-3305.

Leslie, J.F. and B.A. Summerell. 2006. The Fusarium Laboratory Manual Blackwell Publishing. *Ames, Iowa*.

Romberg, M.K. and R.M. Davis. 2007. Host range and phylogeny of *Fusarium solani* f.sp. *eumartiifrom* potato and tomato in California. *Plant Dis*., 91: 585-592.

Rossman, A.Y. and M.E. Palm-Hernández. 2008. Systematics of Plant Pathogenic Fungi: Why it matters. *Plant Disease*, 92: 1376-1386.

Siddiquee, S. 2017. Practical handbook of the biology and molecular diversity of *Trichoderma* species from tropical regions, fungal biology. Springer International Publishing AG. DOI 10.1007/978-3-319-64946-7_2.

Stanis C.S., B.K. Song, T.H. Chua, Y.L. Lau and J. Jelip. 2016. Evaluation of new multiplex PCR primers for the identification of *Plasmodium* species found in Sabah. *Turk. J. Medical Sci*., 46: 207-218.

Walch, G., M. Knapp, G. Rainer and U. Peintner. 2016. Colony-PCR is a rapid method for DNA amplification of Hyphomycetes. *J. Fungi*, 2: 12.

Wang, Y.K., Y.X. Shi, B.J. Li and H.M. Chen. 2008. Studies on identification and expeditious detection of cucumber *Fusarium* wilt. *China Vege*., 11: 18-22.

White, T.J., S. Runs and J.W Taylor. 1990. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Can. J. Bot*., 69(1): 180-190.

Wulff, E.G., J.L. Sorensen, M.L Übeck, K.F. Nielsen, U. Thrane and J. Torp. 2010. *Fusarium* spp., associated with rice bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environ. Microb*., 12: 649-657.

Zakiah, R.M., M.A. Ali, M.A. Shahidul, M.R. Asif, M.B. Rejwan, M.M. Salim, M. Khandakar, A. Iftekharuddaula, M.A. Latif and A.I.K. Mohammad. 2016. Morphological and genetic variability among *Rhizoctonia solani* isolates causing sheath blight disease of rice. *Science Direct*, 23(1): 42-50.

Zhang, S., X. Zhao, Y. Wang, J. Li, X. Chen, A. Wang and J. Li. 2012. Molecular detection of *Fusarium oxysporum* in the infected cucumber plants and soil. *Pak. J. Bot*., 44(4): 1445-1451.

Zhang, Z., S. Schwartz, L. Wagner and W. Miller. 2000. A greedy algorithm for aligning DNA sequences. *J. Computational Biol.*, 7(1-2): 203-214.

(Received for publication 29 August 2018)