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Identification and diversity of *Fusarium* species isolated from tomato fruits

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Abstract: Fruit rot of tomato is a serious disease caused by *Fusarium* species. Sampling was conducted throughout Selangor, Malaysia and fungal species identification was conducted based on morphological and gene encoding translation elongation factor 1-α (*tef1-α*) sequence analysis. Five species of *Fusarium* were discovered namely *F. oxysporum* (including *F. oxysporum* f. sp. *lycopersici*), *F. solani, F. equiseti, F. proliferatum* and *F. verticillioides*. Our results provide additional information regarding the diversity of *Fusarium* species associated with fruit rot disease of tomato.

Key words: diversity, fruit rot, *Fusarium oxysporum*, *Gibberella fujikuroi*, translation elongation factor

Introduction

Tomato (*Lycopersicon esculentum* Mill.) contains many nutrients for human health such as dietary lycopene, β-carotene, carotenoids, vitamins C and E, potassium, fibre and antioxidant elements (Britt and Kristin 2011; Troncoso-Rojas *et al.* 2013). However, tomato plants are often infected by field and storage rot disease pathogens. There are several factors leading to these diseases such as poor packaging and improper management. These factors can cause the fruit at the market to wilt and squash easily (Taskeen-Un-Nisa *et al.* 2011; Xie *et al.* 2012). According to Kristensen *et al.* (2005), the most widely distributed pathogen of tomato fruit rot is *Fusarium* species. *Fusarium* species can produce mycotoxins such as moniliformin, fumonisins B_1, zearalenone, beuvericin and trichothecenes (Wiśniewska *et al.* 2011; Zainudin *et al.* 2015), which are important issues for human health.

Abu Bakar *et al.* (2013) reported that there are five *Fusarium* species, which have only been tentatively identified based on morphological characteristics. They also identified the causal agents of fruit rot of tomato in Malaysia that include *Fusarium semitectum, F. oxysporum, F. equiseti, F. subglutinans* and *F. solani*. Realising that information about the diversity of *Fusarium* species associated with fruit rot of tomato based on gene sequencing is limited in tropical areas we have taken the initiative to characterise some isolates of *Fusarium* species based on gene encoding translation elongation factor 1-α (*tef1-α*) sequence. Translation elongation factor 1-α (*tef1-α*) is an important part of protein translation machinery, which is a good single locus identification tool in *Fusarium* because it shows high sequence polymorphism among closely related species (Nitschke *et al.* 2009; Amatulli *et al.* 2010; Walker *et al.* 2012).

The correct identification of species associated with post-harvest fruit rot of tomato will affect the country's quarantine regulations. Therefore, the aim of this study was to characterise the *Fusarium* strains associated with post-harvest fruit rot of tomato based on morphological characteristics and *tef1-α* gene sequence analysis.

Materials and Methods

Sampling and fungal isolation

Fruit rot of tomato was observed and the tomato samples were obtained from markets throughout Selangor, Malaysia. Ten tomato samples showing fruit rot symptoms were collected from different individual markets. The symptoms observed were similar to those reported in previous literature, namely the rotted tissue was often water-soaked and covered by white to pinkish mycelia externally while the infected tissue was found to be discoloured and watery. Samples, 5 × 5 mm from the border of symptomatic and asymptomatic tissue, were cut. The surface of diseased samples was sterilized with 1% sodium hypochlorite. Samples were then rinsed in sterile distilled water. All disinfected samples were placed onto peptone pentachloronitrobenze agar (PPA) and incubated at 28°C for five days.

Fungal purification and morphological characterization

The fungal cultures were transferred to Water Agar (WA) for single conidial isolation. The conidial suspensions of the cultures were streaked into 4% WA, which was then incubated for 24 h. A hyphal tip derived from a single colony was cut and transferred onto new Potato Dextrose

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Agar (PDA), and then incubated for 7 days. Pure cultures of *Fusarium* isolates were subcultured and maintained as a conidial suspension in 25% glycerol of Complete Medium with Xylose (CMX) at –80°C.

All purified isolates were tentatively identified based on morphological characteristics. The isolates were cultured on PDA and incubated at 25°C with 12 h fluorescent light/12 h darkness for 7 days. Macroscopic characteristics, which were observed included colony features, color and growth rate. The pure isolates were also cultured on Carnation Leaf Agar (CLA). Microscopic characteristics such as conidia shape and size were examined using a microscope (Carl Zeiss, Germany) (Leslie and Summerell 2006).

DNA extraction and polymerase chain reaction (PCR) amplification

DNA extraction was performed using the Ultra Clean® Microbial DNA isolation kit (MO-BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR amplification of *tef1-α* was performed using a TProfessional Standard Thermocycler (Biometra Company); 25 µl reaction master mix that contained 5× PCR buffer, 0.5 µM of primer, 0.2 mM dNTPs, 2.5 mM magnesium chloride ($MgCl_2$), 0.125 unit of Taq Polymerase (Promega Corporation, 2012) and 20 ng of DNA template. A set of primers was used; EF1 (5'-ATGGGTAAGGAGGACAAGAC-3') and EF2 (5'-GGAAGTACCAGTGATCATGTT-3') to amplify 700 bp fragment (Geiser *et al.* 2004). The PCR cycling was conducted as follows: initial denaturation at 94°C for 85 s, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 57°C for 55 s, extension at 72°C for 90 s, final extension at 72°C for 10 min.

Nucleotide sequencing and analysis

Products of amplification were electrophoresed while the gels were stained with ethidium bromide (EtBr) and the DNA bands were visualised under ultraviolet (UV) light. The images were captured with DOC PRINT system (Vilber Lourmat, USA). Target DNA fragment band with sized 700 bp (Nitschke *et al.* 2009) was excised from the gel, followed by the purification of PCR products using QIAquick® Gel Extraction Kit (QIAGEN, USA) following the manufacturer's recommendations.

The purified PCR products were sequenced for the *tef1-α* gene region using ABI3730XL sequencer (MyTACG Bioscience Company, MY). The comparison between *tef1-α* gene sequence of analysed isolates and the sequences in GenBank was done using the Standard Nucleotide BLAST network services for similarities present in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). ClustalW in MEGA software version 6.06 was used to generate the consensus sequences for alignment (Tamura *et al.* 2013).

A phylogenetic tree was also generated using MEGA software analysis version 6.06. Maximum likelihood (ML) analysis was performed and the tree was built with Tamura-Nei model. One thousand bootstrap replicates were performed to assess the stability and robustness of each branch of the phylogenetic tree (Tamura *et al.* 2013). All assembled sequences were then deposited to GeneBank, NCBI (http://www.ncbi.nlm.nih.gov/). A *tef1-α* sequence of *Colletotrichum acutatum* (JQ958570) from GenBank was used as an out-group and sequences of *tef1-α* gene of *Fusarium* spp. from NCBI were used as references for the strains identified in this study.

Results

Morphological characteristics

Based on morphological characteristics, the most important features of *F. oxysporum* are the formation of macroconidia with short-medium length, thick-walled, curved dorsal curvature and a slightly straighter ventral surface, tapered and curved with hook apical cells and foot-shaped basal cells. Microconidia are oval to reniform-shaped and frequently produced in alse heads mycelia on short monophialides. Other characteristics considered are white to pale violet of abundant mycelia and white tinged with purple pigments in the agar. Meanwhile, chlamydospores are oval, which were formed singly and in pairs terminally and intercalary with smooth and rough walls. Macroconidia were observed to obtain 3–5 septa, while microconidia have only 0–1 septum. No difference in morphological characteristics was observed among *F. oxysporum* races.

The most conspicuous microscopic feature of *F. solani* is its ability to produce 0–1 septum oval to reniform-shaped microconidia in false heads mycelia that usually are borne on long monophialides. Macroconidia were found with 3–5 septa, having apical cells that are curved and rounded as well as foot-shaped basal cells. The colonies on PDA were white to cream coloured with sparse mycelia, which produced cream to light yellow pigments. Chlamydospores were found to be singly, intercalary and terminal.

Fusarium proliferatum produced thin-walled, slender and rather straight macroconidia. The apical cell was curved and usually formed with 3–5 septates. Microconidia were oval to obovoid-shaped and arose in false heads and moderate length chains. They were derived from monophialides and also polyphialides conidiogenous cells. Chlamydospores were absent in the culture of *F. proliferatum*. On PDA, *F. proliferatum* initially produced white aerial mycelia and became cottony with a light orange pigment.

Macroconidia of *F. verticillioides* were slender, slightly straight, thin-walled and typically formed with three septates. Their apical cell was blunted and barely notched on the basal cell. The microconidia were oval to obovoid-shaped. Compared with cultures of *F. proliferatum, F. verticillioides* produced longer chains of microconidia, which also arose in false heads. Furthermore, the culture of *F. verticillioides* generated microconidia that were only derived from monophialides conidiogenous cells. No polyphialides were produced by *F. verticillioides. Fusarium verticillioides* culture did not produce chlamydospores. However, they produced swollen cells that can easily be misidentified as chlamydospores. The cultures were cottony flat, which initially grew as white mycelial growth

and covered the PDA plate after nine days of incubation, with orange pigmentation.

The cultures of *F. equiseti* produced long macroconidia, thick-walled, sickle-shaped and curved dorsiventral curvature, tapered, whip-like apical cells and elongated foot-shaped basal cells. Meanwhile, the colonies produced abundant white and brown mycelia and pale brown pigments on PDA. Chlamydospores were found to be oval and formed singly, in pairs, in chains or in clumps. Furthermore, microconidia were formed in false heads.

Molecular characteristics, sequencing and phylogenetic analysis

Species identification of *Fusarium* species was confirmed by the sequences of *tef1-α*. Results showed a clear single band that was amplified and corresponded to the expected molecular fragment size of the *tef1-α* region. No band was observed in the control, where DNA was replaced with water. These PCR amplification results made it possible to predict that all tested strains belonged to the genus *Fusarium*.

The Standard Nucleotide BLAST search for similarities showed that the similarity percentage of the strains ranged from 98 to 99%. The *tef1-α* sequence of all *Fusarium* strains were searched for homology sequences in GeneBank database. The similarities of *tef1-α* sequence of our strains with the sequences from the GeneBank used as references were supported by bootstrap values of more than 70% as stated in this study. All the analysed sequences data were deposited in the GeneBank database (Table 1). The phylogenetic tree was divided into

Table 1. List of *Fusarium* strains isolated from post-harvest fruit rot of tomato in Selangor, Malaysia analysed based on *tef1-α* gene sequence and pathogenicity test

No.	Strains number	Locations	GeneBank accession number	Species identification based on *tef1-α* gene sequence
1	B616T	Serdang	KM886217	*F. solani*
2	B627T	Serdang	KM886223	*F. solani*
3	B664T	Serdang	KM886218	*F. solani*
4	B780T	Sungai Buloh	KM886219	*F. solani*
5	B684T	Kajang	KM886224	*F. solani*
6	B698T	Kajang	KM886220	*F. solani*
7	B777T	Sungai Buloh	KM886221	*F. solani*
8	B722T	Semenyih	KM886222	*F. solani*
9	B702T	Kajang	KM886226	*F. solani*
10	B752T	Bandar Sunway	KM886227	*F. solani*
11	B1349T	Serdang	KM886212	*F. equiseti*
12	B1350T	Serdang	KM886225	*F. solani*
13	B1351T	Ampang	KM886229	*F. solani*
14	B1352T	Ampang	KM886230	*F. solani*
15	B1353T	Ampang	KM886231	*F. solani*
16	B1354T	Kajang	KM886232	*F. solani*
17	B1355T	Ampang	KM886233	*F. solani*
18	B1356T	Kajang	KM886228	*F. solani*
19	B633T	Serdang	KM886238	*F. oxysporum* f. sp. *lycopersici*
20	B645T	Serdang	KM886239	*F. oxysporum* f. sp. *lycopersici*
21	B688T	Kajang	KM873335	*F. oxysporum*
22	B696T	Kajang	KM873336	*F. oxysporum*
23	B711T	Kajang	KM886241	*F. oxysporum* f. sp. *lycopersici*
24	B713T	Kajang	KM886240	*F. oxysporum* f. sp. *lycopersici*
25	B717T	Semenyih	KM886236	*F. oxysporum* f. sp. *lycopersici*
26	B718T	Semenyih	KM886237	*F. oxysporum* f. sp. *lycopersici*
27	B725T	Puchong	KM886213	*F. oxysporum*
28	B635T	Serdang	KM873337	*F. oxysporum*
29	B622T	Serdang	KM886215	*F. oxysporum*
30	B695T	Serdang	KM886216	*F. oxysporum*
31	B1358T	Selayang	KM886214	*F. oxysporum*
32	B1357T	Serdang	KM873334	*F. proliferatum*
33	B1359T	Selayang	KM886234	*F. verticillioides*
34	B1360T	Selayang	KM886235	*F. verticillioides*

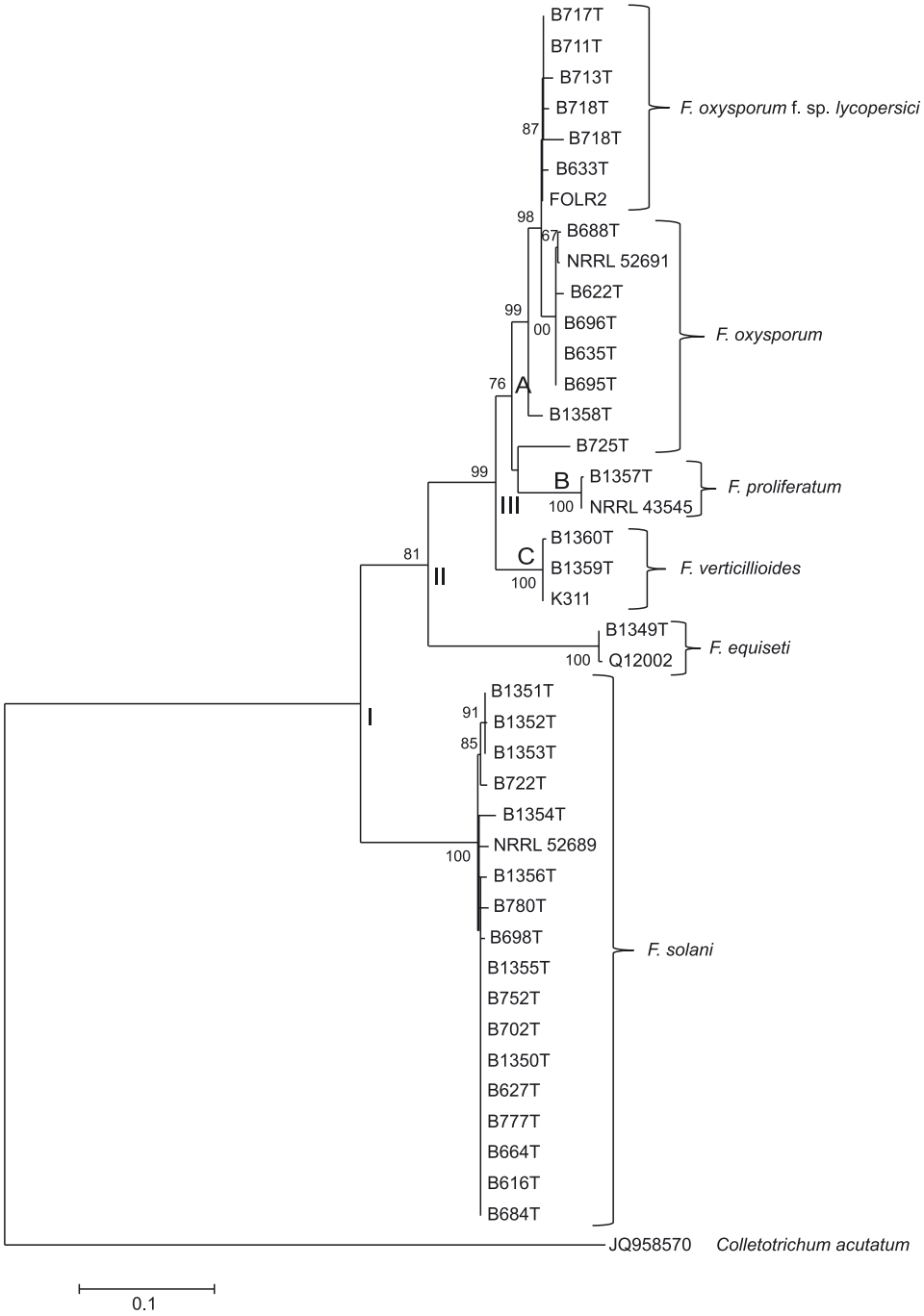

Fig. 1. Phylogenetic tree showing the relationship of 34 *Fusarium* species strains based on *tef1-α* gene sequence using the maximum likelihood method. The percentage of replicate trees in which the linked taxa clustered together in the bootstrap test (1,000 replicates). The bootstraps values > 70% were shown next to the branches. Accession numbers of FOLR2 (DQ837692), NRRL 52691 (JF740776), NRRL 43545 (EF452971), K311 (KF562131), Q12002 (KF208617) and NRRL 52689 (JF740774). *Colletotrichum acutatum* was used as an out-group. I, II, III – clades; A, B, C – subclades

three main clades, known as I, II and III (Fig. 1). Clade I shows that all the strains demonstrated high similarities of *tef1-α* gene sequence to each other and to the referred *F. solani* (NRRL52689). All strains were well supported by the strongest bootstrap value of 100%. On the other hand, Clade II that consists of only single strain B1349T also gave high similarity of *tef1-α* gene sequence to the referred *F. equiseti* (Q12002) from NCBI and was supported by the strongest bootstrap value of 100%. Furthermore, Clade III contains three subclades of A, B and C. Strains grouped in subclade A have high similarities of *tef1-α* gene sequence to each other and to the control strains of *F. oxysporum* (NRRL52691) and *F. oxysporum* f. sp. *lycopersici* (FOLR2). In this analysis, subclade A was supported by the bootstrap value of 76%. This also includes six strains namely B713T, B711T, B718T, B717T, B645T and B633T that belong to the *formae speciales* of *lycopersici* supported by the bootstrap value of 87%. Subclade B contains only a single strain B1357T, which also gave high similarity of *tef1-α* sequence to the referred *F. proliferatum* (NRRL43545) that was supported by the strongest bootstrap value of 100%. Morphology characters confirmed the species identity

of isolate B725T since it was grouped separately from other isolates of *F. oxysporum*. Moreover, subclade C that comprises two strains, B1360T and B1359T also gave the strongest bootstrap value of 100%, which referred with *F. verticillioides* (K311).

Discussion

In the present study, a combination of morphology and *tef1-α* gene sequencing were used to identify the isolates into species levels. Six isolates were identified into *formae speciales* based on *tef1-α* gene sequence. There are limits to the use of morphological characters for identification of species in *Gibberella fujikuroi* species complex and *F. oxysporum* as some species may share similar morphological characteristics, for example *F. proliferatum* and *F. verticillioides*. This study provides an update regarding the diversity of *Fusarium* species associated with fruit rot of tomato. Abu Bakar *et al.* (2013) identified five species of *Fusarium* associated with fruit rot disease of tomato in Malaysia, which are *F. oxysporum, F. solani, F. semitectum, F. subglutinans* and *F. equiseti*. However, in this study, we isolated and identified two other species: *F. proliferatum* and *F. verticillioides*.

Based on morphological characteristics, *F. proliferatum* is most likely to be confused with *F. verticillioides* where they may be found in chains of varying length (Leslie and Summerell 2006). Moreover, relying only on the presence of polyphialides is not rigid enough evidence to differentiate *F. proliferatum* and *F. verticillioides*. Misidentification between these two species of *Fusarium* often occurs since they have similar pigmentation, shape of macroconidia and microconidia and the formation of a false head. Thus, a molecular method, was used to differentiate DNA sequences of gene, to support morphological identification between *F. proliferatum* and *F. verticillioides*. This also happens in the identification of *F. oxysporum* isolates into *formae speciales*. Many *F. oxysporum* isolates appear to be host specific (Leslie and Summerell 2006). Over 100 *formae speciales* and races of *F. oxysporum* have been described. In this study, we found six isolates which belonged to *F. oxysporum* f. sp. *lycopersici*.

Based on the generated phylogenetic tree in this study, *F. oxysporum* including *F. oxysporum* f. sp. *lycopersici, F. verticillioides* and *F. proliferatum* were grouped in Clade III, which was named the *Gibberella* clade that further divided into two different sections, where, *F. oxysporum* was located in section Elegans, while the *F. verticillioides* and *F. proliferatum* were located in section Liseola. They were then grouped into different subclades as A, B, and C, respectively. For Clade I and II, *F. equiseti* and *F. solani* have singly occurred without any subclade grouping. However, the subclades grouping in Clade III showed that the *F. oxysporum*, *F. verticillioides* and *F. proliferatum* are not closely related to each other since they are from a different *Fusarium* species complex. *Fusarium oxysporum* is a member of the *F. oxysporum* species complex, while *F. verticillioides* and *F. proliferatum* are members of the *Gibberella fujikuroi* species complex (GFC) (Kvas *et al.* 2009). Even though *F. verticillioides* and *F. proliferatum* are in the same section of Liseola, based on the generated tree, they were formed by two separated subclades. The GFC is made-up of three different clades known as Asian, American and African clades. On the other hand, *F. verticillioides* is from the African clade, while *F. proliferatum* is from the Asian clade as stated in the previous study conducted by Kvas *et al.* (2009).

In addition, Geiser *et al.* (2004) also classified *F. verticillioides* and *F. proliferatum* as GFC, while the other *Fusarium* species as the member of respective species complex where *F. oxysporum* is *F. oxysporum* and relatives' species complex, *F. solani* is a member of the *F. solani* species complex and *F. equiseti* is a member of the *F. equiseti* species complex. These findings are consistent with the previous study conducted by Watanabe *et al.* (2011), which reported that *F. oxysporum, F. verticillioides* and *F. proliferatum* are in the same clade (*Gibberella*), but in different sections of Elegans and Liseola, respectively. Whereas *F. solani* and *F. equiseti* are in two other clades as well as in a different section of Martiella and Ventricosum, and Gibbosum when referring to their ML tree of *Fusarium* genus and related genera inferred from the combined sequences of rDNA cluster and the *β*-tub and *tef*1-*α* genes. Many taxonomic studies based on morphological characteristics have reported that some "sections" including closely related species share some "synapomorphic" character states. Nitschke *et al.* (2009) also stated in the resulting dendrogram of their study that the *F. oxysporum, F. proliferatum* and *F. verticillioides* are grouped in the same cluster or clade.

As for the *F. solani* and *F. equiseti*, they are totally unrelated to the other three *Fusarium* species in Clade III since they are clearly located in different clades, which are Clade I (section Martiella and Ventricosum) and II (section Gibbosum), respectively. Furthermore, both *Fusarium* species are from different species complexes, where *F. solani* is a member of *F. solani* species complex, while *F. equiseti* is a member of other lineages of the *F. equiseti* species complex.

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