Fungal Microbiology

Morphological and Phylogenetic Analysis of *Fusarium solani* Species Complex in Malaysia

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Received: 20 May 2014 / Accepted: 2 September 2014 / Published online: 20 September 2014
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Abstract Members of *Fusarium solani* species complex (FSSC) have been known as plant, animal, and human pathogens. Nevertheless, the taxonomic status of such an important group of fungi is still very confusing and many new species as well as lineages have been elucidated recently. Unfortunately, most of the new taxa came from temperate and subtropical regions. Therefore, the objectives of the present study were to identify strains of FSSC recovered from different sources in Malaysia. In the present study, 55 strains belonging to the FSSC were examined and phylogenetically analyzed on the basis of internal transcribed spacer (ITS) regions and partial translation elongation factor-1 (TEF-1α) sequences. Based on morphological features, a total of 55 strains were selected for molecular studies. Based on morphological features, the strains were classified into four described *Fusarium* species, namely *Fusarium keratoplasticum*, *Fusarium falciforme*, FSSC5, and *Fusarium cf. ensiforme*, and one unknown phylogenetic species was introduced. Although the data obtained from morphological and molecular studies sufficiently supported each other, the phylogenetic trees based on ITS and TEF-1α dataset clearly distinguished closely related species and distinctly separated all morphological taxa. All members of FSSC in this research were reported for the first time for Malaysian mycoflora.

Introduction

Most members of the *Fusarium solani* species complex (FSSC) are frequently isolated from soils and act as decomposers, but some are putative parasites on plants, insects, humans, and animals [1–4]. Wollenweber and Reinking [5] divided members of *F. solani* into two sections of *Ventricosum* and *Martiella*. Snyder and Hansen [6] represented FSSC as a complex species in the *Martiella* section [1, 7, 8]. Seven mating populations (MPI–VII) were determined for *F. solani* [9–12]. Members of FSSC mating populations MPI, MPV, and MPVI were placed in distinct groups by phylogenetic analysis [13]. Molecular phylogenetic demonstrated that FSSC MPI and MPV as *F. solani* f. sp. *cucurbitae* races 1 and 2, respectively were polyphyletic [14, 15].

Because of the significant role of members of the FSSC in clinical infection and complication in their determination, molecular identification strategies have been emphasized for their identification in the last 20 years [2, 3]. Phylogenetic analysis by 28S ribosomal DNA, internal transcribed spacer (ITS) regions, and *tef1* gene sequences revealed high variability within members of the FSSC, and all 55 diagnosable species were divided into three clades, termed clades 1, 2, and 3 [3, 15–17]. Members of clade 1 comprised two known species (*Fusarium illudens* and *Nectria plagianthi*) from New Zealand. Members of clade 2 included a number of important pathogens that cause sudden death syndrome (SDS) of soybean [18–20]. Nalim et al. [17] used molecular phylogeny to show that members of FSSC in clade 2 are paraphyletic. Molecular phylogeny showed diverse phylogenetic affinities among members of clade 3. This group encompassed many species that are important in agricultural crops and medicine [15]. Members of clade 3 are the most common group of fusaria associated with plant diseases and human infections. Members of *Fusarium falciforme* (FSSC 3+4) and *Fusarium keratoplasticum* as most haplotype-diverse species were placed among clade 3 [2, 3, 15, 21, 22]. Several studies to date have revealed different phylogenetic species within this important evolutionary clade, though little work has been done to improve the taxonomy, and therefore correct identification of species as one of the prerequisites in any disease...
control program has become more challenging. Although the taxonomic status of FSSC from all over the world is being revised and a strong connection has been revealed among strains recovered from humans, insects, and plants [2, 17, 21–24], until today, no attempt has been made to classify members of the FSSC in tropical Southeast Asia, particularly Malaysia. Therefore, the objectives of this study were to identify strains of FSSC recovered from different substrates in Malaysia by using morphological characteristics and sequencing of ITS region and translation elongation factor-1α (TEF-1α) to determine genetic relationship among them.

**Materials and Methods**

**Strains of Fusarium**

A total of 55 strains were selected for the present study. Strains were obtained from *Fusarium* culture collection of the School of Biological Sciences, Universiti Sains Malaysia. A list of species names and culture collection numbers, geographical origins, original substrates, and GenBank accession numbers of the *tef1* and ITS regions of the strains used in this study is in Table 1. To study the pigmentation and growth rates, all strains were transferred onto fresh potato dextrose agar (PDA) plates.

### Table 1

| Culture no. | Species                  | Host/substratum | Plant part (symptom) | Location | *tef1*a  | ITS*b  |
|-------------|--------------------------|-----------------|----------------------|----------|----------|--------|
| USM FSSC-C4383B | *F. c.f. solani*         | Bean            | Root rot             | Pahang   | KC161396 | KC009602|
| USM FSSC-R77O  | *F. c.f. solani*         | Corn            | Seed                 | Perlis   | KC161404 | KC009610|
| USM FSSC-R73O  | *F. c.f. solani*         | Corn            | Leaf (spot)          | Perlis   | KC161402 | KC009608|
| USM FSSC-D8256C | *F. c.f. solani*         | Red chili pepper| Leaf (spot)          | Kelantan | KC161386 | KC009592|
| USM FSSC-T8432C | *F. solani*              | Red chili pepper| Stem (lesion)        | Terengganu| KC161388 | KC009594|
| USM FSSC-Q6002D | *F. solani*              | Pepper          | Stem (lesion)        | Sarawak  | KC161414 | KC009620|
| USM FSSC-Q729D  | *F. solani*              | Pepper          | Stem (lesion)        | Sarawak  | KC161418 | KC009624|
| USM FSSC-Q1033Q | *F. solani*              | Sorghum         | Root (rot)           | Sarawak  | KC161421 | KC009627|
| USM FSSC-Q1017Q | *F. solani*              | Sorghum         | Root (rot)           | Sarawak  | KC161399 | KC009605|
| USM FSSC-P86S   | *F. solani*              | Soil            | –                    | Penang   | KC161393 | KC009599|
| USM FSSC-P104S  | *F. solani*              | Soil            | –                    | Penang   | KC161391 | KC009597|
| USM FSSC-P200S  | *F. solani*              | Soil            | –                    | Penang   | KC161390 | KC009596|
| USM FSSC-A2073S | *F. solani*              | Soil, Watermelon| –                   | Perak    | KC161412 | KC009618|
| USM FSSC-D310T  | *F. solani*              | Tobacco         | Stem (rot)           | Kelantan | KC161394 | KC009600|
| USM FSSC-J872T  | *F. solani*              | Tobacco         | Stem (rot)           | Johor    | KC161400 | KC009606|
| USM FSSC-K600T  | *F. solani*              | Tobacco         | Stem (rot)           | Kuala Lumpur | KC161393 | KC009604|
| USM FSSC-D216T  | *F. solani*              | Tobacco         | Stem (rot)           | Kelantan | KC161392 | KC009598|
| USM FSSC-J3516Gr | *F. solani*              | Grass           | Root (rot)           | Johor    | KC161385 | KC009591|
| USM FSSC-S2253Pu | *F. solani*              | Pumpkin         | Fruit (canker)      | Sabah    | KC161410 | KC009616|
| USM FSSC-D5281Pu | *F. solani*              | Pumpkin         | Fruit (canker)      | Sabah    | KC161409 | KC009615|
| USM FSSC-A1969W | *F. solani*              | Watermelon      | Root (rot)           | Perak    | KC161397 | KC009603|
| USM FSSC-Q1094W | *F. solani*              | Watermelon      | Root (rot)           | Sarawak  | KC161395 | KC009601|
| USM FSSC-T617R  | *F. solani*              | Rice            | Root (rot)           | Terengganu| KC161416 | KC009622|
| USM FSSC-S2257W | *F. solani*              | Wood            | Stem (lesion)        | Sabah    | KC161415 | KC009621|
| USM FSSC-T921T  | *F. solani*              | Tobacco         | Stem (rot)           | Terengganu| KC161403 | KC009609|
| USM FSSC-A1881W | *F. solani*              | Watermelon      | Root (rot)           | Perak    | KC161419 | KC009625|
| USM FSSC-T2550W | *F. solani*              | Watermelon      | Root (rot)           | Terengganu| KC161422 | KC009628|
| USM FSSC-B1770S | *F. falciforme*          | Soil            | Tree bark            | Selangor | JX935592 | JX982559|
| USM FSSC-A1444S | *F. falciforme*          | Soil            | –                    | Perak    | JX935593 | JX982560|
| USM FSSC-D436S  | *F. falciforme*          | Soil            | –                    | Kelantan | JX935594 | JX982561|
| USM FSSC-S2216Ru | *F. falciforme*          | Tree bark       | Tree bark            | Sabah    | JX935586 | JX982553|
| USM FSSC-S2228Tb | *F. falciforme*          | Tree bark       | Tree bark            | Sabah    | JX935588 | JX982555|
| USM FSSC-S2237Ne | *F. falciforme*          | Soil            | –                    | Sabah    | JX935587 | JX982554|
| USM FSSC-S2238Ne | *F. falciforme*          | Soil            | –                    | Sabah    | JX935585 | JX982552|
| USM FSSC-S2236M  | *F. falciforme*          | Soil            | –                    | Sabah    | JX935584 | JX982551|
and incubated under 12 h alternating light (black/white) at 25 ±2 °C for 1 week. For microscopic observations, all strains were transferred to carnation leaf-piece agar (CLA) plates and incubated under 12 h alternating light (black/white) at 25 ±2 °C for 1 week. Thirty randomly selected conidia of each septation class (macro- and microconidia) were measured and analyzed by two-sample t test using MINITAB® 15. For species determination, the descriptions by Summerbell and Schroers, Nalim et al., and Short et al. were adopted.

DNA Extraction

All FSSC strains were grown on PDA with sterile dialysis membranes for 5 days. The mycelium grown over the membranes were harvested and grounded in a sterile mortar with liquid nitrogen to a fine powder. DNA extraction was done by using The DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer’s instructions.

PCR Amplification

Amplification of the TEF-1α gene and ITS regions was conducted using primer pair ef1 and ef2 for TEF-1α [16] and ITS1 and ITS4 for the ITS regions [28]. Polymerase chain reaction (PCR) was performed in a Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) in a total volume of 25 μl for each strain. The PCR mixture contained 4 μl 5× buffer (Promega, Madison, WI, USA), 4 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate (dNTP; Promega), 0.8 μM each primer, 0.75 units of Taq DNA polymerase (Promega), and 6 ng of template DNA. DNA amplification of TEF-1α was performed with an initial denaturation of 1 min at 94 °C followed by 35 cycles of 30 s at 95 °C, 55 s at 59 °C, and 90 s at 72 °C. The PCR for ITS regions was performed at 95 °C (2 min) for a hot start, followed by 35 cycles of 94 °C (1 min), 56 °C (30 s), 72 °C (2 min), and a final extension of 72 °C (10 min). PCR products were purified using Qiagen columns according to the manufacturer’s protocol and sent for sequencing to a service provider.

Sequencing Alignment and Phylogenetic Analysis

The program Molecular Evolutionary Genetic Analysis software, ver. 4.0 (MEGA4.0; http://www.megasoftware.net) was performed in order to edit and align the sequence files, which were manually adjusted. In order to assess the relationships between the major taxa, ambiguous parts of the ITS regions and TEF-1α were removed from further analysis.
A phylogenetic tree was generated using maximum parsimony (MP) in MEGA4.0. Bootstrap values for the maximum parsimony tree (MPT) were calculated for 1,000 replicates. The edited ITS and TEF-α sequences were compared with other available Fusarium species sequences in the GenBank. Furthermore, the sequences of some known species of FSSC were downloaded from GenBank and used to reconstruct a

and more conserved and alignable parts of the region and all genes were used to generate phylogenetic trees containing representative taxa from major groups. The aligned sequences were BLAST in two genome databases, GenBank and Fusarium database, to identify all the 55 strains. In this study, phylogenetic tree was generated using maximum parsimony

| Culture no. | Species identified | Shape of Microconidia | Shape of basal cell and apical cell | Length × width of macroconidia (μm) |
|-------------|--------------------|------------------------|-------------------------------------|-------------------------------------|
| FSSC-R73O   | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 50.3±2.5×5.7±0.5 54.8±2.5×5.9±0.5 |
| FSSC-K600T  | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 47.5±2.5×5.8±0.5 52.5±2.5×5.6±0.5 |
| FSSC-J872T  | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 48.5±2.5×5.6±0.5 54.8±2.5×5.9±0.5 |
| FSSC-R77O   | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 47.5±2.5×5.9±0.5 54.8±2.5×5.9±0.5 |
| FSSC-Q6002D | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 47.0±2.5×5.9±0.5 54.0±2.5×5.9±0.5 |
| FSSC-Q1033Q | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 51.2±2.5×5.6±0.5 52.5±2.5×6.0±0.5 |
| FSSC-Q1071Q | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 48.5±2.5×5.5±0.5 54.8±2.5×5.9±0.5 |
| FSSC-P208S  | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 48.5±2.5×5.5±0.5 54.8±2.5×5.9±0.5 |
| FSSC-C4383B | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 52.2±2.5×5.8±0.5 56.5±2.5×5.9±0.5 |
| FSSC-Q729D  | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 49.3±2.5×5.8±0.5 55.5±2.5×5.9±0.5 |
| FSSC-A2073S | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 48.2±2.5×5.8±0.5 56.5±2.5×5.9±0.5 |
| FSSC-P104S  | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 51.2±2.5×5.8±0.5 56.5±2.5×5.9±0.5 |
| FSSC-Q1094W | *F. cf. solani*    | Oval, reniform, and elongated oval | Foot cell and tapered, curved       | 49.2±2.5×5.8±0.5 56.5±2.5×5.9±0.5 |
| FSSC-J3516 & F. falciforme | Oval, reniform, and elongated oval | Foot cell and tapered, curved | 48.6±2.5×5.6±0.5 57.5±2.5×5.9±0.5 |
| F. falciforme | Oval, reniform, and elongated oval | Foot cell and tapered, curved | 48.0±2.5×5.5 | 5±0.5 | 54.5±2.5×5.9±0.5 |
| FSSC-S2236Mo | *F. falciforme* | Elongated oval and obovoid with a truncate | Barely notched and pointed, curved | 43.5±2.5×5.1±0.5 – |
| FSSC-S2238Ne | *F. falciforme* | Elongated oval and obovoid with a truncate | Barely notched and pointed, curved | 42.5±2.5×5.0±0.5 – |
| FSSC-S2216Ru | *F. falciforme* | Elongated oval and obovoid with a truncate | Barely notched and pointed, curved | 40.5±1.5×5.1±0.5 – |
| FSSC-K418S  | *F. falciforme*    | Elongated oval and obovoid with a truncate | Barely notched and pointed, curved | 41.0±1.0×5.2±0.5 – |
| USM FSSC- P2106S | *F. keratoplasticum* | Oval and elongated and clavate | Notched and blunt | 33.0±2.3×4.9±0.3 – |
| USM FSSC- P2108S | *F. keratoplasticum* | Oval and elongated and clavate | Notched and blunt | 33.0±1.5×4.9±0.3 – |
| USM FSSC- S2138Se | *F. keratoplasticum* | Oval and elongated and clavate | Notched and blunt | 34.0±2.3×4.8±0.3 – |
| USM FSSC- S2126Se | *F. keratoplasticum* | Oval and elongated and clavate | Notched and blunt | 35.0±2.3×4.9±0.3 – |
| USM FSSC- C4651Tb | *F. cf. ensiforme* | Elongated oval | Foot cell and papillate curved | 52.5±2.3×6.1±0.2 60.5±2×6.3±0.3 |
| USM FSSC- C4641Tb | *F. cf. ensiforme* | Elongated oval | Foot cell and papillate curved | 53.5±2.3×6.1±0.2 60.5±2×6.3±0.3 |
| USM FSSC- S2135Tb | *F. cf. ensiforme* | Elongated oval | Foot cell and papillate curved | 52.5±2.3×6.1±0.2 61.5±2×6.3±0.3 |
| USM FSSC- C3496Gr | 5 | Oval, elongated oval, clavate, and reniform | Barely notched and papillate curved | 38.5±1.5×5.3±0.2 45.5±2.5×5.7±0.2 |
| USM FSSC- Q1165Gr | 5 | Oval, elongated oval, clavate, and reniform | Barely notched and papillate curved | 40.5±1.5×5.4±0.2 45.5±2.5×5.8±0.2 |
| USM FSSC- B1409M | 5 | Oval, elongated oval, clavate, and reniform | Barely notched and papillate curved | 41.5±1.5×5.4±0.2 45.5±2.5×5.8±0.2 |

*Mean values of 30 random conidia + standard deviation

**Table 2** Morphological characteristics of individual strains of FSSC collected from different places in Malaysia
Fig. 1 Colonies of Malaysian members of the FSSC grown on PDA incubated under 12 h alternating light (black/white) at 25±2 °C for 2 weeks. a Top view, b reverse view: 1, Fusarium cf. solani (USM FSSC-K600T); 2, Fusarium keratoplasticum (USM FSSC-P2108S); 3, FSSC 5 (USM FSSC-C3496Gr); 4, Fusarium cf. ensiforme (USM FSSC-C4651Tb); 5, F. falciforme (USM FSSC-S2216Ru)
combined ITS region and TEF-α phylogenetic trees. For phylogenetic analysis, 91 taxa were included in the combined dataset and *Fusarium staphyleae* (NRRL 22316) was used as an outgroup.

**Results**

**Morphological Analysis**

The 55 strains were again investigated for their macro- and microscopic characteristics as shown in Table 2. All possible morphological dissimilarities were therefore taken into account and represented by the 55 strains selected. For species determination, the descriptions by Summerbell and Schroers [26], Nalim et al. [17], and Short et al. [21] were adopted. No sexual structures were observed in this study. Based on ITS and TEF-1α sequence data and morphological characteristics (Table 2), five species were identified. Of these, *F. keratoplasticum* (5 strains) and *F. falciforme* (14 strains) were known species. Features showed (Table 2) five isolates belonging to undescribed FSSC (USM FSSC-C3496Gr) as the known phylogenetic species among FSSC and four isolates were identified as *F. cf. ensiforme* (USM FSSC-C4651Tb) that were described by Nalim et al. [17]. Also, 27 strains were identified as *F. cf. solani* (USM FSSC-K600T). Macro- and microscopic characteristics including means and ranges of spore dimensions of individual isolates of FSSC are summarized in Table 2.

In the present study, 27 strains isolated from different crops and soil in Malaysia (Table 1) belonging to the *F. cf. solani* (USM FSSC-K600T) were examined and phylogenetically analyzed on the basis of ITS regions and partial TEF-1α sequences. Strains of *F. cf. solani* (USM FSSC-K600T) exhibited morphological variability in culture. Cultures grew fast, the growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 7.8 to 8.6 mm/day. The hyphae initially were hyaline and sparsely formed. Mycelium becomes yellowish white and yellowish in reverse after 1–2 weeks (Fig. 1 (4)). At 25 °C, abundant, erect, unbranched, or once-branched conidiophores are formed on the surface of the agar. Cream slimy sporodochia is produced on the surface of the agar. Phialides are more or less erect, somewhat swollen in the middle or cylindrical, arising from conidiophores, mainly (28–) 48–85 mm (–115) long and (2.6–) 3.1–4.4 μm (–5.3) at the base. Phialides produced in sporodochia are cylindrical, (13–) 16–21 mm (–26) long, (3.0–) 3.7–5.0 mm (–5.2) in diameter. The macroconidia arising from sporodochia are long, slightly curved with 3–8-septate mostly 5–7-septate, with papillate and somewhat curved apical cell and well-developed foot cell (Fig. 3a, b). The microconidia are oval and elongated oval, mostly 0-septate (Fig. 3c, d). The size of conidia measures as follows: 0-septate=5–11 (–15)×(2.8–) 3.3–4.3 μm (–5.0); 5-septate=(50–) 56–63 (–75)×(5.3–) 6–6.5 μm (–6.8); 6–7-septate=(59–) 64–75 (–86)×(5.0–) 6–6.8 μm (–7.2); and 8-septate=70–80×5.7–6.8 μm. Chlamydospores are smooth walled (Fig. 3e).

Cultures grew fast; the growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 8 to 9 mm/day (Fig. 1 (2)). The hyphae initially were hyaline. Mycelium becomes yellowish white and yellowish in reverse after 1–2 weeks. At 25 °C, aerial conidiophores formed abundantly on CLA, unbranched or branched, up to 100 μm long, 3.5–6.5 μm at base. Phialides are subcylindrical or cylindrical arising from conidiophores. Sporodochial conidiophores are formed on distinctive collarette at the tip. The macroconidia arising from sporodochia are small in length, falcate, dorsiventral, with 3-septate but rarely 4-septate with blunt apical cell and notched basal cell (Fig. 4a, b). The microconidia are typically falcate of their length. The microconidia are oval, elongated oval to sometimes obvoid with a truncate base, mostly 0-septate (Fig. 4c, d). The size of conidia measures as follows: 0-septate=(6–) 8–12 (–13.5)×(3–) 3.4–5.3 μm (–5.6); 1-septate=(9–) 14–16 (–26)×(4–) 4–4.6 μm (–5.8); 3-septate=(35–) 42–48 (–52)×(4.8–) 5.4–5.9 μm (–6.5); 4-septate=(40–) 48–54 (–58)×(5.0–) 5.5–6.0 μm (–6.5); and 5-septate=(44–) 51–57 (–63)×(5.0–) 5.5–6.0 μm (–6.5) (Table 2). Chlamydospores formed relatively abundant in mycelium, mostly globose, subglobose, intercalary or terminal and rough walled, 5–15 μm in diameter, and may occasionally be found within the macroconidia. Chlamydospores formed singly, and in clusters, or in chains (Fig. 2f).

The growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 5.5 to 6.0 mm/day. The hyphae initially were hyaline and sparsely formed. Mycelium tends to become cream to orange in reverse after 1–2 weeks (Fig. 1 (4)). At 25 °C, abundant, erect, unbranched, or once-branched conidiophores are formed on the surface of the agar. Cream slimy sporodochia is produced on the surface of the agar. Phialides are more or less erect, somewhat swollen in the middle or cylindrical, arising from conidiophores, mainly (28–) 48–85 mm (–115) long and (2.6–) 3.1–4.4 μm (–5.3) at the base. Phialides produced in sporodochia are cylindrical, (13–) 16–21 mm (–26) long, (3.0–) 3.7–5.0 mm (–5.2) in diameter. The macroconidia arising from sporodochia are long, slightly curved with 3–8-septate mostly 5–7-septate, with papillate and somewhat curved apical cell and well-developed foot cell (Fig. 3a, b). The microconidia are oval and elongated oval, mostly 0-septate (Fig. 3c, d). The size of conidia measures as follows: 0-septate=(5–) 6–11 (–15)×(2.8–) 3.3–4.3 μm (–5.0); 5-septate=(50–) 56–63 (–75)×(5.3–) 6–6.5 μm (–6.8); 6–7-septate=(59–) 64–75 (–86)×(5.0–) 6–6.8 μm (–7.2); and 8-septate=70–80×5.7–6.8 μm. Chlamydospores are smooth walled (Fig. 3e).

In the present study, 27 strains isolated from different crops and soil in Malaysia (Table 1) belonging to the *F. cf. solani* (USM FSSC-K600T) were examined and phylogenetically analyzed on the basis of ITS regions and partial TEF-1α sequences. Strains of *F. cf. solani* (USM FSSC-K600T) exhibited morphological variability in culture. Cultures grew fast, the growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 7.8 to 8.6 mm/day. The hyphae initially were hyaline and mycelium became yellowish white; green to bluish-gray, and purple in reverse after 1–2 weeks (Fig. 1 (4)). At 25 °C, aerial conidiophores formed abundantly on CLA, unbranched or branched, up to 100 μm long, 3.5–6.5 μm at base. Phialides are subcylindrical or cylindrical arising from conidiophores. Sporodochial conidiophores are formed on distinctive collarette at the tip. The macroconidia arising from sporodochia are small in length, falcate, dorsiventral, with 3-septate but rarely 4-septate with blunt apical cell and notched basal cell (Fig. 4a, b). The macroconidia are typically falcate of their length. The microconidia are oval, elongated oval to sometimes obvoid with a truncate base, mostly 0-septate (Fig. 4c, d). The size of conidia measures as follows: 0-septate=(6–) 8–12 (–13.5)×(3–) 3.4–5.3 μm (–5.6); 1-septate=(9–) 14–16 (–26)×(4–) 4–4.6 μm (–5.8); 3-septate=(35–) 42–48 (–52)×(4.8–) 5.4–5.9 μm (–6.5); 4-septate=(40–) 48–54 (–58)×(5.0–) 5.5–6.0 μm (–6.5); and 5-septate=(44–) 51–57 (–63)×(5.0–) 5.5–6.0 μm (–6.5) (Table 2). Chlamydospores formed relatively abundant in mycelium, mostly globose, subglobose, intercalary or terminal and rough walled, 5–15 μm in diameter.
Chlamydospores are formed singly, and in cluster, or in chains (Fig. 4e–g).

Cultures grew fast, and the growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 8 to 9 mm/day (Fig. 1 (5)). The hyphae initially were hyaline. Mycelium becomes yellowish white and yellowish in reverse after 1–2 weeks. At 25 °C, aerial conidiophores formed abundantly on CLA, unbranched or branched, up to 200 μm long, 3.7–7.0 μm at base. Phialides are subcylindrical or cylindrical arising from conidiophores. Sporodochial conidiophores were formed on distinctive collarete at the tip. The macroconidia are arising from sporodochia falcate, dorsiventral, with 3–4-septate, with pointed apical cell and barely notched basal cell. The macroconidia are slightly curved of their length (Fig. 5a–c). The microconidia were oval, elongated oval to sometimes obovoid with a truncate base mostly 0–1-septate (Fig. 5d, e).

**Fig. 2** *Fusarium cf. solani* (USM FSSC-K600T) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/darkness 12 h. a-e Multiseptate macroconidia produced from sporodochia. d, e, Oval-shaped and reniform conidia formed on conidiophores in hyphae. f Terminal and intercalary chlamydospores. Bar=20 μm for all pictures.
The size of conidia measures as follows: 0-septate=(5.6–12.15)×(2.8–4.8) μm; 1-septate=(10–19)×(4.7–5.3) μm; 3-septate=(35–42)×(5.4–5.9) μm; 4-septate=(38–44)×(5.0) μm; and 5-septate=(38–44)×(5.0) μm. Chlamydospores are formed relatively abundant in the mycelium, mostly globose, subglobose, intercalary or terminal, and rough walled, 8–15 μm in diameter. Chlamydospores are formed singly, and in cluster, or in chains (Fig. 5f).

Phylogenetic Analysis

The tree generated from the combined dataset of ITS regions and TEF-α supported previously inferred clade 3. This also
highlighted the fact that members of clade 3 represent a monophyletic lineage (Fig. 7). MP of 91 taxa (including outgroup) of the *Fusarium* in FSSC inferred from combined ITS and tef1 sequences also revealed diverse phylogenetic affinities among members of FSSC clade 3. All strains isolated from soils and plants represented five different lineages in the clade 3 (Fig. 7). Phylogenetic tree demonstrated that 27 strains isolated from different sources in Malaysia (Table 1) were placed in *F. cf. solani* (USM FSSC-K600T) group with a strong bootstrap support (95 %). All 27 strains are closely related to, but phylogenetically distinct from typical *F. falciforme* and doubtlessly represent a potentially novel phylogenetic species.

The tree showed a sister relationship between *F. keratoplasticum* (NRRL 32959, NRRL 22640, and NRRL 32780) and five new strains included in Table 1, and based on morphological features, all strains were identified as *F. keratoplasticum*. The tree showed a well-supported relationship (88 % MP bootstrap) between *F. falciforme* (FRC S-1973 and FRC S-1953) obtained from GenBank and all 14 new strains included in Table 1, which were identified as *F. falciforme* based on morphological features. Also, the tree showed a monophyly between undescribed species FSSC 5 and five strains isolated from different plants (Table 1). All these five strains represented a putative new lineage within clade 3 with strong phylogenetic affinity (93 % MP.
bootstrap). The phylogenetic tree also represented strains FSSC-C4651Tb, FSSC-C4641Tb, FSSC-S2135Tb, and FSSC-S2256Tb isolated from a tree bark placed in separated lineage within \( F. \text{ensiforme} \) clade with a strong bootstrap support (99 % MP) with \( \text{Fusarium} \) sp. (FRC S-1847). Based on morphological characters, all these strains were identified as \( F. \text{cf. ensiforme} \).

**Discussion**

Due to high morphological variation among members of FSSC in this study and in order to help in defining the species, both molecular and morphological data were taken into consideration. O’Donnell [15] and Aoki et al. [18, 19] found that DNA sequences of ITS regions can clearly present the
evolutionary relationships among this species complex and sequence of TEF-1α gene always offered a finer resolution and separated strains of most *Fusarium* complex species at species rank [30]. Therefore, in this study for accurate identification of FSSC, a molecular systematic study using sequence divergence of ITS regions and TEF-1α gene was used.

As shown by the molecular analysis of combined sequence data of ITS regions and TEF-1α (Fig. 7), members of *F. cf. solani* (USM FSSC-K600T) formed a distinct group. The type of macroconidia in *F. cf. solani* (USM FSSC-K600T) was specific among members of clade 3. The macroconidia arising from sporodochia was typically falcate and mostly 5-septate with papillate, tapered, and curved apical cell and well-
Fig. 7 A maximum parsimony phylogeny for 91 taxa of the FSSC inferred from combined ITS and tef1 sequences. Bootstrap tests were performed with 1,000 replications. *Fusarium staphyleae* (NRRL22316) obtained from GenBank was treated as the outgroup.
Table 3  Comparison of growth rates and range in sporodochial conidia size of individual strains of FSSC used in this study and common species in the FSSC [17, 21]

| Characters                  | FSSC 5 USM FSSC-C496Gr | F. cf. solani USM FSSC-K600T | F. keratoplasticum FRC S-2391 | F. keratoplasticum USM FSSC-P2108S | F. falciforme USM FSSC-S2216Ru | F. cf. ensiforme USM FSSC-C4651Tb | F. cf. ensiforme FRC S-1847 |
|-----------------------------|-------------------------|-------------------------------|-------------------------------|-----------------------------------|---------------------------------|---------------------------------|-----------------------------|
| Colony diameter\(^a\)      | Mean=3 cm White to cream | Mean=3.5 cm White to yellow or green | Mean=3 cm White to yellow | Mean=3 cm White to yellow | Mean=3 cm White to yellow | Mean=3 cm White to yellow | Mean=1.18 cm White to cream |
| Pigmentation on PDA         | Present                 | Present                      | Present                      | Present                          | Present                        | Present                        | Present                     |
| Aerial mycelium             | Oval, elongated oval, clavate, and reniform | Oval, elongated oval, clavate, and reniform | Oval, elongated oval, clavate, and reniform | Oval, elongated oval and obvoid with a truncate base | Oval, elongated oval and obvoid with a truncate base | Oval, elongated oval and obvoid with a truncate base | Oval, elongated oval and obvoid with a truncate base |
| Microconidia Shape          | Typical curved          | Falcate and dorsiventral     | Falcate and dorsiventral     | Falcate and dorsiventral         | Falcate and dorsiventral       | Slightly curved                | Slightly curved             |
| Septa                       | 3–5                     | 3–4 (mostly 3-septate)       | 3–4 (mostly 3-septate)       | 3–4 (mostly 4-septate)           | 3–4 (mostly 4-septate)         | 2–7                            | 2–8                         |
| Length (\(\mu m\))         | \((30–) 35–44\)         | 41.15                        | \((27–) 30–42\)              | \((28–) 31–45\)                 | \((52–) 56–66\)                | \((52–) 56–66\)                | \((52–) 56–66\)              |
| Width (\(\mu m\))          | \((5–) 5.4–5.9\)        | 6.05                         | \((4–) 4.8–5.8\)             | \((5.5–) 6.0–6.7\)              | \((5.5–) 6.0–6.7\)             | \((5.5–) 6.0–6.7\)             | \((5.5–) 6.0–6.7\)           |
| Length                      | 43–50                   | \((44–) 51–57\)              | \(-\)                        | \((50–) 64–73\)                 | \((50–) 66–78\)                | \((62–) 66–78\)                | \((62–) 66–78\)              |
| Width                       | 5.7–6.5                 | \((5.0–) 5.5–6.0\)           | \(-\)                        | \((5.3–) 6–6.8\)                | \((5.0–) 5.7–7.0\)             | \((5.0–) 5.7–7.0\)             | \((5.0–) 5.7–7.0\)           |
| Chlamydospores              | Present                 | Present                      | Present                      | Present                          | Present                        | Present                        | Present                     |

\(^a\) Colonies on PDA at 25 °C in intermittent light after 4 days
\(^b\) Mean length of macroconidia (3–4-septate)
\(^c\) Mean length of macroconidia (5-septate)
\(^d\) Mean length of macroconidia (6–7-septate)
developed foot cell. *F. cf. solani* (USM FSSC-K600T) was found to be the most dominant species from different substrates in Malaysia. The dominance of this species in all sampling sites from different sources was indicative of the revolutionary status of this imperfect fungus in Malaysia. However, molecular phylogeny showed a sister relationship between *F. falciforme* (USM FSSC-S2216Ru) and *F. cf. solani* (USM FSSC-K600T), but the macroconidia of *F. cf. solani* were larger than those of *F. falciforme* and their aerial conidiophores were branched. *Fusarium cf. ensiforme* (FSSC-C4641Tb) had a close morphological resemblance to *Fusarium* sp. (JF433047) that had been described by Nalim et al. [17]. Molecular phylogeny, based on ITS regions and TEF-1α, confirmed that all of these strains formed a monophyletic group with the typical strain of *Fusarium* sp. (JF433047) obtained from GenBank. Based on clear morphological differences and phylogenetic analysis of combined sequence data of ITS regions and TEF-1α (Fig. 7), this group represented a potentially novel phylogenetic species within *F. ensiforme* clade.

*F. keratoplasticum* (USM FSSC-P2108S) is characterized by production of dense hyphae and short 3-septate macroconidia (Tables 2 and 3). Morphological data showed a close morphological resemblance between strains USM FSSC-Q1172Rh, USM FSSC-Q4854D, USM FSSC-S2138Se, USM FSSC-P2108S, and USM FSSC-S2126Se included in Tables 2 and 3 with *F. keratoplasticum* [21]. This result was confirmed by the phylogenetic analysis based on sequence divergent of combined sequence data of ITS regions and TEF-1α. *F. keratoplasticum* (USM FSSC-P2108S) had a close morphological resemblance to *F. falciforme* (USM FSSC-S2216Ru), but the macroconidia in *F. falciforme* was larger than those of *F. keratoplasticum*. This differentiation was confirmed by combined sequence data of ITS regions and TEF-1α (Fig. 7). In this study, *F. falciforme* and *F. keratoplasticum* were reported for the first time for Malaysian mycoflora. The phylogenetic tree presented the monophyly of undescribed species FSSC 5 with strains C3496Gr, Q1165Gr, C1814An, B1409M, and Q1371W (93 % MP bootstrap) (Fig. 7).

According to Short et al. [21, 22], members of *F. falciforme*, *F. keratoplasticum*, and FSSC 5 usually were associated with human infectious diseases, while these strains were associated with plants, and this would be a very interesting finding and would provide phylogenetic context to all of the strains. Combined sequence data of ITS regions and TEF-1α offered higher resolutions and high levels of polymorphism within many species of fungi [3, 15, 30–32]. Phylogenetic analysis of combined sequences of ITS regions and TEF-1α in this study has shown to be a useful marker for identification and characterisation of taxa in FSSC. Since members of the FSSC have caused various types of diseases on plants and notable infections of humans, therefore accurate identification of pathogenic members of FSSC is very important in order to develop proper management practices in pythopathological and medical communities that work on this group of *Fusarium* species.

Acknowledgments Khosrow Chehri acknowledges the Universiti Sains Malaysia, Penang, Malaysia for providing necessary facilities to carry out this research (RU Research grants 1001/PBIOLOGI/811182 and FRGS 203/PBIOLOGI/6711311).

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