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

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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*, FSSC 5, 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

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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 Strains of members of the *Fusarium solani* species complex collected in Malaysia from soil and different substrate

Culture no.	Species	Host/substratum	Plant part (symptom)	Location	*tef1*[a]	ITS[b]
USM FSSC-C4383B	*F.* cf. *solani*	Bean	Root rot	Pahang	KC161396	KC009602
USM FSSC-R77O	*F.* cf. *solani*	Corn	Seed	Perlis	KC161404	KC009610
USM FSSC-R73O	*F.* cf. *solani*	Corn	Leaf (spot)	Perlis	KC161402	KC009608
USM FSSC-D8256C	*F.* cf. *solani*	Red chilli pepper	Leaf (spot)	Kelantan	KC161386	KC009592
USM FSSC-T8432C	*F.* cf. *solani*	Red chilli pepper	Stem (lesion)	Terengganu	KC161388	KC009594
USM FSSC-Q6002D	*F.* cf. *solani*	Pepper	Stem (lesion)	Sarawak	KC161414	KC009620
USM FSSC-Q729D	*F.* cf. *solani*	Pepper	Stem (lesion)	Sarawak	KC161418	KC009624
USM FSSC-Q1033Q	*F.* cf. *solani*	Sorghum	Root (rot)	Sarawak	KC161421	KC009627
USM FSSC-Q1017Q	*F.* cf. *solani*	Sorghum	Root (rot)	Sarawak	KC161399	KC009605
USM FSSC-P86S	*F.* cf. *solani*	Soil	–	Penang	KC161393	KC009599
USM FSSC-P104S	*F.* cf. *solani*	Soil	–	Penang	KC161391	KC009597
USM FSSC-P200S	*F.* cf. *solani*	Soil	–	Penang	KC161390	KC009596
USM FSSC-A2073S	*F.* cf. *solani*	Soil, Watermelon	–	Perak	KC161412	KC009618
USM FSSC-D310T	*F.* cf. *solani*	Tobacco	Stem (rot)	Kelantan	KC161394	KC009600
USM FSSC-J872T	*F.* cf. *solani*	Tobacco	Stem (rot)	Johor	KC161400	KC009606
USM FSSC-K600T	*F.* cf. *solani*	Tobacco	Stem (rot)	Kedah	KC161398	KC009604
USM FSSC-D216T	*F.* cf. *solani*	Tobacco	Stem (rot)	Kelantan	KC161392	KC009598
USM FSSC-J3516Gr	*F.* cf. *solani*	Grass	Root (rot)	Johor	KC161385	KC009591
USM FSSC-S2253Pu	*F.* cf. *solani*	Pumpkin	Fruit (canker)	Sabah	KC161410	KC009616
USM FSSC-D5281Pu	*F.* cf. *solani*	Pumpkin	Fruit (canker)	Kelantan	KC161409	KC009615
USM FSSC-A1969W	*F.* cf. *solani*	Watermelon	Root (rot)	Perak	KC161397	KC009603
USM FSSC-Q1094W	*F.* cf. *solani*	Watermelon	Root (rot)	Sarawak	KC161395	KC009601
USM FSSC-T617R	*F.* cf. *solani*	Rice	Root (rot)	Terengganu	KC161416	KC009622
USM FSSC-S2257W	*F.* cf. *solani*	Wood	Stem (lesion)	Sabah	KC161415	KC009621
USM FSSC-T921T	*F.* cf. *solani*	Tobacco	Stem (rot)	Terengganu	KC161403	KC009609
USM FSSC-A1881W	*F.* cf. *solani*	Watermelon	Root (rot)	Perak	KC161419	KC009625
USM FSSC-T2550W	*F.* cf. *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

Table 1 (continued)

Culture no.	Species	Host/substratum	Plant part (symptom)	Location	*tef1*[a]	ITS[b]
USM FSSC-K1418S	*F. falciforme*	Soil	–	Kedah	JX935589	JX982556
USM FSSC-P1531S	*F. falciforme*	Soil	–	Penang	JX935590	JX982557
USM FSSC-B1765S	*F. falciforme*	Soil	Tree bark	Selangor	JX935591	JX982558
USM FSSC-R43R	*F. falciforme*	Rice	Root (rot)	Perlis	KF836701	KF836673
USM FSSC-S2231Al	*F. falciforme*	Algae	–	Sabah	KF836700	KF836672
USM FSSC-P93S	*F. falciforme*	Soil	–	Penang	KF836702	KF836674
USM FSSC-P2108S	*F. keratoplasticum*	Soil	–	Penang	KF836695	KF836667
USM FSSC-S2138Se	*F. keratoplasticum*	*Azadirachta excelsa*	Root (rot)	Sabah	KF836699	KF836671
USM FSSC-S2126Se	*F. keratoplasticum*	*Azadirachta excelsa*	Root (rot)	Sabah	KF836698	KF836670
USM FSSC-Q1172Rh	*F. keratoplasticum*	*Casuarina equisetifolia*	Root (rot)	Sarawak	KF836696	KF836668
USM FSSC-Q4854D	*F. keratoplasticum*	*Casuarina equisetifolia*	Root (rot)	Sarawak	KF836697	KF836669
USM FSSC-Q1371W	FSSC 5	Watermelon	Root (rot)	Sarawak	KF836691	KF836676
USM FSSC-C3496Gr	FSSC 5	*Echinochloa colonum*	Root (rot)	Pahang	KF836692	KF836677
USM FSSC-Q1165Gr	FSSC 5	Grass	Root (rot)	Sarawak	KF836693	KF836678
USM FSSC-B1409M	FSSC 5	*Mangifera indica*	Fruit (spot)	Mango	KF836690	KF836675
USM FSSC-C1814An	FSSC 5	Grass	Root (rot)	Pahang	KF836694	KF836679
USM FSSC-C4651Tb	*F.* cf. *ensiforme*	Tree bark	Wood (decay)	Sabah	KC161383	KC009589
USM FSSC-C4641Tb	*F.* cf. *ensiforme*	Tree bark	Wood (decay)	Sabah	KC161384	KC009590
USM FSSC-S2135Tb	*F.* cf. *ensiforme*	Tree bark	Wood (decay)	Sabah	KC161382	KC009588
USM FSSC-S2256Tb	*F.* cf. *ensiforme*	Tree bark	Wood (decay)	Sabah	KC161381	KC009587

[a] GenBank numbers for translation elongation factor 1-alpha (*tef1*) partial sequences

[b] GenBank numbers for ITS regions

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) [25] 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 [26], Nalim et al. [17], and Short et al. [21] were adopted.

DNA Extraction

All FSSC strains were grown on PDA with sterile dialysis membranes [27] 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 $MgCl_2$, 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 and a final extension of 10 min 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 [29], 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

Table 2 Morphological characteristics of individual strains of FSSC collected from different places in Malaysia

Culture no.	Species identified	Shape of Microconidia	Shape of basal cell and apical cell	Length×width of macroconidia (μm)[a]	
				3- and 4-septate	5-septate
FSSC-R73O	*F.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	50.2±2.5×5.7±0.5	54.5±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	48.3±2.5×5.6±0.5	54.5±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	54.5±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	54.5±2.5×5.9±0.5
FSSC-Q1033Q	*F.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	51.2±2.5×5.8±0.5	52.5±2.5×6±0.5
FSSC-Q1017Q	*F.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	49.2±2.5×5.8±0.5	54.5±2.5×6±0.5
FSSC-P200S	*F.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	48.5±2.5×5.5±0.5	54.5±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.W	*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.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	50.2±2.5×5.8±0.5	56.5±2.5×5.9±0.5
FSSC-A1969W	*F.* cf. *solani*	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
FSSC-D8256C	*F.* cf. *solani*	Oval, reniform, and elongated oval	Foot cell and tapered, curved	48.0±2.5×5.5±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-K1418S	*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	FSSC 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	FSSC 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	FSSC 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

[a] Mean values of 30 random conidia±standard deviation

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 (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

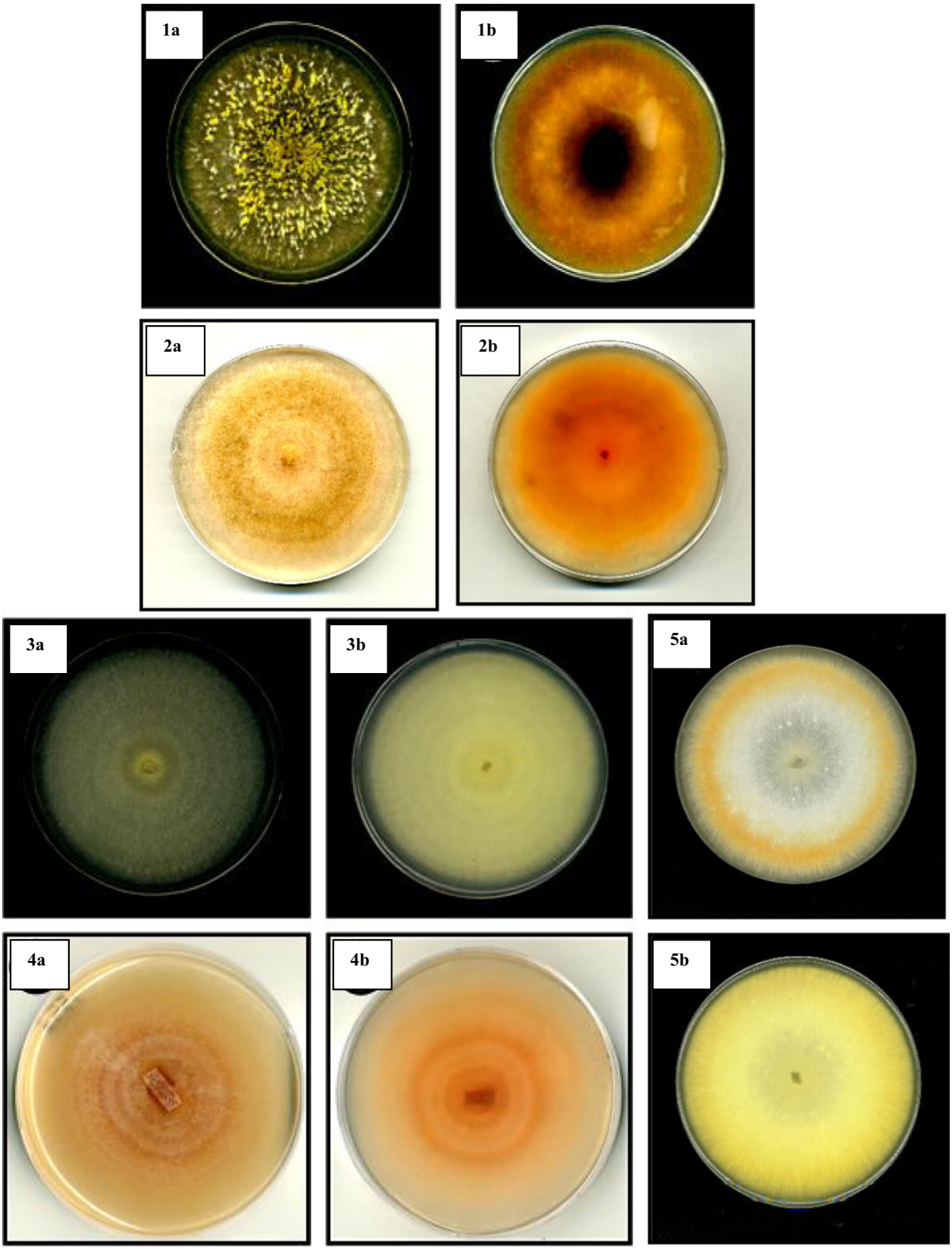

Fig. 1 Colonies of Malaysian members of the FSSC grown on PDA incubated under 12 h alternating light (*black*/*white*) at 25±2 °C for 2 weeks. **a** Top view, **b** reverse view: *1*, *Fusarium* cf. *solani* (USM FSSC-K600T); *2*, *Fusarium keratoplasticum* (USM FSSC-P2108S); *3*, FSSC 5 (USM FSSC-C3496Gr); *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 5 (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 hyaline and mycelium became yellowish white; green to bluish-gray, and purple in reverse after 1–2 weeks (Fig. 1 (1)). At 25 °C, aerial conidiophores formed abundantly on CLA, unbranched or branched, up to 200 μm long, 3.5–7.0 μm at base. Phialides were more or less erect, subcylindrical, or cylindrical arising from conidiophores. Sporodochial conidiophores formed on distinctive collarete at the tip. The macroconidia arising from sporodochia are falcate, dorsiventral, with 3–5-septate and mostly 5-septate, with pointed, tapered, and curved apical cell and well-developed foot cell. The macroconidia are typically falcate, widest in the middle of their length (Fig. 2a–c). The microconidia are oval, reniform, elongated oval to sometimes obovoid with a truncate base, mostly 0-septate (Fig. 2d, e). 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 tend to becomes 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).

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 collarete 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 clavate with a truncate base mostly 0–1-septate (Fig. 4c, d). The size of conidia measures as follows: 0-septate =(3.6–) 6–9.15 (−11.5)×(2.8–) 3.7–5.9 μm (−6.3); 1-septate =(10–) 12–15 (−23)×(4–) 4.6–5 μm (−6.0); 3-septate=(27–) 30–38 (−44)×(4–) 4.8–5.8 μm (−6.4); and 4-septate=36–40×5.7–6.2 μm. Chlamydospores are formed relatively abundant in the mycelium, mostly globose, subglobose, intercalary or terminal, and smooth to rough walled, 8–15 μm in diameter.

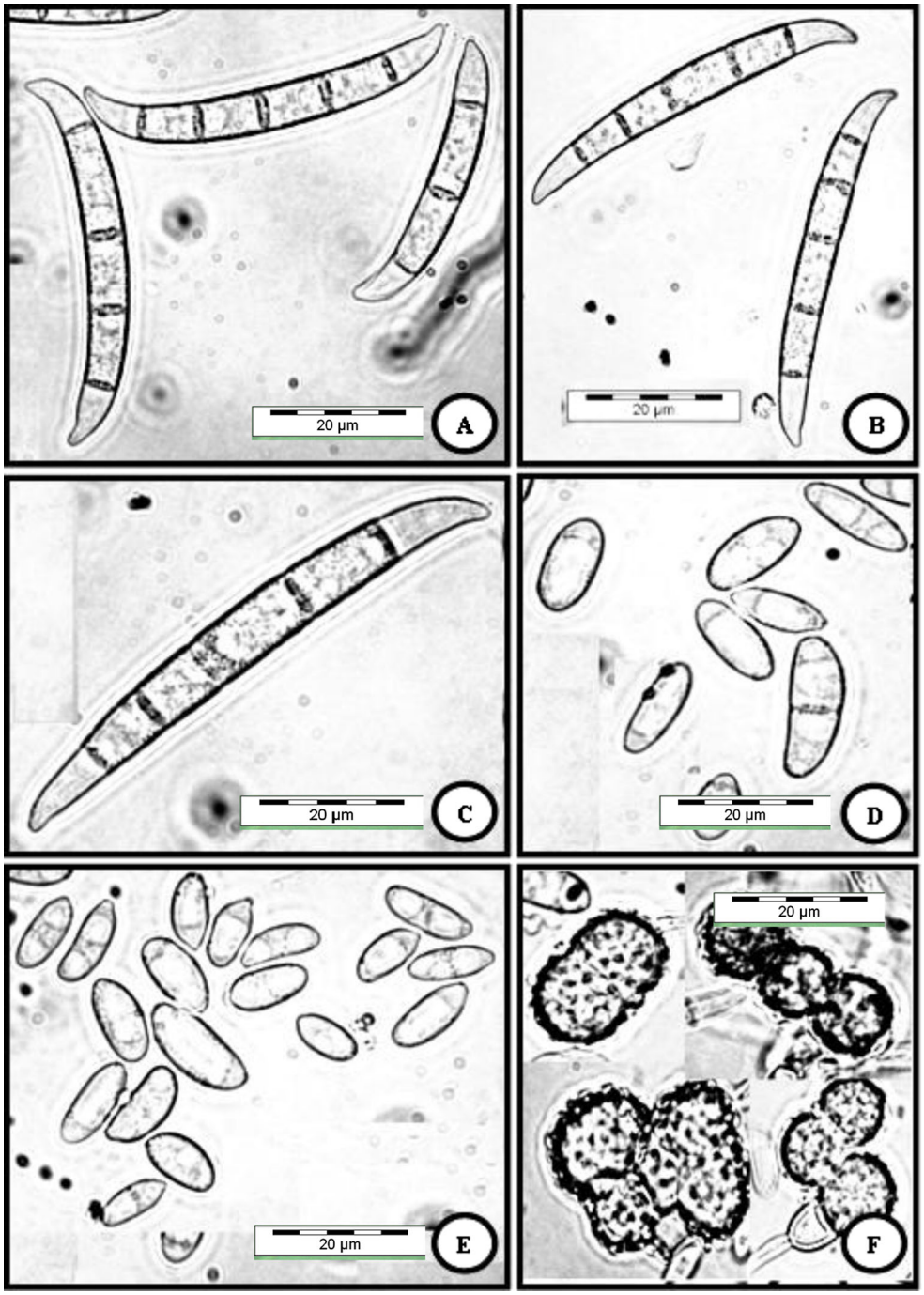

Fig. 2 *Fusarium* cf. *solani* (USM FSSC-K600T) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/darkness 12 h. **a**–**c** Multiseptate macroconidia produced from sporodochia. **d**, **e**, Oval-shaped and reniform conidia formed on conidiophores in hyphae. **f** Terminal and intercalary chlamydospores. *Bar*=20 µm for all pictures

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).

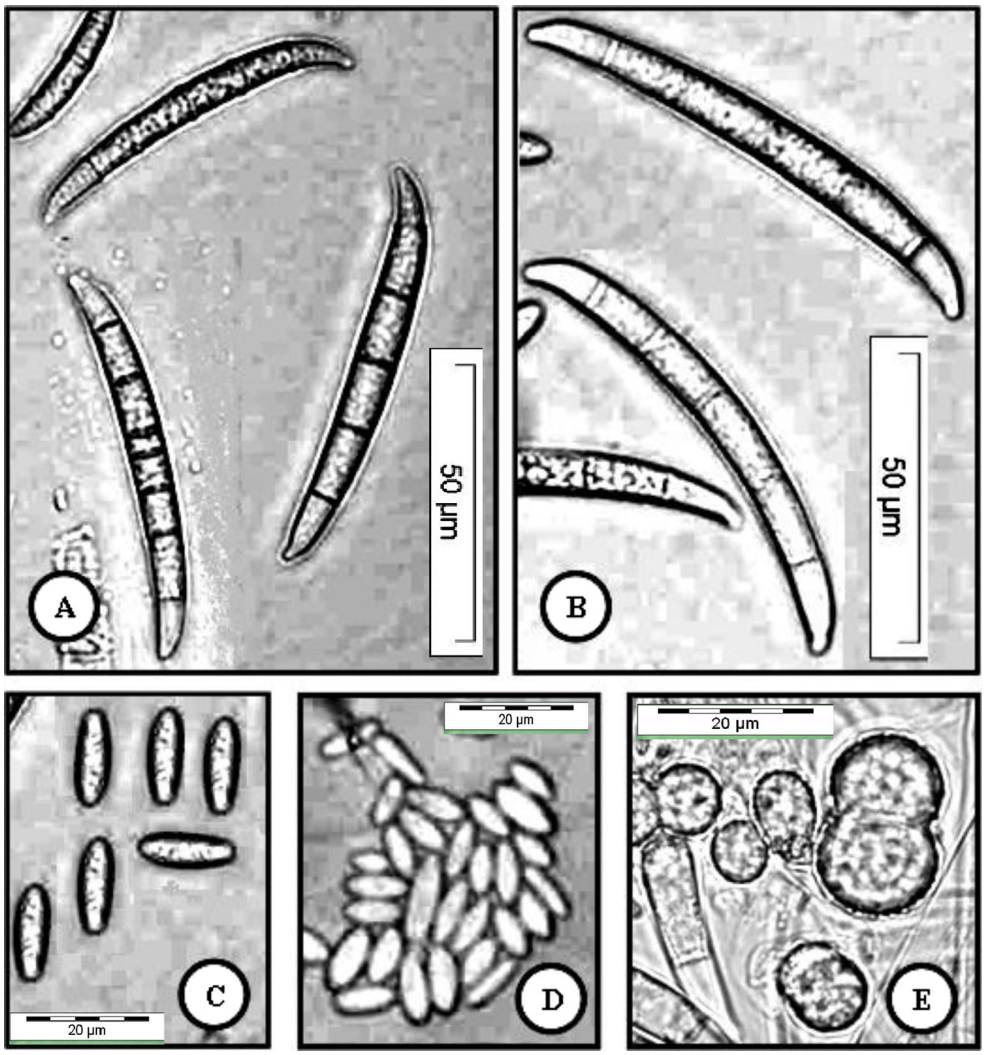

Fig. 3 *Fusarium* cf. *ensiforme* (USM FSSC-C4651Tb) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/ darkness 12 h. **a**, **b** Macroconidia (5–7-septate) produced from sporodochia. **c**, **d** Oval and elongated oval microconidia. **e** Terminal and intercalary chlamydospores. **a**, **b** *Bar*=50 μm; **c**, **d** *Bar*=20 μm

The size of conidia measures as follows: 0-septate=(5.6–) 6–12.15 (−13)×(2.8–) 3.7–4.8 μm (−5.3); 1-septate=(10–) 12–15 (−26)×(4–) 4.2–5 μm (−5.4); 3-septate=(26–) 31–42 (−44)×(4.4–) 4.5–5 μm (−5.5); and 4-septate=39–44×4.6–5.2 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).

The growth rate (mm/day) on PDA at 25 °C in intermittent light ranged from 6.0 to 7.5 mm/day. Aerial mycelium is sparsely formed, arranged in concentric rings, and richly conidial. The mycelium becomes white and cream in reverse after 1–2 weeks (Fig. 1 (3)). At 25 °C, abundant conidiophores, erect or verticillate in the upper part, with two to three phialides arising at each node. On CLA, conidiophores formed abundantly and sometimes on coiled hyphae in the aerial mycelium. Cream to bluish slimy sporodochia is produced adjacent to leaf agar. Phialides more or less erect, somewhat swollen in the middle or cylindrical, arising from conidiophores, mainly 22–150×4–7 μm at base. The macroconidia arising from sporodochia are medium in length, somewhat straight especially in the ventral side with 3–5-septate, with papillate and somewhat curved apical cell and barely notched basal cell (Fig. 6a, b). The microconidia are oval, elongated oval, clavate and reniform, and mostly 0–1-septate (Fig. 6c, d). The size of conidia measures as follows: 0-septate=(3.4–) 6–9.15 (−12.5)×(2.8–) 3.7–5.3 μm (−5.6); 1-septate=(10–) 14–19 (−27)×(4–) 4.7–5.3 μm (−6.0); 3-septate=(30–) 35–42 (−44)×(5–) 5.4–5.9 μm (−6.5); 4-septate =(38–) 40–44(−48)×(5.0–) 5.5–6.0 μm (−6.5); and 5-septate =43–50×5.7–6.5 μm. Chlamydospores are formed singly, and in cluster, or in chains (Fig. 6e, f).

Phylogenetic Analysis

The tree generated from the combined dataset of ITS regions and TEF-α supported previously inferred clade 3. This also

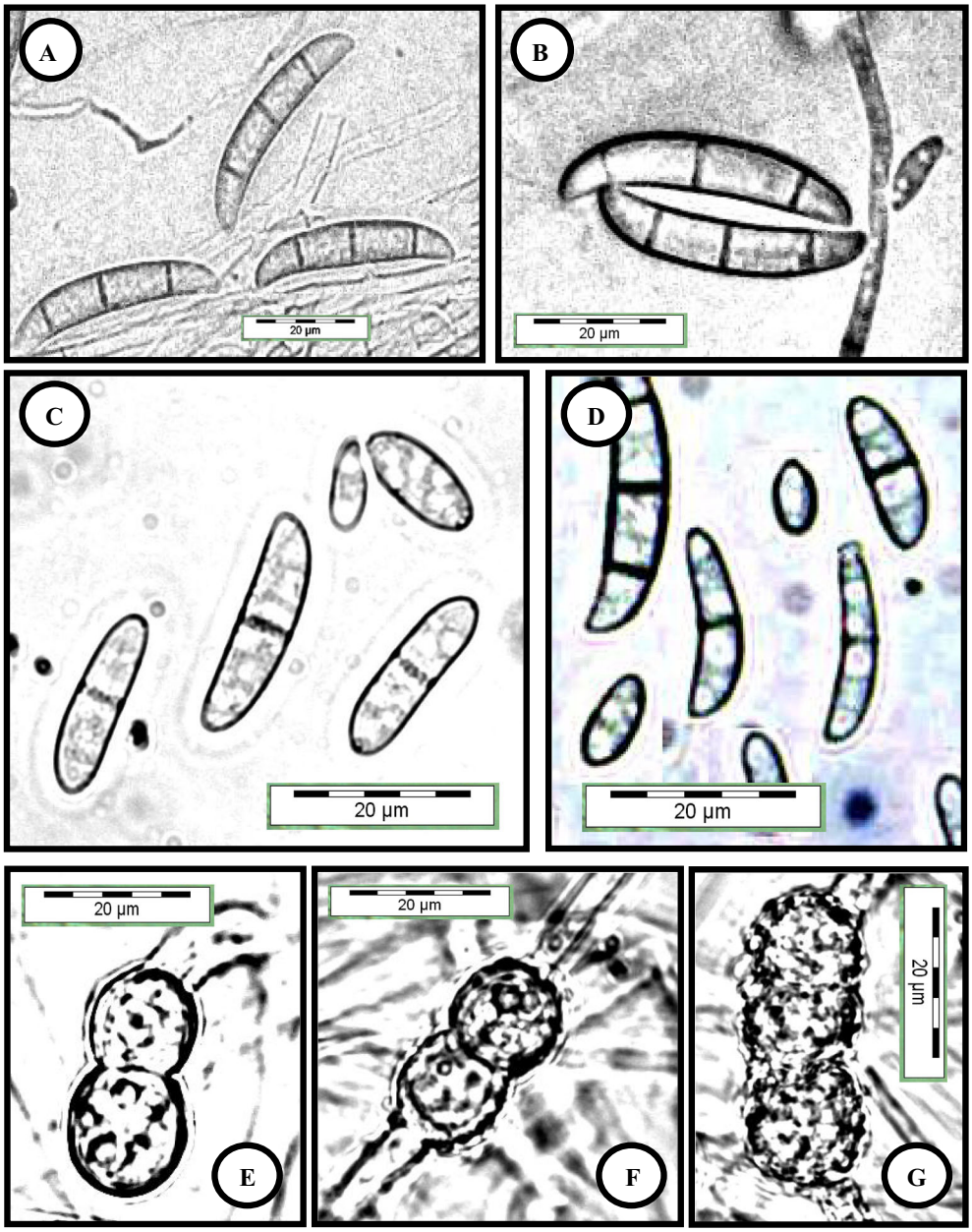

Fig. 4 *Fusarium keratoplasticum* (USM FSSC-P2108S) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/darkness 12 h. **a**, **b** Three septate macroconidia produced from sporodochia. **c**, **d** Oval-shaped and curved cylindrical conidia were formed on conidiophores in hyphae. **e**–**g** Terminal and intercalary chlamydospores. *Bar*=20 µm for all pictures

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

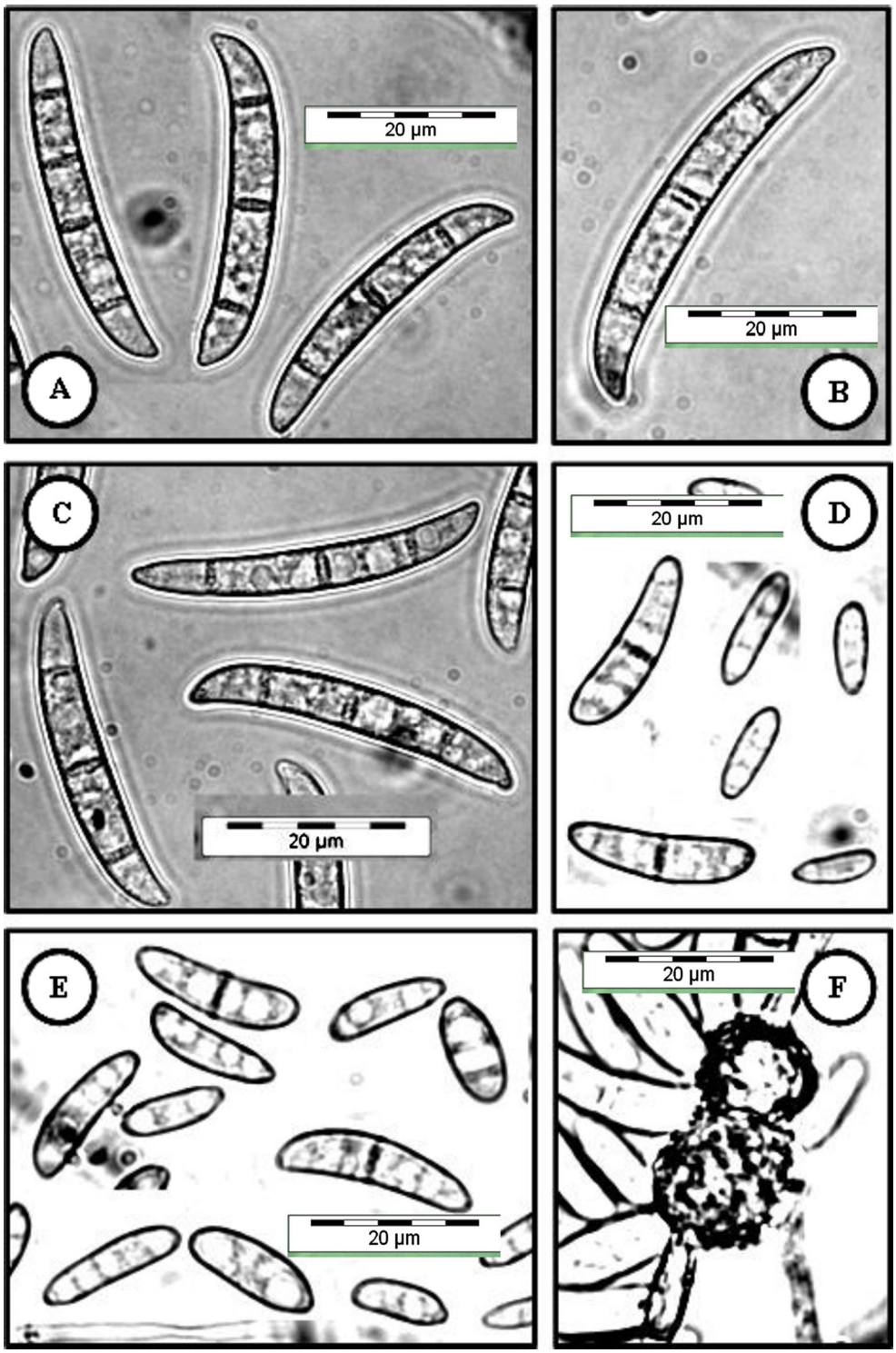

Fig. 5 *Fusarium falciforme* (USM FSSC-S2216Ru) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/darkness 12 h. **a**–**c** Multiseptate macroconidia produced from sporodochia. **d**, **e** Oval-shaped and reniform conidia were formed on conidiophores in hyphae. **f** An intercalary chlamydospores. *Bar* =20 µm for all pictures

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. ensiforme* clade with a strong bootstrap support (99 % MP) with *Fusarium* sp. (FRC S-1847). Based on morphological characters, all these strains were identified as *F.* 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

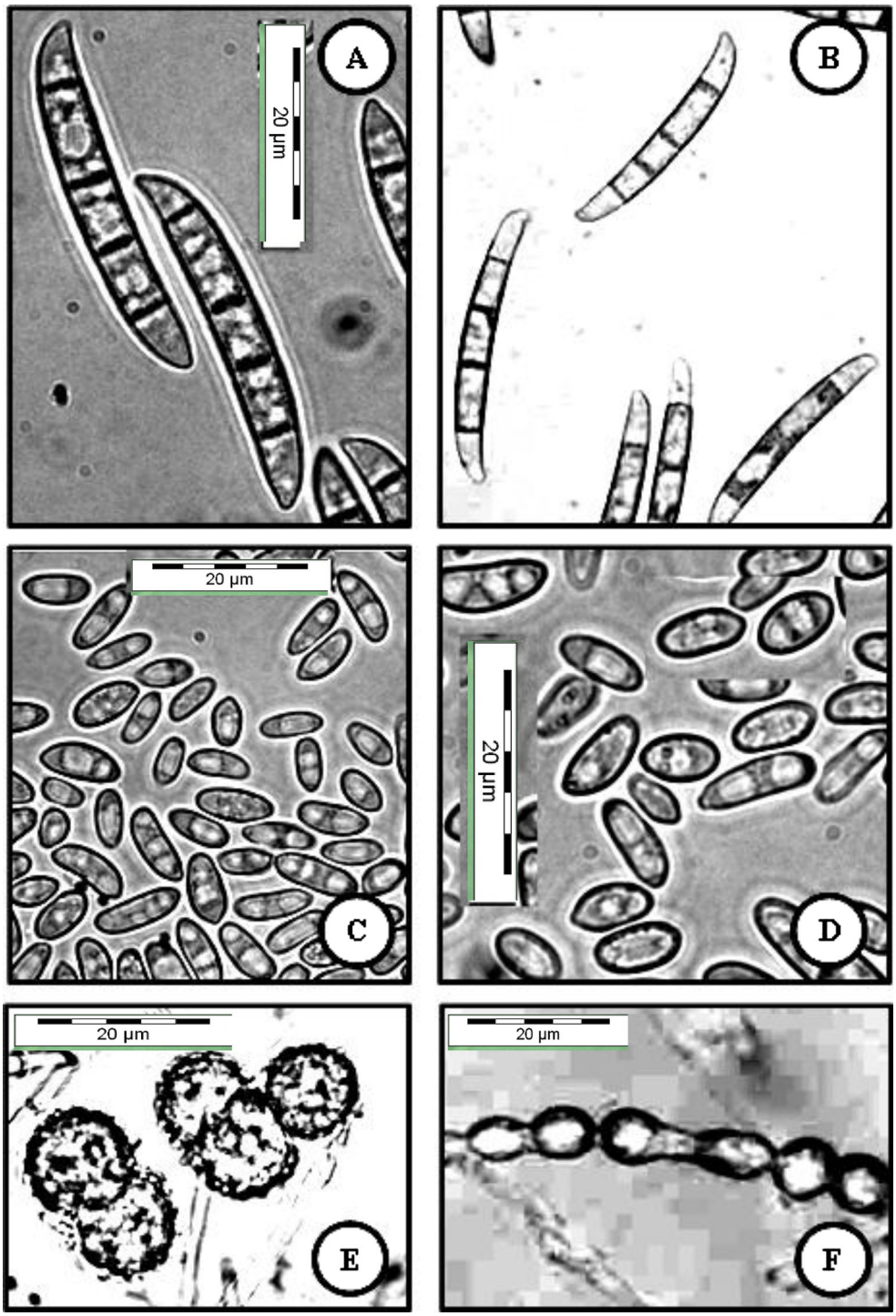

Fig. 6 FSSC 5 (USM FSSC-C3496Gr) grown on CLA, 2 weeks, 25 °C, cool white fluorescent light 12 h/darkness 12 h. **a**, **b** Macroconidia produced from sporodochia. **c**, **d** Oval-shaped and reniform conidia formed on conidiophores in hyphae. **e**, **f** Terminal and intercalary chlamydospores. *Bar*=20 μm for all pictures

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-

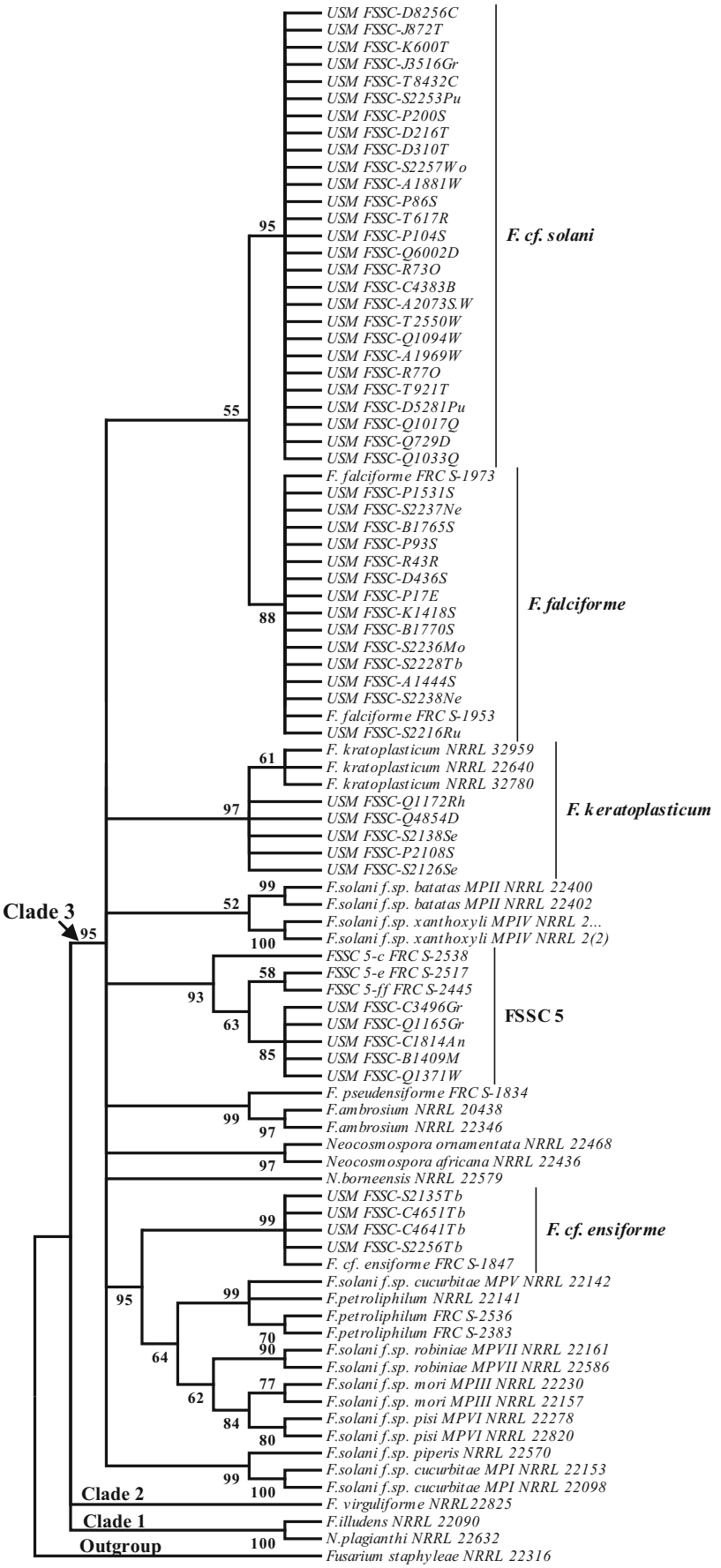

Fig. 7 A maximum parsimony phylogeny for 91 taxa of the FSSC inferred from combined ITS and *tef1* sequences. Bootstrap tests were performed with 1,000 replications. *Fusarium staphyleae* (NRRL22316) obtained from GenBank was treated as the outgroup

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-C3496Gr	*F.* cf. *solani* USM FSSC-K600T	*F. keratoplasticum* FRC S-2391	*F. keratoplasticum* USM FSSC-P2108S	*F. falciforme*	*F. falciforme* USM FSSC-S2216Ru	*F.* cf. *ensiforme* USM FSSC-C4651Tb	*F.* cf. *ensiforme* FRC S-1847
Colony diameter[a]		Mean=3 cm	Mean=3.5 cm	Mean=3 cm	Mean=3 cm	Mean=3 cm	Mean=3 cm	Mean=1.18 cm	Mean=1.18 cm
Pigmentation on PDA		White to cream	White to yellow or green	–	White to yellow	White to yellow	White to yellow	Dark reddish brown	Dark reddish brown
Aerial mycelium		Present	Present	Present	Present	–	Present	Present	Present
Sporodochia	Shape	Oval, elongated oval, clavate, and reniform	Oval, reniform, elongated oval, and obovoid with a truncate base	Oval, elongated oval and obovoid with a truncate base	Oval, elongated oval and obovoid with a truncate base	Elongated oval and obovoid with a truncate base	Elongated oval and obovoid with a truncate base	Ellipsoidal	Ellipsoidal
Microconidia	Shape	Straight to slightly curved	Typically curved	Falcate and dorsiventral	Falcate and dorsiventral	Falcate and dorsiventral	Falcate and dorsiventral	Slightly curved	Slightly curved
	Septa	3–5	3–5 (mostly 5-septate)	3–4	3–4 (mostly 3-septate)	3–4 (mostly 4-septate)	3–4 (mostly 4-septate)	2–7	2–8
μm[b]	Length	(30–) 35–44 (–48)	(35–) 42–54 (–58)	41.15	(27–) 30–42 (–44)	44.3±2.6	(28–) 31–45 (–47)	(52–) 56–66 (–71)	(52–) 56–66 (–71)
μm[c]	Width	(5–) 5.4–5.9 (–6.5)	(4.8–) 5.4–5.9 (–6.5)	6.05	(4–) 4.8–5.8 (–6.4)	5.9–6.1	5.5–6.1	(5.5–) 6.0–6.7 (–7.0)	(5.5–) 6.0–6.7 (–7.0)
	Length	43–50	(44–) 51–57 (–63)	–	–	–	–	(50–) 64–73 (–75)	(62–) 66–78 (–89)
	width	5.7–6.5	(5.0–) 5.5–6.0 (–6.5)	–	–	–	–	(5.3–) 6–6.5(–6.8)	(5.0–) 5.7–7.0 (–7.5)
μm[d]	Length	–	–	–	–	–	–	(59–) 64–75(–86)	(62–) 66–78 (–89)
	Width	–	–	–	–	–	–	(5.0–) 6–6.8 (–7.2)	(5.0–) 5.7–7.0 (–7.5)
Chlamydospores		Present	Present	Present	Present	Present	Present	Present	Present

FRC Fusarium Research Center at Pennsylvania State University, Pennsylvania, *USM* Fusarium culture collection of school of Biological Science, Universiti Sains Malaysia

[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 phytopathological and medical communities that work on this group of *Fusarium* species.

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