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CHARACTERIZATION AND MYCOTOXIN ANALYSIS OF *Fusarium* spp. FROM HIGHLAND AREAS IN MALAYSIA

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ABSTRACT

Fusarium isolates from highland areas in Malaysia were mostly recovered from two species of grasses, *Elyhordeum montanense* and *Paspalum conjugatum*. The isolates were grouped into four morphological groups. Based on TEF-1α sequences, morphotype 1 isolates were molecularly identified as *F. graminearum* species complex, morphotype 2 as *F. venenatum,* morphotype 3 as *F. avenaceum* and morphotype 4 as *F. kyushuense.* Restriction analysis of the Intergenic Spacer region showed high levels of genetic diversity of isolates in *F. graminearum* species complex and *F. venenatum.* For mycotoxin analysis, only *F. avenaceum* and *F. kyushuense* produced beauvericin and moniliformin (0.869 & 0.321 µg/kg, respectively). Zearalenone was produced by 32 isolates of *F. graminearum* species complex (0.002 – 0.437 µg/kg), two isolates of *F. venenatum* (0.006 - 0.014 µg/kg) and *F. kyushuense* (0.006 µg/kg). Only *F. avenaceum* isolate produced fumonisin B1 (0.001 µg/kg). The present study indicates the occurrence of *Fusarium* species commonly reported in highland areas in Malaysia where the weather is cooler and the temperature is lower than in the lowland areas. To our knowledge, this is the first report on the occurrence of phylogenetic species within *F. graminearum* species complex, *F. venenatum, F. avenaceum* and *F. kyushuense* in Malaysia.

Key words: *Fusarium graminearum* species complex, *Fusarium venenatum*, *Fusarium avenaceum*, *Fusarium kyushuense*, highland areas, mycotoxin

INTRODUCTION

The genus *Fusarium* is distributed worldwide and contains species that are important plant pathogens, saprophytes, endophytes and mycotoxin producers. Climate is one of the important factors which can determine the occurrence of *Fusarium* species on a broad, regional scale although many *Fusarium* species are cosmopolitan and are diverse in both tropical and temperate regions (Gordon, 1960) including *F. oxysporum, F. solani, F. semitectum* and *F. proliferatum*. There are also *Fusarium* species which occurred in specific climatic conditions such as those species that only occurred in the temperate region such as *F. avenaceum*, *F. graminearum*, *F. culmorum* and *F. sporotrichioides*. The climate and local variations in weather can limit the range of species observed and also can influence their relative frequency.

Malaysia is located near the equator and according to the Köppen climate classification system, the country has a tropical rainforest climate which is hot and humid with an average temperature of 27 °C. Highland areas have cooler and wetter conditions than the lowland areas with temperatures ranging from 18 °C to 23 °C, and the temperature in the coldest month ranged from 3 °C to 18 °C, and in the warmest month, 23 °C to 24 °C (Reynolds *et al*., 2008).

The highland areas of Malaysia are located at the centre of Peninsular Malaysia (at about 1200 m) and in the interior of Sabah and Sarawak (at about 1200 m to 1800 m) which is a forested mountainous area with alluvial and swampy coastal plains (Andrews & Freestone, 1972; Ooi, 1976). Conifers, laurels, myrtles, oak, and plants from the family Theaceae are among the common vegetation found in the highland areas.

During a biodiversity survey on the occurrence of *Fusarium* species in highland areas in Malaysia, *Fusarium* isolates isolated from various substrates showed variable shades of red pigmentation when cultured on potato dextrose agar. In Malaysia, red pigmented *Fusarium* isolates are not common. Morphological identification particularly the shapes and sizes of macroconidia were used to sort the isolates into groups. Molecular identification using Tanslation Elongation Factor-1α (TEF-1α) gene is highly informative for a single-locus identification of *Fusarium* species and a publicly available database known as Fusarium-ID was created by Geiser *et al*. (2004). To examine the intraspecific genetic variability of the *Fusarium* isolates, restriction analysis of the nuclear ribosomal DNA intergenic

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spacer region (RFLP- IGS) was applied as this region evolves more rapidly and has been used to examine intraspecific relationships among closely related species (Appel & Gordon, 1995; Edel *et al*., 1995). Mycotoxin analysis was also carried out to determine the mycotoxin producer among the isolates. Thus, the objective of this study was to identify and characterize red-pigmented *Fusarium* isolates from highland areas in Malaysia using the TEF-1α gene, RFLP-IGS and mycotoxin analysis of beauvericin (BEA), fumonisin B1 (FB1), moniliformin (MON) and zearalenone (ZEN).

MATERIALS AND METHODS

Isolation of *Fusarium* isolates

A total of 84 isolates of *Fusarium* with reddish pigmentation and one isolate showing brownish orange pigmentation were isolated from various plants from three highland areas in Malaysia, namely Cameron Highlands and Fraser's Hill in the state of Pahang, Peninsular Malaysia and Kinabalu Park in the state of Sabah. The majority of the isolates were recovered from two species of grasses, *Elyhordeum montanense* and *Paspalum conjugatum*. *Fusarium* isolates were also isolated from different types of plants including *Anthurium* sp., *Hibiscus* sp., *Amomum* sp., *Tibouchina semidecandra, Catharanthus roseus, Ipomoea purpurea, Datura suaveolens, Cleome hassleriana, Zingiber* sp., *Impatients walleriana, Lilium longiforum*, *Pinus* sp., moss and fern (*Athyrium filix-femina*, *Gleichenia* sp.). The *Fusarium* isolates were isolated from apparently healthy plant parts.

A direct plating technique was applied and the host plant's tissues were cut into 1.0 – 1.5 cm and directly plated onto Peptone pentachloronitrobenzene (PPA) medium. Eleven isolates used in this study were obtained from the stock culture.

Morphological characterization

For single spore isolation and morphological identification, four media were used, namely potato dextrose agar (PDA), potato sucrose agar (PSA), carnation leaf agar (CLA) and water agar (WA) as described in The *Fusarium* Laboratory Manual (Leslie & Summerell, 2006). For morphological identification, the materials and methods described in The *Fusarium* Laboratory Manual (Leslie & Summerell, 2006) were adopted. The morphological identification was based on the macroscopic and microscopic characteristics of the *Fusarium* isolates as outlined in The *Fusarium* Laboratory Manual. Observation of colony appearance, pigmentations and growth rate was performed using potato dextrose agar (PDA) incubated at 25 ± 1 °C for 2 weeks (Leslie & Summerell, 2006). As for observation of microscopic characteristics, the isolates were grown on CLA incubated at 25 ± 1 °C.

Molecular identification and characterization

TEF-1α gene sequences and RFLP-IGS analysis were used to identify and characterize the *Fusarium* isolates successfully recovered from different host plants in highland areas in Malaysia.

For extraction of genomic DNA, mycelia were grown on PDA, overlaid with a dialysis membrane, and incubated at 25 ± 1 °C for 4-5 days. After incubation, the isolates were harvested and ground using liquid nitrogen using mortar and pestle until they became fine powder. DNA extraction was carried out using DNA Extraction Kit, DNeasy® Mini Plant Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Sequencing of translation elongation Factor-1α gene

TEF-1α gene was amplified using primer pair EF1 (5'-ATG-GGT-AAG-GAG-GAC-AAG-AC-3') and EF2 (5'-GGA-AGT-ACC-AGT-GAT-GAT-GTT-3'), modified from O'Donnell *et al*. (1998a). PCR amplification was carried out in a 50 µL reaction mixture containing 8 µL 1× Buffer, 4 mM $MgCl_2$, 0.8 mM each primer, 200 µM dNTPs mix (Promega, Seattle, WA, USA), 1.5 U *Taq* DNA Polymerase (Promega) and 12 ng genomic DNA and performed using Peltier Thermal Cycler Model PTC-200® (MJ Research, Inc., Watertown, MA, USA) with the following PCR cycles: initial denaturation at 94 °C for 1 min, 35 cycles of denaturation at 95 °C for 21 s, annealing at 59 °C for 33 s, extension at 72 °C for 80 s and final extension for 7 min. The PCR product was detected using 1% agarose gel electrophoresis, run for 90 min, 80 V and 400 mA. The PCR products were then sent to a service provider for sequencing.

After sequencing, consensus sequences from pair-wise alignment were compared with other sequences in Fusarium-ID and Genbank databases using BLAST search. Two databases were used to obtain a more accurate species identity. Multiple sequence alignment was used to align all the sequences in MEGA5 (Tamura *et al*., 2011) and the sequences were visually edited and improved manually. Sequences of several *Fusarium* species downloaded from the Fusarium-ID database were also included in phylogenetic analysis. The species included were *F. mesoamericanum* (FD011320), *F. graminearum* (FD01115), *F. austroamericanum* (FD01134), *F. meridionale* (FD01120), *F. cortaderiae* (FD01027), *F. acaciae-mearnsii* (FD01125), *F. asiaticum* (FD01122), *F. kyushuense* (FD01312), *F. venenatum* (FD01306) and *F. avenaceum* (JX302178).

For phylogenetic analysis, the maximum likelihood (ML) method was used to generate a phylogenetic tree using MEGA5. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The

percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

PCR and RFLP-IGS analysis

The IGS region was amplified using CNL 12 (5′-CTG-AAC-GCC-TCT-AAG-TCA-G-3′) and CNS 1 (5′-GAG-ACA-AGC-ATA-TGA-CTA-CTG-3′) primers (Apple & Gordon, 1995). PCR amplification reaction was carried out in a 50-µL reaction mixture containing 10 µL 1× buffer, 2 mM $MgCl_2$, each primer at a concentration of 0.8 mM, 100 µM dNTPs (Promega, USA), 1.25 U *Taq* DNA Polymerase (Promega) and 8 ng genomic DNA. PCR amplification was performed using a PTC-200® Peltier Thermal Cycler (MJ Research). PCR steps were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 0.35 min, annealing at 59 °C for 0.55 min, extension at 72 °C for 2 min and final extension for 7 min.

The PCR product was detected using 1% agarose gel, run for 90 min, at 80 V and 400 mA. The gel was visualized under a UV transilluminator using the Molecular Imager® Gel Doc XR™ system (Bio-Rad, Hercules, CA, USA). A molecular marker of 1kb (DNA Ladder Plus, Gene RulerTM, Fermentas, Lithuania) was used to determine the size of the PCR products.

Six restriction enzymes, namely *Alu*I, *BsuR*I, *EcoR*I, *Msp*1, *Pst*I and *Taq*1 (Fermentas, Lithuania) were used to digest the PCR products. The digestion mixture containing 7 µL PCR product, 1 µL restriction enzyme, 1 µL buffer, and 6 µL distilled water was carried out in a 15 µL reaction mixture. This mixture was incubated for 1 h based on the restriction enzyme incubation's temperature which was 35 °C for *Alu*I, *BsuR*I, *EcoR*I, *Msp*1 and *Pst*I and 65 °C for *Taq*1. After 1 h incubation, 3 µL of 6X loading dye (6X Loading Dye Solution, Fermentas, Lithuania) was added to the reaction mixture to stop the reaction. The digestion product was detected using 2.5% agarose gel electrophoresis and run for 160 min, 80 V and 400 mA. DNA ladder 100 bp (DNA Ladder Plus, Gene RulerTM, Fermentas, Lithuania) was used as a marker.

Data analysis of restriction bands

The restriction analysis data were analyzed using NTSYS-pc (Numerical Taxonomy System of Multivariable programme) version 2.1 (Rohlf, 2000). The restriction bands were scored based on the presence (1) and absence (0) for each particular band. Simple Matching Coefficient was also employed to construct a similarity matrix and a dendrogram was constructed using Unweighted Pair Group Method with Arithmetic Average (UPGMA) to observe the genetic relationships of the *Fusarium* isolates isolated from various host plants.

Mycotoxin analysis

Thirty-five isolates consisting of 28 isolates of *F. graminearum* species complex, five isolates of *F. venenatum* and one isolate each of *F. avenaceum* and *Fusarium* sp. were chosen for mycotoxin analysis. Four mycotoxins, BEA, FB1, MON and ZEN were analyzed using Ultra Performance Liquid Chromatography (UPLC) (Waters, Milford, MA, USA).

For sample preparation, cleaned corn grit was weighted according to the types of mycotoxins with 45% moisture and placed in 250 mL Erlenmeyer flasks sterilized at 121 °C for 30 min. After 3 days, the sterilized corn grit was inoculated with 1 mL aqueous conidial suspension (10^6 conidia/mL) of monoconidial *Fusarium* isolates. The control for each mycotoxin was treated with the same method except it was inoculated with sterilized distilled water. All the cultures and controls were incubated in the dark at 28 °C for 28 days. The flasks were hand-shaken during the first few days of incubation to avoid clumping.

BEA extraction was performed according to the procedure by Logrieco *et al*. (1998). For FB1, procedures by Nelson *et al*. (1992) were adopted. Modified MON extraction procedures were adapted from Munimbazi & Bullerman (1998). Extraction procedures for ZEN were modified based on Jimenez & Mateo (1997). The four mycotoxins were detected and quantified using UPLC (Waters, Milford, MA, USA).

Waters Acquity UPLC® binary pump connected to a Waters Acquity UPLC® photodiode array (PDA) detector that was set at 229 nm. Chromatographic separations were performed using a C_{18} reversed-phase column (2.1 × 100 mm, 1.7 µm) (Waters, Milford, MA, USA).

RESULTS

Based on macroscopic and microscopic characteristics, the reddish-pigmented *Fusarium* isolates were grouped into four morphological groups or morphotypes designated as morphotype 1 (74 isolates), nine isolates as morphotype 2, one isolates each for morphotypes 3 and 4. The morphological characteristics of the morphotypes are shown in Table 1. All four morphotypes produced different shapes and sizes of macroconidia which might indicate different species (Figure 1**)**. Microconidia were not observed in morphotypes 1 and 2 isolates. Chlamydospores were observed in Morphotypes 1, 2 and 4 isolates (Table 1, Figure 1).

Table 1. Morphological characteristics of four morphotypes of *Fusarium* from highland areas in Malaysia

Morphological characteristics	Morphotype 1	Morphotype 2	Morphotype 3	Morphotype 4
Microscopic characteristics				
Length and width of macroconidia (µm)	42.23 × 5.08	29.64 × 4.58	67.84 × 2.76	28.73 × 4.19
Macroconidia	- stout and thick-walled - tapered apical cell - well-developed foot-shaped basal cell - 3 - 6 septa	- short and stout - curved and tapered apical cell - foot-shaped basal cell - 2 - 5 septa	- long and slender - long apical cell with tapered point - foot-shaped basal cell - 0 - 6 septa	- falcate - curved and tapered apical cell - notched or foot-shaped basal cell - 3 - 5 septa
Microconidia / Mesoconidia	– absent	– absent	– - fusoid microconidia – - present of mesoconidia	– pyriform, napiform, fusoid microconidia – present of mesoconidia – rabbit ear appearance
Chlamydospores	singly, in clusters	singly and in chain	absent	smooth wall, singly and in chain
Sporodochia	hardly found	present - brown colour	present - orange colour	scarce
Macroscopic characteristics				
Colony appearance	white mycelia	white mycelia with concentric rings	white mycelia to light yellow	densely white mycelia
Colony pigmentation	red	red	carmine orange	dark red
Growth diameter	fast growing (8.0 cm)	fast growing (4.0 – 4.6 cm)	slow growing (3.6 cm)	fast growing (7.5 cm)

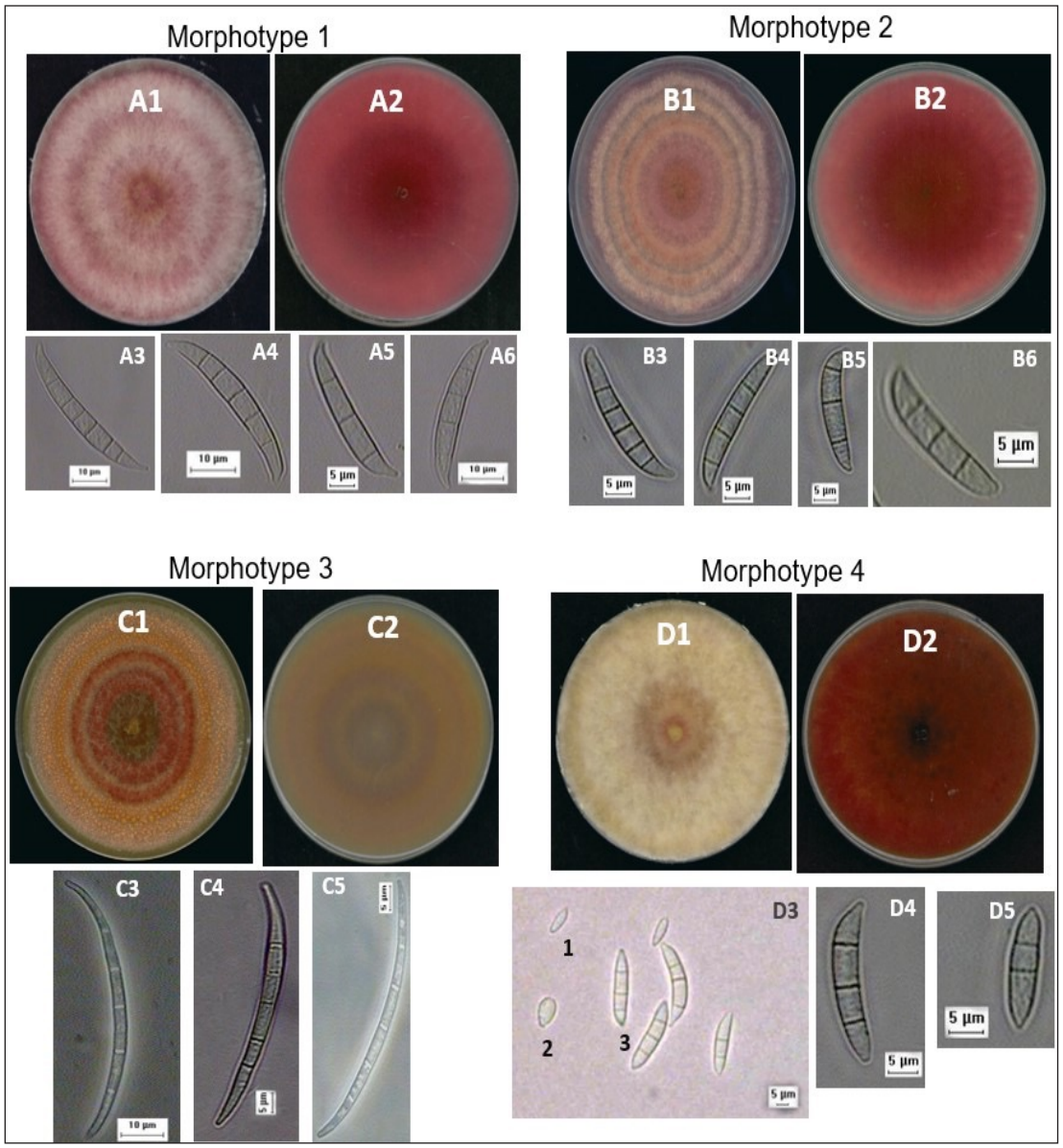

Fig. 1. Macroscopic and microscopic characteristics of *Fusarium* isolates from highland areas in Malaysia. **Morphotype 1:** (A1) Masses of white mycelia; (A2) Red pigmentation; (A3 – A6) Macroconidia; (A3) 6-septa), (A4) 5-septa; (A5) 4-septa; (A6) 3-septa. **Morphotype 2:** (B1) White-reddish colony appearance; (B2) Red pigmentations; (B3 – B6) Macroconidia; (B3) 5-septa; (B4) 4-septa, (B5-B6) 3-;septa. **Morphotype 3:** (C1). Light yellow-orange colony appearance; (C2) Carmine orange pigmentation; (C3-C4) Long and slender macroconidia with 5 septa. **Morphotype 4:** (D1) White-brownish colony appearance; (D2) Dark red pigmentation; (D3) 1: 0-cell fusoid microconidia; 2: Pyriform microconidia; 3: 3-septa mesoconidia; (D4) 3-septa macroconidia; (D5) 2-cell fusoid microconidia.

PCR and sequencing of TEF-1α gene

An approximately 720 bp of TEF-1α gene was amplified from 84 *Fusarium* isolates. Morphotype 1 isolates have 95.4% - 99.8% similarity with several species within *F. graminearum* species complex which were *F. mesoamericanum* (two isolates), *F. acaciae-mearnsii* (two isolates), *F. austroamericanum* (three isolates), *F. meridionale* (six isolates), *F. boothii* (13 isolates), *F. asiaticum* (16 isolates) and *F. cortaderiae* (32 isolates). Therefore, morphotype 1 isolates are referred to as *F. graminearum* species complex. In the present study, the isolates of the *F. graminearum* species complex were commonly isolated from *Elyhordeum montanense* which is wild grass growing on the hillside of Cameron Highlands, Malaysia. Besides grasses, *F. graminearum* species complex isolates were also isolated from flower parts of *Anthurium* sp., *Ipomoea purpurea* and *Begonia* sp., as well as from *Pinus* sp., ferns and mosses (Table 2).

Nine isolates of Morphotype 2 showed 94.2% to 95% similarity with sequences of *F. venenatum*. *Fusarium venetatum* isolates were isolated from several types of flowers (*Anthurium* sp., *Hibiscus* sp., *Amomum* sp., *Tibouchina semidecandra*) from Fraser's Hill and stem of *Pinus* sp. (Table 2).

Morphotype 3 (C5066a) has 99.3% similarity to

Table 2. *Fusarium* isolates recovered from different substrates from several highlands areas in Malaysia

Isolate	Species	Location	Plant parts	Host plant	Common name
Isolates from stock culture					
F1818v F3438v	*F. venenatum*	Fraser's Hill	Flower	*Anthurium* sp.	Anthurium
F1838v	*F. venenatum*	Fraser's Hill	Flower	*Hibiscus* sp.	Hibiscus
F3429v F3432v	*F. venenatum*	Fraser's Hill	Flower	*Amomum* sp.	Cardamom
C8658v C8606v C8779v C8754v	*F. venenatum*	Forest Reserve, Cameron Highlands	Stem	*Pinus* sp.	Pine
C5066a	*F. avenaceum*	Mount Brinchang, Cameron Highlands	Thallus	Moss	Moss
Isolates isolated from different host plants					
C8778s	*F. kyushuense*	Kea Farm, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8919g	*F. austroamericanum*	Mount Brinchang, Cameron Highlands	Leaf blade	*Paspalum conjugatum*	Buffalo grass
C8930g	*F. austroamericanum*	Mount Brinchang, Cameron Highlands	Flower	*Impatients walleriana*	Balsam
C8948g	*F. austroamericanum*	Tanah Rata, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8853g	*F. meridionale*	Mount Brinchang, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8854g	*F. meridionale*	Mount Brinchang, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8656g	*F. meridionale*	Forest Reserve Pine, Cameron Highlands	Pine, stem	*Pinus* sp.	Pine
C8918g	*F. meridionale*	Mount Brinchang, Cameron Highlands	Leaf blade	*Paspalum conjugatum*	Buffalo grass
C8743g	*F. meridionale*	Ringlet, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8949g	*F. meridionale*	Tanah Rata, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8839g	*F. mesoamericanum*	Mount Brinchang, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8941g	*F. mesoamericanum*	Kuala Terla, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8937g	*F. acacia mearnsii*	Tanah Rata, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8945g	*F. acacia mearnsii*	Palas River, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8660g	*F. boothii*	Forest Reserve Pine, Cameron Highlands	Needles	*Pinus* sp.	Pine
C8671g	*F. boothii*	Ringlet, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8756g C8888g C8902g C8843g	*F. boothii*	Ringlet, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8924g	*F. boothii*	Mount Brinchang, Cameron Highlands	Flower	*Lilium longiforum*	Easter Lily
C8928g	*F. boothii*	Mount Brinchang, Cameron Highlands	Flower	*Datura suaveolens*	Angel's Trumpet
C8942g	*F. boothii*	Palas River, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8944g C8947g	*F. boothii*	Palas River, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley

Table 2 continued...

C8950g C8951g	*F. boothii*	Tanah Rata, Cameron Highlands	Flower and Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8607g C8666g	*F. asiaticum*	Forest Reserve, Cameron Highlands	Stem	*Pinus* sp.	Pine
C8655g C8735g	*F. asiaticum*	Ringlet, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
K8697g	*F. asiaticum*	Kinabalu Park, Sabah	Flower	*Catharanthus roseus*	Periwinkle
C8705g	*F. asiaticum*	Forest Reserve, Cameron Highlands	Needles	*Pinus* sp.	Pine
C8838g	*F. asiaticum*	Forest Reserve, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8850g	*F. asiaticum*	Forest Reserve Tringkap, Cameron Highlands	Flower	*Ipomoea purpurea*	Morning Glory
C8855g	*F. asiaticum*	Mount Brinchang, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8929g	*F. asiaticum*	Mount Brinchang, Cameron Highlands	Flower	*Impatients walleriana*	Balsam
C8922g	*F. asiaticum*	Mount Brinchang, Cameron Highlands	Flower	*Cleome hassleriana*	Spider Flower
C8943g	*F. asiaticum*	Blue Valley, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8938g C8946g	*F. asiaticum*	Palas River, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8961g F5472	*F. asiaticum*	Palas River, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8657g C8669g C8670g C8757g C8758g	*F. cortaderiae*	Ringlet, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8662g	*F. cortaderiae*	Mount Irau , Cameron Highlands	Fern	-	Fern
C8737g	*F. cortaderiae*	Ringlet, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8808g	*F. cortaderiae*	Forest Reserve Tringkap, Cameron Highlands	Flower	*Anthurium* sp.	Anthurium
C8809g C8832g	*F. cortaderiae*	Forest Reserve Tringkap, Cameron Highlands	Leaf blade	*Paspalum conjugatum*	Buffalo grass
C4769g C8816g C8826g C8827g C8830g C8844g C8846g C8848g C8856g	*F. cortaderiae*	Mount Brinchang, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8831g C8834g	*F. cortaderiae*	Mount Brinchang, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8845g	*F. cortaderiae*	Forest Reserve Tringkap, Cameron Highlands	Flower	*Hippeastrum* sp.	Jersey Lily
C8917g	*F. cortaderiae*	Mount Brinchang, Cameron Highland	Flower	*Tibouchina semidecandra*	Morning Glory bush

Table 2 continued...

C8927g	*F. cortaderiae*	Mount Brinchang, Cameron Highlands	Flower	*Zingiber* sp.	Ginger
C8931g	*F. cortaderiae*	Tanah rata, Cameron Highlands	Stem	*Elyhordeum montanense*	Mountain Barley
C8932g	*F. cortaderiae*	Blue Valley, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8933g					
C8934g	*F. cortaderiae*	Palas River, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
C8935g					
C8953g	*F. cortaderiae*	Palas River, Cameron Highlands	Leaf blade	*Elyhordeum montanense*	Mountain Barley
C8957g	*F. cortaderiae*	Tanah Rata, Cameron Highlands	Flower	*Elyhordeum montanense*	Mountain Barley
K8788g					

Fusarium sp. (FD 01317) in Fusarium-ID and 99% to *F. avenaceum* based on GenBank. In this study, Morphotype 3 isolate was molecularly identified as *F. avenaceum* which was isolated from moss at Gunung Brinchang, Cameron Highland. Morphotype 4 (C8778s) isolate showed the closest match (97%) to *F. kyushuense* and the isolate was isolated from *Elyhordeum montanense.*

Therefore, based on the closest match of a BLAST search, isolates of morphotype 1 were molecularly identified as *F. graminearum* species complex (with several phylogenetic species), morphotype 2 as *F. venenatum,* morphotype 3 as *F. avenaceum* and morphotype 4 as *F. kyushuense.*

Although BLAST results of several isolates produced a low percentage of similarity, nevertheless the results provide a clue to which species the isolates belong to. The same species isolated from different substrates may show one or few differences in nucleotide sequences with the deposited sequences in both Fusarium ID and GenBank databases (Geiser *et al*., 2004).

A phylogenetic tree based on the ML method was generated based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. In the ML tree, isolates of *F. graminearum* species complex were grouped in clade 1 with many sub-clades (Figure 2). The grouping of the phylogenetic species was not according to the species, host plants or geographical locations. *Fusarium venenatum* isolates clustered in clade 2 and formed several sub-clades. *Fusarium kyushuense* was also grouped in clade 2 but formed a separate sub-clade. *Fusarium avenaceum* was grouped in clade 3.

RFLP- IGS and Cluster analysis of IGS restriction bands

All the *Fusarium* isolates yielded a single PCR product of approximately 2.4 kb except for the *F. avenaceum* isolate which produced an approximately 2.5 kb band. After digestion, different patterns produced by an enzyme were designated by the letter A – N and regarded as different haplotypes (Table 3). Fifty-nine IGS haplotypes were assigned to the isolates after digestion with six different restriction enzymes. Isolates of *F. graminearum* species complex produced the most variable haplotypes with 55 haplotypes (haplotypes 8 - 59) assigned among them. *Fusarium venenatum* isolates produced haplotypes 1 to 5, *F. avenaceum* haplotype 6 and *F. kyushuense* haplotype 7.

Due to variable restriction patterns showed by the *Fusarium* isolates, UPGMA cluster analysis was performed to cluster the isolates and to estimate the intra- and interspecific variability. From the dendrogram, the isolates can be separated into four clusters, 1, 2, 3 and 4 (Figure 3). *Fusarium venenatum* isolates were grouped in cluster 1. Isolates of *F. graminearum* species complex were clustered in cluster 2 and the cluster consisted of many sub-clusters which indicated genetic variability. *Fusarium kyushuense* (C8778s) and *F. avenaceum* (C5066a) were clustered in clusters 3 and 4, respectively (Figure 3).

Mycotoxin analysis

Fusarium avenaceum (C5066a) and *F. kyushuense* (C8778s) produced both BEA and MON. The concentration of BEA produced by *F. avenaceum* was 0.869 µg/kg and 0.321 µg/kg for *Fusarium* sp. For MON, *F. avenaceum* produced 0.004 µg/kg and *Fusarium* sp., 0.014 µg/kg (Table 4).

BEA and MON were not detected from isolates of *F. graminearum* species complex and *F. venenatum*. Only isolate of *F. avenaceum* produced FB_1 with a concentration of 0.001 µg/kg (Table 4). Thirty-two isolates of *F. graminearum* species complex and two isolates of *F. venenatum* produced ZEN except for one isolate of *F. graminearum* species complex (C8737g) and three isolates of *F. venenatum* (F1838v, C8658v, C8779v) did not produce ZEN. The concentration of ZEN produced by 32 isolates of *F. graminearum* species complex ranged from 0.002 – 0.437 µg/kg; *F. venenatum,* 0.006 - 0.014 µg/kg and *F. kyushuense,*

0.006 µg/kg (Table 4). ZEN was not detected in corn grits inoculated with *F. avenaceum*.

DISCUSSION

In the present study, it was very difficult to distinguish the isolates to species level based solely on morphological characteristics although the macroconidia characteristics among the four morphotypes showed different shapes and sizes which suggested the occurrence of different species. Most of the isolates produced relatively stout, thick-walled macroconidia with tapered apical cells and foot-shaped basal cells, and the absence of microconidia Therefore, morphological characteristics were used to sort the isolates into four groups designated as morphotypes.

Based on the closest match of a BLAST search, morphotype 1 contains the largest number of isolates which consisted of several lineages or phylogenetic species in *F. graminearum* species complex, namely *F. mesoamericanum*, *F. acaciae-mearnsii*, *F. austroamericanum*, *F. meridionale, F. boothii, F. asiaticum* and *F. cortaderiae*. The majority of the isolates were isolated from *Elyhordeum montanense.* Previous reports by Inch & Gilbert (2003) and Goswami & Kistler (2004) have shown that *F. graminearum sensu stricto* has also been recovered from wild grasses such as *Agropyron repens* (quack grass), *Echinochloa crusgalli* (barnyard grass),

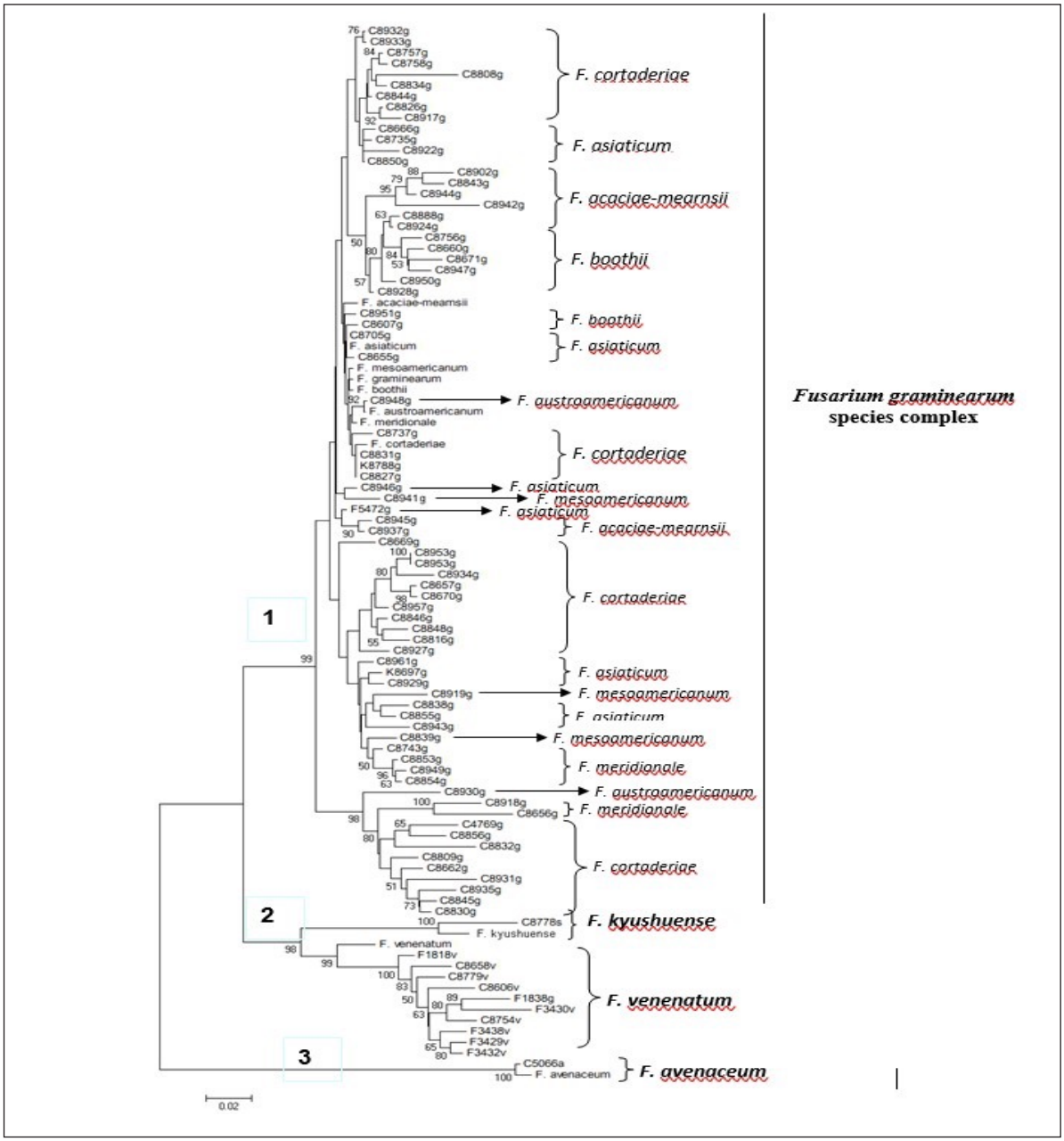

Fig. 2. Maximum Likelihood tree of *Fusarium* isolates from highland areas generated using TEF-1α sequences

Table 3. IGS haplotypes and restriction patterns of *Fusarium* isolates from highland areas in Malaysia

Isolate number	IGS haplotype	Restriction patterns					
		*Alu*I	*BsuRI*	*EcoRI*	*MspI*	*PstI*	*Taq*I
F. venenatum							
F1818v	1	D	B	B	E	G	F
C8606v	1	D	B	B	E	G	F
C8754v	1	D	B	B	E	G	F
C8779v	1	D	B	B	E	G	F
F3432v	1	D	B	B	E	G	F
F3438v	2	D	B	B	E	H	G
F3429v	3	D	B	B	E	H	G
F1838v	4	D	G	B	E	G	F
C8658v	5	D	B	B	E	G	H
F. avenaceum							
C5066a	6	G	H	B	G	I	N
F. kyusuense							
C8778s	7	H	F	B	F	B	F
Fusarium graminearum species complex							
C4769g	8	E	E	F	B	B	B
F5472g	9	A	A	D	A	F	G
C8607g	10	A	A	A	A	B	A
C8944g	10	A	A	A	A	B	A
C8655g	11	B	C	A	B	D	A
C8656g	12	G	B	A	C	B	C
C8657g	13	C	A	B	D	A	C
C8658v	14	D	B	A	E	G	F
C8660g	15	A	A	A	A	A	A
K8697g	15	A	A	A	A	A	A
C8816g	15	A	A	A	A	A	A
C8950g	15	A	A	A	A	A	A
C8662g	16	E	B	A	B	B	H
C8666g	17	A	A	D	A	B	A
C8669g	18	E	C	A	B	B	C
C8670g	18	E	C	A	B	B	C
C8935g	18	E	C	A	B	B	C
C8671g	19	F	A	A	A	B	A
C8843g	20	F	A	A	A	B	A
C8705g	21	E	B	C	D	D	D
C8735g	22	A	A	C	A	C	I
C8737g	23	C	D	A	D	B	H
C8846g	24	C	D	A	D	B	H
C8743g	25	E	C	A	C	B	C
C8756g	26	E	A	A	A	B	A
C8757g	27	E	B	A	C	B	C
C8758g	27	E	B	A	C	B	C
C8834g	27	E	B	A	C	B	C
C8778s	28	H	F	B	F	B	F
K8788g	29	A	A	A	A	C	A
C8808g	30	C	D	C	D	B	H
C8809g	31	C	C	C	D	B	H
C8826g	31	C	D	C	D	B	H
C8830g	31	C	D	C	D	B	H
C8845g	31	C	D	C	D	B	H
C8854g	31	C	D	C	D	B	H
C8827g	32	E	B	A	C	A	C

Table 3 continued...

C8930g	33	G	E	A	C	B	C
C8831g	33	G	E	A	C	B	C
C8948g	33	G	E	A	C	B	C
C8832g	34	C	D	E	D	B	H
C8933g	34	C	D	E	D	B	H
C8838g	35	C	D	C	D	A	H
C8839g	36	E	A	D	C	B	M
C8941g	36	E	A	D	C	B	M
C8844g	37	E	B	D	C	B	B
C8848g	37	E	B	D	C	B	B
C8855g	37	E	B	D	C	B	B
C8953g	37	E	B	D	C	B	B
C8850g	38	A	A	E	A	C	I
C8856g	39	E	B	D	C	B	C
C8888g	40	E	C	D	B	A	C
C8902g	41	F	B	A	A	B	A
C8917g	42	E	B	D	B	B	D
C8918g	43	G	B	A	D	H	K
C8919g	44	E	B	F	B	B	B
C8937g	44	E	B	F	B	B	B
C8931g	44	E	B	F	B	B	B
C8932g	44	E	B	F	B	B	B
C8957g	44	E	B	F	B	B	B
C8922g	45	D	C	C	A	E	E
C8924g	46	E	C	C	D	D	D
C8929g	46	E	C	C	D	D	D
C8927g	47	E	C	E	D	D	D
C8928g	48	D	C	C	A	D	D
C8934g	49	E	A	A	B	F	K
C8938g	50	E	C	C	A	D	D
C8941g	51	F	B	D	C	H	L
C8942g	52	A	B	A	A	B	E
C8943g	53	E	A	E	D	A	D
C8945g	54	E	A	A	B	F	C
C8946g	55	A	A	A	A	B	H
C8947g	56	A	A	D	A	A	A
C8949g	57	E	C	A	D	B	B
C8951g	58	A	A	A	A	A	G
C8961g	59	D	C	C	A	A	E

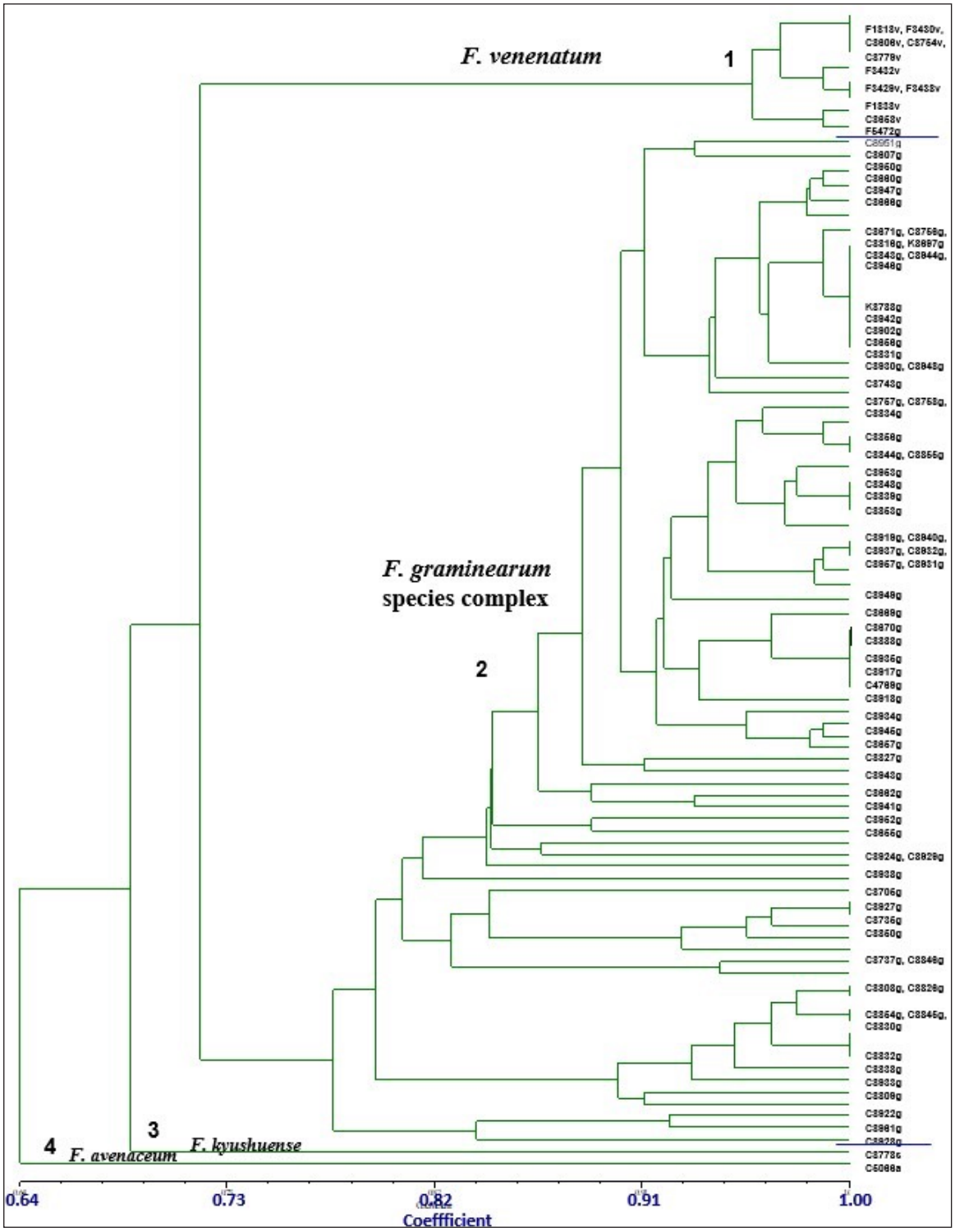

Fig. 3. Dendrogram generated using UPGMA cluster analysis of IGS-RFLP of *Fusarium* isolates from highland areas in Malaysia

Table 4. Mycotoxins produced by *Fusarium* isolates from highland areas in Malaysia

Fusarium species	Isolate	Mycotoxins Concentration (µg/kg)			
		BEA	$FUMB_1$	MON	ZEN
F. graminearum species complex	F5472g	-	-	-	0.182
	C4769g	-	-	-	0.066
	C8662g	-	-	-	0.003
	C8669g	-	-	-	0.002
	K8697g	-	-	-	0.017
	C8735g	-	-	-	0.136
	C8737g	-	-	-	-
	C8758g	-	-	-	0.066
	K8788g	-	-	-	0.005
	C8816g	-	-	-	0.022
	C8826g	-	-	-	0.006
	C8830g	-	-	-	0.003
	C8838g	-	-	-	0.003
	C8839g	-	-	-	0.019
	C8843g	-	-	-	0.067
	C8846g	-	-	-	0.004
	C8855g	-	-	-	0.012
	C8917g	-	-	-	0.035
	C8922g	-	-	-	0.090
	C8927g	-	-	-	0.271
	C8929g	-	-	-	0.437
	C8933g	-	-	-	0.011
	C8935g	-	-	-	0.017
	C8937g	-	-	-	0.051
	C8941g	-	-	-	0.020
	C8943g	-	-	-	0.003
	C8950g	-	-	-	0.033
	C8951g	-	-	-	0.004
	C8957g	-	-	-	0.036
F. kyusuense	C8778s	0.321	-	0.014	0.006
F. avenaceum	C5066a	0.869	0.001	0.004	-
F. venenatum	F1838V	-	-	-	-
	F3432v	-	-	0.006	0.014
	C8658v	-	-	-	-
	C8754v	-	-	0.003	0.006
	C8779V	-	-	0.023	-

*(-) Not detected

Hierochloe odorata (sweet grass) and *Spartina pectinata* (prairie cord grass).

Phylogenetic analysis of TEF-1α and RFLP-IGS analysis was applied to gain insight into the evolutionary relationship and genetic diversity of the isolates within *the F. graminearum* species complex. From both analyses, high levels of genetic diversity were observed and there was no correlation between geographic locations and the grouping of the isolates as well as RFLP-IGS haplotypes produced by the isolates. High levels of genetic diversity have been reported within *F. graminearum* species complex as well as within a phylogenetic species using several molecular markers such as Amplified Fragment Length Polymorphism and Random Amplified Polymorphic DNA (Wang *et al*., 2011). In a study by Sampietro *et al*. (2011) using variable number tandem repeat analysis, high levels of genetic diversity were also observed within the *F. graminearum* species complex and it is much greater than anticipated.

Members of the *F. graminearum* species complex are considered as single cosmopolitan species and morphological characteristics cannot be used to distinguish species in the species complex. The *F. graminearum* species complex has been reported to consist of at least 16 phylogenetic lineages and most of the phylogenetic species have been described (O'Donnell *et al*. 2000, 2004, 2008; Starkey *et al*., 2007; Yli-Mattila *et al*., 2009; Sarver *et al*., 2011) of which several species are pathogen causing head blight of cereal grains such as wheat, corn, barley and rice. *Fusarium graminearum sensu stricto* (lineage 7) which is the dominant species associated with the head blight of cereal grains was not found in the

present study.

Among the phylogenetic species identified in the present study, *F. asiatum* is initially widespread in Asia and recovered from wheat, rice and barley in China, Japan and Korea (O'Donnell *et al*., 2000; Lee *et al*., 2009). Later, *F. asiaticum* spread to North America, Europe and South America indicating the worldwide distribution of this species (Przemieniecki *et al*., 2015). The occurrence of *F. asiaticum* in highland areas in Malaysia might be due to the climatic conditions in the highland areas in which *F. asiaticum* has been reported in areas with temperatures >22ºC quarterly rainfall >320 mm (Backhouse, 2014).

The most common species of the species complex identified in this study was *F. cortaderiae* (32 isolates) of which this species as well as *F. austroamericanum* and *F. meridionale* are common in South America (Starkey *et al*., 2007; Sampietro *et al*., 2011). The isolates of *F. cortaderiae* have been recovered from pampas grass, corn and barley as well from soil and carnation (O'Donnell *et al*., 2004). Most of the isolates in the present study were isolated from grasses and a few from different types of flowers which is similar to previous reports (O'Donnell *et al*., 2004; Starkey *et al*., 2007; Sampietro *et al*., 2011).

In the present study, *F. boothi* (13 isolates) was recovered from pine needles, *Lilium longiforum, Datura suaveolens* and *Elyhordeum montanense*. *Fusarium boothi* has been reported from corn, wheat and rice. According to Backhouse (2014), reports of *F. boothi* are relatively few and the species is widely scattered mostly in Africa and Mexico, which tended to be warm regions, with lower seasonality of temperature, higher seasonality of precipitation and drier conditions.

Mycotoxin analysis showed that isolates of *F. graminearum* species complex produced only ZEN. Among biological active metabolites produced by *F. graminearum* species complex is ZEN and the isolates do not produce BEA and MON (Desjardins, 2006).

Morphotype 2 isolates were molecularly identified as *F. venenatum* and all the isolates produced relatively short and falcate macroconidia with the absence of microconidia and formation of chlamydospore. The morphological characteristics observed were similar to *F. venenatum* described by Nirenberg (1995) of which the species was described as part of *F. sambucinum sensu lato*. In the present study, *F. venenatum* were isolated from several types of flowers and stem of *Pinus* sp. This species is usually found on a variety of plant species such as *Solanum tuberosum*, *Zea mays*, *Humulus lupulus*, and *Triticum tuberosum* and soils especially in Europe (Nirenberg, 1995). *Fusarium venenatum* is a well-known source of mycoprotein for human consumption (O'Donnell *et al*., 1998; Yoder & Christianson, 1998). Plant pathogenic *F. venenatum* was reported by Rigorth *et al*. (2021), infected wheat causing foot and root rot on the cereal grains. Morphotype 2 isolates produced MON and ZEN, however *F. venenatum* is not known to produce both mycotoxins as well as BEA. *Fusarium venenatum* in the present study might be different strains of *F. venenatum* and therefore warranted further studies.

Morphotype 3 isolate (C5066a) was molecularly identified as *F. avenaceum.* In this study, the isolate was recovered from mosses at Mount Brinchang, Cameron Highlands and was probably an endophyte or saprophyte in mosses. *Fusarium avenaceum* has been reported as a pathogen of moss in Japan of which this species caused necrosis and death to the Sunagoke moss (*Racomitrium japonicum*) (Akita *et al*., 2011; Lehtonen *et al*., 2012). *Fusarium avenaceum* is a cosmopolitan soilborne pathogen in temperate regions, infected cereal grains and fruit crops (Leslie & Summerell, 2006). Among the diseases caused by *F. avenaceum* are bulb rot of ornamental onion (*Allium giganteum*) (Zhang *et al*., 2006), ear rot of corn (Ma *et al*., 2019), root rot of raspberry (Wang *et al*., 2017), wilt of black currant (Okorski *et al*., 2020) and branch canker of pear (Polat *et al*., 2022). *Fusarium avenaceum* (C5066a) produced BEA, FB1 and MON. Production of MON by *F. avenaceum* has been reported in Europe, North America and South Africa and the species also produced BEA (Desjardins, 2006). So far, there is no report of *F. avenaceum* that produced FB1.

Morphotype 4 isolate (C8778s) was isolated from *Elyhordeum montanense,* molecularly identified as *F. kyushuense.* Based on morphological characteristics, the isolate produced chlamydospores, pyriform, napiform and fusoid microconidia. The isolate (C8778s) in this study produced chlamydospores and pyriform conidia which were similar to the microscopic characteristics of *F. sporotrichioides* (Gerlach & Nirenberg, 1982; Aoki & O'Donnell, 1998). According to Aoki and O'Donnell (1998), both *F. sporotrichioides* and *F. kyushuense* are morphologically similar except *F. kyushuense* does not produce chlamydospores and has obovate conidia, which contrasted with morphological descriptions of isolate C8778s. Nevertheless, isolate C8778s were tentatively identified as *F. kyushuense* based on TEF-1α sequences and phylogenetic analysis. *Fusarium kyushuense* was described by Aoki & O'Donnell (1998) from diseased wheat and a vinyl plate in Japan. *Fusarium kyushuense* has also been reported to cause wilt of tomatoes (Wang *et al*., 2013), corn ear rot (Wang *et al*., 2014) and wilt of Gerbera daisy (Chen *et al*., 2022). Aoki & O'Donnell (1998) reported *F. kyushuense* produced both Type A and Type B trichothecenes. However, in the present study, isolate C8778s produced ZEN, BEA and MON. On the other hand, *F. sporotrichioides* can produced Type B trichothecene as well as BEA, MON and ZEA (Desjardins, 2006). Thus, to verify the species identity of morphotype 4 (isolate C8778s), further

study is needed particularly using multiple markers.

The present study suggested that the *Fusarium* species isolated from various plant parts might exist in the plant as endophytes as the isolates were recovered from apparently healthy plants and plant parts. A similar study was reported by Postic *et al*. (2012) in which *Fusarium* species from weed samples might be endophytes as the weed samples did not show any disease symptoms. The present study also indicated that the majority of the *Fusarium* species were isolated from two species of grasses, *Elyhordeum montanense* and *Paspalum conjugatum*. Several studies have also indicated that endophytic *Fusarium* species are commonly found as endophytes in Gramineae (Leslie *et al*., 2004; Phan, 2006; Sánchez Márquez *et al*., 2008; Walsh *et al*., 2010; Latiffah & Chua, 2013).

CONCLUSION

Crops from the grass family planted in Malaysia are rice and corn which are common host plants of some species of *F. graminearum* species complex. Rice is one of the major crops planted for domestic consumption, but corn is mainly planted by smallholders. Both crops are planted in low land areas where the climatic conditions are warmer with high humidity. To what extent the species within *F. graminearum* species complex can become potential pathogens to these crops warranted further studies. In the present study, species within *F. graminearum* species complex were recovered from different host plants and isolates tested were able to produce mycotoxin. Thus, infection of these species on crops should be of concern as mycotoxin can contaminate the crops. To our knowledge, this is the first report on the occurrence of phylogenetic species within *F. graminearum* species complex, *F. venenatum* and *F. avenaceum* in Malaysia.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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