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Morphological and molecular detection of *Fusarium chlamydosporum* from root endophytes of *Dendrobium crumenatum*

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Fusarium has a cosmopolitan distribution, with some species able to cause diseases in agricultural crops. A number of isolates of endophytic fungi were isolated from healthy roots of *Dendrobium crumenatum* (Orchidaceae) at Universiti Putra Malaysia campus. The isolates were primarily identified as *Fusarium chlamydosporum* based on morphological characteristics. The cultures were produced in a range of chicory pink to red and brown pigmentation. Only morphological studies of *Fusarium* can not give any guarantee at the species levels of identification but key the putative species names. Therefore, molecular studies based on gene sequencing of the internal transcribed spacer 1 and 2 regions of the ribosomal DNA (rDNA) were carried out. The amplified DNA was sequenced and aligned against the reference sequences, previously identified as *Fusarium* species. Results obtained have shown that all of the isolates putatively identified as *F. chlamydosporum* were in agreement with the ex-type strains of *F. chlamydosporum* gene sequences collected from the GenBank database. Thus, data from the present study suggested that the best conceivable connection between the morphological characters and the molecular characters are to use sequence based analysis of the ITS 1/2 regions of the rDNA of the original specimen at the same times as it could be removed any kind of controversial identification of *F. chlamydosporum* at the species level.

Key word: *Fusarium chlamydosporum*, internal transcribed spacer (ITS), sequencing, polymerase chain reaction (PCR).

INTRODUCTION

Orchids have an intimate association with fungi, at least at some stage of their life cycle (Watling and Lee, 2007; Rasmussen, 1995). Orchid mycorrhizas are known to

promote rapid germination in orchid seeds (Hadley and Williamson, 1972) but non-mycorrhizal endophytes are found in greater abundance and may play more significant role than that recognized thus far (Yuan et al., 2009). An endophytic fungus is one that is in a biological relationship with its host plant, causes no harm and remains asymptomatic to the host (Bacon and Yates, 2005). Their roles in plant life remain obscure; some authors hold the opinion that endophytes may be latent pathogens. According to Kogel et al. (2006), what decides whether an endophyte remains harmless or harmful is the fine balance between the demands of the invading fungus and the plant response; once unbalanced, the endophyte may transform into a pathogen.

Research on endophytic fungi has increased manifolds ever since they have been shown to have potentially

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Abbreviations: ITS, Internal transcribed spacer; PCR, polymerase chain reaction; PDA, potato dextrose agar; SNA, spezieller Nährstoffarmer agar; OA, oatmeal agar; PPA, pentachloronitrobenzene peptone agar; PSA, potato sucrose agar; CLA, carnation leaf agar; WA, water agar; PDB, potato dextrose broth; SDS, sodium dodecyl sulphate; NTS, non-transcribed spacer; ETS, externally transcribed spacer; IGS, intergeneric spacer region.

commercialization properties. Almost three decades later, Strobel et al. (1996) for example, discovered that paclitaxel was produced by a newly described endophytic fungus *Pestalotiopsis microspora*, isolated from the pine needles of *T. wallichiana*. This discovery implies that a large scale production of the natural product paclitaxel can be made at a lower cost just by mass production of the fungus in culture although the paclitaxel currently available is in a generic form. Strobel et al. (2001) found the novel species *Muscodora albus*, a fungal endophyte isolated from *Cinnamomi zeylanicum* as described by Woropong et al. (2001) to produce antifungal volatile compounds that proved lethal, especially to the human pathogens *Aspergillus fumigatus* and *Candida albicans*. Strobel et al. (2008) also discovered that endophyte *Gliocladium roseum* can produce myco-diesel hydrocarbons and is therefore an alternative candidate for the production of bio-diesels. In the agricultural sector, Rubini et al. (2005) isolated the endophyte *Gliocladium catenulatum* from *Theobroma cacao* and found it to hold potential as a biological control agent against *Crinipellis pernicioso*, the causal pathogen of Witches Broom Disease of Cacao.

Thus, there is a need to investigate the endophytic fungal community. Besides testing them for their potential biotechnological values, some isolates may actually be new taxonomic records or at its simplest level, new information. Classical taxonomy plays an important role in the identification of fungi but steps leading to the identification of species can be very complicated, laborious, subjective, and can lead to incorrect identification. The taxonomy of *Fusarium* in particular, can be very complex but should be investigated nevertheless as isolates with some doubtful names are widely cited in literatures although Malaysia and Southeast Asia (Salleh, 1994). While most fungal isolation are made on commercially packaged potato dextrose agar (PDA), accurate microscopic morphological characteristics of *Fusarium* species require appropriate growth media such as Spezieller Nahrstoffarmer Agar (SNA) or Oatmeal Agar (OA) (CBSxx) and allowed to grow for a certain length of time to dole out characteristic features such as the sporodochia, to develop. However, all the cultural steps undertaken do not guarantee that features looked for will be present and the procedure may need to be repeated. Mating studies are recommended for a definitive identification of *Fusarium* (Steenkamp et al., 2006); but this method too is laborious and not without its limitations.

In the light of the above, many researchers turn to molecular systematics as an alternative or a complementary tool to classical taxonomy. Central to this is the decision over which molecular marker to use, that will give the closest interpretation of the conventional morphological data. The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) has become a more popular marker for systematics and phylogenetic studies of closely related species of animals, plants and fungi (Von der Schulenburg et al., 2001). Of the transcribed rDNA region, the non-coding internal transcribed spacers,

described as ITS 1 and ITS 2 are found on each side of the 5.8S rRNA gene. The ITS 1 region only was found to be sufficient in the identification of *Aspergillus* species (Gaskell et al., 1997) and some medically important yeast (Chen et al., 2001). Some researchers used the ITS 2 region only, while others used the full length of the ITS 1 and/ ITS 2 which encompassed the 3' end of the 18S gene, the ITS 1 spacer region, the 5.8S gene, the ITS 2 spacer region and the 5' end of the 28S coding genes (Henry et al., 2000). Chen et al. (2001) found the ITS 1 and/ 2 target site allowed the identification of 35 species of medically important yeasts, whereas only 16 species were distinguished using the ITS 2 region. Henry et al. (2000) described that *Penicillium capsulatum* and *Penicillium glabrum* showed the highest similarity sequence to *Aspergillus* species, but the presence of a 10 bp sequence variation within the ITS 2 region allowed these two species to be readily distinguished from *Aspergillus* species in the main. This distinction was not possible when only the ITS 1 was sequenced.

The objective of this study was to select several fungal endophytic isolates of orchid *Dendrobium crumenatum* which showed an apparently common characteristic of producing a reddish pigmentation on PDA and suspected to be members of *Fusarium* however, at the same time exhibited some slightly different characteristics between each others. A classical taxonomy was performed on these isolates, but no mating studies were made. At the same time, the same isolates were typed at the contiguous internal transcribed spacer region of (18S)-ITS 1-(5.8S)-ITS 2-(28S) of the rDNA and their gene sequence compared to like isolates in the GenBank database for validation. While the aim of this work was not a phylogenetic re-evaluation, a dendrogram was constructed in order to view their genetic distance relationships to other members of the same species, to different species of the same genus and to outgroup samples belonging to other genera using sequences from the GenBank database. It is hoped that this study will show an agreement within and between the classical and molecular data. This study presents the conventional identification of *Fusarium chlamydosporium* and its corresponding gene sequences at the contiguous ITS 1 and 2 regions in the molecular characterization of the species.

MATERIAL AND METHODS

Isolation of endophytes

Wild epiphytic *D. crumenatum* growing in clusters at heights of three meters and above were collected from trees where they inhabit, either by climbing or using poles, from various locations of a 10 mile-radius of the Faculty Science, in the UPM campus. Only healthy, symptomless plants were selected. Three mature, fresh-looking roots per plant were randomly selected and washed under running tap water. The outermost velamen layer were peeled off and the exposed roots cut into 3 mm-length segments starting from about 5 cm away from the root tips. The segments were surface-sterilized in 70% ethanol followed by 0.05% sodium hypochlorite (or

Table 1. Isolates of *Fusarium* sp analysed in this study.

Isolates No.	<i>Fusarium</i> sp	Isolates code	Source	Sampling date	Host genotype
1	<i>F. chlamydosporum</i>	FA001	Nursery1	January, 2007	Orkid 1A
2	<i>F. chlamydosporum</i>	FA003	Nursery4	March, 2007	Orkid 5A
3	<i>F. chlamydosporum</i>	FA007	Nursery3	June, 2007	Orkid 3A
4	<i>F. chlamydosporum</i>	FA005	Nursery1	October, 2007	Orkid 7A
5	<i>F. chlamydosporum</i>	FA004	Nursery2	November, 2007	Orkid 2B
6	<i>F. chlamydosporum</i>	FA002	Nursery2	December, 2007	Orkid 4B
7	<i>F. chlamydosporum</i>	FA006	Nursery5	January, 2008	Orkid 1B

5% Clorox® bleaching agent) for 1 to 2 min, after that were rinsed in sterile water and blotted dry on sterilized filter papers. One or three root segments were then placed onto a potato dextrose agar (PDA) culture plate and incubated for 3 to 10 days under ambient laboratory conditions (28 ± 2°C, 12 h light and 12 h darkness). Fungi that gave reddish pigments in PDA and had spores that appeared to belong to the genus *Fusarium* when examined under the microscope were selected for subsequent macro and micro-morphological studies.

Monoconidial isolation

Mycelial tip fragments from PDA plates were next subcultured on pentachloronitrobenzene peptone agar (PPA) for monoconidial isolation. After approximately 4 day's growth, the conidia were harvested by flooding the lawn in 10 ml sterile water. The suspension was collected and spun at least twice on the vortex for 3 to 5 min, with fresh water changes in between the spins. The spore were then loaded onto an inoculating loop and streaked onto a 2% water agar (WA) in order to get single colonies of monoconidial cultures.

Macroscopic characteristics

The monoconidial colonies obtained were maintained on PDA. They were then subculture on PDA and potato sucrose agar (PSA) for macroscopic analysis. The characteristics observed were growth rates, gross colony features (appearance, texture) and pigmentation on both the top and reverse plates. The Methuen handbook of colour chart (Kornerup and Wancher, 1978) was used to describe the pigmentation colour.

Microscopic characteristics

The isolates were cultured on carnation leaf agar (CLA) for diagnostic micro-morphological characteristics. Fresh, disease-free carnation leaves were cut into 1 cm² pieces and oven-dried for 3 to 4 h at 110°C. Water agar (WA) at 18 g/L was prepared and autoclaved. During pouring into plates, 4 pieces of carnation leaf fragments were placed equidistant to each other over the molten agar and the media left to cool. Mycelial tip fragments of the monoconidial cultures were placed onto CLA which were incubated at 25 - 27°C under ambient conditions. After 10 days, a fragment of the agar culture was mounted on slides in a moist chamber made from sealed Petri dish and incubated for a few days. The slides were observed under light microscope.

The morphological characteristics observed followed that described in Burgess et al. (1994) and Leslie and Summerell (2006). Micrographs were made by using a camera with an image analyzer-SIS programme. The diagnostic characteristics examined

in the identification of species of *Fusarium* encompass the presence/absence of micro-, meso- and macro- conidia; their shapes and sizes if present; the type of phialides bearing microconidia (monophialide and/ or polyphialide) and the presence/ or absence of chlamydospores, microconidial chains and sporodochia on CLA. All of the seven isolates were stored in 10% glycerol at -80°C until needed (Table 1).

DNA extraction

The isolates were cultured in liquid media to obtain mycelia mass for DNA extraction. Agar discs were cut out from an actively growing *Fusarium* colony with a 5 mm-diameter cork borer and placed into 100 ml potato dextrose broth (PDB) (Difco; USA) as starter cultures. The flasks were maintained as still cultures for 10 to 12 days under ambient laboratory conditions. The mycelial mats were harvested by filtration through a double layered muslin cloth, washed several times with sterile water and then ground with a mortar and pestle swabbed with ethanol prior to its use. The slurry obtained was stored at -20°C or used immediately for DNA extraction. Total fungal DNA was extracted by the Phenol-Chloroform Method (Raeder and Broda, 1985). About 50 mg of the ground mycelium was added 500 µl extraction buffer (1 M Tris HCl [pH 8.5], 1 M NaCl [pH 8.5], 1 M EDTA [pH 8.0] and 10% sodium dodecyl sulphate (SDS) and the reaction tubes were placed in a water-bath for 8 h at 40°C. They were next added with 350 µl buffered phenol and 150 µl chloroform, manually mixed in an up-down motion for 10 min and then centrifuged at 13,000 g at 4°C for 10 min. The upper aqueous layer was collected into sterile microcentrifuge tubes to which was added 3 µl RNase solution. The mixture was incubated at 40°C in a water bath for 15 min. An equal volume of chloroform was added to the sample, gently mixed manually for 10 min and then centrifuged a second time (at 13,000 g / 10 min/ 4°C). The upper aqueous phase was again collected into a new microcentrifuge tube and the DNA was precipitated with 250 µl iso-propan-2-ol; the samples were kept overnight at -20°C. The tubes were centrifuged the next day (13,000 g/ 10 min/ 4°C), and the pellet thoroughly washed twice with 500 µl of 70% ethanol, vacuum-dried and diluted in ddH₂O and kept at -20°C or directly proceed to the next step, which was to check the quality of the DNA samples by performing electrophoresis on 1.5% agarose gel using lambda DNA as marker. Electrophoresis was performed in a 1x TBE (0.045 M Tris-borate and 1 mM EDTA [pH 8.2]) running buffer at 70 V for 1 to 2 h. The appearance of bands on the gel after by agarose gel electrophoresis indicated the presence of DNA and thus, allowed the next step, which was preparation of DNA templates for sequencing.

Primers

Two oligonucleotide primers, namely ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC GCT TAT TGA TAT GC 3') as

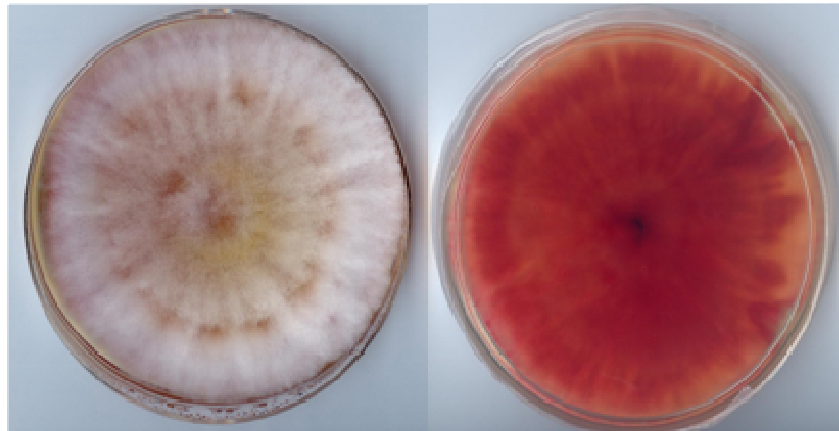


Figure 1. *F. chlamydosporum* colony cultures (observe and reverse) on PDA (smaller concentric ring, white, raised, interspersed by yellow pale purple-pink, outermost yellow, bottom: cherry red with not well-defined concentric rings).

described by White et al. (1990) were used. These two primers amplified the non-coding spacer regions ITS 1 and 2 and the conserved 5.8S; included as well are the partial conserved coding regions of the 18S and 28S genes which are interspersed between the ITS 1 and 2 spacer regions. The ITS1 and ITS2 primers were synthesized by another laboratory through 1st BASE Laboratories Sdn Bhd, Malaysia.

PCR amplifications

The PCR amplifications were directly sequenced using the ITS1 and ITS4 primers. 50 ng was diluted genomic DNA sample in a total reaction volume of 50 μ l, was added 2.5 μ l of PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 10 mM dNTP, 2.5 μ l of 100 mM MgCl₂, 0.5 μ l of 5,000 units/ml *Taq* DNA polymerase (Bioron. net) and 1 μ M each of the primers ITS1 and ITS4. The PCR mixtures were amplified in a Peltier Thermal Cycler-200 and subjected to an initial denaturing at 95°C for 5 min followed by 36 cycles of 1 min at 94°C, an annealing temperature at 55°C for 1 min, extension at 72°C for 3 min and a final extension of 10 min at 72°C. The amplicons were separated by horizontal electrophoresis using 1.7% agarose minigels with a 100 bp molecular marker (Promega®, USA). Electrophoresis was performed in a 1 x TBE (0.045 M Tris-borate and 1 mM EDTA, pH 8.2) running buffer at 70 V for 1 to 2 h. The gels were finally stained with ethidium bromide (0.5 μ l/ml). After 15 to 30 min they were visualized under UV- light and photographed using the Alphamager® 2200 version 5.5 apparatus.

DNA sequencing, alignment and Phylogenetic analysis

Template DNA (50 μ l) for sequencing was prepared purifying the PCR products using a commercial kit (QIAquick® PCR Purification Kit, Canada) with procedures undertaken following the manufacturer's instructions. The purified products (30 μ l) were sent to 1st BASE Laboratories Sdn Bhd, Malaysia for the sequencing of all 7 test isolates. The sequences of ITS 1 and 2 regions of the test isolates thus obtained were next edited in order to generate a consensus sequence from the forward and reverse sequence runs in the amplicon using the BioEdit Sequence Alignment Editor Software (<http://www.bioeditor.sdsc.edu/download.shtml>). Next, the most related corresponding sequences of the 9 strains of *F. chlamydosporum* was selected from the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>), 2 sequences of *Fusarium solani* (AB369490 and EU625405), 3 sequences of *Cylindrocarpon* sp (EU740396, AB369421 and AB369414) and 2 sequences of *Termitomyces heimii* (EU44386 and strain TH02) (Figure 7). Multiple sequence alignments were performed in NEXUS format by using the default option in CLUSTAL-X software version 1.83 (Thompson et al., 1997) based on the algorithm of Waterman (1986). The sequence gaps were treated as a fifth nucleotide (A, C, G, T and gaps) phylogenetic tree and distance matrix were constructed using MEGA software version 3.10 (Kumar et al., 2004) which implemented the Neighbor-Joining (NJ) dendrogram programme of Saitou and Nei (1987). The degree of confidence in phylogenetic branching was assessed using 1,000 bootstrap resamplings based on the method of Felsenstein (1985). The phylogenetic distance was defined as the probability of nucleotide substitutions per site, based on the Kimura 2-parameter model (K2P) (Kimura, 1980).

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RESULTS

Close to 50 fungal endophytes were obtained in total from *D. crumenatum* but only seven gave a reddish pigmentation on PDA with strong indications as being members of *Fusarium* in the preliminary observation.

Macroscopic characteristics

A description of the macroscopic characteristics for each isolate on PDA is given in Figure 1. Generally, the culture grew fast on PDA; the mycelia were floccose, fairly dense, off white and turned lilac in older portions of the colony, some interspersed with yellow pigmentation, in others, the yellow pigmentation formed the outermost ring at the periphery. The reverse cultures showed several shades of red to brown in the 7 isolates (Figure 1). The mycelial characteristics on PSA was not tabulated because they showed similar characteristics on growth on PDA except for 2, which features. The first concerned the yellow pigmentation was brighter, more conspicuous

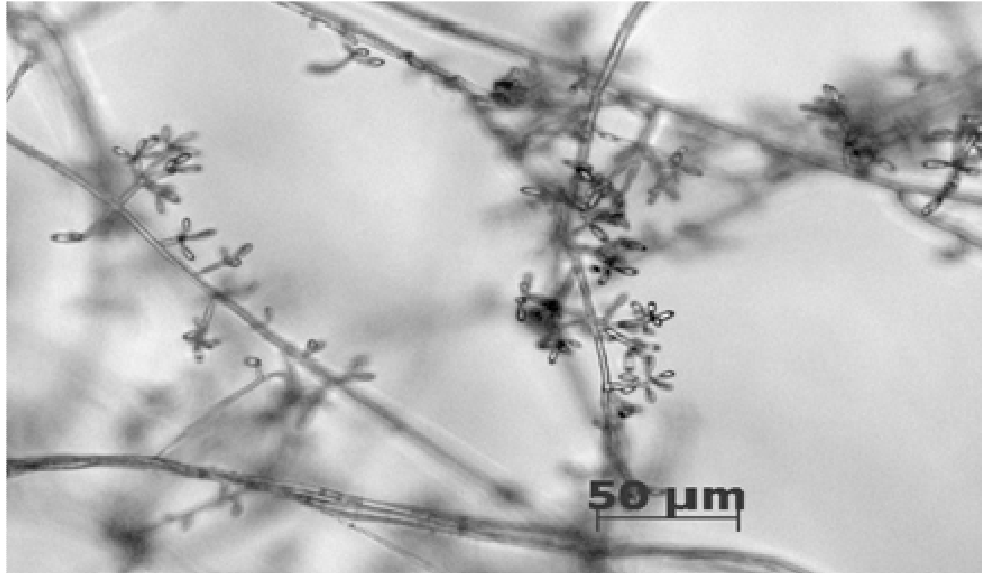


Figure 2. Simple polyphialidic conidiophores of *F. chlamydosporum* aerial mycelium.

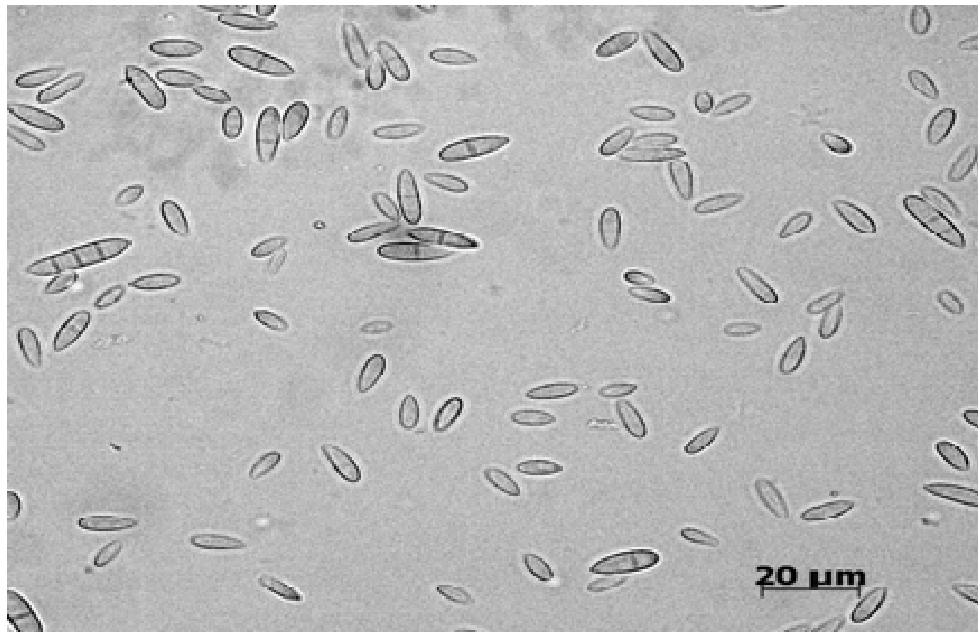


Figure 3. Straight, rare and falcate macroconidia of *F. chlamydosporum* with 2-3 septa per conidium (a), abundant oval to obovate single microconidia with 0 - 1 septa (b) and slightly larger mesoconidia with 1 - 2 septa (c).

and appeared as a wider band at the periphery rather than interspersed in the whitish mycelia. The second concerned the pigmentation on the reverse culture, which was a dark brown in the central portion and shades of reddish brown to a dull brown colour as the mycelia radiates to the periphery. None of the cultures showed bright red pigmentation as was observed for some cultures on PDA.

Microscopic characteristics

Generally, macro-, micro- and meso- conidia were present but no sporodochia were observed even in 9-day old cultures. Conidiophores in the aerial mycelia were mostly short branched (Figure 2). The rare macroconidia were formed straight, rare and falcate with 2-3 spate per conidium (Figure 3). Microconidia were abundant and

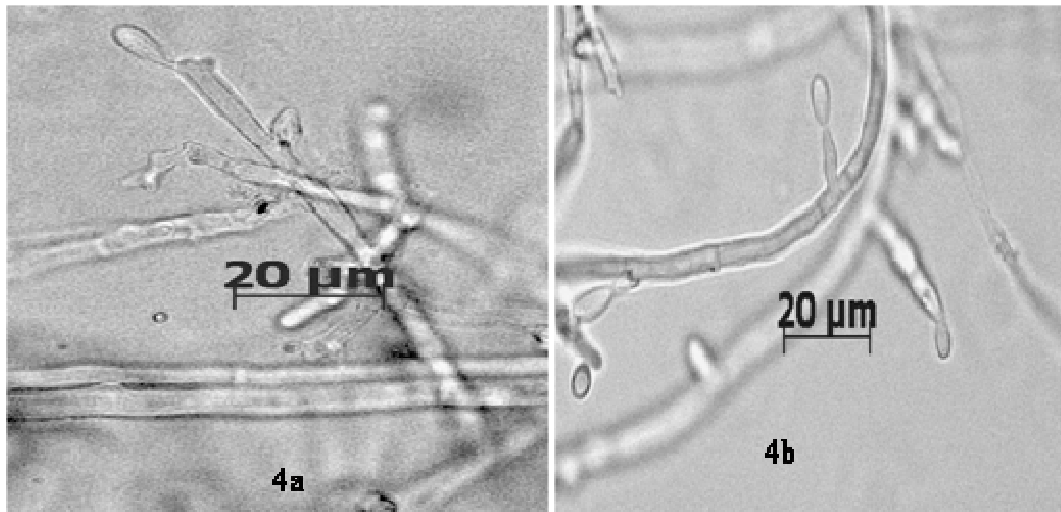


Figure 4. a and b: Simple monophyalidic conidiophores of *F. chlamydosporum* in the aerial mycelium.

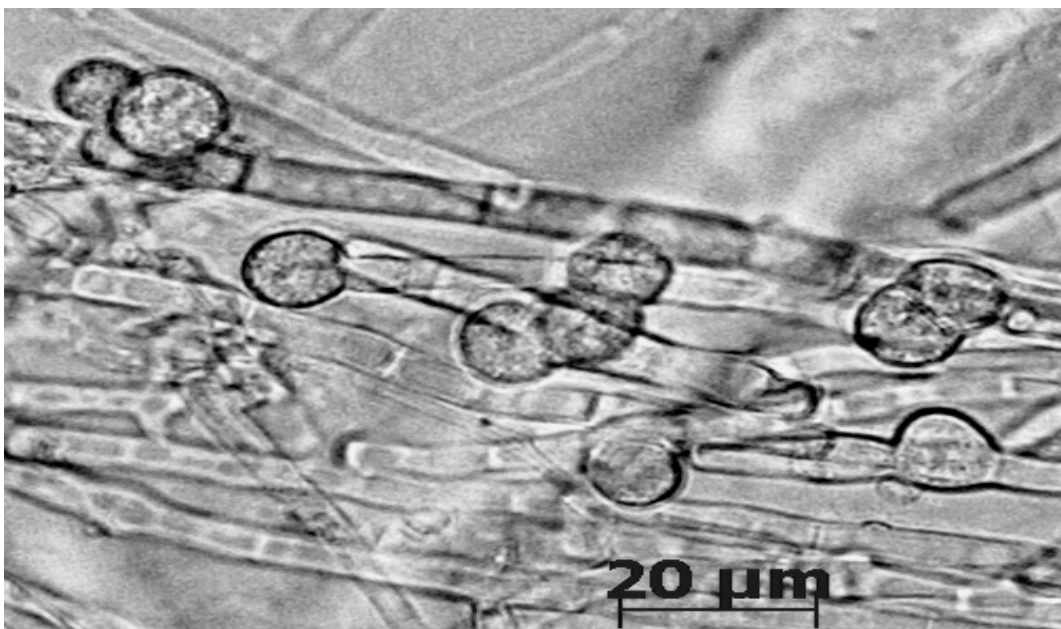


Figure 5. Chlamydoconidia of *F. chlamydosporum* in the aerial mycelium.

occurred singly with oval to obovate shapes (Figure 3). The isolates also produced primary conidia or mesoconidia with 1 - 2 septa. Conidia were borne from both mono- and polyphialides (Figures 4a and b). Chains of microconidia were absent; chlamydoconidia were abundant and formed rapidly, in single, chain or cluster (Figure 5). So, the isolates morphologically putatively identified of *F. chlamydosporum*.

DNA amplifications

Amplification of the rDNA regions with primers ITS1 and

ITS4 yielded products of approximately 600 bp as estimated by agarose gel electrophoresis (Figure 6). The sequences used for the final phylogenetic analysis were 482 to 533 bp after manual counting trimming and corresponded to the ITS 1 and 2 complete regions; the 5' portion of the 18S gene, 5.8S complete sequence and the 3' end of the 28S gene. In addition to ITS 1, 5.8S rDNA and ITS 2, such sequences contained the last base of the 28S rDNA and the first bases of the 18S rDNA. After that each test sequences were compared to the referred sequences collected from the GenBank, similarities values of 90 to 100%.

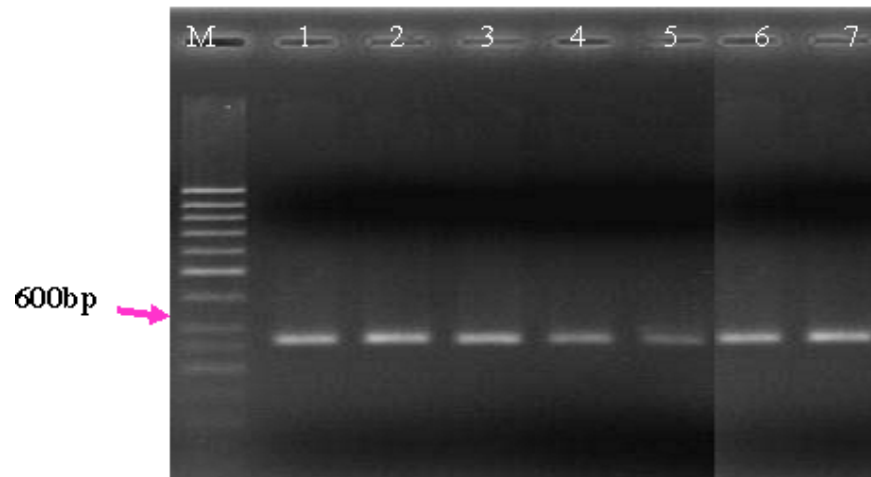


Figure 6. Banding patterns produced by the primer ITS-1 and ITS-4. Lane: 1 - 10. Putative *F. chlamydosporum* isolates: **1)** FA004; **2)** FA002; **3)** FA005; **4)** FA007; **5)** FA001; **6)** FA003 and **7)** FA006. M = 100 bp marker.

Phylogenetic analysis

On the bases of a neighbor-joining (NJ) analysis with 1,000 bootstrap replications, all isolates were divided into two major clusters A and B (Figure 7). Major cluster B was the out-group isolates (strains: TH1 and TH2) belong to *T. heimii* species. Major cluster A consisted of two groups namely group 1 and group 2. Group 2 consisted of the two ex-type strains of *F. solani* representative using for correctly differentiate with *F. chlamydosporum* isolates. Group 1 split into two subgroups (Subgroups 1 and 2). Subgroup 2 consisted of *Cylindrocarpon* sp isolates. The test isolates sequences were then subjected in the same genus namely *F. solani*, *F. chlamydosporum*. The sequences of *Cylindrocarpon* sp were included for difficult to differentiate from *Fusarium* isolates. Sequences of the ITS 1 and 2 regions of the rDNA of the 7 isolates putatively identified as *F. chlamydosporum* (derived from morphological characters), was found to exhibit a similar sequence with the ex-type nine strains gene sequences, collected from the Genbank database obtaining subgroup 1. The resultant NJ dendrogram from this analysis were well-fitted in this group with a bootstrap stability 100% of the test isolates and on the basal position of 87% stability with the referred of ex-type strains. Thus, all test isolates could be referred to synonymy as *F. chlamydosporum*.

DISCUSSION

The anamorphic genus *Fusarium* is a phytopathogenic fungus with a global distribution, capable of infecting a wide range of agricultural crops such as maize, wheat or barley and orchid plants, and also reduced quality and yield of the grain. Many species of the genus *Fusarium*

are causing the most serious diseases affecting root endophytes of orchid plant (Orchidaceae) and in all growing stages, still has not been adequate investigated in Malaysia. *Fusarium* is characterized by the production of septate, hyaline, delicately curved and elongate macroconidia. Traditionally, species have been separated and described based on morphological and cultural characteristics, such as pigment production, presence color of mycelia, phialides, macro-, micro- and mesoconidia shape-size, sporodochia and chlamydospores (Figures 1-5). Cultural methods coupled with morphological details from microscopy, are among the techniques used and assign one to detect exactly which taxon is present (identification). These methodologies are easy, fast and consistent in the given putative species name, however, have limitations which impede a proper assessment.

Davet and Rouxel (1997) mentioned that the traditional methods outlined above tend to overestimate species, while those in mycelial state or those that have slow growth in culture are largely overlooked. These culturable isolates are those that can utilize the energy source under the physical and chemical limitations of the growth medium. Regarding the taxonomic classification of *Fusarium*, fungi still have limitations. There are many species that appear to be similar under cultural conditions and exhibit similar morphology, yet are different. Processing of these cultures can be time-consuming and laborious when a large number of isolates have to be handled. During these processes, the risk of culture contamination is always high and especially the fast-growing fungi overgrow inside the whole medium. Nevertheless, these work multidisciplinary approaches have made for the correct identification of *Fusarium* species.

Morphological characters provide the basis of current fungal systematics. They provide a wealth of information to distinguish taxa and used extensively at different

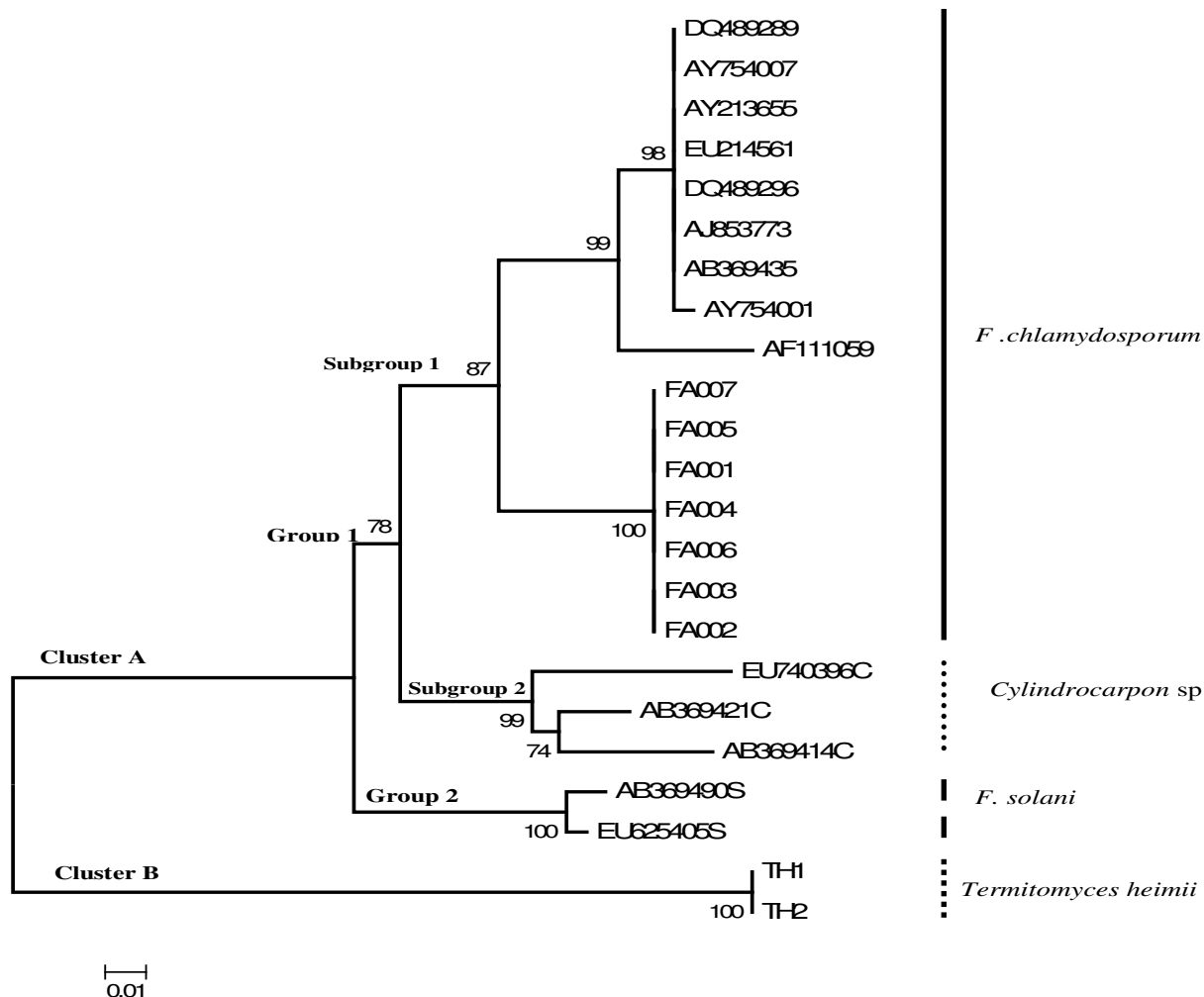


Figure 7. Phylogenetic relationships of *F. chlamydosporum* isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of ITS sequences. Sequences used for this comparison were obtained under the following GenBank accession numbers: AB369435; EU214561; AY213655; DQ489296; DQ489289; AJ853773; AY754007; AY754001; AF111059; AB369490; EU625405.

hierarchies. In some cases, morphological criteria present some problems and fail to resolve taxonomic relationships. This is true in cases where morphological characters are insufficient, convergent, reduced, missing or overlapping. Morales-Rodríguez et al. (2007) reported that *Fusarium nivale* is a misidentification; it does not produce macroconidia and is different from *Fusarium* and its teleomorph (sexual stage), *Monographella nivalis*, belongs to a different group of ascomycetes from the teleomorphs of *Fusarium*. Actually it is placed in the genus *Gerlachia* and also known as *Microdochium nivale*. At present, the morphological features used to establish relationships between species levels- are not phylogenetically decisive, because the lack of clear morphological data or confusion in determining taxa (Bergamaschi et al., 2007).

As a consequence, many taxonomists have not used the combined work at the same time of *Fusarium* isolates

based on morphological and molecular approach to elucidate the taxonomic position. Molecular techniques that have applied successfully in genus *Fusarium* systematics and the application of DNA sequencing attached with phylogenetic tree have significantly extended, owing to the ever-increasing amount of sequences data. Molecular characters offer considerable potential, as it not only closed the gap between the traditional and molecular methods, but also determines relationships between uncultured and cultured fungi. Recently, described *Fusarium* species have initially been discovered by molecular tools used in phylogenetic studies, followed by a formal description of the species (Tan and Niessen, 2003).

In this study, the gene sequence of the ITS 1 and 2 regions of the rDNA of isolates of *Fusarium* were examined. A total of 7 isolates of *Fusarium* were previously identified by morphological as *F. chlamydosporum*. The phylogenetic analysis, Subgroup 1 showed that all of the 7 isolates gave

the same gene sequence to each other and to the referred *F. chlamyosporum* strains from NCBI. The genetic distance among these isolates were virtually 0.0056 (data not shown), thus suggesting that these isolates morphological characters are very similar (i.e are phylogenetically very closely related) to *F. chlamyosporum*. The subgroup 1 was well supported by the bootstrap value of 87% in these analyses.

DNA sequencing is now more of a specific identification of an organism than traditional culture-based methods: identification is refined by including ITSs sequence analyses with phenotypic characterization. For several decades, the nuclear-encoded ribosomal DNA (rDNA) gene has been the gene of interest to review phylogenetic relationships and resolve taxonomic questions at different taxonomic levels (Cai et al., 2005; Jeewon et al., 2004). This is because in eukaryotic cells, the rDNAs are organized in a cluster that includes a small subunit gene (18S), a large subunit gene (28S) and the 5.8S gene that lies in between two internal transcribed spacers (White et al., 1990). The regions that separates the cluster of three genes along the chromosome is called the non-transcribed spacer (NTS) and, prior to where the 18S gene is transcribed, there is another small spacer region called the externally transcribed spacer (ETS). Together, the ETS and NTS regions comprise the intergeneric spacer region (IGS).

The spacer regions of ITS 1 and 2 of the rDNA are widely and routinely used in analysis of species relationships using a phylogenetic reconstruction method in various organisms. It was successfully applied in analysis of phylogenetic relationship between *F. chlamyosporum* species and conclusions from phylogenetic analysis were well in agreement with those from analysis based on morphological systematics and other molecular techniques such as PCR based on the gene sequencing of the internal transcribed spacers (ITSs). Phylogenetic analysis generated trees with similar topologies that were very concurrent with the morphological taxonomy. Therefore, ITSs sequences characteristics are an effective tool in reconstruction of evolutionary relationship among these species, and can be applied in establishment of species relationship (Figure 7).

Most journals publishing molecular phylogenies require that the sequences be deposited in accessible repositories, such as GenBank. However, more molecular data become available; there is a growing concern about the taxonomic origin of these data. In these studies, we had a detail outline for meaningful species identification of *F. chlamyosporum*. Besides the morphological characteristics, additional information provided were: source of isolates, culture of isolates, macro-, micro- and meso-conidia, sporodochia and chlamyospores features (Figures 1 - 5) and molecular phylogenetics, example, liquid culture, DNA extraction, PCR amplification, PCR product purification, etc (Figures 6-7). Molecular data in phylogenetic analyses will, undoubtedly, continue to proliferate in the

future and lead to large amounts of information easily retrievable for researchers. Under these circumstances, the best conceivable connection between the morphological and molecular data of isolates achieved through detailed photograph or drawings of the specimens will be removed any controversial identification of *F. chlamyosporum* at the species level.

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