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SHORT COMMUNICATION

Molecular Characterisation of Endophytic Fungi from Roots of Wild Banana (*Musa acuminata*)

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Abstrak: Kulat endofitik mendiami tisu tumbuhan yang kelihatan sihat dan tersebar luas dalam tumbuhan daratan terutamanya tisu akar yang mengandungi himpunan pelbagai kulat endofitik. Kajian ini memberi tumpuan kepada pemencilan dan pencirian kulat endofitik yang terdapat dalam akar pisang liar (*Musa acuminata*). Sejumlah 31 pencilan kulat endofitik telah dipencilkan daripada 80 fragmen akar. Kulat endofitik tersebut pada mulanya diasing berdasarkan ciri-ciri morfologi dan dikenal pasti menggunakan jujukan gen faktor pemanjangan translasi-1α (TEF-1α) untuk mengenalpasti *Fusarium* spp. dan kawasan *Internal Transcribed Spacer* (ITS) digunakan untuk kulat yang lain. Spesies kulat yang paling lazim dipencilkan adalah *Fusarium* dan pencilan-pencilan tersebut telah dikenal pasti sebagai *F. proliferatum*, *Fusarium* sp., kompleks spesies *F. solani* dan *F. oxysporum*. Kulat endofitik lain yang telah dipencilkan adalah *Curvularia lunata, Trichoderma atroviride, Calonectria gracilis*, *Rhizoctonia solani*, *Bionectria ochroleuca*, dan *Stromatoneurospora phoenix* (Xylariceae). Beberapa genus kulat seperti *Fusarium*, *Trichoderma*, *Rhizoctonia,* dan Xylariceae merupakan antara kulat endofitik lazim yang dilaporkan dalam tumbuhan. Kajian ini menunjukkan bahawa akar pisang liar mengandungi pelbagai kumpulan kulat endofitik.

Kata kunci: Kulat Endofitik, *Musa acuminata*, Akar

Abstract: Endophytic fungi inhabit apparently healthy plant tissues and are prevalent in terrestrial plants, especially root tissues, which harbour a wide assemblage of fungal endophytes. Therefore, this study focused on the isolation and characterisation of endophytic fungi from the roots of wild banana (*Musa acuminata*). A total of 31 isolates of endophytic fungi were isolated from 80 root fragments. The endophytic fungi were initially sorted according to morphological characteristics and identified using the sequences of the translation elongation factor-1α (TEF-1α) gene of *Fusarium* spp. and the Internal Transcribed Spacer (ITS) regions of other fungi. The most common fungal isolates were species of the genus *Fusarium*, which were identified as *F. proliferatum*, *Fusarium* sp., *F. solani* species complex, and *F. oxysporum*. Other isolated endophytic fungi included *Curvularia lunata*, *Trichoderma atroviride, Calonectria gracilis, Rhizoctonia solani, Bionectria ochroleuca,* and *Stromatoneurospora phoenix* (Xylariceae). Several of the fungal genera, such as *Fusarium, Trichoderma, Rhizoctonia*, and Xylariceae, are among the common fungal endophytes reported in plants. This study showed that the roots of wild banana harbour a diverse group of endophytic fungi.

Keywords: Endophytic Fungi, *Musa acuminata*, Root

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Studies on endophytic fungi were initially undertaken mainly in temperate plants. Plant diversity is higher in tropical regions, and these plants might harbour a wider assemblage and more diverse fungal communities. Therefore, the fungal endophytes from tropical plants have received much attention, and most studies indicate that the fungal endophytes from these tropical plants consist of a very diverse and species-rich fungal assemblage (Arnold & Lutzoni 2007; Rungjindamai *et al*. 2008; Tejesvi *et al*. 2009; Huang *et al*. 2009; Rocha *et al*. 2011; Orlandelli *et al*. 2012; Tayung *et al*. 2012). Studies on the occurrence and diversity of fungal endophytes are expected to yield potential sources of natural products and bioactive compounds for medicinal, agricultural and industrial uses, such as new antibiotics as well as novel biological control agents (Molina *et al*. 2012).

Endophytic fungi are usually defined as fungal isolates that grow within plant host tissues without causing any disease symptoms (Schulz *et al*. 1999). Fungal endophytes also refer to fungi within apparently healthy, functional root tissues at the 'moment' of sample collection (Sieber 2002). This concept was supported by Schulz and Boyle (2005) to describe bacteria and fungi that can be detected at a particular 'moment' within the tissues of apparently healthy plant hosts.

Almost all vascular plant species harbour fungal endophytes, which are present in virtually all plant tissues and organs. Some endophytic fungal taxa are regarded as seed-borne endophytes (Hyde & Soytong 2008). Endophytic fungi also contribute to fungal biodiversity, which was estimated to include 5.1 million existing species (Blackwell 2011).

Wild banana (*Musa acuminata*) is in the family Musaceae, which is native to Southeast Asia and is an ancestor of the edible banana. The highest species diversity in the Musaceae family occurs in South East Asia, with representative species found across the Old World Tropics. Studies on the endophytes of *Musa* sp. were conducted in Australia and Hong Kong, mostly on isolations from the leaves of cultivated banana plants (Brown *et al.* 1998; Photita *et al*. 2001). In this study, endophytic fungi were isolated from the roots of wild banana (*M. acuminata*). Many reports have indicated that plant roots harbour diverse groups of fungi and, moreover, the occurrence of endophytic fungi in *Musa* sp. in Malaysia is not well-documented. Therefore, the objective of this study was to isolate and identify the endophytic fungal taxa in the roots of wild banana.

Banana root samples were randomly collected from healthy and symptomless wild banana trees at five locations along Balik Pulau Road, Pulau Pinang. The roots were taken by digging the soil around the banana tree. All root samples were put in plastic bags according to their respective locations.

The soil attached to the roots was removed by washing them with running tap water for 24 hr. To isolate the endophytic fungi, the surfaces were sterilised. The roots were cut into small fragments (2.0–3.0 cm), dipped in 70% ethanol for 30 sec, then dipped in 1% sodium hypochlorite for 3 min, 95% ethanol for 5 min and finally rinsed 3 times with sterile distilled water. The root fragments were then dried using sterile filter paper, cut into much smaller fragments (1.0–1.5 cm) and plated onto potato dextrose agar (PDA).

Four root fragments were plated onto a PDA plate, and a total of 80 root fragments were used for isolation. Before plating onto the PDA plate, the root fragment was imprinted on the PDA plate to detect the presence of epiphytes on the root fragment (Schulz *et al*. 1993). The plates were incubated at 27±1°C and observed every day to detect any fungal growth from the root fragments. Mycelial growths from the roots were sub-cultured onto new PDA plates.

To identify the endophytic fungal isolates, pure cultures from a single spore isolation were used. The fungal isolates were identified using microscopic and macroscopic characters according to the methods and fungal descriptions of Ellis (1971), Barnett and Hunter (2006), and The *Fusarium* Laboratory Manual (Leslie & Summerell 2006). The main purpose of morphological identification is to group the fungal isolates according to similar morphological characteristics. The fungal isolates were considered as belonging to the same group or genus if their morphological characteristics matched the morphological descriptions previously described or reported.

For species confirmation of the morphologically identified isolates, sequencing of the translation elongation factor-1α (TEF-1α) gene and Internal Transcribed Spacer (ITS) regions was performed. TEF-1α sequences were used to confirm the morphologically identified *Fusarium* isolates (Geiser *et al*. 2004); ITS regions were used for the other morphologically identified endophytic fungal isolates. The ITS region is regarded as a DNA barcode marker for the identification of fungi (Schoch *et al.* 2012): therefore, this region was used in this study.

ITS regions were amplified using ITS1 and ITS4 primer pairs (White *et al.* 1990). PCR amplifications were performed in a total volume of 25 µL containing 0.5 µL of 0.5 µM template DNA, 5 µL 5× PCR buffer, 4.0 mM $MgCl_2$, 0.8 mM dNTP mix (Promega, WI, USA) of both forward and reverse primers and 0.625 U of *Taq* polymerase (Promega, WI, USA), in a PTC-100 Peltier Thermal Cycler (MJ Research Inc., MA, USA). PCR cycles began with an initial denaturation at 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

The TEF-1α gene was amplified using EF1 (ATGGGTAAGGAGGACAAGAC) and EF2 (GGAAGTACCAGTGATCATGTT) primers modified from O'Donnell *et al.* (1998). PCR amplification was performed in a 25 µL reaction mixture containing 5 µL 5× PCR buffer, 2.5 mM $MgCl_2$, 0.2 µL template DNA, 0.64 mM of dNTPs mix (Promega), 0.625 unit *Taq* polymerase (Promega), and 0.8 µM of both forward and reverse primers, and the PCR was also performed using a PTC-100 Peltier Thermal Cycler (MJ Research Inc.). The PCR cycles were based on O'Donnell *et al.* (1998) with some modifications: the initial denaturation at 94°C for 1 min was followed by 36 cycles of denaturation at 95°C of 35 sec, annealing at 59°C for 55 sec, and extension at 72°C for 90 sec, followed by a final extension at 72°C for 10 min. The PCR products were visualised using gel electrophoresis.

The PCR products were purified using a QIAquick Purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and the PCR products were sent to a service provider (MyTACG Bioscience Enterprise,

Selangor, Malaysia) for sequencing. After sequencing, pairwise alignment of the sequences was achieved using BioEdit software (Hall 1999). Using Basic Local Alignment Search Tool (BLAST), the consensus sequences of *Fusarium* isolates were compared with other sequences in the Fusarium-ID database and with other fungal isolates using Genbank. Identification of all fungal isolates was based on the closest match of the BLAST search.

A total of 31 fungal isolates (Tables 1 and 2) from 80 fragments of wild banana roots were successfully isolated and identified. Fungal mycelia were not observed on the imprint plates used in the surface sterilisation, indicating the effectiveness of the surface sterilisation method for isolating endophytic fungi from the root fragments. The results also indicated that all the fungal isolates successfully isolated from the roots were endophytes, as epiphytic fungi inhabiting the surface of the root could not grow after surface sterilisation (Schulz *et al*. 1993).

Table 1: Species identities of endophytic *Fusarium* spp. based on TEF-1α sequences.

Isolate	Morphological identification	TEF-1α sequence	% similarity
S1-R	*F. proliferatum*	*F. proliferatum*	98
S1-U	*F. verticillioides*	*F. proliferatum*	99
S1-V	*F. proliferatum*	*F. proliferatum*	99
S1-W	*F. proliferatum*	*F. proliferatum*	96
S1-X	*F. proliferatum*	*Fusarium* sp.	99
S1-Y	*F. proliferatum*	*F. proliferatum*	99
S2-S	*F. proliferatum*	*Fusarium* sp.	99
S2-T	*F. proliferatum*	*Fusarium* sp.	99
S3-S	*F. proliferatum*	*F. proliferatum*	98
S3-U	*F. proliferatum*	*F. proliferatum*	99
S4-N	*F. solani*	*F. solani* species complex	99
S4-R	*F. proliferatum*	*F. proliferatum*	99
S4-S	*F. verticillioides*	*F. proliferatum*	99
S4-T	*F. verticillioides*	*F. proliferatum*	99
S4-U	*F. proliferatum*	*F. proliferatum*	99
S5-R	*F. proliferatum*	*F. proliferatum*	99
S5-S	*F. proliferatum*	*F. proliferatum*	99
S5-U	*F. proliferatum*	*F. proliferatum*	99

The most common endophytic fungal isolates recovered were from the genus *Fusarium*. The isolates were identified molecularly as *F. proliferatum*, *Fusarium* sp., and *F. solani* species complex based on the TEF-1α sequences; three *Fusarium* species were identified on the basis of the ITS regions, namely, *F. oxysporum*, *F. solani*, and *F. proliferatum* (Tables 1 and 2). The three *Fusarium* species, *F. oxysporum*, *F. solani*, and *F. proliferatum*, were morphologically ‘unidentified’, as the microscopic and macroscopic

characteristics observed did not fall within the range of the descriptions of *Fusarium* species; therefore, the ITS regions were used for identification.

Table 2: Species identity of endophytic fungi based on ITS sequences.

Isolate	Morphological identification	ITS sequence	% similarity
S1-S	*Curvularia lunata*	*Curvularia lunata*	99
S3-T	Unidentified	*F. oxysporum*	99
S3-F	Unidentified	*F. solani*	99
S3-I	*Cylindrocladium* sp.	*Calonectria gracilis*	99
S3-N	*Trichoderma viride*	*Trichoderma atroviride*	99
S3-Q	*Cylindrocladium* sp.	*Calonectria gracilis*	99
S2-A	Mycelia sterilia	*Rhizoctonia solani*	95
S2-C	Unidentified	*F. proliferatum*	99
S2-M	Unidentified	*F. oxysporum*	99
S1-E	Mycelia sterilia	*Bionectria ochroleuca*	99
S1-M	*Trichoderma viride*	*Trichoderma atroviride*	99
S3-M	*Trichoderma viride*	*Trichoderma atroviride*	99
S1-L	*Xylaria* sp.	*Stromatoneuspora phoenix*	97

For the TEF-1α sequences, a BLAST search was performed using the Fusarium-ID database, as this database is strongly recommended for the identification of most *Fusarium* species (Geiser *et al.* 2004). Three isolates were identified only as members of the genus *Fusarium* sp., as the correct identity of these isolates remain 'uncertain' until rigorous morphological and multilocus phylogenetic studies are completed (Geiser *et al*. 2004). One isolate was identified as *F. solani* species complex, a species complex within which dozens of phylogenetic species have been recognised (Geiser *et al*. 2004).

Species in the genus *Fusarium* are reported to be among the common root endophytic fungal communities (Sieber 2002). Endophytic *Fusarium* sp. was among the fungal flora identified from the healthy root tissues of wild banana in South China (Cao *et al*. 2002). *Fusarium* sp. has also been isolated from the leaves of mature wild banana from different localities in Sao Paulo, Brazil (Pereira *et al.* 1993) and from young and old leaf tissues of wild banana in Thailand (Photita *et al.* 2001).

In this study, *F. proliferatum* was the most common species isolated and identified from the root fragments. Endophytic *F. proliferatum* has been recovered from *Celastrus angulatus,* a traditional Chinese medicinal plant, (Ji *et al*. 2005); the stems of *Kandelia*, a mangrove plant (Cheng *et al.* 2008); the inner bark tissue of a medicinal plant, *Dysoxylum binectariferum* (Mohana Kumara *et al*. 2012); and the leaves of the oil-seed crop *Jatropha curcas* (Kumar & Kaushik 2013). These studies showed that although *F. proliferatum* is a well-known plant pathogen, the species can also be an endophyte.

Endophytic *F. solani* and *F. oxysporum* have been isolated from the root fragments of wild banana plants (Latiffah & Nur Hidayah 2011), and both species have been isolated from the healthy roots of the Cavendish banana (Athman 2006). *F. solani* has been isolated from the healthy leaf pieces of *Musa* spp. in Hong Kong and South East Queensland (Brown *et al*. 1998). Similar to *F. proliferatum*, both *F. solani* and *F. oxysporum* are well-known plant pathogens and can also be endophytes.

Based on the ITS sequences, other endophytic fungi isolated included *C. lunata*, *T. atroviride, C. gracilis, R. solani, B. ochroleuca* and *S. phoenix* (Table 2). These fungi are reported to be endophytic in different types of host plants.

C. lunata was identified from its morphological characteristics and ITS sequences. *C. lunata* is among the common endophytic fungi isolated from plants, and the occurrence of endophytic *C. lunata* has been reported in several studies, such as Rajagopal *et al*. (2010), Verma *et al.* (2011), Al-Mahi *et al.* (2013), and Tuppad and Shishupala (2013).

T. atroviride was morphologically identified as *T. viride*, but according to the ITS sequences, the isolates exhibit the closest match (99%) to *T. atroviride*. Endophytic *T. atroviride* has been isolated from the roots of red sage (*Salvia miltiorrhiza*), which is used as a Chinese herbal medicine (Ming *et al*. 2013). Xia *et al*. (2011) reported the occurrence of various species of endophytic *Trichoderma* associated with banana roots.

A morphologically identified *Cylindrocladium* sp. was identified as *C. gracilis* (Table 2). The genus *Cylindrocladium* has a *Calonectria* teleomorph, and *Calonectria* species are commonly characterised by their *Cylindrocladium* anamorphs (Crous & Wingfield 1994). The genus *Calonectria* consists of saprophytic and plant pathogenic species, which are associated with a wide range of diseases in various host plants worldwide (Crous 2002). The endophytic *C. gracilis* isolates in this study have a 99% similarity with *C. gracilis* as reported by Lombard *et al.* (2010). *C. gracilis* has not previously been reported to be endophytic.

Two morphologically identified mycelia sterilia were identified as *R. solani* and *B. ochroleuca* on the basis of the ITS sequences. These two isolates did not produce any sporulating structure on PDA and therefore were morphologically identified as mycelia sterilia (Table 2). The two mycelia sterilia isolates showed a high sequence similarity (99%) with *R. solani* and *B. ochroleuca.* Endophytic *R. solani* has been recovered from the leaves of *Taxus mairei* (Wang *et al*. 2008) and from tropical orchids, and the occurrence of endophytic *Rhizoctonia*-like fungi was reported by Otero *et al.* (2002). *Bionectria* is rarely isolated as an endophyte; however, an endophytic *B. ochroleuca* was isolated from *Nothapodytes foetida*, a tree that grows wild in the forests of the Western Ghats (Samaga *et al*. 2013), and from healthy tissues of chili pepper plants (Narayan *et al*. 2013).

A morphologically identified *Xylaria* sp. was identified as *S. phoenix*, a species in the family Xylariaceae, on the basis of the ITS sequences. Endophytic Xylariaceae have been reported in *Magnolia liliifera* (Promputtha *et al*. 2005), *Pinus tabulaeformis* (Wang *et al*. 2005), *Piper aduncum* (Silva *et al*. 2010), and the medicinal plant *Dendrobium* (Chen *et al*. 2013).

This study showed that the roots of wild banana harbour diverse endophytic fungal taxa. The effect of endophytic fungi on the wild banana plant is relatively unknown. However, endophytic fungi are generally positioned between the trophic niches of pathogen and mutualist (Saikkonen *et al*. (1998). Some root endophytes may be latent pathogens, which in later stages can weaken or damage the roots (Schulz *et al.* 1999). Many other root endophytes provide benefits to the host plants by improving plant growth (Schulz *et al*. 2002), assisting in phosphorus uptake (Sieber 2002) and protecting against pests and diseases.

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