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Distinctive Endophytic Fungal Assemblage in Stems of Wild Rice (*Oryza granulata*) in China with Special Reference to Two Species of *Muscodor* (Xylariaceae)[§]

Zhi-lin Yuan[1,2], Zhen-zhu Su[1], Li-juan Mao[3], Yang-qing Peng[4], Guan-mei Yang[1], Fu-cheng Lin[1], and Chu-long Zhang[1*]

[1]*Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, Zhejiang University, Hangzhou 310029, P. R. China*
[2]*Institute of Subtropical Forestry, Chinese Academy of Forestry, Fuyang, Zhejiang Province,311400, P. R. China*
[3]*985-Institute of Agrobiology and Environmental Sciences, Zhejiang University, Hangzhou 310029, P. R. China*
[4]*Department of Applied Biological Science, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, P. R. China*

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Ecological niches in the rhizosphere and phyllosphere of grasses capable of sustaining endophytes have been extensively studied. In contrast, little information regarding the identity and functions of endophytic fungi in stems is available. In this study, we investigated the taxonomic affinities, diversity, and host specificities of culturable endophytes in stems of wild rice (*Oryza granulata*) in China. Seventy-four isolates were recovered. Low recovery rate (11.7%) indicated that there were relatively few sites for fungal infection. Identification using morphology, morphospecies sorting, and molecular techniques resulted in classification into 50 taxa, 36 of which were recovered only once. Nucleotide sequence similarity analysis indicated that 30% of the total taxa recovered were highly divergent from known species and thus may represent lineages new to science. Most of the taxa were classified as members of the classes Sordariomycetes or Dothideomycetes (mainly in Pleosporales). The presence of *Arthrinium* and Magnaporthaceae species, most often associated with poaceous plants, suggested a degree of host specificity. A polyphasic approach was employed to identify two *Muscodor* taxa based on (i) ITS and *RPB2* phylogenies, (ii) volatile compounds produced, and (iii) an *in vitro* bioassay of antifungal activity. This to our knowledge is only the second report regarding the isolation of *Muscodor* spp. in China. Therefore, we hypothesize that wild plants represent a huge reservoir of unknown fungi. The prevalence, novelty, and species-specificity of unique isolates necessitate a reevaluation of their contribution to ecosystem function and fungal biodiversity.

Keywords: endophytic fungi, singletons, host specificity, volatile organic compounds, antibiosis

Endophytic fungi, often presumed to form mutualistic association with plants, occur both in belowground and aboveground plant tissues including roots, stems, leaves and seeds without producing any apparent symptoms. A combination of culturing and culture-free molecular methods is capable of detecting a major proportion of undescribed fungal taxa, indicating the presence of novel functions (Arnold *et al.*, 2007). Recently, class 3 endophytes belonging to the non-clavicipitaceous fungi have been proposed to have extremely high *in planta* biodiversity (Rodriguez *et al.*, 2009); indeed, up to 1,000,000 endophytic fungal species have been estimated to exist (Ganley *et al.*, 2004). Therefore, that an untapped reservoir of previously unexplored endophytic organisms still exists in plants is likely (Kharkwal *et al.*, 2008), some of which may be of use for biological control (Arnold *et al.*, 2003), production of bioactive metabolites (Schulz *et al.*, 2002), extracellular enzymes with industrial application (Qiu and Chen, 2008), and/or myco-diesel production (Strobel *et al.*, 2008), or promotion of plant growth (Deshmukh *et al.*, 2005).

With the unprecedented loss of biodiversity that has occurred in recent years, collection of germplasm from endophytes (especially from endangered plant species) has become imperative (Strobel, 2007). In addition, evidence, albeit limited, supports the hypothesis that non-cultivated plants host unique, diverse, and novel indigenous fungi as a result of continual selective pressure and co-evolution (Thomas *et al.*, 2008; Hung *et al.*, 2009).

Grass-endophyte symbiosis has been recognized for many decades. Most clavicipitaceous endophytes (*Epichloë*/*Neotyphodium*) are generally proposed to be systemic, transmitted vertically via grass seeds, and exclusively infect the leaf sheath (Kuldau and Bacon, 2008). More importantly, horizontally transmitted endophytes have also been identified in grass roots. Dark septate endophytes (DSEs), a paraphyletic fungal group, are ubiquitously distributed in the roots of a wide range of grass species (Porras-Alfaro *et al.*, 2008; Kageyama *et al.*, 2009). To our knowledge, however, data regarding endophytic fungal communities in grass stem tissues remain limited, at least in part due to their inconspicuous infection structures, low colonization rate, and ambiguous ecological roles.

Due to continuous human ecosystem destruction, the distribution of *Oryza granulata*, a wild rice species endemic to China, in nature is shrinking; indeed this species is now nearing extinction. Considering its rarity and the possibility that this

* For correspondence. E-mail: clzhang@zju.edu.cn; Tel: +86-571-8697-1185; Fax: +86-571-8697-1516
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species contains genes useful when introduced to modern rice, protection is essential. Plant-associated endophytes also deserve more attention from professional microbiologists (Jeffries, 2004). Endophytic microbes associated with various species of wild rice have been the focus of several recent studies, but these have concentrated on nitrogen-fixing diazotrophs (Chaintreuil *et al.*, 2000; Peng *et al.*, 2008; Zhang *et al.*, 2008). Endophytic fungal organisms living in wild rice roots are only now being systematically isolated and characterized (Yuan *et al.*, 2010a, 2010b).

Thus, the objective of this study was to investigate the endophytic population of wild rice (*O. granulata*) stem tissues in Yunnan Province, southwestern China. We aimed to address first, the extent of fungal diversity in stem tissues, since current knowledge of endophytes of poaceous plants is lacking; second, the level of taxonomic novelty or rarity of recovered fungi; and finally, any potentially biotechnologically interesting properties of fungi isolated as part of the study. A culture-dependent approach was used to isolate endophytic filamentous fungi for analysis.

Materials and Methods

Sample collection

The site of study was located in Xishuangbanna Nature Reserve, Yunnan province, southwest of China (N 22°04'-22°17'; E 100°32'-100°44'). In September of 2007, 2008 and 2009, we collected 10, 15 and 15 wild rice plants respectively. Healthy and intact plants with bulk soil were packed with a box and carefully transported to laboratory within 48 h.

Fungal isolation, purification and morphological identification

Healthy stems were rinsed with tap water slightly, immersed in ethanol (75%) for 40 sec, immersed in sodium hypochlorite 1% for 5 min and finally rinsed in sterile distilled water with three times. Stems were cut into 0.5 cm length and transferred to a plate with 2% malt extract agar (MEA) medium supplemented with chloromycetin (50 mg/L) to avoid bacteria growth. A total of 634 tissue segments were incubated on plates sealed with Parafilm to avoid desiccation and cultured at 25°C in darkness. Hyphae emerging from segments were sub-cultured onto fresh potato dextrose agar (PDA) medium for purification.

Some isolates readily sporulated on PDA and MEA media after one week inoculation in darkness at 25°C. These sporulating isolates were initially identified to genus and/or species level based on morphological features including colony appearance, conidia morphology and sporulating structures. The microscopic observations were conducted with a light microscopy (Olympus BX51, Japan) and/or cryo-scanning electron microscopy (cryo-SEM, HITACHI S-3000N, Japan). Specimens for light microscopy were mounted in 3% KOH or sterile distilled water for observation. For scanning electronic microscopy, the margin of the culture was sliced out and care was taken not to deform the surface features of the culture, operating between 10 and 15 kV on samples containing a thin layer of gold sputter coated.

Fungal genomic DNA extraction and molecular identification of sterile fungi

Remaining sterile fungal isolates were firstly grouped into ‘morphotypes’ based on similar cultural characters including surface texture, margin characters, colony color and growth rate on PDA medium. To ensure the reliability of morphological identification and ‘morphotype’ sorting, ITS sequences of all recovered isolates were sequenced and subjected to molecular identification. Fungal genomic DNA was extracted using the Multisource Genomic DNA Miniprep kit (Axygen Incorporation) following the manufacturer’s instructions. Primers ITS1 and ITS4 (White *et al.*, 1990) were used for amplification of the fungal rDNA internal transcribed spacer (ITS) regions 1 and 2 of most isolates. With exception of some xylariaceous fungi, ITS1-ITS4 primer set produced only a very faint band. Therefore, ITS1-F and ITS4-A primer pair was used to amplify ITS rDNA region (Larena *et al.*, 1999). For analyzing the molecular phylogeny of two *Muscodor* species, a fragment of RNA polymerase II second largest subunit (*RPB2*) was amplified with primer pairs RPB2-5F and RPB2-7cR (Liu *et al.*, 1999). The PCR reaction conditions and components were listed in supplementary data Table 1. The reaction products were separated in 1.0% (w/v) agarose gel and bands stained with ethidium bromide. The PCR products were then purified using a Gel Band Purification kit (Axygen Incorporation) and sequenced in ABI 3730 sequencer (Applied Biosystems, USA) using the corresponding primer pairs. The consistency between morphotyping and molecular typing was confirmed by assembling ITS sequences with 99% similarity threshold using Sequencher ver. 4.1.4 (www.genecodes.com). Representative sequences of all taxa were queried against GenBank using BLAST program for determination of their putative taxonomic affinities.

Phylogenetic analysis of two *Muscodor* species

For inferring the molecular phylogeny of two *Muscodor* isolates, ITS and *RPB2* sequences were subjected to BLAST analysis. Sequences with high similarity were retrieved, combined with the sequences obtained during this work, aligned using CLUSTAL X, and the alignment manually corrected in GENEDOC. Evolutionary distance was determined using the Jukes-Cantor model to construct phylogenetic trees by the neighbor-joining method using PHYLIP. The resultant trees were analyzed using the program CONSENSE to calculate a majority rule consensus tree. The treefiles then were displayed by Treeview. Bootstrap (1,000 replicates) analysis employed SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in PHYLIP. The GenBank accession no. for distinctive ITS genotypes were as follows: [GQ220331-GQ220366, GU903278-GU903290, and EU636700]. *RPB2* gene fragments of three isolates of *Muscodor* were also deposited in GenBank: *Muscodor* sp. S18-3-1 (FJ480346), *Muscodor albus* S13-1-2 (GQ241929) and *M. albus* (FJ480345) (a living culture provided by professor Strobel GA, Montana State University).

In vitro antifungal test of the volatile compounds production by two *Muscodor* species

The production of antifungal volatile compounds by two *Muscodor* isolates was tested according to Strobel *et al.* (2001), using *Aspergillus clavatus*, *Botrytis cinerea*, *Didymella bryoniae*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *Pytium ultimum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Verticillum dahliae* as plant pathogenic tester strains. A disk of the growing front of *Muscodro* isolates (5 mm diameter) was excised and inoculated on PDA on one section of a two-section Petri dish (a Petri dish has a partition dividing an inside space of the dish into two sections, the height of the partition lower than that of the brim.). After incubation at 25°C, a disk of the growing front of the test fungus (5 mm diameter) was inoculated onto PDA on another section of the Petri dish. The Petri dishes were then wrapped with two layers of Parafilm and incubated at 25°C in darkness. The in-

Table 1. Taxonomic placement of sporulating and sterile morphotypes obtained in this study inferred from BLAST searches and morphological descriptions. Areas shaded in grey indicate the potentially novel fungal lineages

Taxa	Classification	Nearest match	Max Identity	Isolate numbers			Accession Numbers
				2007	2008	2009	
Acremonium sp.	Sordariomycetes Hypocreales	*Nectria mauritiicola* (FJ654435)	98%	0	2	0	GQ220356
Alternaria sp1.	Dothideomycetes Pleosporales	*Alternaria longissima* (FJ971842)	99%	1	0	0	GQ220347
Alternaria sp2.	Dothideomycetes Pleosporales	*Alternaria* sp. (GU187964)	99%	1	0	0	GQ220339
Ampelomyces sp.	Dothideomycetes Pleosporales	*Ampelomyces* sp. (AY513942)	98%	0	0	1	GU903284
Arthrinium aureum	Sordariomycetes Apiosporaceae	*Arthrinium aureum* (AB220246)	100%	2	0	0	GQ220349
Arthrinium phaeospermum	Sordariomycetes Apiosporaceae	*Arthrinium phaeospermum* (FJ462766)	98%	1	0	2	GQ220338
Arthrinium sp.	Sordariomycetes Apiosporaceae	*Arthrinium* sp. (AB462758)	91%	1	0	0	GQ220341
Aspergillus sp.	Eurotiomycetes Eurotiales	*Aspergillus niger* (GU362012)	100%	1	0	0	GQ220334
Beauveria brongniartii	Sordariomycetes Hypocreales	*Cordyceps brongniartii* (DQ153039)	100%	0	1	0	GQ220359
Bionectria ochroleuca	Sordariomycetes Hypocreales	*Bionectria ochroleuca* (FJ025201)	99%	0	1	0	GQ220362
Botryosphaeria dothidea	Dothideomycetes Botryosphaeriales	*Botryosphaeria dothidea* (FJ478129)	99%	0	0	4	GU903279
Cercospora sp.	Dothideomycetes Capnodiales	*Cercospora canescens* (AY266164)	99%	0	0	1	GU903286
Cladosporium sp.	Dothideomycetes Capnodiales	*Cladosporium colocasiae* (EU076964)	99%	0	0	1	GU903289
Cochliobolus lunatus	Dothideomycetes Pleosporales	*Cochliobolus lunatus* (DQ836798)	98%	0	0	1	GU903287
Colletotrichum sp1.	Sordariomycetes Glomerellaceae	*Glomerella magna* (GU358453)	96%	0	1	0	GQ220358
Colletotrichum sp2.	Sordariomycetes Glomerellaceae	*Colletotrichum gloeosporioides* (AJ301979)	99%	0	1	0	GQ220343
Colletotrichum sp3.	Sordariomycetes Glomerellaceae	*Colletotrichum boninense* (AB042313)	99%	1	0	0	GQ220360
Diaporthales sp1.	Sordariomycetes Diaporthales	*Diaporthe* sp. (EF423550)	85%	1	0	0	GQ220333
Diaporthales sp2.	Sordariomycetes Diaporthales	*Ophiognomonia* sp. (EU482284)	83%	0	1	0	GQ220366
Fusarium sp1.	Sordariomycetes Hypocreales	*Fusarium* sp.(EU605879)	99%	0	2	0	GU903290
Fusarium sp2.	Sordariomycetes Hypocreales	*Fusarium equiseti* (GU134899)	99%	0	1	0	GQ220355
Fusarium sp3.	Sordariomycetes Hypocreales	*Gibberella moniliformis* (GU325675)	99%	1	0	0	GQ220332
Gnomoniaceae sp1.	Sordariomycetes Diaporthales	*Ophiognomonia* sp. (EU482284)	93%	1	0	0	GQ220342
Gnomoniaceae sp2.	Sordariomycetes Diaporthales	*Ophiognomonia* sp. (EU482284)	91%	1	0	0	GQ220336
Harpophora sp.	Sordariomycetes Magnaporthales	*Gaeumannomyces graminis* (AY428781)	100%	0	1	0	GQ220365
Leptosphaeria sp.	Dothideomycetes Pleosporales	*Leptosphaeria* sp. (FN394721)	97%	1	3	1	GQ220340
Leptosphaeriaceae sp.	Dothideomycetes Pleosporales	Leptosphaeriaceae sp. (FJ884103)	98%	0	2	0	GQ220352
Magnaporthaceae sp1.	Sordariomycetes Magnaporthales	*Buergenerula spartinae* (AF422962)	86%	2	0	0	GQ220331
Magnaporthaceae sp2.	Sordariomycetes Magnaporthales	*Pyricularia* sp. (AY265323)	92%	0	0	1	GU903288
Magnaporthaceae sp3.	Sordariomycetes Magnaporthales	*Gaeumannomyces* sp. (FJ430719)	89%	1	0	0	GQ220335
Massarinaceae sp.	Dothideomycetes Pleosporales	*Saccharicola bicolor* (AF455415)	93%	0	0	1	GU903280
Microdochium sp1.	Sordariomycetes Xylariales	*Microdochium* sp. (AB255278)	98%	0	2	0	GQ220361
Microdochium sp2.	Sordariomycetes Xylariales	*Microdochium* sp. (AF455402)	93%	0	0	1	GU903285
Muscodor albus	Sordariomycetes Xylariales	*Muscodor albus* (AY927993)	100%	1	0	0	GQ220337
Muscodor sp.	Sordariomycetes Xylariales	*Muscodor yucatanensis* (FJ917287)	98%	1	0	0	EU636700
Paecilomyces lilacinus	Sordariomycetes Hypocreales	*Paecilomyces lilacinus* (EU306174)	100%	1	0	0	GQ220344
Penicillium steckii	Eurotiomycetes Eurotiales	*Penicillium steckii* (EF634431)	99%	0	1	0	GQ220363
Phaeosphaeria sp1.	Dothideomycetes Pleosporales	*Phaeosphaeria* sp. (DQ092510)	99%	0	2	4	GQ220354
Phaeosphaeria sp2.	Dothideomycetes Pleosporales	*Phaeosphaeria* sp. (DQ092510)	94%	0	1	0	GQ220351
Phaeosphaeriaceae sp.	Dothideomycetes Pleosporales	Phaeosphaeriaceae sp. (EF060518)	97%	0	0	1	GU903283
Pleosporales sp1.	Dothideomycetes Pleosporales	Arthopyreniaceae sp. (FJ439584)	92%	0	1	0	GQ220353
Pleosporales sp2.	Dothideomycetes Pleosporales	Pleosporales sp. (AB255289)	93%	1	0	2	GQ220345
Pleosporales sp3.	Dothideomycetes Pleosporales	Pleosporales sp. (AB255255)	91%	0	0	1	GU903282
Ramichloridium sp.	Dothideomycetes Capnodiales	*Ramichloridium apiculatum* (GU214687)	96%	0	0	1	GU903281
Stachybotrys sp.	Sordariomycetes Hypocreales	*Stachybotrys bisbyi* (AF081480)	98%	0	0	1	GU903278
Verticillium sp.	Sordariomycetes Phyllachorales	*Verticillium nigrescens* (AB353349)	100%	0	1	0	GQ220357
Xylaria sp1.	Sordariomycetes Xylariales	*Xylaria curta* (EU715684)	95%	3	0	0	GQ220350
Xylaria sp2.	Sordariomycetes Xylariales	*Xylaria grammica* (AB524025)	99%	1	0	0	GQ220348
Xylariaceae sp1.	Sordariomycetes Xylariales	Xylariaceae sp. (AB440113)	98%	0	1	0	GQ220364
Xylariaceae sp2.	Sordariomycetes Xylariales	*Podosordaria tulasnei* (AY572970)	93%	1	0	0	GQ220346

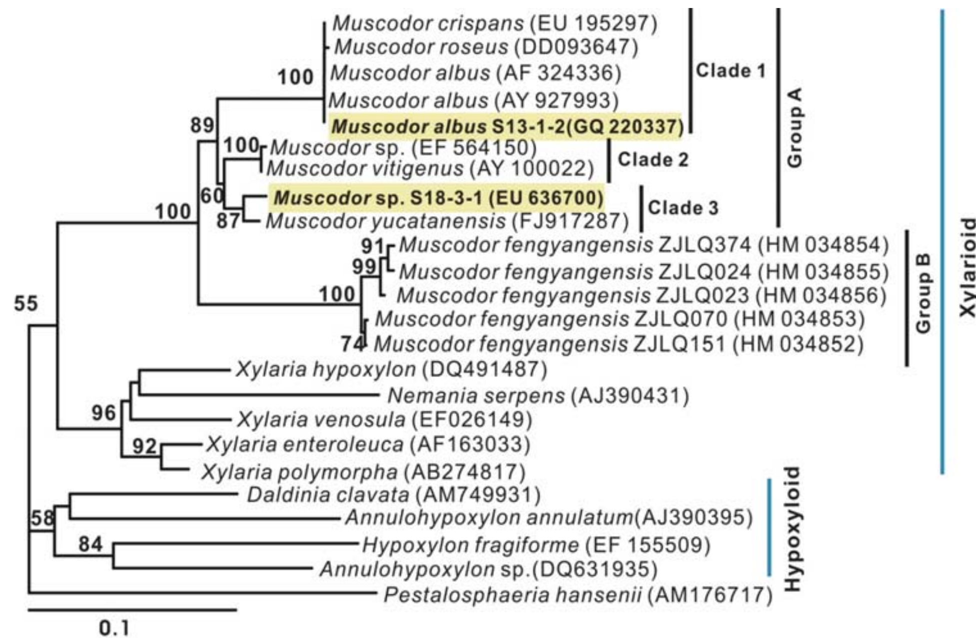

Fig. 1. Neighbor-joining phylogenetic tree based on ITS1-5.8S rDNA-ITS2 gene of *Muscodor* species and other Xylariaceae fungi obtained from GenBank database. Only bootstrap values over 50% are shown above the branches. *Pestalosphaeria hansenii* is designated as the outgroup.

crease in diameter of the growing colony was measured at least three times for a period of 3 days. After the termination of the experiment, the respectively tested fungus was placed onto new PDA to evaluate its viability. Controls were included in which the test fungus was subjected to the same growth conditions but without *Muscodor* isolates.

Solid phase microextraction/Gas chromatograph/Mass spetra (SPME/ GC/MS) analysis of volatile compounds produced by two *Muscodor* species

The analysis of the volatile compounds produced by two *Muscodor* species was performed by SPME/GC/MS methods according to Strobel *et al*. (2001). The gas released from *Muscodor* mycelium growing in Petri plates was extracted with a SPME syringe (SUPELCO, USA), 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/ PDMS) on a StableFlex fiber. Before extracting the volatiles, the SPME fiber was conditioned in the GC at 240°C for 20 min under a flow of He. The SPME needle was passed through a small drilled hole in the side of the Petri plate and the fiber was then exposed in the air space above the mycelium for 45 min immediately. The SPME needle was inserted into the GC injection port and the sample was then desorbed from the fiber for 30 sec. After conditioning was completed, retracted the fiber and removed the needle from the infection port. The volatile compounds were analyzed with a GC with a mass detector (Agilent 6890N/5975B) and the data were analyzed with MSD ChemStation software G1701DA (Agilent, USA) for data acquisition and data processing. A HP-5MS capillary column (5% phenyl methyl siloxane: 30 m×0.25 mm, 0.25 μm film thickness) was used for the separation of the volatiles and He was used as the carrier gas. The column was temperature programmed as follows: 30°C for 2 min, the increasing to 220°C at 5°C per min, post run 270°C for 1 min. The mass spectrum was scanned at a rate of 3.35 scans per sec over a mass range of 20-450 atomic mass unit (a.m.u.). Identification of the volatile compounds was made through comparison with the compounds contained in the NIST05 database (Agilent, USA) on the mass spectrometer. Peak components obtained from control Petri dishes containing only PDA were subtracted from the analyses done on the plates containing the fungus.

Results

General characterization and host-affinity of fungal endophytes

Incubation of 634 sample fragments on artificial media yielded a total of 74 fungal isolates. Overall, the average fungal endophyte colonization frequency was low (11.7%), indicating that their opportunities for penetrating stem tissues, as compared to leaves and roots, was limited.

Sporulating fungi were identified based on morphological characteristics, and important features (such as conidia morphology and conidiophore structure) were recorded (Supplementary data Fig. 1). The remaining sterile isolates were grouped first into 'morphotypes' based on cultural characteristics including colony texture, color, and growth rate. To confirm the reliability of morphological sorting, the ITS rDNA region of isolates was sequenced and compared by assembling ITS sequences using a 99% similarity threshold. Results indicated that morphospecies sorting was consistent with sequence analysis. A putative taxonomic identification of sterile fungi was established using a BLAST search of the GenBank database. In total, 50 distinct taxa were recorded, 36 of which were isolated only once (singletons). The majority of isolates belonged to two Ascomycota classes (Sordariomycetes and Dothideomycetes), with but two exceptions (both Eurotiomycetes). Approximately one-third (32.4%) of isolates belonged to diverse taxa of the order Pleosporales (Table 1). Of the total taxa recovered, 30% were highly divergent from cur-

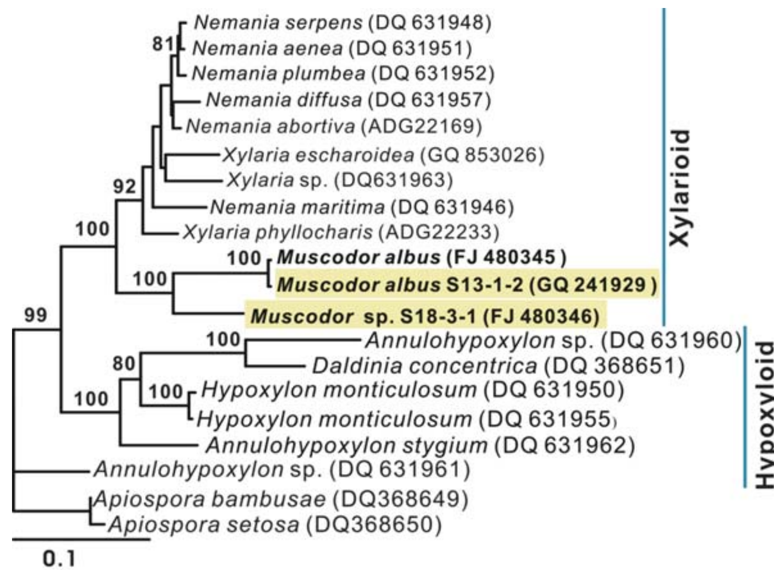

Fig. 2. Neighbor-joining phylogenetic tree based on *RPB2* sequences of three *Muscodor* isolates and other Xylariaceae fungi from GenBank database. Only bootstrap values over 50% are shown above the branches. *Apiospora bambusae* and *A. setosa* are defined as the outgroup.

rently described species (ITS sequence similarity 83-94%), and most remained sterile and unidentifiable to even the genus level. Annual endophytic fungal assembly varied markedly (Table 1), and only one taxon (*Leptosphaeria* sp.) overlapped.

Molecular phylogeny of two *Muscodor* species

Phylogenetic analysis of ITS sequences suggested that two isolates (S13-1-2 and S18-3-1; Fig. 1 and Supplementary data Fig. 2) were similar to the genus *Muscodor*. To confirm this independently, we sequenced a fragment of *RPB2*, which has proven useful in the phylogenetic analysis of fungi at the species and genus level (Malkus *et al*., 2006). Unfortunately, neither *Muscodor* spp. *RPB2* sequences nor cultures are available from sequence databases or fungal culture collections. We therefore sequenced *RPB2* from *M. albus*, which was made available to us by Strobel GA. Results suggested that S13-1-2 was conspecific to *M. albus*, since their ITS and *RPB2* sequences showed 100% similarity. Sequence analysis revealed that S18-3-1 shared a 98% ITS sequence identity with *M. yucatanensis* (FJ 917287), a recently described novel species, while the *RPB2* sequences of *M. albus* were only 83% similar. Both of these data suggest that S18-3-1 is of a novel taxon (Fig. 2). Furthermore, the *Muscodor* lineage can be clearly divided into two groups (Groups A and B). Group A was further separated into three sub-clades with high support values. *M. albus*, *M. crispans*, and *M. roseus* clustered into clade 1 (100% bootstrap support). Clade 2 included *M. vitigenus* and a *Muscodor* isolate. Clade 3 included *M. yucatanensis* and the recovered isolate S18-3-1 (87% bootstrap support). Likewise, molecular phylogeny of *RPB2* gene sequences revealed that the three *Muscodor* isolates clustered together with high support values (Fig. 2) and were closely related to *Xylaria* and *Nemania*.

Data also indicated that the genus *Muscodor* belongs to the Xylarioid group within the Xylariaceae. Phylogenies based upon *RPB2* and rDNA gene sequences suggested that

Table 2. Effects of the volatile compounds of *Muscodor albus* (strain name: S13-1-2) and *Muscodor* sp. (strain name: S18-3-1) on a group of test plant pathogenic fungi

Test pathogenic fungi	Growth after 3d exposure to two *Muscodor* species (% vs control)		Viability after 3d exposure to two *Muscodor* species	
	M. albus	*Muscodor* sp.	*M. albus*	*Muscodor* sp.
Aspergillus clavatus	49.4±7.2	42.2±2.0	Alive	Alive
Botrytis cinerea	–	0	Alive	Dead
Didymella bryoniae	31.5±7.8	0	Alive	Alive
Fusarium oxysporum	33.5±5.9	54.7±1.8	Alive	Alive
Magnaporthe oryzae	63.4±4.7	0	Dead	Dead
Pythium ultimum	0	0	Dead	Dead
Rhizoctonia solani	0	0	Dead	Dead
Sclerotium rolfsii	48.2±8.9	0	Alive	Dead
Verticillum dahliae	41.2±1.6	0	Alive	Alive

Note: tests were repeated three times and means±SD were calculated.

Muscodor, *Xylaria*, and *Nemania* form a distinct clade within the Xylarioid group (Figs. 1 and 2), while *Daldinia*, *Annulohypoxylon*, and *Hypoxylon* constitute the Hypoxyloid group.

Production of volatile compounds is important for identification of the genus *Muscodor*. Therefore, an *in vitro* antifungal assay was performed to further confirm the reliability of the determined taxonomy regarding the two putative *Muscodor* species. As expected, both isolates inhibited a broad range of plant pathogenic fungi (Table 2 and Supplementary data Fig. 3) in our bioassay system. Also, *Muscodor* sp. (S18-3-1) showed greater inhibitory activity against phytopathogenic fungi than *M. albus* (S13-1-2).

Analysis of volatile compounds (VOCs) produced by two *Muscodor* species

SPME/GC/MS analysis revealed that *Muscodor* sp. (S18-3-1) produced a markedly different volatile organic compound (VOC) profile than *M. albus* (Table 3); (2-methyl) propanoic acid was the principal (90.4%) and β-phellandrene (5.43%) one of the major (VOCs) produced by *Muscodor* sp. (S18-3-1), while β-phellandrene production by *M. albus* (S13-1-2) was not detected.

Discussion

Species composition and annual changes in endophytic fungal communities in the stem tissues of wild rice were investigated. Previous studies paid great attention to the nonsystemic endophytic fungal structure in grasses (Vandenkoornhuyse *et al.*, 2002; Sánchez *et al.*, 2007; Maciá-Vicente *et al.*, 2008; Porras-Alfaro *et al.*, 2008; Sánchez *et al.*, 2008; Rosa *et al.*, 2009), however, the authors focused solely on fungal consortia associated with leaves and/or roots, and thus the presence and complexity of fungal communities in stem tissues was overlooked. Recently, a few studies have isolated endophytic fungi from cultivated rice (*Oryza sativa* L.) in China, India, and Italy (Tian *et al.*, 2004; Naik *et al.*, 2009; Vallino *et al.*, 2009), although again, the fungal communities in stem tissues were ignored. Our results underscore the importance of investigating the diverse fungal groups associated with the stem tissues of wild rice.

The community of endophytic fungi in any particular host will comprise both ubiquitous fungal taxa and some host-specific endophytes (Schulz and Boyle, 2005). *Aspergillus*, *Colletotrichum*, *Fusarium*, *Paecilomyces*, and *Penicillium* are cosmopolitan endophytes isolated from many hosts in different climatic regions (Schulz and Boyle, 2005), and xylariaceous fungi have been isolated from a wide range of tropical plants (Davis *et al.*, 2003).

In contrast, fungi belonging to *Arthrinium* and *Gaeumannomyces* (Magnaporthaceae) preferentially colonize poaceous plants. Some species of *Arthrinium* have been isolated from bamboos (Poaceae) (Samuels *et al.*, 1981), and recent exploitation of bamboo-associated endophytic fungi in Japan also suggests that *Arthrinium* is a dominant group (Morakotkarn *et al.*, 2007). In addition, several *Arthrinium* species have been isolated from two other poaceous plants (*Pennisetum purpureum* and *Miscanthus floridulus*) (Wong and Hyde, 2001). Likewise, *Gaeumannomyces* spp. and their anamorphic states are pathogenic and cause the 'take-all' disease of poaceous crops (Wong and Walker, 1975; Henson, 1989; Wong, 2002). Several root DSEs such as *Harpophora* (previously *Phialophora*)

Table 3. Comparison of the volatile compounds produced by *M. albus* (strain name: S13-1-2) and *Muscodor* sp. (strain name: S18-3-1) through SPME/GC/MS analysis

Retention time (min: s)	Possible compound	M_r	Total area (%)	
			Muscodor sp.	*M. albus*
3: 286	*Propanoic acid, 2-methyl-, methyl ester	102	-	4.39
4: 634	1-Butanol, 2-methyl-	88	-	5.11
5: 316	*Propanoic acid, 2-methyl-, ethyl ester	116	-	1.97
5: 763	Acetic acid, 2-methylpropyl ester	116		7.86
7: 092	*Propanoic acid, 2-methyl-	88	90.40	29.12
9: 241	1-Butanol, 2-methyl-, acetate	130	-	3.78
9: 326	*1-Butanol, 3-methyl-, acetate	130	-	18.53
13: 843	*Propanoic acid, 2-methyl-, 2-methylbutyl ester	158	-	0.75
13: 958	*Propanoic acid, 2-methyl-, 3-methylbutyl ester	158	-	0.70
14: 321	.beta.-Phellandrene	136	5.43	-
15: 759	□4-Nonanone	142	-	2.45
16: 366	*2-Nonanone	142	-	1.02
16: 998	*Phenylethyl Alcohol	122	-	1.25
21: 242	*Acetic acid, 2-phenylethyl ester	164	-	2.79
24: 958	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	204	-	0.71
26: 058	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	204	1.45	0.65
26: 147	□Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,4.alpha.,7.alpha.)]-	204	0.40	0.63
27: 839	*Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]- (common name: valencene)	204	-	1.40
31: 297	∓Unknown	204	2.32	13.80

Note: Several minor peaks, making up less than 0.6% of the total detectable VOCs were deleted from the table. Compounds found in the control PDA plate are not included. Symbol *denoted that this component was confirmed in Strobel (2001) and symbol □denoted that this component was observed but not confirmed in Strobel (2001). The other components were observed in this analysis but not consistent with Strobel (2001).

species (Magnaporthaceae) have been detected in herbaceous plant roots, particularly those of the Poacea (Sieber, 2002; Yuan *et al*., 2010a). In this study, three *Arthrinium* and four Magnaporthaceae taxa were isolated. Some of them were not identified due to the absence of reproductive structures and/or the divergence of their ITS rDNA sequences from those of known species (Table 1). These findings suggest a degree of endophyte host-specificity at the level of plant family (Arnold, 2007). Furthermore, these novel lineages may contribute to increasing our understanding of the ecology, evolution, and molecular phylogeny of the Magnaporthaceae family.

A large proportion of singletons (i.e., endophytes isolated only once) were detected in this study. In fact, several studies have documented that singletons represent a high proportion of endophytes recovered from plants (Gallery *et al.*, 2007; Joshee *et al*., 2009). They are usually recognized as opportunistic colonists or contaminants and thus excluded from estimates of total species richness. However, our data imply that some singletons are host-specific and/or novel species and are endowed with properties of potential biotechnological interest. Thus, functional characterization of singletons should not be overlooked when inferring the ecological significance of an endophyitc fungal community. Indeed, some may be unique to their host plant, necessitating their inclusion in estimates of total endophyte biodiversity (Clay, 2004; Stone *et al.*, 2004).

To our knowledge, this is the second report to describe the isolation of *Muscodor* species in China. The presence of unique biological characteristics (production of antimicrobial VOCs) and phylogenetic analyses led to the establishment of this genus. Thus far, application of morphological, biochemical, and genotypic tools has led to the description of six species: *M. albus*, *M. roseus*, *M. vitigenus*, *M. yucatanensis*, *M. crispans*, and *M. fengyangensis* (Worapong *et al*., 2001, 2002; Daisy *et al.*, 2002; Sopalun *et al*., 2003; Mitchell *et al*., 2008; González *et al*., 2009; Zhang *et al*., 2010). The virtual absence of conidia and sporulation structures necessitates the use of molecular techniques to compensate for the lack of morphologically discriminative features. Phylogenetic analysis based on ITS and *RPB2* gene sequences places the genus *Muscodor* into the Xylarioid (but not Hypoxyloid) group. Previous studies have demonstrated that *Xylaria* and *Nemania* also belong to the Xylarioid group (Stadler *et al*., 2008; Tang *et al*., 2009). In addition, VOCs production is useful for discriminating different species. Our data strongly suggest that since the VOCs produced by S18-3-1 differ markedly from those of *M. albus*, this isolate represents a novel endophytic taxon. A phylogenetic and morphological comparison with *M. yucatanensis* would be useful to further confirm this postulate (González *et al*., 2009). Unfortunately, however, *M. yucatanensis* was not available for this study. We also did not perform a thorough physiological characterization of S18-3-1 (such as utilization of carbon sources and growth characteristics) and comparison with *M. yucatanensis*. We therefore cautiously recommend that a polyphasic approach be used for resolving the taxonomy of non-sporulating endophytes. Fungal chemotaxonomy based on extrolite (fungal secondary metabolite) profiles has in the past proven to be of considerable utility (Stadler and Fournier, 2006; Stadler *et al*., 2008; Stadler and Keller, 2008).

The genus *Muscodor* has also been suggested to be preferentially distributed in areas close to the equator (Strobel, 2006). *M. fengyangensis*, a novel species recovered from plants in subtropical regions of China, has recently been described (Zhang *et al*., 2010). Our findings may lead to a reevaluation of the distribution and ecological roles of *Muscodor*. We hypothesize that *Muscodor* species may also be present in high-latitude regions. In addition, *Muscodor* species hold a great biotechnological potential because of their production of volatile antimicrobial compounds. Mycofumigation using *Muscodor* species for controlling soil-borne diseases and for preventing postharvest decay of some fruits have been documented (Strobel, 2006).

In brief, clavicipitaceous endophytes and root fungal symbionts (e.g., mycorrhizal fungi and DSEs) establish intimate relationships with grasses and provide their hosts with many benefits. Study of ecological aspects of fungal communities in stem tissues, however, has thus far been overlooked. Our data support the view that possession of unique ecological niches and growth in the wild makes such plants a reservoir of diverse and previously untapped fungal lineages. Some taxa are thought to be host-specific and thus have coevolved with their host plant. *Muscodor* isolates hold promise as biocontrol agents and so warrant further investigation.

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