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Muscodor fengyangensis sp. nov. from southeast China: morphology, physiology and production of volatile compounds

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ABSTRACT

The fungal genus *Muscodor* was erected on the basis of *Muscodor albus*, an endophytic fungus originally isolated from *Cinnamomum zeylanicum*. It produces a mixture of volatile organic compounds (VOCs) with antimicrobial activity that can be used as mycofumigants. The genus currently comprises five species. Here we describe the isolation and characterization of a new species of *Muscodor* on the basis of five endophytic fungal strains from leaves of *Actinidia chinensis*, *Pseudotaxus chienii* and an unidentified broad leaf tree in the Fengyangshan Nature Reserve, Zhejiang Province, Southeast of China. They exhibit white colonies on potato dextrose agar (PDA) media, rope-like mycelial strands, but did not sporulate. The optimum growth temperature is 25 °C. The results of a phylogenetic analysis based on four loci (ITS1–5.8S–ITS2, 28S rRNA, *rpb2* and *tub1*) are consistent with the hypothesis that these five strains belong to a single taxon. All five strains also produce volatile chemical components with antimicrobial activity *in vitro*, which were different from those previously described for other *Muscodor* species.

Introduction

Muscodor is a genus of endophytic fungi that are known from certain tropical tree and vine species in Central/South America, and South Eastern Asia and Australia (Atmosukarto *et al.* 2005; Daisy *et al.* 2002b; Ezra *et al.* 2004; González *et al.* 2009; Mitchell *et al.* 2008; Sopalun *et al.* 2003; Strobel *et al.* 2007; Worapong *et al.* 2001, 2002). So far five species have, on the basis of morphological, phenetic and genetic features, been described in the genus (i.e. *Muscodor albus*, *Muscodor roseus*, *Muscodor vitigenus*, *Muscodor crispans* and *Muscodor yucatanensis*) (Daisy *et al.* 2002a, 2002b; González *et al.* 2009; Mitchell

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et al. 2008; Worapong et al. 2001, 2002). *Muscodor* spp. also characteristically produce a mixture of volatile organic compounds (VOCs) that consist primarily of various alcohols, acids, esters, ketones, and lipophilic substances that are lethal to a wide variety of plant- and human-pathogenic fungi and bacteria, as well as to nematodes and certain insects (Daisy et al. 2002a; Strobel et al. 2001). *Muscodor* spp. are therefore of high value and promise for biocontrol (Strobel 2006) and the discovery of new isolates and taxa of this genus is of major interest to mycologists.

During a systematic study of the fungal endophytic population of woody plants distributed in the Southeast of China, we isolated five strains that exhibited characteristics typical for *Muscodor*. Here we describe them as a new taxon of *Muscodor*, based on their morphology, the optimum growth temperature, production of volatile chemicals profiles and a multilocus phylogenetic analysis, thereby also expanding the concept of its genus.

Materials and methods

Study site

The site of study was located in Fengyangshan Nature Reserve, Zhejiang province, in the Southeast of China (E119°06′–119°15′, N27°46′–27°58′).

Sampling, isolation of endophytic fungi and maintenance of cultures

Healthy and intact twigs and leaves of *Actinidia chinensis*, *Pseudotaxus chienii* and an unidentified broad leaf tree, respectively, were sampled, placed in sterile plastic bags, stored in an ice box and transported to laboratory within 48 h of sampling.

The plants were then rinsed softly with tap water, thereafter immersed in ethanol (75 %, v/w; 30 s), followed by immersion in sodium hypochlorite (1 %, w/v; 10 min) and finally rinsed thrice with 30 ml of sterile distilled water. The plant tissues were then cut into pieces of 0.6 cm length and six of them placed on a plate containing malt extract agar (2 % w/v; MEA), supplemented with chloromycetin (50 mg l^{-1}) to prevent bacterial growth, and incubated at 25 °C in darkness. After the emerging of fungal hyphae from the tissues, they were transferred to new agar plates and purified by subculturing. For culture maintenance, the isolates were grown on potato dextrose agar (PDA) and either covered with sterile liquid paraffin (at 25 °C) or suspended in GYCG (glucose, 10 g l^{-1}; yeast extract 1 g l^{-1}; acid casein hydrolysate, 1 g l^{-1}; and glycerol 15 %, v/v) and stored at −70 °C.

Scanning electron microscopy (SEM) analysis of the endophytic isolates

For SEM analysis, the fungal strains were grown on PDA medium (10 d, darkness, 25 °C). The peripheral front of the radial cultures was then carefully removed with a scalpel. SEM was performed using cryo-SEM (HITACHI S-3000N microscope, Japan), operating between 10 and 15 kV on samples frozen in liquid nitrogen and coated with a thin layer of gold sputter.

Determination of the optimum growth temperature

The optimum growth temperature was determined on PDA and MEA. The fungal strains were pre-grown on PDA medium for 15 d in darkness at 25 °C. Then an agar disk (5 mm diameter) was excised from the growing front of the fungus and placed into the middle of a new Petri dish and incubated in darkness for 15 d at 10, 15, 20, 25, 28, 30, 35 and 40 °C, respectively. The diameter of the growing colony was measured. We shall note that because of the very slow growth of the tested isolates, they still had not reached the border of the plates after 15 d, and the measurements of different growth rates are thus comparable.

DNA extraction, gene fragment amplification, sequencing and phylogenetic analysis

For DNA isolation, the fungal mycelium was scraped from the margin of the colonies with a sterile needle. Genomic DNA was extracted using the Multisource Genomic DNA Miniprep Kit (Axygen Bioscience, Inc. China) following the manufacturer's instructions. For amplification of the internal transcribed spacer (ITS) and 28S ribosomal RNA (rRNA), the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGGGAAACTTCG-3′), respectively, were used (White et al. 1990). A fragment of the RNA-polymerase subunit B encoding gene *rpb2* was amplified with primer pairs RPB2-5f (5′-GAYGAYMGWGATCAYTTYGG-3′) and RPB2-7cr (5′-CCCATRGCTTGYTTRCCCAT-3′) as described by Liu et al. (1999). A fragment of the beta-tubulin coding gene (*tub1*) was amplified with the primers bena-T1 (5′ -AACA TGCGTGAGATTGTAAGT-3′) and bena-T22 (5′-TCTGGATG TTGTTGGGAATCC -3′) (O'Donnell & Cigelnik 1997). ITS rRNA polymerase chain reaction (PCR) products were separated in 1.0 % (w/v) agarose gels, purified by the aid of a gel band purification kit (Axygen Bioscience, Inc. China), and then sequenced in an ABI 3730 sequencer (Applied Biosystems, USA), using the same primers as for PCR. PCR products of 28S rRNA, *rpb2* and *tub1* were purified as above and ligated into pGEM-T Easy vector (Promega, USA), and transformed into *Escherichia coli* JM109 (Promega) according to the manufacturer's instructions. Positive clones subjected to sequencing with ABI 3730 sequencer.

All sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis at the National Center for Biotechnology Information (NCBI) server. The sequences with highest similarity to each locus were retrieved, combined with the sequences obtained during this work, aligned using Clustal X1.8 (Thompson et al. 1997), and the alignment finally manually corrected using GENEDOC (Nicholas & Nicholas 1997). The alignments were then exported as a NEXUS format and a maximum parsimony analysis was performed in PAUP* 4.0 b 10 (Swofford 2003), using the heuristic search option with tree bisection-reconnection (TBR) branch swapping; stability of clades was tested using 1000 bootstrap replications. Gaps were coded as missing data. Sequences newly obtained during this work were deposited in NCBI GenBank