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ORIGINAL ARTICLE

Existence of *Muscodor vitigenus*, *M. equiseti* and *M. heveae* sp. nov. in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential

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Abstract We isolated volatile metabolite-producing endophytic fungi from the leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.) and studied their antimicrobial competence. A total of three isolates was obtained, and their phenotypic and phylogenetic relationship with the genus *Muscodor* in the family *Xylariaceae* was studied. All isolates could produce volatile metabolites with apparent antimicrobial activity against diverse test microbes (bacteria, yeast and filamentous fungi). An isolate, RTM5IV3, with <86 % similarity with the partial ITS-5.8S rDNA gene as compared to other species of the genus *Muscodor*, was proposed as a novel species with the name *Muscodor heveae* sp. nov. Its bioactive volatile metabolites included 3-methylbutan-1-ol as a major component, followed by 3-methylbutyl acetate and azulene derivatives. The volatile organic compounds (VOCs) produced by the *Muscodor* isolates have the potential for biological control of bacteria, yeast and filamentous fungi. Furthermore, the VOCs of *M. heveae* were active against the pathogenic fungi *Phellinus noxius* and *Rigidoporus microporus* that cause root disease in the rubber tree. We concluded that rubber trees could be an alternative source for discovery of fungi that produce volatile metabolites, and the genus *Muscodor* could be found abundantly in this habitat.

Keywords Endophytic fungi · *Muscodor* · Rubber tree · Taxonomy · Volatile antibiotics

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Introduction

Microbial endophytes are bacteria or fungi which live within plants without causing apparent disease (Huang et al. 2001; Chaverri and Gazis 2011). As symbionts, most endophytes enhance plant growth and a plant's defensive system (Strobel and Daisy 2003; Tejesvi et al. 2007). Because of such benefits, endophytes are one of the promising microbial resources for biocontrol agents applied in agriculture. A mode of action of endophytes in prevention and/or suppression of plant diseases caused by phytopathogens is the production of antimicrobial substances. The bioactive compounds in the form of volatile metabolites produced by endophytes have not been commonly reported. Some fungi that belong to the families *Diaporthaceae*, *Hypocreaceae*, and *Xylariaceae* of the phylum Ascomycota are notable for their capacity to form volatile metabolites with antimicrobial activity (Stinson et al. 2003; Suwannarach et al. 2013b). In the family *Xylariaceae*, *Muscodor albus* is the first known fungal endophyte isolated from *Cinnamomum zeylanicum*, which produces bioactive volatile metabolites (Strobel et al. 2001; Ezra et al. 2004).

The genus *Muscodor* does not produce spores and is classified into the family *Xylariaceae* based on the polyphasic approach (Kudalkar et al. 2011). Although morphological characteristics can be used to classify the members of *Muscodor* at the genetic level, a more appropriate genotypic

classification method is needed for identification at the species level and to understand their phylogenetic lineage. In addition, profiling and comparison of unique volatile metabolites produced by members of the genus *Muscodor* are important criteria for its classification (Strobel 2006; González et al. 2009). The major volatile metabolite produced by the genus *Muscodor* is 2-methylpropanoic acid (Kudalkar et al. 2011; Suwannarach et al. 2013a), which differs from other endophytic genera in the same family, e.g., *Nodulisporium* spp. that produces eucalyptol (Tomsheck et al. 2010). Recently, many novel species of the genus *Muscodor* (*M. cinnamomi*, *M. equiseti*, *M. musae*, *M. oryzae*, and *M. suthepensis*) were isolated from diverse plant hosts growing in northern Thailand (Suwannarach et al. 2010; 2013a). Other species of *Muscodor* have been isolated from tropical trees and vines in Australia, Central and South America, and Central, South and Southeast Asia (Meshram et al. 2013; Suwannarach et al. 2013a; Saxena et al. 2015), while a previous report showed that *Penicillium*, *Pestalotiopsis* and *Trichoderma* were the most frequently isolated fungi from rubber trees (Gazis and Chaverri 2010). However, a diversity study of the genus *Muscodor* that lives in association with rubber trees has not been reported previously.

The rubber tree (*Hevea brasiliensis* Müll.Arg.) is an economic crop that produces natural latex as a major raw material for rubber manufacture. Most of the world's plantation areas of rubber trees are located in countries in South East Asia such as Malaysia, Indonesia and Thailand (van Beilen and Poirier 2007). Although recent farming practices for the rubber tree have achieved a high yield of latex, plant diseases that cause reduction of latex yield and mortality in rubber trees are still common (Jayasuriya and Deacon 1995; Evueh and Ogbebor 2008). In addition, post-harvest products of natural latex in the form of air-dried rubber sheets are commonly contaminated by airborne fungi (Linos and Steinbüchel 2001). This contamination reduces both the quality and price of rubber sheets. Thus, volatile metabolite-producing endophytes may be an alternative biological approach as biofumigation in control of plant diseases and fungal contamination of rubber tree seedlings and their latex products. It is known that sapwood and leaf fragments of rubber trees are a rich source of fungal endophytes (Evueh and Ogbebor 2008; Rocha et al. 2010; Gazis 2012), but few studies have reported their antimicrobial activities (Evueh and Ogbebor 2008; Gazis 2012). Moreover, no volatile metabolite-producing endophytes have yet been reported from rubber trees.

In this study, we aimed to evaluate the variety of volatile metabolite-producing ascomycetes that live in association with rubber trees, giving a high priority to the genus *Muscodor*. Leaves of the rubber tree were used as a source for isolation of fungal endophytes that can produce volatile metabolites. The genetic diversity of such endophytes and the antimicrobial activity of their volatile metabolites were investigated. A novel species of these endophytes was proposed and described in this article. The possible applications of these endophytes as a potent biocontrol agent in rubber farming were also discussed.

Materials and methods

Preparation and surface sterilization of plant materials

Healthy rubber tree leaves were used as a source for isolation of endophytic fungi. A total of 45 leaves were collected from rubber trees planted in Nongbualamphu province, northeastern Thailand (geographical location: 17°12′14″ N, 102°26′26″ E). They were placed in plastic bags and stored in an icebox before transport to the laboratory within 24–48 h after sampling. The age of the rubber trees was approximately 6 years, which is considered as the mature phase for latex harvest. The leaves were prepared and surface sterilized following the method described by Suwannarach et al. (2010). Briefly, plant leaves were rinsed with tap water for 15 min and randomly cut into small segments (25 mm^2). All segments were sterilized by soaking in 75 % ethanol for 30 s, 2 % sodium hypochlorite for 3 min, and 95 % ethanol for 30 s.

Isolation of volatile metabolite-producing endophytic fungi

Endophytic fungi that form volatile metabolites were isolated using a parallel-growth isolation technique (Worapong et al. 2001). Briefly, a part of a two-compartment Petri dish was filled with potato dextrose agar (PDA, Himedia Laboratories, India), while the other side contained half-strength PDA supplemented with rose bengal and chloramphenicol at final concentrations of 0.033 g L^{-1} and 50 mg L^{-1}, respectively. A strain producing volatile antibiotic metabolites, *M. cinnamomi* (MB518008) (Suwannarach et al. 2010) was used as a reference strain for this isolation. An agar plug (6-mm diameter) of *M. cinnamomi* grown previously on PDA at ambient temperature (25±2 °C) for 7 days was placed on the PDA side of the prepared two-compartment Petri dish. This inoculation was incubated at 25±2 °C for 4 days. Then, five surface sterilized plant segments were placed on the half strength PDA, where they were exposed to the volatile metabolites produced by *M. cinnamomi*. The Petri dish was sealed with parafilm® M, and incubated at 25±2 °C for 2 weeks. The production of its VOCs facilitated selection pressure, allowing the growth of only fungal species that tolerated these volatiles. Then, the hyphal tips of endophytic fungi that grew out from the plant segments were aseptically transferred to a separate PDA plate. This technique can be used to select for other isolates of *Muscodor* (Worapong et al. 2001; Mitchell et al. 2008). The endophytic fungi were tested with *Colletotrichum gloeosporioides*, following a protocol described by Strobel et al. 2001, if they produced volatile antibiotics. Spore

production of endophytic fungi was studied on four different media, including corn meal agar (CMA), malt agar (MA), PDA and water agar (WA). The microscopic structures, such as hyphal characteristics, cellular bodies and spore production, were observed under a light microscope (Olympus SZ40, Japan). The colony texture, color and fruiting body were observed under a stereomicroscope (Olympus CH30, Japan). The pure cultures of endophytic fungi were subsequently stored in 20-% (v/v) glycerol at −20 °C and deposited at the Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, and the BIOTEC Culture Collection (BCC), Bangkok, Thailand.

Phenotypic classification

Colony and hyphal characteristics of all endophytic fungal isolates were initially observed with a light microscope (Olympus CH30, Japan). The morphological characteristics were observed also with a scanning electron microscope (JEOL JSM- 5910LV, Japan), following a protocol described by Ezra et al. (2004). All endophytic fungal isolates whose morphology was typical of the genus *Muscodor* were grouped and selected for further classification.

Genotypic classification

All *Muscodor* isolates were grown on PDA at 25±2 °C for 10 d. The aerial mycelium of each isolate was scraped from the PDS surface. This fungal biomass was then freeze-dried and ground into a fine powder with a pestle and mortar. A modified SDS-CTAB method (Suwannarach et al. 2013a) was used for the DNA extraction. The internal transcribed spacer regions 1 and 2 including 5.8S rDNA (ITS1-5.8-ITS2 rDNA) was a targeted nucleotide sequence for the amplification using a pair of universal primers (ITS4 and ITS5). The amplification was carried out using a GeneAmp 9700 thermal cycler (Applied Biosystems) with the following polymerase chain reaction (PCR) conditions: initial denaturization at 95 °C (2 min), 30 cycles of reaction [denaturization at 95 °C (30 s), annealing at 50 °C (30 s), and extension at 72 °C (1 min)], and final extension at 72 °C (10 min). The reaction mixture (25 μL) contained 1 μL of the DNA template, 0.2 μM dNTP, 0.2 μL of FastTaq (Applied Biosystems), 0.2 μM each primer, 2.5 μL of $MgCl_2$ buffer, and sterile water to bring the volume to 25 μL. The PCR product was confirmed by gel electrophoresis and purified with a PCR cleanup Gel Extraction NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Germany) following the manufacturer's manual. The purified PCR product was then sequenced by 1st Base, Malaysia. All DNA sequences obtained were aligned and compared to the available sequences in the GenBank database using the BLASTN facility within the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis of all sequences was conducted by the neighbor-joining method using MEGA6 software (Tamura et al. 2013).

In vitro antimicrobial assay of VOCs

Antagonistic activity of all *Muscodor* isolates was tested against a set of pathogenic microbes listed in Table 1. The test microbes were comprised of bacteria, yeast and filamentous fungi. The parallel-growth isolation technique was adapted for the antagonism test. Briefly, part of a two-compartment Petri dish was filled with PDA, while the other side contained nutrient agar for the test bacteria, yeast extract-malt extract agar for the test yeast, or PDA for the test filamentous fungi. An agar plug from the mycelial margin of each *Muscodor* isolate growing on PDA was inoculated on the PDA part of the Petri dish, and allowed to grow at 25±2 °C for 4 days. Then, each test bacterium or yeast was individually streaked on its respective agar medium in the Petri dish. For the filamentous fungi test, an agar plug (6-mm diameter) of their 4-day-old PDA culture was inoculated on PDA on the opposite side of the Petri dish. All Petri dishes were wrapped with parafilm® M, and incubated at 25±2 °C for 6 days. The percent inhibition of fungal growth after the dual culture test was calculated with the following equation: $[(R_1\text{-}R_2)\times 100] \div R_1$, where R_1 is the average colony radius of each test microbe measured in the control plates (without *Muscodor* isolate), and R_2 is the average colony radius calculated from the test plates. In the case of bacteria and yeast, the microbes were checked for visible growth and viability. The viability of the test microbes was observed by transferring them from the test plates and re-growing in fresh media (Strobel et al. 2001).

Profiling and comparison of volatile metabolites

Only the *Muscodor* isolate that was a possible novel species (having a <86-% similarity to the ITS1-5.8-ITS2 rDNA sequence of other members of the genus *Muscodor*), was analyzed for its profile of volatile metabolites. The mixture of volatile metabolites in the headspace of the *Muscodor* isolate growing on PDA at 25±2 °C for 10 days was identified by a modified gas chromatography–mass spectrometry (GC-MS) procedure with solid phase microextraction (SPME; Strobel et al. 2001). The SPME equipped with a syringe consisting of gray hub fiber material made by divinylbenzene/carboxen (50/30) on polydimethylsiloxane affixed on a stable flex fiber was used for trapping the volatile metabolites produced in the headspace of the *Muscodor* culture for 45 min. The syringe was inserted into the splitless injection port of an Agilent 7890A gas chromatograph equipped with a mass spectrometer MSD 5975C (EI) mass selective detector. A DB-wax capillary column (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm was used for separation of volatile metabolites. The column

Table 1 Antimicrobial activity of volatile metabolite-producing *Muscodor* isolates

Test microbes	Percent inhibition of microbial growth (viability) after exposure to different isolates of *Muscodor*		
	RTM5IV1	RTM5IV2	RTM5IV3
Gram positive bacteria			
Bacillus cereus	100 (Dead)	0 (Alive)	100 (Alive)
Methicillin-resistant *Staphylococcus aureus* (MRSA)	0 (Alive)	0 (Alive)	100 (Dead)
Micrococcus luteus	0 (Alive)	0 (Alive)	100 (Dead)
Staphylococcus aureus ATCC 29213	100 (Dead)	0 (Alive)	100 (Dead)
Gram negative bacteria			
Enterobacter aerogenes	100 (Dead)	0 (Alive)	100 (Dead)
Enterococcus faecalis	0 (Alive)	100 (Dead)	100 (Dead)
Escherichia coli ATCC 35218	0 (Alive)	0 (Alive)	100 (Dead)
Klebsiella pneumoniae (ESBL+)	0 (Alive)	0 (Alive)	100 (Dead)
Proteus mirabilis	0 (Alive)	100 (Dead)	100 (Dead)
Pseudomonas aeruginosa ATCC 27859	0 (Alive)	0 (Alive)	0 (Alive)
Salmonella sp. Group D	0 (Alive)	0 (Alive)	100 (Dead)
Yeast			
Cryptococcus neoformans	100 (Dead)	0 (Alive)	100 (Dead)
Filamentous fungi			
Aspergillus flavus	100 (Alive)	98.4±1.3 (Alive)	100 (Dead)
Aspergillus niger	100 (Dead)	100 (Dead)	100 (Dead)
Colletotrichum gloeosporioides	100 (Dead)	100 (Dead)	100 (Dead)
Fusarium oxysporum f. sp. *vasinfectum*	39.8±2.2 (Alive)	47.4±1.5 (Alive)	62.8±2.2 (Alive)
Ganoderma australe BCC22321	100 (Dead)	100 (Dead)	100 (Dead)
Phellinus noxius BCC26237	100 (Dead)	100 (Dead)	100 (Dead)
Phytophthora parasitica BCC15560	100 (Dead)	97.1±1.3 (Alive)	100 (Dead)
Rhizoctonia solani AG-2	100 (Alive)	71.1±2.1 (Alive)	100 (Dead)
Rigidoporus microporus	100 (Dead)	100 (Dead)	100 (Dead)

All tests were done in triplicate and repeated twice, the % inhibition is shown as the mean±standard deviation (SD) while without SD refers to the absence of the mean difference

was initiated with a thermal program of 40 °C for 2 min, increasing to 200 °C at a rate of 5 °C min^{-1}. Ultra-high purity helium was used as a carrier gas with an initial column head pressure of 60 kPa. Before trapping the volatile metabolites, the fiber material was conditioned at 250 °C for 34 min under a flow of helium gas. A 30-s injection time was used to introduce the adsorbed volatile metabolites into the GC. The volatile metabolites were identified through a library comparison with the National Institute of Standards and Technology (NIST) database by considering their quality match (80 % or higher).

Results

Volatile metabolite-producing endophytic fungi of the rubber tree leaf

A total of three isolates of volatile metabolite-producing endophytic fungi was isolated from leaves of rubber trees, using the parallel-growth isolation technique with *M. cinnamomi* as a reference strain. All isolates (100 %) were classified morphologically into the genus *Muscodor*. This phylogenetic relationship was supported by 100 % similarity of the gene sequence and morphological characteristics. The phylogenetic analysis of ITS1-5.8-ITS2 rDNA sequences derived from all *Muscodor* isolates revealed the different closest related species and displayed three major clades, A, B, and C, containing all known species of *Muscodor*, and all isolates from this study with a high bootstrap support (91 %) (Fig. 4). Isolate RTM5IV3 showed the lowest sequence similarity (86 %) to *M. albus* cz620[1], *M. cinnamomi*, *M. musae*, and *M. oryzae*. In clade A, it separated from the *M. albus* group, *M. strobelii* and *M. suthepensis* with 93-% bootstrap support (Fig. 4). Isolates RTM5IV1 and RTM5IV2 showed a maximum homology of 100 % with *M. vitigenus* and *M. equiseti*, respectively, and were classified into clade B with 99 % bootstrap support (Fig. 4). Morphological data and bioinformatics were insufficient to place the *Muscodor* isolates into exact lineages.

Nevertheless, we could suppose that the isolates giving a % sequence similarity lower than 100 % could be novel members of the genus *Muscodor*. With this hypothesis, one of the *Muscodor* isolates (33.3 %) found in this work was possibly a novel species isolated from rubber tree leaves.

Volatile analysis of *Muscodor* spp.

Isolate RTM5IV3 produced a mixture of 12 volatile metabolites which were identified by GC/MS through a library comparison with the NIST database (Table 3). Its volatile profile was compared with its closest phylogenetic species; *M. albus* cz620[1], *M. cinnanomi*, *M. musae*, and *M. oryzae*. The volatile compound produced by isolate RTM5IV3 with the highest percentage peak area (11.26) was 3-methylbutan-1-ol, the same as produced by *M. albus* cz620 and *M. oryzae*. The minor volatile compounds were 3-methylbutyl acetate and 2-methylpropanoic acid. These volatile compounds are also produced by *M. albus* cz620. However, the volatile profile of isolate RTM5IV3 was different from other *Muscodor* spp. (Table 3). In addition, isolate RTM5IV3 produced two volatile compounds, 1,1,9-trimethyl-5-methylidenespiro[5.5]undec-9-ene and methyl (*Z*)-*N*-hydroxybenzenecarboximidate, compounds that are not found in other *Muscodor* spp. (Table 3).

Antimicrobial activity of volatile metabolites produced by *Muscodor* isolates

The volatile metabolites produces by different *Muscodor* isolates were tested for their antimicrobial activity against a set of test microbes (Table 1). The volatile metabolites produced by *Muscodor* isolates RTM5IV1 and RTM5IV3 exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria, yeast and filamentous fungi, while isolate RTM5IV2 inhibited only the growth of some Gram-negative bacteria and filamentous fungi. Isolate RTM5IV3 showed the greatest antimicrobial activity with all test organisms. *Fusarium oxysporum* f. sp. *vasinfectum* was more susceptible to the volatile metabolites of RTM5IV3 than those of other isolates. *Pseudomonas aeruginosa* ATCC 27859, the most resistant test microbe, was insensitive to the volatile metabolites produced by every isolate of *Muscodor*. All the *Muscodor* volatiles exhibited significant control of all tested pathogenic fungi of the rubber tree (*C. gloeosporioides, Phellinus noxius* and *Rigidoporus microporus*) compared with other tested fungi.

Taxonomic description of a novel species

Muscodor isolate RTM5IV3 did not produce spores on any medium, similar to the original *Muscodor*, and had rope-like mycelium with coiled structures (Strobel 2011). Although the isolate formed pale orange colonies when exposed to natural light similar to *M. cinnamomi* and *M. oryzae*, its mycelial morphology was wavy and hair-like at the mycelial edge and produced a unique unidentified structure (Fig. 1 and Table 2). The isolate RTM5IV3 was classified and identified with the polyphasic approach. Both phenotype and genotype revealed that the isolate belongs to the genus *Muscodor*. It shared relatively low sequence similarity of ITS1-5.8-ITS2 rDNA gene (86 %) with many species of the genus *Muscodor*. The phylogenetic tree showed isolate RTM5IV3 was separated from the other 12 *Muscodor* spp. in clade A with high bootstrap support (Fig. 4). The phylogenetic dendrogram also supported the node separating isolate RTM5IV3 in clade A from five species of *Muscodor* in clade B and C with 88 % and 91 % bootstrap support, respectively. The isolate RTM5IV3 was able to form volatile metabolites with antimicrobial activity against a diverse set

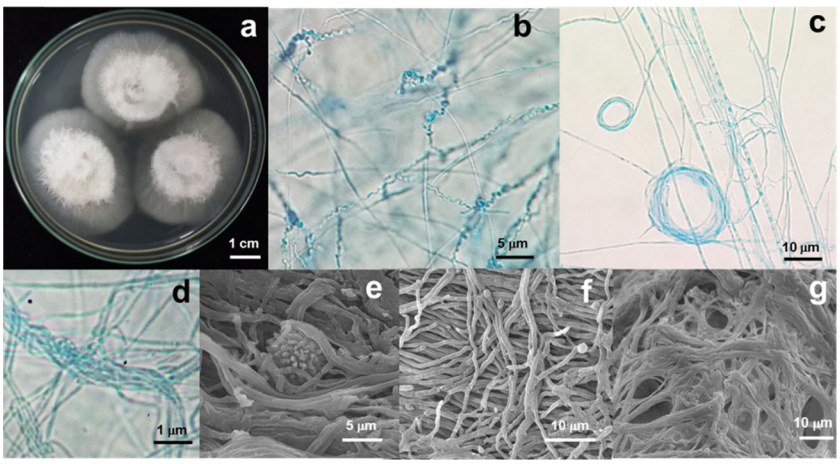

Fig. 1 Morphological characteristics of *M. heveae* (RTM5IV3) isolated from leaves of the rubber tree. A difference in colony morphology was observed after growing on PDA in the dark (**a**) for 10 days. The hyphal morphology of *M. heveae* was observed further with light (**b**–**d**) and scanning electron (**e**–**g**) microscopes. The wavy mycelium from the colony edge (**b**), formation of coiling hypha (**c**), rope-like mycelium (**d**, **g**), unique unidentified structure (**e**), and swollen-like hyphal tips (**f**) were found as a typical morphology of *M. heveae*

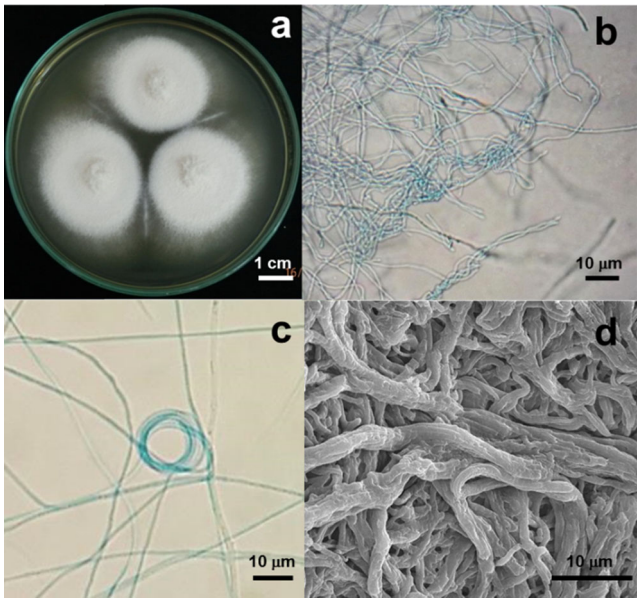

Fig. 2 Morphological characteristics of *M. vitigenus* (RTM5IV1) isolated from leaves of the rubber tree. A difference in colony morphology was observed after growing on PDA in the dark (**a**) for 10 days. The hyphal morphology of *M. vitigenus* was observed further with light (**b**–**c**) and scanning electron (**d**) microscopes. The formation of rope-like mycelium (**b**, **d**), and coiling hypha were found as a typical morphology of *M. vitigenus*

of test microbes, which is a typical phenotype of the genus *Muscodor* (Table 1). However, the profile of volatile metabolites produced by the isolate was different than its closely related phylogenetic species. However, the major component of the volatile metabolites was 3-methylbutan-1-ol, which was the same as the type species of the genus *Muscodor*, *M. albus* cz620[1] (Table 3). Based on the phenotypic, genotypic and phylogenetic evidence, isolate RTM5IV3 represents a novel species of the genus *Muscodor*, for which the name *Muscodor heveae* sp. nov. was proposed. The taxonomic description includes the following:

Taxonomy

Muscodor heveae S. Siri-Udom & S. Lumyong, sp. nov. (Fig. 1)

MycoBank: MB809310.

Diagnosis: Pale orange colonies on PDA in natural light, hyphae 1.2 - 3.7 μm thick, coils 14.8 - 27.1×19.7 - 39.4 μm in diameter, and producing a fruity odor.

Etymology: *heveae*, refers to the name of the host plant, *Hevea brasiliensis*.

Holotype: THAILAND, Nongbualamphu province, Chiang Mai, Chiang Mai University, from a healthy leaf of *Hevea brasiliensis* (*Euphorbiaceae*) during May 2011-December 2012. (ex-type living culture BCC 70461 and SDBR-CMU RTM5IV3), GenBank sequence KF850712.

Teleomorph: Unknown.

Description: In nature, the fungus is associated with *Hevea brasiliensis*. It is an ascomycete and does not produce spores. Colony color of the fungus on PDA in natural light is pale orange but becomes whitish in the dark (Fig. 1a). The edge of colony was wavy mycelium (b). Hyphae (1.2 - 3.7 μm thick) with coils (14.8 - 27.1×19.7 - 39.4 μm diameter; Fig. 1c), common intertwining of mycelium into a twisted cable-like strand and a rope like strand (4.9 - 7.4 μm thick; Fig. 1d, 1 g). Mycelium forms unique unidentified structures (3.8 - 7.5 μm diameter; Fig. 1e) and swollen cells at hyphal tips (2.5 - 4.9 μm diameter; Fig. 1f). A culture on PDA produced a fruity odor, which contains 3-methylbutan-1-ol as a major

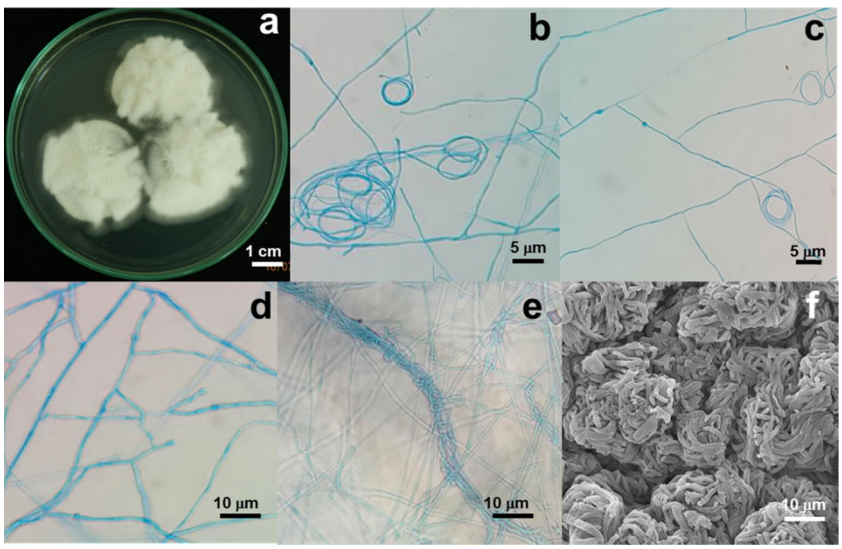

Fig. 3 Morphological characteristic of M. *equiseti* (RTM5IV2) isolated from leaves of the rubber tree. A difference in colony morphology was observed after growing on PDA in the dark (**a**) for 10 days. The hyphal morphology of *M. equiseti* was observed further with light (**b**–**e**) and scanning electron (**f**) microscopes. The formation of coiling hyphae (**b**–**c**), a triangular branching pattern (**d**), swollen cells (**c**–**d**), rope-like mycelium (**e**), and cottony-like mycelium (**f**) were found as a typical morphology of *M. equiseti*

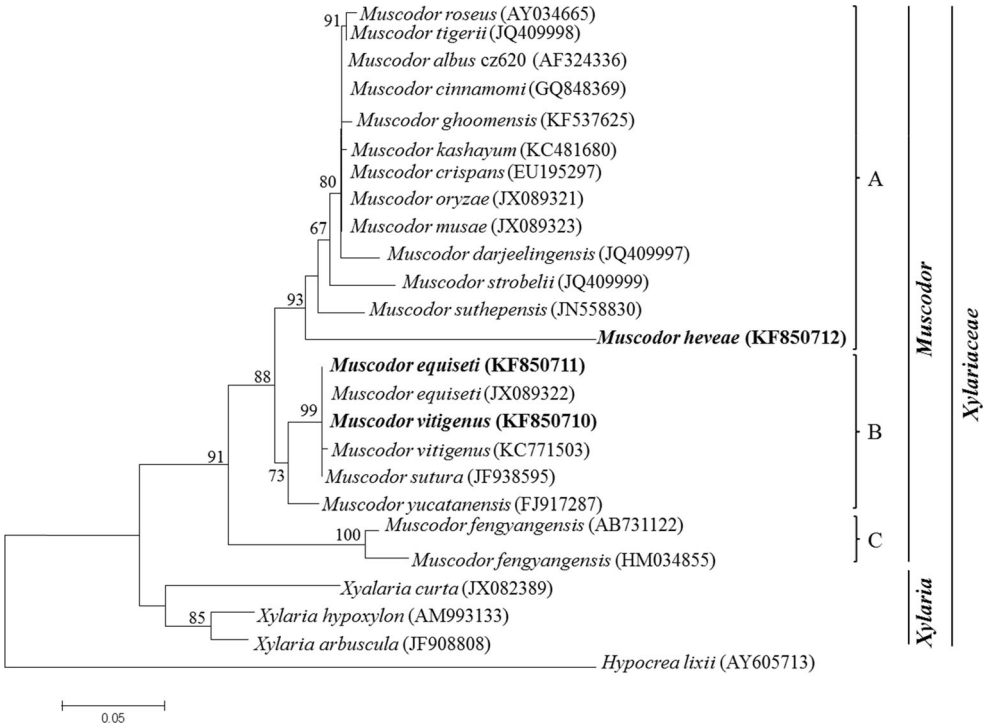

Fig. 4 Maximum parsimony tree of *Muscodor* spp. based on ITS1-5.8S rDNA-ITS2 sequence alignment of 25 sequences. *Hypocrea lixii* was used as an out group. The isolate RTM5IV3 was proposed as a novel species, *M. heveae* (see text for the description of this novel species). The isolate RTM5IV1 and RTM5IV2 were proposed as *M. vitigenus* and *M. equiseti*, respectively. The codes indicated in parentheses refer to the accession numbers of the sequences available in the GenBank database

component. Spores and other fruiting bodies did not appear under any of the test conditions.

The other two isolates of *Muscodor* tested, isolates RTM5IV1 and RTM5IV2, were identified as *M. vitigenus* (Mycobank: MB373747) (Fig. 2) and *M. equiseti* (Mycobank: MB800814) (Fig. 3), respectively, based on morphology and phylogenetic analysis. The description of *M. vitigenus* and *M. equiseti* were showed in previous publications of Daisy et al. (2002) and Suwannarach et al. (2013a), respectively.

Discussion

It is known that ascomycetes including the following notable genera, *Colletotrichum*, *Penicillium*, *Pestalotiopsis*, and *Trichoderma* are dominant fungal endophytes of the rubber tree (Gazis and Chaverri 2010; Gazis 2012). However, there is no report of volatile metabolite-producing endophytes that live associated with the rubber tree. We were the first to find that the rubber tree can be a source for discovering volatile metabolite-producing endophytes. The isolation technique

Table 2 Morphology and bioactivity of *M. heveae* and its closely related phylogenetic species

Morphology and bioactivity	*M. heveae*	*M. albus* cz620[1]	*M. cinnamomi*	*M. musae*	*M. oryzae*
Mycelial growth	Rope-like strand and swollen cells with a coil structure	Rope-like with a coil structure	Rope-like with cauliflower-like bodies	Rope-like with a coil structure	Rope-like with a coil structure
Hyphal growth at colony front	Wavy and hairy-like mycelium	Straight	Straight	Straight and hairy-like mycelium	Straight
Pigment production					
in the light	Pale orange	Whitish	Pale orange	Whitish	Pale orange
in the dark	Whitish	ND	Whitish	Whitish	Pale orange
Host	*Hevea brasiliensis*	*Cinnamomum zeylanicumin*	*Cinnamomum bejolghota*	*Musa acuminata*	*Oryza rufipogon*
Bioactivity	Antifungal/ antibacterial	Antifungal/ antibacterial	Antifungal/antibacterial	Antifungal/ antibacterial	Antifungal/ antibacterial
Reference	This work	Worapong et al. (2001)	Suwannarach et al. (2010)	Suwannarach et al. (2013a)	Suwannarach et al. (2013a)

ND refers to not determined

Table 3 Composition of the volatile metabolites produced by *Muscodor* spp. as determined via GC/MS

Retention time (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
5.41	cyclopentane	C_5H_{10}	7.69	0	0	0	7.69	0	0
5.56	ethyl acetate	$C_4H_8O_2$	88	0	0	7.63	0	0	0
5.60	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0.76/86*	0	0	0	0
6.03	3-methylbutyl acetate	$C_7H_{14}O_2$	130	0	6.92/90*	0	0	0	0
6.38	methyl 2-methylbutanoate	$C_6H_{12}O_2$	116	0	0	0	14.90	0	0
6.51	methyl propanoate	$C_4H_8O_2$	102	0	0	0.31	0	0	0
7.07	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0	0	0	0	3.48
7.16	ethanol	C_4H_6O	ND	0	0	6.24	0	0	0
7.77	3-methylbutanoyl acetate	ND	130	0	0	0	0	20.41	4.70
8.03	ethyl 2-methylpropanoate	$C_6H_{12}O_2$	116	0	0	2.07	0	0	0
8.29	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	11.26/83*	0	3.12	0	0
10.32	2-methylbutyl 2-methylpropanoate	$C_9H_{18}O_2$	158	0	0	0	0	0.21	0
10.88	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	0	0	0	13.34	32.69
11.45	2-methylpropyl 2-methylpropanoate	$C_8H_{16}O_2$	ND	0	0	0.58	0	0	0
12.05	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0	2.06	0	0	0
12.10	3-methyl-3-buten-1-ol	$C_5H_{10}O$	86	0	0	0	0	0	0.27
12.20	ethyl 2-hydroxy-2-methylpropanoate	$C_6H_{12}O_3$	132	0	0	0	0	0	0.88
12.50	3-methylbutyl acetate	$C_7H_{14}O_2$	ND	0	0	22.24	0	0	0
12.66	1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-2,4,6,8,10,12-hexaoxa-1,3,5,7,9,11-hexasilacyclododecane	$C_{12}H_{36}O_6Si_6$	445	0.67/91*	0	0	0	0	0
12.97	3-hydroxybutan-2-one	$C_4H_8O_2$	88	0	0	0	0	0.59	2.00
14.57	3-methylbutyl 2-methylpropanoate	$C_9H_{18}O_2$	ND	0	0	1.53	0	0	0
15.28	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	0	22.99	0	0	0
15.88	benzaldehyde	C_7H_6O	106	4.61/96*	0	0	0	0	0
16.08	2-pentylfuran	$C_9H_{14}O$	138	0	0	0.29	0	0	0
16.19	2,4-dimethyl-1-heptene	C_9H_{18}	126	0	0	0	0	0	0.13
16.31	1-isobutoxy-2-ethylhexane	$C_{12}H_{26}O$	186	0	0	0	0	0	0.31
17.13	2-methylpropanoic acid	$C_4H_8O_2$	88	0	4.73/91*	0	0	0	0
19.04	(1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0] undec-4-ene	$C_{15}H_{24}$	204	0	0.41/93*	0	0	0	0
19.43	isopropyl-4-piperidone	$C_8H_{15}NO$	141	0	0	0	0	0.23	0
19.50	4,5-dimethyl-1,3-cyclopentanedione	$C_7H_{10}O_2$	126	0	0	0	0	0	0.37
19.77	1,1,9-trimethyl-5-methylidenespiro [5.5]undec-9-ene	$C_{15}H_{24}$	204	0	0.51/95*	0	0	0	0
20.38	nonan-2-one	$C_9H_{18}O$	142	0	0	0.41	0	0	0
20.75	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	2.03/99*	0	0	0	0
21.03	2-methylpropanoic acid	$C_4H_8O_2$	88	0	0	0	0	33.17	15.41
21.07	8α-methyl-4-methylidene-6-propan-2-ylidene-2,3,4α,5,7,8-hexahydro-1*H*-naphthalene	$C_{15}H_{24}$	204	0	0	0.30	0	0	0
21.69	[1*S*-(1. α.,4. α.,7. α)]-1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1methylethenyl)-azulene	$C_{15}H_{24}$	204	0	0	0	0	0.29	0
21.81	(4*Z*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	$C_{15}H_{24}$	204	0	0	0	0	0	2.19
21.92	methyl (*Z*)-*N*-hydroxybenzenecarboximidate	$C_8H_9NO_2$	151	0	0.31/83*	0	0	0	0
22.54	azulene	$C_{10}H_8$	204	0	0	1.51	0	0	0
22.61	2-phenylethyl acetate	$C_{10}H_{12}O_2$	164	0	0.9/83*	0	0	0	0

Table 3 (continued)

Retention time (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
23.16	1-methyl-4-[(2*E*)-6-methylhepta-2,5-dien-2-yl]cyclohexene	$C_{15}H_{24}$	204	0	0	0.94	0	0	0
23.76	2-methylbutanoic acid	$C_{15}H_{10}O_2$	102	0	0	0	0	1.10	0
24.72	2-phenylethanol	$C_8H_{10}O$	122	0	1.28/97*	0	0	0	0
24.75	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	0	0	1.01	0.44
25.20	1*H*-3α,7-methanoazulene, 2,3,4,7,8,8 α-hexahydro-3,6,8,8 tetramethyl-, [1*R*-(1.α., 4α.α., 8α.α.)]	$C_{15}H_{24}$	204	0	0	3.63	0	0	0
25.30	2-methylpropanoic acid	$C_4H_8O_2$	88	0	0	6.08	0	0	0
25.72	3,7-dimethyl-1,6-octadiene	$C_{10}H_{18}$	138	0	0	0	0	0.05	0
26.04	(1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	$C_{15}H_{24}$	204	0	0	0.48	0	0	0
26.45	phenol	C_6H_6O	94	1.80/95*	0	0	0	0	0
26.91	2-methyl-propanamide	C_4H_9NO	87	0	0	0	0	0.07	0
27.05	2-phenylethyl acetate	$C_{10}H_{12}O_2$	164	0	0	0	0	3.28	1.46
27.27	6-nitro-2-picoline	$C_6H_6N_2O_2$	138	0	0	0	0	0.12	0
27.42	(1*E*,5*E*)-1,4,4-trimethyl-8-methylidenecycloundeca-1,5-diene	$C_{15}H_{24}$	204	0	0	0	3.23	0	0
27.55	naphthalene,1,2,4α,5,6,8α-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,[1*R*-(1.α., 4α.α., 8α.α.)]	$C_{15}H_{24}$	204	0	0	0.34	0	0	0
28.34	(*R*)-11-methylene-3,7,7-trimethylspiro[5.5]undec-2-ene	$C_{15}H_{24}$	204	0	0	0.36	0	0	0
28.50	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	1.07	0	0	0
28.57	(2*R*)-8,8,8α-trimethyl-2-prop-1-en-2-yl-1,2,3,4,6,7-hexahydronaphthalene	$C_{15}H_{24}$	204	0	0	3.24	0	0	0
30.18	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	3.30/95*	0	0	0	0
30.89	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	0	8.58	0	0
30.90	(3*R*,α *S*,α *R*)-α -methyl-5-methylidene-3-prop-1-en-2-yl-1,2,3,4,α ,6,7,8-octahydronaphthalene	$C_{15}H_{24}$	107	0	0	0	7.32	0	0
31.12	2-phenylethyl acetate	$C_{10}H_{12}O_2$	ND	0	0	1.74	0	0	0
31.72	2-(2-methyl-2-propenyl)-2-cyclohexane-1-one	ND	150	0	0	0	0	0.25	0
33.17	2-phenylethanol	$C_8H_{10}O$	122	0	0	1.06	0	0	0

The volatile metabolites produced by *M. heveae* (MH) and its closest phylogenetic species; *M. albus* cz620[1] (MA), *M. cinnamomi* (MC), *M. musae* (MM), and *M. oryzae* (MO) were identified by GC-MS. The results were reported with retention time (RT), possible compound (with IUPAC name), molecular formula, mass to charge ratio (M/z), % total area and % quality*. The data may be obtained from [a] this study or be taken from [b] Strobel (2011), [c] Suwannarach et al. (2010), and [d] Suwannarach et al. (2013a). The *red highlight* refers to the major composition in each profile of the volatile metabolites. Compounds presented in a control PDA plate are not included in this Table. The analysis for *M. heveae* was done in triplicate

used in this study was highly selective for the genus *Muscodor*. This was similar to a number of previous studies that used the same technique for isolation of volatile metabolite-producing endophytic fungi (Suwannarach et al. 2010; 2013a). Moreover, there was no contamination found during such isolations, suggesting that the volatile metabolites produced by either the reference strain and the emerging isolates exhibited strong and broad-spectrum antimicrobial activity. In addition, determining the distribution of volatile metabolite-producing endophytes in different plant tissues of rubber trees would be a further study to understand their spatial and temporal dynamics within the plant host.

In this study, we proposed a novel species, *M. heveae*, that has a phenotypic and phylogenetic relationship with the genus *Muscodor*, but showing a distinct morphology, genotype, and profile of volatile metabolites produced. The phylogenetic analysis based on partial sequences of 18S rDNA, ITS1-5.8-ITS2 rDNA was required for accurate genetic classification. These sequences have been demonstrated to be highly conserved regions of DNA and very useful in the classification of fungi (von der Schulenburg et al. 2001). The sequence data obtained on *Muscodor* indicated that it was a xylariaceous fungus with incomplete molecular identity to other fungi in family *Xylariacea* (Ezra et al. 2004). In addition, a high percent similarity (99-100 %) of ITS1-5.8-ITS2 rDNA sequences among several emerging species of the genus *Muscodor* was often found (Suwannarach et al. 2010; Kudalkar et al. 2011). However, the authors are still confident that their isolates could be proposed as a novel species. The most notable property of *Muscodor* was its ability to produce a mixture of volatile metabolites with antibiotic activity against variety of pathogenic fungi and bacteria (Ezra et al. 2004). Thus, detailed phenotypes of the isolates, volatile gas composition analysis and comparison with their closely related phylogenetic species are priority criteria for assigning their novelty. At the early stage, colony and mycelial characteristics are the only available phenotypes to identify the *Muscodor* species (Zhang et al. 2010). Subsequently, analysis and comparison of volatile metabolites produced by *Muscodor* isolates have been used as an important tool for their classification and novelty determination. Variation in the volatile metabolite profile is commonly found across member species of the genus *Muscodor*, but they often share the same major volatile components (Kudalkar et al. 2011; Suwannarach et al. 2013a). The volatile profiles (Table 3) of four *Muscodor* spp. have been compared with *M. heveae*. All species are prevalent in Thailand except *M. albus* cz620. However, previous reports have shown that there are many isolates of *M. albus* in local host plants in many countries (Ezra et al. 2004; Atmosukarto et al. 2005; Strobel et al. 2007; Banerjee et al. 2010). One such isolate is *M. albus* MFC2, which has been isolated from *Myristica fragrans* in Thailand and produced bulnesene, ledol, (−)-globulol and azulene derivatives as major volatiles (Sopalun et al. 2003). Variable profiles of volatile metabolites may be a result of a diversity factors, including the growing media and environment, and enzyme activity for biosynthesis of such metabolites by the test microbes (Morath et al. 2012). The bioactive volatile components for the genus *Muscodor* are 2-methylpropanoic acid, 3-methylbutyl acetate and 3-methylbutan-1-ol (Strobel 2006; Mitchell et al. 2010), which are in agreement to those detected in *M. heveae*.

In nature, *Muscodor* spp. live within host plants under symbiotic conditions, where they produce and expose their volatile metabolites into the surrounding environments of the host plants for preventing the invasion of pathogens (Mitchell et al. 2010; Zhang et al. 2010). A number of endophytes produce extremely biologically active compounds against pathogenic microbes (Yang et al. 1994). Thus, *Muscodor* spp. seem to be beneficial to the host by providing protection from plant pathogens. Such volatile metabolites are formed as a mixture, and not all volatile metabolites exhibit antimicrobial activity. However, applying the mixture of volatile metabolites for antimicrobial activity has been suggested to be more potent than using them individually (Strobel et al. 2001). The major volatile metabolites in the mixture are not always the bioactive ones, and this is different between either species or taxa. In the genus *Muscodor*, the major volatile antimicrobial metabolite is 2-methylpropanoic acid (Mitchell et al. 2010). This bioactive volatile metabolite has been found to be formed by the yeast *Saccharomyces cerevisiae*, but the most active compounds of its mixture of volatiles were 2-methyl-1-butanol and 3-methylbutan-1-ol (Fialho et al. 2011). Most pathogenic fungi in diverse economic plants were inhibited after exposure to the volatile metabolites produced by *Muscodor* isolates. The inhibitory mechanism on fungal growth indicate it is likely that volatile metabolites influence protein expression and the function of metabolic enzymes (Humphris et al. 2002; Fialho et al. 2011).

Since bioactive volatile metabolites are found in the genus *Muscodor*, they have been considered for uses in agricultural, medical and industrial applications (Strobel 2006). Biological control via volatile metabolite-producing fungi could be a sustainable alternative method to prevent plant diseases at the nursery and postharvest stages, with reduced used of chemical fungicides (Mercier and Manker 2005). Biological fumigation was a strategy for applying the volatile metabolite-producing fungi, which has been approved for controlling diverse postharvest infections caused by *Botrytis cinerea, Penicillium expansum,* and *Sclerotinia sclerotiorum* (Ramin et al. 2005). The applications of biological fumigation using the genus *Muscodor* for controlling root diseases has also been demonstrated (Worapong and Strobel 2009; Suwannarach et al. 2012). Based on the potent antimicrobial activity of the volatile metabolite-producing fungi that we observed, the *Muscodor* isolates could be used effectively for controlling infectious diseases in rubber trees. Further evaluation of the *Muscodor* isolates for their antimicrobial activity in applied biofumigation are required not only for agricultural aspects but also for medical and industrial relevance.

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