Ann Microbiol (2013) 63:1341–1351
DOI 10.1007/s13213-012-0593-6

ORIGINAL ARTICLE

Molecular and morphological evidence support four new species in the genus *Muscodor* from northern Thailand

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Received: 1 August 2012 / Accepted: 13 December 2012 / Published online: 9 January 2013

Abstract The genus *Muscodor* comprises fungal endophytes which produce mixtures of volatile compounds (VOCs) with antimicrobial activities. In the present study, four novel species, *Muscodor musae*, *M. oryzae*, *M. suthepensis* and *M. equiseti* were isolated from *Musa acuminata*, *Oryza rufipogon*, *Cinnamomum bejolghota* and *Equisetum debile*, respectively; these are medicinal plants of northern Thailand. The new *Muscodor* species are distinguished based on morphological and physiological characteristics and on molecular analysis of ITS-rDNA. Volatile compound analysis showed that 2-methylpropanoic acid was the main VOCs produced by *M. musae*, *M. suthepensis* and *M. equiseti*. The mixed volatiles from each fungus showed in vitro antimicrobial activity. *Muscodor suthepensis* had the highest antifungal activity.

Keywords Endophytes · Sterile ascomycete · Taxonomy · Volatile compounds

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Introduction

Endophytes colonize healthy inter- and intracellular living tissue of host plants, typically without causing any visible symptoms of disease (Azevedo et al. 2000; Saikkonen et al. 2004; Hyde and Soytong 2008). Endophytes also protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Azevedo et al. 2000; Gao et al. 2010). The highest plant biodiversity biomes are found in tropical and temperate rainforest regions, and plants in these areas also possess a high endophyte diversity (Strobel 2003). *Muscodor* species are volatile, producing endophytes known from certain tropical tree and vine species in Australia, Central and South America, and Central and Southeast Asia (Worapong et al. 2001, 2002; Daisy et al. 2002; Sopalun et al. 2003; Ezra et al. 2004; Atmosukarto et al. 2005; Strobel et al. 2007; Mitchell et al. 2008; González et al. 2009; Suwannarach et al. 2010; Zhang et al. 2010). The volatile organic compounds (VOCs) produced by *Muscodor* species are active against many plant pathogenic fungi and Gram-positive and Gram-negative bacteria, and the genus is thus potentially important for biocontrol (Strobel et al. 2001; Strobel and Daisy 2003; Macías-Rubalcava et al. 2010).

Eight species of *Muscodor* have been described on the basis of morphological, physiological, biological and genetic features (www.MycoBank.org; Kudalkar et al. 2012). These fungi are classified in the family *Xylariaceae* and have a unique molecular identity as compared to other genera in the family (Ezra et al. 2004; Zhang et al. 2010). Gas chromatography and mass spectrometry (GC/MS) can also be used to identify *Muscodor* species; this is based on the differences in the VOCs that they produce (Strobel et al. 2001).

We are investigating the endophytes of various plants, including medicinal plants in northern Thailand. The purpose of the present paper is to introduce four new species of *Muscodor* isolated from medicinal plants based on their

morphology, antimicrobial activities, volatile chemical composition and phylogeny.

Materials and methods

Study sites

Study sites were Chiang Mai University (18°47′74″N, 98° 57′41″E, altitude 328 m), Queen Sirikit Botanic Garden (18° 53′93″N, 98°51′11″E, altitude 640 m) and Medicinal Plant Garden, Doi Suthep-Pui National Park (18°48′22″N, 98°54′ 51″E, altitude 1,076 m), located in Chiang Mai Province, northern Thailand. Specimens were collected during November 2010 to May 2011.

Fungal isolation

The endophytic fungi were isolated from four plant species, *Cinnamomum bejolghota* and *Musa acuminata* (Medicinal Plant Garden site), *Equisetum debile* (Queen Sirikit Botanic Garden site) and *Oryza rufipogon* (Chiang Mai University site) by previously described methods (Mitchell et al. 2008). The plant samples (leaf and stem) were collected, placed in sterile plastic bags, stored in an icebox and transported to the laboratory within 48 h of sampling. A total of 40 samples were cut into segments (leaf, 5 mm × 5 mm; stem, 5 mm long). All segments were surface-sterilized in 75 % ethanol for 30 s, 2 % sodium hypochlorite for 3 min and 95 % ethanol for 30 s under a laminar flow hood. Potato dextrose agar (PDA) was poured into a half-plate of two-compartment plastic plates (90 mm × 15 mm). The other side of each plate contained ½ strength PDA, 0.05 % streptomycin sulfate and 0.03 % rose bengal. An agar plug (6 mm diam) of actively growing culture of *Muscodor cinnamomi* was placed into the PDA. The plant tissues then were placed onto ½PDA on the other side. The Petri dishes were sealed with Parafilm M and incubated at room temperature for 2 weeks. Fungi growing out from the samples were aseptically transferred to PDA. Pure culture isolates were maintained in PDA slants and tested three times by exposure to *M. cinnamomi* in order to exclude false positive growth in the initial isolation. The fungi were then grown on PDA for 12 days before small squares of colony were cut and placed into vials containing sterile 20 % glycerol, distilled water or mineral oil. The former vials were stored at −20 °C, while the latter two vials were stored at 4 °C, and pure fungi were deposited to the Research Laboratory for Excellence in Sustainable Development of Biological Resources (SDBR), Faculty of Science, Chiang Mai University, Thailand and Japan Collection of Microorganisms (JCM), Japan.

Fungal DNA extraction

Genomic DNA was extracted by a SDS-CTAB method (Suwannarach et al. 2010). *Muscodor* species were subcultured onto PDA and incubated for 10 days. Mycelium was harvested, freeze-dried, and ground into a fine powder with a pestle and mortar. About 15 mg of powdered mycelium was suspended in 1 mL of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris–HCl, pH 7.4, 20 mg/mL proteinase K), transferred into a 1.5-mL Eppendorf tube and kept at 4 °C to prevent endonuclease activity during rehydration of the sample. SDS was added to a final concentration of 2 %, vortexed and incubated for 30 min at 65 °C. After centrifugation for 15 min at 14,000 rpm, the supernatant was transferred to a new sterile 1.5-mL Eppendorf tube. The volume of supernatant was measured and the NaCl concentration was adjusted to 1.4 M, and one-tenth volume of 10 % CTAB buffer (10 % CTAB, 500 mM Tris–HCl, 100 μM EDTA, pH 8.0) was added. The solution was thoroughly mixed and incubated for 10 min at 65 °C. After cooling for 2 min at 15 °C, an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, thoroughly mixed, and the tube was centrifuged for 15 min at 14,000 rpm. The extraction was repeated until the interface was clear. The supernatant was moved to a new Eppendorf tube, containing 2 volumes of cold 100 % ethanol. After DNA precipitation, the pellet was centrifuged for 15 min at 14,000 rpm and 4 °C. The pellet was washed with 70 % ethanol and dried at room temperature. It was resuspended in 100 mL of 0.002 % RNase (5 mg/mL) in TE buffer and incubated for 1 h at 37 °C. The suspension was stored at −20 °C pending use for PCR amplification.

ITS sequencing and phylogenetic analysis

The internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA, were separately amplified in a 25-μL reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems) under these reaction conditions: 1 μL of template DNA extraction, 0.2 μM dNTP, 0.2 μL of FastTaq (Applied Biosystems), 0.2 μM each of primers, 2.5 μL of the supplied PCR buffer with $MgCl_2$, and sterile water to bring the volume to 25 μL. The ITS regions were amplified using ITS4 and ITS5 primers. Amplification of ITS regions was initialed by denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1 % agarose gels in TAE buffer (20 mM Tris-Acetate, 1 mM EDTA, pH 8.0) and viewed by staining with ethidium bromide. PCR products were purified using PCR clean up Gel Extraction NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced.

Sequencing reactions were performed and sequences determined automatically in a genetic analyzer (1st Base, Malaysia) using the PCR primers mentioned above. Sequences obtained in this study were compared to those from GenBank database using the BLAST software on the NCBI website: (http://www.ncbi.nlm.nih.gov/BLAST/). A multiple sequence alignment was carried out using the alignment subroutines in Clustal X (Thompson et al. 1997) and deposited at TreeBASE under study number 12911. The data was analyzed to determine the phylogenetic relationship based on distance and parsimony criteria phylogenetic tree was inferred with PAUP beta 10 software program, v.4.0 (Swofford 2002).

Bioassay tests

A dual culture volatile assay was used to investigate antimicrobial activity against 5 bacteria, 2 yeasts and 14 fungi test microorganisms. One side of a two-compartment plastic plate was loaded with PDA while the other side was loaded with nutrient agar (NA), PDA or yeast extract peptone dextrose agar (YPDA) for bacteria, fungi, and yeast tests, respectively. A 12-day-old *Muscodor* isolate was inoculated on the PDA side of the plate and grown for 3 days at room temperature (25 ±2 °C). Twenty-one strains including 14 plant pathogenic fungi, 2 yeasts, 3 Gram-positive bacteria, and 2 Gram-negative bacteria were used in this experiment (Table 3). An agar plug taken from the margin of 7-day-old fungal pathogen mycelium was placed into the other compartment containing PDA. Bacteria and yeasts were streaked (1.5-cm-long streak) onto either NA or YPDA. All incubated plates were sealed with Parafilm and incubated at room temperature for 6 days. Percentage inhibition of growth of each tested microorganism was then recorded and the viability of the tested microorganisms were investigated by subculture of each microorganism in the tested plate onto fresh medium. The experiment was repeated two times with three replicates.

Qualitative analysis of fungal volatiles

Analyses of volatiles produced by *Muscodor* spp. grown for 1 week at 25±2 °C on PDA were carried out according to the protocols described earlier (Strobel et al. 2001). A baked solid phase micro-extraction (SPME) fiber consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a stable flex fiber was exposed for 45 min to the vapor phase of the headspace of the grown culture. The fiber was inserted into the splitless injection port of a gas chromatograph GC 2010 (Shimadzu, Japan) equipped with a 30 m×0.25 mm I.D. DB-Wax capillary column with a film thickness of 0.25 μm. The column was subjected to a thermal program as follows: 40 °C for 2 min increased to 200 °C at 5 °C/min. Ultra-high purity helium was used as a carrier gas with an initial column head pressure of 60 kPa. The fiber was conditioned at 250 °C for 57 min under a flow of helium gas prior to trapping the volatiles. A 30-s injection time was used to introduce the adsorbed volatiles into the GC. The gas chromatograph was interfaced to a mass spectrometer MS-QP2010 Plus (Shimadzu) mass selective detector mass spectrometer operating at unit resolution. Data acquisition and data processing were performed on the software system. The compounds produced by *Muscodor* spp. were tentatively identified through a library comparison with the NIST database, hence all chemical compounds described in this report use the NIST database chemical terminology and are compared to the type strains (Daisy et al. 2002; González et al. 2009; Kudalkar et al. 2012; Mitchell et al. 2008; Suwannarach et al. 2010; Worapong et al. 2001, 2002; Zhang et al. 2010).

Results

Fungal isolation

Four volatile-producing endophytic fungal isolates, *Muscodor equiseti*, *M. musae*, *M. oryzae* and *M. suthepensis* were isolated from medicinal plants, *Equisetum debile*, *Musa acuminata*, *Oryza rufipogon* and *Cinnamomum bejolghota*, respectively. Two isolates produced white mycelium on PDA, while isolate *M. suthepensis* developed a pale pink-colored mycelium in natural light, and isolate *M. oryzae* produced pale orange mycelium (Table 1). All isolates produced rope-like and coiled hyphae. Only swollen hyphae cells were found in isolate *M. equiseti*. The morphology characteristics of all isolates were different from other *Muscodor* species reported previously (Table 1) and therefore are introduced as new species. In addition, the morphological taxonomy was confirmed by molecular and physiological analysis. The characteristics of the *Muscodor* species are shown in Figs. 1, 2, 3 and 4.

Molecular phylogeny analysis

The ITS sequences data of the four isolates of *Muscodor* were obtained and compared with species downloaded from GenBank. The aligned dataset of 32 sequences consisted of 745 characters, of which 324 characters were constant, 184 variable characters were parsimony uninformative, and 237 characters were parsimony informative. Heuristic searches resulted in six equally parsimonious trees with a length of 904 steps, CI=0.683, RI=0.752, RC=0.531 and HI=0.318. One of the maximum parsimony trees is shown in Fig. 5. *Muscodor* species from GenBank formed a monophyletic clade with a high bootstrap support (83 %) and are closely related to other members within *Xylariaceae*. A phylogenetic dendrogram strongly supported the node separating *M. musae* and *M. suthepensis* from other *Muscodor* species with 96 and 100 % bootstrap support, respectively

Table 1 Biological and culture characteristic comparison of *Muscodor* species

Taxon	Host	Mycelium pigment production	Hyphal growth at colony front	Mycelial growth	Major compounds	Bioactivity
M. albus[a,b]	*Cinnamomum zeylanicum*	White	Straight	Rope-like	2-Methylpropanoic acid; naphthalene and azulene derivatives	Antifungal and antibacterial
M. cinnamomi[c]	*Cinnamomum bejolghota*	White in the dark and pale orange in the light	Straight	Rope-like with cauliflower- like bodies	Ethyl 2-methylpropanoate	Antifungal and antibacterial
M. crispans[d]	*Ananas ananassoides*	White in the dark and pink in the light	Wavy growing	Rope-like with cauliflower-like bodies	2-Methylpropanoic acid	Antifungal and antibacterial
M. equiseti[e]	*Equisetum debile*	White	Cottony-like pattern	Rope-like with coils structure and swollen cell	2-Methylpropanoic acid	Antifungal and antibacterial
M. fengyangensis[f]	*Actinidia chinensis*	White	Straight	Rope-like with coils structure	2-Methylpropanoic acid	Antifungal and antibacterial
M. musae[e]	*Musa acuminata*	White	Straight and hairy-like mycelium	Rope-like with coils structure	2-Methylpropanoic acid	Antifungal and antibacterial
M. oryzae[e]	*Oryza rufipogon*	Pale orange	Straight	Rope-like with coils structure	3-Methylbutan-1-ol	Antifungal and antibacterial
M. roseus[g]	*Grevillea pteridifolia*	Lightly rose	Felt-like mycelium	Forming rope-like stands and coils structure	Ethyl 2-butenoate and 1,2,4-trimethylbenzene	Antifungal
M. suthepensis[e]	*Cinnamomum bejolghota*	White in the dark and pale pink in the light	Straight	Rope-like with coils structure	2-Methylpropanoic acid	Antifungal and antibacterial
M. sutura[h]	*Prestonia trifidi*	White in the light and reddish in the dark	Suture-like pattern	Rope-like nondescript extracellular bodies	2-Methylpropanoic acid	Antifungal
M. vitigenus[i]	*Paullinia paullinioides*	White	Straight	Rope-like	Naphthalene	Anti-insect
M. yucatanensis[j]	*Bursera simaruba*	White	Flocculose pattern	Rope-like with coils structure and swollen cell	1R,4S,7S,11R-2,2,4,8-Tetramethyltricyclo [5.3.1.0(4,11)]undec-8-ene	Phytoinhibitory activity

[a] Worapong et al. (2001), [b] Ezra et al. (2004), [c] Suwannarach et al. (2010), [d] Mitchell et al. (2008), [e] present study, [f] Zhang et al. (2010), [g] Worapong et al. (2002), [h] Kudalkar et al. (2012), [i] Daisy et al. (2002), [j] González et al. (2009)

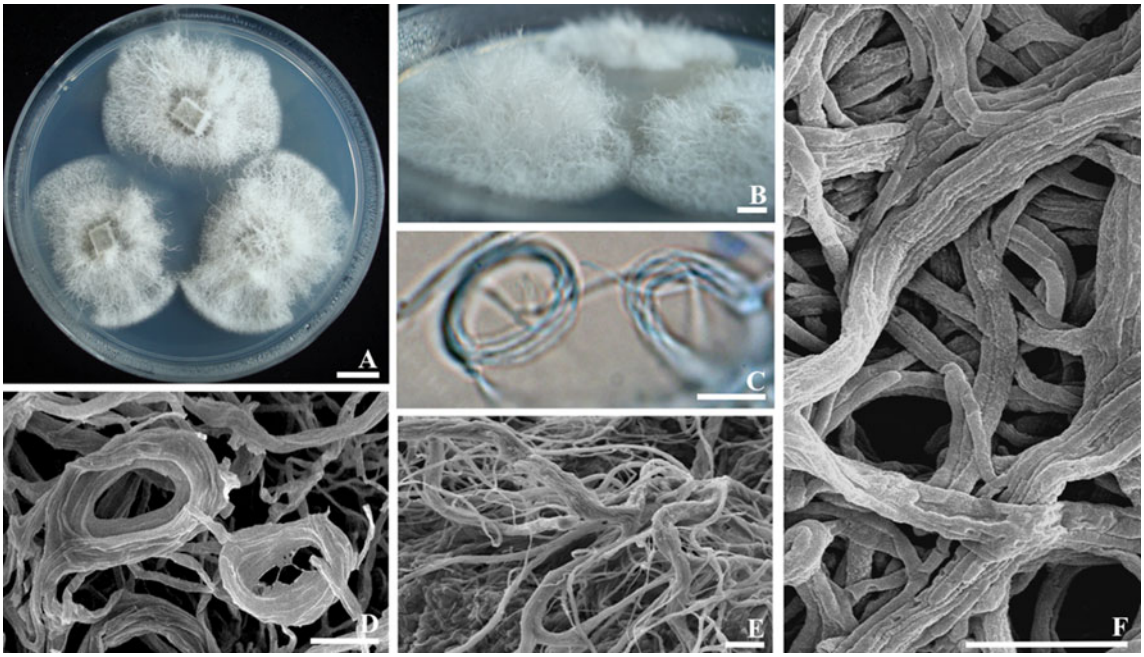

Fig. 1 *Muscodor musae* (CMU-MU3) **a**, **b** culture on PDA, *bars* 1 cm; **c** light microscope micrographs of coiling formation of fungal hyphae, *bar* 10 μm; **d**–**f** scanning electron micrographs, *bars* 10 μm: **d** coiling formation of fungal hyphae; **e**, **f** hyphal cells from the colony edge showing fused, rope-like hyphal cells

(Fig. 5). In addition, *M. equiseti* formed a sister group with 100 % bootstrap support to *M. vitigenus* and *M. sutura*. The remaining novel species, *M. oryzae*, was distant from the other novel species.

Antimicrobial activity

Twenty-one strains including 14 plant pathogenic fungi, 2 yeasts, 3 Gram-positive bacteria and 2 Gram-negative

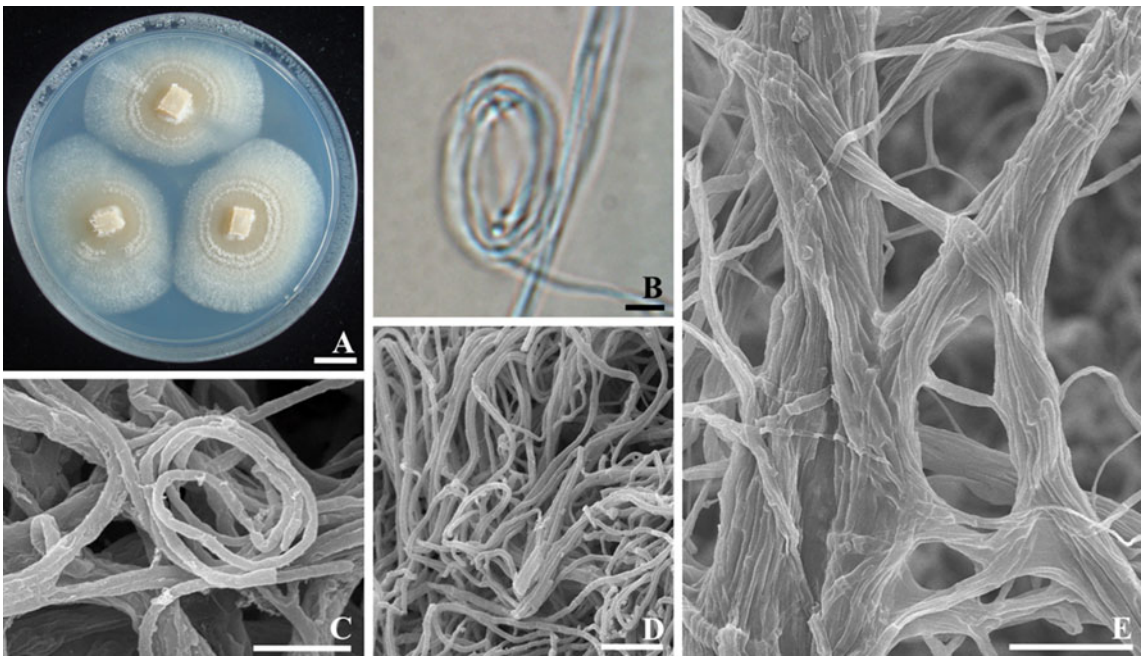

Fig. 2 *Muscodor oryzae* (CMU-WR2) **a** culture on PDA, *bar* 1 cm; **b** light microscope micrographs of coiling formation of fungal hyphae, *bar* 10 μm; **c**–**e** scanning electron micrographs, *bars* 10 μm: **c** coiling formation of fungal hyphae; **d**, **e** hyphal cells from the colony edge showing fused, rope-like hyphal cells

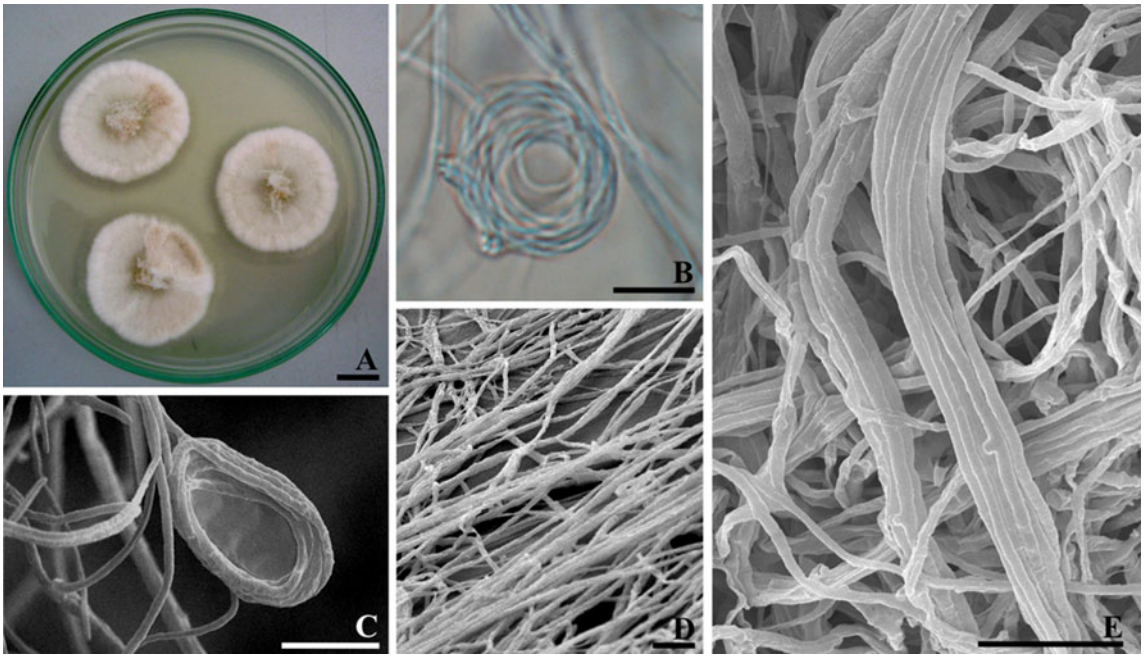

Fig. 3 *Muscodor suthepensis* (CMU-Cib462) **a** culture on PDA, *bar* 1 cm; **b** light microscope micrographs of coiling formation of fungal hyphae, *bar* 10 μm; **c**–**e** scanning electron micrographs, *bars* 10 μm: **c** coiling formation of fungal hyphae; **d**, **e** hyphal cells from the colony edge showing fused, rope-like hyphal cells

bacteria were tested. The growth and viability of the tested microorganisms were recorded after 7 days of exposure to volatile compounds from each novel *Muscodor* species. All new species have inhibitory activity on all tested microorganisms (Table 2). The volatile compounds produced by all *Muscodor* species inhibited the growth (100 %) and caused the death of the tested yeasts and bacteria. Percentage inhibition of tested plant pathogenic fungi ranged from 62.3 ± 2.1 to 100 % (Table 2).

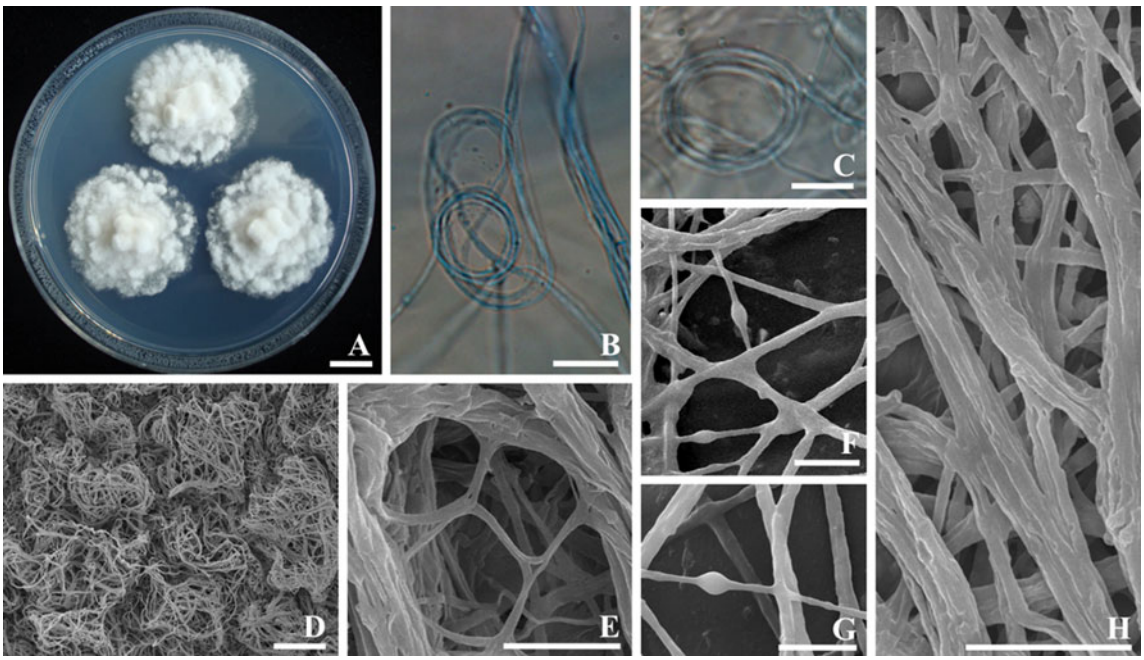

Fig. 4 *Muscodor equiseti* (CMU-M2) **a** culture on PDA, *bar* 1 cm; **b**, **c** light microscope micrographs of coiling formation of fungal hyphae, *bars* 10 μm; **d**–**h** scanning electron micrographs: **d** cottony-like mycelium, *bar* 50 μm; **e** triangular branching pattern; **f**, **g** swollen cells, *bars* 10 μm; **h** hyphal cells from the colony edge showing fused, rope-like hyphal cells, *bar* 10 μm

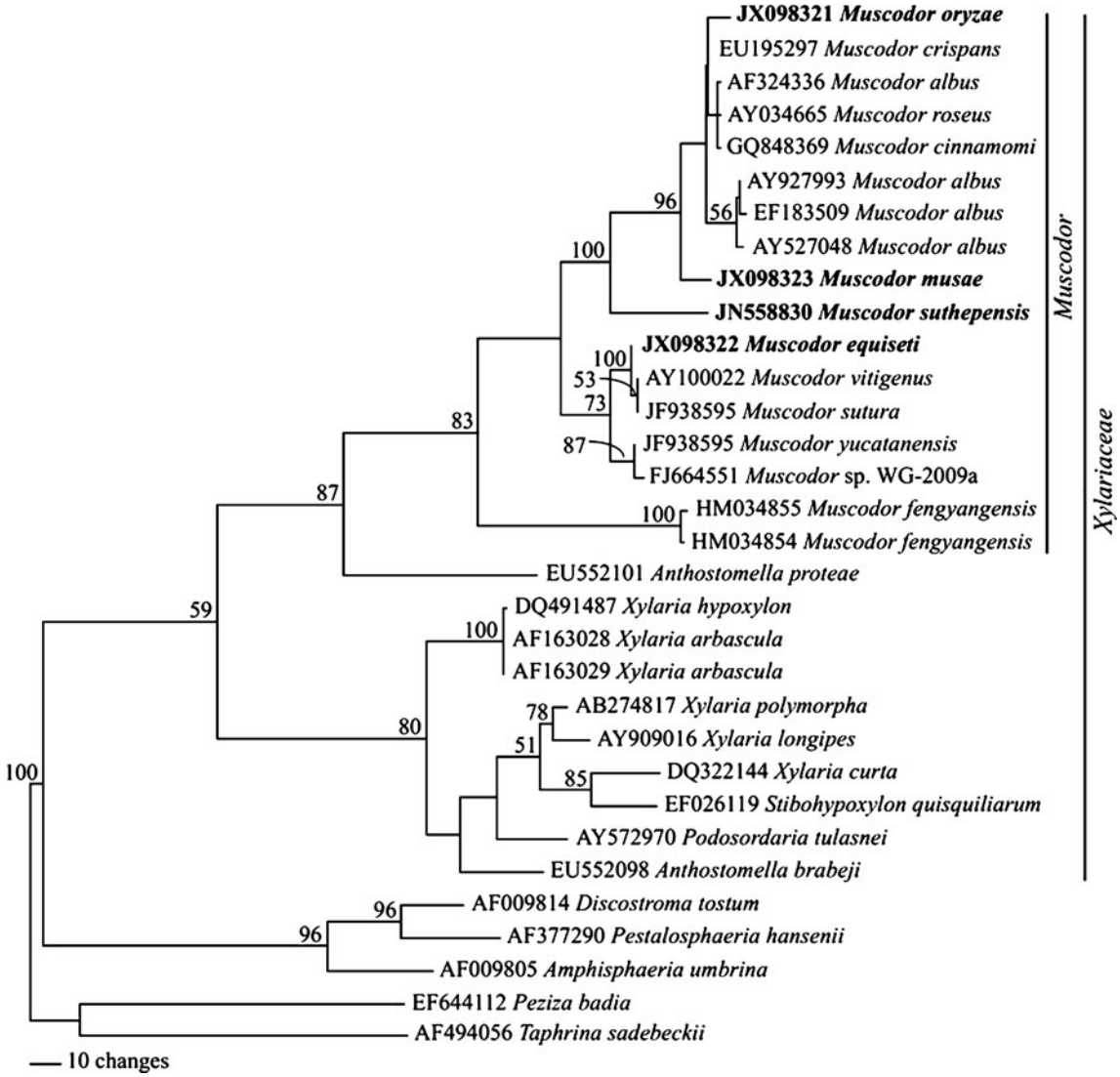

Fig. 5 Maximum parsimonious trees inferred from a heuristic search of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequence alignment of 32 sequences. *Peziza badia* and *Taphrina sadebeckii* were used to root the tree. Branches with bootstrap values ≥50 % are shown at each branch and the *bar* represents 10 substitutions per nucleotide position. New species are in *bold*

Most pathogens were killed by most *Muscodor* species. *Muscodor suthepensis* showed 100 % inhibition and killed all tested plant pathogenic fungi.

Volatile compound analysis

SPME-GC/MS analysis of 1-week-old cultures of the four *Muscodor* species grown on PDA revealed strikingly different patterns of volatile compounds production (Table 3). The four novel *Muscodor* species produced VOCs consisting mainly of esters, alcohols and small molecular weight acids. *Muscodor suthepensis* produced 27 VOCs which was the highest number, followed by *M. musae* (18 VOCs), *M. equiseti* (16 VOCs) and *M. oryzae* (15 VOCs). 2-methylpropanoic acid was the main VOC produced by *M. musae*, *M. suthepensis* and *M. equiseti*, whereas *M. oryzae* produced 3-methylbutan-1-ol as the major VOCs. Most taxa produced azulene derivatives, except for *M. equiseti*. Only naphthalene derivative compounds were detected in *M. suthepensis* cultures.

Taxonomy

Muscodor musae N. Suwannarach & S. Lumyong, sp. nov. Fig. 1

MycoBank No.: MB800811
GenBank No.: JX098323

Table 2 Comparison IUPAC name of the volatile compounds produced by novel *Muscodor* species through SPME-GC/MS analysis

RT (min)	Possible compound	*M/z*	Total area (%)			
			M. musae	*M. oryzae*	*M. suthepensis*	*M. equiseti*
3.42	Ethyl 2-methylpropanoate	116	2.96	0.57	2.03	0.82
4.30	Methyl 2-methylbutanoate	116	3.52	–	1.82	0.62
4.39	2-Methylpropyl acetate	116	4.91	9.75	1.3	0.95
4.54	Methyl 3-methylbutanoate	116	0.18	–	0.39	–
6.72	2-Methylpropyl propanoate	144	–	–	0.20	–
7.07	2-Methylpropan-1-ol	74	–	3.48	0.57	0.17
7.77	3-Methylbutanoyl acetate	130	20.41	4.70	3.31	5.60
10.32	2-Methylbutyl 2-methylpropanoate	158	0.21	–	0.97	0.32
10.88	3-Methylbutan-1-ol	88	13.34	32.69	11.00	7.36
12.10	3-Methyl-3-buten-1-ol	86	–	0.27	–	–
12.20	Ethyl 2-hydroxy-2-methylpropanoate	132	–	0.88	–	–
12.97	3-Hydroxy-2-butanone	88	0.59	2.00	0.58	–
13.78	3-Ethyl-2-methylpentane	114	–	–	0.07	–
14.73	6-Methyl-5-hepten-2-one	126	–	–	0.05	–
14.46	2-Methylpropanoyl chloride	106	–	–	–	0.35
16.18	3-Methylhexane	100	–	–	0.04	0.42
16.19	2,4-Dimethyl-1-heptene	126	–	0.13	–	–
16.26	1-(2-Propenyloxy)-heptane	156	–	–	0.12	–
16.31	1-Isobutoxy-2-ethylhexane	186	–	0.31	–	–
19.43	Isopropyl-4-piperidone	141	0.23	–	–	–
19.50	4,5-Dimethyl-1,3-cyclopentanedione	126	–	0.37	0.26	2.05
21.03	2-Methylpropanoic acid,	88	33.17	15.41	57.81	34.63
21.50	Ethyl 2-hydroxypropanoate	118	–	–	0.03	0.70
21.69	[1S-(1. α.,4. α.,7. α.)]-1,2,3,4,5,6,7,8-Octahydro-1,4-dimethyl-7-(1-methylethenyl)-azulene	204	0.29	–	0.25	–
21.81	Caryophyllene	204	–	2.19	0.12	–
23.76	Butanoic acid, 2-methyl-	102	1.10	–	0.62	1.02
24.55	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a. α.,7. α.,8a. β.)]-	204	–	–	0.32	–
24.75	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1. α.,7. α.,8a. β.)]-	204	1.01	0.44	1.99	–
24.99	Spiro[3.4]octan-5-one	124	–	–	0.36	0.72
26.91	2-Methyl-propanamide	87	0.07	–	0.05	–
25.72	3,7-Dimethyl-1,6-octadiene	138	0.05	–	–	–
27.05	2-Phenylethyl acetate	164	3.28	1.46	1.45	1.15
27.27	6-Nitro-2-picoline	138	0.12	–	–	–
31.37	Pentyl 2-methyl-2-propenoate	156		–	0.07	0.24
31.72	2-(2-Methyl-2-propenyl)-2-cyclohexen-1-one	150	0.25	–	0.86	–

Compounds present in a control PDA have been subtracted from the data. Unknown compounds represent those with a quality % value less than 80

RT retention time; *M/z* mass to charge ratio; – not detected

Diagnosis: Colonies cotton white, form a hairy mycelium pattern on PDA. Hyphae 0.9–3.4 μm thick, coils 24–28.9 × 26–27.4 μm diam and produced a musty odor.

Etymology: *musae*, refers to the name of the host plant, *Musa acuminata*.

Holotype: THAILAND, Chiang Mai Province, Doi Suthep-Pui, Medicinal Plant Garden, from a leaf of *Musa acuminata* (*Musaceae*), May 2011, Nakarin Suwannarach, dried culture (SDBR CMU-MU3), ex-type living culture JCM 18230.

Teleomorph: Unknown

Table 3 Effects of volatile compounds from the novel *Muscodor* species on the test microorganisms after 7 days exposure

Test microorganisms	Growth after exposure to *Muscodor* (% inhibition)				Viability after exposure to *Muscodor* culture			
	M. musae	*M. oryzae*	*M. suthepensis*	*M. equiseti*	*M. musae*	*M. oryzae*	*M. suthepensis*	*M. equiseti*
Alternaria porri	100	100	100	100	Dead	Dead	Dead	Dead
Alternaria solani	100	100	100	100	Dead	Dead	Dead	Dead
Aspergillus flavus	66.1±1.9	100	100	63.0±1.3	Alive	Dead	Dead	Alive
Botrytis cinerea	68.7±1.2	100	100	100	Alive	Dead	Dead	Dead
Colletotrichum capsici	100	100	100	100	Dead	Dead	Dead	Dead
Colletotrichum gloeosporioides	100	100	100	100	Dead	Dead	Dead	Dead
Colletotrichum musae	100	100	100	100	Dead	Dead	Dead	Dead
Fusarium oxysporum	76.8±2.8	81.3±2.4	100	100	Alive	Alive	Dead	Dead
Fusarium solani	81.2±1.6	86.7±1.9	100	100	Alive	Alive	Dead	Dead
Nigrospora oryzae	100	100	100	100	Dead	Dead	Dead	Dead
Penicillium digitatum	100	96.4±0.9	100	100	Dead	Alive	Dead	Dead
Penicillium expansum	62.3±2.1	87.3±2.1	100	73.2±1.5	Alive	Alive	Dead	Alive
Rhizoctonia solani	100	100	100	100	Dead	Dead	Dead	Dead
Sclerotium rolfsii	100	100	100	100	Dead	Dead	Dead	Dead
Candida albicans	100	100	100	100	Dead	Dead	Dead	Dead
Cryptococcus neoformans	100	100	100	100	Dead	Dead	Dead	Dead
Escherichia coli	100	100	100	100	Dead	Dead	Dead	Dead
Enterococcus faecalis	100	100	100	100	Dead	Dead	Dead	Dead
Proteus mirabilis	100	100	100	100	Dead	Dead	Dead	Dead
Staphylococcus aureus	100	100	100	100	Dead	Dead	Dead	Dead
Streptococcus pneumoniae	100	100	100	100	Dead	Dead	Dead	Dead

Tests were repeated two times with three replicates and means ± SD were calculated

Description: In nature, the fungus is associated with *Musa acuminata* and is an ascomycete with sterile mycelium. Fungal colonies are cotton white and form a hairy mycelium pattern on PDA when grown in darkness and natural light (Fig. 1a, b). Hyphae (0.9–3.4 μm thick) with coils (24–28.9 × 26–27.4 μm diam.; Fig. 1c, d), commonly appearing as fused rope-like hyphal strands and branching (5.3–16.1 μm thick; Fig. 1e, f). Mycelium on PDA has a growth rate of 3.50±0.35 mm day^{-1}, producing a musty odor. Spores and other fruiting bodies did not develop under any conditions tested.

Muscodor oryzae N. Suwannarach & S. Lumyong, sp. nov. Fig. 2

MycoBank No.: MB800812

GenBank No.: JX098321

Diagnosis: Colonies pale orange on PDA. Hyphae 1.1–3.9 μm thick, coils 19.6–39.4 × 18.1–36.7 μm diam and produced a fruity odor.

Etymology: *oryzae*, refers to the name of the host plant, *Oryza rufipogon*.

Holotype: THAILAND, Chiang Mai Province, Chiang Mai, Chiang Mai University, from a leaf of *Oryza rufipogon* (*Poaceae*), May 2011, Nakarin Suwannarach and Jaturong Kumla, dried culture (SDBR CMU-WR2), ex-type living culture JCM 18231.

Teleomorph: Unknown

Description: In nature, the fungus is associated with *Oryza rufipogon* and is an ascomycete with sterile mycelium. Fungal colonies pale orange on PDA when grown in darkness and natural light (Fig. 2a). Hyphae (1.1–3.9 μm thick) with coils (19.6–39.4 × 18.1–36.7 μm diam.; Fig. 2b, c); commonly appearing as fused rope-like hyphal strands and branching (6.3–20.9 μm thick; Fig. 2d, e). Mycelium on PDA has a growth rate of 3.65±0.30 mm day^{-1}, producing a fruity odor. Spores and other fruiting bodies did not develop under any conditions tested.

Muscodor suthepensis N. Suwannarach & S. Lumyong, sp. nov. Fig. 3

MycoBank No.: MB800813

GenBank No.: JN558830

Diagnosis: Colonies pale pink in natural light and white in dark on PDA. Hyphae 1–4.6 μm thick, coils 16.1–31.8 × 13.4–29.5 μm diam and produced a fruity odor.

Etymology: *suthepensis*, refers to the collection site, Doi Suthep-Pui National Park.

Holotype: THAILAND, Chiang Mai Province, Doi Suthep-Pui, Medicinal Plant Garden, from a stem of *Cinnamomum bejolghota* (*Lauraceae*), November 2010, Nakarin Suwannarach, dried culture (SDBR CMU-Cib462), ex-type living culture JCM 18232.

Teleomorph: Unknown

Description: In nature, the fungus is associated with *Cinnamomum bejolghota* and is an ascomycete with sterile mycelium. Fungal colonies pale pink on PDA when grown in natural light (Fig. 3a), and white mycelium when grown in dark condition. Hyphae (1–4.6 μm thick) have coils (16.1–31.8 × 13.4–29.5 μm diam.; Fig. 3b, c); commonly appearing as fused rope-like strands and branching (8.2–35.6 μm thick; Fig. 3d, e). Mycelium on PDA has a growth rate of 3.00±0.61 mm day^{-1}, producing a fruity odor. Spores and other fruiting bodies did not develop under any conditions tested.

Muscodor equiseti N. Suwannarach & S. Lumyong, sp. nov. Fig. 4

MycoBank No.: MB800814

GenBank No.: JX098322

Diagnosis: Colonies cottony pattern, white on PDA. Hyphae 0.9–2.9 μm thick, coils 16.5–29.4 × 19.1–26.9 μm diam, tri-angle branching, subglobose swollen cells (1–4.2 × 1.8–6.7 μm and produced a fruity odor.

Etymology: *equiseti*, refers to the name of the host plant, *Equisetum debile*.

Holotype: THAILAND, Chiang Mai Province, Queen Sirikit Botanic Garden, from a stem of *Equisetum debile* (*Equsetaceae*), May 2011, Nakarin Suwannarach, dried culture (SDBR CMU-M2), ex-type living culture JCM 18233.

Teleomorph: Unknown

Description: In nature, the fungus is associated with *Equisetum debile* and is an ascomycete with sterile mycelium. Fungal colonies are cotton white on PDA when grown in darkness (Fig. 4a). Hyphae (0.9–2.9 μm thick) with coils (16.5–29.4 × 19.1–26.9 μm diam.; Fig. 4b, c) and a cottony pattern (Fig. 4d); tri-angle branching (Fig. 4e); subglobose swollen cells (1–4.2 × 1.8–6.7 μm; Fig. 4f, g) commonly appearing as fused rope-like strands and branching (5.3–17.1 μm thick; Fig. 4h). Mycelium on PDA has a growth rate of 2.82±0.06 mm day^{-1}, producing a fruity odor. Spores and other fruiting bodies did not develop under any conditions tested.

Discussion

Muscodor is essentially a genus of endophytic volatile-producing tropical fungi. Its diversity, host range and habitats are being expanded (Atmosukarto et al. 2005; Strobel et al. 2007). The morphological identification of *Muscodor* species is difficult because the genus does not produce any reproductive structrues on any substrate or medium tested. Colony and mycelial characters are the only available morphological traits (Strobel et al. 2001; Zhang et al. 2010). Consequently, the physiological characteristics such as VOC production, biological activities and molecular analysis are necessary tools for characterization. We have described four novel *Muscodor* species, based on differences in colony and hyphal morphology, VOC production and ITS sequence data. In general, *Muscodor* species produce white mycelium (Worapong et al. 2001; Daisy et al. 2002; Ezra et al. 2004; Mitchell et al. 2008; González et al. 2009; Suwannarach et al. 2010; Zhang et al. 2010; Kudorka et al. 2012), However, *M. roseus* produces a light rose-colored mycelium (Worapong et al. 2002). *Muscodor oryzae* produced a pale orange mycelial pigment similar to *M. cinnamomi*, but *M. cinnamomi* produces pigment only under natural light conditions (Suwannarach et al. 2010). *Muscodor suthepensis* produced pale pink pigment in natural light similar to *M. crispans*, but *M. crispans* produced wavy growing hypha and cauliflower-like bodies (Mitchell et al. 2008). In addition, hyphal growth of *M. equiseti* on agar had a cottony-like pattern, while *M. musae* produced hairy-like mycelium in contrast to other species of *Muscodor* (Table 1). The ITS sequence analysis of the rDNA of the four novel species showed that they are clearly separated from the other *Muscodor* species (Fig. 5). A phylogenetic dendrogram of the *Muscodor* group is in concordance with previous studies that show *Muscodor* as a member of the *Xylariaceae*, but in a district phylogenetic clade (González et al. 2009; Suwannarach et al. 2010; Zhang et al. 2010).

GC/MS of *Muscodor* species consistently found alcohols, esters and small molecular weight acid in the gas phase, when grown on PDA. Both major and minor VOCs produced by different *Muscodor* species are distinguishable (Table 1). The most abundant VOC produced by *M. suthepensis*, *M. equiseti* and *M. musae* was 2-methylpropanoic acid which is similar to *M. albus*, *M. crispans*, *M. sutura* and *M. fengyangensis* (Worapong et al. 2001; Ezra et al. 2004; Mitchell et al. 2008; Zhang et al. 2010; Kudorka et al. 2012). However, in the present study *M. oryzae* produced 3-methylbutan-1-ol as a major volatile compound. *Muscodor suthepensis* produced both azulene and naphthalene derivatives similar to *M. albus*, *M. roseus*, *M. vitigenus* and *M. fengyangensis* (Worapong et al. 2001, 2002; Daisy et al. 2002; Ezra et al. 2004; Zhang et al. 2010). In addition, *M. musae* and *M. oryzae* produced only azulene derivatives similar to *M. cinnamomi* (Suwannarach et al. 2010). Neither

azulene nor naphthalene derivatives were found in *M. equiseti* culture which is similar to *M. crispans* and *M. yucatanensis* (Mitchell et al. 2008; González et al. 2009). The biological activity of mixed volatile compounds produced by the novel species showed antifungal and antibacterial activity (Tables 1 and 2). This result agrees with previous studies that mixed VOCs produced by *Muscodor* spp. were inhibitory to growth and killed tested bacteria and fungi (Worapong et al. 2001; Ezra et al. 2004; Mitchell et al. 2008; Suwannarach et al. 2010; Zhang et al. 2010). *Muscodor roseus* and *M. sutura* showed only antifungal activities (Kudalkar et al. 2012; Worapong et al. 2002). Only *M. yucatanensis* has been reported to have phytoinhibitory activity (González et al. 2009). Moreover, Worapong et al. (2002) reported that *M. vitigenus* has anti-insect activity. In addition, previous studies reported that 8 VOCs: aciphyllene, 3-methylbutanoyl acetate, 2-butanone, 2-methylbutan-1-ol, ethyl butyrate, 2-methyl furan, isobutyric acid, and tetrahydofuran from *M. albus* showed antibiotic activity (Atmosukarto et al. 2005; Ramin et al. 2005; Mercier et al. 2007). Each VOC produced by the four novel *Muscodor* species but not produced by *M. albus* will be further investigated using biological tests. Further studies are necessary to determine their potential for biological control, food storage, agricultural production, and industrial applications.

Acknowledgments This work was supported by The Higher Education Commission, Thailand, under the National Research University (A1) program, Chiang Mai University, and TRF Research-Team promotion Grant RTA5580007. We are grateful to Keegan Kennedy for improving the English text.

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