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Article
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Confirming the phylogenetic position of the genus *Muscodor* and the description of a new *Muscodor* species

Chen JJ[1#], Feng XX[2#], Xia CY[1], Kong DD[2], Qi ZY[2], Liu F[3], Chen D[3], Lin FC[1], Zhang CL[1*]

[1] *State Key Laboratory for Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou, Zhejiang, China*
[2] *Agricultural Experiment Station, Zhejiang University, Hangzhou, Zhejiang, China*
[3] *Naban River Watershed National Nature Reserve, Jinghong, Yunnan, China*

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Abstract

It has been suggested that the genus *Muscodor* should be rejected, while many new species recently introduced are based on chemical profiles of volatile organic compounds and insufficient phylogenetic analyses. The ITS rRNA gene was used for identification of *Muscodor* species, but has limitations. A four-locus (ITS rRNA, 28S rRNA, RPB2 and TUB1 gene) combined phylogenetic tree has been reconstructed in the current study to confirm that the genus *Muscodor* is phylogenetically distinct from other closely related genera. A new *Muscodor* species named *Muscodor yunnanensis* is described based on phylogenetic analyses and culture characteristics.

Key words – 1 new species – *Muscodor* – phylogenetic position – new species – four-loci

Introduction

Muscodor is a genus introduced by the discovery of *Muscodor albus* isolated from branches of *Cinnamomum zeylanicum* from Honduras (Worapong et al. 2001), which comprises endophytes placed in Xylariales genera *incertae sedis* (Xylariales, Sordariomycetes, Ascomycota) (Maharachchikumbura et al. 2016, Daranagama et al. 2018) or Sordariomycetes genera *incertae sedis* (Wijayawardene et al. 2017, 2018). The genus is known for producing volatile organic compounds (VOCs). VOCs produced by *Muscodor* species can induce lethal effects against a broad range of plant and human fungal pathogens, nematodes and insects (Strobel 2006, Mercier & Tamezguerra 2007, Kudalkar et al. 2012) and is therefore considered to be a promising agent of myco-fumigation in agriculture and industry (Mitchell et al. 2001, Atmosukarto et al. 2005, Strobel 2006, Suwannarach et al. 2016).

To date, 21 species (Zhang et al. 2010, Meshram et al. 2015, Siri-udom et al. 2016, Roskov et al. 2018) have been recorded in this genus based on culture characteristics, chemical profiles of VOCs and molecular phylogenetic analyses. However, the identification of *Muscodor* remains ambiguous and the position of this genus remains controversial. There are several reasons for this: 1) *Muscodor* species are completely sterile as reproductive structures have never been observed in any medium, and these characters are not available for identification. Thus, characterization is only based on colonies and mycelial characteristics, even though some species may exhibit a particular

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#These authors contributed equally to this work
Corresponding Author: Zhang Chulong – e-mail – clzhang@zju.edu.cn

hyphal arrangement (Mitchell et al. 2001, Zhang et al. 2010), 2) the profile of unique volatile metabolites produced by *Muscodor* species analysed through gas chromatography coupled to mass spectrometry (GC–MS) are used as criteria for classification (Strobel 2006, MaríAc et al. 2009), while no other taxa in Xylariales have been compared based on volatile profiles as with *Muscodor* species (Stadler et al. 2013), 3) for most *Muscodor* species, ITS rRNA gene sequences are the only molecular phylogenetic data available and the limitations for reconstruction of the phylogenetic relationships within the Xylariales have been discussed (Tang et al. 2009, Stadler et al. 2013).

Since one genus can only have one name (Hawksworth 2011, Gams et al. 2012a, b, Hawksworth 2012), the new nomenclature calls for abandoning some ill-defined asexual morph genera including *Muscodor*. Stadler et al. (2013) doubted the definition of *Muscodor*, but did not advise to integrate the younger asexual morph genus *Muscodor* into any of the older genera. Wendt et al. (2018) even suggested to reject the genus for not following good taxonomic standards as presented in Stadler et al. (2013). Therefore, in the current study, we attempt to clarify the classification status of *Muscodor* though further identified materials and a more appropriate multi-gene genealogy. During our investigation of the endophytic fungi in some gramineous plants in the southwest of China, we found nine strains belonging to the genus *Muscodor* and one, numbered W–S–38, is described as a novel *Muscodor* species, named *Muscodor yunnanensis* according to multi-loci phylogenetic evidence. These nine strains, along with 13 strains including *Muscodor fengyangensis* (Zhang et al. 2010) were analysed by single ITS rRNA gene, RPB2 gene and four-loci (ITS rRNA, 28S rRNA, RPB2 and TUB1 genes) combined phylogenies, which demonstrate the phylogenetic position of *Muscodor*.

Materials & Methods

Isolation of fungi

The grasses were sampled during August 2015 from the Naban River Watershed National Natural Reserve (E 100°32'–100°44', N 22°04'–22°17') in Yunnan Province of China, which is one of biodiversity hot spots of the world (Shen et al. 2017). The samples were packed into a box with ice. Within 48 hours after sampling, healthy roots, stems and leaves were rinsed first with tap water, then sterilized with 75% ethanol for 5 minutes, followed by immersion into 1% sodium hypochlorite (NaOCl) for 10 minutes and finally rinsed again three times with sterile-distilled water. The plant tissues were cut into about 5 mm long segments and every ten segments of each part were placed on a plate of potato dextrose agar (PDA) supplemented with 50 mg/L^{-1} chloramphenicol to prevent bacterial growth. The plates were then incubated at 25 °C in darkness and observed every 12 hours and arising fungal cultures transferred to fresh PDA plates for sub-culturing.

Morphology

The cultures were incubated on PDA at 25 °C in darkness for growth rates measurements every day and culture characteristics observations.

Scanning electron microscopy (SEM) analysis

For SEM analysis, the fungal strain was incubated on PDA at 25 °C in darkness for 7 days. The peripheral front of the radial culture was then carefully removed with a scalpel. SEM was performed using HITACHI SU8000 microscope.

Fungal DNA extraction, PCR and sequencing

Seven days old colonies grown on PDA were used for genomic DNA extraction following the protocol of Cubero et al. (1999). ITS rRNA gene was sequenced for nine strains isolated in current study and three loci including 28S rRNA, RPB2 and TUB1 genes were sequenced for 5 selected strains as ITS rRNA gene sequences of strains Y–L–54, W–T–27 were same as strains W–S–41, Y–S–35 respectively and ITS rRNA gene sequences of strains W–S–35, Y–L–43 were same as strain W–S–38. The primers and annealing temperatures used for amplification and sequencing were in

summarised in Table 1. The PCR products were sequenced in an ABI 3730 sequencer using the same primers as PCR.

Table 1 Primers and annealing temperatures used for amplification and sequencing.

Locus	Annealing temp (°C)	Cycles	Primer and Primer sequence (5' – 3')	Reference
ITS	52	35	ITS1 TCCGTAGGTGAACCTGCGG ITS4 TC CTCCGCTTATTGATATGC	White et al. 1990
28S	48	35	LR0R ACCCGCTGAACTTAAGC LR5 TCCTGAGGGAAACTTCG	White et al. 1990
RPB2	48	35	RPB2-5f GAYGAYMGWGATCAYTTYGG RPB2-7cr CCCATRGCTTGYTTRCCCAT	Liu et al. 1999
TUB1	48	35	bena-T1 AACATGCGTGAGATTGTAAGT bena-T22 TCTGGATGTTGTTGGGAATCC	O'Donnell et al. 1997

Sequence alignment and phylogenetic analyses

The sequences were subjected BLAST analysis at NCBI and aligned with reference sequences of *Muscodor* strains using CLUSTAL X 2.1 (Larkin et al. 2007). Isolates used in this study and GenBank accessions are listed in Table 2. The alignment was manually modified in GENEDOC (Nicholas & Nicholas 1997) to remove extra 5' and 3' sequences from where the sequences were overlapped. DAMBE5 (Xia 2013) was used to perform the substitution saturation test. Phylogenetic analyses were conducted by maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) analyses using PAUP v.4.0a152 (Swofford 2002), MrBayes v.3.2.6 (Ronquist et al. 2012) and IQ-TREE v.1.6.3 (Nguyen et al. 2015), respectively. MP analysis was performed with heuristic search algorithm (1000 random sequence stepwise additions) with a tree-bisection-reconnection (TBR) branch swapping. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. The branch support values of MP analysis were calculated using a bootstrapping method with 1000 replicates. BI analysis was using a Markov Chain Monte Carlo (MCMC) algorithm. The best-fit nucleotide substitution model of BI and ML analyses was specified by jModelTest 2.1.7 (Darriba et al. 2012) under default settings followed by Akaike information criterion (AIC). Trees were sampled every 100 generations from 5,000,000 generations resulting in total 50,000 trees. The first 12,500 trees (first 25 % samples by default in the software) were discarded representing the burn-in phase and the remaining 37,500 trees were for calculating posterior probabilities (PP) values in the majority rule consensus tree. In ML analysis, we obtained branch supports with the ultrafast bootstrap (Hoang et al. 2017) implemented in the IQ-TREE. The p–distance was calculated by MEGA version 6 (Tamura et al. 2013) with substitutions: Transitions + Transversions.

Table 2 Strains used in this study and GenBank accessions.

Strain	ITS	28S	RPB2	Tub1
Annulohypoxylon moriforme var. *microdiscum* JF TH–28–01	DQ631935	DQ840061	DQ631960	DQ840095
Annulohypoxylon nitens ST2313/YMJ 91022108	DQ223751	DQ840063	–	AY951663
Annulohypoxylon stygium CM AT–010	AJ390409	DQ840064	DQ631962	–
Anthostomella brabeji	EU552098	–	–	–
Anthostomella eucalyptorum	DQ890026	–	–	–
Anthostomella proteae	EU552101	–	–	–
Astrocystis cocoes	AY862571	–	–	–
Bartalinia robillardoides (outgroup)	AF405301	AF382366	DQ368653	–
Biscogniauxia capnodes CM AT–015/YMJ 142	DQ631933	DQ840055	–	AY951674

Table 2 Continued.

Strain	ITS	28S	RPB2	Tub1
Biscogniauxia mediterranea CBS 280.61	AJ390413	–	–	AY951684
Biscogniauxia simplicior B73C/YMJ 136	AJ390416	–	–	AY951686
Camillea tinctor C83C	AJ390421	–	–	–
Camillea tinctor C84M	AJ390422	–	–	–
Collodiscula japonica	JF440974	–	–	–
Creosphaeria sassafras CM AT–018	DQ631934	DQ840056	DQ631964	–
Creosphaeria sassafras Cr90M	AJ390424	–	–	–
Creosphaeria sassafras Cr91M	AJ390425	–	–	–
Daldinia concentrica M-0066225	AY616681	–	DQ368651	–
Daldinia loculata HJ108	AF176959	–	–	AY951698
Daldinia petriniae H Knudsen s.n.	AF176970	–	–	AY951699
Entoleuca mammata ATCC 58108	AF201713	–	–	–
Entoleuca mammata E.MAMM1	AJ246232	–	–	–
Entoleuca mammata E.MAMM3	AJ246235	–	–	–
Fungal endophyte isolate 1128	EU686807	–	–	–
Fungal endophyte isolate 1138	EU686808	–	–	–
Fungal endophyte isolate 1155	EU686810	–	–	–
Fungal endophyte isolate 1157	EU686811	–	–	–
Fungal endophyte isolate 1730	EU686946	–	–	–
Fungal endophyte isolate 1744	EU686949	–	–	–
Fungal endophyte isolate 2067	EU687018	–	–	–
Fungal endophyte isolate 2161	EU687035	–	–	–
Fungal endophyte sp. ECD–2008	EU686114	–	–	–
Fungal endophyte sp. P1907B	EU977281	–	–	–
Fungal endophyte sp. P913A	EU977208	–	–	–
Fungal sp. R15	AY699660	–	–	–
Fungal sp. ZH S13–1–2	GQ220337	HM034864	GQ241929	HM034845
Hypoxylon fragiforme agrD459/HKUCC 1022	AY616690	AY083829	-	–
Hypoxylon monticulosum GZ AT–M050	DQ631936	DQ840067	DQ631955	–
Hypoxylon monticulosum HK AT–PTC015	DQ631939	–	DQ631950	DQ840096
Kretzschmaria clavus CBS 826.72	AJ390435	–	–	–
Kretzschmaria clavus JP 3113	AJ390434	–	–	–
Kretzschmaria clavus K171C	AJ390437	–	–	–
Muscodor albus	AF324336	–	–	–
Muscodor albus 141–3s	AY927993	–	–	–
Muscodor albus 9–6	HM034857	HM034865	KC243321	HM034844
Muscodor albus aa3	JN426991	–	–	–
Muscodor albus GP100	AY555731	–	–	–
Muscodor albus GP115	AY527048	–	–	–
Muscodor albus GP206	AY527047	–	–	–
Muscodor albus isolate E–6	EF183509	–	–	–
Muscodor albus KN26	AY527044	–	–	–
Muscodor albus KN27	AY527046	–	–	–
Muscodor albus MFC2	AY244622	–	–	–
Muscodor albus MOW12	JX469138	–	–	–
Muscodor albus TP21	AY527045	–	–	–
Muscodor camphorae 1639CCSTITD	KC481681	–	–	–

Table 2 Continued.

Strain	ITS	28S	RPB2	Tub1
Muscodor cinnanomi strain CMU–Cib 461	GQ848369	–	–	–
Muscodor coffeanum isolate CDA743	KP862879	–	KP862880	–
Muscodor coffeanum strain CDA739	KM514680	–	–	–
Muscodor coffeanum strain CDA741	KM514681	–	–	–
Muscodor crispans isolate B–23	EU195297	–	–	–
Muscodor darjeelingensis 1CCSTITD	JQ409997	–	–	–
Muscodor equiseti strain CMU–M2	JX089322	–	–	–
Muscodor fengyangensis ZJLQ023	HM034856	HM034859	HM034849	HM034843
Muscodor fengyangensis ZJLQ024	HM034855	HM034861	HM034851	HM034842
Muscodor fengyangensis ZJLQ070	HM034853	HM034858	HM034847	HM034841
Muscodor fengyangensis ZJLQ151	HM034852	HM034860	HM034848	HM034839
Muscodor fengyangensis ZJLQ374	HM034854	HM034862	HM034850	HM034840
Muscodor ghoomensis 6CCSTITD	KF537625	–	–	–
Muscodor heveae RTM5–IV3	KF850712	–	–	–
Muscodor indicus 6(b)CCSTITD	KF537626	–	–	–
Muscodor kashayum 16AMLWLS	KC481680	–	–	–
Muscodor musae strain CMU–MU3	JX089323	–	–	–
Muscodor musae strain ORE8	KR011970	–	–	–
Muscodor oryzae strain CMU–WR2	JX089321	–	–	–
Muscodor roseus A3–5	AH010859	–	–	–
Muscodor sp. 16AMLWLS	KC481680	–	–	–
Muscodor sp. 1CCSTITD	JQ409997	–	–	–
Muscodor sp. 2CCSTITD	JQ409998	–	–	–
Muscodor sp. 3_4_3	KF269183	–	–	–
Muscodor sp. A3–5	AY034665	–	–	–
Muscodor sp. AB–2011	JN426991	–	–	–
Muscodor sp. CA–01	KF229758	–	–	–
Muscodor sp. CMU20	GQ924909	–	–	–
Muscodor sp. D31	EF564148	–	–	–
Muscodor sp. E6710b	HM999898	–	–	–
Muscodor sp. E8514i	HQ117854	–	–	–
Muscodor sp. EXH1_22	KF227856	–	–	–
Muscodor sp. Fun50W1	KF496182	–	–	–
Muscodor sp. Fun56W3	KF496186	–	–	–
Muscodor sp. GBA	GU797134	–	–	–
Muscodor sp. GS3_3_4	KF128788	–	–	–
Muscodor sp. GS5_3_5	KF269187	–	–	–
Muscodor sp. GSH5_3_5	KF128812	–	–	–
Muscodor sp. GSM5_5_7	KF128852	–	–	–
Muscodor sp. M112	HM595541	MG866043	HM595625	MG866071
Muscodor sp. M153	MG866055	MG866044	MG866062	MG866072
Muscodor sp. M21	HM595540	MG866041	HM595624	MG866069
Muscodor sp. M211	MG866056	MG866045	MG866063	MG866073
Muscodor sp. M25	HM595539	MG866042	HM595623	MG866070
Muscodor sp. M7	EF564149	–	–	–
Muscodor sp. N128	KP689118	–	–	–
Muscodor sp. N190	KP689119	–	–	–

Table 2 Continued.

Strain	ITS	28S	RPB2	Tub1
Muscodor sp. N28	EF564150	–	–	–
Muscodor sp. OM–01	KF229762	–	–	–
Muscodor sp. RTM5–IV1	KF850710	–	–	–
Muscodor sp. RTM5–IV2	KF850711	–	–	–
Muscodor sp. RTM5–IV4	KF850713	–	–	–
Muscodor sp. S18–3v1	EU636700	HM034863	FJ480346	HM034846
Muscodor sp. SR–2011	JF938595	–	–	–
Muscodor sp. UBSX	KJ425599	–	–	–
Muscodor sp. UFMGCB 4666	JN031052	–	–	–
Muscodor sp. VC–01	KF229754	–	–	–
Muscodor sp. WG–2009a	FJ664551	–	–	–
Muscodor strobelii 6610CMSTITBRT	JQ409999	–	–	–
Muscodor suthepensis strain CMU462	JN558830	–	–	–
Muscodor sutura SR–2011	JF938595	–	–	–
Muscodor tigerii 2CCSTITD	JQ409998	–	–	–
Muscodor vitigenus	AY100022	–	–	–
Muscodor yucatanensis strain B110	FJ917287	–	–	–
Muscodor yunnanensis W–S–38	MG866046	MG866038	MG866059	MG866066
Nemania aenea JF 02118	-	DQ840070	DQ631951	DQ840085
Nemania diffusa (FR) FR AT–113	DQ658238	DQ840073	DQ631947	DQ840088
Nemania serpens FR AT–114	DQ631942	DQ840075	DQ631948	DQ840086
N–L–7	MG866054	MG866040	MG866061	MG866068
Rosellinia corticium GZ-AT-F004	DQ631940	DQ840078	–	DQ840091
Stilbohypoxylon quisquiliarum CM AT–016	DQ631937	DQ840079	–	–
Uncultured Sordariomycetidae clone D11	DQ273343	–	–	–
Whalleya microplaca W80M	AJ390419	–	–	–
Whalleya microplaca W81M	AJ390420	–	–	–
W–S–35	MG866052	–	–	–
W–S–41	MG866047	MG866036	MG866057	MG866064
W–T–27	MG866051	–	–	–
Xylaria grammica XT09009	DQ631944	DQ840081	DQ631956	DQ840090
Xylaria hypoxylon CBS 499.80	AJ309350	U47841	DQ368652	–
Xylaria sp. XT09003	DQ631945	DQ840080	DQ631953	–
Xylariaceae sp. IZ-1249	AM921731	–	–	–
Xylariaceae sp. JF TH–06–04	DQ631943	DQ840069	DQ631954	DQ840097
Y–L–43	MG866053	–	–	–
Y–L–54	MG866049	–	–	–
Y–L–56	MG866048	MG866039	MG866060	MG866067
Y–S–35	MG866050	MG866037	MG866058	MG866065

Results

Phylogenetic position of *Muscodor*

The substitution saturation test performed by DAMBE5 showed that the substitution was unsaturated. Sequence data and substitution models of ITS rRNA gene, RPB2 gene and combined four-loci are showed in Table 3. The trees reconstructed by MP, BI and ML analyses were similar in topology and a tree reconstructed by BI analysis with BI posterior probabilities, MP bootstrap support values and ML ultrafast bootstrap supports are showed in Figs 1, 2, 3. For the ITS

phylogenetic tree (Fig. 1), nine strains isolated in the current study were included along with other *Muscodor* species and closely related genera. *Muscodor* split into three clades (clade A, B and C) and was well separated from other genera with high statistical supports (BI/MP/ML = 1/75/100, BI/MP/ML = 1/83/97 and BI/MP/ML = 1/99/100). The 22 strains included in all three clades could represent *Muscodor* using the RPB2 gene and four–loci combined phylogenetic trees. The closest phylogenetic affinities of *Muscodor* were Fungal endophyte sp. ECD–2008 and *Anthostomella proteae*, but other *Anthostomella* species *Anthostomella eucalyptorum* and *Anthostomella brabeji* clustered in another distant clade, while *A. brabeji* and *A. proteae* clustered as singletons and were separated with other *Anthostomella* species (Daranagama et al. 2015). Moreover, the phylogenetic relationships among *Muscodor* species were not satisfactory, because different species clustered in the same clade like *Muscodor oryzae, Muscodor crispans* and *Muscodor musae*, and same species split into different clades, especially in clade A, such as *Muscodor albus*.

The RPB2 gene phylogenetic tree in current study (Fig. 2) demonstrated the monophyly of *Muscodor* and the inclusion of three clades (clade A, B and C) with high statistical support (BI/MP/ML = 1/100/100, BI/MP/ML = 1/100/96 and BI/MP/ML = 1/100/100). The clade of *Muscodor* was well separated from the closest clade containing the genera *Nemania* and *Xylaria* with high support (BI/MP/ML = 1/100/100). Because of a lack of RPB2 sequence data for most of *Muscodor* species, only one extra species, *Muscodor coffeanum* strain CDA739 was added. Consistent with the ITS phylogenetic tree, *Muscodor coffeanum* strain CDA739 clustered in clade B falling between *Muscodor* sp. S18–3–1 and *Muscodor* sp. M25.

The four-loci combined phylogenetic tree (Fig. 3) confirmed the phylogenetic position and the three clades (clade A, B and C) of species of *Muscodor* with high statistical support (BI/MP/ML = 1/100/100, BI/MP/ML = 1/100/100 and BI/MP/ML = 1/100/100). The clade of *Muscodor* was well separated from the closest clade containing genera *Nemania*, *Stilbohypoxylon*, *Rosellinia* and *Xylaria* with high support (BI/MP/ML = 1/100/100). In conclusion, the single ITS gene can identify a strain up to the genus *Muscodor*, but cannot resolve it to species. Moreover, for purpose of the accurate phylogenetic analysis, four or more loci should be used to understand the phylogenies of *Muscodor* species.

Molecular phylogenetic identification of the new *Muscodor* species

The ITS rRNA gene phylogenetic tree (Fig. 1) shows that strain W–S–38 formed an independent lineage with high statistical support (BI/MP/ML = 1/*/91). The four–loci phylogenetic tree (Fig. 3) showed that strain W–S–38 also formed an independent clade with high statistical support (BI/MP/ML = 1/100/100) and there were sufficient genetic distances (Table 4) (Zhang et al. 2010) among strain W–S–38 with phylogenetically close species such as *Muscodor suthepensis* strain CMU462, *Muscodor coffeanum* strain CDA741 and *Muscodor yucatanensis* strain B110. As a result, strain W–S–38 is recognized as a novel species.

Taxonomy

Muscodor yunnanensis C.L. Zhang, sp. nov. Fig. 4

Mycobank: MB824602; Facesoffungi Number: FoF04824

Etymology – named by habitat

Sexual morph: undetermined. Asexual morph: unicellular, hyphae, septate, ramose. The width of the mycelium was 0.9–1.7 μm and mycelium frequently intertwined, forming rope-like strands.

Culture characteristics – Cultures incubated on PDA at 25 °C in darkness, with a linear growth rate of 4.29 mm diam/day in first 7 days and then be slower. Upper colony white, with longitudinal and sunken stripes, then developing zonate and mycelium in the ring collapsing, reverse colony white and the ring obvious; no conidia and sporulation structure were observed under laboratory conditions.

Holotype – W-S-38, Naban River Watershed National Nature Reserve (E100°32'–100°44', N22°04'–22°17'), Yunnan Province, China, from leaf sheath of *Oplismenus undulatifolius*, Aug. 2015, deposited in China General Microbiological Culture Collection Center (accession number: CGMCC 3.18908). The accession number of GenBank database for ITS rRNA, 28S rRNA, RPB2 and TUB1 genes sequences were MG866046, MG866038, MG866059 and MG866066, respectively.

Habitat and distribution – in leaf sheath of *Oplismenus undulatifolius*, Yunnan province, China.

Notes – Strain W-S-38 belonging to this novel species was isolated as a plant endophyte. It is differentiated from other *Muscodor* species by significant phylogenetic support ((BI/MP/ML = 1/100/100) and high p-distances (Table 4). As for culture characteristics, differences with other phylogenetically close *Muscodor* species were in Table 5.

Table 3 Sequence data and substitution models of ITS rRNA, RPB2 gene and combined four-loci (ITS rRNA, 28S rRNA, RPB2 and TUB1 genes).

Sequence	Nucleotide characters including gaps	Parsimony informative sites	Variable and parsimony uninformative sites	Constant sites	Best-fit nucleotide substitution model for BI and ML analyses
ITS	566	361	66	139	TIM3ef+I+G
RPB2	891	427	43	421	TIM2+G
Combined four-loci	3735	1479	331	1925	TIM2+I+G

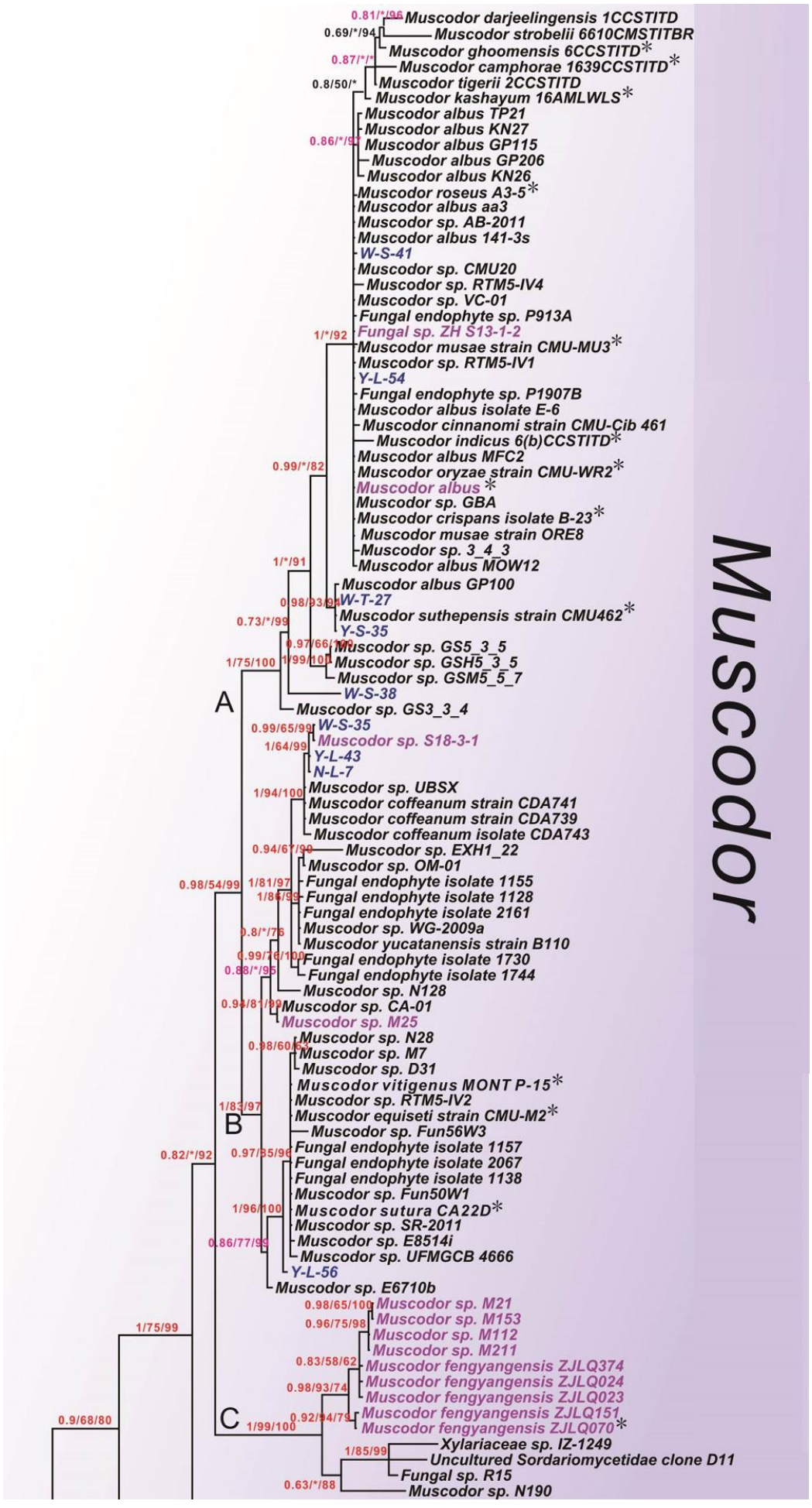
Table 4 p-Distance of nucleotide sites among the four-loci sequences compared between W-S-38 and close species.

	***M. suthepensis* strain CMU462**	**W-S-38**	***M. coffeanum* strain CDA741**	***M. yucatanensis* strain B110**
M. suthepensis strain CMU462				
W-S-38	0.050			
M. coffeanum strain CDA741	0.066	0.084		
M. yucatanensis strain B110	0.061	0.082	0.014	

Table 5 Culture characteristic comparison with several phylogenetically close *Muscodor* species.

Taxon	Host	Mycelium pigment production	Colonial morphology	Mycelial growth
M. albus[a,b]	*Cinnamomum zeylanicum*	White	Felt-like mycelium	Rope-like
M. suthepensis[c]	*Cinnamomum bejolghota*	White in the dark and pale pink in the light	No description	Rope-like with coils structure
M. yucatanensis[d]	*Bursera simaruba*	Whitish and ivory-white for older colonies	Flocculose pattern and reverse uncolored, slightly funiculose	Rope-like with coils structure and swollen cell
W-S-38	*Oplismenutls undulatifolius*	White	With longitudinal and sunken stripes, then developing zonate and mycelium in the ring collapsing,	Rope-like

[a]Worapong et al. 2001, [b]Ezra et al. 2004, [c]Suwannarach et al. 2013, [d]González et al. 2009


Muscodor darjeelingensis 1CCSTITD
Muscodor strobelii 6610CMSTITBR
Muscodor ghoomensis 6CCSTITD*
Muscodor camphorae 1639CCSTITD*
Muscodor tigerii 2CCSTITD
Muscodor kashayum 16AMLWLS*
Muscodor albus TP21
Muscodor albus KN27
Muscodor albus GP115
Muscodor albus GP206
Muscodor albus KN26
Muscodor roseus A3-5*
Muscodor albus aa3
Muscodor sp. AB-2011
Muscodor albus 141-3s
W-S-41
Muscodor sp. CMU20
Muscodor sp. RTM5-IV4
Muscodor sp. VC-01
Fungal endophyte sp. P913A
Fungal sp. ZH S13-1-2
Muscodor musae strain CMU-MU3*
Muscodor sp. RTM5-IV1
Y-L-54
Fungal endophyte sp. P1907B
Muscodor albus isolate E-6
Muscodor cinnanomi strain CMU-Cib 461
Muscodor indicus 6(b)CCSTITD*
Muscodor albus MFC2
Muscodor oryzae strain CMU-WR2*
Muscodor albus*
Muscodor sp. GBA
Muscodor crispans isolate B-23*
Muscodor musae strain ORE8
Muscodor sp. 3_4_3
Muscodor albus MOW12
Muscodor albus GP100
W-T-27
Muscodor suthepensis strain CMU462*
Y-S-35
Muscodor sp. GS5_3_5
Muscodor sp. GSH5_3_5
Muscodor sp. GSM5_5_7
W-S-38
Muscodor sp. GS3_3_4
W-S-35
Muscodor sp. S18-3-1
Y-L-43
N-L-7
Muscodor sp. UBSX
Muscodor coffeanum strain CDA741
Muscodor coffeanum strain CDA739
Muscodor coffeanum isolate CDA743
Muscodor sp. EXH1_22
Muscodor sp. OM-01
Fungal endophyte isolate 1155
Fungal endophyte isolate 1128
Fungal endophyte isolate 2161
Muscodor sp. WG-2009a
Muscodor yucatanensis strain B110
Fungal endophyte isolate 1730
Fungal endophyte isolate 1744
Muscodor sp. N128
Muscodor sp. CA-01
Muscodor sp. M25
Muscodor sp. N28
Muscodor sp. M7
Muscodor sp. D31
Muscodor vitigenus MONT P-15*
Muscodor sp. RTM5-IV2
Muscodor equiseti strain CMU-M2*
Muscodor sp. Fun56W3
Fungal endophyte isolate 1157
Fungal endophyte isolate 2067
Fungal endophyte isolate 1138
Muscodor sp. Fun50W1
Muscodor sutura CA22D*
Muscodor sp. SR-2011
Muscodor sp. E8514i
Muscodor sp. UFMGCB 4666
Y-L-56
Muscodor sp. E6710b
Muscodor sp. M21
Muscodor sp. M153
Muscodor sp. M112
Muscodor sp. M211
Muscodor fengyangensis ZJLQ374
Muscodor fengyangensis ZJLQ024
Muscodor fengyangensis ZJLQ023
Muscodor fengyangensis ZJLQ151
Muscodor fengyangensis ZJLQ070*
Xylariaceae sp. IZ-1249
Uncultured Sordariomycetidae clone D11
Fungal sp. R15
Muscodor sp. N190
A
B
C
Muscodor

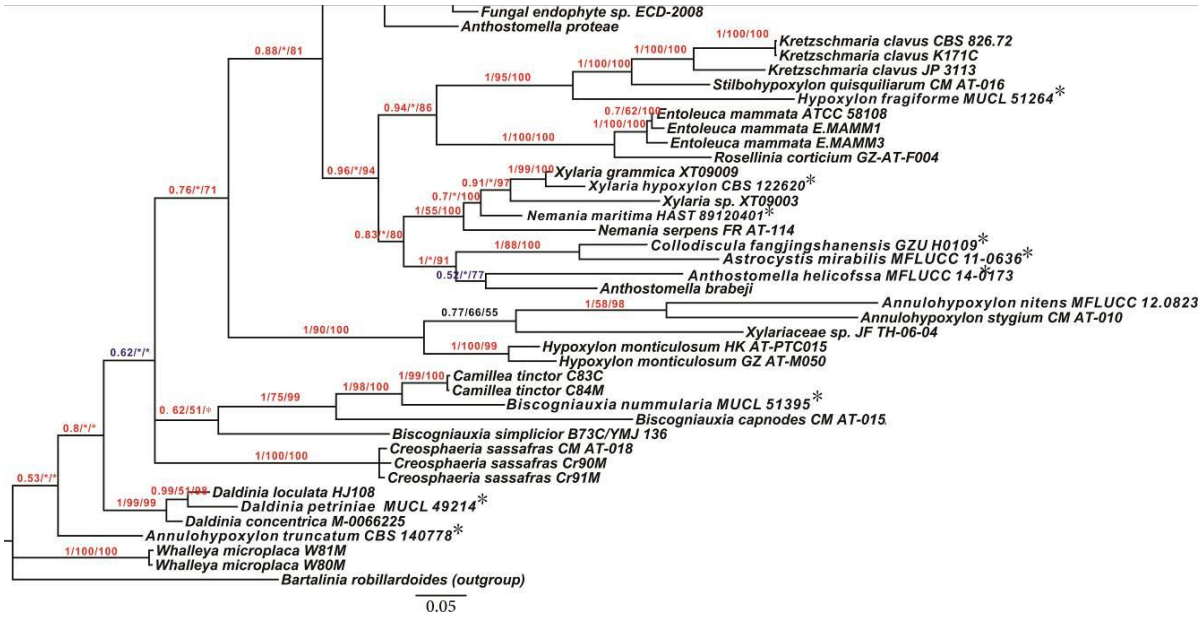


Figure 1 – The phylogenetic tree inferred from MP, BI and ML analyses based on ITS rRNA gene dataset. BI posterior probabilities, MP bootstrap support values and ML ultrafast bootstrap supports are shown at the first, second and third position above branches. *Bartalinia robillardoides* is defined as an outgroup. * above branches indicates MP and ML phylogenetic tree is not consistent with BI phylogenetic tree here. The bar represents 0.05 substitution per site. * after strains indicates type strains.

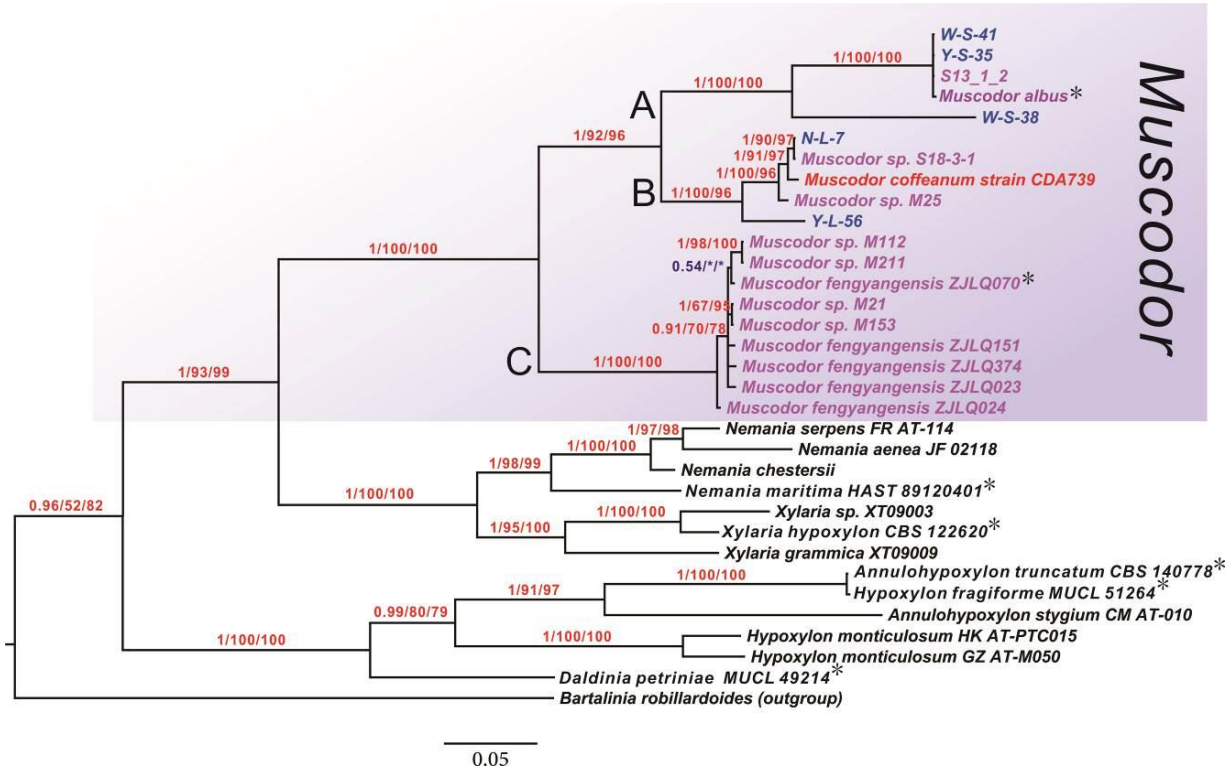


Figure 2 – The phylogenetic tree inferred from MP, BI and ML analyses based on RPB2 gene dataset. BI posterior probabilities, MP bootstrap support values and ML ultrafast bootstrap supports are shown at the first, second and third position above branches. *Bartalinia robillardoides* is defined as an outgroup. * above branches indicates MP and ML phylogenetic tree is not consistent with BI phylogenetic tree here. The bar represents 0.05 substitution per site. * after strains indicates type strains.

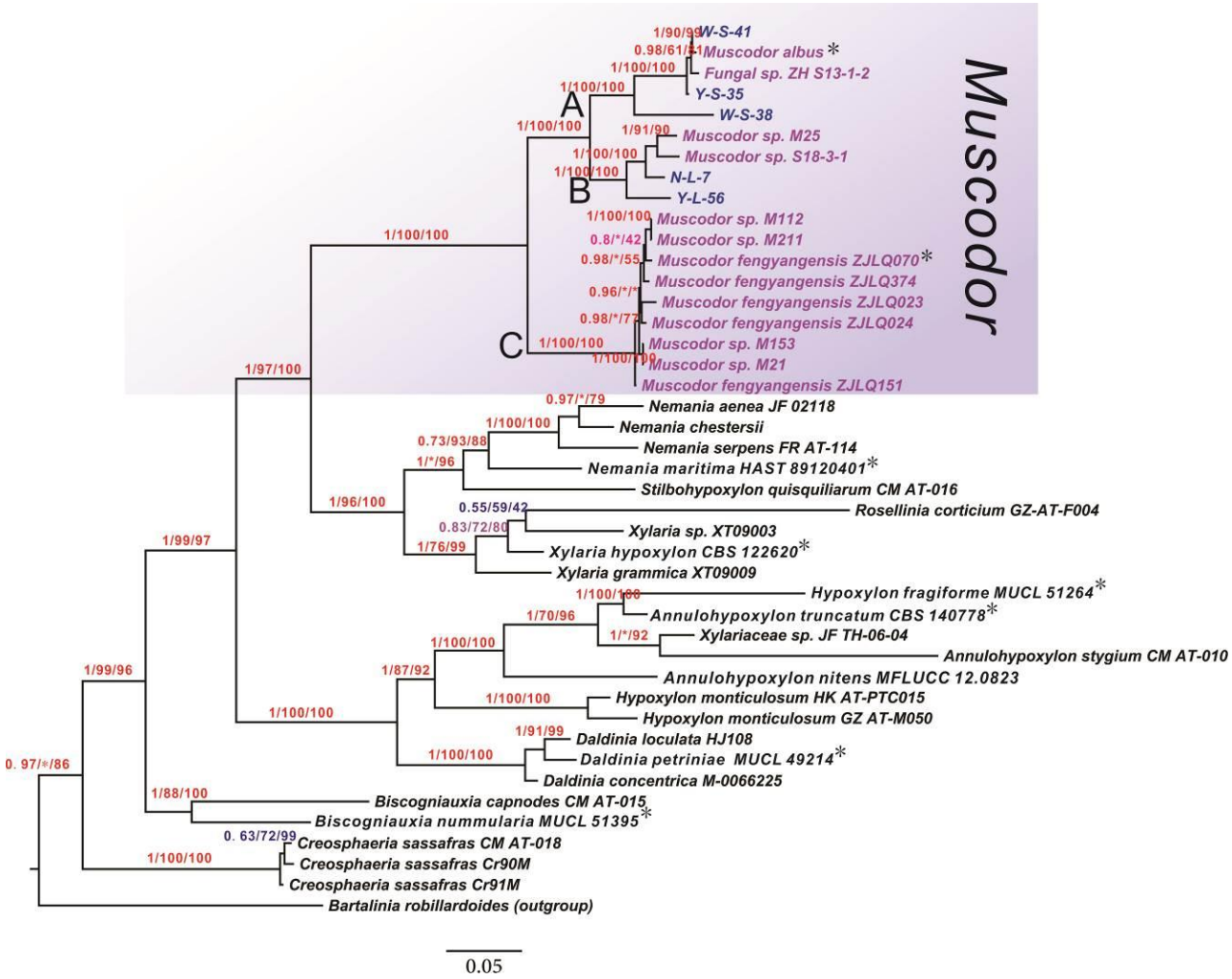


Figure 3 – The phylogenetic tree inferred from MP, BI and ML analyses based on four-loci (ITS rRNA, 28S rRNA, RPB2 and TUB1 genes) dataset. BI posterior probabilities, MP bootstrap support values and ML ultrafast bootstrap supports are shown at the first, second and third position above branches. *Bartalinia robillardoides* is defined as an outgroup. * above branches indicates MP and ML phylogenetic tree is not consistent with BI phylogenetic tree here. The bar represents 0.05 substitution per site. * after strains indicates type strains.

Discussion

New nomenclature calls for more accurate identification (Stadler et al. 2013), however, because of the lack of reproductive structures, the identification of *Muscodor* species mainly relies on phylogenetic analyses and other criteria, such as the differences of their profile of volatiles. For the lack of taxon sampling and reference materials, the rationale that authors used to introduce a new genus appears highly questionable (Stadler et al. 2013). In order to clarify the position of *Muscodor*, a four-loci phylogenetic tree was reconstructed in this study, which demonstrated that all 18 strains are distributed in three clades in *Muscodor* that clustered as a monophyletic group. Meshram et al. (2013) separated the genus *Muscodor* into two clades based on maximum likelihood analysis of ITS1-5.8S-ITS2 region and Siri-udom et al. (2016) separated *Muscodor* into three major clades based on maximum parsimony analysis of ITS1-5.8S rDNA-ITS2 region. The phylogenetic trees in this study (Figs 1, 2, 3) were topologically similar with the tree in Siri-udom et al. (2016) and the three separate clades were also consistent. The result should be convincing although some sequences in four-loci data were lacking and majority of *Muscodor* species were sequenced only by ITS.

The p-distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. Zhang et al. (2010) used this approach to determine genetic relatedness of

isolates, as the p-distance among the five novel *Muscodor fengyangensis* isolates was the level of millesimal or lower and was always 10- to 20-fold lower than that between *Muscodor* spp. where sequences for all named *Muscodor* spp. available was in the same range of the level of hundredth. Saxena et al. (2015) also used p-distance to distinguish *Muscodor tigerii* within *Sordariomycetes*, *Xylaria* and *Muscodor* spp. In the present study, p-distance of nucleotide sites among the four-loci sequences between W-S-38 and similar species was in the same range as that between *Muscodor* spp. and was in the level of hundredth, which provided strong evidence to demonstrate that W-S-38 is a novel species.

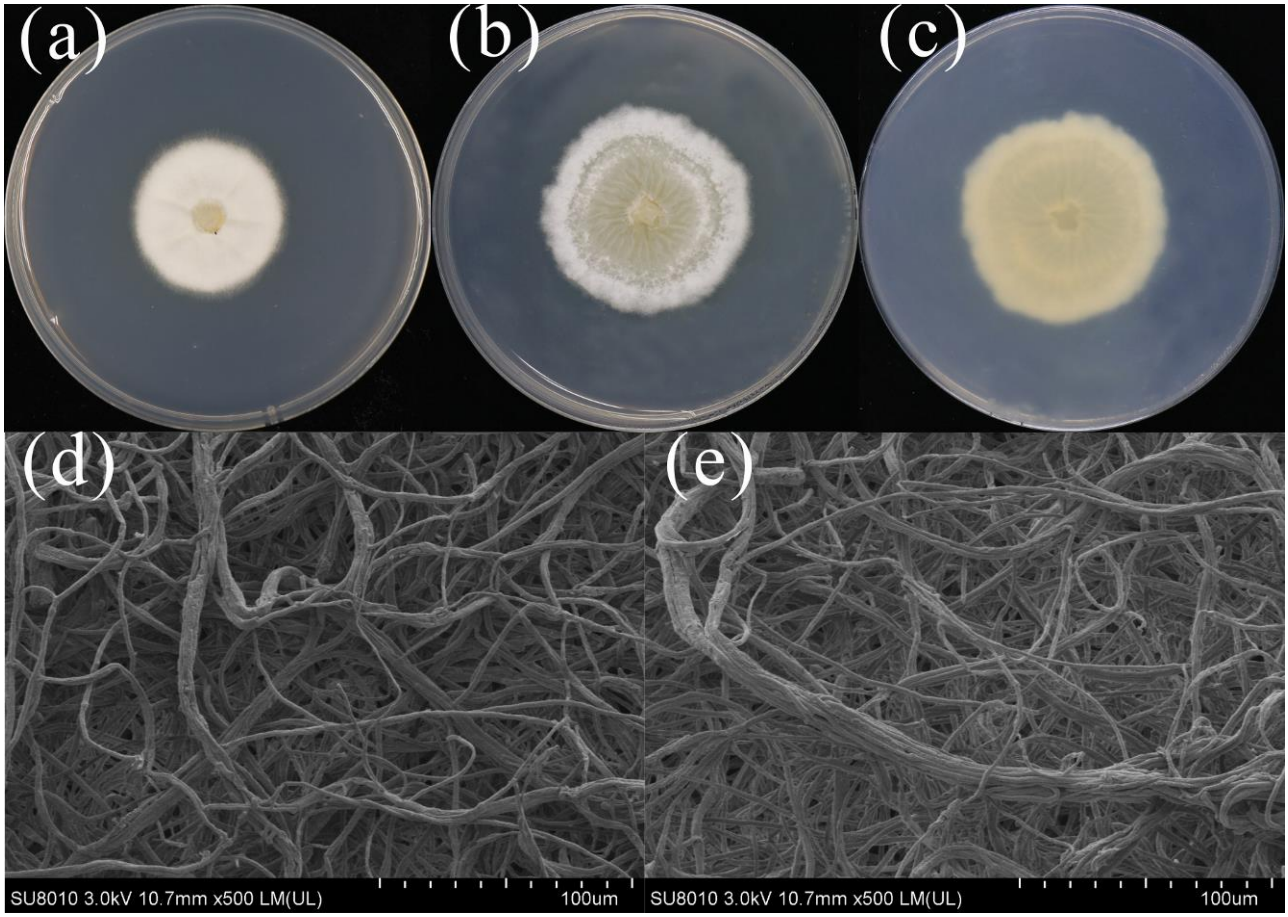


Figure 4 – Morphological characteristics of *Muscodor yunnanensis*. a colony of strain W–S–38 on PDA after 7 d at 25 °C in darkness. b colony of W-S-38 on PDA after 30 d at 25 °C in darkness. c reverse colony of W-S-38 on PDA after 30 d at 25 °C in darkness. d scanning electron micrograph of the mycelial arrangement and morphology. e scanning electron micrograph of the fused rope-like hyphae.

There have been decades that ITS rRNA gene was used for diagnostics and molecular phylogenetic identifications in fungi (White et al. 1990, Henrion et al. 1992) and has been proved to be effective in a large quantity of taxonomic groups, and was also proposed to be a universal DNA barcode marker for fungi (Begerow et al. 2010, Schoch et al. 2012). Nonetheless, there were limitations in some aspects and use of only ITS rRNA gene sequences to infer phylogenetic relationships of Xylariales genera *incertae sedis* was found to be inappropriate (Mazzaglia et al. 2001, Suwannasai et al. 2005, Peláez et al. 2008, Tang et al. 2009, Stadler et al. 2013). More taxa included did not result in better phylogenetic resolution and some taxa could not be well separated (Tang et al. 2009, Stadler et al. 2013). It was also reflected in the current study that *Muscodor* species could not be separated well from each other at some level and the genera *Hypoxylon* and *Annulohypoxylon* were not well separated (Fig. 1).

In addition, some protein-coding genes such as RPB2 (Liu et al. 1999) were used for phylogeny. In some cases, RPB2 gene provided the higher proportion of informative characters and the phylogeny based on it appeared to be highly resolved (Stadler et al. 2013). Moreover, when RPB2 gene was analysed in combination with other genes, such as with ITS rRNA gene, the phylogenetic tree obtained would be more reliable. Therefore, RPB2 gene would be a promising candidate for identification within Xylariales genera *incertae sedis*.

Acknowledgements

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