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Mycosphere Essays 2. *Myrothecium*

Chen Y[1], Ran SF[1], Dai DQ[2], Wang Y[1], Hyde KD[2], Wu YM[3] and Jiang YL[1]

[1] *Department of Plant Pathology, Agricultural College of Guizhou University, Huaxi District, Guiyang City, Guizhou Province 550025, China*
[2] *Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[3] *Department of Plant Pathology, Shandong Agricultural University, Taian, 271018, China*

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Abstract

Myrothecium (family *Stachybotryaceae*) has a worldwide distribution. Species in this genus were previously classified based on the morphology of the asexual morph, especially characters of conidia and conidiophores. Morphology-based identification alone is imprecise as there are few characters to differentiate species within the genus and, therefore, molecular sequence data is important in identifying species. In this review we discuss the history and significance of the genus, illustrate the morphology and discuss its role as a plant pathogen and biological control agent. We illustrate the type species *Myrothecium inundatum* with a line diagram and *M. uttaradiensis* with photo plates and discuss species numbers in the genus. The genus is re-evaluated based on molecular analyses of ITS and EF1-α sequence data, as well as a combined ATP6, EF1-α, LSU, RPB1 and SSU dataset. The combined gene analysis proved more suitable for resolving the taxonomic placement of this genus. Results indicate that *Myrothecium* species are polyphyletic within *Stachybotryaceae*. We suggest future studies needed for the genus.

Key words – biocontrol – mycotoxins – opportunistic pathogens – phylogeny – Sordariomycetes – Stachybotryaceae

The significance of *Myrothecium*

Myrothecium species are saprobes in the soil or weak plant pathogens (Ellis & Ellis 1985, Watanabe 1994). More than 30 species has been reported worldwide (Seifert et al. 2011), while there are 83 records in Index Fungorum (2016).

The identification of species in this genus is of particular importance as some species have medical or public health significance. Studies have investigated the toxicity of verrucarins and roridins, which are macrocyclic trichothecene mycotoxins produced by *Myrothecium* species (Ueno 1983, Moss 1996). The macrocyclic trichothecenes are extremely toxic (Yang & Johanning 1997), and include stra-toxins produced by *Stachybotrys atra* Corda (Levetin & Shaughnessy 1997). Because *Myrothecium* species are capable of growing on walls in houses and produce mycotoxins that are similar to *Stachybotrys* toxins, they can cause a variety of adverse health effects, including inhibition of protein synthesis, immune suppression, and impairment of alveolar macrophage function (Yang & Johanning 1997). The presence of this genus as an indoor air contaminant must be regarded as a potential health hazard (Levetin & Shaughnessy 1997). However, no study has assessed the risk to humans.

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Corresponding author: Yu-Lan Jiang – e-mail – yljchsd@163.com

The positive actions of *Myrothecium* species include compounds that are able to resist tumour activity and inhibit the activity of liver cancer and promyelocytic leukemia cells (Koyama et al. 1989, Lu et al. 2010, Zhu et al. 2012, Liu et al. 2014). If the medicinal properties of *Myrothecium* species are further explored, this may provide advantages for human health.

Myrothecium as a plant pathogen

The first report of disease caused by *Myrothecium* was on a tomato in America (Stevenson et al. 1947). *Myrothecium roridum* Tode was also found to infect coffee causing bark canker (Schieber & Zentmyer 1968, Tulloch 1972). *Myrothecium verrucaria* (Alb. & Schwein) Ditmar, was reported as a weak pathogen within the seeds of rice and soybean (Neergaard 1979). *Myrothecium* species are also pathogens of mulberry (*Myrothecium* leaf spot of mulberry) (Du et al. 1988, Murakami R et al. 2005, Ben et al. 2009) and chilli seed (Liang 1989), and many other hosts, including *Ficus elastica*. (*Myrothecium* leaf spot) (Norman & Ali 2013), *Dieffenbachia picta* cv. Compacta (*Myrothecium* leaf spot) (Mcmillan 2010). *Myrothecium* species cause disease on the leaves of different types of vegetables (Li et al. 2009). Because *Myrothecium roridum* infects many commercial crops and a wide range of hosts, it is an important plant pathogen (Li et al. 2009). The development of the disease can take advantage of wet soil conditions. Thus, in order to reduce economic losses in commercial cultivation, it is important to consider watering practices and soil sanitation (Han et al. 2014).

The potential of _Myrothecium_ as a bio-control agent

The genus *Myrothecium* is a prolific producer of bioactive secondary metabolites, with more than 20 reported compounds from *M. roridum*, and more than 30 compounds from *M. verrucaria* (Wagenaar & Clardy 2001). *Myrothecium verrucaria* is particularly virulent against several weedy plant species and is potentially useful as a bio-herbicide (Walker & Tilley 1997). *Myrothecium verrucaria* has high activity against extracellular insect cuticles and produces chitinases, proteinases and lipases (Kobayashi et al. 1996). Thus, the cuticle degrading enzyme complex produced by *M. verrucaria* has potential for the bio-control of mosquitoes (Mendonsa et al. 1996). Mou (1975) showed that some insects (such as *Macrotermes barneyi*, termites and *Dendrolimus punctatus*) are parasitized by taxa, which are morphologically similar to *M. roridum*. *Myrothecium verrucaria* and its metabolites show nematode activity, which could control plant damage from nematodes by reducing egg hatching, inhibiting development, or even killing the nematodes directly (Lamovšek et al. 2013).

Taxonomic history and phylogenetic understanding of _Myrothecium_

The genus *Myrothecium* was introduced by Tode (1790) who described five species: *M. inundatum*, *M. roridum* and three others which were later stated by Fries (1829) to belong to other genera. The genus was adopted in an emended form by Fries (1829) with four species, *M. roridum*, *M. verrucaria*, *M. inundatum* Tode and *M. scybalorum* (Schum.) Fr. Its original diagnosis (Tode 1790) was of a cup-shaped fungus with the cups surrounded by a sheath filled with sticky spores. Tode (1790) and Preston (1943, 1948, 1961) published a series of papers on *Myrothecium* and provided detailed descriptions and illustrations. Pidoplichko (1953) distinguished *Myrothecium* from the similar genera, *Dendrodochium Chaetostroma*, *Verticilliodochum* and *Volutella*. Nicot & Olivry (1961) studied *Myrothecium* species and concluded that the genus is common in soils. Tulloch (1972) defined the key generic characters of *Myrothecium* and accepted 13 species after checking numerous collections and descriptions of 55 species. Additionally, she summarized the morphological characters of species of *Myrothecium* and provided a key for the genus. Wu (1991) compared the morphology of *M. roridum* on different hosts.

We consider that the identification of *Myrothecium* species using morphology is imprecise, due to the following reasons: 1) morphology cannot clearly differentiate species and 2) the genus probably comprises species complexes. We therefore generated a phylogenetic tree for species of *Myrothecium* based on ATP6, EF1-α, ITS, LSU, RPB1 and SSU sequence data downloaded from

GenBank (Figs 3, 4, 5), which shows the genus to be polyphyletic in Stachybotryaceae. Morphology cannot effectively reflect phylogenetic relationships in the genus, because of the few characters available to differentiate species (Judd et al. 2002).

Gene sequence data from fungi are changing our understanding of species and genera or of higher taxonomic levels (Shenoy et al. 2007, Udayanga et al. 2011, Wikee et al. 2011, Hyde et al. 2013, Wijayawardene et al. 2014). Fekete et al. (1997) utilized the *Tri5* gene for identification of *Myrothecium* and *Stachybotrys* species and strains that produced trichothecenes received strong bootstrap support (*Myrothecium*, *Stachybotrys*, *Trichoderma*, and *Trichothecium*). Seifert et al. (2003) isolated a strain of *Myrothecium* and LSU sequence data indicated that it was related to *Myrothecium inundatum*. Recently, several Chinese mycologists have identified *Myrothecium* species by combining morphology and analysis of ITS sequence data (Li et al. 2009, Zhao et al. 2009, Liu et al. 2014, Liu et al. 2015).

Morphology of *Myrothecium*

Type species: *Myrothecium inundatum* Tode, Fung. mecklend. Sel. (Lüne burg) 1:25 (1790). (Fig. 1) Facesoffungi number FoF 01961.

Morphological characters – *Sporodochia* sessile or stalked, viscid, horny when dry, with a green to black mass of conidia, usually surrounded by a white zone, with flocculent hyphae from which setae project in some species. *Stroma* often present**.** *Setae* when present unbranched, colourless or pale. *Hyphopodia* absent. *Conidiophores* macronematous, mononematous, closely packed together to form sporodochia, branched, with the branched apex arranged penicillately, straight or flexuous, colourless or olivaceous, smooth or verruculose. *Conidiogenous cells* monophialidic, discrete, cylindrical, clavate or subulate. *Conidia* aggregated in dark green or black, slimy masses, semi-endogenous or acrogenous, simple, hyaline to pale olive, 1-celled, cylindrical with rounded ends, navicular, limoniform or broadly ellipsoidal, often with a projecting truncate base, smooth or striate (Ellis 1971).

Notes – *Myrothecium inundatum* was introduced as the type species of this genus by Tode (1790), Link provided a revised description in 1809, while Preston (1943) make a comparison of morphology with *M. inundatum*. *Myrothecium inundatum* has been reported from dead agarics, e.g. *Russula adusta* in Europe (1971), soil from a forest in Yunnan Province, China (Xu 2006), soil from conifer-broadleaf mixed forest in Liuba, Zhouzhi, China, farmland in Mei Xian, China, and flowerpots in Xi'an, China (Yu 2008). ATP, EF1-α, ITS, LSU and SSU sequence data are available for *M. inundatum* (IMI 158855) in GenBank, and the LSU sequence as the first gene was released by Rossman (2007). We provide a line drawing of *M. inundatum* (Fig. 1) from Yu (2008) and a figure of *M. uttaradiensis* (Fig. 2) from Dai et al. (in press).

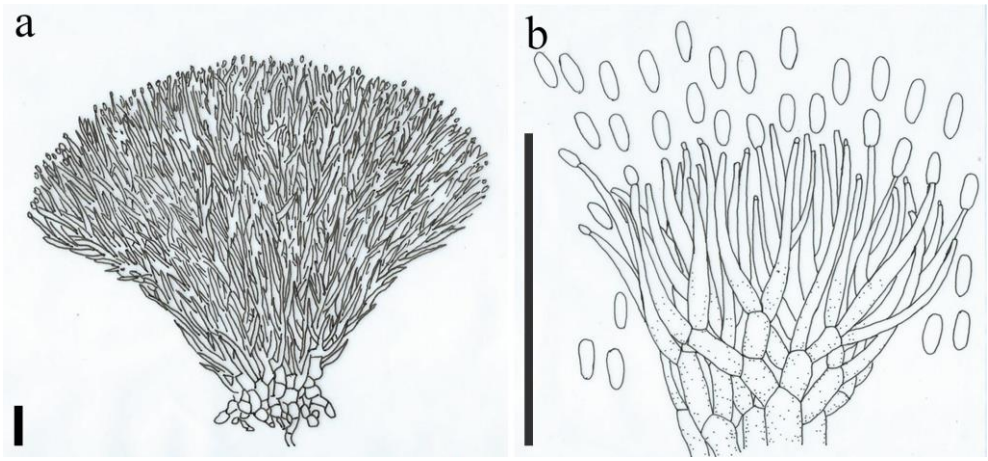

Fig. 1 – *Myrothecium inundatum*. (redrawn from Yu 2008). a Sporodochium. b Conidia and conidiogenous cells. – Scale bars: a, b = 25μm.

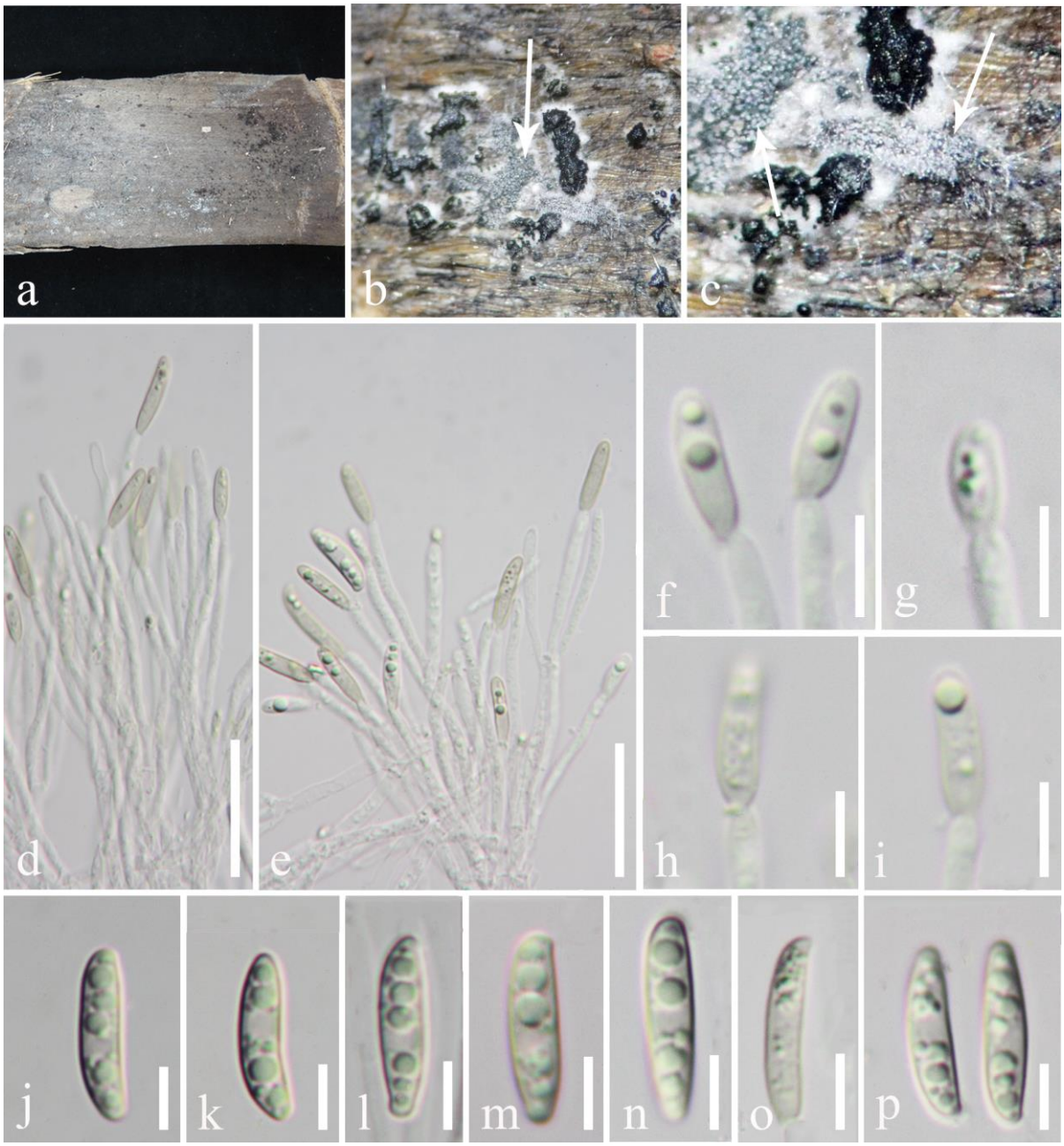

Fig. 2 – *Myrothecium uttaraditensis* (THAILAND, Uttaradit, on dead culm of bamboo, 28 October 2011, Dong-Qin Dai DDQ00216 (MFLU 15–1197, holotype) (Dai et al. (2016)). a Mycelia on bamboo. b, c Mycelia and sporodochia. d, e Conidiophores and conidia. f-i Conidia and conidiogenous cells. j-p Conidia. **–** Scale bars: d, e = 20mm, f, g, h, I, j, k, l, m, n, o, p = 5mm.

Phylogenetic studies on *Myrothecium*

Materials and Methods

Phylogenetic analyses were performed based on sequence data available in GenBank with the accession and strain numbers listed in Table 1 and used by Castlebury et al. 2004, Wang et al. 2015, Maharachchikumbura et al. 2015. This study used ATP6, EF1-α, SSU, LSU, RPB1 sequences data and EF1-α+ITS sequences data for combined gene analyses and ITS for single gene analyses. Multiple sequences alignments were generated with BioEdit v. 7.0.9.0 or MEGA 6.06 (Kumar et al. 2012); Maximum Likelihood (ML) phylogenies analyses of each aligned dataset, generated by RAxML-HPC BlackBox v. 8.2.4 on CIPRES using default conditions (Miller et al. 2010). The results were viewed in TreeView v. 1.6.6 (Page 1996).

Table 1 Strains used in phylogenetic analyses and their corresponding GenBank accession numbers.

Species	***Strain (or isolate) No.***	***GenBank acc. No.***					
		ITS	**EF1-α**	**SSU**	**LSU**	**RPB1**	**ATP6**
Bionectria ochroleuca	CBS 114056		AY489611	AY489684	AY489176		
B. ochroleuca	Sin 80	KF055399					
B. pityrodes	ATCC 208842		AY489623	AY489696	AY489728	AY489658	AY489589
Cosmospora coccinea	CBS 341.70	HQ897827					
C. coccinea	A. R. 2743	KJ676141					
Didymostilbe echinofibrosa	A. R. 2824	DQ135999	AY489601	AY489674	AY489706	AY489634	AY489567
Hypomyces polyporinus	ATCC 76479		AF543784	AF543771	AF543793	AY489663	AY489593
Myrothecium sp.	E31	KP401889					
Myrothecium sp.	BAB-5430	KT281599					
Myrothecium sp.	JZ-45	HQ637275					
Myrothecium sp.	BCC9829	DQ135992					
Myrothecium sp.	SC0265	KM086710					
Myrothecium sp.	DoF25	JQ388270					
Myrothecium sp.	C101	JQ936269					
Myrothecium sp.	ASR-261	GU973803					
Myrothecium sp.	30382	KP714371					
Myrothecium sp.	CNN22	DQ092541					
Myrothecium sp.	HKB28	DQ092520					
Myrothecium sp.	M7-CA-302	JX984613					
Myrothecium sp.	E6	KJ862068					
Myrothecium sp.	Sn342	KP006363					
Myrothecium sp.	E2	KF887082					
Myrothecium sp.	M9-CA-360	JX984615					
M. atroviride	BBA 71016	AJ302002					
M. atroviride	wb256	AF455507					
M. atrum	CBS 338.97	AY254160					
M. cinctum	ATCC 18947	DQ135997					
M. cinctum	BCC8249	DQ135995					
M. cinctum	ATCC 32918	DQ135998					
M. cinctum	ATCC 22270		AY489605	AY489678	AY489710	AY489638	AY489571
M. cylindrosporum	MFLUCC 11-00392	KP744448					
M. carmichaelii	IMI 199044	AY254150					
M. gramineum	C41	JQ936265					
M. gramineum	LCJ 177	KF414681					

Species	*Strain (or isolate) No.*	*GenBank acc. No.*					
		ITS	**EF1-α**	**SSU**	**LSU**	**RPB1**	**ATP6**
M. gramineum	BCC9458	FJ825374		FJ825369	FJ825379		
M. gramineum	A243	GQ373154					
M. gramineum	CBS 324.54	AY254151					
M. gramineum	WS11720	JX406554					
M. inundatum	CBS 582.93	AY254152					
M. inundatum	BBA 71019	AJ302005					
M. inundatum	F216	KM979993					
M. inundatum	IMI 158855		AY489626	AY489699	AY489731		AY489594
M. inundatum	SCSGAF0095	JN851023					
M. lachastrae	IMI 273160	AY254159					
M. leucotrichum	AR3506		AY489602	AY489675	AY489707	AY489635	AY489568
M. leucotrichum	BBA 71014	AJ302000					
M. leucotrichum	BBA 65577	AJ301992					
M. masonii	ATCC 24426	AY254153					
M. prestonii	CBS 175.73	AY254154					
M. roridum	MA-20	JF724152	JF724141				
M. roridum	A243	GQ351270					
M. roridum	781	JF724155	JF724132				
M. roridum	MA-83	JF724154	JF724149				
M. roridum	UAMH 1369		DQ676613	DQ680056			
M. roridum	ATCC 16297		AY489603	AY489676	AY489708	AY489636	AY489569
M. roridum	MA_73	JF724153	JF724144				
M. roridum	801	JF724151					
M. roridum	794	JF724158	JF724140				
M. roridum	784	JF724157	JF724134				
M. roridum	ATCC 52485		DQ676603	DQ680047			
M. roridum	IMI 394934	GQ853401					
M. roridum	ICMP:20424	KJ909289					
M. roridum	Myr2-2	KC469695					
M. roridum	DUCC4002	KC581914					
M. roridum	CDA725	KJ815095					
M. roridum	KKFC400	AB823652					
M. roridum	UB:2246	KJ494661					
M. roridum	BCRC 34581	GU929191					
M. roridum	RDCCT07091402	KP942366					
M. roridum	F04	HQ839773					
M. roridum	CBS 331.51	HQ115647					
M. roridum	ATCC 52801	AY254155					
M. roridum	KUAB1MRCD	KF171528					

Species	*Strain (or isolate) No.*	*GenBank acc. No.*					
		ITS	**EF1-α**	**SSU**	**LSU**	**RPB1**	**ATP6**
M. setiramosum	CBS 534.88	AY254156					
M. tongaense	CBS 873.85	AY254157					
M. uttaraditensis	MFLUCC 11-00216	In press					
M. verrucaria	NRRL 52420	GU183129					
M. verrucaria	PTCC 799	KC140228					
M. verrucaria	KAUEF26	HF548712					
M. verrucaria	HGUP 0731	KC806230					
M. verrucaria	E21	KF887115					
M. verrucaria	ATCC 9095		AY489608	AY489681	AY489713	AY489641	AY489574
M. verrucaria	AR346	HQ596904					
Neonectria ditissima	CBS 226.31	JF735309					
N. major	CBS 240.29	JF735308					
N. ramulariae	CBS 151.29	JF735313					
N. ramulariae	CBS 182.36	JF735314					
N. ramulariae	ATCC 16237	HM364297					
Nectria cinnabarina	CBS 255.47	HM484710					
N. cinnabarina	AR 4477	HM484548					
N. cinnabarina	CBS 189.87	HM484699					
Nectriopsis violacea	CBS 424.64			AY489687	AY489719	AY489579	AY489579
Niesslia exilis	CBS 357.70		AY489613	AY489686	AY489718	AY489645	AY489578
Ochronectria calami	CBS 125.87		AY489612	AY489685	AY489717	AY489644	AY489577
Parasarcopodium ceratocarvi	CBS 110664	AY344479					
Peethambara spirostraita	CBS 110115		AY489619	AY489692	AY489724	AY489654	AY489585
Stachybotrys albipes(=*Melanopsamma pomiformis*)	ATCC 18873		AY489604	AY489677	AY489709	AY489637	AY489570
S. chartarum	ATCC 9182		AY489609	AY489682	AY489714	AY489642	AY489575
S. chartarum	ATCC 66238 (=UAMH 6417, =CBS 25089)		AY489607	AY489680	AY489712	AY489640	AY489573
S. chlorochalonata	CBS 109285	AY180261.					
Stachybotrys dichroa	ATCC 18913	AF081472					
S. echinata (=Memnoniella echinata)	UAMH 6594		AY489631	AY489704	AY489736	AY489672	AY489599
S. eucylindrospora	ATCC 18851	AF081474					

Species	*Strain (or isolate) No.*	*GenBank acc. No.*					
		ITS	**EF1-α**	**SSU**	**LSU**	**RPB1**	**ATP6**
S. parvispora	ATCC 18877	AF081483					
S. subsimplex (=*M. subsimplex*)	ATCC 32888	AF205439	AY489606	AY489679	AY489711	AY489639	AY489572
S. subcylindrospora	HGUP 0201	JX998163					
S. subreniformis	HGUP 1051	KC305344					
Stilbocrea macrostoma	CBS 114375		AY489620	AY489693	AY489725	AY489655	AY489586
Stephanonectria keithii	CBS 114057		AY489622	AY489695	AY489727	AY489657	AY489588
Torrubiella wallacei	CBS 101237	EF469153	EF469073				

Bayesian Analyses (PP) was performed using MrBayes 3.2.0 (MrBayes v. 3.2.1; Ronquist et al. 2012). The optimal models for the Bayesian analysis were preliminarily chosen using models of nucleotide substitution for each gene and combined genes, as determined using MrModeltest v. 2.2 (Nylander 2004). The GTR+I+G model was selected for each multi-loci analyses and single gene analyses. The analyses of four simultaneous Markov Chain Monte Carlo (MCMC) chains were run from random trees for 1,000,000 generations and sampled every 100 generations. The temperature value was lower than 0.15, burn-in was set to 0.25, and the run was automatically stopped as soon as the average standard deviation of split frequencies reached below 0.01 (Maharachchikumbura et al. 2015). The resulting trees were printed with Figtree v 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

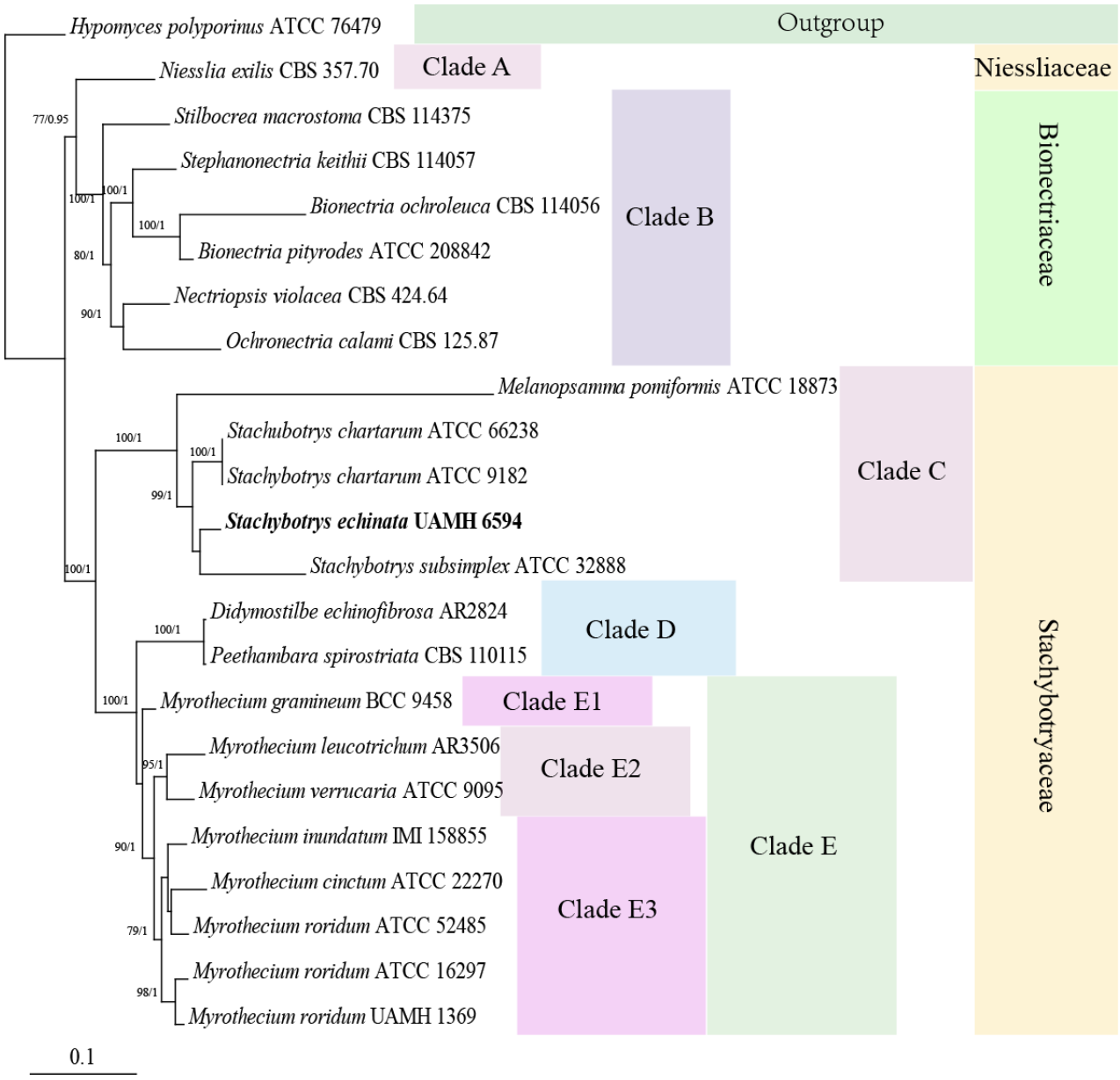

Fig. 3 – Maximum Likelihood (ML) majority rule consensus tree based on a combined SSU, LSU, ATP6, EF1-α and RPB1 sequence data. ML bootstrap support values higher than 70% and Bayesian posterior probabilities (PP) above 95% are given above or under the branches (ML/PP). The tree is rooted with *Hypomyces polyporinus* (ATCC 76479). Ex-type strains are in bold.

Myrothecium at the species level

ITS sequences data can be considered as the primary barcode for *Myrothecium* species because its sequence data can reliably identify 73% of taxa studied across kingdom Fungi and has a high sequence and PCR success rate (Bridge et al. 2005, Schoch et al. 2012, Nilsson et al. 2014). ITS can be used as a rough and quick identification guide (Udayanga et al. 2012). ITS sequence data provides persuasive evidence for species delineation with a few distantly related taxa analyzed, but confusion occurs when a large number of species from a wide range of host species (or the same host, but different distributions) are analyzed (Udayanga et al. 2012). Branches in the phylogenetic trees should be bifurcate. Any node that has only two intermediate decedents is said to be resolved. Its internal nodes are polytomous (more than two descendents i.e., sister taxa), then relationships are unclear (Udayanga et al. 2011, 2012).

ITS sequence data has been most useful for molecular systematics at the species level, and even within species (e.g. to identify geographic races) (Wang et al. 2015). Furthermore, combined

gene tree could be provided more precise classification system. Udayanga et al. (2012) evaluated the phylogenetic species recognition of *Diaporthe* based on ITS, EF1-α, TUB and CAL sequence data, individually and in combination, to establish a robust concept to circumscribe species in the genus. The result shows that the combined gene analysis provides robust support to delineate cryptic species at the terminal node, and to recognize subclades of closely related taxa across the genus *Diaporthe*. Similarly, Maharachchikumbura et al. (2012) analyzed sequence data from three gene regions (ITS, TUB and EF1-α), individually and in combination, to evaluation their ability to resolve species limits of *Pestalotiopsis*. Combined multi-genes analysis successfully resolved most of the *Pestalotiopsis* species with high bootstrap support. Furthermore, Wikee et al. (2011) identified the species in the genus *Phyllosticta* by combining ITS, ACT and EF1-α. They recommend the improvement of the multi-loci phylogenetic analysis by using more phylogenetically informative genes.

Multi-genes trees have basically resolved the problems of phylogenetic species in *Diaporthe*, *Pestalotiopsis* and *Phyllosticta* and many other phytopathogenic genera (Hyde et al. 2014). In this study, combined analysis of ITS and EF1-α sequence data was also used to evaluate the species of *Myrothecium*.

ITS gene analyses

The ITS gene tree is presented in Fig. 4 from strains of *Didymostilbe, Melanopsamma*, *Peethambara* and *Stachybotrys*. Some Nectriaceae species which previously grouped near the family Stachybotryaceae, in Hypocreales, in the study by Maharachchikumbura et al. (2015), were used in the phylogenetic analyses.

There are 194 ITS sequences for 15 putatively named *Myrothecium* species in GenBank: *M. atroviride* M.C. Tulloch, *M. atrum* M.C. Tulloch, *M. carmichaelii* Grev., *M. cinctum* Sacc., *M. gramineum* Libert, *M. cylindrosporum* (D.Q. Dai & K.D. Hyde 2016), *M. inundatum*, *M. lachastrae* Sacc., *M. leucotrichum* M.C. Tulloch, *M. masonii* M.C. Tulloch, *M. prestonii* M.C. Tulloch, *M. roridum*, *M. setiramosum* R.F. Castañeda, *M. tongaense* W.B. Kendr, DiCosmo & Michaelides, *M. verrucaria*. However, only *M. prestonii* and *M. cylindrosporum* are represented by ex-type strains. Additionally, three uncultured *Myrothecium* isolates and 72 isolates named as *Myrothecium* sp. were obtained from GenBank. We used these data to build a Maximum Likelihood (ML) tree and Bayesian Analyses (PP) (Fig. 4) with *Myrothecium uttaradiensis* strain from Dai et al. (in press), based on ITS sequence data from 66 strains and 16 species of *Myrothecium*, with *Bionectria ochrolenca* (Schwein.) Schroers & Samuels as the outgroup taxon (Table) (Castlebury et al. 2004, Wang et al. 2015, Maharachchikumbura et al. 2015). *Myrothecium* is polyphyletic (Fig. 4) and clustered in seven clades (Clades A-G).

Clade A comprises seven *Myrothecium* strains and *Parasarcopodium ceratocaryi*, Mel'nik, S.J. Lee & Crous. Clade B comprises four *Myrothecium* strains (BBA 65577, HKB 28, WS-11720 and CBS 324.54) and clustered with *Stachybotrys* species. Clades C-G comprise *Myrothecium* strains and species plus *Didymostilbe echinofibrosa* in Clade D. The phylogenetic placement of *Myrothecium* is cryptic, because these species do not clearly separate from other genera. Therefore, using only ITS sequence data for the genealogical classification and analyses of lineages is not reliable. Clade A can be also separated two subclades: Clade A1 as *M. cinctum* complex group and Clade A2 possibly a new species together. This needs to be confirmed by further research.

Family placement of *Myrothecium*

In this study, the generic placement of *Myrothecium* in Stachybotryaceae was evaluated by generating a phylogenetic tree using five genes dataset with *Hypomyces polyporinus* Peck as the outgroup taxon (Fig 3) (Castlebury et al. 2004).

Myrothecium species cluster in the family Stachybotryaceae (Wang et al. 2015) with *Didymostilbe*, *Peethambara* and *Stachybotrys*. The *Stachybotrys* group (Clade C) has high bootstrap support. Clade D comprises *Didymostilbe echinofibrosa* and *Peethambara spirostriata*

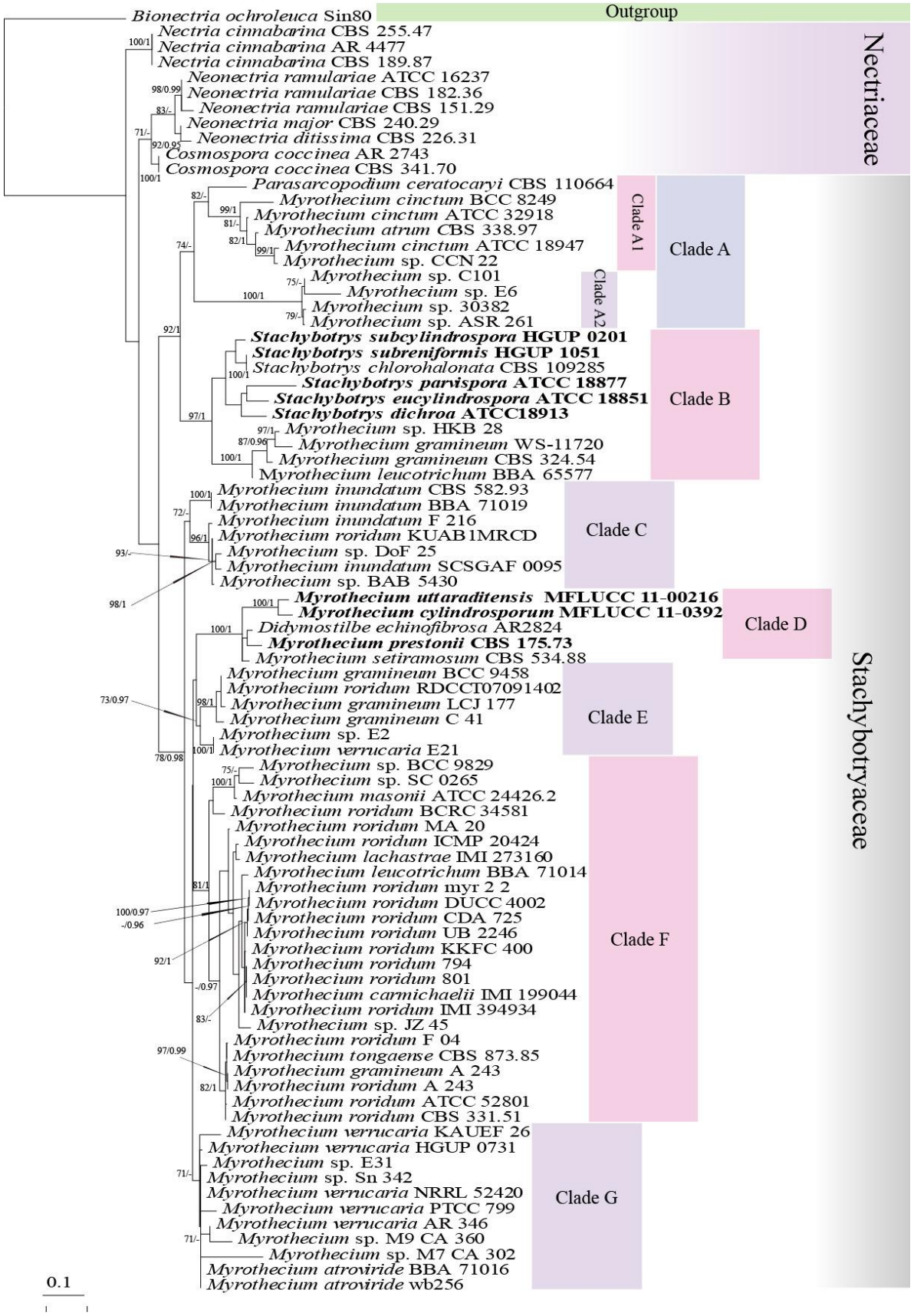

Fig. 4 – Phylogenetic tree of *Stachybotryaceae* species from analysis of ITS sequence data using Maximum Likelihood (ML) method with *Bionectria ochrolenca* as the outgroup taxon. ML bootstrap support values higher than 70% and Bayesian posterior probabilities (PP) above 95% are given above or under the branches (ML/PP). Type or ex-type strains are in bold.

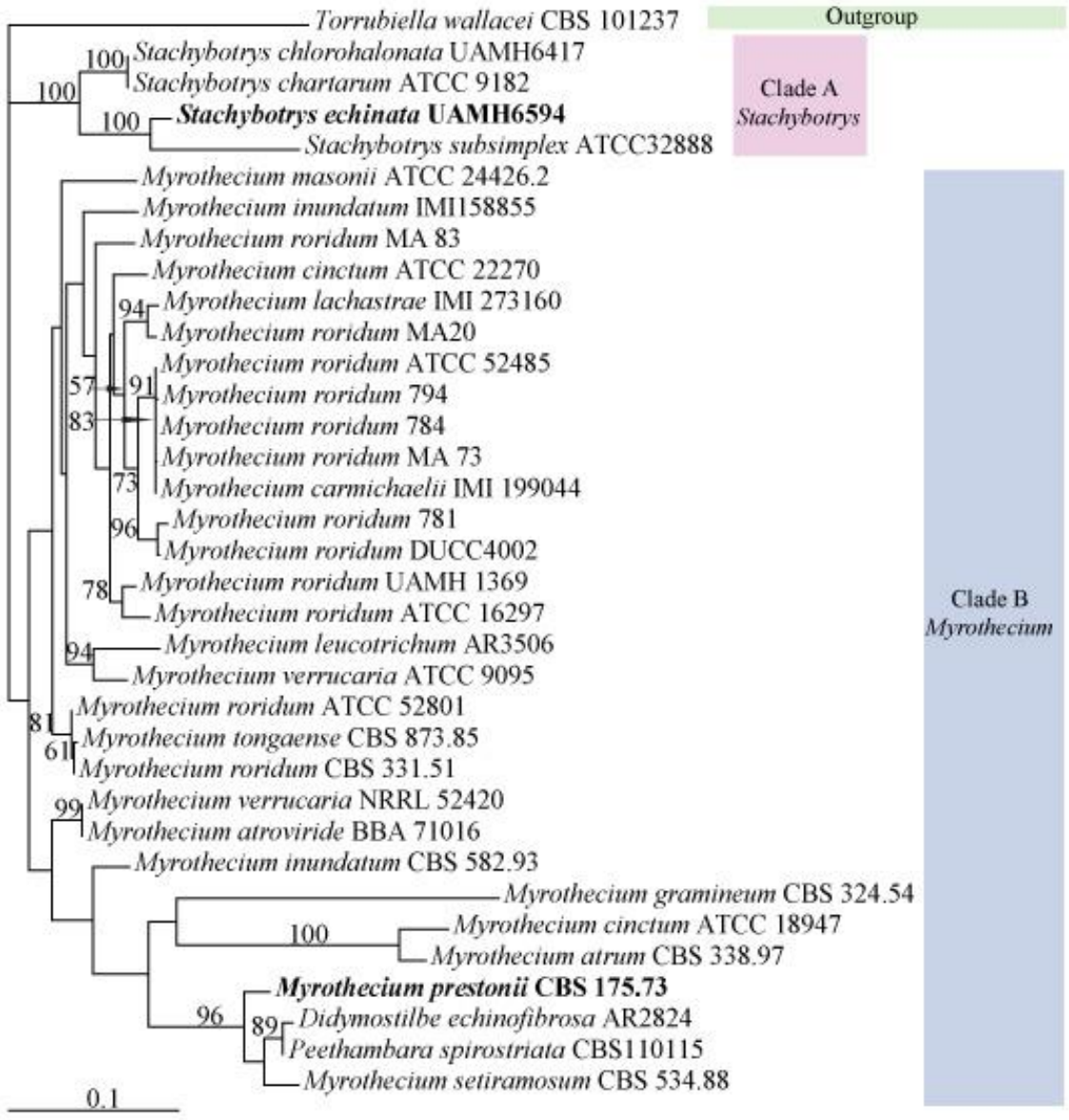

Fig. 5 – Phylogenetic tree of *Myrothecium* species from GenBank generated from combined ITS and EF1-α sequence data based on ML method with *Torrubiella wallacei* (CBS 101237) as outgroup taxon. ML bootstrap support values higher than 50% are given above or under the branches (ML). Type or ex-type strains are in bold.

again with strong support. *Myrothecium* clusters in Clade E which can be separated into three subclades, however there support is relatively low. Clades C, D and E formed an independent lineage, which attained 100% (ML) and 1 (PP) bootstrap value, differentiated from other families in the order Hypocreales (Castlebury et al. 2004, Summerbell et al. 2011).

Clades C, D, E, F and G clustered in one large clade with 78% (ML) and 0.98 (PP) bootstrap support. Within this large clade, strains of variously putatively named *Myrothecium* species are confused, e.g. *M. gramineum* and *M. roridum*, and are likely to be species complexes and / or wrongly named. Clade D includes one type species *M. prestonii* (CBS 175.73), and two ex-type species *M. cylindrosporum* (MFLUCC 11–0392) and *M. uttaraditensis* (MFLUCC 11–00216). *Didymostilbe echinofibrosa* (Finley) Rossman (AR2824) clustered in this clade with three *Myrothecium* species. Thus, *D. echinofibrosa* may be a synonym of *Myrothecium*, or this strain may be wrongly named. The other four Clades (C, E, F and G) comprise a mixture of putatively named *Myrothecium* species. Until the species are epitypified it is unclear which clade represents which

species, although Clade C may comprise *M. inundatum*, Clade E may comprise *M. gramineum*, Clade F may be *M. roridum*, and Clade G may be *M. verrucaria* and all are probably species complexes. *Myrothecium* species in Clades A and B cluster with other genera and this is confusing.

Combined gene analyses of *Myrothecium*

Combined gene analyses were evaluated using ITS and EF1-α sequence data with *Torrubiella wallacei* H.C. Evans as outgroup taxon in Fig. 5 (Maharachchikumbura et al. 2015). The phylogenetic tree illustrated that multi-gene tree could resolve classification better than single gene trees of ITS and EF1-α respectively (data not shown). In Fig. 5, *Myrothecium* species cluster in Clade B, but there is a low bootstap support mainly because of 75 percent of *Myrothecium* strainslack gene. *Didymostilbe echinofibrosa* (AR2824) and *Peethambara spirostriata* (CBS 110115) classify near *M. prestonii* (CBS 175.73) and *M. setiramosum* (CBS 534.88), the same as the analyses of ITS gene tree. Some species are differently placed in the genus in the ITS tree, such as *M. gramineum* (CBS 324.54) which separates from *Stachybotrys* (Clade A), and *M. atrum* (CBS 338.97) and *M. cinctum* (ATCC 18947) which cluster into the large *Myrothecium* clade (Clade B). Even though many species of *Myrothecium* lack sequence data in GenBank, the analyses used in this study suggests that combine genes provide a better solution to determine species of *Myrothecium*.

Research prospects

Species of *Myrothecium* are presently difficult to resolve because of few distinct morphological characters and lack of voucher specimens with molecular data. The type species, *M. inundatum* is in urgent need of epitypification or being provided with a reference specimen (*sensu* Ariyawansa et al. 2014). The majority of species in GenBank only have ITS sequence data and this does not resolve species well, EF1-α is also available for some *Myrothecium* species and this maybe a better gene candidate. Multi-gene sequence analyses can better resolve species in the genus, however, as only few ex-type strains are available and most strains in GenBank lack more than ITS sequence data, a thorough study of the genus is needed.

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