



## *Sarocladium brachiariae* sp. nov., an endophytic fungus isolated from *Brachiaria brizantha*

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### Abstract

In a survey on the diversity of endophytic fungi of *Brachiaria brizantha*, a new species of *Sarocladium* was isolated and proposed here as *Sarocladium brachiariae*. According to the LSU and ITS rDNA sequences and culture morphology and micromorphology, the species differed from the species hitherto described in *Sarocladium*, and is characterized as a new species. The new species can produce hyphal coils and slimy heads. In addition, raised, cottony, moist to slimy colonies on PDA, and adelophialides, branched conidiophores were also useful characters for distinguishing species from other species of *Sarocladium*.

**Keywords** – Endophytes – *Sarocladium* – Phylogeny – Taxonomy

### Introduction

*Sarocladium* was established by Gams and Hawksworth (1976) based on a species described by Sawada in Taiwan, firstly including two fungal pathogens causing sheath rot of rice. Based on a recent molecular phylogenetic study of rDNA sequences, Summerbell et al. (2011) transferred seven species, formerly named *Acremonium kiliense*, *A. strictum*, *A. zae*, *A. bacillisporum*, *A. bactrocephalum*, *A. glaucum* and *A. ochraceum*, to the genus. Yeh & Kirschner (2014) introduced a new species named *Sarocladium spinificis*. Giraldo et al. (2015) used multilocus phylogenetic inferences combined with phenotypic data, to introduce *S. bifurcatum*, *S. gamsii*, *S. hominis*, *S. pseudostrictum*, *S. subulatum* and *S. summerbellii*, and re-allocated *A. implicatum* and *A. terricola* to the genus. *Sarocladium attenuatum* is confirmed as synonym of the type species of the genus, *S. oryzae*.

*Sarocladium* presently encompasses 18 species (Summerbell et al. 2011, Giraldo et al. 2015). Although *Sarocladium* species have traditionally been considered as important phytopathogens (Gams and Hawksworth 1976, Ayyadurai et al. 2005), the genus also contains opportunistic human pathogens (Fincher et al. 1991, Perdomo et al. 2011), and other properties of applied value. For example, *S. oryzae* produces antibiotics (Bills et al. 2004), and *S. zae* (Gams & Sumner, 1971) is considered as a mutualistic endophyte in maize (Wicklow et al. 2008). Endophytic fungi form nonpathogenic and intercellular associations with host, completing their entire life cycle in the plant

(Kelemu et al. 2001). Species of the genus *Sarocladium* are commonly associated with members of Poaceae (Yeh & Kirschner 2014, Giraldo et al. 2015). Some endophytic fungi are potential biological control agents for plant pathogens (Kelemu et al. 2001). Endophytic-infected plants also possess other properties such as growth stimulation, improved survival, and drought tolerance (Lim et al. 2000, Yang et al. 2014). *Brachiaria* are commercially important forage grasses in tropical area. In this study, we collected endophytic fungi associated with *Brachiaria* spp. in China, and a species was identified as a member of *Sarocladium*, but was not identical to any of the previously described species.

## Materials & Methods

### Plant tissue staining, fungal isolations, and culture maintenance

In 2005, healthy grass leaves and sheaths of *Brachiaria brizantha* were collected from Danzhou, China. Plant tissue staining and endophytic fungal isolation were conducted according to the method described by Kelemu et al. (2001). Samples which appeared to have intercellularly growing hyphae were noted, and plants from which the samples originated were used for further fungal isolation. Small pieces (5 mm) of tissues that surface sterilized were plated on potato-dextrose agar, and incubated for 4 to 6 weeks at 28 °C. Cultures were effectively maintained either on PDA supplemented with 10 µg/mL tetracycline, or, for long-term storage, by lyophilization according to the method described by Kelemu et al. (2001).

### DNA extraction, amplification and sequencing

Isolates grow on PDA for 10 days at 25 °C. Genomic DNA was extracted using Genomic DNA Spin Kit (Plant), according to the modified manufacturer's protocol (TIANGEN Co., Ltd., China). The internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) and D1/D2 domains of the large-subunit nrRNA were amplified with the primer pairs ITS1/ITS4 and NL1/NL4b, respectively (White et al. 1990, O'Donnell 1993). The PCR products were visualised on an electrophoresis gel after GoldView (Solarbio, China) staining. PCR products of the expected size were purified with E.Z.N.A.<sup>TM</sup> Gel Extraction Kit I (OMEGA) according to the manufacturer's instructions. DNA sequencing was done by BGI Biotech (Shenzhen, China) with 3730 DNA Analyzer (Applied Biosystems).

### Alignment and phylogenetic analysis

DNA Sequences were assembled and edited using SeqMan II software (DNASStar, Inc., Madison, Wis.). Related DNA sequences of ITS and LSU rDNA were compared using the BLAST function of GenBank. For phylogenetic analysis, sequences retrieved from the BLAST search and the taxon sampling based on ITS and LSU rDNA sequences (Yeh & Kirschner 2014, Giraldo et al. 2015) were used (Table1). In addition to sequences of *Sarocladium* spp., *Acremonium* species closely related with *Sarocladium* spp., but outside the *Sarocladium* clade, were included as outgroup (Giraldo et al. 2012). Multiple sequence alignments were performed with Clustal W using MEGA 5 (Tamura et al. 2011) and manually corrected where necessary. Nucleotide substitution models were generated using MrModeltest v. 2.3 (Nylander et al. 2008). A maximum likelihood phylogenetic analysis of the dataset was performed with PAUP v.4.0b10 (Swofford 2002). Markov Chain Monte Carlo (MCMC) sampling was used to reconstruct phylogenies in MrBayes v. 3.2 (Ronquist & Huelsenbeck 2003). Analyses of 2 MCMC chains based on the full dataset were run for  $1 \times 10^7$  generations and sampled every 100 generations. The alignments and trees in TreeBASE ([http://purl.org/phylo/treebase/phyloids/study/TB2:S20969?x-access-code=e79cef78c47c3d70ebcb\\_7314c8a991ab&format=html](http://purl.org/phylo/treebase/phyloids/study/TB2:S20969?x-access-code=e79cef78c47c3d70ebcb_7314c8a991ab&format=html)), and taxonomic novelties in MycoBank (Crous et al. 2004).

## Phenotypic studies

Morphological characterization of the fungal isolates was carried out based on cultures grown on potato dextrose agar (PDA), oatmeal agar (OA; filtered oat flakes after 1 h of simmering 30 g, agar 20 g, distilled water to final volume of 1000 mL) and malt extract agar (malt extract 20 g, peptone 1 g, glucose 20 g, agar 15 g, H<sub>2</sub>O 1000 ml). Cultures were incubated at 25 ± 1 °C in the dark and periodically examined each 7 days up to 4 weeks. Colony diameters were measured after 14 days of growth, and colony colours determined using the colour charts of Kornerup & Wanscher (1978). In addition, the ability of the isolates to grow at 15, 20, 25, 30, 35, 37 and 40 °C was determined on PDA. Conidiophore morphology was observed on cultures grown on dilute malt agar (malt extract 5 g, 12 g agar, 1000 ml distilled H<sub>2</sub>O) supported on microscope coverslips, other microscopic features were examined and measured by slide cultures on OA, using an Ni-E light microscope (Nikon Corporation, Japan). Photomicrographs were made with a Nikon DS-Ri1 light microscope (Nikon Corporation, Japan). Scanning electron microscope (SEM) micrographs were obtained with a S-3000N scanning electron microscope (Hitachi, Japan).

## Results

### Phylogenetic analysis

The BLAST query revealed that the isolate was shown to be closely related to *Sarocladium* on the basis of its D1/D2 and the ITS regions. Sequences of the D1/D2 (889 bp) and ITS (585 bp) were deposited in GenBank as KP715271 and EU880834, respectively. When comparing the BLAST search results among sequences exceeding a length of 831 bp of D1/D2 fragments, the highest similarity between our isolate and other identified *Sarocladium* species was 99%, namely with one strain of *Sarocladium oryzae* (GenBank number HG965047, Giraldo et al. 2015). The highest similarity of ITS rDNA fragments was 95%, namely with one strain of *Sarocladium kiliense* (Genbank number LN864540, Asadzadeh 2015). The systematic positions of *Sarocladium* sp. was estimated by the combined analysis from ITS and D1/D2 partial gene consisted of 980 characters including alignment gaps. The phylogenetic analysis allowed distributing the isolates included in this study into 18 lineages (Fig. 1). These lineages were phylogenetically distant enough to be considered as different species.

### Taxonomy

On the basis of phylogenetic analysis and phenotypic features, we propose that the isolated fungus *Sarocladium* sp. is different from any previously described species in this genus and therefore are proposed as new.

*Sarocladium brachiariae* X.B. Liu, G.X. Huang & Z.K Guo sp. nov.

MycoBank number: MB 814539, Facesoffungi number: FoF 03372

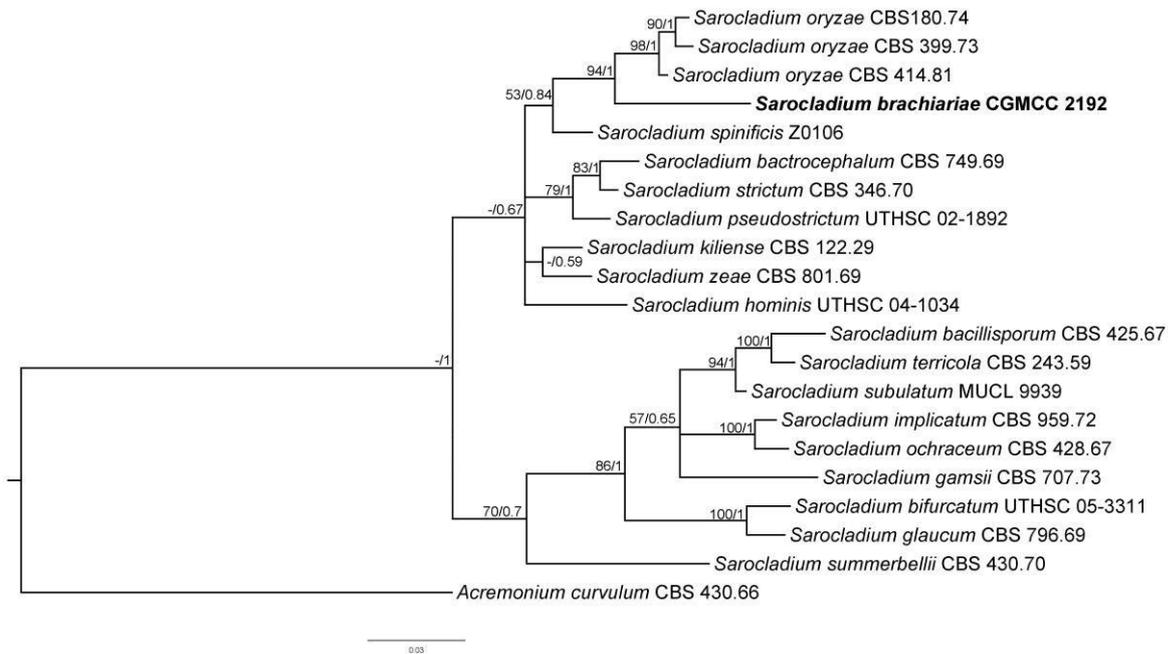
Etymology – Referring to the host (*Brachiaria brizantha*).

Fig. 2A-K

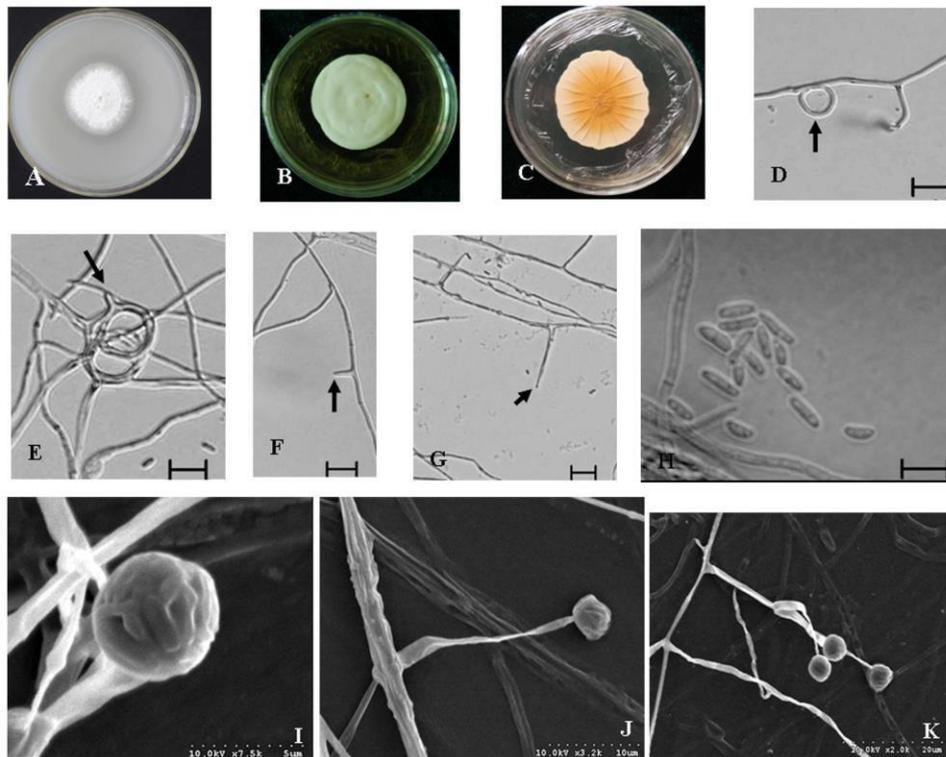
**Table1** Species and strains included in the study, their origin and GenBank accession numbers.

Species	Strain	Source	GenBank accession no.		
			D1/D2 domains of 28SrRNA gene	ITS region	References
<i>Sarocladium bacillisporum</i>	CBS 425.67 <sup>T</sup>	Soil, Ontario, Canada	HE608658	HE608639	Giraldo et al. 2015
<i>Sarocladium bactrocephalum</i>	CBS 749.69 <sup>T</sup>	Ustilago sp., Canada	HQ231994	HG965006	Giraldo et al. 2015
<i>Sarocladium bifurcatum</i>	UTHSC05-3311 <sup>T</sup>	Bronchoalveolar lavage fluid, USA	HG965057	HG965009	Giraldo et al. 2015
<i>Sarocladium gamsii</i>	CBS 707.73 <sup>T</sup>	Dead stem of Pandanus lerum, Sri Lanka	HG965063	HG965015	Giraldo et al. 2015
<i>Sarocladium glaucum</i>	CBS 796.69 <sup>T</sup>	Woolen overcoat, Solomon Islands	HE608657	FN691454	Giraldo et al. 2015
<i>Sarocladium hominis</i>	UTHSC04-1034 <sup>T</sup>	Right calf tissue, USA	HG965060	HG965012	Giraldo et al. 2015
<i>Sarocladium implicatum</i>	CBS 959.72 <sup>NT</sup>	Desert soil, Egypt	HG965072	HG965023	Giraldo et al. 2015
<i>Sarocladium kiliense</i>	CBS 122.29 <sup>T</sup>	Skin, Germany	HQ232052	FN691446	Giraldo et al. 2015
<i>Sarocladium mycophilum</i>	CBS 166.92 <sup>T</sup>	Cortinarius subsertipes, Germany	HG965046	HG965024	Giraldo et al. 2015
<i>Sarocladium ochraceum</i>	CBS 428.67 <sup>T</sup>	Zea mays, Kenya	HQ232070	HG965025	Giraldo et al. 2015
<i>Sarocladium oryzae</i>	CBS180.74 <sup>ET</sup>	Oryza sativa, India	HG965047	HG965026	Giraldo et al. 2015
<i>Sarocladium oryzae</i>	CBS 399.73	Oryza sativa, India	HG965048	HG965027	Giraldo et al. 2015
<i>Sarocladium oryzae</i>	CBS 414.81	Oryza sativa, Nigeria	HG965049	HG965028	Giraldo et al. 2015
<i>Sarocladium pseudostrictum</i>	UTHSC 02-1892 <sup>T</sup>	Sputum, USA	HG965073	HG965029	Giraldo et al. 2015
<i>Sarocladium strictum</i>	CBS 346.70 <sup>T</sup>	Triticum aestivum, Germany	HQ232141	FN691453	Giraldo et al. 2015
<i>Sarocladium subulatum</i>	MUCL9939 <sup>T</sup>	Soil, Egypt	HG965075	HG965031	Giraldo et al. 2015
<i>Sarocladium summerbellii</i>	CBS 430.70 <sup>T</sup>	Soil from greenhouse, The Netherlands	HG965078	HG965034	Giraldo et al. 2015
<i>Sarocladium terricola</i>	CBS 243.59 <sup>T</sup>	Forest soil, USA	HE608659	FN706553	Giraldo et al. 2015
<i>Sarocladium zeae</i>	CBS 800.69 <sup>T</sup>	Zea mays stalk, USA	HQ232152	FN691451	Giraldo et al. 2015
<i>Sarocladium spinificis</i>	Z0106 Ex-type	Root of Spinifex littoreus, Taiwan	JQ954463	KF269096	Yeh & Kirschner 2014
<i>Sarocladium</i> sp. (= <i>Sarocladium brachiariae</i> )	CGMCC 2192 <sup>T</sup>	Leaves of <i>Brachiaria brizantha</i> , China	KP715271	EU880834	This study
<i>Acremonium curvulum</i>	CBS 430.66 <sup>T</sup>	Wheat field soil, Germany	HE608656	HE608638	Giraldo et al. 2012

Note: <sup>ET</sup> = Epitype strain; <sup>NT</sup>=Neotype strain ; <sup>T</sup> = type strain



**Fig. 1** Maximum-likelihood (ML) tree and Bayesian analysis obtained from the combined DNA sequence data from two loci (D1/D2 and ITS). Bootstrap support values above 50 % and Bayesian posterior values above 0.5 are shown at each node (ML/PP) are shown at the nodes; *Acremonium curvulum* is used as outgroup.



**Fig. 2** *Sarocladium brachiariae* (CGMCC 2192<sup>T</sup>). A. Colonies on OA after 14 days at 25 C; B, C. colonies on PDA after 14 days at 25 C; D. Hyphal coil (arrow); E. Adelophialides in a hyphal coil (arrow); F. adelophialides (arrow); G. phialide with distinct periclinal thickening at the apex (arrow); H. conidia; I. conidia arranged in slimy heads; J, K. simple and branched conidiophores with conidia arranged in slimy heads. — Scale bars: D–H = 10  $\mu$ m.

Type – HND5, CGMCC 2192.

Colonies on OA at 25 °C attaining 30–35 mm in 14 days, white, flat, powdery. On PDA attaining 30–37 mm at 25 °C after 14 days in the dark, white to yellowish white, raised, cottony, moist to slimy, reverse pale orange, sometime radially folded. Vegetative hyphae septate, hyaline, smooth- and thin-walled, 1–2 µm wide, sometimes bound together into ropes, could produce hyphal coil and net form structure. Conidiophores erect, arising directly from vegetative hyphae, simple or poorly branched, hyaline to subhyaline. Phialides straight or slightly flexuous, subulate, 14–30 (–40) µm long, 2–2.5 µm wide at the base, with a distinct periclinal thickening at the conidiogenous locus, hyaline, thin- and smooth-walled; adelophialides present on OA, 2–8 µm long, 1.5 µm wide at the base. Conidia unicellular, cylindrical with rounded ends, 3–8 (–11) × 1–1.5µm, hyaline to subhyaline, thin- and smooth-walled, arranged in slimy heads. Chlamydospores and sexual morph not observed.

Material examined – CHINA. Hainan province: Danzhou, Chinese Academy of Tropical Agricultural, on leaves of *Brachiararia brizantha*, 10 September 2005, X.B Liu, HND5 (CGMCC 2192, type).

## Discussion

*Sarocladium* is a hyphomycetous genus belonging in the family *Hypocreaceae*, order *Hypocreales* and class *Sordariomycetes* (Maharachchikumbura et al. 2015, 2016) and has been well-studied by various authors (Summerbell et al. 2011, Yeh & Kirschner 2014, Giraldo et al. 2015) Based on the combination of a multilocus analysis and phenotypic features, we propose and describe a new species of *Sarocladium*, *S. brachiarariae*. Apart from DNA sequence differences, the species is proposed as new because of raised, cottony and moist to slimy colonies on PDA, and branching conidiophore, adelophialides, cylindrical conidia arranged in slimy heads. Key morphological feature of the fungi is the production of hyphal coil. Giraldo et al. (2015) showed that all the *Sarocladium* species producing cylindrical conidia arranged in slimy heads, grouped in the same lineage, while those species with fusiform conidia arranged in chains, or/and slimy heads were distributed in other clades. This distribution is in agreement with our study. *Sarocladium brachiarariae* together with *S. strictum*, *S. hominis*, *S. pseudostrictum*, *S. bactrocephalum*, *S. kiliense*, *S. spinificis*, *S. oryzae* and *S. zae* formed a clade morphologically characterized by cylindrical or ellipsoidal conidia arranged in slimy heads.

*Sarocladium brachiarariae* can be differentiated from *S. hominis*, *S. pseudostrictum*, *S. bactrocephalum* and *S. zae* by its faster growth rate on OA and PDA at 25 C, its ability to grow at 37 C, and its adelophialides (Giraldo et al. 2015); *Sarocladium kiliense* differs in the formation of chlamydospores and appears as dirty orange to pale ochraceous colonies on OA (Summerbell et al. 2011); *S. spinificis* differs from *S. brachiarariae* in the absent of branched conidiophores and having longer conidia (Yeh & Kirschner 2014); *S. brachiarariae* nests in a well-supported clade together with *S. oryzae*, but can be differentiated in the following features: *S. oryzae* produces repeatedly branched conidiophores and colonies with a dull buff grey to dull bluish green reverse on PDA (Bills et al. 2004). *S. brachiarariae* is morphologically very similar with *S. strictum*, such as raised, cottony and moist to slimy colonies on PDA, hyphae bound together into ropes, branched conidiophores, adelophialides, conidia, and able to grow at 37 C, but *S. brachiarariae* can produce hyphal coils, which is specific to this species.

Traditionally, species of *Sarocladium* have been reported as plant pathogens or as saprobes (Gams & Hawksworth 1976, Chen et al. 1986). However, numerous studies have demonstrated that some also be involved in endophyte of plant, which displayed significant antifungal activity against plant pathogenic fungi (McGee et al. 1991, Kelemu et al. 2001, Wicklow et al. 2005, Summerbell et al. 2011, Yeh & Kirschner 2014). Surprisingly, the new species prominently shows broad-spectrum inhibition to growth of many tested plant pathogenic fungi on solid media plate, including *Colletotrichum gloeosporioides* of mango trees, *Fusarium oxysporium* f.sp. cubense, *Gloeosporium musarum*, *Colletotrichum gloeosporioides* of rubber trees, *Corynespora cassicola* of

papaya, *Fusarium oxysporium* of bamboo, *Magnaporthe grisea*, *Bipolaris oryzae* Shoem, *Colletotrichum falcatum* Went and *Drechslera* sp. (data no shown).

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