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A NEW ENDOPHYTIC ASCOMYCETE ASSOCIATED WITH THE MEDICINAL PLANT, ACHYRANTHES *BIDENTATA BLUME* (AMARANTHACEAE)

BING-DA SUN[1,2#], AMANDA-JUAN CHEN[1#], WEI-WEI GAO[1*], YU-GUANG ZHOU[2], M. USMAN GHAZANFAR[3] AND WAQAS WAKIL[4]

[1]Institute of Medicinal Plant Development, Chinese Academy of medical Sciences and Peking Union Medical College, Beijing 100193, P.R. China; [2]Institute of Microbiology, Chinese Academy of Sciences, Beichen West Road, Chaoyang District, Beijing 100101, P.R. China; [3]Department of Plant Pathology, University College of Agriculture, University of Sargodha 40100; [4]Department of Agri. Entomology, University of Agriculture, Faisalabad 38000, Pakistan
[*]Corresponding author, e-mail address: wwgao411@sina.com
[#]These authors contributed equally to this study

Abstract

During a survey of the endophytic fungi associated with the Chinese traditional medicinal plant *Achyranthes bidentata* Blume (Amaranthaceae), a new fungus was isolated from the stem of this plant. Cultures of this fungus on PDA form grey floccose colony with a reddish-brown reverse and mycelium that develops mostly right-angled branches and form rope-like strands and coils. This endophytic fungus does not form reproductive structures on artificial media but can produce conidiomata on host leaves. Based on morphological and DNA sequence analyses, this fungus is proposed to be a new member of the ascomycete genus, *Edenia* and the name *E. achyranthi* is introduced.

Introduction

The genus *Edenia* was firstly introduced for a sterile endophytic fungus isolated from leaves of *Callicarpa acuminata* (Verbenaceae) in Mexico (González *et al.,* 2007). The genus was characterized by producing numerous sterile, whitish mycelial strands and coils on PDA. Only one species has been reported in this genus till this time. Crous *et al*., (2009) found an *Edenia* isolate from *Cassia alata* (Leguminosae) in Philippines and the isolate became fertile on Oatmeal agar (OA). Also, the Philippine isolate was thought to be pathogen of *C. alata* because it was associated with a hyphomycete sporulating on leaf spots of the plant. The isolates from Mexico and Philippines were believed to belong to same fungus because of the same colony characteristics and the identical DNA sequence data.

Achyranthes bidentata Blume (Amaranthaceae) is a traditional Chinese medicinal plant that has wide distribution in the north part of China that other medicinal plants harbour endophytic mycoflora (Khan *et al*., 2010). It is usually prescribed by practitioners of traditional Chinese medicines for the treatment of osteodynia of lumbar and knees, spasm and flaccidity of limbs (Anon., 2005). Root disease was seldom found during the planting of *A. bidentata* and it could be cropped continuously (Li, 2008). Plants in Amaranthaceae were thought to be less infected by mycorrhizal fungi (Peterson *et al.,* 1985; Shanker *et al.,* 1990), so the mutualistic effects of endophytic fungi on *A. bidentata* might play important role in environmental adapting of the plant. However, few studies have been focused on the endophytic fungi associated with Amaranthaceae plants and nothing is known about the fungal endophytes in *A. bidentata*. During the year 2008 and 2009 a project to study the biodiversity of endophytic fungi associated with *A. bidentata* was undertaken. Based on this study, we are describing a new endophytic fungal species to the genus *Edenia*.

Materials and Methods

Sample collection: The sample collection was conducted in monoculture fields at Anguo county (N 38°23', E 115°18', Hebei province) on September 7th 2009. A total of 30 asymptomatic plants (including leaves, stems and roots) were collected from three fields. After taking back to the laboratory, the samples were stored at 4°C and processed within 2 d of collection.

Isolation of endophytic fungi: Two segments with eustipes at both ends (large segment) were collected randomly from each of plants and three short segments of 0.5cm in length were selected from each large segment. A total of 100 short stem segments were screened for the occurrence of endophytic fungi.

Plant materials were thoroughly washed in distilled water before surface sterilization. Surface sterilization was performed by the following immersion sequence: 75% ethanol for 1 min, NaClO (3% available chlorine) for 3 min and 75% ethanol for 1 min (Khan *et al.,* 2010). The samples were then dried on sterilized paper before cutting into small segments or slices. Four segments were evenly placed in each 90mm Petri dish containing 2% malt extract agar (MEA) supplemented with chloromycetin (100 mg/L) and Rose Bengal (33 mg/L) (Photita *et al.,* 2005). Petri dishes were sealed, incubated for 2 weeks at 25°C. The pure endophytic fungi strains were transferred to new MEA slants.

Description and preservation: Among the fungi recovered was an interesting isolate named AS-60. This fungus was cultured with potato dextrose agar (PDA: scrubbed and diced potatoes 200g, dextrose 20g, agar 15g, distilled water 1L) and malt extract agar (MEA: malt extract 20g, dextrose 20g, peptone 1g, agar 15g, distilled water 1L) for the examination of colony characteristics. We attempted to induce formation of reproductive structures by inoculating the fungus on oatmeal agar (OA: oatmeal 30g, agar 15g, distilled water 1L) and small

pieces of sterilized leaves of *A. bidentata*. The morphology of this fungus was examined using light microscopy and photomicrographs were taken with Zeiss Stemi 200-C and Zeiss Axioplan 2 imaging microscopes. For preservation, a living culture of this new endophytic ascomycete was stored in liquid nitrogen vapor and at -80°C in cryoprotectant (15% (v/v) glycerol in distilled water) in China general microbiological culture collection center (CGMCC) with the accession number 3.14305. Dried cultures have been deposited in the Herbarium MycoloGicum Academiae Sinicae (HMAS) under the accession number 242793.

Molecular procedures: Nuclear ribosomal DNA internal transcribed spacer (ITS) regions (ITS1-5.8S rDNA-ITS2) of strain AS-60 were amplified and sequenced using primers ITS5 and ITS4 following the procedure of White *et al*., (1990). Nucleotide-nucleotide BLAST (megablast) search of the 507bp amplicon against the nr database of NCBI (www.ncbi.nlm.nih.gov) suggested strain AS-60 was a member of the genus *Edenia*. This was confirmed by phylogenetic tree constructed with the sequences of AS-60 and similar taxa retrieved by BLAST search (Table 1). The software MEGA version 4.0 (Tamura *et al.,* 2007) was used for the phylogenetic analyzing processing (neighbor joining method) and two *Aureobasidium pullulans* sequences (Table 1) were chosen as out group.

Table 1. Sequences retrieved from GenBank for the construction of phylogenetic tree.

Fungal species	Strain No.	Host plant	Location	GenBank accession No.	Cited in publication
Aureobasidium pullulans	CBS 105.22	unknown	unknown	FJ150886	Yes, Zalar *et al.* , 2008
Aureobasidium pullulans	CBS 584.75	*Vitis vinifera*	France	FJ150906	Yes, Zalar *et al.* , 2008
Coniothyrium palmarum	CBS 400.71	*Chamaerops humilis*	Italy	AY720708	Yes, Lennox *et al.* , 2004
Didymella bryoniae	CBS 233.52	*Trifolium repens*	Germany	EU167573	Yes, Simon *et al.* , 2009
Didymella rabiei	CBS 581.83A	*Cicer arietinum*	Syria	EU573020	Yes, Simon *et al.* , 2009
Edenia gomezpompae	C1c	*Callicarpa acuminata*	Mexico	EF565744	Yes, González *et al.* , 2007
Edenia gomezpompae	CBS 124106	*Senna alata*	Philippine	FJ839619	Yes, Crous *et al.* , 2009
Edenia gomezpompae	UFMGCB 2177	*Solanum cernuum*	Brazil	HM997129	No, only present study
Leptospora rubella	CPC 11006	unknown	unknown	DQ195780	Yes, Crous *et al.* , 2006
Phaeosphaeriopsis musae	CBS 120026	*Musa* sp.	Mauritius	DQ885894	Yes, Crous *et al.* , 2009
Phaeosphaeriopsis sp.	TMS-2011	*Miscanthus* sp.	American	HQ630983	Yes, Shrestha *et al.* , 2011
Phaeosphaeria nodorum	DAOM 215173	unknown cereal plant	unknown	U04237	Yes, Morales *et al.* , 1995
Phoma exigua var. *exigua*	CBS 118.94	*Trifolium repens*	Germany	EU167567	Yes, Simon *et al.* , 2009
Phoma pinodella	CBS 318.90	*Pisum sativum*	Netherlands	EU573028	Yes, Irinyi *et al.* , 2009
Phoma sojicola	CBS 567.97	*Glycine max*	Hungary	EU573026	Yes, Irinyi *et al.* , 2009
Pyrenochaeta sp.	YS-2010	*Stipa grandis*	China	HM007089	Yes, Su *et al.* , 2010

Results

Phylogenetic analysis: Nucleotide-nucleotide BLAST search using the sequence of AS-60 (GenBank accession #**JF737856**) against the nr database of NCBI showed that sequences belonging to the genera of Pleosporales (including *Didymella*, *Edenia*, *Phaeosphaeria*, *Phaeosphaeriopsis*, *Phoma* and *Pyrenochaeta* etc.) produced significant alignments. The optimal Neighbor-Joining tree (Fig. 1) constructed using sequences of these genera and that of AS-60 had the sum of branch length of 0.990, which was computed using the Maximum Composite Likelihood method. The sequence of AS-60 together with three *Edenia gomezpompae* sequences (**EF565744**, **FJ839619** and **HM997129**) was clustered into one clade. Although the 3 *E. gomezpompae* isolates were from different countries and different hosts, they had almost identical ITS sequences. The isolate AS-60 was separated from the three *E. gomezpompae* isolates with 100% bootstrap support (1000 replications).

Taxonomic Description

Edenia achyranthi B.D. Sun, A.J. Chen & W.W. Gao anam. **sp. nov**. Fig. 2-11.
MycoBank #**563137**, GenBank #**JF737856**

Etymology: The epithet achyranthi refers to the genus of the host plant.

Coloniae in agaro decocto tuberorum (PDA), celeriter crescentes 55-60 mm diametro in 14 diebus 25°C, primo albae, deinde cinerae cum mycelio fasciculus nigero, intermidius, abundus; reversum rubro-brunnea. Hyphae hyalinae, leptodermica, aequata, septatae, saepe ramificatione in angulis 90° plerumque, flexuosae, convolventes, fila funiformia et spiras formantes. Conidia semipellucida, aequata, leptodermica, elliptica vel paulo constrictae in basi, 3.5–6.1×1.7–2.5 µm.

Colonies grow fast on PDA, attaining 55-60 mm diam in 14 d at 25°C, at first whitish, later becoming gray, velvety to floccose, with abundant dark hyphal bundles in central part, reverse reddish-brown. Mycelium sterile, asexual and sexual spores and soporiferous structures unknown. Hyphae hyaline, thin-walled, smooth to undulate, septate, 1.0-3.7 µm diam, frequently developing by 90° angle branching, intertwining and forming rope-like strands and coils.

Colonies on MEA whitish to olive-gray, attaining 60-65 mm diam in 14 d at 25°C, reverse cinnamon rufous; on OA whitish, thin, attaining 50-55 mm diam in 14 d at 25°C, reverse colorless; only poorly sporolated on sterilized leaves of *A. bidentata*, Conidia subhyaline, smooth, thin-walled, ellipsoidal or slightly narrowed at base, 3.5-6.1×1.7-2.5µm. Teleomorph unknown.

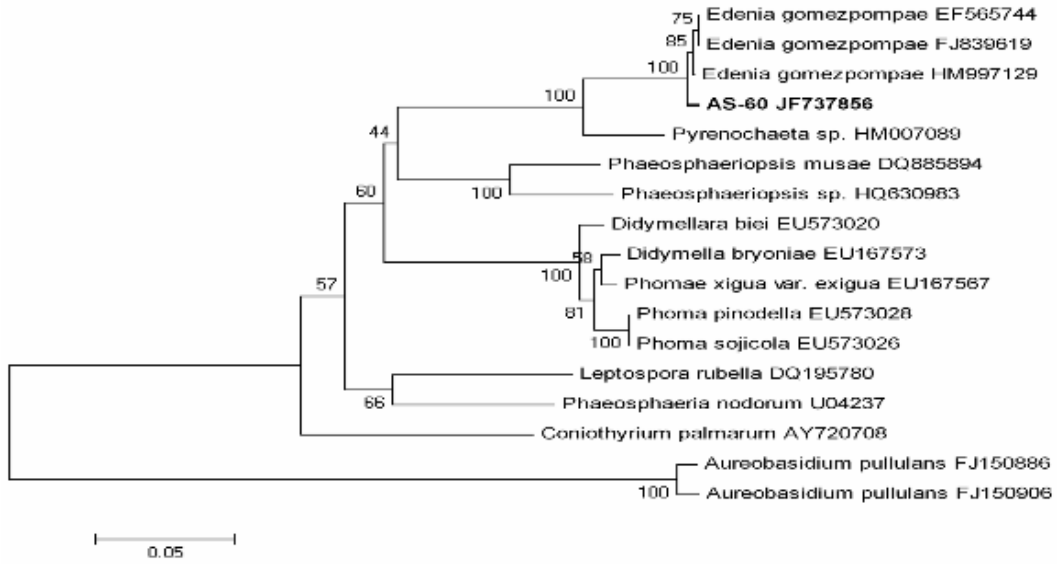

Fig. 1. The optimal Neighbor-Joining tree constructed using sequences of Pleosporales genera and that of AS-60 (bold).

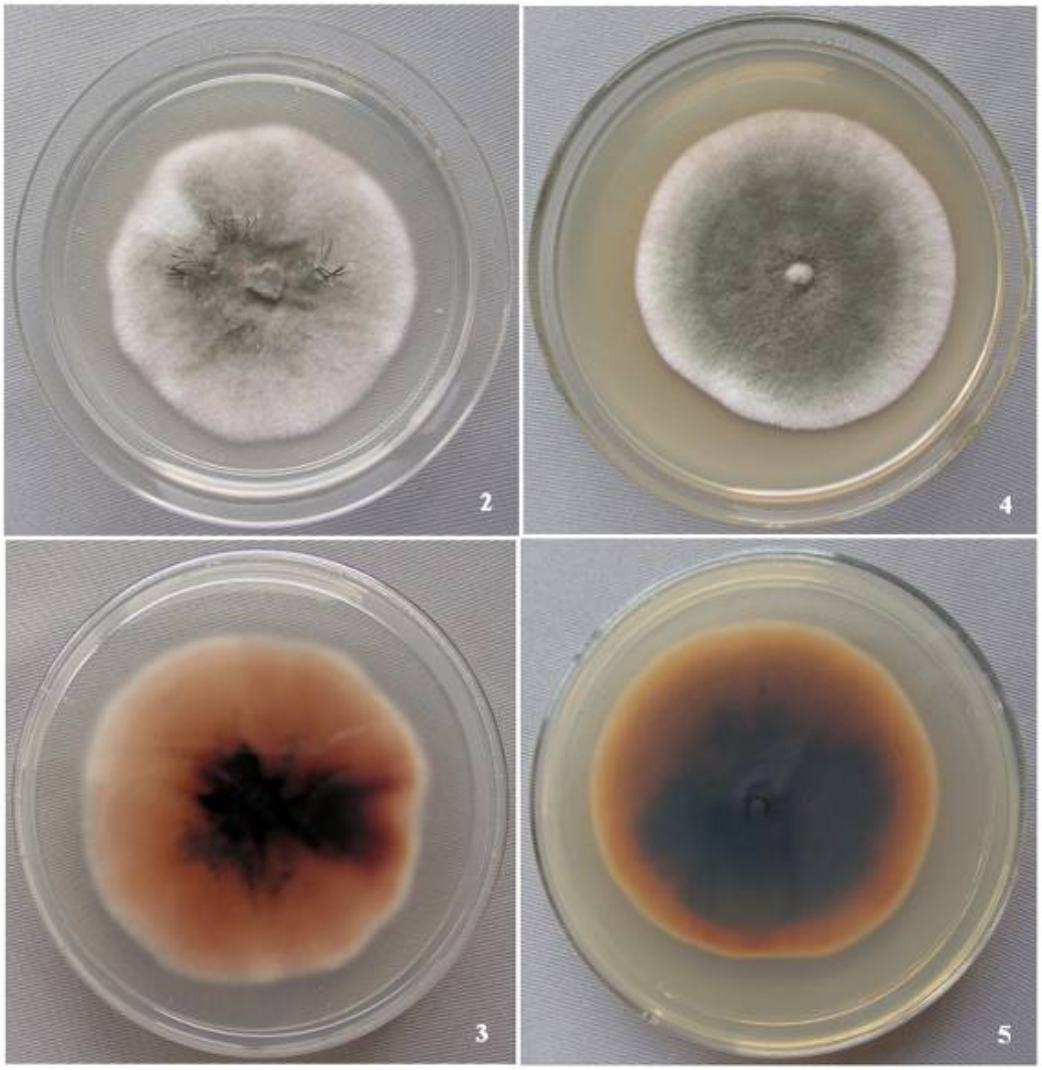

Fig. 2-5. *Edenia achyranthi*. 2, 3, Colony appearance on PDA after 14 days at 25 °C. 4, 5, Colony appearance on MEA after 14 days at 25 °C.

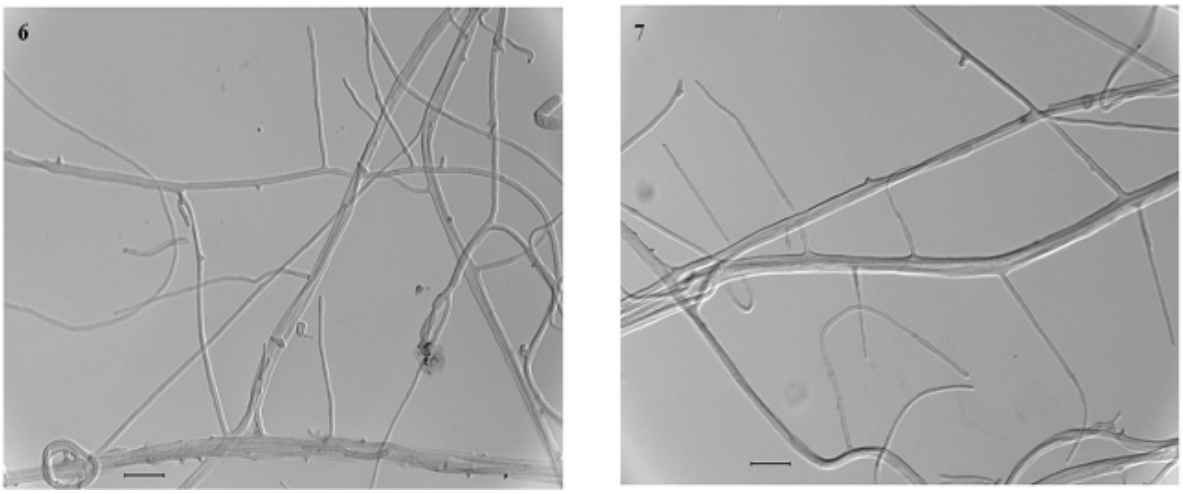

Fig. 6-7. *Edenia achyranthi*. 6, Rope-like strands and coil. 7, Hyphae with 90° and other angle branching developing.

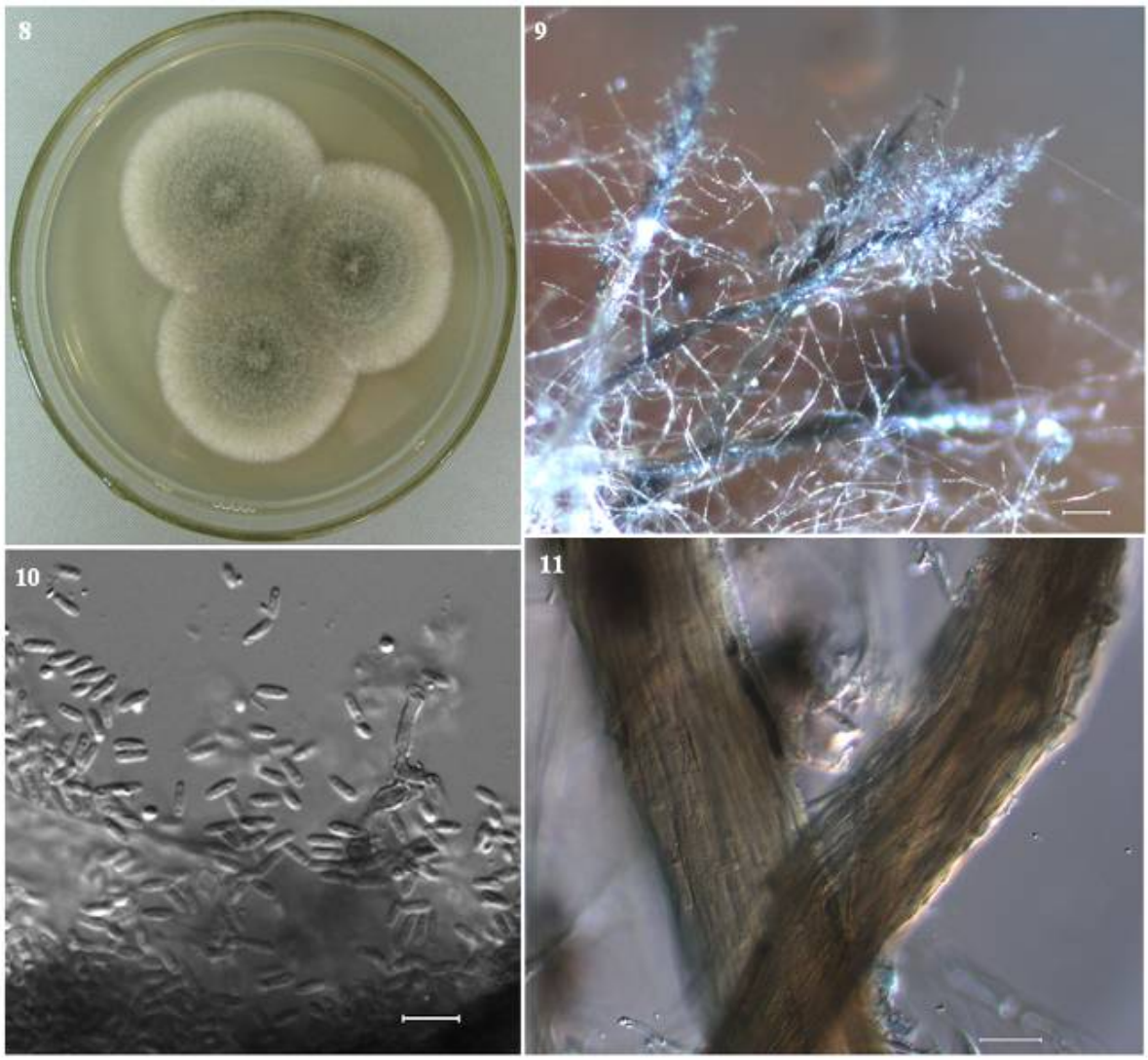

Fig. 8-11. *Edenia achyranthi*. 8, Colonies on MEA after 7 days at 25°C. 9, Hyphal bundles under stero microscope, bar = 200 μm. 10, Condia formed on sterilized leaves of *A. bidentata*, bar = 10 μm. 11, Hyphal bundles under microscope, bar = 20 μm.

Habitat: Anamorphic ascomycete endophytic within living stems of *Achyranthes bidentata*.
SPECIMENS EXAMINDED-China, Hebei province, Anguo (N 38°23', E 115°18'), from stems of *Achyranthes bidentata*, September 2009, B.D. Sun, A.J. Chen. (HOLOTYPE **HMAS 242793**)

Disscussion

E. gomezpompae, *E. achyranthi* were isolated as plant endophytic fungus. The hyphal branches of the two *Edenia* species are both mainly developing by 90° angle. They both formed hyphal strands and the reverse colorations of colonies are both showed reddish-brown. However, they can be easily distinguished by the obvious smaller conidia and darker colonies of the later species. The difference also present in the blackish hyphal bundles formed by *E. achyranthi* on PDA.

Although the present description of *E. achyranthi* is based on an endophytic fungal isolates, it seemed that this fungus has more divergent ecological distribution. The nuclear ribosomal DNA internal transcribed spacer region of this fungus has identical sequence with 3 sequences in GenBank which are associated with fungal isolates other than plant endophytes: **AY303602** and **AY303611**, from 2 unidentified fungal isolates from foraging soil sheetings built by termites (Senegal); **GU073117**, from pathogenic fungus of gramineous grass (Beijing). As for *E. gomezpompae*, the reported isolations were all from tropical forest plants (González *et al.,* 2007; Crous *et al.,* 2009).

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References

Anonymous. 2005. *Pharmacopoeia of the People's Republic of China*, Chemical Industry Press, Beijing, pp. 49.

Crous, P.W., U. Braun, M.J. Wingfield, A.R. Wood, H.D. Shin, B.A. Summerell, A.C. Alfenas, C.J.R. Cumagun and J.Z. Groenewald. 2009. Phylogeny and taxonomy of obscure genera of microfungi. *Persoonia*, 22: 139-161.

Crous, P.W., G.J.M. Verkley and J.Z. Zroenewald. 2006. Eucalyptus microfungi known from culture. 1. *Cladoriella* and *Fulvoflamma* genera nova, with notes on some other poorly known taxa. *Stud. Mycol.*, 55: 53-63.

González, M.C., A.L. Anaya, A.E. Glenn, A. Saucedo-Garcia and M.L. Macias-Rubalcava. 2007. A new endophytic ascomycete from El Eden Ecological Reserve, Quintana Roo, Mexico. *Mycotaxon*, 101: 251-260.

Khan, R., S. Shahzad, M. I. Choudhary, S.A. Khan and A. Ahmad. 2010. Communities of endophytic fungi in medicinal plant *Withania somnifera*. *Pak. J. Bot.*, 42(2): 1281-1287.

Irinyi, L., G.J. Kövics and E. Sándor. 2009. Taxonomical re-evaluation of *Phoma*-like soybean pathogenic fungi. *Mycol. Res.*, 113(2): 249-260.

Lennox, C.L., M. Serdani, J.Z. Groenewald and P.W. Crous. 2004. *Prosopidicola mexicana* gen. et. sp. nov., causing a new pod disease of *Prosopis* species. *Stud. Mycol.*, 50: 187-194.

Li, Z.F. 2008. *Positively allelopathic effect and its molecular mechanism of Achyranthes bidentata Blume mediated by continuous cropping soils*. Master degree dissertation, Fujian Agricultural and Forest University.

Morales, V.M., C.A. Jasalavich, L.E. Pelcher, G.A. Petrie and J.L. Taylor. 1995. Phylogenetic relationship among several *Leptosphaeria* species based on their ribosomal DNA sequences. *Mycol. Res.*, 99(5): 593-603.

Peterson, R.L., A.E. Ashford and W.G. Allaway. 1985. Vesicular-arbuscularmycorrhizal association of vascular plants on Heron Island, a Great Barrier Reef coral ray. *Aust. J. Bot.*, 33: 669-676.

Photita, W., P.W.J. Taylor, R. Ford, K.D. Hyde and S. Lumyong. 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Divers.*, 18: 117-133.

Shanker, N.A., J. Mathew and A. Varma. 1990. Occurrence of vesicular-arbuscular mycorrhizae with Amaranthaceae in soils of the Indian semi-arid region. *Biol. Fert. Soils*, 11: 140-144.

Shrestha, P., T.M. Szaro, T.D. Bruns and J.W. Taylor. 2011. Systematic search for cultivatable fungi that best deconstruct cell walls of *Miscanthus* and sugarcane in the field. *Appl. Environ. Microb.*, 77(15): 5490-5504.

Simon, U.K., J.Z. Groenewald and P.W. Crous. 2009. *Cymadothea trifolii*, an obligate biotrophic leaf parasite of *Trifolium*, belongs to Mycosphaerellaceae as shown by nuclear ribosomal DNA analyses. *Persoonia*, 22: 49-55.

Su, Y.Y., L.D. Guo and K.D. Hyde. 2010. Response of endophytic fungi of *Stipa grandis* to experimental plant function group removal in Inner Mongolia steppe, China. *Fungal Divers.*, 43:93-101.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 24: 1596-1599.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A Guide to Methods and Applications*. (Eds.): M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White. Academic Press, New York, 315-322.

Zalar, P., C. Gostinčar, G.S. de Hoog, V. Uršič, M. Sudhadham and N.G. Cimerman. 2008. Redefinition of *Aureobasidium pullulans* and its varieties. *Stud. Mycol.*, 61: 21-38.

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