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

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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 Amarantaceae 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 Amarantaceae 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.

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

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Strain No.</th>
<th>Host plant</th>
<th>Location</th>
<th>GenBank accession No.</th>
<th>Cited in publication</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aureobasidium pullulans</em></td>
<td>CBS 105.22</td>
<td>unknown</td>
<td>unknown</td>
<td>FJ50886</td>
<td>Yes, Zalar et al., 2008</td>
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<td><em>Aureobasidium pullulans</em></td>
<td>CBS 584.75</td>
<td><em>Vitis vinifera</em></td>
<td>France</td>
<td>FJ150906</td>
<td>Yes, Zalar et al., 2008</td>
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<td>Coniothyrium palmarum</td>
<td>CBS 400.71</td>
<td><em>Chamaecops humilis</em></td>
<td>Italy</td>
<td>AJY27008</td>
<td>Yes, Lennox et al., 2004</td>
</tr>
<tr>
<td>Didymella bryoniae</td>
<td>CBS 233.52</td>
<td><em>Trifolium repens</em></td>
<td>Germany</td>
<td>EU167573</td>
<td>Yes, Simon et al., 2009</td>
</tr>
<tr>
<td>Didymella rabei</td>
<td>CBS 581.83A</td>
<td><em>Cicer arietinum</em></td>
<td>Syria</td>
<td>EU573020</td>
<td>Yes, Simon et al., 2009</td>
</tr>
<tr>
<td>Edenia gomezpompea</td>
<td>C1c</td>
<td><em>Callicarpa acuminata</em></td>
<td>Mexico</td>
<td>EF565744</td>
<td>Yes, González et al., 2007</td>
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<tr>
<td>Edenia gomezpompea</td>
<td>CBS 124106</td>
<td><em>Senna alata</em></td>
<td>Filipinae</td>
<td>FJ389619</td>
<td>Yes, Crous et al., 2009</td>
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<tr>
<td>Edenia gomezpompea</td>
<td>UFMGCB 2177</td>
<td><em>Solanum cernuum</em></td>
<td>Brazil</td>
<td>HM997129</td>
<td>No, only present study</td>
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<td>Leptospora rubella</td>
<td>CPC 1106</td>
<td>unknown</td>
<td>unknown</td>
<td>DQ195780</td>
<td>Yes, Crous et al., 2006</td>
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<td>Phaeosphaeriopsis musae</td>
<td>CBS 120026</td>
<td><em>Musa sp.</em></td>
<td>Mauritius</td>
<td>DQ858984</td>
<td>Yes, Crous et al., 2006</td>
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<td>Phaeosphaeriopsis sp.</td>
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<td><em>Miscanthus sp.</em></td>
<td>American</td>
<td>HQ630983</td>
<td>Yes, Shrestha et al., 2011</td>
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<tr>
<td>Phaeosphaeria nodorum</td>
<td>DAOM 215173</td>
<td>unknown cereal plant</td>
<td>unknown</td>
<td>U04237</td>
<td>Yes, Morales et al., 1995</td>
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<td>Phoma exigua var. exigua</td>
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<td><em>Trifolium repens</em></td>
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<td>Phoma pinodella</td>
<td>CBS 318.90</td>
<td><em>Pisum sativum</em></td>
<td>Netherlands</td>
<td>EU573028</td>
<td>Yes, Irinyi et al., 2009</td>
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<td>Phoma sojicola</td>
<td>CBS 567.97</td>
<td><em>Glycine max</em></td>
<td>Hungary</td>
<td>EU573026</td>
<td>Yes, Irinyi et al., 2009</td>
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<td>Pyrenochaeta sp.</td>
<td>YS-2010</td>
<td><em>Stipa grandis</em></td>
<td>China</td>
<td>HM007089</td>
<td>Yes, Su et al., 2010</td>
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</table>

**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 gomezpompea sequences (EF565744, FJ839619 and HM997129) was clustered into one clade. Although the 3 E. gomezpompea isolates were from different countries and different hosts, they had almost identical ITS sequences. The isolate AS-60 was separated from the three E. gomezpompea 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

**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.

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

Colonial in agaro decocto tuberorum (PDA), celeriter crescentes 55-60 mm diametro in 14 diebus 25°C, primo albae, deinde cinerae cum mycelio fasciculatus nigero, intermidius, abundus; reversum rubro-brunnea. Hyphae hyalinae, leptoderma, aequata, septatae, saepe ramificatione in angulis 90° plerumque, flexuosae, convergentes, fila funiformia et spiras formantes. Conidia undulate, septate, 1.0-3.7 μm diam, frequently developing by 90° angle branching, intertwining and forming rope-like strands and coils. Conidia semipellucida, aequata, leptoderma, 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, leptoderma, aequata, septatae, saepe ramificatione in angulis 90° plerumque, floccosae, convolventes, fila funiformia et spiras formantes. Conidia semipellucida, aequata, leptoderma, elliptica vel paulo constrictae in basi, 3.5-6.1×1.7-2.5 μm. Teleomorph unknown.
A NEW ENDOPHYTIC ASCOMYCETE ASSOCIATED WITH THE MEDICINAL PLANT

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 stereo microscope, bar = 200 μm. 10, Conidia 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 EXAMINED—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**)

Discussion

*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


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