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A new species of *Scopulariopsis* and its synergistic effect on pathogenicity of *Verticillium dahliae* on cotton plants

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ABSTRACT

A new species, *Scopulariopsis gossypii*, was found to be present in the vascular bundles of cotton plants (*Gossypium hirsutum*) infected by *Verticillium dahliae* which is an economically important pathogen in Hangzhou, China. The fungus was only present in the diseased plants, but it never became isolated from the healthy plants. Scanning electron microscopy showed that the fungus was present in parenchyma cells enclosing vessels in dark brown vascular tissues of stems, and produced asexual conidia within the tissues. Phylogenetic analysis of combined nuclear ribosomal D1/D2 region of the 28S rDNA as well as translation elongation factor 1-alpha (*TEF1*) and beta-tubulin (*TUB*) gene showed that *S. gossypii* represents an undescribed species of *Scopulariopsis*, *Microascaceae*. In this study, characteristics of sexual and asexual stages of the fungus were described, illustrated and compared with similar taxa. In addition, the molecular and morphological analyses indicated that *S. gossypii* was a distinct species of *Scopulariopsis*. The pathogenicity tests proved by inoculation of wounded roots confirmed that *S. gossypii* was an opportunistic pathogen causing leaf interveinal chlorosis and vascular browning of cotton plants. However *S. gossypii* did not infect host with undamaged roots. Moreover, coinoculation with *S. gossypii* and *V. dahliae* significantly increased disease severity.

1. Introduction

Among 107 species of genus *Scopulariopsis* and 61 *Microascus* listed in Index Fungorum, 86 and 51 species have been accepted presently in *Scopulariopsis* and *Microascus*, respectively. *Scopulariopsis* was erected by Bainier (1907) with *S. brevicaulis* as type species, which was originally described as *Penicillium brevicaule* by Saccardo (1882). The teleomorph of *S. brevicaulis* was firstly reported by Abbott et al. (1998) and described as *Microascus brevicaulis*. According to morphological characteristics, mating type studies and molecular data, it is known that sexual morphs of *Scopulariopsis* spp. belong to the genus *Microascus* (Curzi, 1930, 1931; Abbott et al., 1998; Abbott and Sigler, 2001; Issakainen et al., 2003). However, a recent research has demonstrated that the genera *Microascus* and *Scopulariopsis* are different and separated into two distinct lineages by three loci (D1/D2 region of the 28S rDNA, translation elongation factor 1-α (*TEF1*) and β-tubulin (*TUB*) (Sandoval-Denis et al., 2016).

In morphology, two genera *Scopulariopsis* and *Microascus* are delimitated by the features of colonies, conidiogenous cells and conidia (Sandoval-Denis et al., 2016). In *Scopulariopsis*, the colonies are white, pale grey, tan or brown and mycelium is mostly hyaline. The conidiogenous cells are cylindrical and hyaline, with a wide flat opening, and form on penicillate conidiophores. Conidia are hyaline or pale brown which often show a protruding base. The colonies of *Microascus* are dark-coloured. In addition, mycelia, conidiogenous apparatus and conidia are mostly brown to green-brown. The conidiogenous cells are ampulliform or lageniform. The conidiophores are often branched up to 80 μm long, and ascospores have a germ pore (Sandoval-Denis et al., 2016). This research established a new classification framework for *Microascus, Scopulariopsis* and allied genera.

The genus *Scopulariopsis* currently comprises species with a worldwide distribution that are commonly isolated from soil, air, plant debris and dung (Domsch et al., 2007). Moreover, some species have been described as colonizers or pathogens of insects and mammals including humans (de Hoog et al., 2000; Iwen et al., 2012; Sandoval-Denis et al., 2013). In 2014, Verticillium wilt of cotton (*Gossypium hirsutum*) seriously occurred in Hangzhou, Zhejiang province, China, and the pathogen was identified as defoliation pathotype of *Verticillium dahliae* (Sun et al., 2016). In addition to the pathogen, a *Scopulariopsis* sp. was usually isolated from dark brown vascular tissues in living root and stems of plants. Therefore, different tests were conducted to determine endophytic or pathogenic role of the fungus.

The resulted isolates of *Scopulariopsis* sp. were able to produce the sexual structures in culture and they were primitively recognized as a

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species of *Microascus*, but based on new taxonomic criteria described by Sandoval-Denis et al. (2016), it belonged to *Scopulariopsis*. However, it differs morphologically and phylogenetically from other currently accepted species in the genus. In this study, we described and illustrated a new species of *Scopulariopsis* and compared it with morphologically and molecularly from three loci (TEF1, D1/D2 and TUB) to other species of *Scopulariopsis*. In addition, inoculation tests were conducted to confirm the pathogenic role of the new *Scopulariopsis* sp. to cotton plants, and fungal sporulation properties in the host tissues were assessed using Scanning electron microscopy (SEM).

2. Materials and methods

2.1. Fungal isolation

All fungi were isolated from healthy and diseased cotton plants (cv. CCR1-49) to detect the presence of *Scopulariopsis* sp. when symptoms of Verticillium wilt became serious during flowering period. The samples were collected from an experimental field of Zhejiang University, Hangzhou, China in the early July, 2014. Each stem was cut apart and then two pieces (approximately 0.5 × 0.5 cm) were cut again from brown or healthy vascular tissues of the middle part of a stem in 50 diseased and 50 healthy plants. Pieces were disinfected in 0.5% NaOCl for 5 min, moved into Potato Dextrose Agar (PDA) and incubated at 25 °C for 5–7 d. The morphological characteristics of the emerging colonies were evaluated. Moreover, single-spore isolation of *Scopulariopsis* sp. was performed as described by Chomnunti et al. (2014). Single-spore isolates were stored in 1.5 mL tubes with 20% glycerol at −70 °C in the Culture Collection of Biotechnology Institute, Zhejiang University, Zhejiang Province, China. After the isolates were identified, they were deposited at China General Microbiological Culture Collection Center (CGMCC).

2.2. Scanning electron microscope

The pieces (0.5 × 1.0 cm^3) taken from discolored parts of vascular tissue of diseased plants were cut as described above and placed into glutaraldehyde (2.5% vol/vol) for 48 h at 4 °C. The samples were washed in phosphate buffer prior to post fixation in 1% (w/v) osmium tetraoxide in 0.1 mol/L phosphate buffer for 2 h, and then rinsed thoroughly with 0.2 M phosphate buffer (pH 6.8) and dehydrated in a graded ethanol series, followed by a few changes of isoamyl acetate for 30 min. Before the critical-point drying, the samples were longitudinally cut; the reverse side of a cut section was mounted onto an aluminum stub with double-sided sticky film, sputter-coated with gold, and examined and photographed in a SEM (SU8010, HITACHI, Japan).

2.3. DNA extraction and PCR amplification

Three isolates of *Scopulariopsis* sp. (HJC001, HJC002 and HJC003) were selected for PCR amplification and sequencing. Fungal isolates were grown on PDA for 7 d at 25 °C, mycelia were scraped from the surfaces of media and grounded to a fine powder in liquid nitrogen. Genomic DNA was extracted using a protocol described by Zhang and Li (2009). The obtained genomic DNA was resuspended in 50 μL TE buffer and stored at −20 °C.

Three DNA fragments were amplified in an automated thermal cycler (Eppendorf AG, Germany). The primers NL1/NL4 amplified the D1/D2 region of the large subunit (LSU) rDNA gene (O'Donnell, 1993), and primers 983F/2218R were used to amplify the sequence fragments of translation elongation factor 1 alpha (*TEF1*) gene (Rehner and Buckley, 2005). In addition, the primers BT2a/BT2b amplified sequence fragments of beta-tubulin gene (*TUB*) (Glass and Donaldson, 1995). The amplification was performed in a 50 μL reaction volume which contained 5 μL 10 × PCR buffer, 2 μL of each primer (20 μM), 2 μL template DNA(2 μg/μL), and 0.5 μL Taq DNA polymerase (5U/μL) (TaKaRa Bio Inc., Japan). The thermal cycling program was performed with 35 cycles after an initial denaturation at 95 °C for 4 min. Each cycle included a denaturation step at 95 °C for 1 min, annealing at a suitable temperature for 1 min, and an extension step at 72 °C for 1.5 min. Annealing temperatures for each reaction were 51 °C for D1/D2 region, 57 °C for *TEF1* and *TUB*. The PCR products for *EF-1α* were inserted into pUCm-T Vector (Sangon Biotech Company, Shanghai, China) and transformed into *Escherichia coli* DH5α competent cells. The positive clones were identified by PCR and restriction endonuclease enzyme digestion and their plasmids were extracted. The recombinant plasmids and purified PCR products were submitted to the Sangon Biotech Company Limited (Shanghai, China) for sequencing in both directions.

2.4. Sequence alignment and phylogenetic analysis

The obtained Sequences were assembled and edited using BioEdit software (Hall, 1999), and they were deposited in GenBank sequence database (KU523865, KU523866, KU523867, KU523859, KU523860, KU523861, KU523868, KU523869 and KU523870). We downloaded available data for species of *Scopulariopsis* and *Microascus* from GenBank, including 45 ingroup and two outgroup taxa (*Cephalotrichum stemonitis* CBS 103.19 and *Wardomycopsis inopinata* FMR 10306) (Sandoval-Denis et al., 2016; Jagielski et al., 2016). Sequences of each gene were aligned with MAFFT 7.273 (Katoh and Standley, 2013) and trimmed with BioEdit. The resulted alignment was put into Gblocks 0.91b to eliminate the ambiguously aligned positions and divergent regions prior to phylogenetic analyses (Katoh et al., 2005).

Molecular phylogenies were constructed with maximum likelihood (ML) and Bayesian inference (BI). The model of evolution applied to each alignment was estimated by using jModel Test 2.1.7 (Darriba et al., 2012) and the model chosen according to the Akaike information criterion. The best GTR + I + G model was selected for *TEF1* and TrN + I + G for D1/D2 region and *TUB*. ML analyses were performed on the concatenated *TEF1* + *TUB* + D1/D2 data with RaxmlGUI v. 1.5 (Silvestro and Michalak 2012). ML bootstrap (ML-BS) analysis for each ML tree was performed with 1000 fast bootstrap replicates with the same parameter settings using the GTR+ I + G model of nucleotide substitution. A threshold of ≧80% was used as the cut-off for significantly supported nodes.

BI analyses were conducted with MrBayes v. 3.2.6 run by partitioning codon positions (Ronquist et al., 2011). The Markov-chain Monte-Carlo searches were performed with four chains, each of which was run for 10,000,000 generations, with trees sampled every 100 generations. The initial 25% of trees from each run were discarded as burn-in, and the remaining trees were combined into one tree with 50% majority rule consensus tree. BI posterior probability (BI-PP) values equal or above 0.95 were found to be significant. The resulted trees were imported into FigTree v.1.3.1. The alignments and phylogenetic trees were deposited in TreeBase (www.treebase.org) under Accession no. S19843.

2.5. Morphological studies

Isolates were grown on oatmeal agar (OA; 30 g oatmeal, 20 g agar, 1 L distilled water) and potato-carrot agar (PCA; 20 g each of potatoes and carrots, 20 g agar, 1 L distilled water) at 25 °C. They were assessed at 7–15 d for asexual structures and colony growth rates, and at 15–25 d for sexual development. The fungal asexual and sexual structures (conidiophores, conidiogenous cells, conidia and asci and ascospores) were observed and measured using a Zeiss Axiophot 2 microscopy with Axiocam CCD camera and Axiovision digital imaging software (AxioVision Software Release 3.1., v.3–2002; Carl Zeiss Vision Imaging Systems). For SEM, 0.5 × 0.5 cm^3 agar pieces containing asexual and sexual structures were cut and fixed in glutaraldehyde. Procedures of sample treatment included post fixation, dehydration and