Phylogenetic Study of *Curvularia* on Sorghum from Indonesia Based on ITS rDNA Sequence

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Abstract

Seven species of *Curvularia* were recorded from Indonesia, including species that pathogen to sorghum (*Sorghum bicolor*). The present study aims to determine four isolates of *Curvularia* isolated from sorghum in Indonesia using a combination of molecular phylogenetic analysis based on internal transcribed spacer (ITS) rDNA sequence and morphological examination. The results showed that the four isolates were genetically closed to *C. lunata* and *C. dactyloctenicola*. The morphological examination also insufficient to determine the identity of the four *Curvularia* isolates from sorghum until species level. Therefore, additional sequences from the partial fragments of the glyceraldehyde-3-phosphate dehydrogenase and the translation elongation factor 1-a genes are necessary to determine the identity of the *Curvularia* sequences isolated from sorghum in Indonesia until species level.

Keywords: ITS rDNA, Indonesia, leaf spot, phylogenetic, sorghum

Introduction

*Curvularia* Boedijn is an asexual morph genus characterized by having curved conidia with two or three central darkened cells and hyaline apical cells, one of which is enlarged and contributes to the curvature (Shoemaker 1959). Members of *Curvularia* have been known as pathogen to human and plants as well as saprobes on various substrates (Ellis 1971, Manamgoda et al. 2015). In Indonesia, seven species of *Curvularia* have been recorded (Farr & Rossman 2019). These species include *C. andropogonis* (Zimm.) Boedijn, *C. crustacea* (Henn.) Y.P. Tan & R.G. Shivas, *C. eragrostidis* (Henn.) J.A. Mey., *C. geniculata* (Tracy & Earle) Boedijn (*Cochliobolus geniculatus* R.R. Nelson), *C. lunata* (Wakker) Boedijn, *C. pallescens* Boedijn, and *C. verruculosa* Tandon & Bilgrami ex M.B. Ellis (Farr & Rossman 2019).

In sorghum (*Sorghum bicolor* (L.) Moench), 12 species of *Curvularia* have been reported from sorghum, viz, these include *C. boreriae* (Viégas) M.B. Ellis, *C. clavata* B.L. Jain, *C. eragrostidis*, *C. fallax* Boedijn, *C. geniculata* (*C. geniculatus*), *C. lunata*, *C. muehlenbeckiae* Madrid, K.C. Cunha, Gené, Guaro & Crous, *C. pallescens*, *C. penniseti* (Mitra) Boedijn, *C. sorghina* R.G. Shivas & Sivan., *C. trifolii* (Kauffman) Boedijn, *C. tritici* S.M. Kumar & Nema (Farr & Rossman 2019, Index Fungorum 2019). In this study, we examined the morphological characters of *Curvularia* isolated from sorghum and analyzed their phylogenetic affinities with closely related species based on the ITS rDNA sequence data.

Materials and Methods

Collection of specimens and isolation

Specimen collection was conducted at Cibinong Science Center, Cibinong, West Java, Indonesia in 26 February 2019. The sorghum specimen was collected by cutting off the
symptomatic leaves and placed them in paper bags. The paper bags were sealed and labelled with the name of the host, collection site, date, and collector/s. All materials were kept in ice boxes prior to isolation in the laboratory. The isolation protocol of the fungal colony on the sorghum leaf was conducted according to the method described by Hidayat (2017). The growth of germination tube was observed after 24 h for everyday. The growing colonies were purified using hyphal tip isolation method to get a pure culture. Culture isolates obtained in this study were deposited at the Indonesian Culture Collection (InaCC) (Table 1).

Table 1 Additional information of new Curvularia isolates on Sorghum from Cibinong, Indonesia

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain code</th>
<th>Host</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lunata</td>
<td>IR-1</td>
<td>S. bicolor</td>
<td>Cibinong, Indonesia</td>
</tr>
<tr>
<td>C. lunata</td>
<td>IR-2</td>
<td>S. bicolor</td>
<td>Cibinong, Indonesia</td>
</tr>
<tr>
<td>C. lunata</td>
<td>IR-3</td>
<td>S. bicolor</td>
<td>Cibinong, Indonesia</td>
</tr>
<tr>
<td>C. lunata</td>
<td>IR-4</td>
<td>S. bicolor</td>
<td>Cibinong, Indonesia</td>
</tr>
</tbody>
</table>

Morphological examination

The micromorphological structures of Curvularia were observed using Olympus BX51 (Olympus, Japan) (Fig. 1). For cultural characterization, isolates were grown on PDA at room temperature in the dark.

DNA isolation, PCR amplification and sequencing

The fungal genomic DNA was obtained by using Phytopure™ DNA extraction kit (GE Healthcare, UK). A total 25 µL of PCR mix was prepared as follow: 1.25 µL of 10 µM ITS5 (forward) (5’–TCCTCCGCTTATTGATATGC–3’) and ITS4 (reverse) (5’–TCCGTAGGTGAACCTGCGC–3’) (White et al. 1990) primer pairs, 2 µL (10-100 ng) of DNA template, 25 µL GoTaq® green mastermix (Promega, USA), and 20.5 µL ultrapure water RNase free. PCR reaction was conducted using Thermalcycler (Takara Shuzo Co., Ltd., Shiga, Japan) according to the following setting: 90 s at 95°C for initial denaturation, followed by 35 cycles of 30 s at 95°C denaturation, 30 s at 55 °C annealing, 90 s at 72 °C extension, and 5 min at 72 °C for the final extension. PCR products were run in 1% agarose gel by electrophoresis at 100 V for 30 min, and soaked in ethidium bromide (EtBr) for 15 min. Gel was then visualized using Gel Doc™XR system (BIO-RAD, Germany). PCR products were sent to 1stBASE (Malaysia) for sequencing.

Phylogenetic analysis

Nucleotide sequence obtained from the respective primer, ITS5 and ITS4, were assembled in Chromas Pro 1.41 (Technelysium Pty Ltd., Australia). The sequences were aligned with sequences retrieved from DNA databases (DDBJ, NCBI) using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (Edgar 2004) in MEGA 7 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2016). GenBank accession number, strain code, and taxon name used in this study are given in figure. Phylogenetic analysis was conducted using the neighbour joining (NJ) method implemented in MEGA 7. Maximum composite likelihood was used as the substitution model for the current analysis. Strength of the internal branches of the phylogenetic trees was tested with bootstrap (BS) analysis (Felsenstein 1985) using 1000 replications. Other parameters used in the NJ analysis were
selected according to the default standard in MEGA 7 software. Bootstrap values of 50% or higher were shown. GenBank accession number, sequence name and strain code used in the phylogenetic analysis were showed in Figure 2.

Results and Discussion
Morphological examination

Figure 1 Curvularia dactyloctenicola. a, d–h conidiophores and conidia; b colony obverse on PDA after 1 week; c colony reverse on PDA after 1 week. Scale bars: a, d–h = 10 μm.

Description – Asexual morphology on WA and PDA: Hyphae hyaline, branched, septate, 2–5 μm diam. Conidiophores arising singly or in groups, septate, straight, often geniculate in the upper part, size of cells not decreasing towards apex, sometimes branched, cells walls thicker
than those of vegetative hyphae, mononematous, semi- to macronematous, pale brown to brown, sometimes swollen at the apex and at the base, 80–120 μm tall. *Conidiogenous cells* smooth-walled to slightly verruculose, terminal or intercalary, proliferating sympodially, pale brown to brown, subcylindrical to swollen at the apex, 6–12.5 × 2.5–5 μm. *Conidia* slightly verruculose, curved, middle cells unequally enlarged, ellipsoidal to obovoid, pale brown to brown, apical and basal cells slightly paler than the middle cells, (2–)3-distoseptate, 12–20 × 5–8.5 μm. *Microconidiation*, *chlamydospores* and *sexual morph* not observed.

Cultural characteristics – Colonies on PDA reaching 57–59 mm diam in 1 week, with aerial mycelium, velvety at the edge and cottony at the center, filiform margin, pale brown to pale grey in obverse, pale brown to grey in reverse.

Host – *Sorghum bicolor*.

Geographical distribution – Indonesia.

Material examined – INDONESIA, West Java, Cibinong, Cibinong Science Center (CSC), InaCC greenhouse, on *S. bicolor* leaf, 26 February 2019, I. Hidayat and I. Ramadhani, (IR-1, IR-2, IR-3, IR-4).

**Phylogenetic analysis**

The NJ tree of *Curvularia* species from sorghum showed that four new sequences of *Curvularia* from Indonesia nested in the same clade with *C. lunata* strain CBS 730.96 (NR 138223) with 100% BS (Fig. 2). This data indicates that the new *Curvularia* sequences isolated from sorghum in Indonesia belong to *C. lunata*.

The phylogenetic tree analysis involving homologous sequences (TYPE sequences) from the BLAST results showed that the new *Curvularia* sequences isolated from sorghum in Indonesia nested in the same clade with *C. chiangmaiensis* strain CPC 28829 (MF 490814), *C. lunata* strain CBS 730.96 (NR 138223), and *C. dactyloctenicola* strain CPC 28810 (MF 490815) (Fig. 3). The new *Curvularia* sequences isolated from sorghum in Indonesia are identical with the *C. lunata* strain CBS 730.96 and *C. dactyloctenicola* strain CPC 28810 sequences. Additional sequences from the partial fragments of the glyceraldehyde-3-phosphate dehydrogenase and the translation elongation factor 1-a genes are necessary to determine the identity of *Curvularia* sequences isolated from sorghum in Indonesia.
Figure 2 Neighbour Joining (NJ) tree showing relationship between *Curvularia* spp. on sorghum based on the ITS rDNA sequences. Bootstrap value > 50% is shown at the branches node.
The current phylogenetic study showed that Curvularia sequences from Indonesia is phylogenetically close to C. lunata and C. dactyloctenicola (Figs. 2-3), and their ITS sequences are identical. Curvularia lunata was commonly found as pathogen and saprobes on various plants in temperate and tropical regions (Farr & Rossman 2019), while C. dactyloctenicola was only found on Dactyloctenium aegyptium (L.) Willd. in Thailand (Marin-Felix et al. 2017). The morphological examination of the Curvularia specimens from sorghum in Indonesia with C. lunata and C. dactyloctenicola showed a closer relationship with C. dactyloctenicola. The conidiophores of Curvularia from sorghum in Indonesia (80–120 μm) is shorter than C. lunata (up to 650 μm) or C. dactyloctenicola (up to 400 μm), and the conidial size of the Curvularia from sorghum in Indonesia is also smaller (5–8.5 μm) than those of C. lunata (9–15 μm) and C. dactyloctenicola (7–9 μm).

The current morphological examination and phylogenetic analysis based on the ITS rDNA sequence failed to determine the identity of the Curvularia specimens from sorghum in Indonesia. Therefore, additional sequences from the partial fragments of the glyceraldehyde-3-phosphate dehydrogenase and the translation elongation factor 1-a genes are necessary to determine the identity of the Curvularia sequences isolated from sorghum in Indonesia.

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