RESEARCH ARTICLE

Morphological and molecular characterization of two graminicolous *Exserohilum* species associated with cultivated rice and early barnyard grass from Sri Lanka

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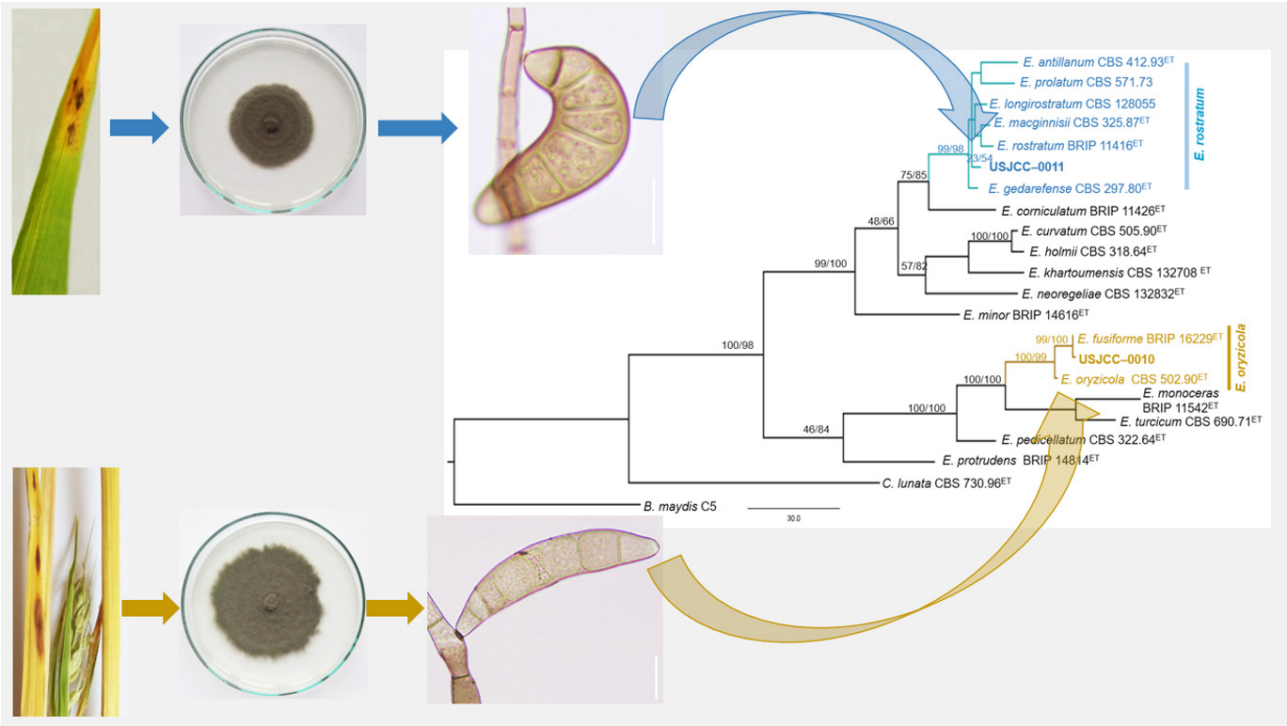

Highlights

- *Exserohilum rostratum* and *E. oryzicola* were characterized from cultivated rice and early barnyard grass in Sri Lanka
- Evolutionary relationships were inferred based on multi-locus phylogeny
- Both records are novel plant-fungal associations from Sri Lanka.

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RESEARCH ARTICLE

Morphological and molecular characterization of two graminicolous *Exserohilum* species associated with cultivated rice and early barnyard grass from Sri Lanka

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Abstract: The genus *Exserohilum* (Order Pleosporales, Class Dothideomycetes) comprises plant pathogenic hyphomycetous fungi, associated with poaceous hosts. Although numerous pathogenic species of *Exserohilum* are known globally, only *E. turcicum* and *E. rostratum* have been reported from Sri Lanka. In the present study, samples showing the symptoms of leaf blight of *Oryza sativa* (cultivated rice) and sheath blight of *Echinochloa oryzoides* (early barnyard grass) were collected and causal agents were primarily identified as *Exserohilum* spp. based on morphological characters. Molecular phylogenetic analyses based on three loci namely, nuclear ribosomal internal transcribed spacer (ITS), partial glyceraldehyde 3-phosphate dehydrogenase (GPDH) and translational elongation factor (TEF-1α) were used to infer evolutionary relationships and accurate identification. These isolates from *O. sativa* and *Echinochloa oryzoides* were identified as *Exserohilum rostratum* and *E. oryzicola* respectively. Both records are novel plant-fungal associations from Sri Lanka based on available data. This study suggests the need for morphological and molecular reassessments of emerging and poorly known species of fungi associated with cereals, their wild relatives and other economically important hosts in Sri Lanka.

Keywords: Cereal pathogens; emerging species; hyphomycetes; molecular phylogeny.

INTRODUCTION

The hyphomycetous fungi associated with grass hosts, previously known as "graminicolous *Helmonthosporium* species", include six genera belonging to order Pleosporales namely *Curvularia*, *Bipolaris*, *Exserohilum*, *Drechslera*, *Johnalcornia* and *Porocercospora* (Manamgoda *et al*., 2012; Amaradasa *et al*., 2014; Tan *et al*., 2014; Hernandez-Restrepo *et al*., 2018). The genus *Exserohilum* which contains a number of plant, human pathogenic and saprobic fungi, has been introduced with the type species, *E. turcicum* (syn. *Helminthosporium turcicum)* (Leonard and Suggs 1974; Passerini, 1876). The sexual morph of *Exserohilum* was previously characterized under *Setosphaeria* (Leonard and Suggs, 1974). Species of this genus are frequently encountered as asexual morphs in nature, although the sexual morphs were often obtained by mating compatible strains (Hernandez-Restrepo *et al*., 2018).

Recent molecular phylogenetic assessments have resulted in considerable taxonomic refinements of numerous species in the genus *Exserohilum*. For instance, previously known two species, *E. heteropogonicola* and *E. inaequale* are now placed in the genus *Curvularia* as *C. heteropogonicola* and *C. crassiseptum*, respectively (Alcorn, 1991; Zhang *et al*., 2004; Hernandez-Restrepo *et al*., 2018). Based on molecular phylogenetic analysis, six formerly known taxa namely *E. antillanum, E. gedarefense, E. leptochloae, E. longirostratum, E. macginnisii* and *E. prolatum* were found to be conspecific with commonly encountered taxon *E. rostratum* (Hernandez-Restrepo *et al*., 2018). In the same study, *E. curvatum* was synonymized with *E. holmii,* and *E. fusiforme* with *E. oryzicola* (Hernandez-Restrepo *et al*., 2018).

Exserohilum species are encountered as pathogenic fungi of humans and plants and also frequently found as saprobic, endophytic and soil-borne fungi. Human pathogenic *Exserohilum* spp. are generally opportunistic fungi which may also cause life-threatening infections in immune-compromised humans. The most commonly reported human pathogenic species is *E. rostratum*, whereas some cases are attributed to *E. longirostratum* and *E. macginnisii* (McGinnis *et al*., 1986; De Hoog *et al*., 2000; Al-Attar *et al*., 2006). These pathogens have been reported on immune-compromised patients causing skin and corneal infection, invasive diseases, and allergic fungal sinusitis (Adler *et al*., 2006).

The plant family Poaceae comprises of important cereal crops such as rice, wheat, millet and corn which provide major dietary needs of the human population. Pleosporalean fungal pathogens, bearing brown asexual spores, are often associated with cereal crops, their wild relatives and weeds in the family Poaceae in different life styles including, epiphytes, endophytes, saprophytes or pathogens (Hernandez-Restrepo *et al*., 2018). Understanding the host associations and host ranges of fungi is important due to the possibilities of host shift of these species from weed hosts to important crops, as observed in many species. For example, *E. fusiforme* (syn. *E. oryzicola*) has originally been identified as pathogenic on the weed, *Echinochloa*

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crus-galli, causing numerous small leaf lesions and later known to cause small linear spots on, cultivated rice plants (Alcorn, 1991).

Majority of *Exserohilum* species are associated with grasses and important crops in the family Poaceae causing leaf blights of corn and millet, leaf spots and foot rots of wheat and damping-off of sugarcane seedlings (Sivanesan, 1987). The type species of the genus, *Exserohilum turcicum,* is the causative agent of northern leaf blight of corn which is a widespread foliar disease characterized by oblong, straw-colored to greyish necrotic lesions and causing significant death of foliar tissue. The reduction of effective photosynthetic area of leaves may lead to severe cases of grain yield losses of 20–25 % (Smith *et al*., 1988).

Although *Exserohilum* species are widely known emerging fungi on cereal hosts and weeds with worldwide distribution, only two species, *E. turcicum* and *E. rostratum*, have been recorded so far from Sri Lanka (Farr and Rossman, 2020). Information on the diversity and DNA sequence data of these common cereal pathogenic fungi is important to establish control measures for emerging fungal diseases (Udayanga, 2019). Therefore, the major aim of this study was to use molecular and morphological data to characterize freshly collected isolates of *Exserohilum* species associated with rice and associated grass species collected from two selected locations in Sri Lanka.

MATERIALS AND METHODS

Sample collection, isolation and morphological studies

Samples were collected from field surveys carried out in Kegalle and Gampaha districts and all the specimen information (date of collection, collector, locality, host and symptomatology) were recorded and the samples were brought to the laboratory for further processing.

Fresh specimens were observed under stereomicroscope (Optika, LAB 30) and, instances where fungal structures were not visible, they were incubated for another 24 h in a moist chamber. Single spore isolation was done from the sporulating samples to isolate fungi (Chomnunti *et al*., 2011). Pure cultures were prepared on Potato Dextrose Agar (PDA) and stock cultures were maintained on Corn Meal Agar (CMA) slants. To determine colony morphology, cultures were triplicated on several media; PDA, CMA and Malt Extract Agar (MEA), and incubated at 25 °C for 12 h each in light and dark conditions. The color notations were recorded according to the standard color charts (Rayner, 1970). Micro-morphological characters were observed under compound light microscope (Optika, B 290) and measurements of structures were obtained under imaging facility. At least 30 length and width measurements were made from conidia of each isolate. Digital microscopic images were generated to illustrate the morphological characteristics. For all morphological measurements, statistical data (mean, minimum, maximum and standard deviation) were calculated and used in taxonomic descriptions. The specimens collected were dried and preserved as reference herbarium material at the herbarium, University of Sri Jayewardenepura (USJ) and the cultures are maintained at the fungal collection (USJCC) at the Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.

DNA extraction, PCR amplification and sequencing

Genomic DNA were extracted from the morphologically identified *Exserohilum* fungal isolates following the modified Sodium Dodecyl Sulphate (SDS) method as described in Arnold and Lutzoni (2007). The PCR amplifications were carried out in the BIORAD T 100 Thermal cycler according to the protocols described in Manamgoda *et al*. (2012) with the primer pairs for ITS region with ITS1 and ITS4 (White *et al*., 1990), GPDH with gpd1 and gpd2 (Berbee *et al*., 1999) and TEF1-α with EF1-983F and EF1-2218R (Rehner and Buckley, 2005). The PCR products were visualized on 2 % agarose gel electrophoresis. PCR product purification and Sanger sequencing of the successfully amplified samples were carried out in Macrogen Inc, Korea.

Sequence alignment, phylogenetic analyses and species recognition

Raw sequences were assembled on BioEdit v7.0.5 programme for windows. Initial alignments of assembled DNA sequences were accomplished using BioEdit v7.0.5, optimized with MAFFT v. 7 using default settings (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley, 2013). Preliminary identification of the isolates was carried out using newly generated ITS, GPDH and TEF1-α sequences with all available ex-type sequences from the GenBank as listed in Table 1.

Phylogenetic analyses were performed in two different criteria; Maximum Parsimony (MP) and Maximum Likelihood (ML) in order to infer evolutionary relationships among closely related species. Sequence data generated in this study were deposited in GenBank (Table 1).

Maximum Parsimony was performed with PAUP v. 4.0b10 (Swofford, 2003). Trees were inferred using the heuristic search option with 1000 random sequence additions. Descriptive tree statistics for parsimony [Tree length (TL), Consistency Index (CI), Retention Index (RI), Rescaled Consistency Index (RC) and Homoplasy Index (HI)] were calculated for trees generated in the parsimony analysis. Maximum likelihood trees were constructed using the RAxML v.7.4.2 Black Box (Stamatakis *et al*., 2008) in the CIPRES Science Gateway platform (Miller *et al.,* 2010). For the combined dataset all free model parameters were obtained using RAxML with ML estimate of 25 per site rate categories. Phylogenetic trees generated were visualized by FigTree v. 1.4 (Rambaut and Drummond, 2008).

RESULTS AND DISCUSSION

Molecular Phylogeny

In the present study, two different *Exserohilum* species from rice and early barnyard grass were accurately identified. The updated backbone phylogenetic tree for the genus *Exserohilum* presented in Figure 1 includes

Table 1: GenBank accession numbers and culture collection details of the stains used in this study.

Species	Strain no.	Host/Substratum	Country	GenBank accessions			Reference(s)
				ITS	GPDH	TEF1-α	
E. antillanum	CBS 412.93^{ET}	Soil	Cuba	MH862427	LT715894	LT883556	Vu *et al*., 2019
E. corniculatum	BRIP 11426^{ET}	*Oryza sativa*	Australia	LT837453	LT883533	LT883558	Hernandez-Restrepo *et al*., 2018
E. curvatum	CBS 505.90^{ET}	*Sorghum vulgare*	Venezuela	KT265252	LT715889	LT883560	Hernandez-Restrepo *et al*., 2018
E. fusiforme	BRIP 16229^{ET}	*Echinochloa crus-galli*	Australia	KJ415560	KJ415386	KJ415433	Tan *et al*., 2014
E. gedarefense	CBS 297.80^{ET}	*Sorghum bicolor*	Sudan	LT631323	LT715895	LT883563	Hernandez-Restrepo *et al*., 2018
E. holmii	CBS 318.64^{ET}	*Dactyloctenium aegyptium*	Unknown	LT837457	LT883537	LT883565	Hernandez-Restrepo *et al*., 2018
E. khartoumensis	CBS 132708^{ET}	*Sorghum bicolor var. mayo*	Sudan	LT837461	LT715888	LT883569	Hernandez-Restrepo *et al*., 2018
E. longirostratum	CBS 128055	*Acacia mellifera subsp. detinens*	Namibia	LT837478	LT883549	LT896609	Hernandez-Restrepo *et al*., 2018
E. macginnisii	CBS 325.87^{ET}	*Homo sapiens*	USA	KT265237	LT715898	HE664082	Hernandez-Restrepo *et al*., 2018
E. minor	BRIP 14616^{ET}	*Dactyloctenium aegyptium*	Australia	LT837470	LT883545	LT883580	Hernandez-Restrepo *et al*., 2018
E. monoceras	BRIP 11542^{ET}	*Setaria italica*	Australia	LT837473	LT883546	LT896604	Hernandez-Restrepo *et al*., 2018
E. neoregeliae	CBS 132832^{ET}	*Neoregelia carolinae*	Japan	LT837476	LT715886	LT896607	Hernandez-Restrepo *et al*., 2018
E. oryzicola	CBS 502.90^{ET}	*Oryza sativa*	Colombia	HF934949	LT715878	LT896629	Hernandez-Restrepo *et al*., 2018
	USJCC–0010	*Echinochloa oryzoides*	Sri Lanka	MN860001	MN962922	MN962924	This study
E. paspali	CBS 128057	*Paspalum conjugatum*	Brazil	LT837854	LT715857	-	Hernandez-Restrepo *et al*., 2018
E. pedicellatum	CBS 322.64^{ET}	*Triticum aestivum*	USA	KT265258	LT715902	LT896630	Hernandez-Restrepo *et al*., 2018
E. prolata	CBS 571.73	*Zea mays*	USA	LT837831	LT715892	LT896646	Hernandez-Restrepo *et al*., 2018
E. protrudens	BRIP 14814^{ET}	*Dactyloctenium aegyptium*	Australia	KJ415561	LT715880	KJ415432	Tan *et al*., 2014; Hernandez-Restrepo *et al*., 2018
E. rostratum	BRIP 11416^{ET}	*Zea mays*	Australia	LT837466	LT883543	LT883576	Hernandez-Restrepo *et al*., 2018
	USJCC–0011	*Oryza sativa*	Sri Lanka	MN860002	MN962923	-	This study
E. turcicum	CBS 690.71^{ET}	*Zea mays*	Germany	LT837487	LT882581	LT896618	Hernandez-Restrepo *et al*., 2018

BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity

Institute, Utrecht, The Netherlands; USJCC: University of Sri Jayewardenepura Culture Collection, Sri Lanka. ET: ex-type.

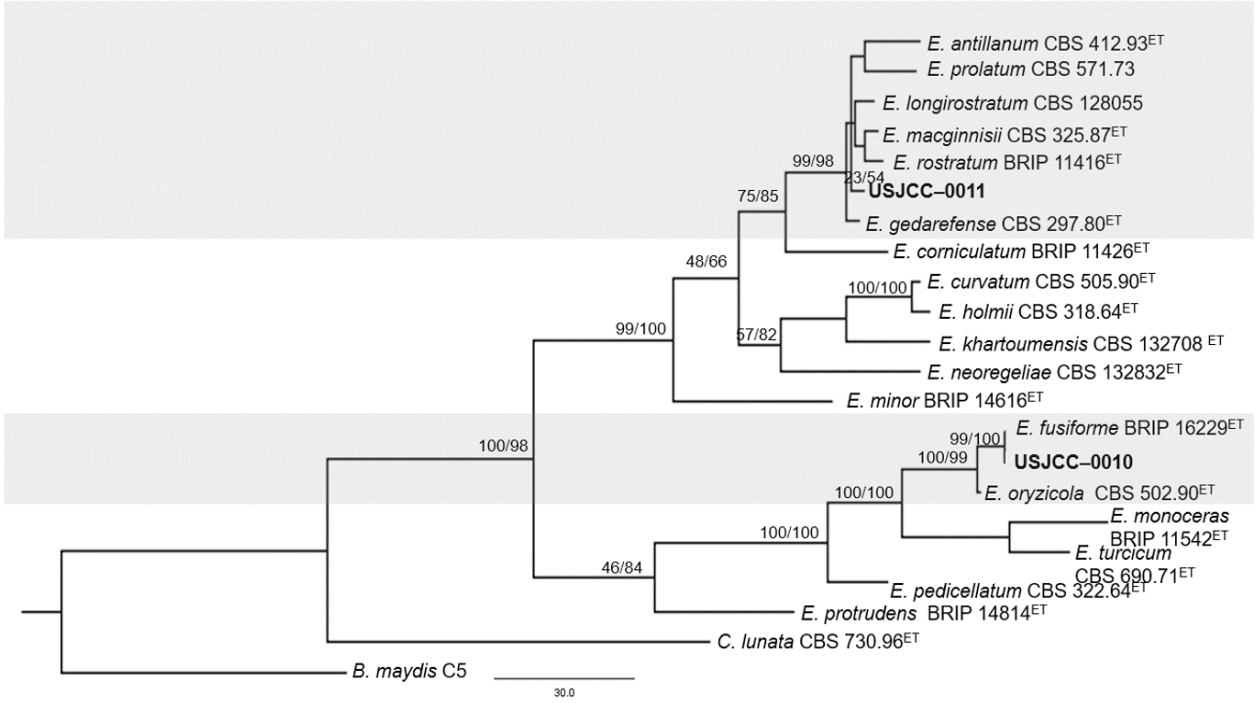

Figure 1: A Maximum Parsimony phylogenetic tree generated based on the combined ITS, GPDH and TEF1-α sequence alignment. MP/ML bootstrap support values are indicated on the branches respectively. Fresh collections from the current study are in bold. The tree is rooted with *Bipolaris maydis*. ET: ex-type.

all currently available ex-type or reference sequences of the species. The phylogram is based on multi-locus concatenated alignment of 21 in-group taxa with *Bipolaris maydis* as the out-group taxon (Hernández-Restrepo *et al*., 2018). The phylogram consists of 18 taxa of *Exserohilum* spp. from GenBank and two *Exserohilum* isolates collected in this study and *Curvularia lunata* ex-type (CBS 730.96). Maximum parsimony analysis revealed that 1661 characters are constant, 175 variable characters are parsimony–uninformative, while 241 characters are parsimony informative out of 2077 total characters. The analysis generated three compatible parsimonious trees and the best tree with the tree statistics: TL = 708, CI = 0.726, RI = 0.792, RC = 0.575, HI = 0.274, is presented (Figure 1).

According to the phylogram generated, two isolates clustered in distinct clades within the genus representing two distinct species. The strain USJCC–0010 isolated from the host *Echinochloa oryzoides*, grouped as more closely related with *Exserohilum fusiforme*. Recent phylogenetic assessments of the genus *Exserohilum* (Hernández-Restrepo *et al*., 2018) have shown that *E. fusiforme* is conspecific with closely related *E. oryzicola.* Therefore the isolate USJCC–0010 was identified as *E. oryzicola*. Similarly, Hernández-Restrepo *et al*. (2018) revealed that *E. antillanum*, *E. gedarefense*, *E. longirostratum*, *E. macginnisii* and *E. prolatum* are conspecific with *E. rostratum*. Therefore, the isolate USJCC–0011, which clustered in the broadly classified "*rostratum* clade" was hereby determined as *E. rostratum*. Although the aforementioned five species are determined to be one species, *E. rostratum,* by Hernández-Restrepo *et al.* (2018) in the phylogeny, sequence variabilities are observed within all three gene loci. Therefore, these species may be segregated in to different taxa if the sampling and gene regions are increased in future studies.

Taxonomy

Based on both morphological characteristics and molecular phylogenetic data, updated taxonomic descriptions are provided below with full illustrations, notes on habitats and recorded hosts and geographic distribution.

Exserohilum oryzicola Sivan., Transactions of the British Mycological Society **83**(2): 325 (1984) (Figure 2)

= *Exserohilum fusiforme* Alcorn, Mycotaxon **41**: 337. 1991.

Sheath blight on *Echinochloa oryzoides*: Linear to irregular, dark brown to brown, elongated lesions. Asexual morph: *Hyphae* pale brown, branched. *Conidiophores* (391–) 431–653 (–638) µm long and 8–10 µm wide (av. = 542, SD = 111, n = 8; av. = 9, SD = 1, n = 5), macronematous, simple, septate, thicker than the vegetative hyphae, straight, rarely flexuous, swollen at the base, dark brown, pale brown to hyaline at the upper part. *Conidia* on CMA (67–) 81–107 (–118) × (12–) 14–18 (–20) µm (av. = 94, SD = 13, n = 30; av. = 16, SD = 2, n = 30), fusiform, straight to slightly curved, pale to dark olivaceous brown, singly or clusters, produced abundantly on CMA, 4–10-distoseptate, pale brown septa. *Hila* strongly protruding.

Colony characteristics: *Colonies* on PDA cottony appearance, olivaceous green, concentric growth ring pattern, convex, irregular margin slightly undulated, abundant aerial mycelia, reaching 6.5 cm diam. in 7-d of incubation. *Colonies* on MEA, dark green and olivaceous

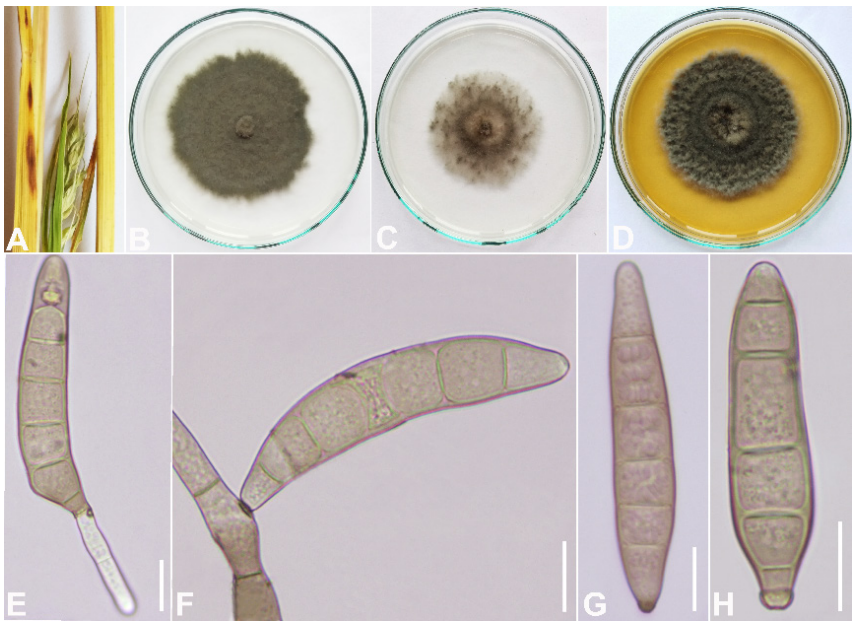

Figure 2: Morphological characters of *Exserohilum oryzicola* (isolate USJCC–0010). A. lesions on *Echinochloa oryzoides*. B. 7-d old colony on PDA C. 7-d old colony on CMA D. 7-d old colony on MEA. E. Germinating conidium. F–H. Conidia. (Scale bars: E–H = 10 µm).

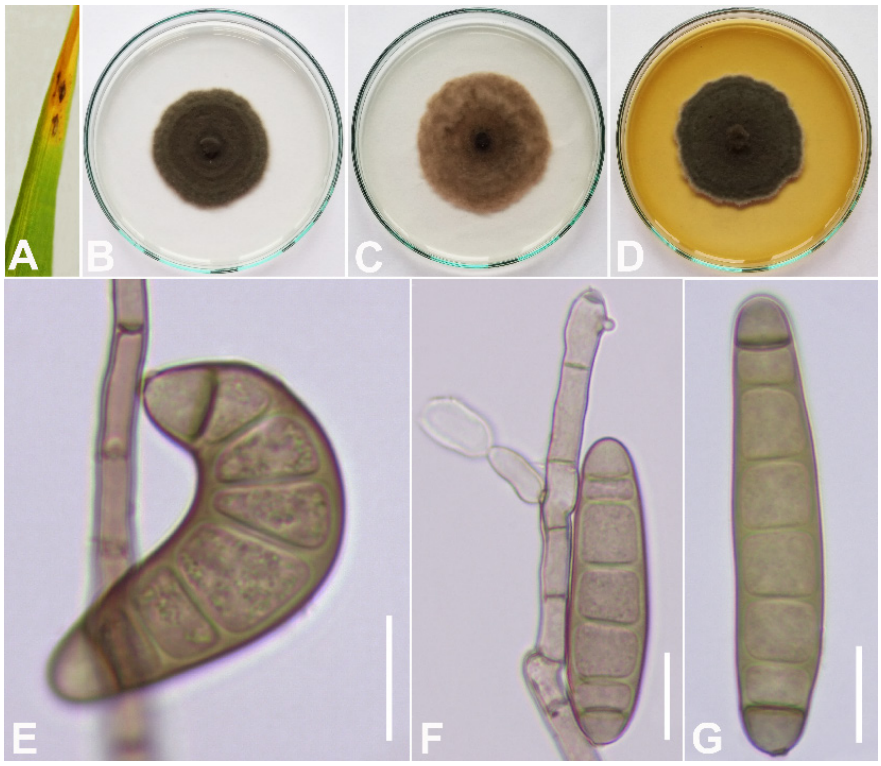

Figure 3: Morphological characters of *Exserohilum rostratum* (isolate USJCC–0011). A. Lesions on host *Oryza sativa*. B. 7-d old colony on PDA C. 7-d old colony on CMA D. 7-d old colony on MEA. E–G. Conidia. (Scale bars: E–G = 10 µm).

green with mouse grey center, irregular margins, attaining approximately 6.9 cm diam.; on CMA dull green aerial mycelia, convex, approximately 5.8 cm in 7-d.

Type specimen: Colombia, Meta, Villavicencio, on leaves of *Oryza sativa*, 2nd Nov. 1982, *E.A. Urresta* (IMI 273194 holotype; CBS 502.90 culture ex-isotype).

Specimens examined: Sri Lanka, Kegalle, Udugama, (N 7°10'23.41801'', E 80°17' 32.01914''), on sheath of *Echinochloa oryzoides*, 19th Feb. 2019, *H.S. Ferdinandez*.

Recorded hosts and geographic distribution: Australia – *Echinochloa crus-galli*; Colombia – *Oryza sativa*; Turkey – *Oryza sativa* (Farr and Rossman, 2020).

Exserohilum rostratum (Drechsler) K.J. Leonard & Suggs, Mycologia **66**: 290 (1974) (Figure 3)

Basionym. Helminthosporium rostratum Drechsler, J. Agric. Res. **24**: 724. 1923.

= *Bipolaris rostrata* (Drechsler) Shoemaker, Canad. J. Bot. **37**: 883. 1959.

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≡ *Exserohilum halodes* (Drechsler) K.J. Leonard & Suggs, Mycologia **66**: 290. 1974.

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= *Helminthosporium longirostratum* Subram., J. Indian Bot. Soc. **35**: 463. 1957.

= *Exserohilum longirostratum* (Subram.) Sivan., Trans. Brit. Mycol. Soc.**83** (2): 328. 1984.

= *Exserohilum prolatum* K.J. Leonard & Suggs, Mycologia **66**: 290. 1974.

=*Setosphaeria prolata* K.J. Leonard & Suggs, Mycologia **66**: 294. 1974.

= *Setosphaeria rostrata* K.J. Leonard, Mycologia 68: 409. 1976.

= *Exserohilum gedarefense* (El Shafie) Alcorn, as 'gedarefensis', Mycotaxon **17**: 68. 1983.

= *Exserohilum macginnisii* A.A. Padhye & Ajello, as 'mcginnisii ', J. Clin. Microbiol. 24: 247. 1986.

=*Exserohilum antillanum* R.F. Castañeda, Guarro & Cano, Mycol. Res. **99**: 825. 1995.

Leaf tip blight on *Oryza sativa*; brown color lesions surrounded by yellow halo. Asexual morph: *Hyphae* pale brown, septate, branched. *Conidiophores* (295–) 341–549 (–656) µm × (4–) 6–8 (–9) µm (av. = 445, SD = 104, n = 8; av. = 7, SD = 1, n = 5), macronematous, simple, septate, thicker than the vegetative hyphae, straight to flexuous, dark brown, pale brown to hyaline at the upper part. *Conidia* on CMA (45–) 50–66 (–77) × (12–) 15–19 (–21) µm (av. = 58, SD = 8, n = 30; av. = 17, SD = 2, n = 30), fusiform, elongated, curved, ellipsoidal, pale to dark olivaceous brown, basal and apical cells often delimited by a dark septum, pale brown middle septa, singly or clusters, produced abundantly on CMA, 5–8-distoseptate. *Hila* slightly protruding.

Colony characteristics: *Colonies* on PDA dark greenish center and olivaceous green to the periphery, flat, entire margin slightly undulated, sparse aerial mycelia, reaching 4.4 cm diam. after 7-d of incubation. *Colonies* on MEA dark green and olivaceous green concentric rings, flat, attaining approximately 5.8 cm diam.; on CMA brownish aerial mycelia, flat colony, approximately 7.9 cm in 7-d.

Type specimen: USA, Washington DC, on dry leaves of *Eragrostis major*, Sept. 1921, *C. Drechsler* BPI 430144 holotype.

Specimen examined: Sri Lanka, Gampaha, (N 7°4'45.19956'', E 79°54' 21.72463''), on leaf of *Oryza sativa*, 31st Jan. 2019, *H.S. Ferdinandez*.

Recorded hosts and geographic distribution: Australia – *Areca catechu*, *Cenchrus setigerus, Chloris barbata, Chrysalidocarpus lutescens, Croton* sp., *Cymbopogon citratus*, *Dactyloctenium aegyptium, Dinebra retroflexa*; Barbados – *Cynodon dactylon*; Brazil – *Brachiaria ruziziensis*; China– *Ananas comosus, Cynodon × dactylon-transvaalensis*; India – *Acacia auriculiformis, Eleusine coracana*, *Sorghum vulgare*, *Triticum aestivum*, *Vigna sinensis*; United States: Florida– *Aechmea fasciata*, *Aloe vera*, *Bromelia* sp., *Caryota mitis*, *Chamaedorea elegans, Chamaedorea seifrizii,* Hawaii– *Dendrobium* sp., North Carolina– *Cannabis sativa*, *Cassia obtusifolia*, *Cyprus*, Texas– *Panicum texanum;* Namibia – *Acacia mellifera* subsp. *detinens*; Oman– *Citrus aurantiifolia;* Taiwan– *Bromus inermis*, *Oryza sativa*; Thailand– *Zea mays*; Sri Lanka– *Coix lacryma* (Farr and Rossman, 2020).

Based on the available sources and databases, we confirm that the two species *Exserohilum oryzicola* on *Echinochloa oryzoides* and *Exserohilum rostratum* on *Oryza sativa* are novel plant-fungal association records. This study highlights the potential occurrence of *Exserohilum* associated with rice and associated weeds in Sri Lanka. Further studies in combination with phytopathological surveys incorporated with molecular data could reveal many unknown fungi and fungal-hosts associations, significance in agriculture and biosecurity. Therefore, this study urges the need for molecular identification and taxonomic studies in Sri Lanka for the control of emerging plant and human pathogens and also to update quarantine measures, pathogen lists for cereal and fiber crops and weeds.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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