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First report of *Pyrrhoderma noxium* from rice in northern Thailand

Absalan S[1,2], Jayawardena RS[2,3*], Bundhun D[2,3], Jungkhun N[4,5], and Lumyong S[1,6,7*]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand
[3]School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand
[4]Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[5]Rice Department, Chiang Rai Rice Research Center, Phan, Chiang Rai 57120
[6]Research Center of Microbial Diversity and Sustainable Utilization, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[7]Academy of Science, The Royal Society of Thailand, 10300 Bangkok, Thailand

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Abstract

Endophytic fungi live in plant tissues without causing disease symptoms. However, they can cause disease on their host by changing their life style. Moreover, endophytic fungi can enhance plant growth and health by antagonistic relationships with the host plant. Rice is one of the most important crops in many tropical countries, particularly in Thailand. Therefore, isolation and identification of fungal endophytes associated with rice can be useful for understanding about fungal diversity on rice, disease management and future studies on endophytes. In this study, *Pyrrhoderma noxium*, formerly known as *Phellinus noxius* has been isolated from rice variety RD10, collected from Chiang Rai province, Thailand. Identification was based on morphology and phylogenetic analyses, including the ITS and LSU loci. This is the first report of *P. noxium* associated with rice.

Keywords – Basidiomycota – Fungal endophyte – *Oryza sativa* – Phylogeny – Taxonomy

Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population (Liang et al. 2019). In Thailand, rice is the most important economic crop, with about 10,407,272 hectares under its cultivation (Kongcharoen et al. 2020). More than 33 million tons of rice were produced in 2021 for domestic consumption and exported to international markets (FAO 2021).

Rice plant is associated with many microorganism communities that play a significant role in its growth and health (Okubo et al. 2014). Some even are pathogens, while some are beneficial to the host (González-Teuber 2016). Endophytes are microorganisms that live within plants' tissues or organs, such as seeds, leaves, flowers, twigs, stems, and roots, without causing apparent harm (Wijesooriya & Deshappriya 2016). They have a variety of relationships with their host, including symbiotic, commensalistic, and mutualistic (Ikeda et al. 2014). Fungal endophytes are known for

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Corresponding Author: Saisamorn Lumyong – e-mail – scboi009@gmail.com,
Ruvishika S Jayawardena – e-mail – ruvishika.jay@mfu.ac.th

their ability to provide many hidden benefits for the host in different ways, including promoting growth, inducing host plants to tolerate biotic and abiotic stresses, producing bioactive secondary metabolites, good yields, and disease resistance (Maciá-Vicente et al. 2009, Hartley et al. 2015). There are few studies on the biodiversity of fungal endophytes, especially in developing countries. However, researchers have begun to take notice of them (Arnold 2007, Rodriguez et al. 2009). One study in Bangkok, Thailand, on endophytic fungi isolated from different parts of healthy plants of two rice varieties, revealed that most of the endophytic fungi isolated from leaves were identified as *Chaetomium cupreum*, *Colletotrichum* sp., *Curvularia lunata*, *Fusarium solani*, *Penicillium* sp., *Trichoderma* sp. Endophytic strains isolated from stems belonged to *Ch. globosum*, *Ch. brasiliense*, *F. oxysporum*, *Penicillium* sp. and *T. harzianum*, *Aspergillus niger*, *A. flavus*, *Pythium* sp. and *Rhizopus* sp. were isolated from the roots (Leewijit et al. 2016).

Pyrrhoderma, typified by *P. sendaiense*is, is a genus with only 14 taxa in *Hymenochaetaceae* that was established by Imazeki (1966). *Hymenochaetaceae* belongs to *Hymenochaetales* which is a large order in Basidiomycota with more than 900 species and over 80 presently identified genera, placed in six families (Cao et al. 2021, Wijayawardene et al. 2021). Zhou et al. (2018) resolved the relationships among the members of this family based on both morphology and phylogeny analysis. They used ITS and LSU to show that the species of *Pyrrhoderma* sensu stricto were placed into two clades. One of them includes six described species of *Pyrrhoderma* and the other clade represents a new genus as *Fulvoderma* with two species. *Pyrrhoderma noxium* formerly known as *Phellinus noxius*, is a destructive tree pathogen that is the main cause of the widespread disease known as brown root rot in tropical and subtropical areas all over the world (Chung et al. 2017).

Although many studies have been conducted on endophytic fungi around the world, a concentrated research on endophytic fungi of rice has not been performed in Thailand. In the present study we identified and illustrated a fungal endophyte from rice.

Materials & Methods

Sample collection, isolation and examination

A rice panicle was collected from Chiang Rai province, Thailand, in November 2021. The rice seed coat was cleaned under running tap water and cut into 1 cm segments. Surface sterilization was done by washing with 70% ethanol for 2 min, 10% sodium hypochlorite solution for 2 min, followed by three rinses in sterile distilled water. After these treatments, the segments were placed on 9 cm petri dishes with potato dextrose agar (PDA) medium that had been supplemented with an antibiotic to inhibit bacterial growth. The petri dishes were then incubated at 26 °C for a week (Senanayake et al. 2020). Fungi grown from plant tissues were transferred to another new PDA culture medium. Finally, fungal isolate was obtained using the hyphal tip isolation method and then purified on a new plate (Senanayake et al. 2020).

Micro-morphological study was conducted using a Nikon ECLIPSE 80i compound microscope and photographs were taken with Canon 750D digital camera attached to the microscope. The Tarosoft (R) Image Frame Work tool was used to take measurements, and Adobe Photoshop CS6 Extended version 10.0 was used to arrange the images (Adobe Systems, USA).

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from 200 mg mycelia scraped from two-week-old culture. The DNA extraction process was carried out using the OMEGA E.Z.N.A.® Forensic DNA Kit according to the manufacturer's protocols.

Two loci, the internal transcribed spacer (ITS) and partial 28S large subunit (LSU) were amplified by polymerase chain reaction (PCR) using ITS5/ITS4 (White et al. 1990) and LR0R/LR5 primers (Vilgalys & Hester 1990) respectively. Agarose gel electrophoresis was conducted to check the positive amplicons from PCR products. Then were visualized on the agarose gel stained with Cybergreen under UV light using a molecular imaging system. The positive PCR products was sent to SolGent Co., South Korea for purifying and sequencing using the same primer pairs.

Phylogenetic analyses

Phylogenetic analyses were carried out following Dissanayake et al. (2020). Sequence data for both loci were subjected to BLASTn search (Basic Local Alignment Search Tool, https//:blast.ncbi.nlm.nih.gov/Blast.cgi), following which related sequences were downloaded from NCBI (National Center for Biotechnology Information) (Table 1). Each individual locus was aligned using MAFFT v. 7.036 (http://mafft.cbrc.jp/alignment/server/large.html, Katoh et al. 2019) with default settings. Sequences were then improved manually when necessary, using BioEdit v. 7.0.5.2 (Hall 1999).

Maximum Likelihood (ML) analysis for the combined alignment was carried out using RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis et al. 2008, Stamatakis 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) with the GTRGAMMA evolution model and bootstrap supports with 1000 replicates.

Bayesian posterior probability (BYPP) analysis was conducted using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001) by Markov Chain Monte Carlo sampling (MCMC) based on four simultaneous Markov chains, with 1,000,000 generations and sampling every 100th generation. The first 25% trees of the burn-in phase were discarded, and the remaining 75% trees were used for calculating the posterior probability (PP). The phylogenetic trees were customized with FigTree v.1.4.0 (Rambaut 2011) and rearranged in Adobe Illustrator CC 22.0.0 (Adobe Systems, USA).

Table 1 Strains and related GenBank accession numbers of *Pyrrhoderma* species used in this study. The ex-type cultures are indicated with superscript "T", and the newly generated sequence is indicated in bold.

Species	**Strains**	**GenBank accession numbres**	
		ITS	**LSU**
Pyrrhoderma hainanense	IFP 019153 ^{T}	NR_158943	NG_064468
P. hainanense	LWZ 20150530-1	MF860794	MF860739
P. lamaense	Dai 16227	MF860802	MF860803
P. lamaense	Dai 16292	MF860803	MF860744
P. lamaense	LWZ 20140617-4	MF860806	MF860746
P. lamaense	Dai 17500	MF860804	MF860748
P. lamaense	Dai 17877	MF860805	MF860749
P. noxium	**MFLUCC 22-0174**	**OP785060**	**OP785091**
P. noxium	P91902W.1	MG645094	MG734573
P. noxium	R29329	MG645116	MG734572
P. noxium	UOG12	MG645089	MG734570
P. noxium	UOG15	MG645090	MG734571
P. noxium	UOG17	MG645091	MG734562
P. noxium	Yap5D	MG645102	MG734558
P. noxium	Yap5B	MG645071	MG734557
P. noxium	R2530	MG645132	MG734553
P. noxium	FRIMPN686	MG645107	MG734551
P. noxium	PNCHINAE35	MG645108	MG734550
P. noxium	AusPn15	MG645155	MG734525
P. noxium	AusPn18	MG645096	MG734527
P. noxium	AusPn20	MG645104	MG734539
P. noxium	AusPn25	MG645129	MG734540
P. noxium	PNCHINAE13	MG645159	MG734515
P. noxium	PNCHINAYMT97	MG645120	MG734514
P. noxium	FRIMPN727	MG645114	MG734511
P. thailandicum	IFP 019159 ^{T}	NR_158944	NG_064469
P. thailandicum	LWZ 20140731-17	MF860812	MF860753
P. yunnanense	IFP 019160 ^{T}	NR_158945	NG_064470
P. yunnanense	LWZ 20140719-12	MF860814	MF860755

Table 1 Continued.

Species	Strains	GenBank accession numbres	
		ITS	LSU
P. yunnanense	LWZ 20140719-13	MF860815	MF860756
Fulvoderma australe	IFP 019150	NR_158428	NG_064467
F. scaurum	LWZ 20130909-2	MF860780	MF860731

Results

Phylogenetic analyses

The combined ITS–LSU alignment comprised 30 strains, including our collection (Table 1). *Fulvoderma scaurum* (LWZ 20130909-2) and *F. australe* (IFP 019150) were used as outgroup taxa. The best scoring RaxML tree (Fig. 1) with value of -3628.501783 as a final ML optimization likelihood is presented. The matrix had 150 distinct alignment patterns with 0.48% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.261444, C = 0.188232, G = 0.263987, T= 0.286338, substitution rates AC = 1.075784, AG = 5.525158, AT = 1.893750, CG = 0.600407, CT = 7.252712, GT = 1.000000. The strain MFLUCC 22-0174 isolated in the present study clustered with other existing strains of *P. noxium* with 54% ML and 0.90 BYPP statistical support (Fig. 1).

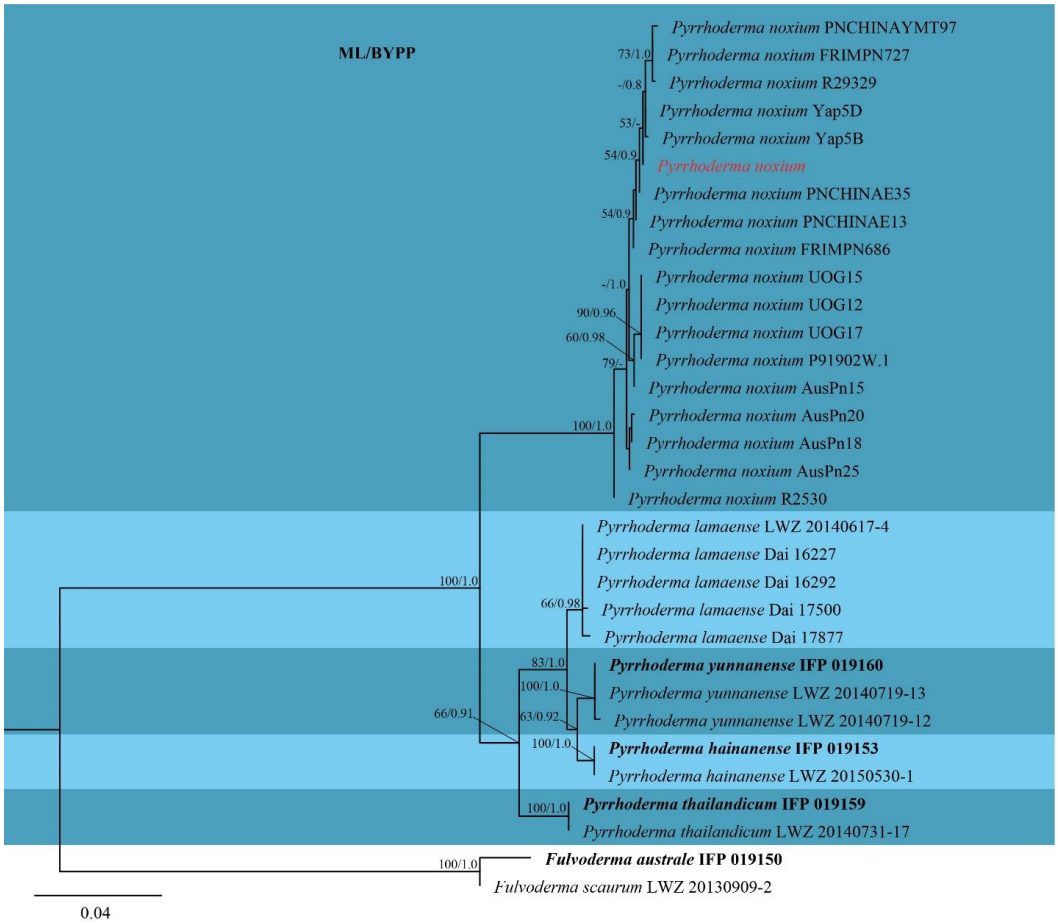

Fig. 1 – RAxML tree of *Pyrrhoderma* based on concotenated ITS–LSU sequence data. Bootstrap support values for ML ≥50% and BYPP >0.8 are shown as ML/BYPP near the nodes. The isolate used in the present study is shown in red. Type strains are indicated in black bold. The tree is rooted using *Fulvoderma scaurum* (LWZ 20130909-2) and *F. australe* (IFP 019150). The scale bar represents the expected number of nucleotide substitutions per site.

Taxonomy

Pyrrhoderma noxium (Corner) L.W. Zhou & Y.C. Dai, in Zhou, Ji, Vlasák & Dai, Mycologia 110(5): 882 (2018)

Endophytic on *Oryza sativa* Asexual morph: *Mycelium* mostly superficial with reddish brown to dark brown, septate, branched, with fairly thick-walled and partly swollen hyphae. Staghorn-like hyphae are produced on irregular lines or patches after two months, sometimes branched or swollen, becoming aggregated with age (Fig. 2). Sexual morph: Not observed.

Culture characteristics – Colonies on PDA, reaching 75 mm diam. after a week at 28 °C. Fluffy to cottony, slightly raised, medium in dense, circular, entire margin. Initially white, later turning brown with irregular dark brown lines or patches. Reverse same color (Fig. 2).

Material examined – THAILAND, Chiang Rai province, Wiang Chiang Rung District, from healthy tissue part of rice seed coat, 25 October 2021, Nootjarin Jungkhun, NS01-3a, (MFLU23-0137; MFLUCC 22-0174).

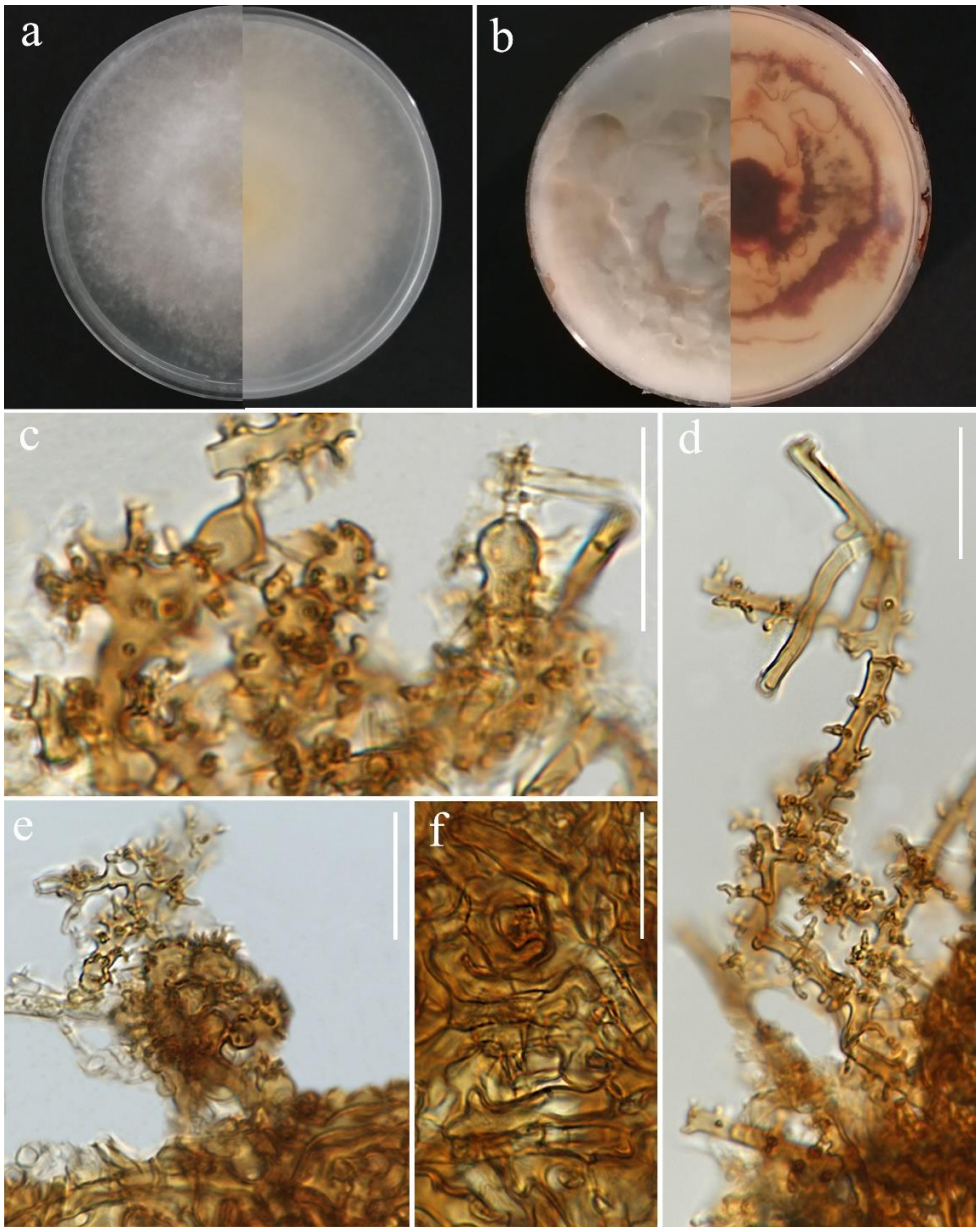

Fig. 2 – a Culture character on PDA after 7 days. b Culture character on PDA after 8 weeks. c, d Staghorn-like hyphae. e Aggregate structure of swollen hyphae. f Patch structure. Scale bars: c–f = 20 µm

Notes – Based on the phylogenetic tree (Fig. 1), all strains of *P. noxium* clustered together with a high bootstap values (100% BS/1.00 PP), indicating high interspecific variations. On one hand, no type strain was established for *P. noxium* (Zhou et al. 2018), and on the other hand, culture morphology of *P. noxium* strains shown in the tree has not been illustrated. However, morphological comparison with strain PN-3 (Ann & Ko 1992) confirmed that our strain belonged to *P. noxium* species.

Discussions

Previous studies indicated that this fungus had a wide range of hosts as the pathogen, mostly woody plants including forest and fruit trees, such as litchi, sugar-apple, plum, pear, loquat, persimmon, carambola, wax apple, grape, jelly fig (Ann et al. 1999), longan (Ann & Ko 1992), and tea (*Camellia sinensis*) (Pegler & Waterston 1968). Some herbaceous plants can be infected by this pathogen (Ann et al. 2002). It was isolated as an endophyte from the medicinal plant *Huperzia serrata* in China (Chen et al. 2011). However, *P. noxium* has never been reported as the endophyte from Thailand.

Although *P. noxium* has been subjected of a few studies as an endophytic fungus, it has not been documented from rice. In the present study, this species was isolated as an endophyte for the first time from rice.

Based on the study of Chi et al. (2019), *P. noxium* isolated as an endophyte from *Acanthus ilicifolius* var. *xiamenensis*, has antifungal activity against two pathogens *Candida albicans* and *Cryptococcus neoformans*. Since rice is an economically important crop in tropical regions, particularly in Thailand, study of rice-associated endophytic fungi can be useful to improve insights on diversity, phylogenetic relationships, lifestyle and function of endophytes, aiming to enhance future researches and improve potential applications.

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