Journal of Agricultural Technology 2009, V.5(1): 129-142

Diversity of filamentous fungi on brown rice from Pattalung Province, Thailand

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Lapmak, K., Lumyong, S., Wangspa, R. and Sardsud, U. (2009). Diversity of filamentous fungi on brown rice from Pattalung Province, Thailand. Journal of Agricultural Technology 5(1): 129-142.

This study emphasized on the diversity of filamentous fungi associated with brown rice from Pattalung province. Two hundred and forty-nine sporulating isolates were identified to 10 genera (*Acremonium, Aspergillus, Bipolaris, Colletotrichum, Curvularia, Drechslera, Fusarium, Geotrichum, Nigrospora,* and *Penicillium*) using morphological characters. Among these, the most common genus was *Colletotrichum*. Two hundred and sixty-seven non-spore forming isolates were grouped to three morphospecies; NS1 (193 isolates), NS2 (20 isolates) and NS3 (54 isolates). Characterization of non-sporulating isolates were based on 28S rDNA sequence analysis. Isolate NS2 was closely related to *Didymella* and *Phoma* (Pleosporales) and NS3 was claded together with *Persiciospora* (Ceratostomataceae).

Key words: brown rice, filamentous fungi, morphospecies, non-sporulating fungi, Pattalung province

Introduction

The number of fungi discovered in Thailand is currently estimated as ca. 6000 which is lower than some other Asian countries (Jones and Hyde, 2004). Thus, further studies on fungal diversity are needed. Several studies on fungal diversity on various substrates or diverse ecological habitats in Thailand and tropical countries has been reported such as bamboo (Hyde *et al.*, 2001), banana (Photita *et al.,* 2001), gingers (Bussabun *et al*., 2001), insects (Jones, 2004; Aung, *et al.,* 2008), Magnoliaceae (Kodsueb, *et al.,* 2008), marine (Sakayaroj *et al.,* 2004), palms (Fröhlich and Hyde, 1999; Yanna and Hyde, 2001; Pinnoi *et al.,* 2006), and Pandanaceae (Whitton, 1999; Thongkantha *et al.,* 2008). Fungal diversity from seed and soil fungi were also investigated (Manoch, 2004; Somrithipol *et al*., 2004). There is only a little information on the diversity of fungal flora in Thai rice grains. However, there have not been investigated fungal communities on brown rice.

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Since during processing, only the hull of the rice kernel was removed, brown rice retained most of its nutritional value that probably as a good habitat for fungi. Brown rice consists mainly of the embryo and endosperm. It has three brownish pericarp layers, two tegmen layers and the aleurone layers. The nutrition facts indicated that 100 g of brown rice contain 2.4 g total fat, 12 mg calcium, 255 mg phosphorus, 326 mg potassium, 0.26 mg vitamin B1, 0.04 mg vitamin B2, 5.5 g niacin and 7.4 g protein while white rice has 0.8 g total fat, 8 mg calcium, 87 mg phosphorus, 111 mg potassium,0.07 mg vitamin B1, 0.02 mg vitamin B2, 1.8 g niacin and 6 g protein (Institute of Nutrition, Mahidol University, 1999). Investigation of the fungi on brown rice should provide answers on global fungal number and diversity of fungi in Thai rice, and the isolates obtained from this study might be undescribed species or unidentified fungi.

Sporulation of fungi is the most important characteristic for fungal identification. However, most fungal isolates in this study cannot identify due to the lack of sporulation. Non-sporulating isolates are generally termed 'mycelia sterilia' and grouped as 'morphospecies' based on similarity in cultural characteristics such as colony surface texture, hyphal pigments, exudates, margin shapes and growth rates (Lacap *et al.,* 2003). Because morphospecies are not real taxonomic entities (Guo *et al.,* 2003), molecular techniques have been used successfully for resolve this problem. Guo *et al.* (2003) identified 18 morphotypes from *Pinus tabulaeformis* to various taxonomic levels based on nrDNA sequence analysis. Lacap *et al.* (2003) compared nucleotide ITS and 5.8S rDNA sequence similarities and identified 6 morphotypes to genus and verified 'morphotypes' as taxonomic groups. Promputtha *et al.* (2005) identified 31 morphospecies to generic level based on ITS and 5.8S regions. During the conducted of the study, we encountered numerous mycelia sterilia, which was grouped in to 3 morphospecies. To determine the phylogenetic placement of mycelia sterilia we used 28S rDNA, because it is highly conserved.

The objectives of this study were 1) to investigate the diversity of fungi associated with brown rice from three varieties of *Oryza sativa* L. ;var. Chiang Pattalung, Leb Nok Pattani and Sang Yod and 2) to identify non-sporulating fungal isolates to familial and generic level by using molecular datas. This study was the first report of fungi associated with brown rice in Thailand.

Materials and methods

Sample collection

Three varieties of paddy rice; Chiang Pattalung (CP), Leb Nok Pattani (LNP) and Sang Yod (SY) were obtained from Pattalung Rice Research Center (PRRC), Thailand in April, 2006. The samples were kept in paper bags and

transported to the laboratory and divided into randomly into portions of 200 g each for fungal isolation.

Isolation of fungi

The rice grains (200 g) were blown and then immersed in 1% NaOCl (w/v) with tween 80 for 2 minutes. Finally, the grains were rinsed three times with sterilized distilled water. Excess water on the grains was mopped using steriled tissue paper. The surface-sterilized samples were aseptically dehulled for more than three hundred dehulled grains (brown rice; BR). One hundred grains were then separated to embryonic (EB) and endosperm (ED) parts. The samples were placed directly on Potato Dextrose Agar (PDA), 20 grains/pieces per 90 mm diameter Petri dish and incubated at 25° C for 3-7 days. Hyphal tips of the developing fungal colonies were transferred onto PDA and subcultured several times until purity. The fungal isolates were kept on PDA slant and in sterilized distilled water tubes.

Identification

The isolated fungi were examined under the microscope and identified on the basis of their morphological characteristics using several keys *i.e.* Watanabe (2001), Pitt and Hocking (1997), Carmichael *et al.* (1980) and von Arx (1981). Mycelia sterilia (non-sporulating isolates) found in these were identified by using molecular techniques.

Statistical analysis

The fungi found in several brown rice and each parts are presented in terms of percentage occurrence by the following formula

$$\text{Percentage occurrence} = \frac{\text{Number of samples which the fungus is detected}}{\text{Total number of samples examined at each sampling}} \times 100$$

The percentage abundance for each species was calculated by using the following formula

$$\text{Percentage abundance of taxon A} = \frac{\text{abundance of taxon A}}{\text{abundance of all taxa}} \times 100$$

Shannon (H′) and Simpson (1-D) diversity indices were used to express the diversity of fungi on different hosts and parts of hosts (Shannon and Weaver, 1949; Simpson, 1949). Index of similarity was estimated using Sorensen's formula to determine the similarity in species occurrence (Odum, 1971). The similarity values ranging from 0 to 1 (1 meaning very similar, 0 indicating no

similarity) were calculated using the formula S′=2C/(A+B) where A and B are the number of species in host 1 and 2 respectively, C is the number of species in common in both hosts.

Phylogenetic analysis for non-spore forming fungi

DNA extraction

Fungal cultures were grown on PDA plates at 25°C for 10 days and total genomic DNA was extracted from fresh mycelium using the protocol outlined by Jeewon *et al.* (2003, 2004) and Lacap *et al*. (2003).

PCR amplification and sequencing of LSU rDNA

The primer pair LROR and LR5 (White *et al.,* 1990) were used to amplify the 28S rDNA. Amplification was carried out in a 50 µl reaction volume. The thermal cycles consisted of 5 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 55°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. Size and purify of PCR products were examined by electrophoresis in 1% (w/v) agarose gel with ethidium bromide (10 mg/ml). PCR products were purified using GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Catalog no. 27-9602-01) following the manufacturer's protocol. The purified PCR products were directly sequenced in an automated sequencer at Macrogen Sequencing System, Korea. The PCR primer pair; LROR and LR5, were used as sequencing primer.

Phylogenetic analysis

The fungal taxa were used in phylogenetic analysis for non-spore forming fungi coded NS2 (accession no. AJ358496) which are listed in Table 1 and mycelia sterilia coded NS3 (accession no. AJ358495) was placed in tree base of Zhang and Blackwell (2002) which added fungal accession numbers AB067709, EF590327 and DQ017375 from GenBank. Nucleotide sequences were aligned using ClustalW program (Thompson *et al.*, 1994). Alignments were manually edited where necessary. Maximum Parsimony method (MP) was used in construction of a phylogenetic tree using the program in MEGA4 (Tamura *et al.*, 2007). The topological analysis was performed with 1000 bootstrap replicates.

Results and discussions

Fungal taxonomic composition

There are few information about diversity of fungi associated with brown rice in the tropical regions including Thailand, while diversity of fungi on

paddy rice (Tonon *et al.,* 1997) or polished rice has been investigated (Tonon *et al.,* 1997; Taligoola *et al.,* 2004; Park *et al.,* 2005). Comparative studies on the diversity of various fungi composite in brown rice are still undocumented.

In this study, brown rice from three rice varieties of *O. sativa* L. obtained from PRRC was examined for fungal diversity. Percentage occurrence of fungi from each samples are given in Table 2 BR parts of every rice varieties had high occurrence while EB part showed lower than the other two parts. Five-hundred and sixteen fungal records were identified including 11 taxa (249records) and 3 morphospecies (NS1, NS2 and NS3) (267 records). The list of taxa and percentage abundance are shown in Table 3 The most common taxa were non-spore forming fungi sp.1 (37.4% of all records), *Colletotrichum* sp. (20.35%), non-spore forming fungi sp.3 (10.47%), *Fusarium oxysporum* (7.75%) and *Curvularia* sp. (5.23%) (Table 3). Sometime, it is necessary to grow the fungus on host substrates to induce sporulation (Guo *et al.,* 1998; Fröhlich *et al.,* 2000). The dominant fungi differed from those found to be common paddy rice and milled rice. In paddy rice, *Fusarium oxysporum* was the most prevalent toxigenic fungal species recorded, followed by *F. verticillioides* and *Aspergillus flavus* respectively. Tonon *et al.* (1997) reported *Penicillium citrinum, P. islandicum, P. aurantiogriseum, F. semitectum* and *A. niger* as a dominant fungi associated with paddy rice while milled rice showed the major fungi were *P. citrinum* and *P. islandicum*.

The most prevalent fungi associated with milled rice are the genera of *Aspergillus, Penicillium, Eurotium, Fusarium, Cladosporium* and *Cochliobolus* (Taligoola *et al.,* 2004), *Penicillium, Aspergillus* and *Alternaria (*Park *et al.,* 2005). Species richness (S), speciec evenness (E_H), Shannon-Wiener diversity index (H′) and Simpson diversity index (1-D) were estimated (Table 4). Similarity index of fungal taxa composition between different hosts and number of overlapping taxa are represented in Table 5 and Table 6.

Fungal communities on different hosts

Three-dimensional correspondence analysis of fungi obtained from three hosts is presented in Fig. 1. The percentage of variance explained by the model is 71.35%. Similarity index among hosts indicated the highest between CP and LNP (0.92%), followed by LNP and SY (0.8%) and CP and SY (0.73%) respectively. According to the similarity index and 3D correspondence analysis, the results from present study suggested the different fungal communities occurred on different hosts but parts of hosts have no effect on fungal occurrence (Fig.1 and Table 5). The overlapping taxa between three hosts were high modulating (8 species out of 14).

Surprisingly, in this study, fungal species on brown rice are made from CP provided the greatest species richness while from LNP showed higher Shannon

Table 1. Fungal taxa used for phylogentic analysis of isolate NS2.

Species	Isolates/strain	GenBank accession number
Bimuria_novae-zelandiae	CBS 107.79	AY016356
Cochliobolus heterostrophus	AFTOL-ID 54	AY544645
Curvularia brachyspora	ATCC 58872	AF279380
Didymella cucurbitacearum	IMI 373225	AY293792
Dothidea ribesia	CBS 195.58	AY016360
Dothidea sambuci	AFTOL-ID 274	AY544681
Helicomyces roseus	BCC3381	AY787932
Karstenula rhodostoma	CBS 690.94	AY787933
Letendraea eurotioides	CBS 212.31	AY787935
Letendraea helminthicola	CBS 884.85	AY016362
Massarina arundinacea	CBS 619.86	DQ813509
Massarina phragmiticola	CBS 110446	DQ813510
Non-sporulating fungi sp.2 (NS2)	BR 488	AJ358496
Phaeosphaeria avenaria	AFTOL-ID 280	AY544684
Phoma herbarum	ATCC 12569	AY293791
Pleospora ambigua	CBS 366.52	AY787937
Pleospora herbarum var. herbarum	CBS 191.86	AF382386
Preussia terricola	AFTOL-ID 282	AY544686
Pyrenophora tritici-repentis	AFTOL-ID 173	AY544672
Repetophragma ontariense	HKUCC 10830	DQ408575
Setomelanomma holmii	CBS110217	AF525678
Setosphaeria monoceras	CBS 154.26	AY016368
Spirosphaera floriformis	A80	AY616238
Sporidesmiella fusiformis	HKUCC 10831	DQ408577
Stylodothis puccinioides	CBS 193.58	AY004342
Thaxteriella helicoma	JCM2739	AY787939
Trematosphaeria heterospora	CBS 644.86	AY016369
Tubeufia amazonensis	ATCC 42524	AY787938
Westerdykella cylindrica	CBS 454.72	AY004343
*Mycosphaerella suttoniae*_outgroup		AF309587

Table 2. Percentage occurrence of fungi on different brown rice.

% occurrence								
CP_BR	**CP_EB**	**CP_ED**	**LNP_BR**	**LNP_EB**	**LNP_ED**	**SY_BR**	**SP_EB**	**SP_ED**
94	75	91	87	58	79	17	3	14

CP: Chiang pattalung, LNP: Leb nok pattani, SY: Sang yod, BR: whole grain of brown rice, EB: embryo, ED: endosperm

Table 4. Diversity indices of fungi recovered from different brown rice.

	CP	LNP	SY
Species richness (S)	14	12	8
Shannon-Wiener indices (H′)	1.71	2.07	1.61
Simpson indices (1-D)	0.7	0.85	0.76
Species evenness (E_H)	0.65	0.83	0.77

CP: Chiang Pattalung, LNP: Leb Nok Pattani, SY: Sang Yodand Simpson diversity index than the other two varieties (Table 4). Rice variety SY showed lowest species richness, Shannon and Simpson diversity index (Table 4). These could be due to it has red pigment aleurone layer may cause hinder fungi accessibility to the food source.

Taxonomic placement of mycelia sterilia NS2 and NS3

Identification for non spore forming fungi, the DNA sequences of LSU region of these fungi were amplified and sequenced. Blast search for similar LSU regions sequence in GenBank were estimated. The evolutionary distances were computed using the Maximum parsimony (MP) method. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Maximum parsimony analysis revealed that NS2 (*Genera incertae sedis*) and NS3 belongs to the order *Pleosporales* and family *Ceratostomataceae* (*Nectriaceae*) respectively. NS2 formed a clade with *Didymella cucurbitacearum* and *Phoma herbarum* with bootstrap support of 97% while NS3 clustered with *Persiciospora africana* with 52% bootstrap support. The heuristic search of NS2 under MP criterion yielded tree (Fig 2) with tree length (TL) of 561, a consistency index (CI) of 0.509, a retention index (RI) of 0.761 and a composite index of 0.439. NS3 belongs to family *Ceratostomataceae*, genus *Persiciospora*. The parsimonious tree of NS3 had TL of 705, CI of 0.384, RI of 0.709 and a compo-site index of 0.294 (Fig. 3). *Persiciospora* was first described by Doguet (1955) as *Melanospora moraui.* Most of *Melanospora* are parasitic on or closely associated with other fungi.

Table 5. Similarity indices of fungal taxa composition between different hosts (overlapping taxa represent in brackets).

	Sørensen indices	
	***Oryza sativa* L. var. Leb Nok Pattani**	***Oryza sativa* L. var. Sang Yod**
Oryza sativa L. var. Chiang Pattalung	0.92 (12)	0.73 (8)
Oryza sativa L. var. Leb Nok Pattani		0.8 (8)

***** overlapping between all hosts = 8 species

Table 6. Similarity indices of fungal taxa composition between different parts (overlapping taxa represent in brackets).

	Sørensen indices	
	Embryo (EB)	**Endosperm (ED)**
Whole brown rice grain (BR)	0.88 (11)	0.92 (11)
Embryo (EB)		0.87 (10)

* overlapping between all parts = 10 species

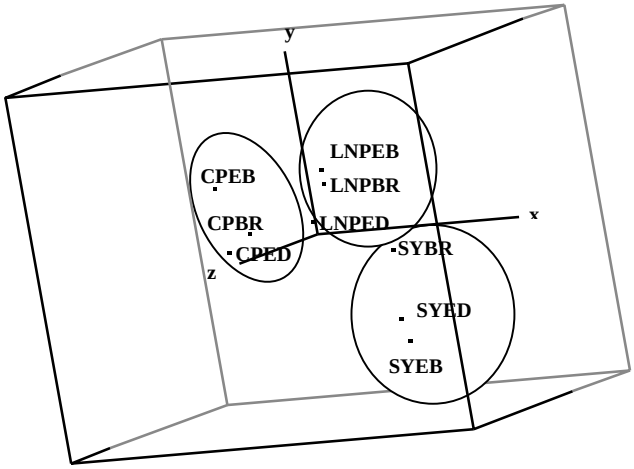

Fig. 1. Three dimensional correspondence analysis of fungal taxa occurring on three brown rice (CP = Chiang Pattalung, LNP = Leb Nok Pattani, SY = Sang Yod, BR = whole brown rice grain, EB = embryo, ED = endosperm).

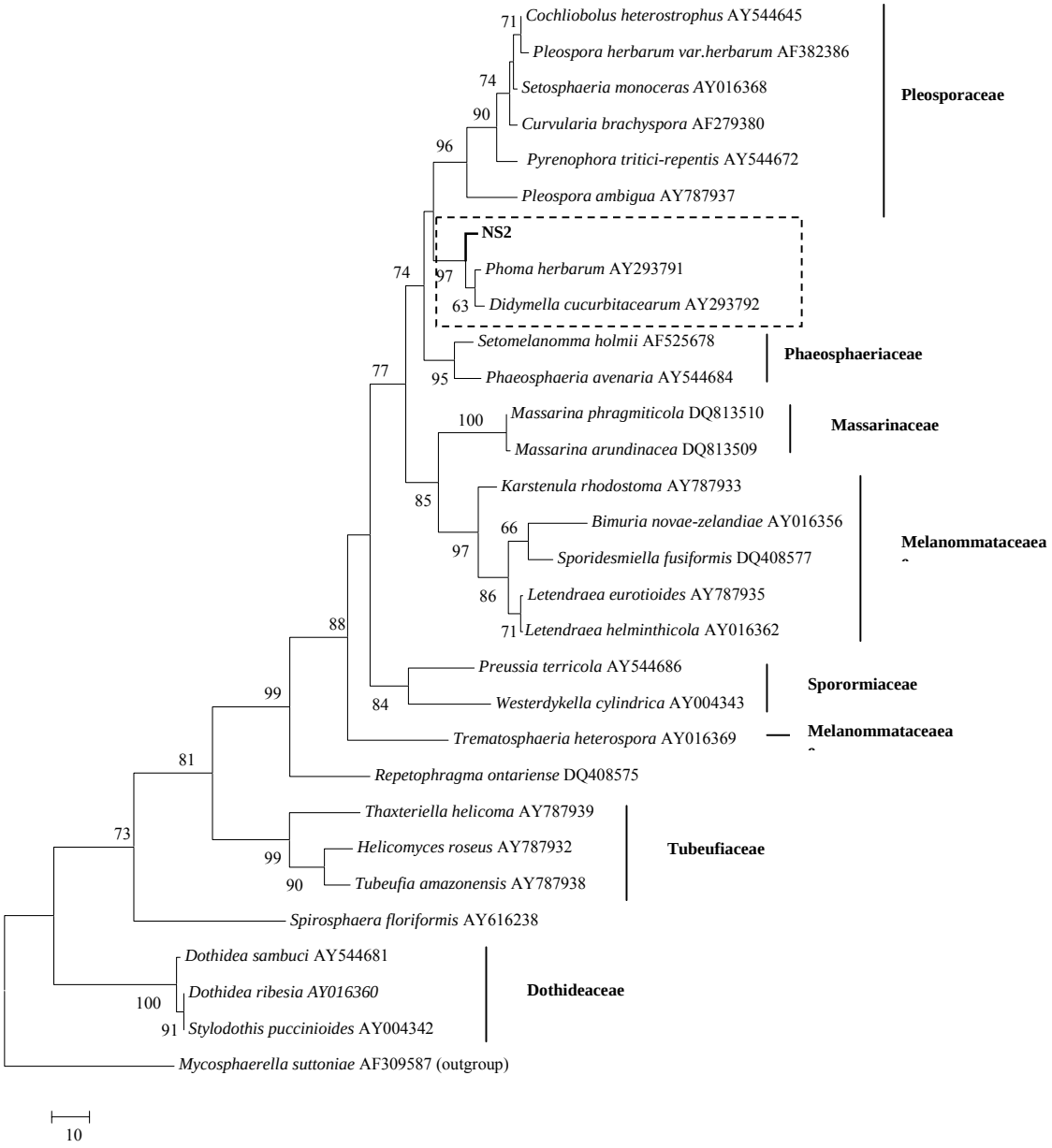

Fig. 2. Maximum parsimony tree generated from the LSU sequences of 30 taxa showing the relationships of NS2 with reference taxa. The tree was rooted with *Mycosphaerella suttoniae* (TL = 561, CI = 0.509, RI = 0.761 and a composite index = 0.439). Bootstrap support based on 1000 replicates for each clade are shown**.**

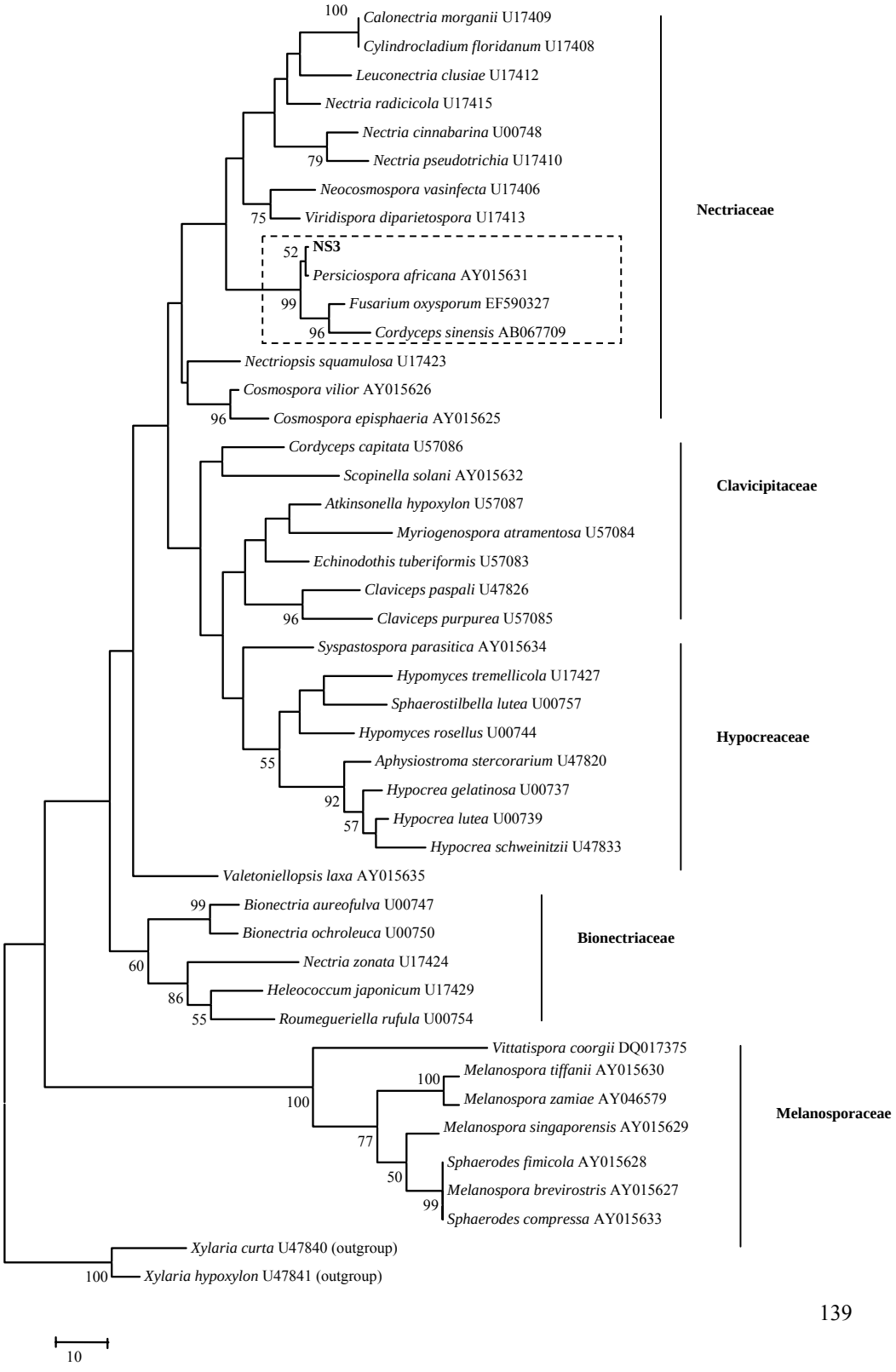
100
Calonectria morganii U17409
Cylindrocladium floridanum U17408
Leuconectria clusiae U17412
Nectria radicicola U17415
Nectria cinnabarina U00748
79
Nectria pseudotrichia U17410
Neocosmospora vasinfecta U17406
75
Viridispora diparietospora U17413
52
NS3
Persiciospora africana AY015631
99
Fusarium oxysporum EF590327
96
Cordyceps sinensis AB067709
Nectriopsis squamulosa U17423
Cosmospora vilior AY015626
96
Cosmospora episphaeria AY015625
Nectriaceae
Cordyceps capitata U57086
Scopinella solani AY015632
Atkinsonella hypoxylon U57087
Myriogenospora atramentosa U57084
Echinodothis tuberiformis U57083
Claviceps paspali U47826
96
Claviceps purpurea U57085
Clavicipitaceae
Syspastospora parasitica AY015634
Hypomyces tremellicola U17427
Sphaerostilbella lutea U00757
Hypomyces rosellus U00744
55
Aphysiostroma stercorarium U47820
Hypocrea gelatinosa U00737
92
Hypocrea lutea U00739
57
Hypocrea schweinitzii U47833
Hypocreaceae
Valetoniellopsis laxa AY015635
99
Bionectria aureofulva U00747
Bionectria ochroleuca U00750
60
Nectria zonata U17424
86
Heleococcum japonicum U17429
55
Roumegueriella rufula U00754
Bionectriaceae
Vittatispora coorgii DQ017375
100
Melanospora tiffanii AY015630
100
Melanospora zamiae AY046579
Melanospora singaporensis AY015629
77
Sphaerodes fimicola AY015628
50
Melanospora brevirostris AY015627
99
Sphaerodes compressa AY015633
Melanosporaceae
Xylaria curta U47840 (outgroup)
100
Xylaria hypoxylon U47841 (outgroup)
10

Fig. 3. Maximum parsimony consensus tree generated with the LSU data set (TL = 705, CI = 0.384, RI = 0.709, composite index = 0.294). Designated outgroup are *Xylaria curta* and *Xylaria hypoxylon*. Bootstrap values higher than or equal to 50% based on 1000 replicates are shown at branches.fuse with the host protoplasts to obtain nutrients, an interaction called fusion biotrophism (Zhang and Blackwell, 2002). Cannon and Howksworth (1982) revised the genus *Melanospora* based on shape and ornamentation of ascospores and described two new species, *P. moreaui* and *P. masonii* and separated *Persiciospora* from *Melanospora.* Nowaday, *Perisiciospora* including four species, *P. moreaui* and *P. masonii* (Cannon and Howksworth, 1982), *P. japonica* from Japan (Horie *et al.*, 1986) and *P. africana* from South Africa (Krug, 1988). Harveson and Kimbrough (2000) investigated *Persiciospora moreaui* that infected watermelon roots in associated with *Fusarium* wilt pathogen and reported first of the relationship of *Persiciospora moreaui* as mutualism or commensalism of *Fusarium oxysporum*. To induce sporulation of *Persiciospora*, Harveson and Kimbrough recommended grow it in the presence of *Fusarium* or some metabolites produced from *Fusarium*.

Acknowledgements

The research project was supported by the Commission on Higher Education, Thailand; Uttaradit Rajabhat University; the Graduated School of Chiang Mai University; Postharvest Technology Institute, Chinag Mai University and The Conservation and Utilization of Biodiversity Project, Chiang Mai University. The paddy rice in this study was supported by Pattalung Rice Research Center, Thailand.

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(Received 14 October 2008; accepted 17 February 2009)

Table 3. Abundance of fungal taxa found on different brown rice.

Taxa	Number of records									Percentage abundance
	BR			EB			ED			
	CP	LNP	SY	CP	LNP	SY	CP	LNP	SY	
Acremonium sp.	1	8	1	4	5	-	2	2	-	4.46
Aspergillus niger	3	-	-	1	-	-	2	-	-	1.16
Bipolaris sp.	1	-	-	4	-	-	-	-	-	0.96
Colletotrichum sp.	15	13	6	13	13	-	18	25	2	20.35
Curvularia sp.	7	4	-	1	6	-	4	5	-	5.23
Drechslera sp.	1	-	-	-	-	-	1	1	1	0.77
Fusarium moniliforme	4	5	-	7	1	-	1	3	-	4.07
Fusarium oxysporum	1-	9	1	5	5	-	2	7	1	7.75
Geotrichum sp.	2	4	1	-	-	-	-	-	-	1.36
Nigrospora oryzae	-	-	-	1	3	-	-	-	-	0.77
Penicillium sp.	-	2	-	2	1	-	2	-	-	1.36
non sporulation sp.1* (NS1)	43	23	1	34	13	1	54	21	3	37.4
non sporulation sp.2 (NS2)	1	8	-	-	4	-	-	6	1	3.88
non sporulation sp.3 (NS3)	6	11	5	3	7	2	5	9	6	10.47
Total records	94	87	15	75	58	3	91	79	14	100

CP: Chiang Pattalung, LNP: Leb Nok Pattani, SY: Sang Yod, BR: whole brown rice grain, EB: embryo, ED: endosperm, - : not found
*identification of non sporulation sp.1 is further study.