POTENTIAL APPLICATION OF FUNGAL ISOLATES FROM PALMS

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FINAL REPORT

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1. SUMMARY OF REPORT:

In screening for antagonist production, the selected fungi isolated from palms were tested for their ability to inhibit the growth and overgrowth of colonies of *Ganoderma boninense*, the cause of basal stem rot of oil palm trees by dual culture test in a yeast extract agar medium. Twenty fungi were tested for their radial growth, and antagonism towards *G. boninense*, with 2 strains showing inhibition of 50 %, of the radial growth of the pathogen. A paper disk agar diffusion assay method was used to check the activity of resulting supernatants from potato dextrose broth. Seventeen strains were selected for study and the results showed that the growth of *Ganoderma boninense* was most strongly inhibited by endophytes no.104, 14.14, 148.148, 16.16, 191.191, 6.6, and 129.129 (clear zone of paper disc are 2.0 mm.). The utilization of oil palm waste for the production of edible mushrooms by local community farmers was also studied.

OBJECTIVES OF THIS STUDY:

1. To evaluate the potential of saprobic and endophytic palm fungi as antagonists of the oil palm pathogen *Ganoderma boninense* (Part I).
2. Utilization of oil palm waste for the production of edible mushrooms by local community farmers (Part II).

Part I: The potential of saprobic and endophytic palm fungi as antagonists of the oil palm pathogen *Ganoderma boninense*

1.1. MATERIAL AND METHODS

1.1.1. Location

Samples of living and decaying palms were collected from various locations in Thailand and examined for saprobic fungi, isolates were made and are deposited in the BIOTEC Culture Collection. Endophytic fungi were also isolated from selected palms to increase the diversity species for the experimental work.

1.1.2. Sample collection

Nine visits were made in September, October, and November 2005 and January, March, November 2006, March, November, 2007 and July 2008 to collect palm materials and for the isolation of endophytic and saprophytic fungi.

1.1.3. Dual culture test

1.1.3.1. Fungi to be tested

Twenty palm fungi were selected to test for antagonism towards *G. boninense*.

1.1.3.2. Radial growth of fungi

Twenty palm fungi were grown in Yeast Extract Agar to determine their radial growth as well as those of the pathogenic strains. This will enable the timing of inoculation of antagonist and pathogenic isolates of *G. boninense*. The radial growth was measured and divided into slow growth (0.01-2.49 cm into 2 weeks), moderate growth (2.50-2.90 cm into 2 weeks), and fast growth (5.91-7.20 cm into 2 weeks) (Tables 1, 2).

1.1.3.3. Dual culture method

The method used to study interactions between the palm fungi and the pathogen was similar to that used by Cavalcante and Eaton (1981). Mycelial plugs removed from the growing margins of cultures of tested fungi were placed at one side of a Petri dish containing yeast extract agar (with added 10 g/L glucose) and incubated at 25°C for 1 day (for faster growth fungi) and 3 days (for slower growing fungi). After this time, cores removed from the margins of actively growing cultures of the pathogen, *G. boninense*, were placed at the opposite sides of the dishes and the plates incubated under the same conditions. Three replicate pairings of each isolate were made, including self-paired controls. Plates were examined daily to determine the outcome of interactions between the organisms.

$$\text{Percentage inhibition} = \frac{r_1 - r_2}{r_1} \times 100$$

r_1 = radius of pathogen in control plate

r_2 = radius of pathogen in dual culture plate (Figure 1).

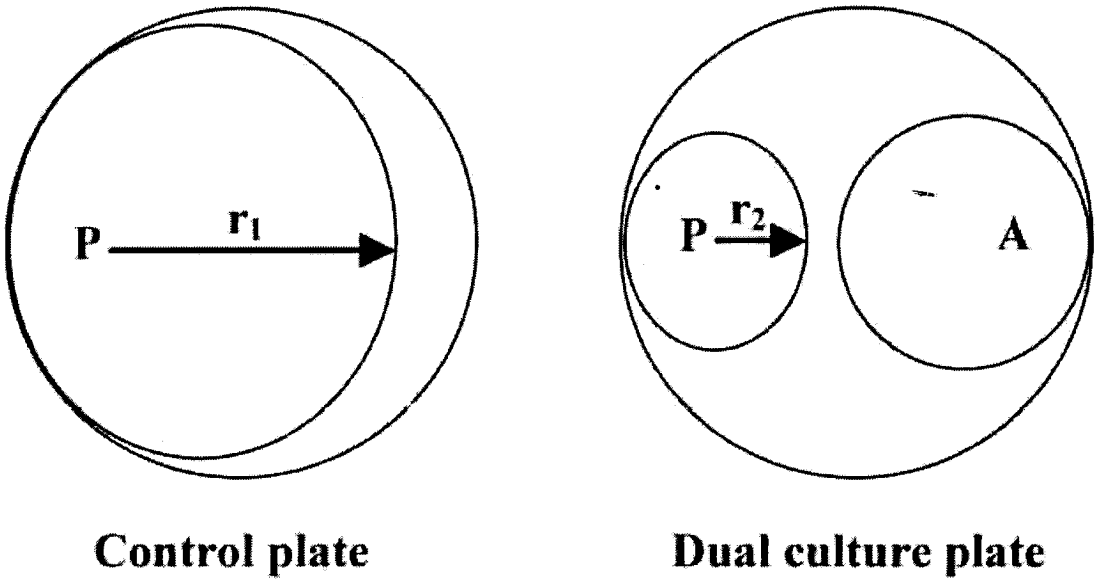


Fig 1. Percentage inhibition in dual culture test: P = Pathogen, A = selected palm fungus, r_1 = radius of pathogen in control plate, r_2 = radius of pathogen in dual culture plate.

Antagonists showing high specificity to *Ganoderma* will be further screened for bioactive activity (Figures 2, 3).

1.1.4. Paper disk agar diffusion assay

Seventeen strains selected from the isolates showing high antagonist activity from the dual culture test (the percentage inhibition over 80%, the results from the 1st and 2nd years) (Table 4.)

1.1.4.1. Preparation of assay plates

Ganoderma boninense was cultured and maintained at room temperature on half potato dextrose agar.

1.1.4.2. Screening for anti-microbial production

Potato dextrose broth was used as the liquid medium for fermentation. Seventeen strains of selected palm fungi were individually subcultured on to PDA plates and incubated for 7 days. One 5 mm diameter plug was cut from the growing edge of each colony and inoculated into a flask containing 100 ml fermentation medium. The fermentation flasks were incubated at room temperature (25–30 °C) for 21 days on a reciprocal shaker at 200 rotations per minute (2 replications/ medium/ test fungus).

1.1.4.3. Extraction

After fermentation, the culture was harvested by filtration. The culture broths were then extracted twice with equal volumes of ethyl acetate (EtOAc). The EtOAc extracts were pooled and dried by rotary evaporator (BÜCHI witzerland) at 45 °C under reduced pressure. The extract residue was dissolved in dimetyl sulfoxide (DMSO) and stored at 4 °C until the bioassays could be conducted.

1.1.4.4. Paper disk diffusion assay method

Sterile paper disks (5 mm diameter) were dipped into each extract, allowed to air dry, and placed on the assay plates. Controls consisted of disks impregnated with solvents and allowed to air dry. All assay plates were then incubated at room temperature for 2–3 days. After incubation the diameter of fungal growth inhibition zone was measured (Figures 4-5).

1.2. RESULTS

1.2.1. Dual culture test

1.2.1.1. Radial growth of fungi

Twenty palm fungi were studied to determine their radial growth and are listed in Tables 1 (slow growing species), and 2 (m oderately fast growing strains). The interactions between the organisms are showed in Table 3.

Table 1. Fungi showing slow growth (0.01-2.49 cm at two weeks) on freshwater yeast extract agar.

No.	morph	radial growth (cm)
1	15	2.42
2	22	1.93
3	12.12	0.42
4	20.20	0.53
5	28.28	2.03
6	40.40	1.10
7	130.130	0.80
8	64.64	0.43
9	156.156	2.43
10	158.158	1.97
11	239.239	2.33
12	260.260	2.43
13	271.271	0.47

Table 2. Fungi showing moderate growth (2.50-5.90 cm at two weeks) on freshwater yeast extract agar.

No.	morph	radial growth (cm)
1	5.5	3.87
2	23.23	2.77
3	29.29	2.60
4	30.30	2.80
5	32.32	3.10
5	288.288	4.83
6	298.298	5.70

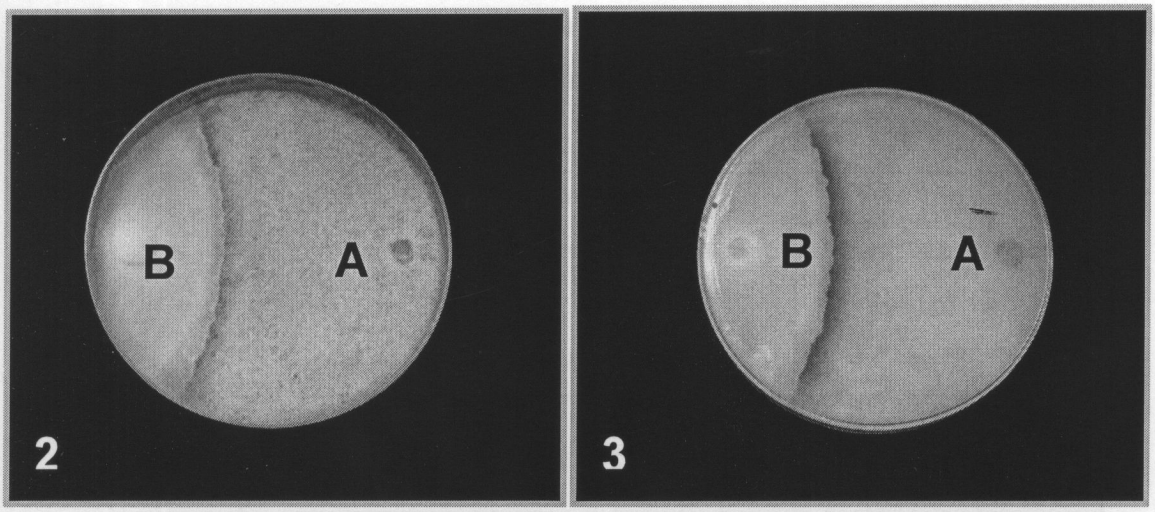
1.2.1.2. Dual culture of antagonists and pathogen on Yeast extract agar

In the dual culture study, a wide variety of reactions were noted by selected palm fungi against the oil palm pathogen *Ganoderma boninense* (Table 3). The results show that two strains produced percentage inhibition of radial growth by over 50%. Sample antifungal activity by the dual culture method are illustrated in Figures 1-2. This set of results brings the total number of isolates screened to 300 (over 1.5 year period), of which 86 showed over 65% inhibition of the pathogen.

Table 3. Growth of *Ganoderma boninense* when grown on media with selected antagonists.

No.	Test Fungi (no.)	Radial growth of *Ganoderma boninense* (cm) control = 7.2	% inhibition of radial mycelial growth
1	15	4.2	41.7
2	22	6.2	13.9
3	12.12	7.2	0.0
4	20.20	7.2	0.0
5	23.23	4.3	40.3
6	28.28	4.5	37.5
7	29.29	3.7	48.6
8	30.30	4.0	44.4
9	32.32	3.6	50.0
10	40.40	7.2	0.0
11	130.130	7.2	0.0
12	64.64	7.2	0.0
13	5.5	4.7	34.7
14	156.156	4.2	41.7
15	158.158	6.0	16.7
16	239.239	7.2	0.0
17	260.260	4.0	44.4
18	271.271	7.2	0.0
19	**288.288**	**3.5**	**51.4**
20	**298.298**	**3.0**	**58.3**

Note: Bold font = percentage inhibition of radial growth by over 50%.



Figs 2-3. Antifungal activity of endophytic fungi (A) against *G. boninense (B)* by dual culture method. 2. surface view 3. back view.

1.2.2. Paper disk agar diffusion assay

1.2.2.1. Selected fungi for test

Seventeen strains showing percentage inhibition over 80% by dual culture test were selected.

Table 4. The seventeen strains selected for the paper disk agar diffusion assay, based on their antagonistic activity against *Ganoderma boninense*.

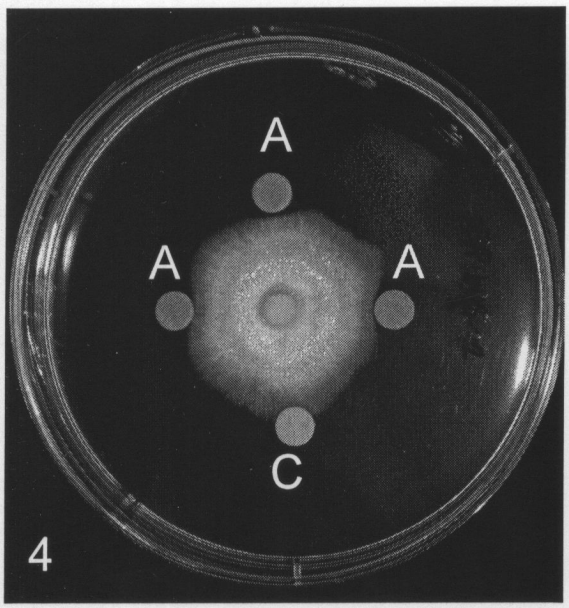
No.	test endophyte (no.)	growth rate of . Ganoderma boninense (cm) control = 7.2	% inhibition of radial mycelial growth (Over 80 %)
1	148.148	0.5	93.1
2	129.129	0.6	91.7
3	90.90	0.7	90.3
4	104	0.8	88.9
5	235.235	0.9	87.5
6	16.16	0.9	87.5
7	197.197	0.9	87.5
8	6.6	1.0	86.1
9	14.14	1.0	86.1
10	138.138	1.0	86.1
11	59.59	1.0	86.1
12	179.179	1.0	86.1
13	101.101	1.2	83.3
14	232.232	1.2	83.3
15	149.149	1.2	83.3
16	191.191	1.4	80.6
17	333.333	1.4	80.6

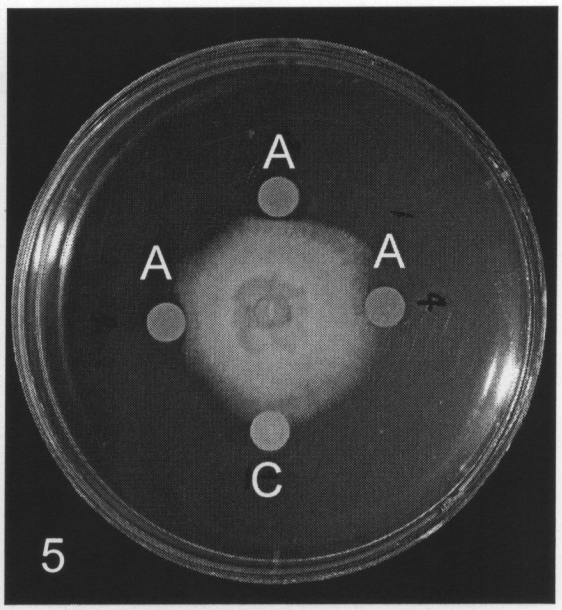
1.2.2.2. Screening for anti-microbial production

All fungal isolates showed inhibition against *Ganoderma boninense* (Table 5). Growth of *Ganoderma boninense* was most strongly inhibited by endophytes no.104, 14.14, 148.148, 16.16, 191.191, 6.6, and 129.129 (clear zone of paper disc are 2.0 mm.) These were selected for further experimentation.

Table 5. The qualitative antimicrobial activity of selected palm fungi against *Ganoderma boninense*.

No.	Original code	Clear zone of paper disc	Activity
1	104	2.0 mm.	Strongly active
2	14.14	2.0 mm.	Strongly active
3	148.148	2.0 mm.	Strongly active
4	16.16	2.3 mm.	Strongly active
5	191.191	2.1 mm.	Strongly active
6	6.6	2.0 mm.	Strongly active
7	129.129	2.0 mm.	Strongly active
8	149.149	1.0 mm.	Moderately active
9	59.59	1.5 mm.	Moderately active
10	90.90	1.0 mm.	Moderately active
11	179.179	1.5 mm.	Moderately active
12	232.232	1.0 mm.	Moderately active
13	197.197	1.2 mm.	Moderately active
14	235.235	1.0 mm.	Moderately active
15	333.333	1.5 mm.	Moderately active
16	138.138	0.5 mm.	Weakly active
17	101.101	0.0 mm.	Weakly active





Figs 4-5. Screening for antimicrobial production by paper disk agar diffusion assay on half PDA placed with *Ganoderma boninense*. Sterile paper disks dipped into fungal extract (A) Controls (C) 4. surface view 5. back view.

Part II: The utilization of oil palm waste for the production of edible mushrooms by local community farmers.

2.1. MATERIAL AND METHODS

2.1.1. Substrate preparation

1. Oil palm fronds were collected from Suksomboon oil palm plantation of oil palm factory, Chonburi province and used for mushroom cultivation comparison with parawood sawdust.
2. Cut off all leaflet from oil palm fronds.
3. Grind oil palm fronds to produce a fine pulp.

2.1.2. Mixture substrate

Three media were prepared from the oil palm frond pulp.

1. Recipe 1 (control)

- Parawood sawdust 100 kg
- Rice bran 7.5 kg
- Calcium sulphate ($CaSO_4$) 2 kg
- Calcium carbonate [$Ca(CO_3)_2$] 1 kg
- Magnesium sulphate ($MgSO_4$) 200 g
- Water : mixture substrate 1:1

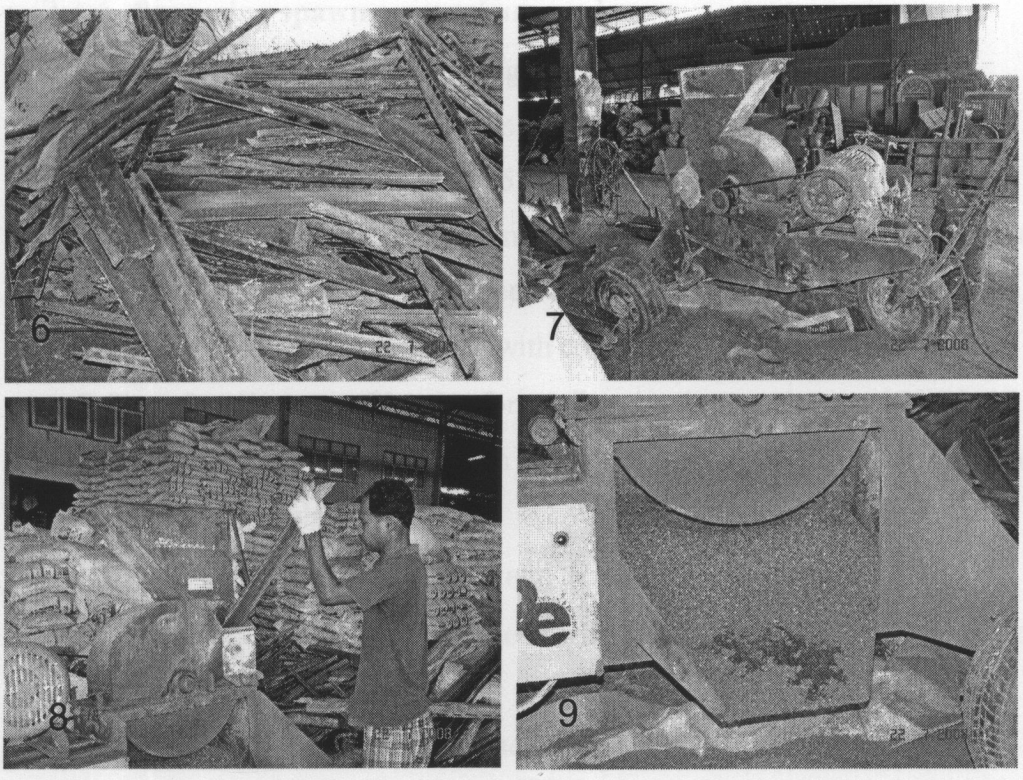
2. Recipe 2 (nutrients similar with recipe1 but used oil palm frond pulp)

- Oil palm frond pulp 100 kg
- Rice bran 7.5 kg
- Calcium sulphate ($CaSO_4$) 2 kg
- Calcium carbonate [$Ca(CO_3)_2$] 1 kg
- Magnesium sulphate ($MgSO_4$) 200 g
- Water : mixture substrate 1:1

3. Recipe 3 (nutrients similar with recipe 2 but without rice bran)

- Oil palm frond pulp 100 kg
- Calcium sulphate ($CaSO_4$) 2 kg
- Calcium carbonate ($CaCO_3$) 1 kg
- Magnesium sulphate ($MgSO_4$) 200 g
- Water : mixture substrate 1:1

1. Mix nutrients and substrate for each recipe together by using blending machine or manual methodand bagged in polythene bags.
2. Each bag contains 800 g medium.
3. Sterilization of all substrate bags by streaming at 100 C°, for 3.5 hours or 121 C°, 15 min. by autoclaving.
4. Bags were seeded and fruit bodies initiated following standard protocols.



Figs. 6-9 Raw material of oil palm petioles, grinding machine and grinding processes of raw material.

2.1.3. Isolation of edible basidiomycetes

Basidiomycetes are isolated from basidiospores by allowing them to eject on to a culture medium or alternatively, removal of sample tissue from the basidiocarp trama. Media used for isolation was Malt Extract Agar (MEA) or Potato Dextrose Agar (PDA) with the addition of 50 ppm streptomycin (to suppress bacterial growth) + 2 ppm benomyl (to suppress the growth of anamorphic fungi). When growth is good the strain is transferred to PDA without antibiotics.

2.1.4. Growth of fungi

Three commercial basidiomycetes in Thailand; *Schizophyllum commune*, *Pleurotus sajor-caju*, and *Lentinus squarrosulus* were selected and grown on potato dextrose agar in flat bottles.

2.1.5. **Preparing spawn on sorghum seeds:**

1. Soak sorghum seeds for one night.
2. Wash and strain sorghum seeds to remove all water.
3. Steam sorghum seeds for 30-45 minutes to soften grain.
4. Drain water and spread sorghum seeds to cool down and decrease moisture.
5. Fill ¾ of bottle with sorghum seeds.
6. Tightly plug mouth of bottles with cotton wool and leave to ventilate.
7. Transfer all prepared bottles for sterilization in autoclave at 121 °C, 15 minutes.
8. After bottles are cooled down inoculate sorghum seeds with a disc of mushroom from an agar plate.
10. Place paper over cotton wool and tie with a rubber band.
11. Sorghum seed spawn was used to inoculate the palm frond pulp in the polythene bags.
12. Shake the bottle to separate the seeds colonized with the mycelium.
13. A few sorghum seed spawn (about 20 to 30 seeds) are poured into the substrate bag.

2.1.6. Harvesting basidiocarps methods

For *S*chizophyllum *commune* and *Lentinus squarrosulus* harvesting by using razor blade or thin blade cut under basal part of aggregate basidiocarps nearest the bag, do not pull out the basidiocarps because if we pull out the aggregate basidiocarps it might be make the vegetative mycelium very slow or not form basidiocarps for the next round.

For *Pleurotus sajor-caju* harvesting is by pulling out aggregate basidiocarps directly from the top of cultivated bags, after we pull out the aggregate basidiocarps using spoon handle picked the stipes residue out from bags. If we not do this the stipes residue will rot and become contaminated by bacteria and other anamorphic fungi.

2.1.7. Yield of mushrooms

Mushrooms were weighed at specific times to determine yield and then compared with the growth of the basidiomycetes on commercial media.

2.2. RESULT

Table 6 list the growth parameters for the three selected edible basidiomycetes. Tables 7-10 summarize the yields of the basidiomycetes on the different media.

Table 6. Incubation time for the three selected edible basidiomycetes at room temperature.

Taxa	Growth on PDA in flat bottle (days)	Growth on sorghum seed spawn (days)	Incubation time until initial forming basidiocarps (days)
Schizophyllum commune	8	6	23
Lentinus squarrosulus	7	8	22
Pleurotus sajor-caju	7	11	23

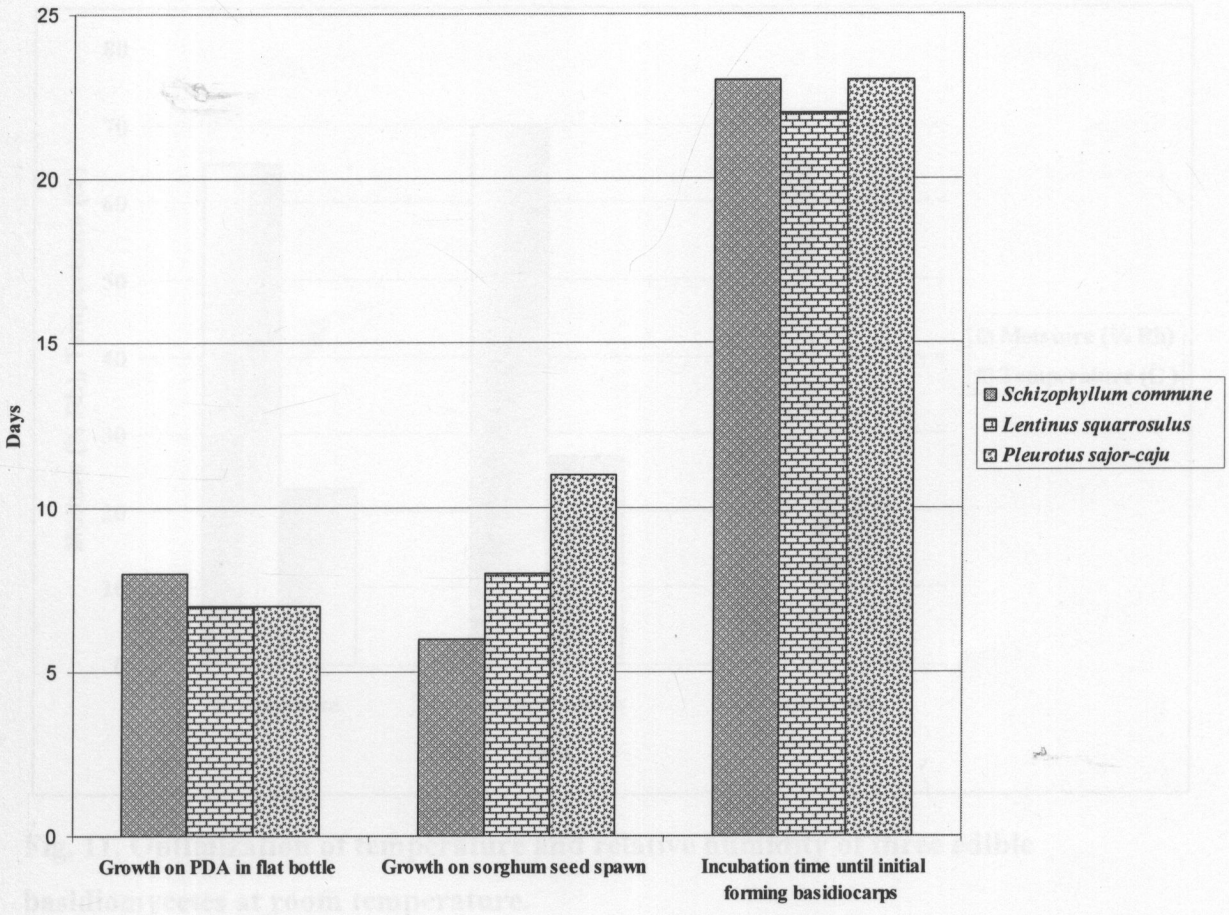


Fig. 10. The incubation time of three selected fungi at room temperature.

Incubation time for the three selected edible basidiomycetes at room temperature from started inoculation to initial forming basidiocarps are similar, but a few different growth in sorghum seed spawn.

Table 7. Incubation time for the three selected edible basidiomycetes at room temperature.

Condition	Taxa		
	S. commune	*L. squarrosulus*	*P. sajor-caju*
Moisture (%Rh)	65	70	65
Temperature (°C)	23	27	23

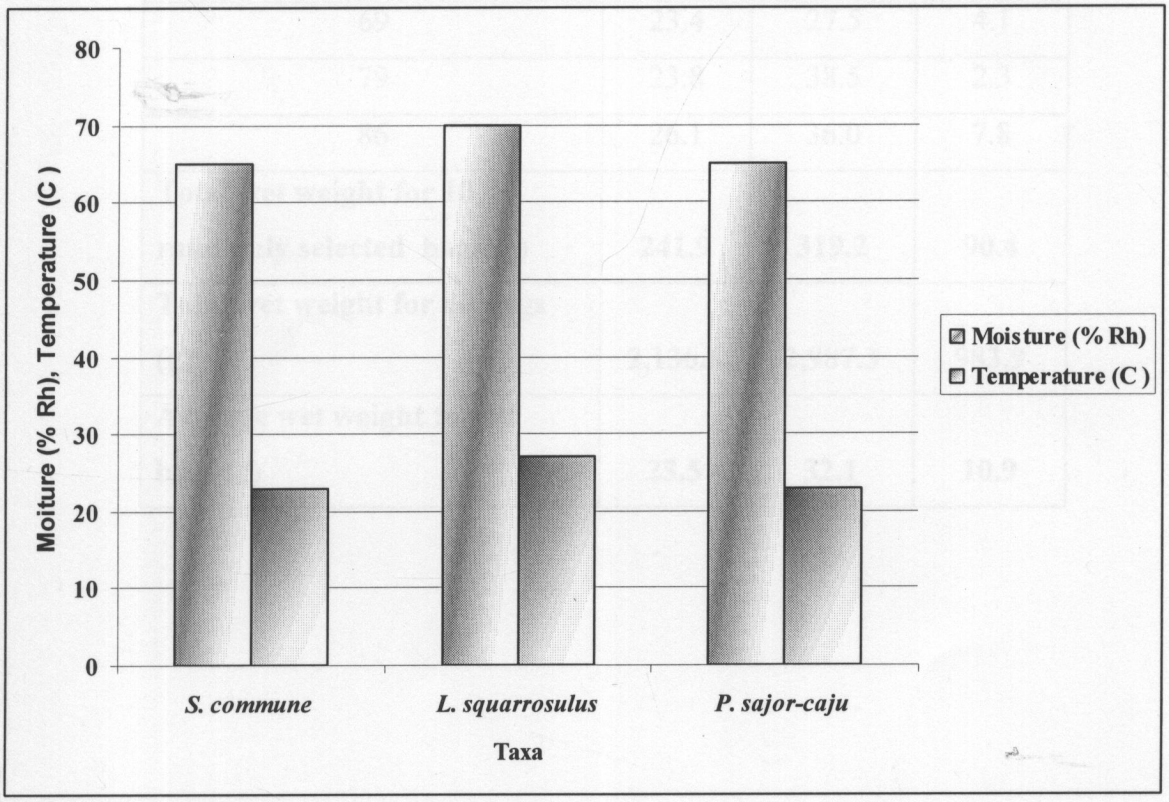


Fig. 11. Optimization of temperature and relative humidity of three edible basidiomycetes at room temperature.

The optimization of temperature and relative humidity (%Rh) for growth of *Schizophyllum commune* and *Pleurotus sajor-caju* is about 23 °C, 65% while *Lentinus squarrosulus* is about 27 °C, 70%.

Table 8. Wet weight of *Schizophyllum commune* for the three recipes from 10 bags selected at random.

Number	Recipe 1	Recipe 2	Recipe 3
5	23.6	27.2	6.5
19	26.7	25.2	12.4
26	24.5	33.3	13.0
35	27.4	34.4	11.0
39	23.1	31.7	13.4
52	19.9	29.6	10.3
57	23.6	35.7	9.7
69	23.4	27.5	4.1
79	23.8	38.5	2.3
86	26.1	36.0	7.8
Total wet weight for 10 randomly selected bags (g)	**241.9**	**319.2**	**90.4**
Total wet weight for all bags (g)	**2,136.6**	**2,987.3**	**983.9**
Average wet weight for all bags (g)	**23.5**	**32.1**	**10.9**

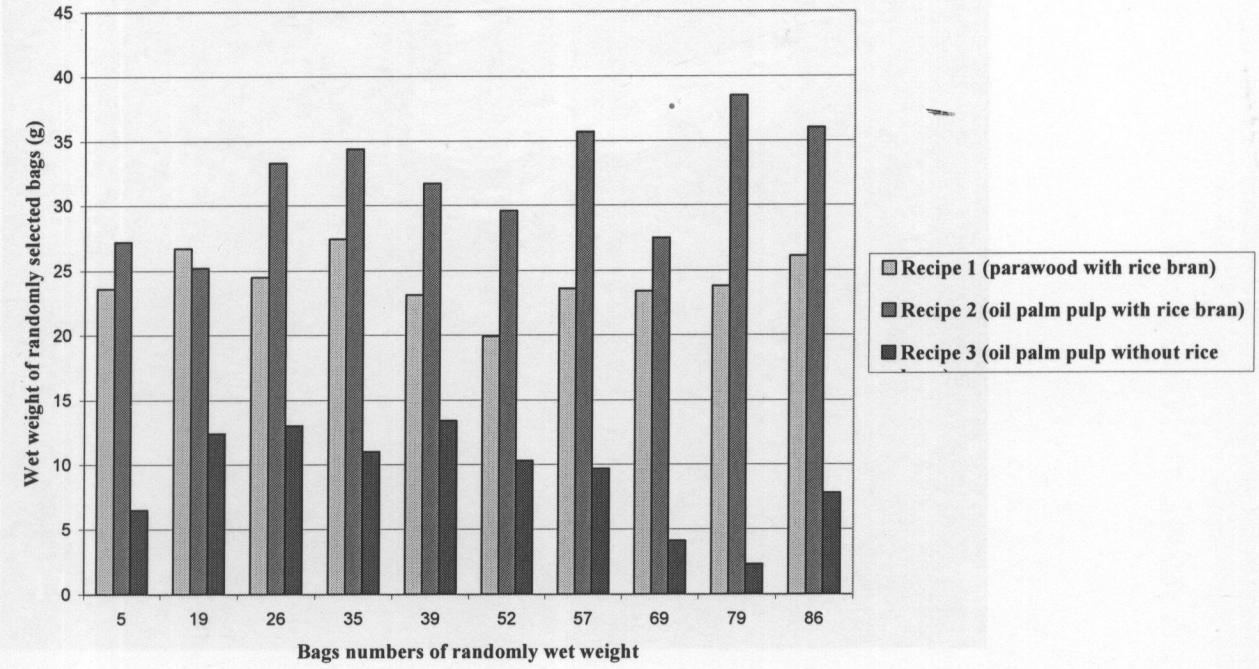


Fig. 12. Comparative wet weight of *Schizophyllum commune* from three recipes.

Schizophyllum commune on recipe 1 from the 91 growth bags yielded 2,136.6 g of fruit bodies, with an average of 23.5 g per bag. On recipe 2 from 93 bags, the yield was 2,987.3 g, an average of 32.1 g per bag. Recipe 3 from 90 bags yielded 983.9 g with an average of 10.9 g per bag.

Of the three media used for the growth of *S. commune*, the best yield was for recipe 2; 32.1 g per bag, the lowest yielded was recipe 3; 10.9 g per bag. The difference between recipes 2-3 is the addition of rice bran to recipe 2, and this is thought to promote basidiocarp production of *S. commune*.

Fig. 13 Harvesting basidiocarps of *Schizophyllum commune*.

Problem and suggestions: The problem for *S. commune* cultivation is firstly, a short harvesting period (about two rounds harvested). After the second round basidiocarps will be very small or not produced. Secondly, they were contaminated by *Trichoderma* spp.(might be contaminated from tap water from the farm). Some species of Myxomycetes such as *Stemonitis fusca* and *Cribaria* sp. infected *S. commune* after the second round of harvesting basidiocarps, it probably because of the high humidity in the substrate. Thirdly, the problem from mite and insect, so after the second round if *S. commune* not produce basidiocarps, we should dry by sunlight, or burn substrate for protect from mite and insect, and keep the area clean.

Table 9. Wet weight of *Pleurotus sajor-caju* for three recipes of substrate from 10 bags selected at random.

No.	Recipe 1 (g)	Recipe 2 (g)	Recipe 3 (g)
5	152.85	144.54	112.27
19	108.33	83.97	68.61
26	169.81	290.28	149.99
35	257.07	147.61	142.01
39	226.54	18.57	67.06
52	120.62	169.49	110.99
57	149.02	-	98.02
69	167.15	178.44	93.09
79	150.93	110.36	104.36
86	219.56	319.83	119.84
Total wet weight for 10 randomly selected bags (g)	**1,721.9**	**1,463.1**	**694.6**
Total wet weight all (g)	**11,722.89**	**11,914.82**	**8,774.89**
Average wet weight all (g)	**147.8**	**143.9**	**105.2**

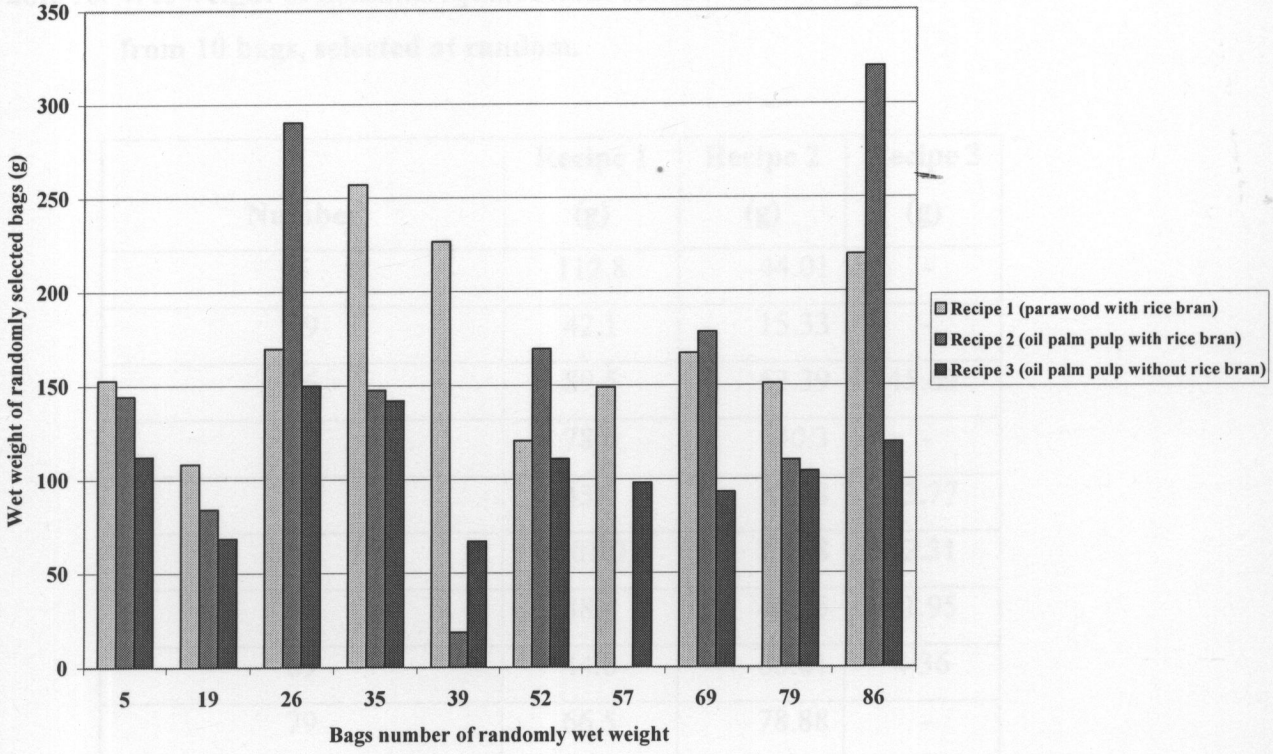


Fig. 14. Comparative wet weight of *Pleurotus sajor-caju* for the three recipes.

Pleurotus sajor-caju on recipe 1 from the 91 growth bags, yielded 11,722.9 g of fruit bodies, with an average of 147.8 g per bag. On recipe 2 from 93 bags, the yield was 11,914.8 g, an average of 143.9 g per bag. Recipe 3 from 90 bags, yielded 8,774.9 g with an average of 105.2 g per bag.

Of the three media used for the growth of *P. sajor-caju*, the best yield was for recipe 1; 147.8 g per bag, the lowest yield was recipe 3; 105.2 g per bag. The differs between recipes 1-3 is the addition of rice bran to recipe 1, and this is thought to promote basidiocarp production of *P. sajor-caju*.

Table 10. Wet weight of *Lentinus squarrosulus* for the three recipes of substrate from 10 bags, selected at random.

Number	Recipe 1 (g)	Recipe 2 (g)	Recipe 3 (g)
5	112.8	44.01	-
19	42.1	15.33	-
26	89.5	63.39	45.69
35	78.5	40.3	-
39	43.1	38.58	13.77
52	101.0	27.28	12.31
57	88.4	48.15	11.95
69	44.0	65.01	4.36
79	66.5	78.88	-
86	58.5	12.06	19.17
Total wet weight for 10 randomly selected bags (g)	**724.3**	**433.0**	**107.3**
Total wet weight all (g)	**7,116.6**	**3,269.0**	**1,379.9**
Average wet weight all (g)	**78.2**	**25.2**	**15.3**

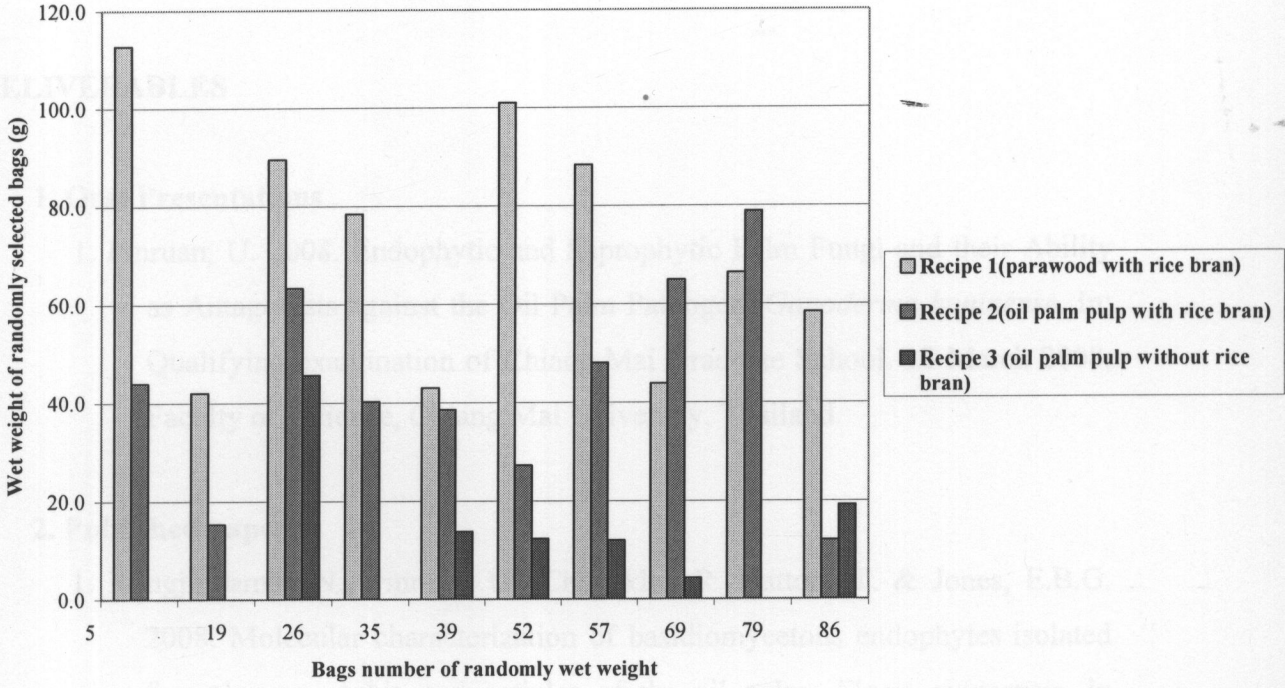


Fig. 15. Comparative wet weight of *Lentinus squarrosulus* for the three recipes.

Lentinus squarrosulus on recipe 1 from the 91 growth bags, yielded 7,116.6 g of fruit bodies, with an average of 78.2 g per bag. On recipe 2 from 94 bags, the yield was 3,269.0 g, an average of is 25.2 g per bag. Recipe 3 from 90 bags yielded 1,379.9 g with an average of 15.3 g per bag.

Of the three media used for the growth of *L. squarrosulus*, the best yield was for recipe 1; 78.2 g per bag, the lowest yield was recipe 3; 15.3 g per bag. The differs between recipes 1-3 is the addition of rice bran to recipe 1, and this is thought to promote basidiocarp production of *L. squarrosulus*.

Problem and suggestions: *Lentinus squarrosulus* prefers a high temperature (about 30 °C) and high relative humidity (about 70%) to promote basidiocarp production, so this fungus should be cultivated in the dry season.

In conclusion the best medium for the growth of *L. squarrosulus* and *P. sajor-caju* was on recipe 1 which contained parawood sawdust, while *S. commune* grew best on the medium with oil palm pulp. The poorest medium for all three basidiomycetes was recipe 3 with only oil palm pulp as the major lignocellulose substrate. Protein from

rice (rice bran), which is rich in phenylalanine, leucine, isoleucine, and valine is important in increasing mushroom yield (Nwanze et al., 2005).

DELIVERABLES

1. Oral Presentations

1. Pinruan, U. 2008. Endophytic and Saprophytic Palm Fungi and their Ability as Antagonists against the Oil Palm Pathogen, *Ganoderma boninense*. In: Qualifying examination of Chiang Mai Graduate School. 18 March 2008, Faculty of Science, Chiang Mai University, Thailand.

2. Published paper

1. Rungjindamai, N., Pinruan, U., Choeyklin, R. Hattori, T. & Jones, E.B.G. 2008. Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis,* in Thailand. Fungal Diversity 33: 139-161.

3. Paper in preparation

1. Pinruan, U., Choeyklin, R., Rungjindamai, N., Lumyong, S. & Jones, E.B.G. 2009. Endophytic fungi from palms in Thailand. Fungal Diversity.

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Nwanze, P.I., Khan, A.U., Ameh, J.B. and Umoh, V.J. 2005. The effect of spawn grains, culture media, oil types and rates on carpophore production of *Lentinus squarrosulus* (Mont.) Singer. African Journal of Biotechnology 4(6): 472-477.

Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand

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Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T. and Jones, E.B.G. (2008). Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. Fungal Diversity 33: 139-161

Most endophytes isolated from plants and algae are members of the Ascomycota or their anamorphs, with only a few reports of basidiomycetous endophytes, these often being orchid mycorrhizas. Fungal endophyets were isolated from healthy leaves, rachis and petioles of the oil palm *Elaeis guineensis* in a Thai plantation. In two experiments 892 and 917 endophytes were isolated yielding 162 and 178 morphotypes, respectively. Non-sporulating isolates were grouped into 162 morphotypes according to their colony morphology. Many of these morphotypes were shown to be basidiomycetes as clamp connections were present and some produced basidia and basidiospores in culture. Thirteen basidiomycetous morphotypes were therefore further characterized by molecular analysis using ribosomal DNA sequences. The LSU region was used to clarify the ordinal taxonomic level status of these isolates. The phylogenetic position of the basidiomycetous endophytes was separated into two major lineages, two and eleven in the *Agaricales* and *Polyporales*, respectively. Based on ITS sequence analysis the two *Agaricales* strains grouped with *Schizophyllum* species and showed a close relationship with *S. commune*. Within the *Polyporales* two and nine strains had an affinity with the *Polyporaceae* and *Fomitopsidaceae*, respectively. One of the endophytic *Polyporaceae* strains was monophyletic with seven sequences of *Pycnoporus sanguineus*, while another isolate grouped with a fungal endophyte DQ979682 and *Trametes elegans*. The largest fungal assemblage was within the *Fomitopsidaceae*, four endophytic isolates clustered with *Fomitopsis* species (*F. ostreiformis, F. palustris*), two and three isolates grouped with *Fomitopsis pinicola* and *Fomitopsis meliae*, respectively. Numerous genera of the Basidiomycota are reported herein as endophytes and are the first report of basidiomycete endophytes from oil palm. Our analysis demonstrated that LSU and ITS data are powerful tools to resolve the taxonomy of basidiomycetous endophytes. The biological role of these endophytes is discussed.

Key words: Agaricomycotina, Basidiomycota, *Elaeis guineensis*, endophyte, *Fomitopsis*, *Pycnoporus*, rDNA phylogeny, systematics, *Schizophyllum*, *Trametes*

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Introduction

The oil palm *Elaeis guineensis*, a native of West Africa, was introduced to Java by the Dutch and by the British into Malaysia in 1910. Oil palms are widely planted in Thailand and were introduced in 1920 and have been cultivated on a commercial basis since 1968 (Likhitekaraj and Tummakate, 2000). The oil palm is a source of edible vegetable oil yielding some 28 million tones' in 2004 (Stevenson, 2006). Nowadays, demand of oil palm consumption is increasing as a precursor in biodiesel production. Therefore oil palm has become an important economic plant for industrial exploitation as an alternative energy source. However, in recent years, oil palms have been prone to fungal attack by *Ganoderma boninense* and a number of studies have been undertaken to find biofungicides that can control infestations (Abdullah, 2000; Ariffin *et al.*, 2000; Flood *et al.*, 2000, 2005; Likhittekaraj and Tummakate, 2000; Paterson, 2007). Sieber *et al.* (1991) and Petrini *et al.*

a second immersion in 95% ethanol for 30 seconds, followed by washing in sterile distilled water. Leaf discs were transferred to Petri dishes (9 cm diam.) containing potato dextrose agar (PDA) and corn meal agar (CMA) with added streptomycin sulphate. Five discs were placed in each dish. The same procedure was applied to the 5 mm segments from the petiole and rachis, but were dipped in 95% ethanol for 90 seconds, Chlorox for 7 minutes, then 30 seconds in ethanol, and then washed in sterile distilled water. Petri dishes were incubated at 25°C for up to one week, and mycelium growing from the tissues sub-cultured on to PDA and CMA in 6 cm diam Petri dischs and incubated at 25°C. Isolates were identified by their sporulation structures on the media, while non sporlulating strains were characterized by their colony morphology into morphotypes.

From examination of the non sporulating strains 19 strains were identified as basidiomycetes by their clamp connections. Thirteen of these strains were selected for this molecular study.

DNA extraction and PCR amplification

Fungi were inoculated on potato dextrose agar (PDA) for three weeks and then transfered into potato dextrose broth (PDB) at room temperature for one week. Mycelium was filtered and washed with sterilized water. Biomass was frozen and ground into fine powder with mortar and pestle. Genomic DNA was extracted using CTAB method (O' Donnell *et al.*, 1997) with some modification. Partial large subunit (LSU) and complete internal transcribed spacer (ITS) were amplified with fungal specific primer: LROR, LR7 and ITS5, ITS4, respectively (White *et al.*, 1990, Bunyard *et al.*, 1994) using Fermentas, *Tag* DNA Polymerase (recombinant) kit (Fermentas, Ontario, Canada). The PCR amplification cycles were performed following White *et al.* (1990) and Bunyard *et al.* (1994) with a DNA Engine DYAD ALD 1244 Thermocycler (MJ Research, Waltham, MA). Amplified PCR fragments were purified with NucleoSpin Extract DNA purification kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instruction and then sequenced by Macrogen (Seoul, Korea) using the same primers as for amplification.

Sequence alignment and phylogenetic analysis

LSU and ITS regions were employed to search the closest sequences from the GenBank database (http://www.ncbi.nlm.nih.gov) using a BLAST search (Altschul *et al.*, 1990). The LSU region was initially blasted in order to determine the familial and ordinal level. The phylogenetic construction of LSU sequence was performed based on the study of Moncalvo *et al.* (2002) and Hibbett *et al.* (2007). Further LSU sequences from different major classes, orders and families of the Agaricomycetes were included in data matrix. The ITS region was used to clarify the generic and species level of the isolates. Our endophytic sequences were compared with relatedness from BLAST search. DNA sequences were multiple aligned using Clustal W 1.6 (Thompson *et al.*, 1994) and adjusted manually to maximize alignment using BioEdit 7.5.0.3 (Hall, 2006).

The aligned dataset was subsequently analysed using MP in PAUP* 4.0b10 (Swofford, 2002), for the most parsimonious trees (MPTs). Heuristic searches algorithm with tree-bisection-reconnection (TBR) branch swapping, 100 replicates of random stepwise sequence addition, were performed. Gaps were treated as missing data and given equal weight. The tree length, consistency indices (CI) and retention indices (RI) were calculated for each tree generated. The Kishino-Hasegawa (K-H) test was used for estimation of the best tree topology (Kishino and Hasegawa, 1989).

Bayesian phylogenetic inference was calculated with MrBayes 3.0b4 with general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites (Huelsenbeck and Ronquist, 2001). Four Markov chains were run from random starting trees for 5 M generations and sampled every 100 generations. The first 500K generations were discarded as burn-in of the chain. A majority rule consensus tree of all remaining trees was calculated.

Statistical support for the internal branches was estimated by bootstrapping

Table 2. Selected list of basidiomycetous endophytes reported in the literature.

Plant host	Order	Fungal identification	Host plant	Reference
Orchid	***Cantharellales***	*Ceratobasidium cornigerum*	*Plantanthera obtusata*	Currah and Sherburne, 1992
		Ceratobasidium obscurum	*Amerorchis rotundifolia*	Currah and Sherburne, 1992
		Epulorhiza anaticula	*Calypso bulbosa*	Currah and Sherburne, 1992
		Epulorhiza repens	*Platanthera obtusata*	Currah and Sherburne, 1992
		Epulorhiza repens	*Acianthus* spp.	Bougoure *et al.*, 2005
		Moniliopsis anomala	*Coeloglossum viride*	Currah and Sherburne, 1992
		Sistotrema sp.	*Pipperia unalascensis*	Currah and Sherburne, 1992
		Thanatephorus pennatus	*Calypso bulbosa*	Currah and Sherburne, 1992
		Tulasnella calospora	*Diuris maculata*	Warcup, 1971
		Tulasnella sp.	*Neuwiedia veratrifolia*	Kristiansen *et al.*, 2004
		Thanatephorus sp.	*Neuwiedia veratrifolia*	Kristiansen *et al.*, 2004
		Thanatephorus sp.	*Pterostyulis* spp.	Bougoure *et al.*, 2005
	Sebacinales	*Sebacina vermifera*	*Nicotiana attenuata*	Barazani *et al.*, 2007
		Sebacina vermifera	*Caladenia* spp.	Warcup, 1971
			Glossodia major	
			Elythranthera brunonis	
			Elythranthera emarginata	
			Eriochilus cucullatus	
		Sebacina sp.	*Bletilla ochracea*	Tao *et al.*, 2008
		Sebacina sp.	*Platanthera obtusata*	Currah and Sherburne, 1992
Liverworts	***Cantharellales***	*Tulasnella* sp.	*Cryptothallus mirabilis*	Bidartondo *et al.*, 2003
			Aneura pinguis	
		Tulasnella sp.	*Aneura pinguis*	Kottke *et al.*, 2003
	Sebacinales	*sebacinoid*	*Lophozia incisa*	Weiss *et al.*, 2004
		sebacinoid	*Lophozia sudetica*	Weiss *et al.*, 2004
		sebacinoid	*Calypogeia muelleriana*	Weiss *et al.*, 2004
		sebacinoid	*Lophozia ibcisao*	Kottke *et al.*, 2003
		sebacinoid	*Lophozoia sudetica*	Kottke *et al.*, 2003
	Incertae sedis	Basidiomycete associations	*Jungermanniales*	Duckett *et al.*, 2006

Table 2 (continue). Selected list of basidiomycetous endophytes reported in the literature.

Plant host	Order	Fungal identification	Host plant	Reference
Monocotyledon and Dicotyledon		*Pycnoporus* sp. 1-2	*Theobroma cacao*	Crozier *et al.*, 2006
		cf. Pycnoporus sp.	*Theobroma gileri*	Thomas *et al.*, 2008
		Trametes sp.	*Theobroma gileri*	Evans *et al.*, 2003
		Trametes hirsuta	*Podophyllum hexandrum*	Puri *et al.*, 2006
	Russulales	*Lachnocladiaceae* sp.	*Theobroma gileri*	Thomas *et al.*, 2008
		Wrightoporia sp.	*Theobroma gileri*	Thomas *et al.*, 2008
	Sebacinales	*Piriformospora indica*	*Hordeum vulgare*	Waller *et al.*, 2005
	Incertae sedis	Basidiomycetes sp. 1-4	*Theobroma gileri*	Evans *et al.*, 2003
		Basidiomycete spp.	*Theobroma gileri*	Evans *et al.*, 2003
		Basidiomycete P1-9	*Livistona chienesis*	Guo *et al.*, 2001
		Bjerkkandera sp.	*Drimys winteri*	Oses *et al.*, 2006
		Mycelia sterilia	*Theobroma gileri*	Evans *et al.*, 2003
		Tulasnella	*Cryptothallus mirabilis*	Bidartondo *et al.*, 2003
		Unidentified basidiomycete	*Prumnopitys andina*	Oses *et al.*, 2006

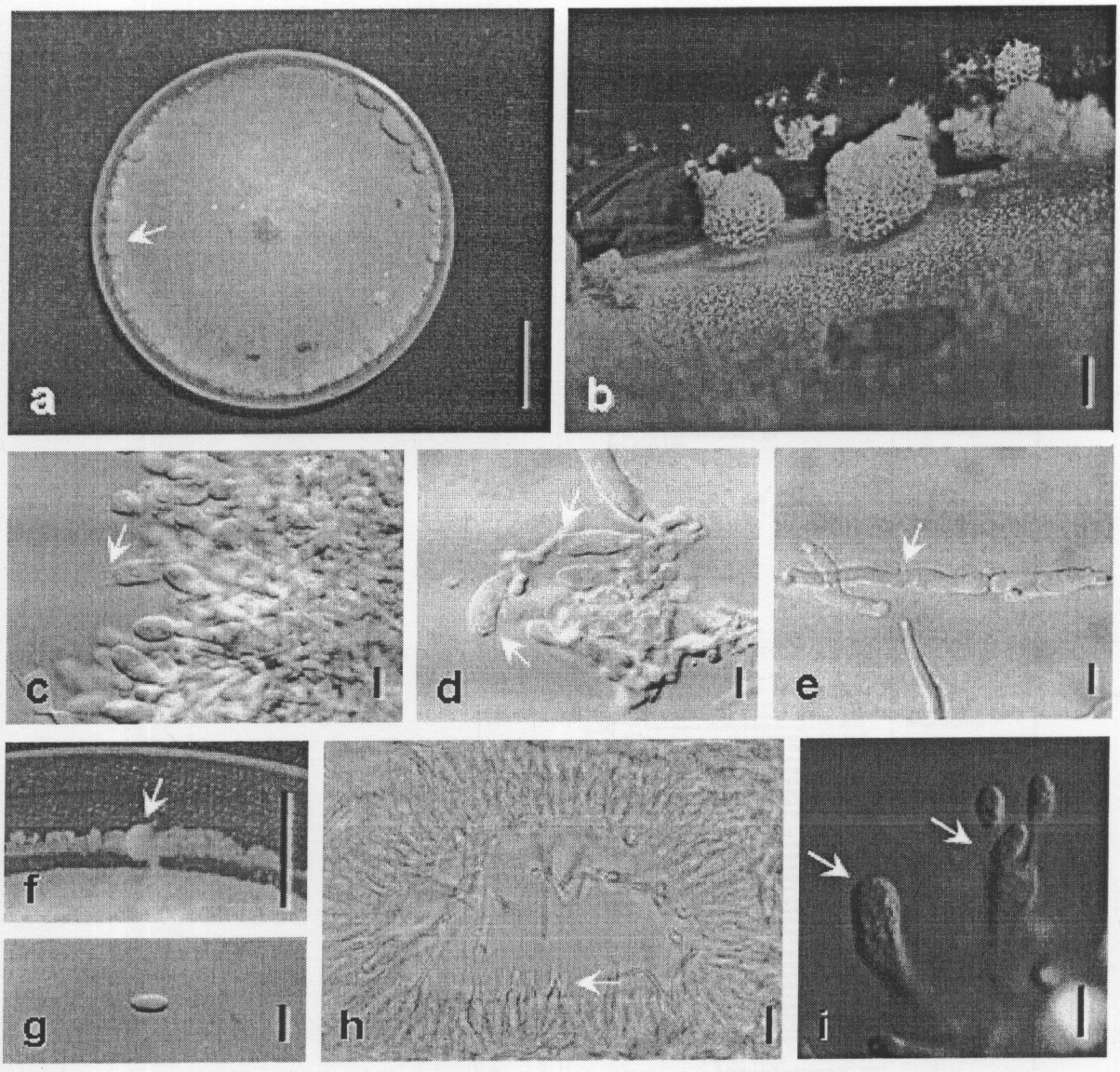
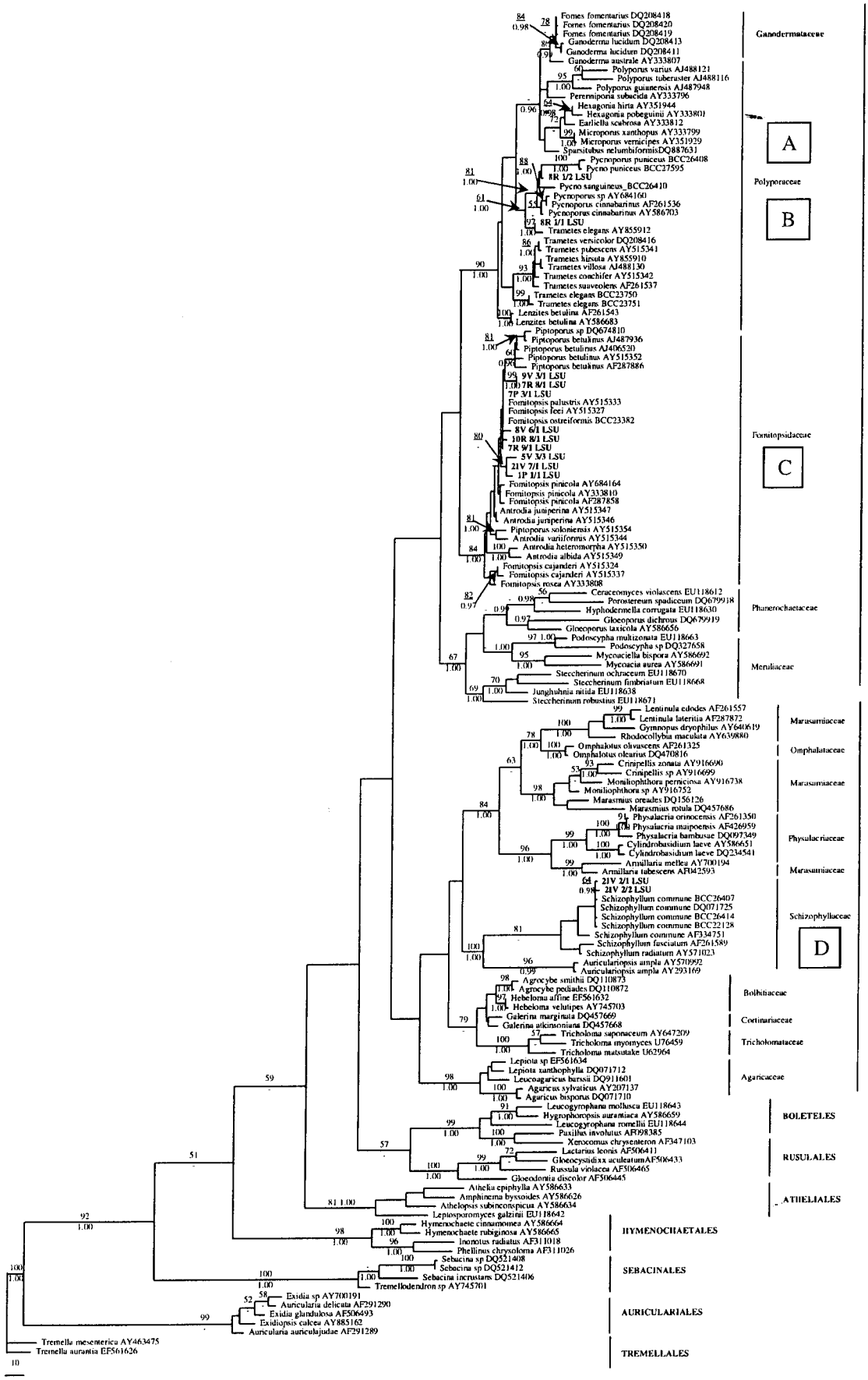


Fig. 1. Isolate 8R 1/1 a-b. Basidiomes on PDA culture formed on side of Petri dish (arrowed). **c-d.** Basidia (arrowed). **e.** Generative hyphae with clamp connection (arrowed). **Isolate 5V 3/3. f.** Basidiomes on PDA culture on Petri dish side (arrowed). **g.** Basidiospore. **h.** Crossection of a pore in culture material with cystidia (arrowed). **i.** Basidia with basidiospores (arrowed), Bars: a = 1 cm, b = 1 mm, c-e = 5 μm, f = 1 cm, g, i = 5 μm, h = 10 μm

Isolate 8R 1/1 clustered with *Trametes elegans* with high support (97 %BS and 1.00 PP) (Fig. 3 subclade B), while 8R 1/2 formed a clade with *Pycnoporus* sequences, although the statistical support is low (Fig. 3 subclade A). Nine endophyte isolates grouped with the *Fomitopsidaceae* with good statistical support (84% BS and 1.00 PP) (Fig. 3 subclade C). However, the statistical support within this group is low. Four isolates (8V 6/1, 10R 8/1, 7P 3/1 and 7R 9/1) clustered with three *Fomitopsis* species. Two strains (7R 8/1 and 9V 3/1) grouped together with 99 % BS and 1.00 PP but showed no relationship to any known taxa. These two sequences formed a sister group with various *Piptoporus* species. Three strains including the endophytes 5V 3/3, 2IV 7/1 and 1P 1/1 grouped together with low support and showed no affinity with any subclade. Two endophytic fungi (2IV 2/1 and 2IV 2/2) grouped with members of the *Schizophyllaceae*, in the *Agaricales*, with high statistical support (100 % BS and 1.00 PP) (Fig. 3 subclade D).


Fomes fomentarius DQ208418
Fomes fomentarius DQ208420
Fomes fomentarius DQ208419
Ganoderma lucidum DQ208413
Ganoderma lucidum DQ208411
Ganoderma australe AY333807
Polyporus varius AJ488121
Polyporus tuberaster AJ488116
Polyporus guianensis AJ487948
Perenniporia subacida AY333796
Hexagonia hirta AY351944
Hexagonia pobeguinii AY333801
Earliella scabrosa AY333812
Microporus xanthopus AY333799
Microporus vernicipes AY351929
Sparsitubus nelumbiformisDQ887631
Pycnoporus puniceus BCC26408
Pycno puniceus BCC27595
8R 1/2 LSU
Pycno sanguineus_BCC26410
Pycnoporus sp AY684160
Pycnoporus cinnabarinus AF261536
Pycnoporus cinnabarinus AY586703
8R 1/1 LSU
Trametes elegans AY855912
Trametes versicolor DQ208416
Trametes pubescens AY515341
Trametes hirsuta AY855910
Trametes villosa AJ488130
Trametes conchifer AY515342
Trametes suaveolens AF261537
Trametes elegans BCC23750
Trametes elegans BCC23751
Lenzites betulina AF261543
Lenzites betulina AY586683
Piptoporus sp DQ674810
Piptoporus betulinus AJ487936
Piptoporus betulinus AJ406520
Piptoporus betulinus AY515352
Piptoporus betulinus AF287886
9V 3/1 LSU
7R 8/1 LSU
7P 3/1 LSU
Fomitopsis pulustris AY515333
Fomitopsis feei AY515327
Fomitopsis ostreiformis BCC23382
8V 6/1 LSU
10R 8/1 LSU
7R 9/1 LSU
5V 3/3 LSU
21V 7/1 LSU
1P 1/1 LSU
Fomitopsis pinicola AY684164
Fomitopsis pinicola AY333810
Fomitopsis pinicola AF287858
Antrodia juniperina AY515347
Antrodia juniperina AY515346
Piptoporus soloniensis AY515354
Antrodia variiformis AY515344
Antrodia heteromorpha AY515350
Antrodia albida AY515349
Fomitopsis cajanderi AY515324
Fomitopsis cajanderi AY515337
Fomitopsis rosea AY333808
Ceraceomyces violascens EU118612
Porostereum spadiceum DQ679918
Hyphoderonella corrugata EU118630
Gloeoporus dichrous DQ679919
Gloeoporus taxicola AY586656
Podoscypha multizonata EU118663
Podoscypha sp DQ327658
Mycoacia bispora AY586692
Mycoacia aurea AY586691
Steccherinum ochraceum EU118670
Steccherinum fimbriatum EU118668
Junghuhnia nitida EU118638
Steccherinum robustius EU118671
Lentinula edodes AF261557
Lentinula lateritia AF287872
Gymnopus dryophilus AY640619
Rhodocollybia maculata AY639880
Omphalotus olivascens AF261325
Omphalotus olearius DQ470816
Crinipellis zonata AY916690
Crinipellis sp AY916699
Moniliophthora perniciosa AY916738
Moniliophthora sp AY916752
Marasmius oreades DQ156126
Marasmius rotula DQ457686
Physalacria orinocensis AF261350
Physalacria maipoensis AF426959
Physalacria bambusae DQ097349
Cylindrobasidium laeve AY586651
Cylindrobasidium laeve DQ234541
Armillaria mellea AY700194
Armillaria tabescens AF042593
21V 2/1 LSU
21V 2/2 LSU
Schizophyllum commune BCC26407
Schizophyllum commune DQ071725
Schizophyllum commune BCC26414
Schizophyllum commune BCC22128
Schizophyllum commune AF334751
Schizophyllum fasciatum AF261589
Schizophyllum radiatum AY571023
Auriculariopsis ampla AY570992
Auriculariopsis ampla AY293169
Agrocybe smithii DQ110873
Agrocybe pediades DQ110872
Hebeloma affine EF561632
Hebeloma velutipes AY745703
Galerina marginata DQ457669
Galerina atkinsoniana DQ457668
Tricholoma saponaceum AY647209
Tricholoma myomyces U76459
Tricholoma matsutake U62964
Lepiota sp EF561634
Lepiota xanthophylla DQ071712
Leucoagaricus barssii DQ911601
Agaricus sylvaticus AY207137
Agaricus bisporus DQ071710
Leucogyrophana mollusca EU118643
Hygrophoropsis aurantiaca AY586659
Leucogyrophana romellii EU118644
Paxillus involutus AF098385
Xerocomus chrysenteron AF347103
Lactarius leonis AF506411
Gloeocystidiixx aculeatumAF506433
Russula violacea AF506465
Gloeodontia discolor AF506445
Athelia epiphylla AY586633
Amphinema byssoides AY586626
Athelopsis subinconspicua AY586634
Leptosporomyces galzinii EU118642
Hymenochaete cinnamomea AY586664
Hymenochaete rubiginosa AY586665
Inonotus radiatus AF311018
Phellinus chrysoloma AF311026
Sebacina sp DQ521408
Sebacina sp DQ521412
Sebacina incrustans DQ521406
Tremellodendron sp AY745701
Exidia sp AY700191
Auricularia delicata AF291290
Exidia glandulosa AF506493
Exidiopsis calcea AY885162
Auricularia auriculajudae AF291289
Tremella mesenterica AY463475
Tremella aurantia EF561626
Ganodermataceae
Polyporaceae
A
B
POLYPORALES
Fomitopsidaceae
C
Phanerochaetaceae
Meruliaceae
Marasmiaceae
Omphalotaceae
Marasmiaceae
AGARICALES
Physalacriaceae
Marasmiaceae
Schizophyllaceae
D
Bolbitiaceae
Cortinariaceae
Tricholomataceae
Agaricaceae
BOLETELES
RUSULALES
ATHELIALES
HYMENOCHAETALES
SEBACINALES
AURICULARIALES
TREMELLALES

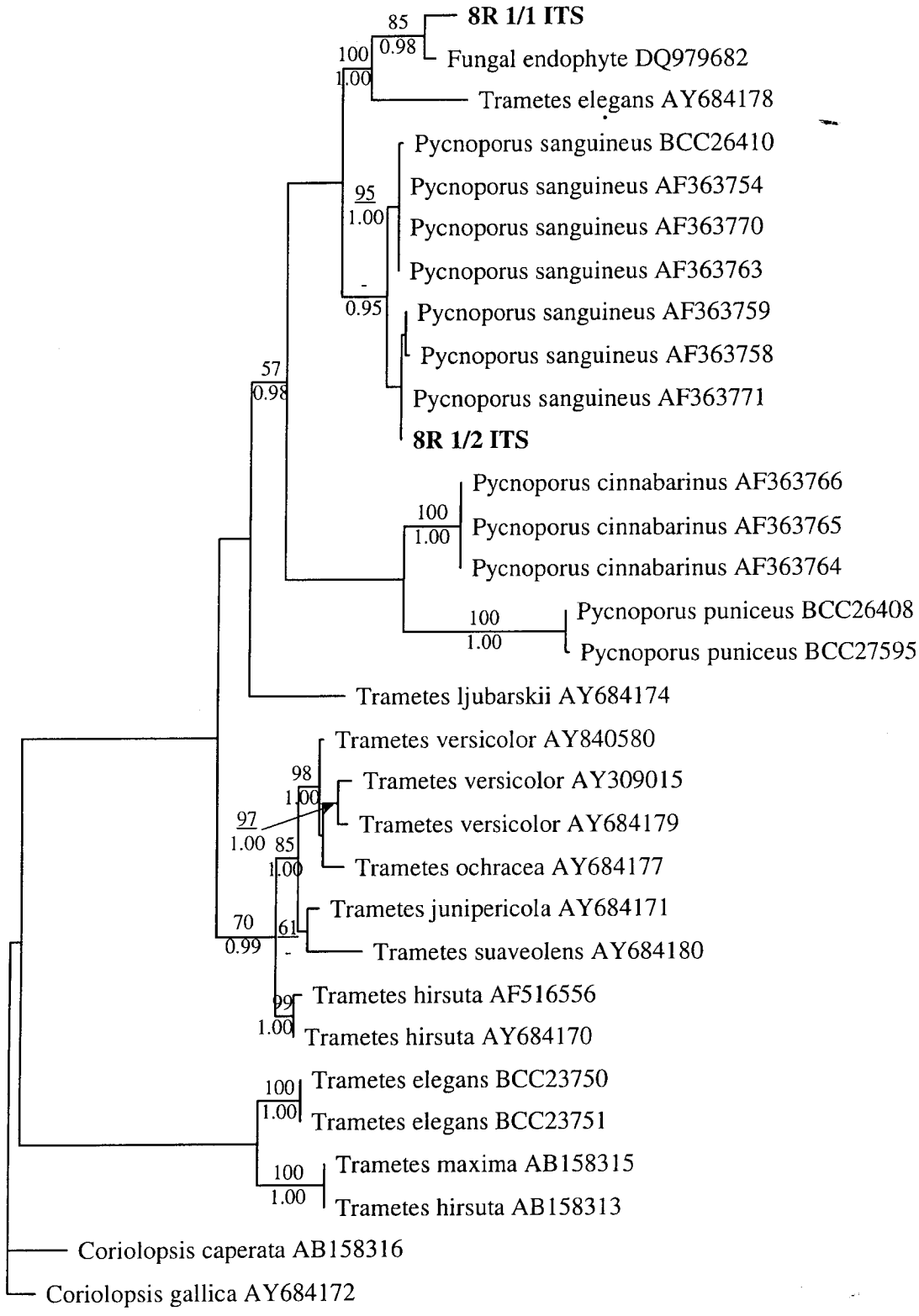


Fig. 4. One of 62 MPTs inferred from ITS sequenes of two isolates of the *Polyporaceae* isolated from *Elaeis guineensis*. The MP value (≥50%) and Bayesian PP (≥0.95) are shown above and below the branches, respectively (tree length= 335 steps, CI = 716, RI=0.884). Basidiomycete endophytes sequenced in this study are printed in bold. Bar = number of changes per nucleotide position.

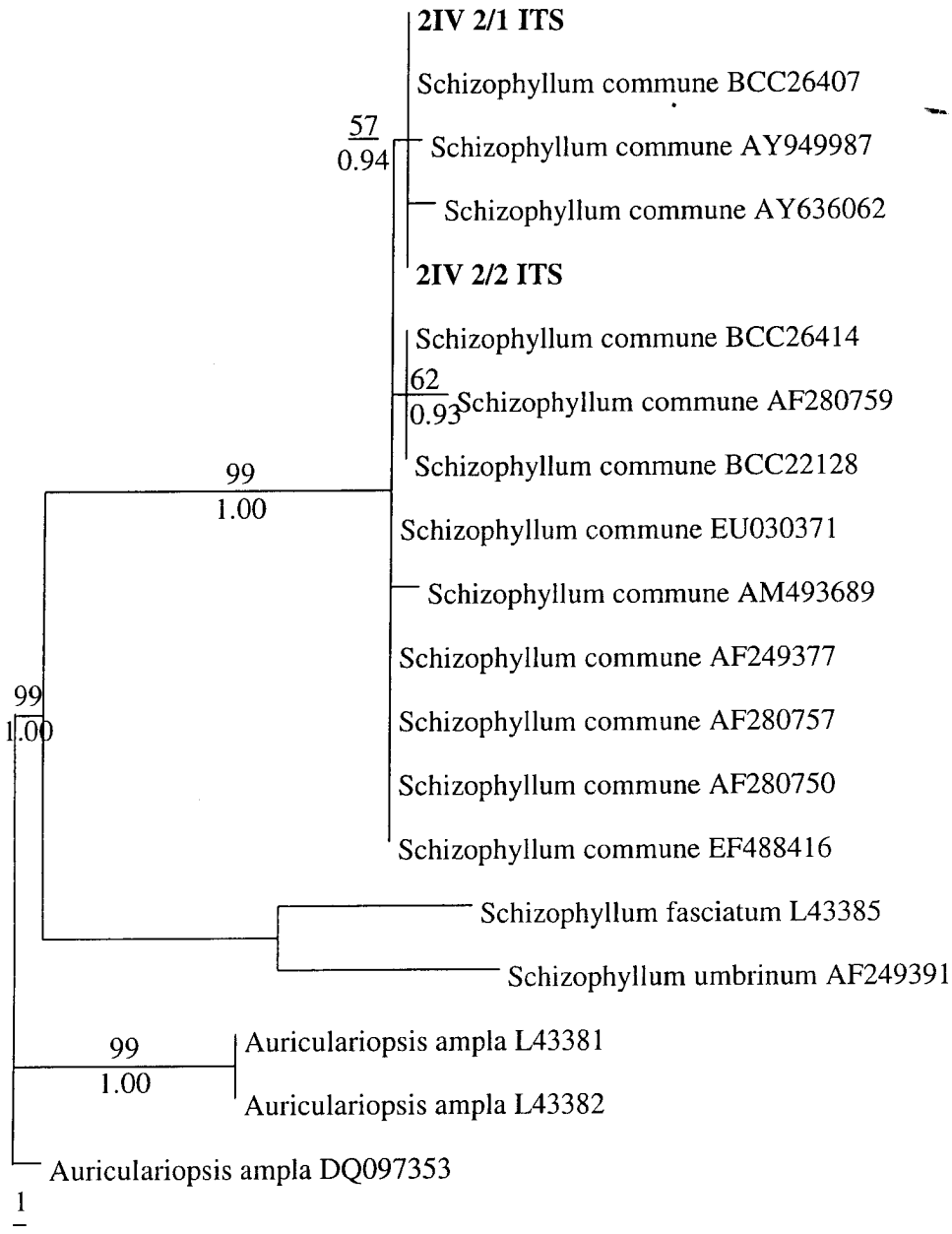


Fig. 6. One of 72 MPTs inferred from ITS sequenes of two isolates of the *Schizophyllaceae* isolated from *Elaeis guineensis*. The MP value (≥50%) and Bayesian PP (≥0.95) are shown above and below the branches, respectively (tree length= 84 steps, CI = 0.942, RI=0.924). Basidiomycete endophytes sequenced in this study are printed in bold. Bar = number of changes per nucleotide position

the ultrastructure of the fungal endophytes within the cells of the hosts, while Kottke *et al.* (2003) used both septal pore ultrastructure and molecular studies to resolve the identity of these basidiomcyete endophytes. For example, the mycobiont from the liverwort *Aneura pinguis* clustered in the *Tulasnella* clade, while microbionts of *Calypogeia muelleriana, Lophozia incisa* and *L. sudetica*, grouped with the *Sebacinaceae* (Kottke *et al.*, 2003).

There are various definitions of what constitutes an endophyte (e.g. Arnold, 2007). Generally mycorrhizal fungi are excluded (Rogers, 2000), as they are restricted to plant roots and derive nutrients from the soil by

The greatest number of palm endophytes grouped in the *Fomitopsidaceae*, *Polyporales* and the genus *Fomitopsis*. These are reported for the first time as endophytes (Table 2). *Fomitopsis* species are active brown rot fungi and cosmopolitan in their distribution in boreal and temperate zones (Ryvarden and Gilbertson, 1993; Kim *et al.*, 2005, 2007). *Fomitopsis* is phylogenetically heterogeneous, which Kim *et al.* (2005) divided into three subgroups, but none well-supported by bootstrap support. Kim *et al.* (2007) described a new *Fomitopsis* (*F. incarnatus*) which groups with *F. rosea* (*Rhodofomes*) and *F. cajanderi*, in a well-supported clade. However, the phylogenetic position of the *Fomitopsis* species is not fully resolved.

In our phylogenetic analysis, *Fomitopsis* species separated in to three clades: (1). Four isolates (7R 9/1, 7P 3/1, 8V 6/1, 10R 8/1) forming a subclade with *F. ostreiformis,* with *F. palustris* as a sister group. However, Kim *et al.* (2005) report *F. feei* and *F. palustris* grouping together with *Piptoporus portentosus*, and *Daedalea quercina*, but the relationship was not resolved. (2). Two isolates (7R 8/1, 9V 3/1) formed a well supported sister group to *F. pinicola.* However Kim *et al.* (2005) show that *F. pinicola* formed a monophyletic group with *Piptoporus betulinus* as a sister group. (3). Three isolates (1P 1/1, 2IV 7/1, 5V 3/3) group with *Fomitopsis meliae* with high support, which has an affinity with *F. pinicola*, *P. betulinus* and *F. palustris* (Kim *et al.*, 2007), and this is also reflected in our study. *Fomitopsis meliae* is sometimes regarded as a synonym of *Fomes meliae* (Index Fungorum) but does not belong in that genus because it is a brown rot species (Hattori, pers. comm.) *Fomitopsis meliae* is often regarded as an allied species of *F. palustris* (Kim *et al.*, 2007) and refered by Kotlaba and Pouzar (1990) to the genus *Pilatoporus*. However, in our data *F. meliae* and *F. palustris* are not monophyletic. *Fomitopsis meliae* is an American species and occurs in tropical Asia as well. Of some 43 recognized *Fomitopsis* species (Index Fungorum), *F. pinicola* and *F. pseudopetchii* are known from Thailand, both collected in the north of the country (Hjortstrom and Ryvarden, 1982; Phanichapol, 1968), while Corner (1989) reported *F. euosma* and *F. pseudopetchii* from Malaysia. Therefore the data recorded here adds to our knowledge of *Fomitopsis* in tropical areas.

Induction of basidiomycete fruiting bodies

Initially our basidiomycete isolates did not sporulate under laboratory conditions, but eventually five strains produced minute poroid basidiomes (Figs 1a-b, 2b-e). The endophyte strains were inoculated with test blocks of palm petioles and small basidiomes formed after 12 months of incubation.

Fruiting body induction in basidiomycetes is variable with *Schizophyllum commune* producing prolific basidiomes on sawdust media in plastic bags (Thaithatgoon *et al.*, 2004; Vikineswary *et al.*, 2007) after 4 weeks. Lomascolo *et al.* (2002) induced basidium-producing areas of *Pycnoporus* strains as "reddish-orange granules" on malt extract broth after 4-5 weeks incubation at 20-24°C. Similar observations are repeated here (Figs 1a-b). Basidiomycete endophytes may well have been overlooked in previous studies as the mycelium was not examined for the presence of clamp connections, or the induction of fruiting bodies under laboratory conditions. For the latter, a prolonged incubation period may be necessary.

Role of endophytic basidiomycetes

The documentation of a wider range of basidiomycetes as endophytes raises the questions as to their role in nature. Chapela and Boddy (1988a,b) pointed out that endophytes (particularly basidiomycetes), may be precursors to a saprobic phase. They drew attention to the rapid growth of these fungi on senescence of the woody tissue, and ultimately a saprobic regime. This hypothesis has been revisited by Oses *et al.* (2006), who isolated two basidiomycete endophytes from Chilean tree species (*Drimys winter* and *Prumnopitys andina*) and evaluated their ability to produce lignocellulotytic enzymes. The *Bjerkandera* sp. produced phenoloxidase and cellulase, with a weight loss of wood

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