Endophytic fungi from *Rafflesia cantleyi*: species diversity and antimicrobial activity

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The *Rafflesia* flower is the largest single flower in the world, a parasitic flowering plant found only in Borneo, Indonesia, Malaysia and Philippines. This study was undertaken to isolate, identify and evaluate antimicrobial activity of endophytic fungi from *Rafflesia cantleyi*, a Malaysian endemic species. Different parts of the flower and bud collected in Perak, Malaysia, were surface sterilized and plated onto potato dextrose agar. Fungal isolates recovered were cultured in potato dextrose broth for antimicrobial activity screening and solvent extraction. Fungal 5.8S gene and flanking internal transcribed spacer regions of rDNA were sequenced for construction of phylogenetic trees. Eight endophytic strains obtained from *R. cantleyi* were categorized as seven morphotypes. Three isolates inhibited the growth of *Candida albicans* with IC_{50} values of 3.5–8.2 µg/ml for crude fungal extracts. Based on morphological study, the endophytes were identified as belonging to *Colletotrichum*, *Cytospora* and *Gliocladiopsis*. Phylogenetic analysis of fungal rDNA internal transcribed spacer sequences confirmed the three active isolates were *Colletotrichum siamense*, *Colletotrichum* sp. and *Cytospora* sp. while other isolates were identified as *Colletotrichum siamense*, *Colletotrichum* sp. and *Gliocladiopsis* sp. Endophytic fungi isolated from *Rafflesia cantleyi* produce bioactive metabolites which may contribute to the plant's medicinal properties.

Key words – Anti *Candida* – *Colletotrichum siamense* – rDNA phylogeny

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Introduction

Endophytic fungi

Endophytic fungi are fungal microorganisms which asymptomatically inhabit plant tissues and have been isolated from many species of woody plants and grasses (Petrini 1991, Hyde & Soytong 2008). Endophytes may contribute to their host plant by producing a plethora of compounds that provide protection and survival value to the plant (Carroll & Carroll 1978, Strobel 2003, Aly et al. 2010, Xu et al. 2010). Ultimately, these compounds, once isolated and characterized, may also have potential use in modern medicine. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of compounds produced by endophytes (Strobel et al. 2005, Aly et al. 2010). Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. Plants from unique environmental settings, with an ethno botanical history or which are endemic are likely to house novel endophytic microorganisms as well as microorganisms making novel bioactive products (Strobel & Daisy 2003).

Rafflesia is a rare, endemic plant in South East Asia which has not been previously studied for its endophytic component.

The unique *Rafflesia*

Rafflesia is a genus of the parasitic flowering plant family, Rafflesiaceae, discovered in the Indonesian rain forest by a local guide working for Dr. Joseph Arnold in 1818, and named after Sir Thomas Stamford Raffles, the leader of the expedition (Nais 2004).The genus *Rafflesia* is distributed from north of the Kra isthmus of Thailand through peninsular Malaysia, the Philippines, Borneo to Sumatra and Java (Bänziger 1991, Salleh 1991, Meijer 1997). *Rafflesia* flowers are of various sizes from a few inches to a meter in diameter and 20 known species have been recorded. In some species, such as *Rafflesia arnoldii*, the flower may be over 107 cm in diameter (Nais 2004). In Malaysia, *R. cantleyi* is an endemic Malaysian species, commonly observed in bloom around the Gopeng and Taiping area in the state of Perak (Figs 1–3) (Sabah Forestry Department 2008). *Rafflesia* has been used for centuries by the indigenous people (Orang Asli) in Peninsular Malaysia to treat various ailments (Thulaja 2003, Kanchanapoom et al. 2007). *Rafflesia* is an understory plant, which is first evident as a small protuberance emerging from the roots or near-ground stems of several species of the vine *Tetrastigma* (Vitaceae). After 6–12 months it takes the form of a pink-brownish 'cabbage', which blooms into an ephemeral flower but lacks leaves or photosynthetic tissue, stems or roots, the only vegetative parts being fine filaments that penetrate the tissue of the vine host (Meijer 1985, Ismail 1988, Nais & Wilcock 1998). The flowers are unisexual, smelling like 'rotten flesh' or 'festering sore', and attract several species of carrion flies or blowflies of the genus *Lucilia* and *Chrysomya* (Calliphoridae), which pollinate them (Beaman et al. 1988, Bänziger 1991, 1996). If pollinated, after 6–9 months the structure below the column that holds the ovary of the female flower becomes the fruit, holding many thousands of miniature seeds that are likely to be dispersed by small mammals such as squirrels and tree shrews (Meijer 1985). How the seeds germinate and penetrate the host is still unclear (Patiño et al. 2002). To further our understanding of this unique flower, we conducted the present study to isolate endophytic fungi from *R. cantleyi*, evaluate the bioactivity of these isolates and identify them based on morphological and molecular characteristics.

Materials and methods

Plant sampling and fungal isolation

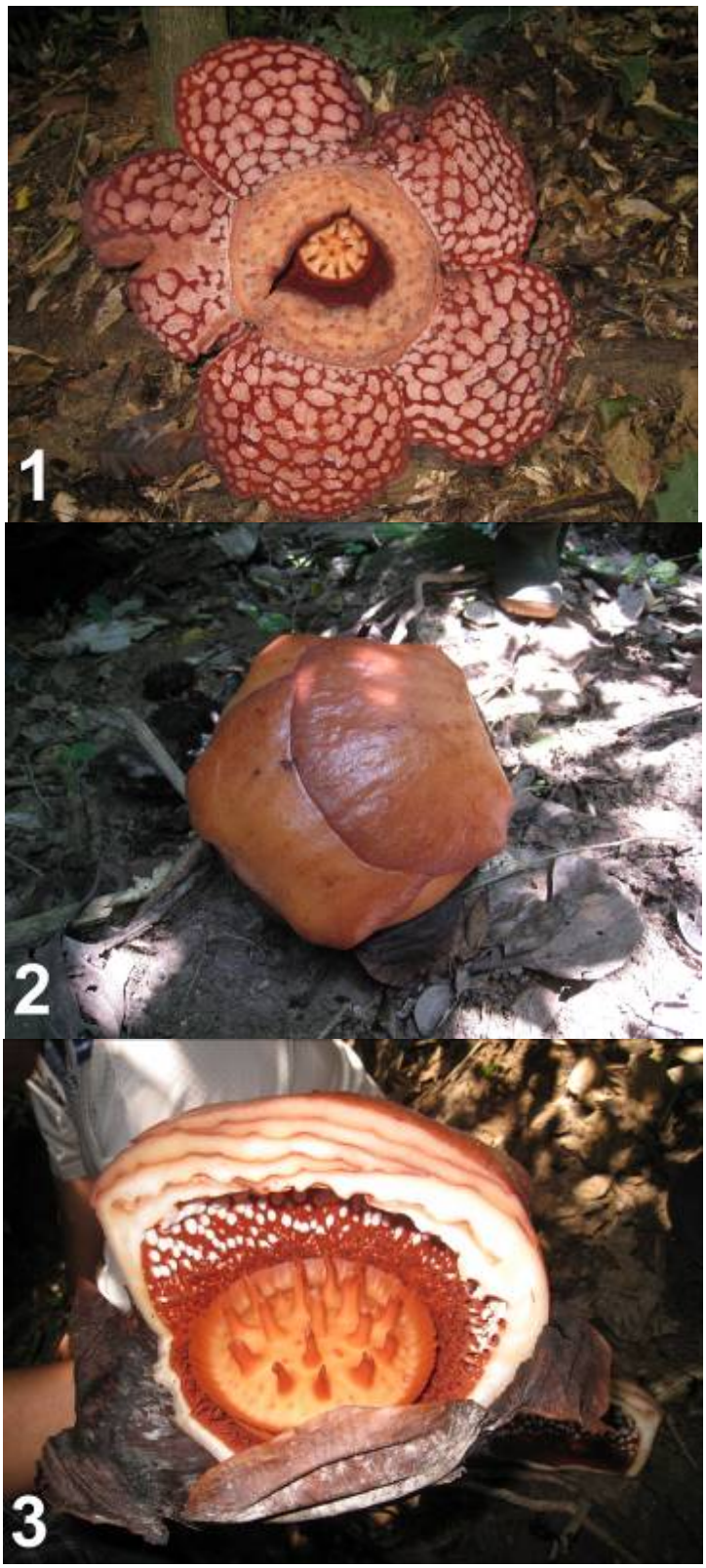
R. cantleyi was collected from Sungai Kapar, Pos Dipang, Perak, Malaysia (Figs 1–3). Different parts of the flower (petal, upper well wall, raised disc, vertical spines and bud) were cut into small fragments (1×1 cm^2) using a sterile surgical blade. Small fragments were successively surface sterilized by immersion in 97% ethanol for 1 min., 2.6% sodium hypochlorite solution for 3 min., 97% ethanol for 30sec. followed by rinsing with sterile distilled water for 30sec. A total of 50 pieces of flower and bud parts were placed on both potato dextrose agar (PDA) and water agar (WA) at room temperature (25–28°C) until outgrowth of endophytic fungi was discerned. Fungal tips that emerged from plant segments were transferred onto PDA.

Fungal preservation

All fungal isolates were preserved by placing small pieces of PDA supporting fungal growth in sterile distilled water at 4°C and in 10% glycerol (v/v) at –80°C. Sterilized wheat grains colonized by fungi were also stored at –80°C (Ezra et al. 2004).

Evaluation of Bioactivity

Each fungal isolate was grown in 20 mL potato dextrose broth (PDB) at room temperature for two weeks under stationary conditions. The broth cultures were filtered with sterile filter paper, to separate the filtrate and mycelia. The culture filtrate was used for preliminary evaluation of antimicrobial activity using an agar diffusion method (Lorian 1996) against nine pathogenic species of bacteria and fungi (*Aspergillus fumigatus*, *Aspergillus niger*, *Bacillus subtilus*, *Candida albicans*, *Escherichia coli*, *Fusarium solani*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *and Trichoderma viridae*). Filtrate (20 µL) was placed in wells punched into a PDA plate, streaked with a suspension of pathogenic organism. After



Figs 1–**3** – *Rafflesia cantleyi*. **1** Flower, **2** Bud, **3** Inside the bud (raised disc and vertical spines).

incubation at 37°C for 18 h for bacteria, 24–48 h for the yeast and at room temperature for 1–4 days for moulds, the inhibition zones were recorded. Fungal isolates showing antimicrobial activity were cultured in 200 mL of PDB at room temperature for two weeks. The broth and mycelia were separated via filtration, the filtrates extracted with an equal volume of ethyl acetate overnight and the solvent evaporated to obtain a crude fungal extract. The crude extract was assayed for its IC_{50} value based on the standard M38-A method from

National Committee for Clinical Laboratory Standards (NCCLS) with amphotericin B (Sigma) as a positive control. Briefly, extract in 10% methanol was added to microtitre plate wells with a suspension of the test organism (0.5×10^3–2.5×10^3 CFU/mL) in RPMI medium and incubated at 37C for 24 hours (for *Candida albicans*). An MTT (3-(4,5Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (20µl; 5mg/ml Sigma) was added to all wells, incubated at 37°C for 4 hours and the resulting formazon product dissolved in DMSO (100 µL, Merck). Cell viability percentage was determined by measuring absorbance of every well at 540 nm and subtracting the absorbance value of extract free control.

Morphological identification

All endophytic fungi isolated from *R. cantleyi* were subcultured onto three different media, PDA, corn meal agar (CMA) and malt extract agar (MEA) at room temperature. The microscopic features such as size and shape of hyphae and conidia were examined, measured and recorded using a light microscope. Active isolates were subjected to a detailed morphological study.

The identity of *Colletotrichum* species was determined by size and shape of conidia and appressoria; presence or absence of setae, sclerotia, acervuli and teleomorph state and cultural characters such as colony colour, growth rate and texture (Simmonds 1965, Smith & Black 1990, Sutton 1992, TeBeest et al. 1997, Photita et al. 2005, Than et al. 2008a,b,c, Thaung 2008). Appressoria were produced using a slide culture technique, with 10 mm^2 squares of PDA placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip placed over the inoculated agar (Johnston & Jones 1997).

Molecular identification

Fungal DNA isolation

Fungal isolates were cultured in 300 mL PDB for 2–3 weeks at room temperature. Fungal mycelium separated by filtration was washed with warm water (60°C) and, excess water removed with sterile paper towels, prior to freezing, at –20°C for at least 30–60 min or overnight. Frozen mycelia was crushed using a pestle and mortar whilst adding CTAB lysis buffer (100mM trisHCL, 25mM EDTA, 1.4 M NaCl, 2% CTAB, pH=8.4) and sterile sand (white quartz, –50+70 mesh, Sigma-Aldrich). Crushed mycelia was heated at 65–70°C in a water bath for one hour and the lysate extracted several times with phenol-chloroform-isoamyl alcohol (25:24:1). DNA precipitated with cold ammonium acetate (7.5 M) (1/2 vol.) and ethanol (6×vol.) was resuspended in nanopure water, to be used for polymerase chain reaction (PCR).

Amplification of ITS and 5.8S rDNA sequences

Fungal ITS and 5.8 S rDNA regions were amplified by PCR using the universal ITS primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC -3') (White et al 1990). PCR was performed in a 50 µl reaction containing 0.1–1 µg of genomic DNA, 0.2 µM of each primer, 0.2 mM of dNTPs, 5 units Taq polymerrase (Fermentas), 1.5 mM $MgCl_2$ in buffer (Fermentas) containing 100mM tris-HCL(pH 8.8 at 25°C), 500 mM KCl and 0.8% Nonidet P_4O. PCR cycle consisted of denaturation at 94°C for 1.5 min, annealing at 48.4°C for 2 min and extension at 72°C for 3 min. for 35 cycles, with a final extension at 72°C for 10 min. The amplified DNA fragments were purified with QIAquick PCR Purification Kit (Qiagen) and sequenced by Medigene (Seoul, Korea) or 1st Base (Malaysia) using the same primers as for amplification.

BLAST search and Phylogenetic analysis

All ITS sequences were submitted to GenBank to obtain accession numbers. A BLAST search was used to search for closest matched sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) (Altscul et al. 1990). The most similar reference sequences. with query sequences were obtained and used for subsequent phylogenetic analysis along with selected taxonomic reference sequences. Comparison and alignment of query and reference sequences were done by using MUSCLE program version 3.6 (Multiple sequence comparison by log-expectation) (Edgar 2004) or CLUSTAL W 1.6 (Thompson et al. 1994),

followed by manual alignment using BioEdit 7.5.0.3 (Hall 2006) to determine sequence homology. For phylogenetic analysis, maximum parsimony bootstrap method (Flesenstein 1985) with heuristic search was performed using PAUP*version 4.0b10 (Swofford 2002).The bootstrap analysis was set with 1000 replications, tree bisection-reconnection branch swapping, and random sequence addition. 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 & Hasegawa 1989).

Phylogenetic analysis using ITS sequence data is a useful tool to give a preliminarily identification for *Colletotrichum* species or place them in species complexes. However, caution must be taken here as the majority of the ITS sequences deposited in GenBank are wrongly named. A phylogenetic tree for *Colletotrichum* spp. was constructed by using a backbone tree generated by Cai et al (2009).

Results

Fungal isolates and bioactivity

Eight endophytic fungal isolates coded RP1, RP1WA, RP2, RP3, RP4, RP5, RP6, and RFL1 were obtained from *R. cantleyi* and categorized as seven morphotypes as RP2 and RP1WA had the same morphological features. Preliminary antimicrobial assays revealed that three isolates, RP3, RP4 and RP6 inhibited growth of only *Candida albicans* (Fig. 4), while their crude fungal extracts showed IC_{50} values of 3.501 µg/mL, 6.048 µg/mL and 8.241 µg/mL respectively against this pathogenic yeast species.

Morphological identification

Based on fungal morphology and culture characteristics, such as, growth rate, type of conidiophores, size, shape of conidia, RP1, RP1 WA, RP2, RP3, RP5 and RP6 were identified as *Colletotrichum* species, RP4 as *Cytospora* species and RFL1 as *Gliocladiopsis* species. A detailed description of the isolates characteristics and identifying features is presented here for each genus.

Fig. 4 – Agar diffusion assay showing inhibition zone produced by RP-6 culture broth filtrate against *Candida albicans*.

Colletotrichum species

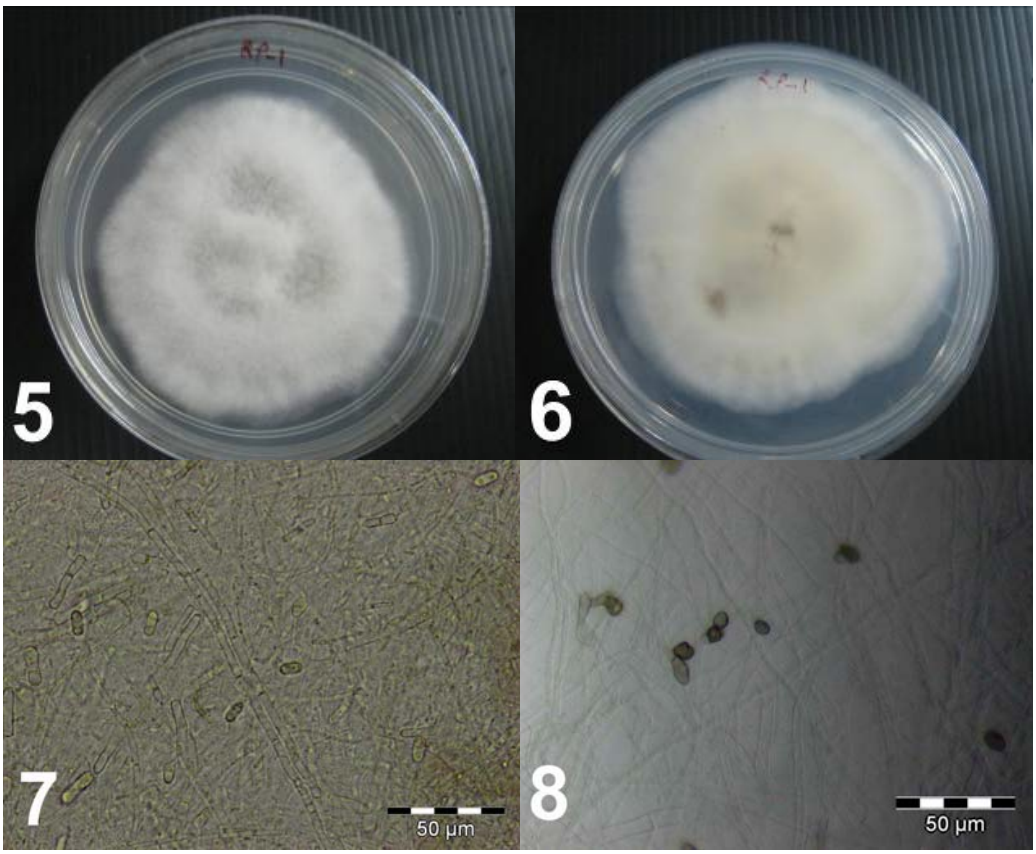
Description and images of the six isolates (RP1, RP1WA, RP2, RP3, RP5, and RP6) identified as *Colletotrichum* species are shown in Table 1 and Figs 5–30.

Cytospora species

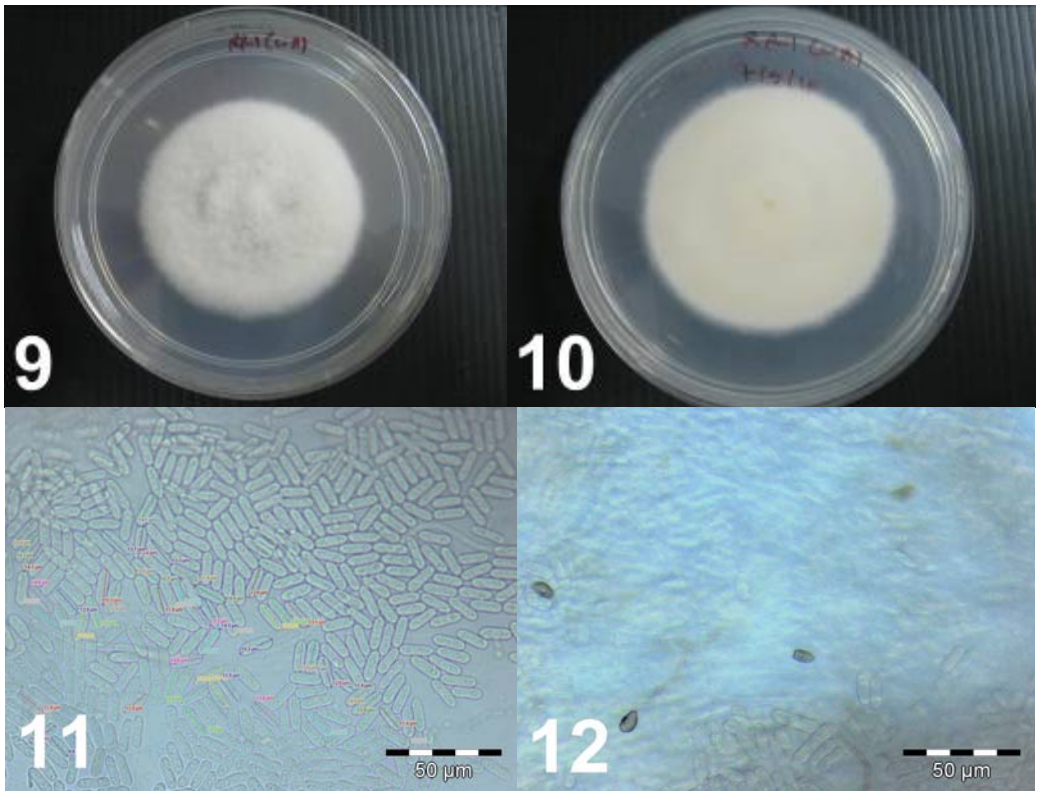
RP4 was identified as *Cytospora* sp. based on its colony morphology and microscopic features. On PDA, fungal growth rate was moderately rapid (11 mm/day) and the colony matured within 7–10 days. The colony was flat and yellow becoming light brown with a fringed border and yellow centre. Colony reverse was yellow and grey. The strain sporulated forming light brown to dark brown pycnidia. Conidiogenous cells were enteroblastic and phialidic with extensive branching. Conidia were hyaline, aseptate, eguttulate and allantoid (Figs 31–35).

Gliocladiopsis species

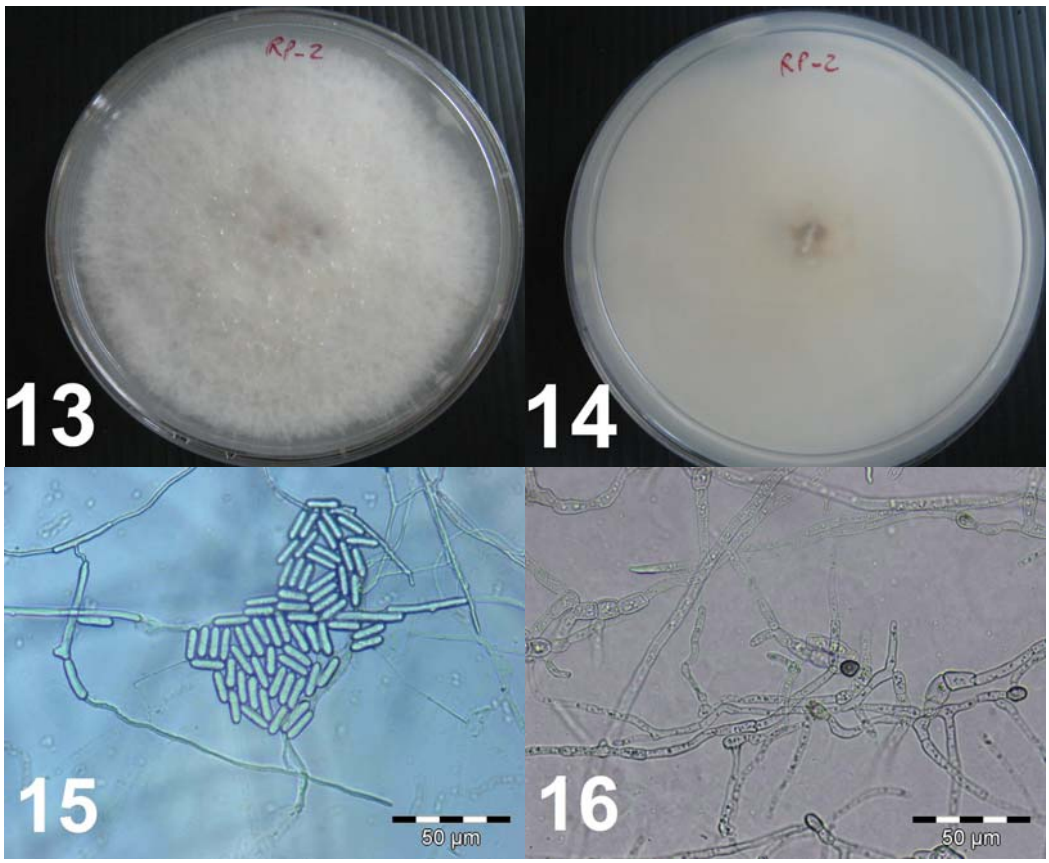
The endophyte RFL1 was identified as *Gliocladiopsis* sp. based on its anamorphic stage characterised by its penicillate conidiophores with numerous branches, primarily forming 1-septate conidia. The colony on PDA was slow growing (4 mm/day) and matured in 10–14 days. At first it was yellow velvety with aerial hyphae and white border then beige with brown rings. Conidiogenous cells were phialidic, hyaline and branched at the upper portion. Conidia were hyaline, cylindrical 1-septate and with a subtruncate base (Figs 36–40).



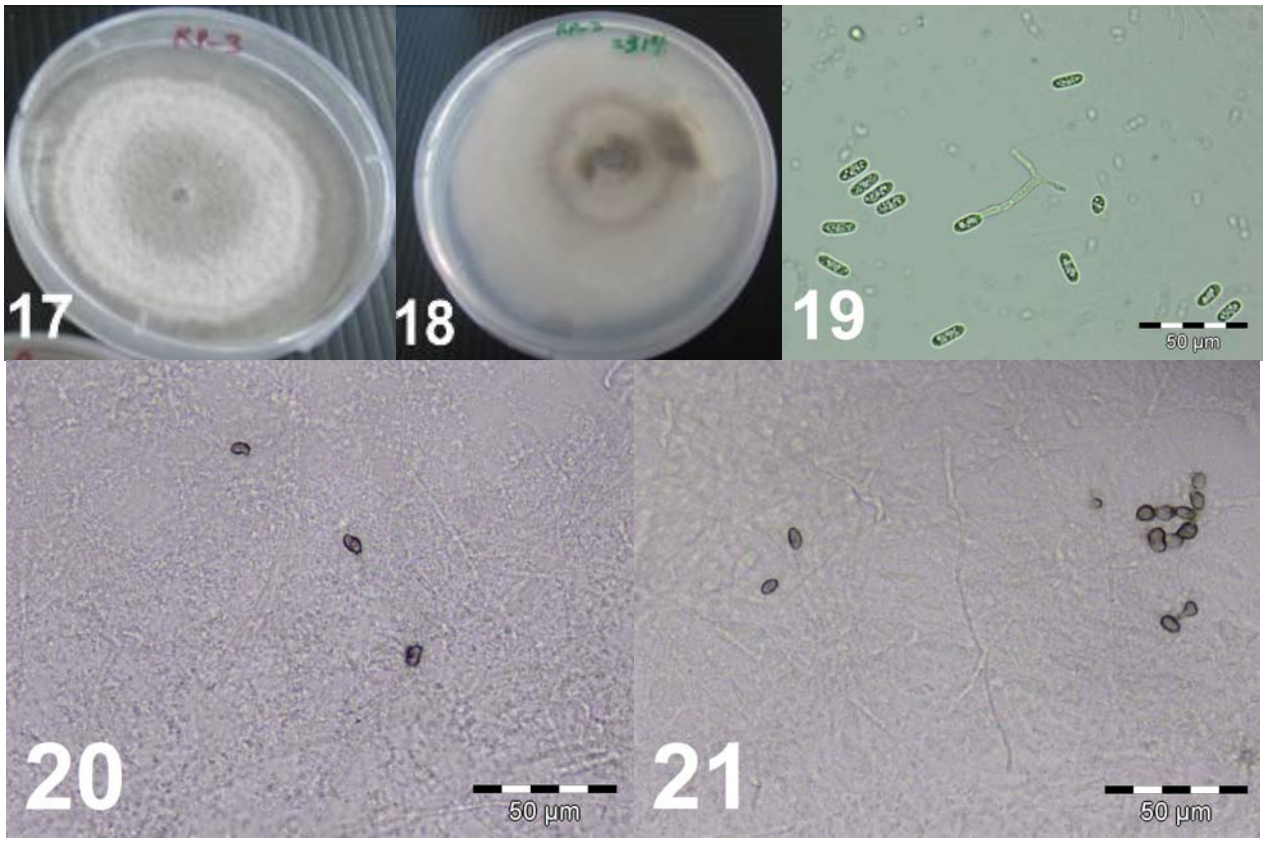
Figs 5–8 – Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP1) on PDA. **5** Colony surface, **6** Colony reverse, **7** Conidia, **8** Appressoria.



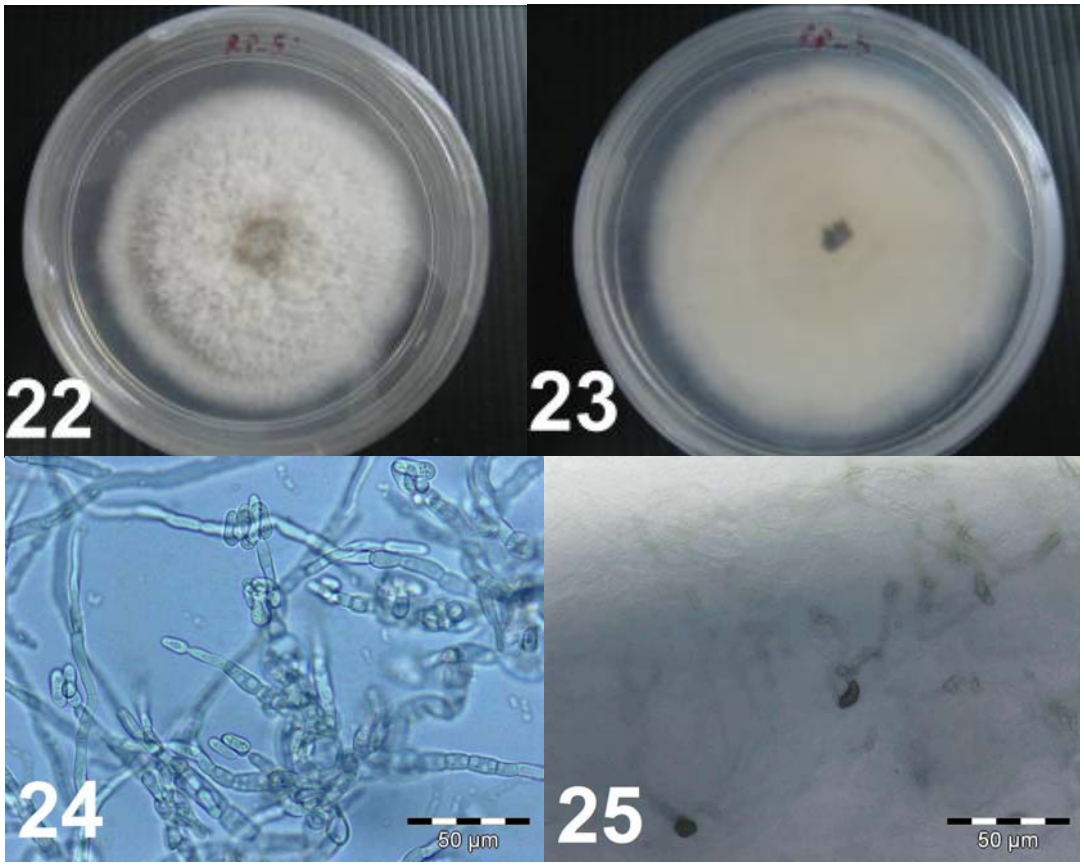
Figs 9–12 – Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP1WA) on PDA. **9** Colony surface, **10** Colony reverse, **11** Conidia, **12** Appressoria.



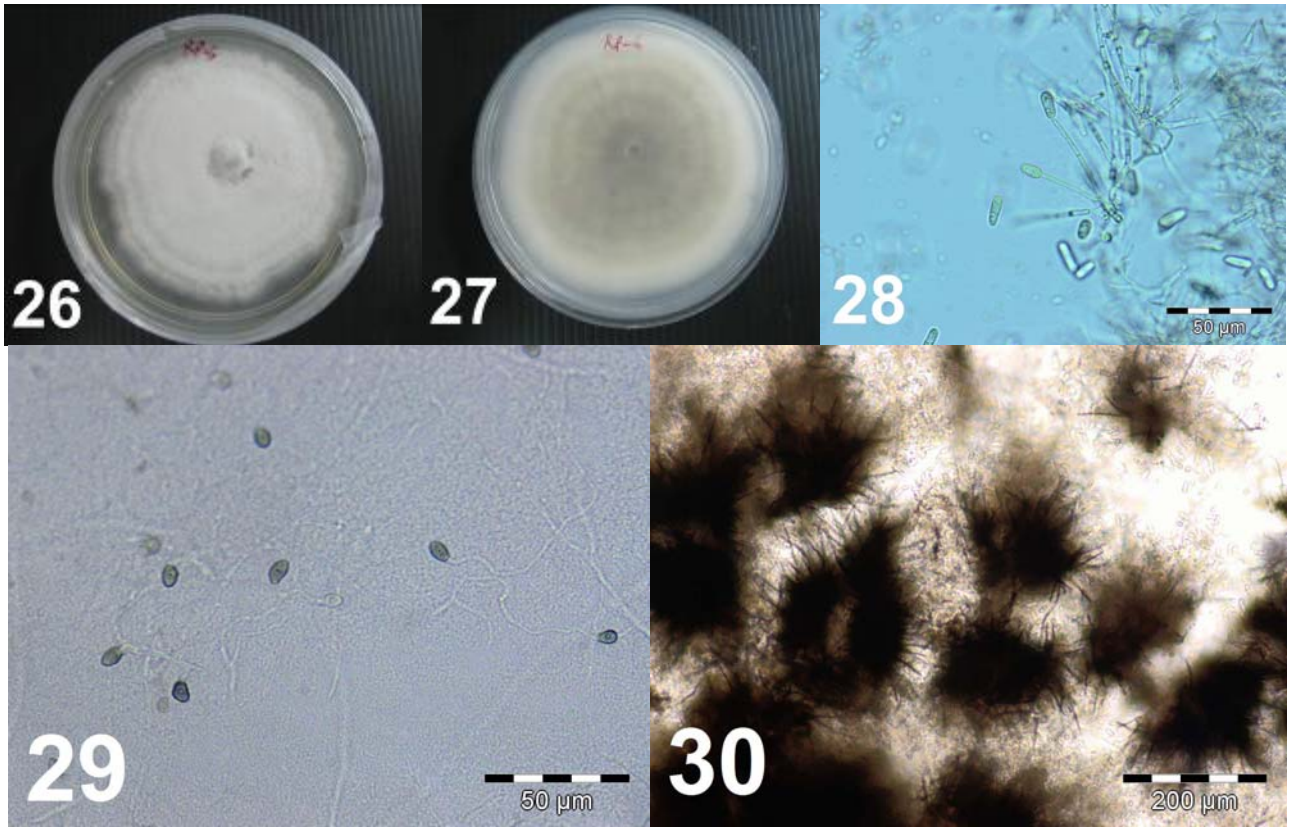
Figs 13–16– Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP2) on PDA. **13** Colony surface, **14** Colony reverse, **15** Conidia, **16** Appressoria.



Figs 17–21 – Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP3) on PDA. **17** Colony surface, **18** Colony reverse, **19** Conidia, **20** Appressoria, **21** Secondary appressoria.



Figs 22–25 – Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP5) on PDA. **22** Colony surface, **23** Colony reverse, **24** Conidia, **25** Appressoria.

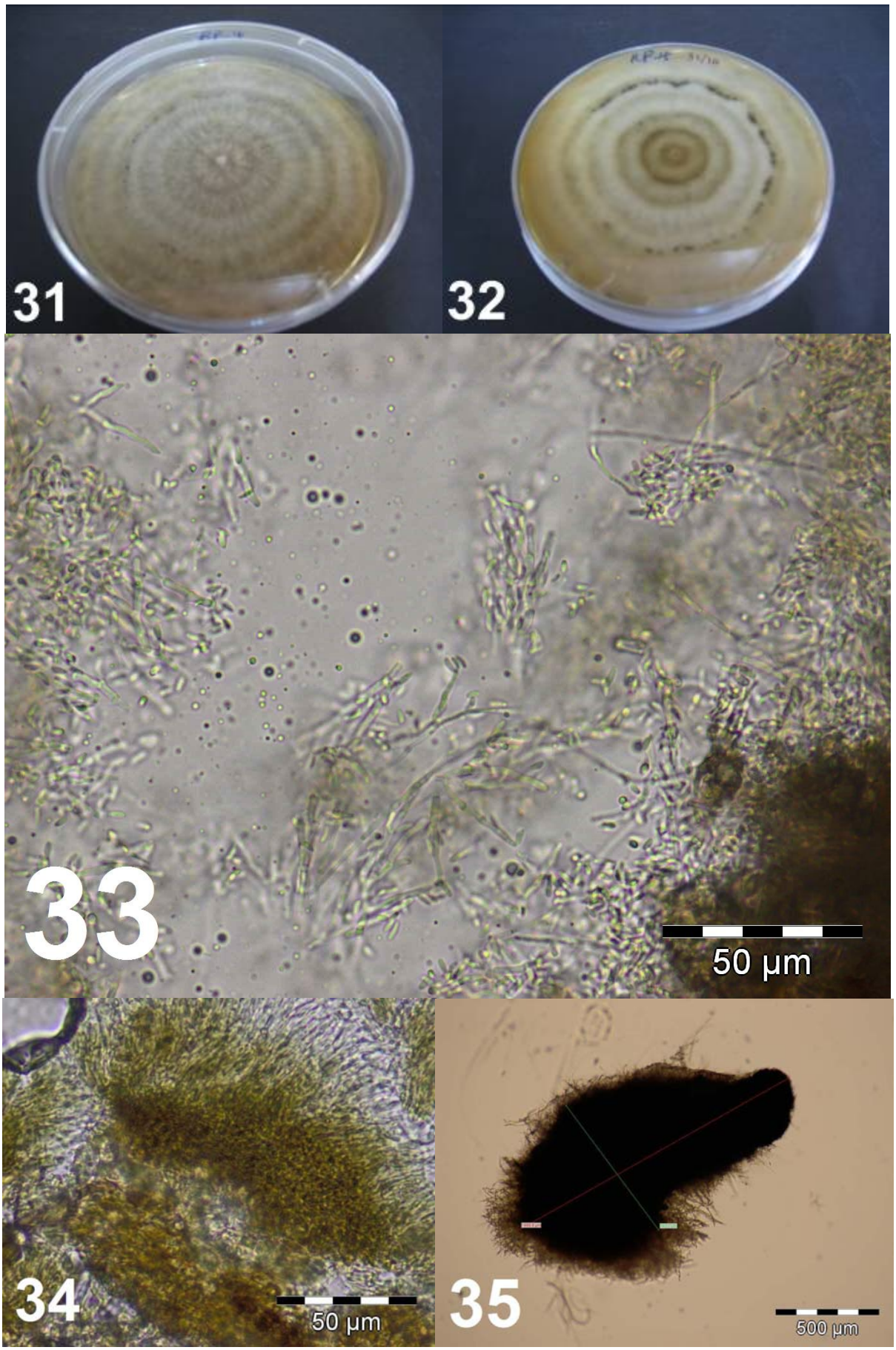


Figs 26–30 – Macroscopic and microscopic morphology of *Colletotrichum* isolate (RP6) on PDA. **26** Colony surface. **27** Colony reverse. **28** Conidia. **29** Appressoria. **30** Conidiomata with setae.

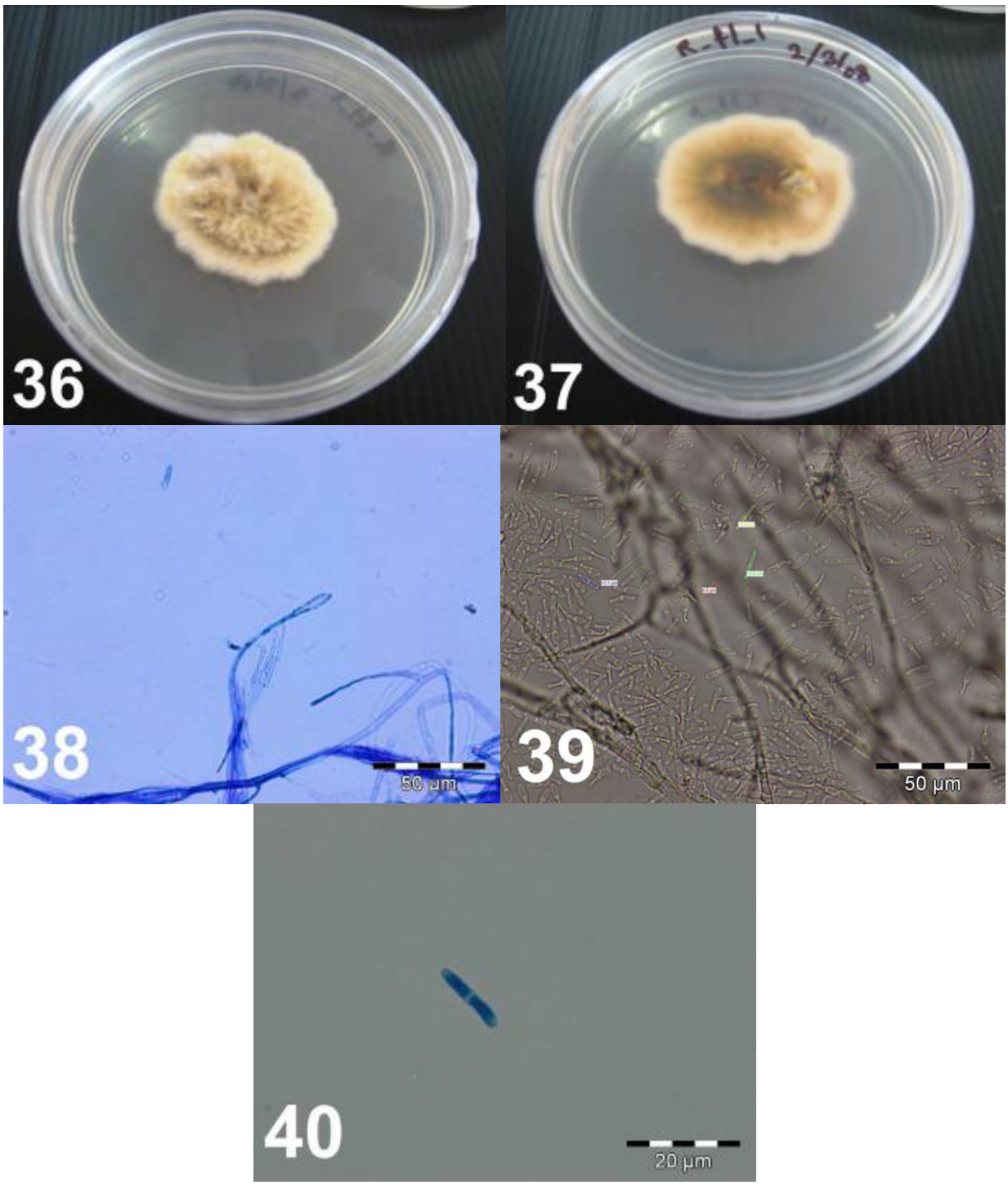
Table 1 Culture and conidia characteristics of *Colletotrichum* isolates**.**

Fungal isolate	Growth rate on PDA (mm/day)	Colony morphology on PDA	Conidia length (µm)	Conidia width (µm)	Conidia shape	Appressoria (in slide cultures)	Presence of setae
RP1	8.6	White, fluffy becoming olive, reverse yellow and white	10.22±1.34	4.63±0.63	Fusiform	Dark brown, aseptate, solitary, sometimes in group of two, elliptical to bullet shape	–
RP1WA	7.1	White, cottony with grey centre becoming powdery with orange spots, reverse pale yellow	13.94±1.55	4.54±0.5	Cylindrical	Medium brown, aseptate, solitary, bullet shape to clavate	–
RP2	10.7	White, cottony with grey centre becoming powdery with orange spots, reverse pale yellow	14.64±1.13	4.05±0.52	Cylindrical	Pale to medium brown, aseptate, solitary, elliptical to clavate	–
RP3*	11.4	White, then grey velvety at the centre with white border becoming grey, with circles and orange spots, reverse white then grey	11.10±1.34	4.92±0.73	Fusiform	Dark brown, aseptate, solitary, sometimes in chain, elliptical	–
RP5	8.6	White and grey, velvety with olive circles and orange spots, reverse pale yellow and black	12.04±1.55	4.68±0.76	Fusiform	Dark brown, one or two celled, elliptical to bean shaped, sometimes crenate	–
RP6*	9.3	White, cottony then grey velvety with some green and orange spots, reverse pale yellow and grey	12.57±1.81	4.87±0.68	Fusiform to cylindrical	Medium brown, aseptate, solitary, elliptical to clavate	+

*Active isolates



Figs 31–35 – Macroscopic and microscopic morphology of *Cytospora* isolate (RP4) on PDA. **31** Colony surface. **32** Colony reverse. **33** Conidia & conidiogenous cells. **34** Conidioma. **35** Pycnidia.



Figs 36–40 – Macroscopic & microscopic morphology of *Gliocladiopsis* isolate (RFL1). **36** Colony surface on PDA. **37** Colony reverse on PDA. **38** Conidiogenous cells. **39, 40** Conidia.

Molecular identification

Nucleotide BLAST analysis

Molecular identification correlated very well with morphological identification of the endophytic fungal isolates. Table 2 shows the most closely matched sequences obtained from GenBank with a nucleotide BLAST analysis of the fungal ITS sequences. Isolates RP1WA and RP2 which had been categorized as the same morphotype showed 100% homology in their sequences.

Phylogenetic tree construction

Separate phylogenetic trees constructed for each fungal genus identified, enabled taxonomic placement of the endophyte isolates.

Table 2 Identification of the endophyte fungal isolates based on ITS region sequence.

Fungal isolate (GenBank accession number)	Morphological identity	Molecular identity (Closest match in GenBank)	Nucleotide homology (%)
RP1 (HM368438)	*Colletotrichum*	*Colletotrichum gloeosporioides* (GU222375)	99.8
RP1WA(HM368439)	*Colletotrichum*	*Colletotrichum gloeosporioides* (FJ968592)	100
RP2 (HM368440)	*Colletotrichum*	*Colletotrichum gloeosporioides* (FJ968592)	100
RP3 (HM368441)	*Colletotrichum*	*Glomerella* sp. (GQ352482) (Anamorph: *Colletotrichum* sp.)	99.2
RP4 (HM368442)	*Cytospora*	*Cytospora* sp. (FJ904827)	98.8
RP5 (HM368443)	*Colletotrichum*	*Colletotrichum gloeosporioides* (FJ968596)	99.3
RP6(HM368444)	*Colletotrichum*	*Colletotrichum* sp.(GQ496383)	100
RFL1(HM368445)	*Gliocladiopsis*	*Glionectria* sp. (Anamorph: *Gliocladiopsis sp*.) (GU827507)	100

Colletotrichum isolates

To produce a phylogenetic tree for *Colletotrichum* spp. (Phyllachorales) a total of 38 sequences were aligned with *Fusarium oxysporum* as the outgroup. Out of 609 characters, 90 are parsimony informative, 95 are parsimony uninformative and 429 are constant characters (tree length = 282, C.I. = 0.8121, R.I. = 0.9047) (Fig. 41). Six *Colletotrichum* isolates separated into two groups, group one (RP1WA, RP2 and RP6) and group two (RP1, RP3 and RP5). Group one (RP1WA, RP2 and RP6) clustered together with *C. siamense* with weak support. Based on sequence identity matrix RP1WA, RP2 and RP6 ITS sequences showed 100% similarity to two GenBank *C. siamense* isolates (FJ972613and FJ972614). This tree also showed that RP1WA, RP2 and RP6 formed a monophyletic clade with three *Colletotrichum* species, *C. siamense*, *C. gloeosporioides* and *C. fructicola*.

For group two isolates, RP3 and RP5 clustered together with *C. hymenocalidis* with weak support. RP1, RP3 and RP5 clustered together within a monophyletic clade with five *Colletotrichum* species (*C. hymenocalidis*, *C. siamense*, *C. gloeosporioides*, *C. fructicola and C. asianum*). RP1, RP3 and RP5 ITS sequences showed the most homology at 99.5%, 98.8% and 99.1% with *C. hymenocalidis* isolate (GQ 485601) respectively.

Cytospora species isolate

A phylogenetic tree was constructed for *Cytospora* sp. (Valsaceae) by using 31 ITS rDNA sequences aligned with *Pestalotiopsis uvicola* as the outgroup (Fig. 42). Out of 689 characters, 250 are parsimony informative, 49 are parsimony uninformative and 390 are constant characters (Tree length = 737, (CI) = 0.6364, (RI) = 0.7985).RP4 grouped with *Cytospora* sp. (FJ904827) with moderate support in a sister group to two isolates of *Cytospora nitschkii*. After calculating sequence identity matrix we found that RP4 is most similar to *Cytospora* sp. (FJ904827) (98.8%) and *Cytospora nitschkii* (AY347356) (97.8%).

Gliocladiopsis species isolate

Gliocladiopsis is a genus assigned to the order *Hypocreales*. To construct a phylogenetic tree, 13 ITS rDNA sequence were aligned with *Bionectria grammicospora* as the outgroup (Fig. 43). Of 540 characters, 65 are parsimony informative, 55 are parsimony uninformative and 420 are constant characters (Tree length = 173, (CI) = 0.8497, (RI) = 0.8785). RFL1 grouped with a *Glionectria* sp. (GU827507) in a well supported *Glionectria* clade (100% BS). RFL1 and *Glionectria* sp. (GU827507) showed 100% homology in ITS sequences.

Discussion

Although there are many studies on endophytic fungi from various plant types, plant parts and climatic regions, this is the first report on endophytic fungi from *Rafflesia*, a unique, geographically limited parasitic plant. The indigenous Orang Asli people in Malaysia use *Rafflesia* as a remedy for fever and it is especially prescribed for women after child-birth (Thulaja 2003, Kanchanapoom et al 2007). Antimicrobial activity of *Rafflesia* extracts was described by Wiart et al (2004) against four bacterial species: *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. As secondary metabolites from endophytic fungi may contribute to the host plant's

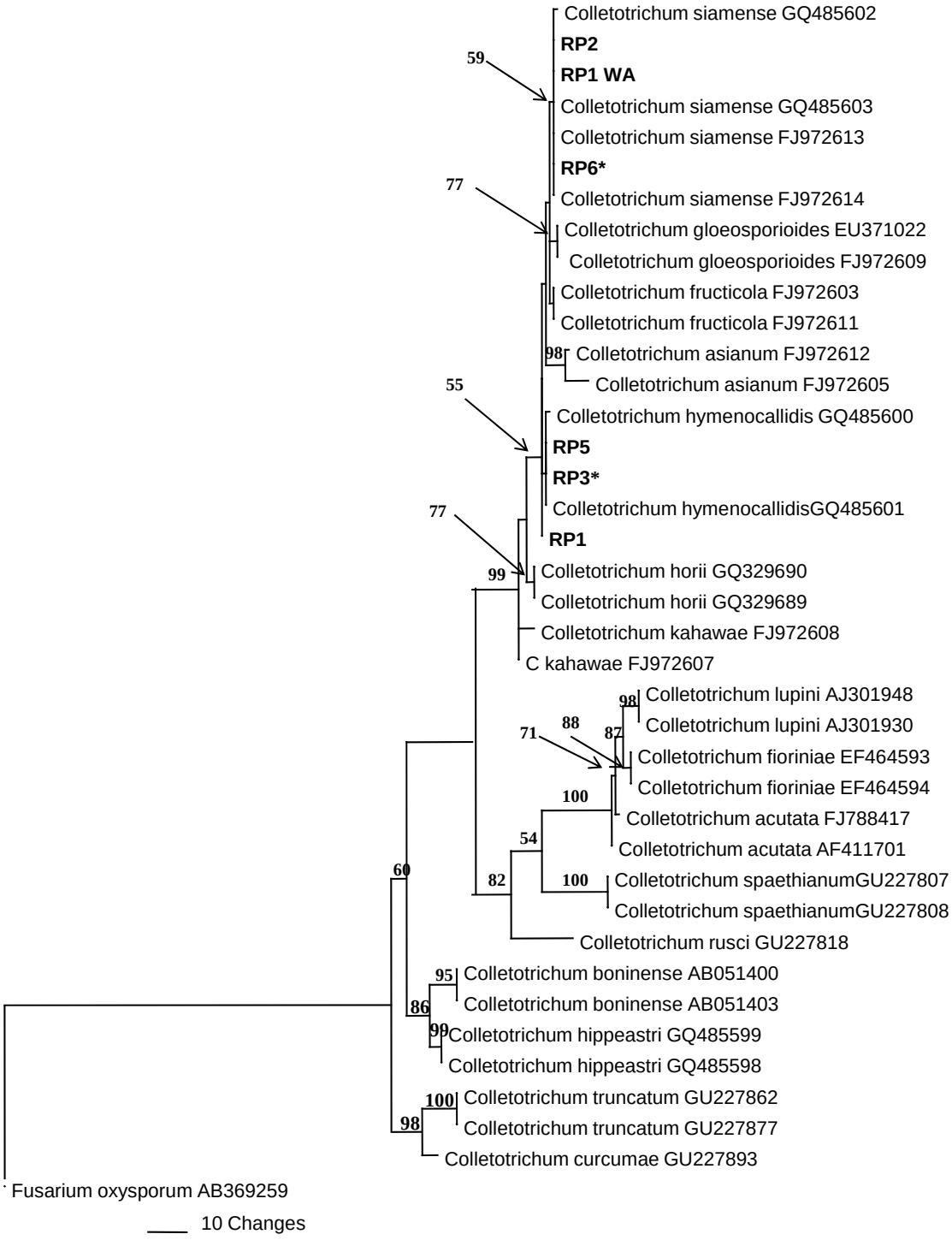


Fig. 41 – Phylogram generated from parsimony analysis based on rDNA ITS sequence data. Bootstrap values ≥50% are shown above the branches. The tree is rooted with *Fusarium oxysporum*. *Rafflesia* endophytes (RP1,RP1(WA),RP2,RP3, RP5 and RP6) sequenced in this study are printed in bold. *denotes active isolates, Bar = number of changes per nucleotide position.

biological activity, we isolated and characterized endophytic fungi from *Rafflesia* to determine their identity and antimicrobial activity.

In this study, seven morphologically different endophytes (RP1, RP1WA, RP3, RP4, RP5, RP6, and RFL1) belonging to three genera, *Colletotrichum*, *Cytospora* and *Gliocladiopsis* were isolated from *Rafflesia cantleyi*. Generally plants yield a high number of endophyte isolates mainly from stems and leaves. For example in a study on biodiversity of endophytic fungi associated with 29 traditional Chinese

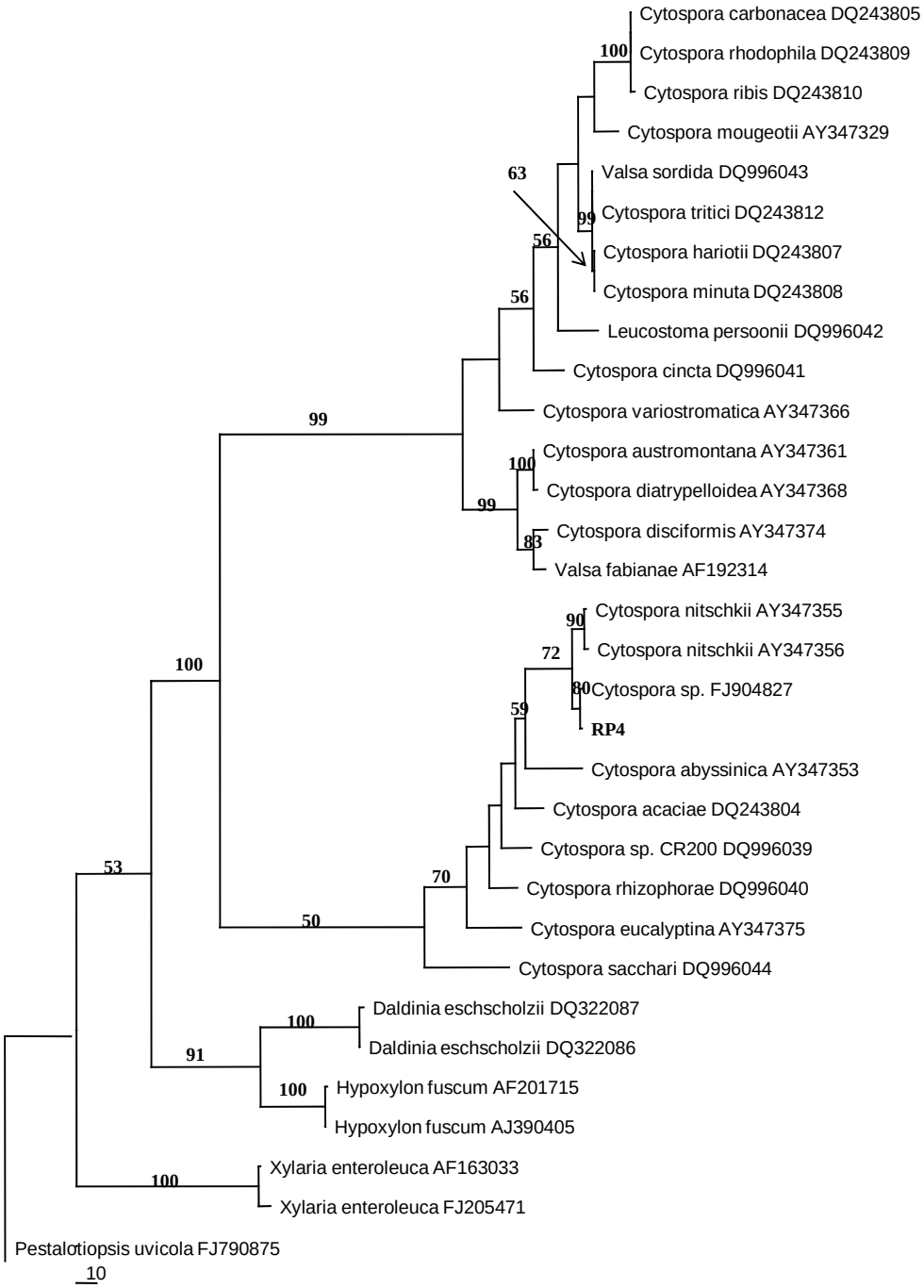


Fig. 42 – Phylogram generated from parsimony analysis based on rDNA ITS sequence data. Bootstrap values ≥50% are shown above the branches. The tree is rooted with *Pestalotiopsis uvicola*. *Rafflesia* endophyte (RP4) sequenced in this study is printed in bold. Bar = number of changes per nucleotide position.

medicinal plants, the number of morphospecies isolated for each plant varied from 23 to 83, however on average only 5–6 isolates were obtained from each flower species (Huang et al. 2008). The low number of endophytes obtained in our study may also be accounted for by the limited material sampled as compared to the very large flower size. Besides this, the *Rafflesia* flower produces copious amounts of CO_2 and volatile compounds (Meeuse 1966, 1975, 1978, Buggeln et al 1971) which may not beconducive for fungal growth. In our study, the endophytes were mainly recovered from petals, which is the most exposed part of the plant (Fig. 1). The central part of the flower (comprising raised disc, upper well wall and

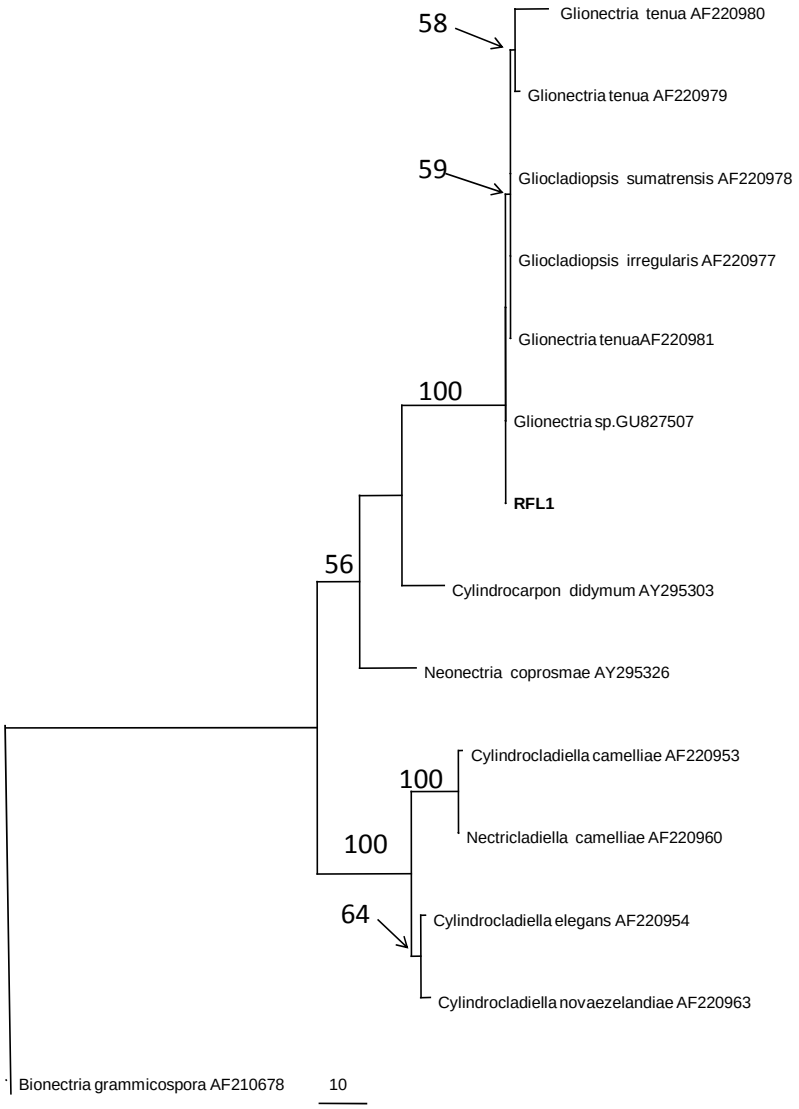


Fig. 43 – Phylogram generated from parsimony analysis based on rDNA ITS sequence data. Bootstrap values ≥50% are shown above the branches. The tree is rooted with *Bionectria gramicospora*. *Rafflesia* endophyte (RFL1) sequenced in this study is printed in bold. Bar = number of changes per nucleotide position.

vertical spines) is partially enclosed and is more likely to contain a higher concentration of CO_2 and volatile compounds. Only one fungal isolate (RFL1) was recovered from the central part of the flower.

Endophytic fungal species vary according to plant species, parts, and habitats (Arnold 2007). *Colletotrichum* species have been frequently identified as endophytes (Photita et al. 2005, Devarajan & Suryanarayanan 2006) and were the second most common taxa isolated from 26 traditional Chinese medicinal plants (Huang et al 2008). It has been proven that *Colletotrichum* metabolites have activity against bacteria and fungi (Table 3). Lu et al (2000) isolated a *Colletotrichum* endophyte from *Artemisia annua* that produced metabolites with inhibitory effects against *Candida albicans* and *Aspergillus niger*. *Colletotrichum dematium*, an endophytic fungus recovered from *Pteromischum* sp. in Costa Rica, produced a novel antimycotic peptide, colutellin A, which was active against *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Ren et al. 2008) (Table 3). *Cytospora* spp. have also been isolated as endophytic fungi in different studies (Singh et al. 2007**,** Abreu et al. 2010) and some species have been shown to possess bioactivity (Table 3). In a systematic screening of fungi in the United Kingdom for anti-microbial and

Table 3 Bioactivity and metabolites of *Colletotrichum* and *Cytosora* species.

Fungal genus	Metabolites produced	Organism inhibited
Colletotrichum	[a]6-isoprenylindole-3-carboxylic acid	*Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Pseudomonas* sp.
	[a]3β, 5α-dihydroxy-6β-acetoxy-ergosta-7, 22-diene	*Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Pseudomonas* sp.
	[a]3β, 5α-dihydroxy-6β-phenylacetyloxy-ergosta-7, 22-diene	*Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Pseudomonas* sp.
	[a]3β, 5α -dihydroxy-6β-acetoxy-ergosta-7, 22-diene+3β, 5α -dihydroxy-6β-phenylacetyloxy-ergosta-7, 22-diene + 3β-hydroxy-ergosta-5-ene + 3β-hydroxy-5α,8α-epidioxy-ergosta-6, 22-diene	*Candida albicans*, *Aspergillus niger*
	[b]colletotric acid	*Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea*, *Helminthosporium sativum*
	[c]Colutellin A	*Botrytis cinerea* ,*Sclerotinia sclerotiorum*
Cytospora	[d]Cytoskyrin A	Gram positive bacteria, *E.coli*
	[d]Cytosporon D	Gram positive bacteria, yeast
	[d]Cytosporon E	Gram positive bacteria, yeast
	[e]3, 5-dimethyl-8-hydroxy-7-methoxy-3, 4-dihydroisocoumarin	Gram positive bacteria, fungi
	[e]3, 5-dimethyl-8-methoxy-3, 4-dihydroisocoumarin	Gram positive bacteria, fungi

[a] Lu et al. 2000, [b] Zou et al. 2000, [c] Ren et al. 2008, [d] Singh et al. 2007, [e] Kokubun et al. 2003.

anti-insect activities, *Cytospora eucalypticola* was found to produce anti-fungal and anti-bacterial metabolites (Kokubun et al. 2003). In another study involving endophytic fungi from Costa Rica, *Cytospora* sp., isolated from a buttonwood tree, produced compounds that inhibited the growth of Gram-positive bacteria, including antibiotic-resistant strains (Singh et al. 2007). Although *Gliocladiopsis* is known as a plant pathogen, it has also been recovered as an endophytic fungus from rhizomes of *Paris polyphylla* (Li et al. 2008).

In our study, two *Colletotrichum* spp. (RP3 and RP6) and one *Cytospora* sp. (RP4) isolates inhibited the growth of *C. albicans*, a pathogenic yeast, in a preliminary screening assay. While no antibacterial activity was noted, the crude extracts from broth cultures of these fungal isolates (RP3, RP6 and RP4) showed potent bioactivity against *C. albicans* with IC_{50} values of 3.501 µg/mL, 6.048 µg/mL and 8.241 µg/mL, respectively. Although initial screening of RP3 culture only showed activity against *C. albicans*, modification of culture conditions and media resulted in antimicrobial activity detected against *A. niger* (unpublished data). Since both of these genera have been established as endophytic fungi producing bioactive metabolites (Zou et al. 2000, Singh et al. 2007), we endeavoured to resolve our isolates at the species level. Morphological characters of RP3 showed close similarity with *Colletotrichum hymenocalidis* as described by Yang et al (2009). After constructing a phylogenetic tree, RP3 grouped with *C. hymenocalidis* species with a sequence identity of 98.8% with *C. hymenocalidis* (GQ485600) and (GQ485601) (Fig. 41).When comparing RP6 with the description of *Colletotrichum siamense* by Prihastuti et al (2009), numerous similar characteristics were found. The phylogenetic tree placed RP6 in a clade with *C. siamense* (Fig. 41). Comparative analysis of partial ITS 1 & 2 and 5.8S rDNA sequences of RP6 and *C. siamense* (FJ972613) and (FJ972614) showed 100% similarity. *C. siamense* Prihastuti, L. Cai & K.D. Hyde was reported as a new species associated with coffee berries in northern Thailand by Prihastuti et al (2009).This is the first report of *C. siamense* from Malaysia. Based on morphological characters, RP4 was identified as *Cytospora* species. After constructing a tree, RP4 was found to be closely related to an unclassified *Cytospora* species

FJ904827 (Fig. 42). At present ITS data exist for less than 30 of the more than 300 species of *Cytospora* that have been described. Thus the isolate RP4 could not be resolved to species level. All other endophyte isolates, including the *Gliocladiopsis* sp. did not show any bioactivity. It is interesting to note that isolates RP1WA, RP2 and RP6, which were subsequently identified as belonging to the same species (*Colletotrichum siamense*), did not display the same bioactivity, indicating the importance of strain differences in fungal secondary metabolite production. Both RP1-WA and RP2 were morphologically very similar and produced no bioactive metabolites, while RP6 was morphologically different (Table 1, Figs 5–12, Figs 26–30) and showed activity.

Conclusion

This is the first study to report on endophytes from *Rafflesia* sp., but only a few isolates were recovered. These were identified as *Colletotrichum* spp. (six isolates), *Cytospora* sp. and *Gliocladiopsis* sp. Three *Colletotrichum* isolates were resolved to species level and identified as *C. siamense* based on molecular evidence. Antimicrobial activity against *Candida albicans* was detected in one isolate of each of *C. siamense*. *Colletotrichum* sp. and *Cytospora* sp.

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