CHAPTER 4

Classification of Nematophagous Fungi using Morphology Characteristics and Molecular Techniques

4.1 Introduction

Classification is act of forming into groups, such as classes, orders, families, etc., according to some common relations or affinities. There are several models to categorize organisms depending on frame of reference and objective. For example, taxonomic classification groups organisms into genera and species; statistical classification requires observational data while chemical classification utilizes elemental or compound composition. Currently, molecular techniques have been developed to demonstrate the phylogenetic relationships of organisms based on heredity and gene expression.

Nematophagous fungi are carnivorous fungi and around 160 species are known (Wikipedia, 2012d). They are found in most fungal taxa: Ascomycetes and their hyphomycete anamorphs, Basidiomycetes, Zygomycetes, Chytridiomycetes and Oomycetes (78 Steps Health Journal, 2012). These fungi can be classified into four major classes according to their infective strategies: trapping, endoparasitic, opportunistic and toxic fungi (Xue-Mei & Zhang, 2011). They use special mycelial structures before penetration of the nematode cuticle, invasion and digestion of nematodes (Nordbring-Hertz *et al*., 2006). In addition, morphology of conidiophore branching patterns, arrangement of conidia and dictyochlamydospore production,

growth and sporulation are also principal characteristics to identify fungal species. Annemarthe (no date) improved “Keys to the nematode-trapping genera of hyphomycetes and some similar genera”. Philip (2002) developed the “Nematophagous Fungi Guide” for preliminary classification into species of these fungi based on their special mycelial types. Recently, International Mycological Association (IMA), established an online database program called “Mycobank” to describe nomenclature and fungal species.

Taxonomy of the nematophagous fungi is being redefined with the development of new molecular techniques, for instance, Juan (2009) identified the nematophagous fungi obtained from *Rotylenchulus reniformis* plantings based on morphological characters and the ITS regions and 5.8S rDNA amplified by PCR using the primers ITS1 and ITS4. The ITS region is now perhaps the most widely sequenced DNA region used for fungal identification. It has typically been most useful for molecular systematic at the species level, and even within species e.g., to identify geographic races (Vilgalys lab, 2012). Moreover, molecular data have greatly improved our understanding of the taxonomy of these fungi and their physiological, biochemical activity and ecology, including their function as bio-control agents.

The objectives of this chapter were as follows:

1. To study the taxonomy of nematophagous fungi damaging root-knot nematode populations, *in vitro*.

2. To classify the species of selected nematophagous fungi based on morphological characteristics and molecular techniques.

4.2 Material and method

4.2.1 Identification of nematophagous fungi based on morphological characteristics

Seven isolates of selected nematophagous fungi, *Monacrosporium* sp. isolate JDI1-001, *Arthrobotrys* sp. isolate MTI2-001, *Arthrobotrys* sp. isolate MSO1-001, *Monacrosporium* sp. isolate MPI1-003, *Arthrobotrys* sp. isolate API3-001, *Pochonia* sp. isolate KJO1-003 and *Paecilomyces* sp. isolate WJI1-003 which showed a high percentage of damaged nematodes from previous experimental result (Chapter 3) and an effective fungus *Arthrobotrys* sp. isolate DLO1-001 obtained from Lumphun province (old stocked isolate) were studied.

Each fungal culture was maintained on corn meal agar (CMA) at 27ºC for 10 days to observe morphological characteristics including height, texture and color of colony surfaces. For microscopic characterization, micro-cultures were prepared on slides covered by a thin layer of water agar and incubated in a wet chamber at 25^{o}C for a week after inoculation of the fungi. The isolates were analyzed and described using the keys and species descriptions based on conidiophore branching patterns, arrangement and mode of conidia production using the online database programs “Mycobank (http://www.mycobank.org/)” and “Index Fungorum (http://www.indexfungorum.org/)” and “Keys to the nematode-trapping genera of hyphomycetes and some similar genera” described by Annemarthe (no date).

4.2.2 Identification of nematophagous fungi based on molecular techniques

DNA extraction: Eight isolates of nematophagous fungi were grown on PDA at room temperature (27±3^{o}C). For each isolate, the mycelia were ground in liquid nitrogen with mortar and pestle to a fine powder. The genomic DNA was extracted using the DNA Trap I (DNA TEC Cat NO.100-1009) according to the manufacturer's instructions. Powdered mycelium was suspended in detergent solution (700 µl of extraction buffer), incubated at 65°C for 60 min and put on ice box for 5 min. Extraction was with 120 µl neutralizer, mixed and put on ice box for 10 min. The cellular debris was pelleted by centrifugation for 5 min at 10,000 rpm. One point five ml of supernatant was mixed with 500 µl of trapping buffer and left at room temperature for 10 min. The mixture was centrifuged at 10,000 rpm for 1 min to harvest any pellets after discarding the supernatant. Samples were washed with 50 µl of washing buffer I and centrifuged at 10,000 rpm for 1 min to harvest whole pellets. The previous step was repeated with washing buffer II. The pellets were dried in an incubator at 65°C and 10 µl of elution buffer was added before rotation. The mixture was incubated at 65°C for 30 min and centrifuged at 10,000 rpm for 5 min. The supernatant with DNA was kept at -20°C prior to final characterization.

Determination of DNA concentration: DNA quality and quantity were determined by comparing with standard DNA intensity using an agarose gel electrophoresis protocol. A standard 1% (w/v) agarose gel prepared in Tris-Acetate-EDTA (TAE) electrophoresis buffer was used for analysis of total DNA preparations from fungal isolates and PCR amplicons. One gram of agarose powder was dissolved in

100 ml of 1X TAE buffer and microwaved for 2 min. The gel was casted with a sample slot comb. After approximately 30 min, the gel had solidified sufficiently to allow comb removal. TAE buffer was added in electrophoresis tank after submerging the gel. The samples in 6X gel-loading buffer were loaded into individual gel slots and run at 100 volts for 30 min. The gel was stained with ethidium bromide solution (10 µl /100 ml of buffer) for 10 min before viewing on long wave UV transilluminator and photographing.

PCR amplification of the ITS region: PCR reaction and digestion of amplified fragments were performed according to the procedures of Korabecna (2007) and Esteve-Zarzoso *et al*. (1999). The 5.8S-ITS2-28S rDNA gene (Figure 4.1) was amplified by PCR using the internal transcribed spacer primers ITS1 (5´TCC GTA GGT GAA CCT GCG G 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) (White, 1990). The PCR amplification was carried out using the GeneAmp^{R} PCR System 9700 (Applied Biosystems). Twenty µl of reaction mixture contained 10 ng of template DNA, 0.25 µM of each ITS1 and ITS4 primer, PCR buffer which was comprised of 100 mM Tris-HCL (pH 9.0), 500 mM KCl, 2.0 mM $MgCl_2$), 200 µM dNTPs and 0.6 unit of *Taq* DNA polymerase. The PCR amplification was programmed to carry out an initial denaturation step at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 7 min, followed by 1% (w/v) agarose gel electrophoresis and purification with PCR kit.

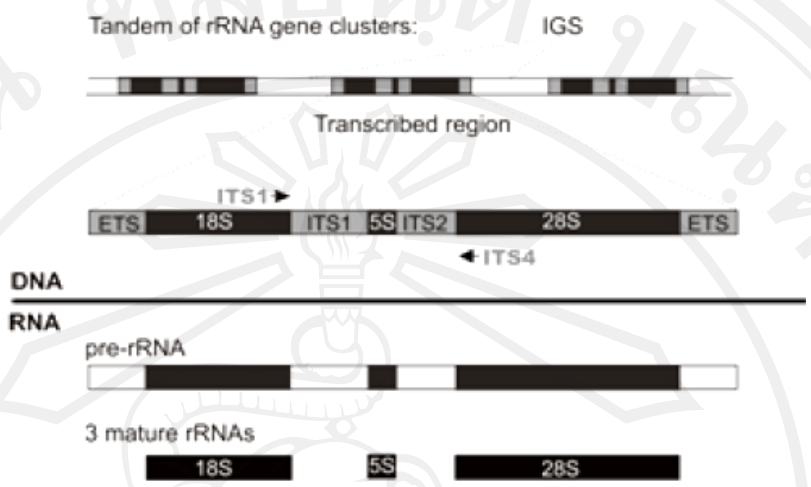


Figure 4.1 Schematic representation of rDNA with primer ITS1 and ITS4 localization (arrows). ITS= internal transcribed spacers. ETS= external transcribed spacers. IGS= non-transcribed intergenic spacers.

Source: Korabecna (2007)

Sequencing and phylogenetic analysis: Sequences of PCR products were obtained from both strands with ITS1 and ITS4 primers using the dideoxy chain termination method. The PCR products generation was carried out with the BigDye® Terminator v3.1 cycle sequencing kit, (1st BASE, Singapore) and automated DNA sequencer following the manufacturer' s instructions. The Sequencher version4.7 software was used to assemble, edit and generate high-quality sequences. Sequence similarity analyses were performed using the Basic Local Alignment Search Tool (BLAST) in GenBank or databases of National Center for Biotechnology Information: NCBI BLAST Assembled RefSeqGenomesprogram (NCBI, 2012).

The multiple sequence alignment program: MAFFT version 6 and GeneDoc version 2.7 was used to align nucleotide sequences. The phylogenetic tree was obtained from data using one of three equally parsimonious trees obtained through 1,000 replications of an heuristic search with random, stepwise sequence addition by PAUP version 4.0b10 (Phylogenetic Analysis Using Parsimony). Additional ITS sequences of nematophagous fungi were retrieved from GenBank.

4.3 Result

4.3.1 Identification of nematophagous fungi based on morphological characteristics

Fungal isolate DLO1-001 and MTI2-001 were classified as *Arthrobotrys oligospora*. Colony textures on corn meal agar (CMA) of these fungi were fuzzy and powdery, respectively, with dirty white surface color, but the reverse side of the colonies was colorless. Colonies grew rapidly nearing a diameter of 9 cm after 5 days at 27°C and had smooth margins. Mycelia grew shallowly in light concentric zones and produced a wooly pattern. DLO1-001 produced aerial mycelia while the mycelia of MTI2-001 was thin (Table 4.1 and Figure 4.2). Conidiophores were simple and erect, ranged in length from 200-450 µm, proliferated repeatedly and sporulated heavily. Conidia were obovoidal to pyriform. Submedially, 1-2 septa were observed which sometimes were site of slight constrictions. The conidia of DLO1-001 were 33.10±1.41 µm long ×12.90±0.85 µm wide (Table 4.2 and Figure 4.3) while conidia of MTI2-001 were 29.10±1.55 µm

long×12.25±0.85 µm wide (Table 4.2 and Figure 4.4). These fungi formed adhesive nets for trapping nematodes.

Fungal isolate API3-001 was grouped in *Arthrobotrys conoides*. Colony texture of this fungus was similar to that of MTI2-001 and was powdery with a dirty white color. It grew rapidly reaching 9 cm in diameter after 7 days at 27°C. In addition, API3-001 produced a colorless mycelium substrate and thin aerial mycelia (Table 4.1 and Figure 4.2). Conidiophores were erect, rarely branched, up to 400 µm in length, and proliferated repeatedly causing heavy conidial production. The conidiogenous heads were irregularly swollen, sometimes elongate and had short denticles. Conidia were elongate-obovoidal, with one median septum and slight constriction and measured 38.10±1.07 µm in length ×12.90±1.07 µm in width (Table 4.2 and Figure 4.5). Nematodes were trapped by adhesive nets.

Monacrosporium thaumasium was the designation of fungal isolate JDI1-001 and isolate MPI1-003. The surface and reverse colony color of these fungi were white and colorless, respectively. Colony textures were powdery, but differences in zonation were noted; JDI1-001 had a wooly format and MPI1-003 had slight radial furrowing. Both fungi grew moderately fast reaching 9 cm after 7 days at 27°C. Their mycelia had shallow growth and smooth margins with a thin aerial layer (Table 4.1 and Figure 4.2). Conidiophores were mostly simple, 150-300 µm in length, had 1-2 small perpendicular branches, and consequently these fungi produced a moderate number of conidia. Spindle-shaped conidia were detected on media. At their widest part conidia measured

23.15±1.09 µm and most often had two, equidistant septa (Table 4.2 and Figure 4.6-4.7). Three-dimensional networks were used for trapping nematodes.

Fungal isolate MSO1-001 was classified as *Arthrobotrys musiformis*. This fungus partially resembled isolate DLO1-001 in colony texture which was fuzzy and dirty white in color, but had limited growth in the center. Furthermore, the mycelium substrate was colorless and thin. This fungus grew rapidly (9 cm after 10 days) with slight radial furrowing texture (Table 4.1 and Figure 4.2). Microscopically and by measurement the fungus was most similar to the genus *Arthobotrys* in that it had erect conidiophores, averaging 272.50±54.95 µm in length. However, this isolate rarely produced side branches and proliferated subapically to produce a candelabrum-like branching system, each branch bearing a single terminal conidium. Conidia were elongate-obovoidal to ellipsoidal and 1-septate slightly below the middle. Conidia averaged 30.85±1.35 µm long×13.05±0.94 µm wide (Table 4.2 and Figure 4.8). The fungus trapped nematodes by means of adhesive nets.

Fungal isolate WJI1-003 was categorized as *Paecilomyces lilacinus*. Colonies were relatively slow-growing, often producing a diameter of <57 mm after 5 days at 27°C. Colony surface texture was velvety with a light concentric pattern consisting of numerous conidiophores and heavy sporulation. Aerial mycelium was at first white and changed to shades of light purple or sometimes was uncolored (Table 4.1 and Figure 4.2). Conidiophores were 30.25±7.34 µm in length, occasionally forming 2-4 layers of loose synnemata which had stalks with roughened thick walls. Verticillate branches with whorls of 2-4 phialides were often abundant. Phialides were 26-30±6-8 µm in length,

consisting of a swollen basal portion tapering into a short distinct neck. Conidia in divergent chains were ellipsoidal to fusiform. They were smooth-walled to slightly roughened, hyaline, but purple in mass. Conidia were 3.125±0.22 µm long×3.05±0.15µm wide (Table 4.2 and Figure 4.9). This fungus formed a special hyphal tip to damage nematode eggs.

Fungal isolate KJO1-003 was identified as *Pochonia chlamydosporia.* A creamish white and slight cottony colony was observed on CMA. Colony texture was wooly. This fungus grew relatively slowly as did as isolate WJI1-003 (*Paecilomyces lilacinus*) reaching 5.0-5.5 cm diameter after 5 days at 27°C. Its aerial mycelium had shallow growth and a thin form. Conidiophores were usually prostrate and little differentiated from the vegetative hyphae, but sometimes erect and differentiated (Table 4.1 and Figure 4.2). Conidiogenous cells were phialides, tapered to a narrow tip, and were hardly visible and solitary. Conidia were transversely positioned on phialides and formed in small slimy heads. Phialides originated from prostrate hyphae, were solitary and up to five per node. Conidia were subglobose, ellipsoidal to rod-shaped, isodiametric-polyhedric, or falcate with blunt ends, 3-3.5 µm wide and mostly adhered on globose heads or chains (Table 4.2 and Figure 4.10).

Table 4.1 Colony characterization of eight nematophagous fungi

Fungal isolate	Genera and species	Colonydiam[1/] 5 days (cm)	Colony character			Zonation	Sporulation
			Texture	Surface color	Reverse color		
DLO1-001	*Arthrobotrys oligospora*	7.5-8.0	Fuzzy	Dirty white	colorless	Light concentric zones	Heavy
MTI2-001	*Arthrobotrys oligospora*	7.5-8.0	Powdery	Dirty white	colorless	Wooly	Heavy
API3-001	*Arthrobotrys conoides*	7.5-8.0	Powdery	Dirty white	colorless	Wooly	Heavy
JDI1-001	*Monacrosporium thaumasium*	6.5-7.0	Powdery	White	colorless	Wooly	Moderate
MPI1-003	*Monacrosporium thaumasium*	6.5-7.0	Powdery	White	colorless	Slightly radially furrowed	Moderate
MSO1-001	*Arthrobotrys musiformis*	7.5-8.0	Central fuzzy	Dirty white	colorless	Slightly radially furrowed	Moderate
WJI1-003	*Paecilomyces lilacinus*	5.5-5.7	Velvety	Light purplewhite	Slightly purple	Light concentric zones	Heavy
KJO1-003	*Pochonia chlamydosporia*	5.0-5.5	Slight cottony	Creamish white	Slightly creamish	Wooly	Moderate

[1/] Data represents mean of four replicates

Table 4.2 Conidiophore and conidium measurements of nematophagous fungi

Fungal isolate[1/]	Conidiophores[2/]		Conidia[2/]		Genera and species
	Length mean ± standard deviation, range in um	Width mean ± standard deviation, range in um	Length mean ± standard deviation, range in um	Width mean ± standard deviation, range in um	
DLO1-001	337.50±64.12	9.30±0.98	33.10±1.41	12.90±0.85	*Arthrobotrys oligospora*
MTI2-001	290.00±30.78	9.10±1.02	29.10±1.55	12.25±0.85	*Arthrobotrys oligospora*
API3-001	252.50±80.25	5.00±0.79	38.10±1.07	12.90±1.07	*Arthrobotrys conoides*
JDI1-001	225.00±41.36	5.00±0.00	45.95±1.70	22.55±1.85	*Monacrosporium thaumasium*
MPI1-003	237.50±39.32	5.00±0.00	47.05±1.36	23.15±1.09	*Monacrosporium thaumasium*
MSO1-001	272.50±54.95	6.80±1.74	30.85±1.35	13.05±0.94	*Arthrobotrys musiformis*
WJI1-003	30.25±7.34	2.55±0.51	3.125±0.22	3.05±0.15	*Paecilomyces lilacinus*
KJO1-003	48.50±20.84	2.60±0.50	3.15±0.24	3.025±0.11	*Pochonia chlamydosporia*

[1/] Each fungal isolate was inoculated on corn meal agar (CMA).
[2/] Data represents mean of twenty replicates ± SE.

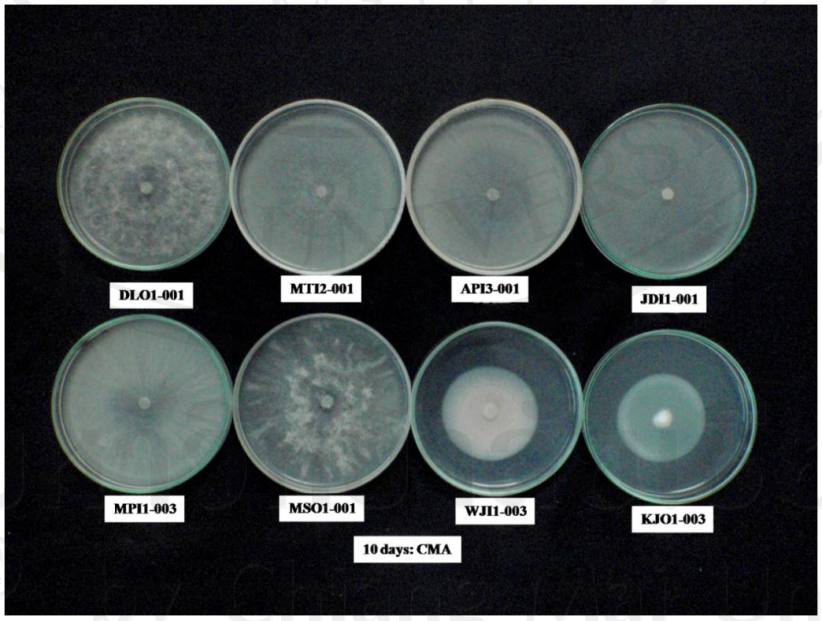


Figure 4.2 Characteristics of eight fungal colony textures on corn meal agar 10 days after incubation

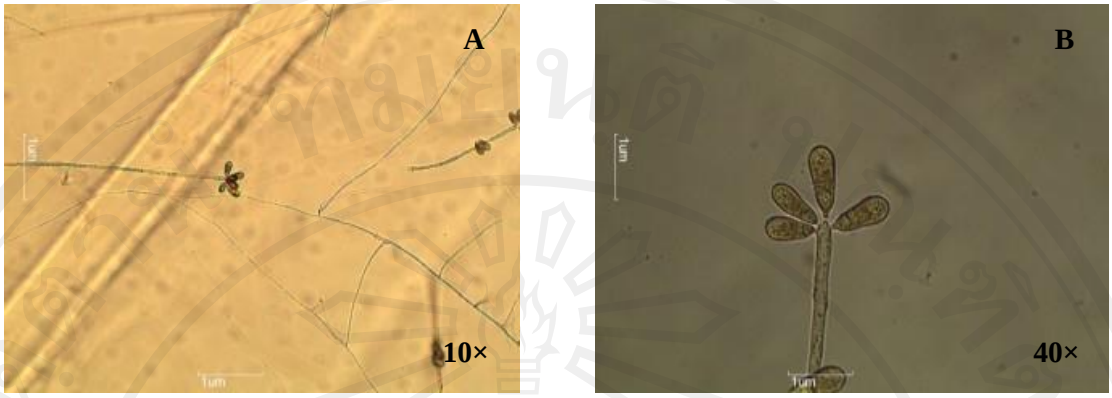
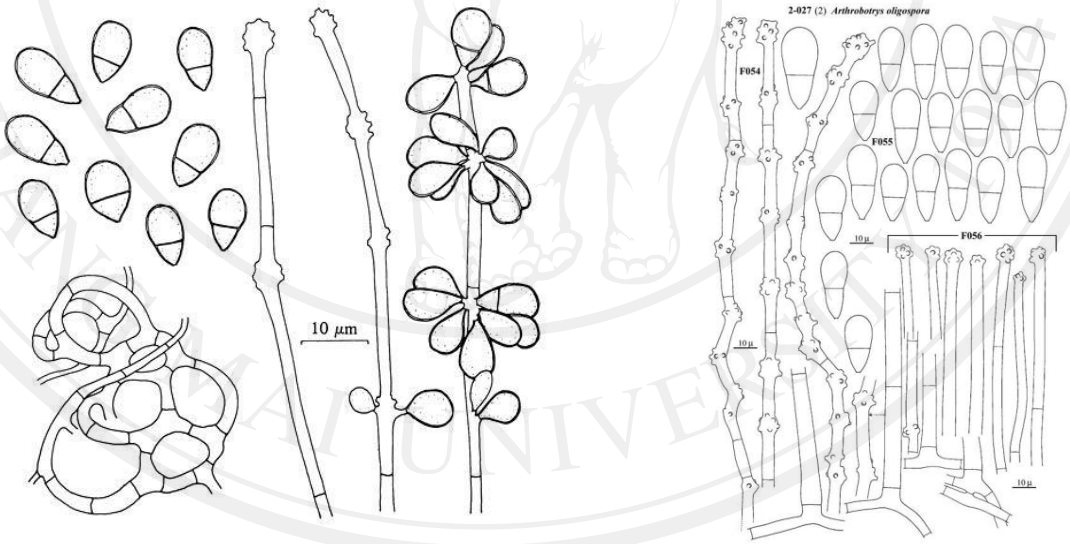


Figure 4.3 Conidiophore patterns (A) and conidia (B) of *Arthrobotrys oligospora* isolate DLO1-001 on micro-culture technique



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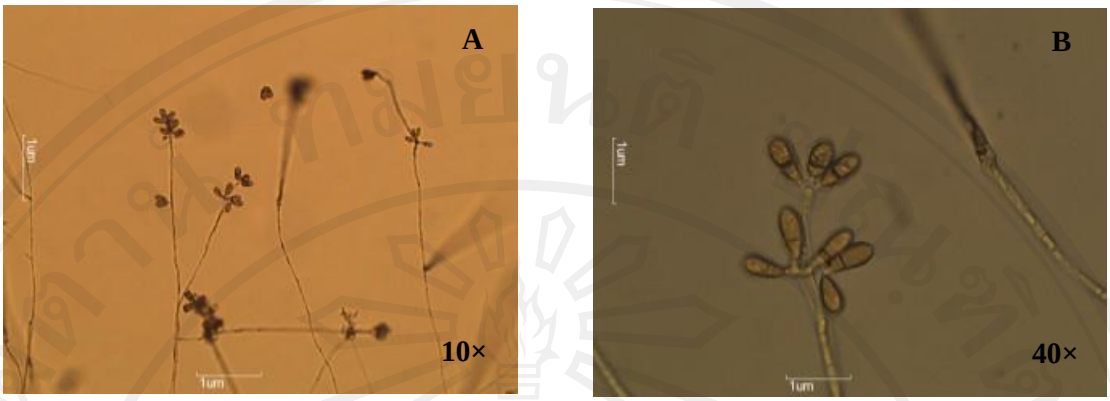
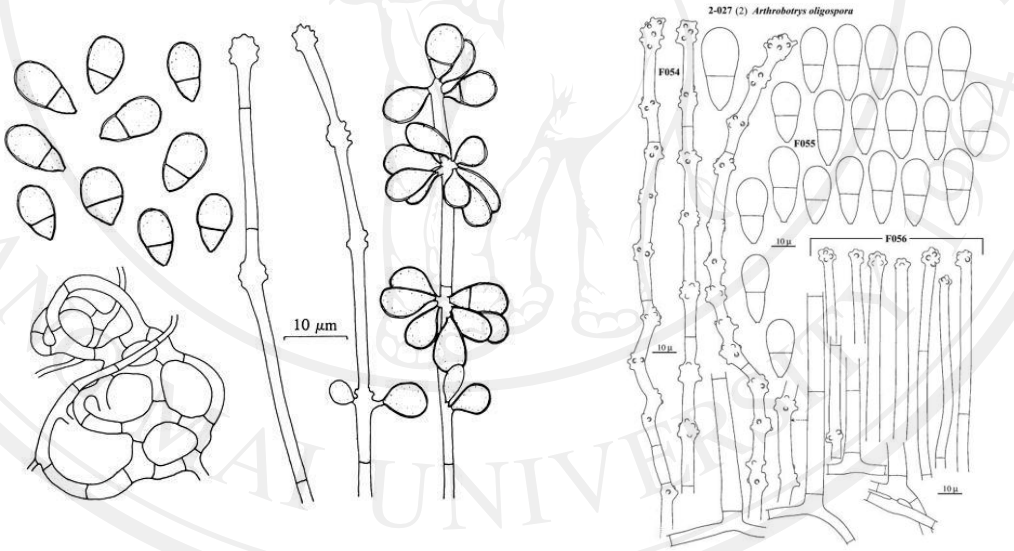


Figure 4.4 Conidiophore patterns (A) and conidia (B) of *Arthrobotrys oligospora* isolate MTI2-001 on micro-culture technique



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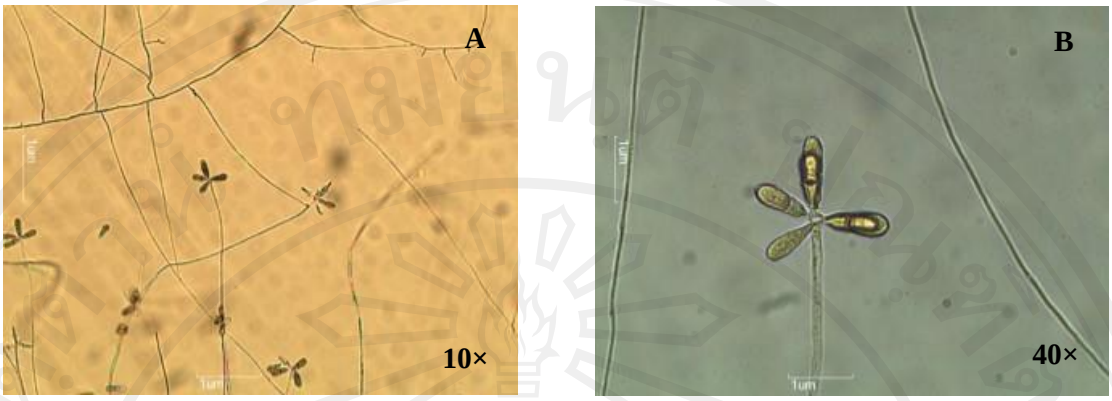
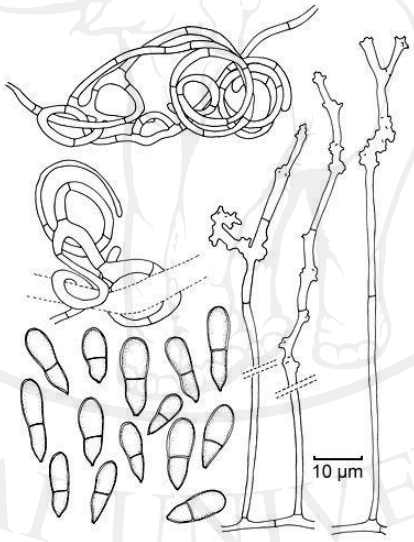


Figure 4.5 Conidiophore patterns (A) and conidia (B) of *Arthrobotrys conoides* isolate API3-001 on micro-culture technique



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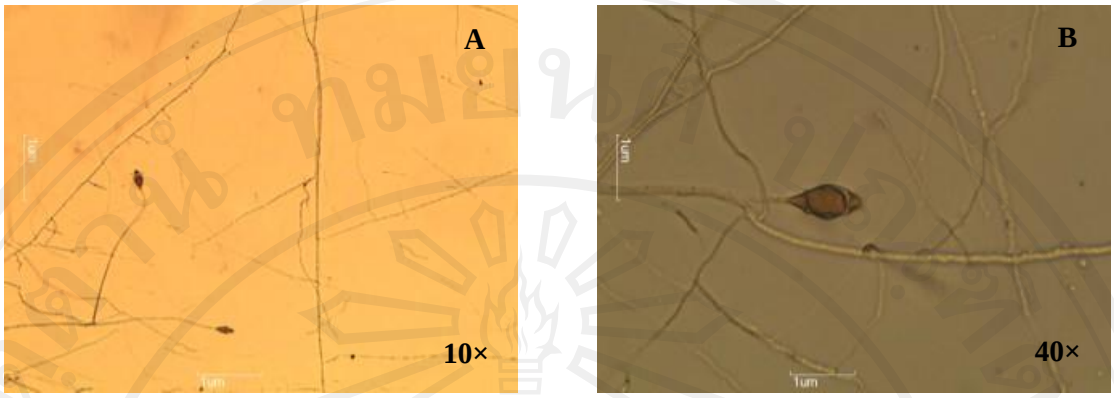
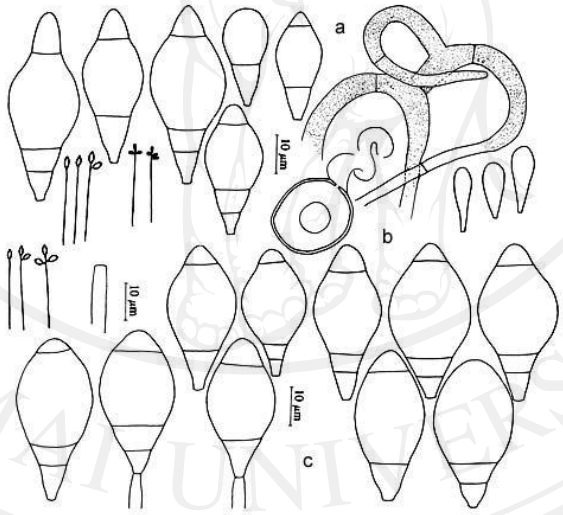


Figure 4.6 Conidiophore patterns (A) and conidia (B) of *Monacrosporium thaumasium* isolate JDI1-001 on micro-culture technique



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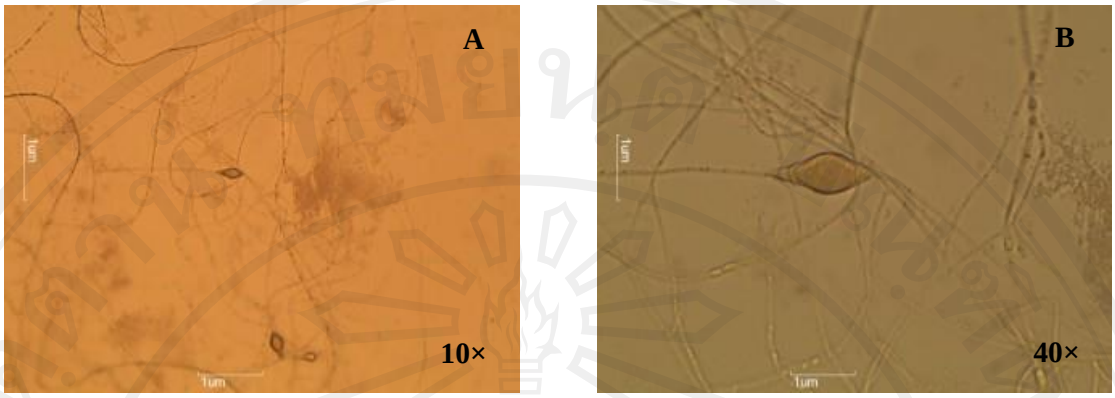
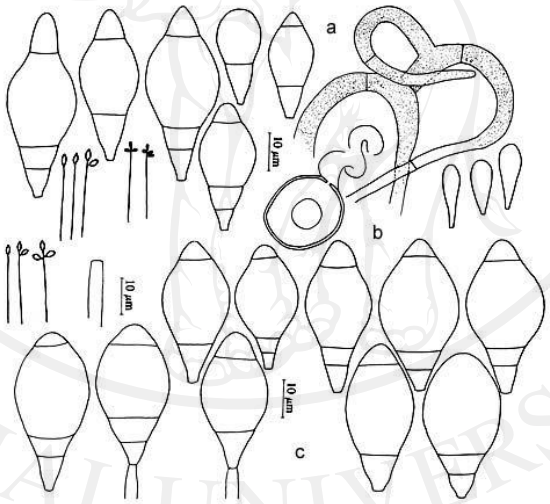


Figure 4.7 Conidiophore patterns (A) and conidia (B) of *Monacrosporium thaumasium* isolate MPI1-003 on micro-culture technique



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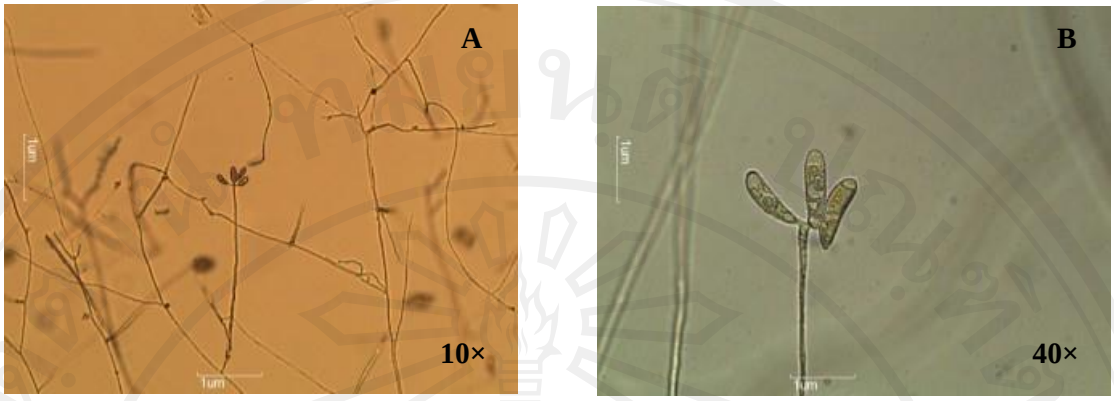
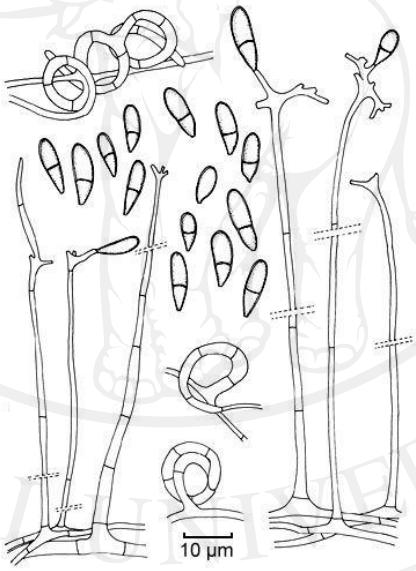


Figure 4.8 Conidiophore patterns (A) and conidia (B) of *Arthrobotrys musiformis* isolate MSO1-001 on micro-culture technique



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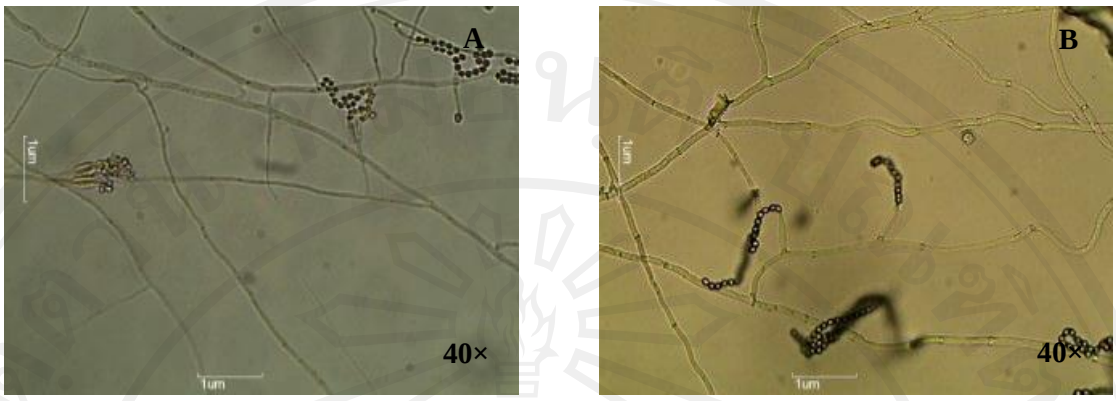
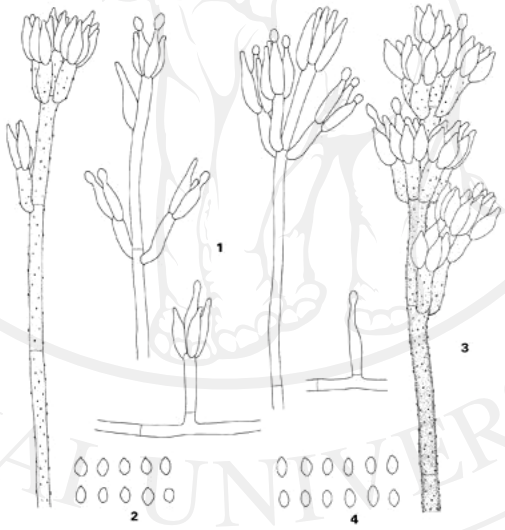


Figure 4.9 Conidiophore patterns (A) and conidia (B) of *Paecilomyces lilacinus* isolate WJI1-003 on micro-culture technique



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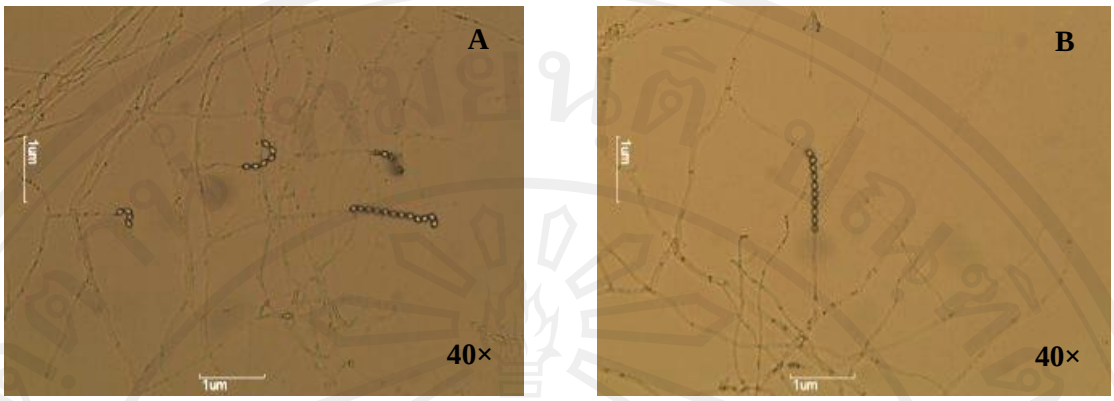
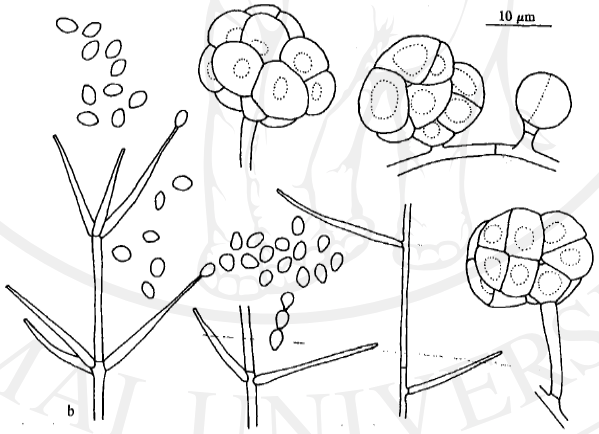


Figure 4.10 Conidiophore patterns (A) and conidia (B) of *Pochonia chlamydosporia* isolate KJO1-003 on micro-culture technique



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4.3.2 Identification of nematophagous fungi based on molecular techniques

PCR products of the ITS gene: The species of eight isolates of nematophagous fungi, *Arthrobotrys* sp. isolate DLO1-001, *Monacrosporium* sp. isolate JDI1-001, *Arthrobotrys* sp. isolate MSO1-001, *Arthrobotrys* sp. isolate API3-001, *Monacrosporium* sp. isolate MPI1-003, *Pochonia* sp. isolate KJO1-003, *Arthrobotrys* sp. isolate MTI2-001 and *Paecilomyces* sp. isolate WJI1-003, were confirmed by molecular techniques. The DNA of each fungal isolate was extracted to digest amplified fragments. The 5.8S-ITS2-28S rDNA gene was amplified using the internal transcribed spacer primers: ITS1 and ITS4. The PCR amplified region and the PCR products ranged 670-740 bp. The fungal isolates could be divided into three groups based on PCR products: (1) isolate DLO1-001, MTI2-001 and API3-001 (740 bp), (2) isolate JDI1-001 and MSO1-001(690 bp) and (3) isolate MPI1-003, KJO1-003 and WJI1-003 (670 bp) (Figure 4.11).

Nucleotide comparisons of these fungi with the implemented GenBank and NCBI databases using the BLASTN 2.2.26 program by Zhang *et al*. (2000) at highly similar sequences (megablast) indicated that isolate DLO1-001 and MTI2-001 were *Arthrobotrys oligospora* (91% and 90% homology, respectively) following the report of Swe *et al*. (unpublished). However, there was some divergence in the sequences of isolate DLO1-001 and MTI2-001 producing significant misalignments. Blast results identified isolate JDI1-001 as *Arthobotrys thaumasia* with a maximum score (741 bits). But the same maximum identity (90%), subordinate alignment analyzed this fungus as *Monacrosporium thaumasium* (715 bits) and *Monacrosporium megalosporum* (706 bits). Isolate MPI1-003 was identified as either *Monacrosporium*

thaumasium (601 bits) or *Arthobotrys thaumasia* (597 bits) at the similar maximum identity, 87%. Five hundred and eighty nucleic acid query length of isolate MSO1-001 was significantly aligned (91%) and identified this fungus as *Arthrobotrys musiformis*. Isolate API3-001 had highly similar sequences with *Arthrobotrys conoides* following the report of Falbo *et al*. (2011) who referenced *A. conoides* on GenBank accession number JN191309. Isolate KJO1-003 and WJI1-003 had no significant similarity with any genera or species based on molecular data. The details of all results are shown in Table 4.3.

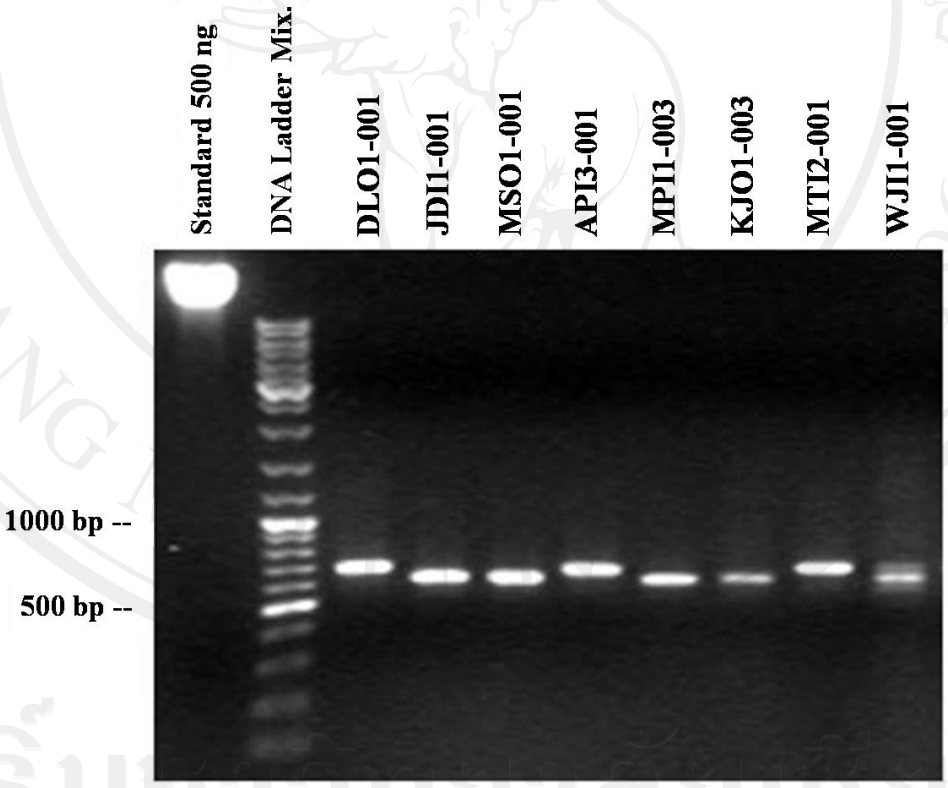

Figure 4.11 Amplification of the partial ITS region for species identification of eight nematophagous fungi by PCR using the internal transcribed spacer primers: ITS1 and ITS4. Markers are the 100 bp ladder, and the arrow on the left side indicates the 500 bp and 1,000 bp position.

Table 4.3 The blast result of rDNA ITS sequences from nematophagous fungi and their closely related sequences in GenBank during June 2012

Taxon [1/]	Trap type	Example code	ITS Blast result [2/]	Maximum Score[3/]	Identity[3/]	Gap[3/]	Accession#[3/]	Reference[3/]
Arthrobotrys oligospora	Adhesive nets	DLO1-001	*Arthrobotrys oligospora*	802	529/580 (91%)	1/580 (0%)	EU977526	Swe *et al*. (Unpublished)
Arthrobotrys oligospora	Adhesive nets	MTI2-001	*Arthrobotrys oligospora*	817	565/631 (90%)	2/631 (0%)	HQ649929	Macia-Vicente *et al*. (Unpublished)
Arthrobotrys conoides	Adhesive nets	API3-001	*Arthrobotrys conoides*	817	552/616 (90%)	0/616 (0%)	JN191309	Falbo *et al*. (2011)
Monacrosporium thaumasium	Adhesive nets	JDI1-001	*Arthrobotrys thaumasia*	741	509/564 (90%)	4/564 (1%)	AF106526	Hagedorn & Scholler (1999)
Monacrosporium thaumasium	Adhesive nets	MPI1-003	*Monacrosporium thaumasium*	601	464/535 (87%)	4/535 (1%)	FJ380934	Kuo *et al*. (2009)
Arthrobotrys musiformis	Adhesive nets	MSO1-001	*Arthrobotrys musiformis*	745	497/574 (91%)	2/574 (0%)	U51948	Liou & Tzean (1997)
Paecilomyces lilacinus	Hyphal tips	WJI1-003	Non matched	-	-	-	-	-
Pochonia chlamydosporia	Appressoria	KJO1-003	Non matched	-	-	-	-	-

[1/] Isolated from agricultural soil, Thailand
[2/] Referenced program by Zhang *et al*. (2000)
[3/] Reported first sequences producing significant alignment

Phylogenetic analysis: In this study, ITS sequences of selected fungal isolates were compared with those of 36 published nematophagous fungi; *Arthrobotrys* spp., *Monacrosporium* spp. and *Duddingtonia* sp. retrieved from GenBank. *Neurospora pannonica* and *Sordaria fimicola* were used as the outgroup (Table 4.4).

Multiple sequence alignment was used to infer the maximum likelihood tree. Out of 821 included characters, 244 characters were constant and 203 variable characters were parsimony-uninformative so a number of parsimony-informative included characters were 374. Nucleotide sequences based on the rDNA ITS region indicated a relationship between genotypes of some fungal isolates. Most of selected nematophagous fungi were harmoniously clustered as blast groups. The phylogenetic relationships of Orbiliaceae which include *Arthrobotrys oligospora* isolate DLO1-001 and MTI2-001, *A. conoides* isolate API3-001 and *A. musiformis* isolate MSO1-001 were well defined and consecutively had bootstrap values (BSV) of 100%, 99% and 94%. While the phylogenetic relationships of isolate JDI1-001 and MPI1-003 were not clear, they had 99% BSV and nearby groups with *A. thaumasia*, *Monacrosporium thaumasium*, *M. microscaphoides*, *A. multisecun* and *M. eudermatum* as shown in Figure 4.12. However, the selected maximum parsimonious tree showed BSV and phylogenetic relationships of Orbiliaceae close to Neighbor Joining trees using BioNJ method and Kimura 2- parameter which had 821 included characters and 810 excluded characters (data not shown).

Table 4.4 ITS sequence used in phylogenic analyses from GenBank

Taxon	Trapping devices	Geographic origin	GenBank Accession Numbers
Arthrobotrys oligospora	Adhesive nets	P.R. China	FJ557237
Arthrobotrys oligospora	Adhesive nets	P.R. China	EU977526
Arthrobotrys oligospora	Adhesive nets	Taiwan	FJ380939
Arthrobotrys oligospora	Adhesive nets	Germany	AM412778
Arthrobotrys conoides	Adhesive nets	Brazil	JN191309
Arthrobotrys conoides	Adhesive nets	P.R. China	AY773455
Arthrobotrys musiformis	Adhesive nets	P.R. China	U51948
Arthrobotrys musiformis	Adhesive nets	P.R. China	EU977517
Arthrobotrys musiformis	Adhesive nets	Taiwan	FJ380938
Arthrobotrys thaumasia	Adhesive nets	P.R. China	EU977538
Arthrobotrys thaumasia	Adhesive nets	P.R. China	EU977529
Arthrobotrys thaumasia	Adhesive nets	P.R. China	EU977535
Arthrobotrys vermicola	Adhesive nets	P.R. China	HQ595346
Arthrobotrys vermicola	Adhesive nets	P.R. China	GQ121416
Arthrobotrys vermicola	Adhesive nets	P.R. China	AY773454
Arthrobotrys vermicola	Adhesive nets	P.R. China	EU977508
Arthrobotrys multisecun	Adhesive nets	P.R. China	EF059815
Arthrobotrys sinensis	Adhesive nets	P.R. China	AY773445
Arthrobotrys scaphoides	Adhesive nets	P.R. China	GU171370
Arthrobotrys scaphoides	Adhesive nets	P.R. China	GU171371
Arthrobotrys cladodes	Adhesive nets	P.R. China	U51945
Arthrobotrys cladodes	Adhesive nets	P.R. China	U51952
Arthrobotrys janus	Adhesive nets	P.R. China	AY773459
Arthrobotrys flagrans	Adhesive nets	Germany	AF106520
Arthrobotrys amerospora	Adhesive nets	Taiwan	FJ380931
Arthrobotrys amerospora	Adhesive nets	UK	EF192178
Arthrobotrys javanica	Adhesive nets	P.R. China	U51947
Arthrobotrys javanica	Adhesive nets	Taiwan	FJ380932
Arthrobotrys pyriformis	Adhesive nets	P.R. China	EU977541
Arthrobotrys superba	Adhesive nets	P.R. China	EU977558
Monacrosporium thaumasium	Adhesive nets	P.R. China	U51972
Monacrosporium microscaphoides	Adhesive nets	P.R. China	EF059816
Monacrosporium eudermatum	Adhesive nets	P.R. China	U51975
Monacrosporium megalosporum	Adhesive nets	Japan	AB114475
Monacrosporium ellipsosporum	Adhesive knobs	P.R. China	AY965759
Duddingtonia flagrans	Adhesive nets	New Zealand	AY095103
Neurospora pannonica	-	Netherlands	GQ922532
Sordaria fimicola	-	USA	DQ518178

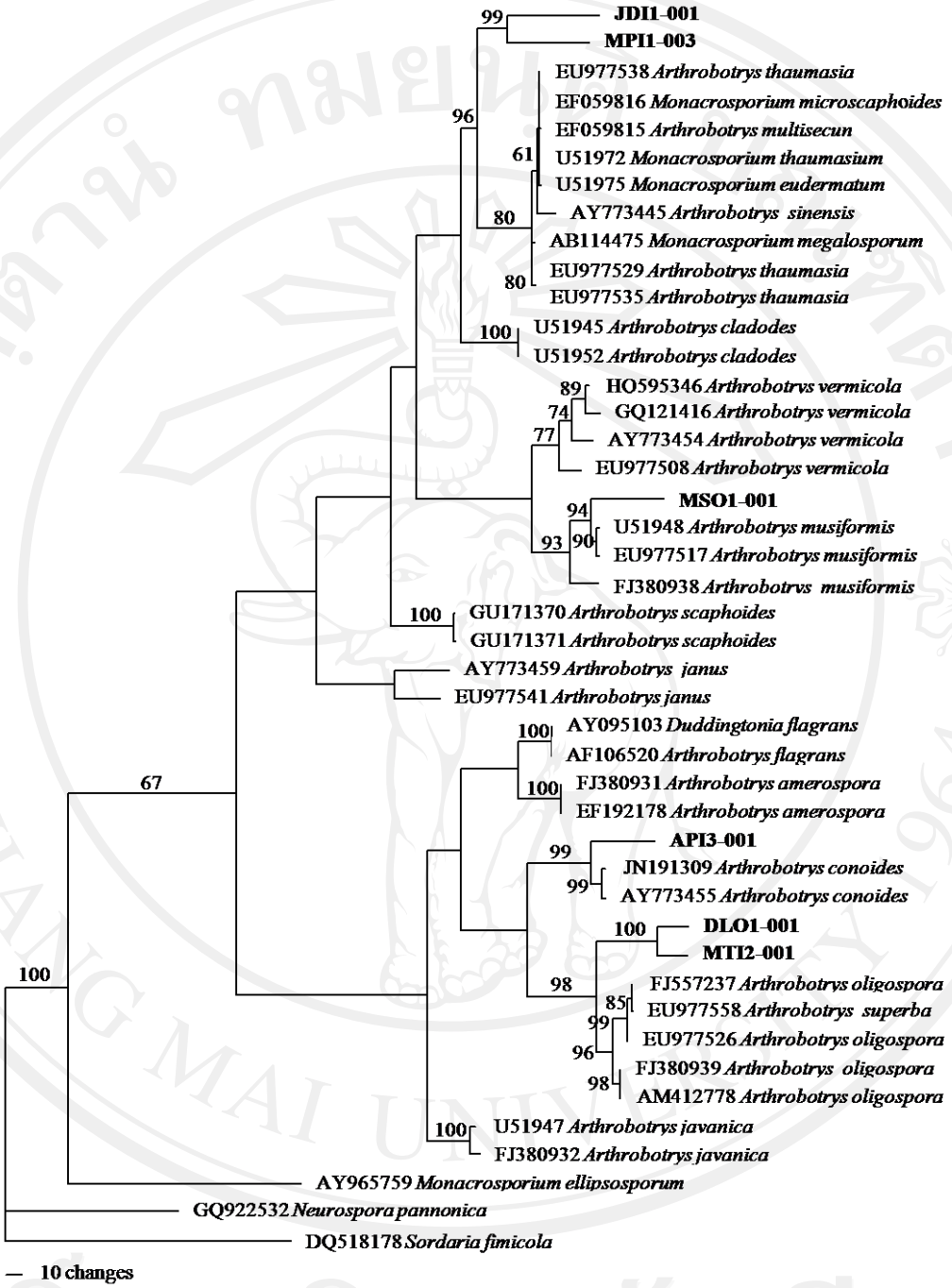

Figure 4.12 One of 6 equally most parsimonious trees inferred from a heuristic search of the ITS1-5.8S-ITS2 rDNA sequences alignment of 44 isolates of *Arthrobotrys* and related genera. The size of the branches is indicated with a scale bar. Length=1,525, Cl=0.609 and Rl=0.723

4.4 Discussion

Morphological classification of the nematophagous fungi described in this research was based on such characteristics as colony diameter, culture appearance (texture, surface and reverse colouration, zonation) and colony growth rate. It is understood that the specific colony characteristics of each fungus may be different depending on a type of culture medium used. Sharma & Pandey (2010) found that the type of growth medium greatly influenced the colony diameter, culture characteristics and sporulation of 10 selected fungal isolates grown potato dextrose agar (PDA), czapek's dox mixed yeast extract agar (CYA) and lignocellulose agar (LCA).

Results of morphological and molecular identification were generally concordant. The morphological and molecular data were in agreement for four fungal isolates, DLO1-001 (*Arthrobotrys oligospora*), isolate MTI2-001 (*Arthrobotrys oligospora*), isolate API3-001 (*Arthrobotrys conoides*) and isolate MSO1-001 (*Arthrobotrys musiformis*). The cases of isolate JDI1-001 and MPI1-003 were less clear cut. The conidiophore patterns and conidia classified isolate JDI1-001 and MPI1-003 as *Monacrosporium thaumasium*. Nevertheless, the 5.8S-ITS2-28S rDNA sequence data using ITS1 and ITS4 primers aligned them with *Arthrobotrys thaumasia* and *M. thaumasium* with equal identity percentages. However, Index Fungorum (2012) reported that *M. thaumasium* and *A. thaumasia* (Drechsler) S. Schenck, W.B. Kendr. & Pramer, were actually synonymous *Can. J. Bot.* 55(8): 984 (1977).

The phylogenetic patterns of this study showed a concordant relationship between fungal genera and their infection structures that was in agreement with the suggestion of Scholler *et al*. (1999) that trapping devices might be associated with

other morphological features. Scholler *et al*. (1999) divided predatory anamorphic Orbiliaceae fungi into four genera with the genus *Arthrobotrys* typified by formation of adhesive networks to capture nematodes.

Fungal isolate WJI1-003 and KJO1-003 were morphologically categorized as *Paecilomyces lilacinus* and *Pochonia chlamydosporia*, but could not be classified molecularly based on nucleotide blast format. This result may be based on the unsuitability of the sequence region and primer selection, and/or the PCR protocols used. Peter & Myrian (2006) selected DNA protocols and used internal transcribed spacer (ITS1 and ITS2) for sequencing some entomogenous *Paecilomyces* species at the 5.8S rDNA and observed polyphyly in the genus and showed the existence of *Paecilomyces* cryptic species. Ciancio *et al*. (2005) designed specific fluorescent molecular probes for detecting the nematode-egg parasitic fungus *P. chlamydosporia* var. *chlamydosporia* through a nucleotide sequencing of a conserved region unique in the ITS2 rDNA gene. The primers were NBRC 9249 U1 (5′-GAGGTGAAATT CTTGGATTTATTG-3′), NBRC 9249 D (5′-CGCCGAAGCAACGGTTGTAAATG G-3′), NBRC 9242 U2 (5′-GCTTGGGCTCCAGGGGGAGTATGGT-3′), NBR29242 U3 (5′-GCTTGCGTTGATTACGTCCCTG CC-3′) and NBRC 9242 U4 (5′-GGAAA CTCACCAGGTCCAGACACA-3′). In addition, fungal isolates *Arthrobotrys oligospora, A. conoides, Monacrosporium thaumasium* and *A. musiformis* are nematode trapping fungi within the Orbiliales (Ascomycota) forming adhesive nets while *P. lilacinus* and *P. chlamydosporia* are egg parasites. It is therefore not surprising that the molecular characteristics of the two types of nematode parasites were divergent and in the cases of *P. lilacinus* and *P. chlamydosporia* may require different molecular identification techniques.

The genera *Paecilomyces* and *Pochonia* include a few species that damage nematodes and they had distinct colony characteristics on most growing media so molecular identification may not be necessary. On the other hand, the genus *Arthrobotrys* contains a world wild distribution of nematode-trapping fungi including 71 species that can kill second stage juveniles (Wikipedia, 2012c) and *Monacrosporium* contains 53 species that can trap nematodes (Wikipedia, 2012f). Moreover, cultural and microscopic characteristics overlap which may lead to possible errors in fungal taxon classification. For this reason, ITS region which had higher degree of variation than other genetic regions of rDNA was used for molecular systematics at the species level, and even within species to identify geographic races (Wikipedia, 2012e). Afterwards of this study, fungal isolate WJI1-003 and KJO1-003 were used *Paecilomyces* sp. isolate WJI1-003 and *Pochonia* sp. isolate KJO1-003 as a result of unidentified species by molecular technique referring GenBank sequence database.

4.5 Conclusion

This study found and characterized four nematophagous fungal isolates of genus *Arthrobotrys* which classified as *Arthrobotrys oligospora* (DLO1-001 and MTI2-001), *Arthrobotrys conoides* (API3-001) and *Arthrobotrys musiformis* (MSO1-001), two isolates of *Monacrosporium thaumasium* were actually synonymous with *Arthrobotrys thaumasia* so that isolate JDI1-001 and MPI1-003 were *A. thaumasia* and single isolate of *Paecilomyces* sp. (WJI1-003) and *Pochonia* sp. (KJO1-003) from Thailand. *Arthrobotrys* and *Monacrosporium* are trapping fungi belong to the Orbiliaceae. They formed adhesive nets to capture and kill second stage juveniles of

root-knot nematodes while *Paecilomyces* used hyphal tips and *Pochonia* applied appressoria to damage eggs.

Morphological characteristics used for species identification of isolates included colony growth and culture characteristics on corn meal agar, conidiophore branching pattern and arrangement including conidia morphology and quantity. These criteria were generally useful for species identification but in some cases interspecies overlap occurred. Therefore, optimal molecular techniques are useful in confirmation of cultural and morphological species identification.