การคัดแยก Pythiaceae จากยางพารา *Hevea brasiliensis* ในภาคตะวันออกของประเทศไทยเพื่อการระบุไวรัส Pythiaceae

นางสาวชนกนันท์ หัตถพณิชพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ภาควิชาจุลชีววิทยา
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ปีการศึกษา 2559

ISOLATION OF PYTHIACEAE FROM PARA RUBBER *Hevea brasiliensis* IN EASTERN THAI LAND FOR IDENTIFICATION OF PYTHIACEAE VIRUS

Miss Chanoknan Hattapanichaporn

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By	Miss Chanoknan Hattapanichaporn
Field of Study	Microbiology and Microbial Technology
Thesis Advisor	Thanyanuch Kriangkripipat, Ph.D.
Thesis Co-Advisor	Associate Professor Wanchai Assavalapsakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science

(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Tanapat Palaga, Ph.D.)

.....Thesis Advisor

(Thanyanuch Kriangkripipat, Ph.D.)

.....Thesis Co-Advisor

(Associate Professor Wanchai Assavalapsakul, Ph.D.)

.....Examiner

(Assistant Professor Panan Rerngsamran, Ph.D.)

.....External Examiner

(Professor Prakitsin Sihanonth, Ph.D.)

ชนกนันท์ หัตถพณิชพร : การคัดแยก Pythiaceae จากยางพารา *Hevea brasiliensis* ในภาคตะวันออกของประเทศไทยเพื่อการระบุไวรัส Pythiaceae (ISOLATION OF PYTHIACEAE FROM PARA RUBBER *Hevea brasiliensis* IN EASTERN THAILAND FOR IDENTIFICATION OF PYTHIACEAE VIRUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. ธัญนุช เกรียงไกรพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.วันชัย อัศวลาภสกุล, 131 หน้า.

ไมคอไวรัส คือ ไวรัสในรา จีโนมของไมคอไวรัส ส่วนใหญ่มีสารพันธุกรรมเป็นแบบอาร์เอ็นเอสายคู่ ราที่มีไมคอไวรัสอาจก่อโรคได้รุนแรงเพิ่มขึ้นหรือน้อยลง งานวิจัยนี้มีจุดมุ่งหมายเพื่อหาอาร์เอ็นเอสายคู่ในรากลุ่ม Pythiaceae ซึ่งเป็นสิ่งมีชีวิตคล้ายรา ในวงศ์ดังกล่าวประกอบด้วย *Phytophthora*, *Pythium* และ *Phytopythium* โดยมีรายงานว่า *Phytophthora* ก่อโรคใบร่วงในยางพารา (*Hevea brasiliensis*) ราในวงศ์ Pythiaceae 79 ไอโซเลต ที่แยกได้จากใบยางพาราที่แสดงอาการของโรคใบร่วงจากพื้นที่ปลูกในจังหวัดจันทบุรี และระยอง เมื่อนำไอโซเลตทั้งหมดมาตรวจชิ้นส่วนดีเอ็นเอและอาร์เอ็นเอสายคู่ที่ไม่ใช่สารพันธุกรรมของราเจ้าบ้าน พบว่า ไอโซเลต R84 มีอาร์เอ็นเอสายคู่ 3 ขนาด คือ 8.0, 3.7 และ 2.3 กิโลเบส โดยอาร์เอ็นเอสายคู่ขนาด 8.0 กิโลเบส เป็นชิ้นเดียวที่พบได้ในทุกรุ่นของการต่อเชื้อ ผลการตรวจสอบอาร์เอ็นเอสายคู่โดยสร้าง cDNA เพื่อตรวจจับด้วยไพรเมอร์ที่จำเพาะต่อไวรัสของ Pythiaceae และวิเคราะห์ลำดับเบสของ cDNA พบว่า อาร์เอ็นเอสายคู่ที่พบในไอโซเลต R84 ไม่ตรงกับไวรัสของ Pythiaceae ที่มีผู้รายงานมาก่อน การศึกษาชิ้นส่วน cDNA ขนาด 233 คู่เบส ซึ่งเป็นส่วนหนึ่งของอาร์เอ็นเอขนาด 8.0 กิโลเบส พบว่า มียีนที่แปลรหัสคล้ายกับโปรตีน ALG2 mannosyltranferase แต่ไม่สามารถระบุสกุลของอาร์เอ็นเอสายคู่ได้ ส่วนไอโซเลต R84 ซึ่งเป็นเจ้าบ้านของอาร์เอ็นเอสายคู่ สามารถระบุว่าเป็น *Pythium cucurbitacearum* ซึ่งยังไม่มีผู้รายงานว่า ราสกุลดังกล่าวก่อโรคในยางพารามาก่อน เมื่อศึกษาการทนต่อ hymexazol ซึ่งเป็นสารฆ่ารา ของไอโซเลต R84 พบว่า ทนต่อ hymexazol ที่ความเข้มข้นสูงได้ อาร์เอ็นเอสายคู่ที่พบในงานวิจัยนี้อาจเกี่ยวข้องกับคุณสมบัติการทนต่อสารฆ่าราของ *Py. cucurbitacearum* ไอโซเลต R84

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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CHANOKNAN HATTAPANICHAPORN: ISOLATION OF PYTHIACEAE FROM PARA RUBBER *Hevea brasiliensis* IN EASTERN THAILAND FOR IDENTIFICATION OF PYTHIACEAE VIRUS. ADVISOR: THANYANUCH KRIANGKRIPIPAT, Ph. D. , CO- ADVISOR: ASSOC. PROF. WANCHAI ASSAVALAPSAKUL, Ph.D., 131 pp.

Mycoviruses are viruses infecting fungi of which dsRNA genome is the most common. Mycovirus infection may confer the hyper and hypo-virulence phenotypes of fungi. This study focused on isolation of virus in Pythiaceae, a family of fungus-like organism. The members of this family are plant pathogens including *Phytophthora*, *Pythium* and *Phytopythium*. Species in *Phytophthora* cause abnormal leaf fall disease (ALF) in para rubber tree (*Hevea brasiliensis*). Pythiaceae were isolated from rubber leaves showing ALF symptoms collected from plantations in Chanthaburi and Rayong province, Thailand. All 79 Pythiaceae isolates were screened for extrachromosomal DNA and dsRNA elements. One isolate, R84 was found harboring 3 dsRNA element approximately 8.0, 3.7 and 2.3 kb. However, upon consecutive subcultures the only largest segment remained. Two-step reverse transcription using Pythiaceae virus specific primers revealed that the dsRNA was not one of reported Pythiaceae viruses. A partial cDNA clone of 233 nt out of the largest 8.0 kb dsRNA element contained a fragment of a gene encoding ALG2 mannosyltransferase-like protein but the genus of the dsRNA could not be specified. The dsRNA host was identified as *Pythium cucurbitacearum*, which has never been reported associated with rubber diseases. Isolate R84 showed hyper-resistance to hymexazol fungicide. The dsRNA found in this study might be responsible for resistant to fungicide of *Py. cucurbitacearum* isolate R84.

Department:	Microbiology	Student's Signature .....
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature .....
		Co-Advisor's Signature .....
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CHAPTER I
INTRODUCTION

According to statistic data recorded by Office of Agricultural Economics (2015), Thailand is the world's largest rubber exporter, amounting to 4.46 million tons of natural rubber was exported by Thailand totaled 197 billion baht in 2015. Rubber tree requires warm temperature around 25-28°C, well-distributed rainfall of 100-150 days through the year with 2000–4000 mm of annual precipitation, and high humidity ranging between 67-82% for high rubber yield (Priyadarshan, 2003; Yu *et al.*, 2014). These optimal conditions for latex production are similar to the climate in the south and the east region of Thailand (RAOT, 2011). The most planted rubber clone in Thailand is RRIM 600, which has been recommended by Rubber Research Institute of Thailand (RRIT) for high latex yield (RAOT, 2011; Pethin *et al.*, 2015). However, this rubber clone is susceptible to various rubber diseases, especially *Phytophthora* spp. causing abnormal leaf fall (ALF) and black stripe disease (RAOT, 2016).

Pythiaceae is the family of oomycetes, the fungi-like organism or water molds (van den Berg *et al.*, 2013; de Cock *et al.*, 2015). Physiology and habitat of oomycetes are similar to those of filamentous fungi but they are more related to brown-algae and diatoms. Despite the fact, oomycetes are actively studied by mycologists (Baldauf *et al.*, 2000; Webster and Weber, 2007). Three genus in the family Pythiaceae including *Phytophthora* (*P.*), *Pythium* (*Py.*) and *Phytopythium* (*Phy.*) are recognized among plant pathologists as devastating plant pathogens (Drenth and Guest, 2004a; Baten *et al.*, 2014; Jankowiak *et al.*, 2015). Species of Pythiaceae reported causing rubber diseases are *P. capsici*, *P. citrophthora*, *P. meadii*, *P. nicotianae*, *P. palmivora* and *Phy. vexans* (previously named *Py. vexans*), all 6 species of the genus *Phytophthora* have been reported worldwide, while *Phy. vexans* was mentioned only in China (Holliday, 1980; Gupta and Gupta, 1992; Zeng *et al.*, 2005). In Thailand, *P. palmivora* and *P. botryosa* are common (Johston, 1989). Species in Pythiaceae produce different types of spores to complete their life cycle (Drenth, 1994). In asexual stage, sporangia or zoosporangia are produced and

germinated through plant's stomata and released motile zoospores which responsible for primary infection and disease outbreak in rainy season (Drenth, 1994; Ristaino and Gumpertz, 2000). Other asexual spore is chlamydospore. This type of spore is responsible for long term survival in stress condition as same as oospores in sexual cycle (Erwin and Ribeiro, 1996b). Chlamydospore survives in dead plants and soil for many years (Drenth and Guest, 2004a). In optimum condition, oospore germinates to form sporangium completing its life cycle by repeating asexual stage and infects other plants (Nowicki *et al.*, 2011). To control rubber diseases caused by species in Pythiaceae, prophylactic fungicidal spraying on rubber plants is the normal practice (Priyadarshan, 2011). However, the fungal pathogens showed resistance development to the fungicide (Cohen and Coffey, 1986). ALF and black stripe diseases caused by species in Pythiaceae may reduce field latex yield by up to 30-50% (Chee, 1969a).

Mycovirus is the virus that infects fungi and has been commonly found in all major taxonomic groups of fungi (Nuss, 2005; Ran *et al.*, 2016). Mycovirus with either an RNA or a DNA genome have been reported (King *et al.*, 2011). Most mycoviruses have the genomes of double-stranded RNA (dsRNA) or positive single-stranded RNA (ssRNA) (Pearson *et al.*, 2009). Only two mycovirus species have a ssDNA and a dsDNA genome, respectively (Yu *et al.*, 2010; Kraberger *et al.*, 2013). The characteristic type of mycovirus is their transmission route. Transmission mode of mycovirus has been known to lack extracellular transmission and limited to hyphal fusion (anastomosis) and sporulation (Son *et al.*, 2015). There has been only an exception in a ssDNA virus found in the plant fungus, *Sclerotinia scelerotiorum* causing rot disease in rapeseed which showed extracellular transmission when virus-free strain could be converted to virus-infected strain after inoculation of intact hyphae with viral particles (Yu *et al.*, 2010, 2013). Almost all mycoviruses live quietly without interfering with their host's phenotype but some may be hypovirulence or hypervirulence (Pearson *et al.*, 2009). Hypovirulence refers to mycoviruses that reduce virulence of their fungal hosts, while hypervirulence means mycoviruses that enhance virulence of their hosts (Nuss, 2005; Ghabrial and Suzuki, 2009). Introduction of *Cryphonectria* hypoviruses in field condition showed a successful

control of chestnut disease in Europe (Dawe and Nuss, 2001). This hypovirulence study stimulated the search for other mycoviruses which hold a potential to be used as natural fungicides (Ghabrial *et al*., 2015). In case of hypervirulence, the hyperviruses were reported associated with changing in some characteristics of their fungal hosts including abundant sporulation, increased radial growth, interference with signal-transduction, and increased disease incidence and virulence (Ahn and Lee, 2001; Özkan and Coutts, 2015; Kotta-Loizou and Coutts, 2017). The study of fungal phenotypic changes caused by mycoviruses may reveal more information about different traits of pathogenicity for understanding of mechanisms of fungal virulence (Ahn and Lee, 2001).

This study aims to isolate extrachromosomal DNA and dsRNA elements which are expected to be a mycovirus from Pythiaceae causing ALF disease in rubber tree from plantations in eastern Thailand, to study some phenotypic changes of the host, and to identify the genome of those elements and the host. These may provide the basic knowledge of virus in Pythiaceae for the further study.

CHAPTER II
LITERATURE REVIEW

2.1 Oomycetes (Pythiaceae)

Oomycota or oomycetes are often referred to as water molds (van den Berg *et al*., 2013). Based on morphology, oomycetes were once sorted in the group of fungi because of their typical filamentous growth but have been taxonomically classified later in the eukaryote supergroup Chromalveolata together with brown algae and diatoms based on molecular, biochemical and ultrastructure studies (Sogin and Silberman, 1998; Baldauf *et al*., 2000; Cai *et al*., 2009; Shen *et al*., 2011). Major characteristics that differentiate oomycetes from true fungi are aseptate (coenocytic) diploid vegetative hyphae, cell wall containing cellulose and β-glucans, and biflagellated motile asexual spores, whereas characteristics of true fungi are usually haploid vegetative hyphae or dikaryotic vegetative hyphae, presence of chitin in cell wall and non-motile spores except in chytrids (Cai *et al*., 2009).

The important destructive plant-pathogen oomycetes are members in the family Pythiaceae, including genus *Phytophthora* (*P*.), *Pythium* (*Py*.) and *Phytopythium* (*Phy*.) (Drenth and Guest, 2004a; Baten *et al*., 2014; de Cock *et al*., 2015; Jankowiak *et al*., 2015). About 60 described species in the genus *Phytophthora* are pathogens of over 1,000 plant species (Drenth and Guest, 2004b). Approximately 80 species of *Pythium* have been described as an important plant pathogen and were reported worldwide distribution (Matsumoto *et al*., 1999). As a plant pathogen, however, *Pythium* is a weaker parasite and more saprophytic than *Phytophthora* (Erwin and Ribeiro, 1996c). Some species of *Pythium* were found living as parasites in *Phytophthora* (Larousse and Galiana, 2017). *Phytopythium* is a new genus in family Pythiaceae reviewed by Bala and colleagues (2010). It was first identified as the genus *Pythium* but was classified later using molecular-based study that supported a separate phylogenetic relationship of *Phytopythium* and *Pythium* (de Cock *et al*., 2015).

A general life cycle of the species in the family Pythiaceae is shown in Figure 2.1. Life cycle of *Phytophthora* involves asexual and sexual stages and produces several types of spores to complete the cycle. In asexual stages, at low temperature, sporangiophores germinate to sporangium or zoosporangium from the plant's stomata and release zoospores, which are biflagellated motile and responsible for primary infection (Drenth, 1994; Ristaino and Gumpertz, 2000). At higher temperature, direct germination of sporangium to hyphae occurs and penetrates through a host cell beside stomatal guard cells. After attachment of a zoospore to a new host, cyst is formed and a germtube with appressoria to infect host plants germinated (Drenth, 1994). In many *Phytophthora* species, resting asexual chlamydospores with thick-walled are produced for long term survival (Drenth and Guest, 2004b; Judelson and Blanco, 2005). For sexual stage, members in the genus *Phytophthora* were recorded both homothallic and/or heterothallic (Erwin and Ribeiro, 1996c). Homothallic refers to self-fertilize strain, which can produce oogonia and oospores in the same culture without seeking a mate, while heterothallic produces oospores only after mating between different mating-type strains (Erwin and Ribeiro, 1996a; Coppin *et al*., 1997; Alby *et al*., 2009).

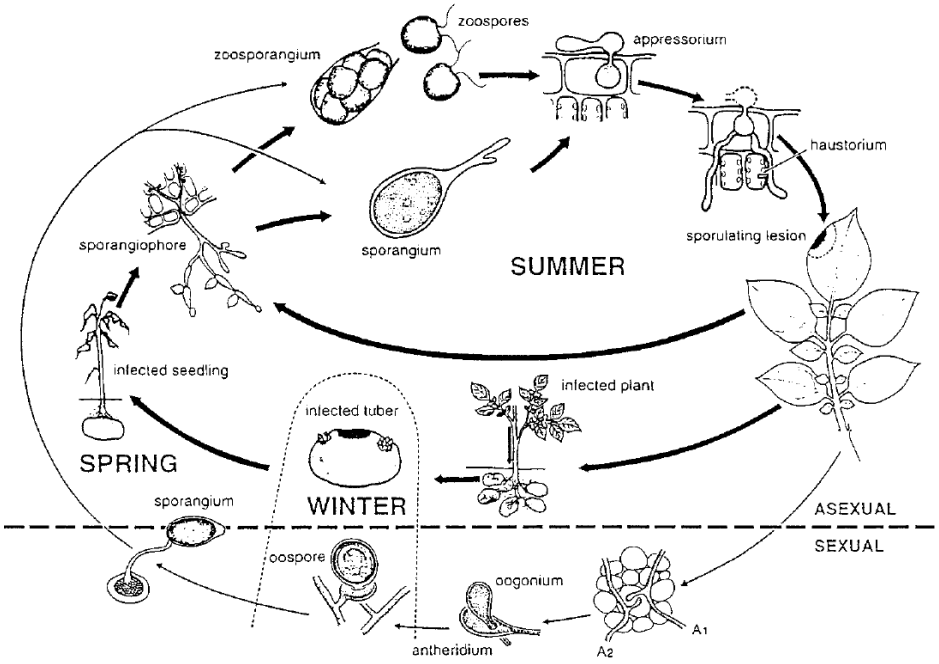

Figure 2.1 Disease cycle of *Phytophthora infestans* (Drenth, 1994)

Different types of spores play important roles in dispersal or resting functions (Judelson and Blanco, 2005). Zoospores are specialized in dispersal but its survival time usually less than 24 hours and may travel several kilometers (Aylor, 2003; Kong and Hong, 2014). One sporangium produces 8-32 zoospores (Drenth and Sendall, 2001). In air and waterborne species, sporangium shows caducous or deciduous characteristic (Garbelotto and Hayden, 2012). Caducous means sporangium can be sheded from stalk making dispersion in wind-driven rain easy (Erwin and Ribeiro, 1996c; Ristaino and Gumpertz, 2000). Chlamydospores are present in unsuitable environmental conditions for survival by some species (McCarren *et al.*, 2005). These dormant thick-wall spores are separated from hypha by a septum and function as resting spores (Drenth and Sendall, 2001). Oosprores produced in sexual stage are also the resting structure. These two resting spores can survive in stress conditions but usually remain in their habitat (Judelson and Blanco, 2005). Chlamydospores may survive for 6 years, while oospores may remain viable for 13 years (Erwin and Ribeiro, 1996c).

2.1.1 Morphological characteristics of Pythiaceae

1) Mycelium

Hyaline-branched and coenocytic (nonseptate) are typical characteristics of hypha but septa can be seen in old cultures (Erwin and Ribeiro, 1996c). Mycelium may be aerial or appressed in the medium surface (Jeffers, 2016). Diameter and appearance of the mycelium depend on the chemical property of the medium, rendering mycelium characteristics not useful enough as criteria to differentiate species (Erwin and Ribeiro, 1996c; Kotov *et al.*, 2005).

2) Colony patterns

Colony types are variable in different species and on different media (Erwin and Ribeiro, 1996c; Jung *et al.*, 2011). Four types of colony patterns are common. Petaloid shows narrow sectors like a chrysanthemum. Rosaceous is broadly rounded sectors. Stellate type refers to the colony radiates like a star. Cottony is a densely fluffy colony (Figure 2.2) (Erwin, 1965; Pratt and Mitchell, 1973; Waterhouse *et al.*, 1983;

Stack and Millar, 1985; Erwin and Li, 1986). Colony pattern of the same species may be varied on different media as shown in Figure 2.3. Caten and Jinks (1968) found that subcultures of *P. infestans* using single hyphal tips or single sporangium as inoculums gave more uniform of colony morphology than single zoospore cultures. Asexual reproduction via hyphal fragmentation is different from zoospores in that single zoospore is uninucleate homokaryon but coenocytic hypha is multinucleate heterokaryon, making phenotype of progeny from zoospore germination a homozygous trait (Dick, 1972). These may be concluded that variation of colony pattern in the same species is under cytoplasmic control (Caten and Jinks, 1968). Patterns of colony types depend on angle, frequency and extent of hypha branching (Waterhouse, 1970).

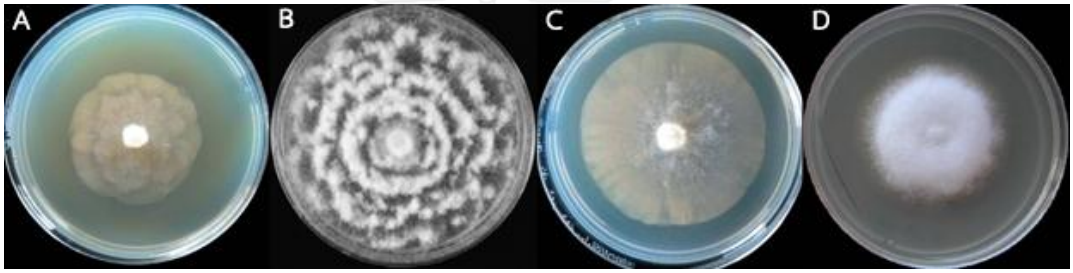

Figure 2.2 Typical colony patterns of Pythiaceae.(A) Petallate colony of *Phytophthora litoralis* on malt extract agar (Jung *et al*., 2011). (B) Rosaceous colony of *Phytophthora cinnamomi* on potato dextrose agar (Eggers *et al*., 2012). (C) Stellate colony of *Phytophthora litoralis* on V8 agar (Jung *et al*., 2011). (D) Cottony colony of *Phytophthora austrocedrae* on corn meal agar supplemented with β-sitosterol (Greslebin *et al*., 2007).

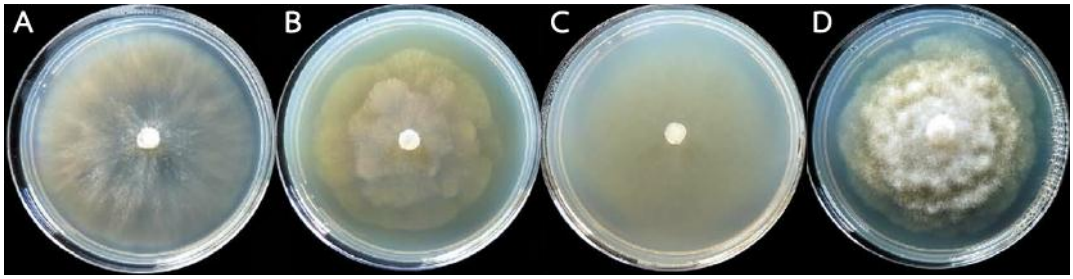

Figure 2.3 Different colony patterns of *Phytophthora megasperma* on different media. (A) Stellate colony on V8 agar. (B) Petallate colony on malt extract agar. (C) Uniform submerged colony on corn meal agar. (D) Rosaceous-cottony colony on potato dextrose agar (Jung *et al*., 2011).

Sporangium morphology is a useful feature for species differentiation (Drenth and Sendall, 2001). Important terms of sporangium characteristics are:

• Sporangiophore branching

Stalks of sporangium are sporangiophores (Drenth and Sendall, 2001). Patterns of sporangiophore branching are shown in Figure 2.4. Simple sympodium occurs when a new sporangium was stalked form the same base of the previous sporangium. Compound sympodium is shed sporangia by branched sporangiophore from which the hyphae initiate. An invert umbrella-like cluster of sporangia on the long sporangiophores is the umbel type (Erwin and Ribeiro, 1996c).

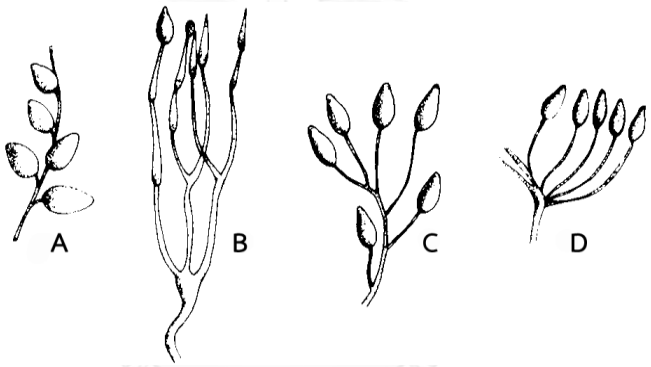

Figure 2.4 Types of sporangiophore branching. (A) Simple sympodium. (B) and (C) Compound sympodium. (D) Umbel type. (Erwin and Ribeiro, 1996c)

• Sporangium shape

Sporangium shapes are usually classified as ellipsoid, limoniform, obovoid, obpyriform, obturbinate, ovoid, pyriform, or subspherical (Erwin and Ribeiro, 1996b). Different shapes of sporangia are shown in Figure 2.5. Some Pythiaceae species do not produce sporangium in homogeneous shape and size such as *P. nicotianae*, *Py. perplexum* and *Phy. aichiense* (Erwin and Ribeiro, 1996d; Galland and Paul, 2001; Baten *et al.*, 2015). Environmental conditions required for sporangium production were reported to be various in different species, some produce sporangium only on agar-based medium while some require incubation in aqueous solutions (Erwin and Ribeiro, 1996c). Effect of light, atmospheric oxygen, presence of cations and co-

incubation with bacteria are also associated with formation of sporangium (Ayers and Zentmyer, 1971; Duniway, 1975; Halsall and Forrester, 1977; Ribeiro, 1983).

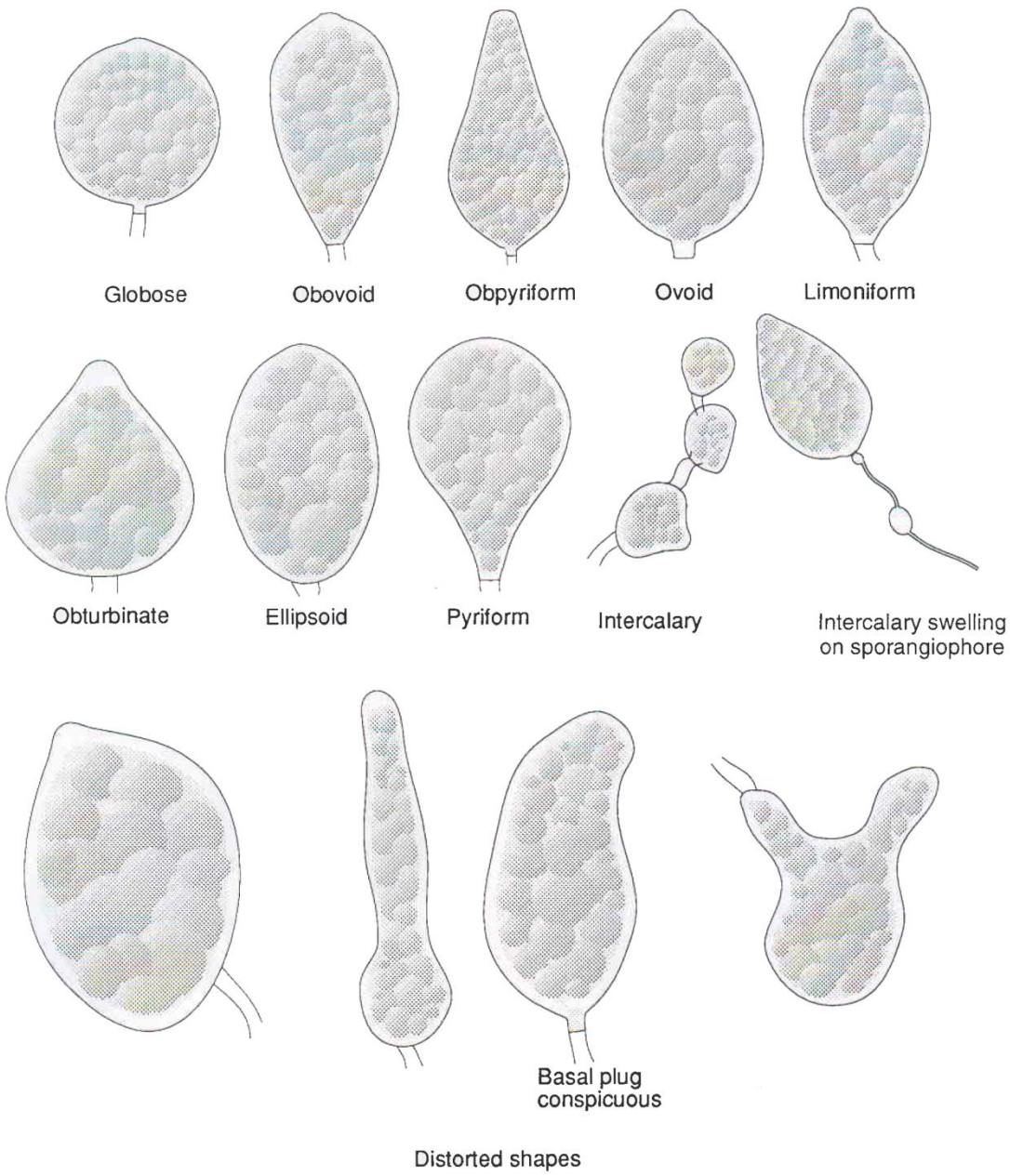

Figure 2.5 Different shapes of sporangia. (Erwin and Ribeiro, 1996c)

• Papillation

Papilla is the apical thickening feature on the sporangium. Three terms of papillation are classified (Figure 2.6). Papillate sporangium shows a nipplelike prominence about 3-5 μm of the apical thickening. Semipapillate sporangium has a shallow thickening less than 3 μm. Nonpapillate sporangium implies a sporangium without a clear area between protoplast and outer wall at the tip (Chapman and Vujicic, 1965; Erwin and Ribeiro, 1996c).

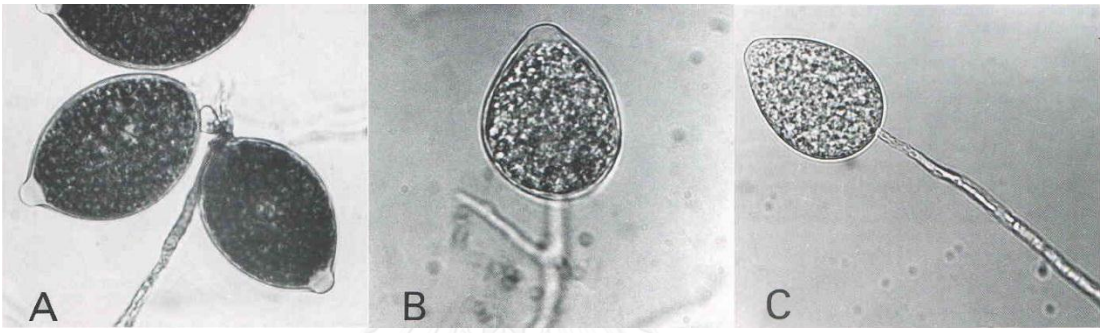

Figure 2.6 Papillation characteristic of sporangium. (A) Papillate sporangium. (B) Semi-papillate sporangium. (C) Non-papillate sporangium. (Erwin and Ribeiro, 1996c)

• Caducity

Detachment of sporangium from sporangiophore defines as caducity (Beales, 2012). Non-caducous is the undetachable sporangium. Caducous sporangium is shed from hypha and left with a stalk or a pedicel when it is flooded with water as shown in Figure 2.7 (Kellam and Zentmyer, 1985; Yang *et al.*, 2016). All caducous species are waterborne plant pathogens (Erwin and Ribeiro, 1996c).

• Pedicel

The stalk of caducous sporangium is called pedicel (Figure 2.7A) (Surujdeo-Maharaj *et al.*, 2016). The length of pedicel varies among different species and can be divided into 3 categories. Short pedicel is less than 5 μm in length. Intermediate is in the range between 5-20 μm, while long pedicel is more than 20 μm in length (Erwin and Ribeiro, 1996c).

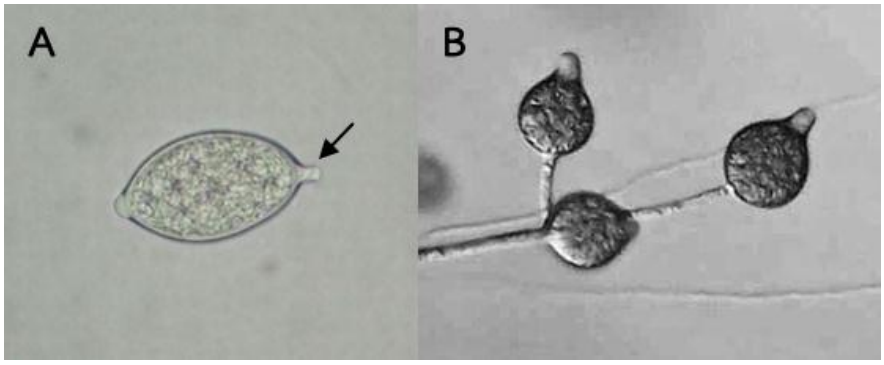

Figure 2.7 Caducity of sporangium. (A) Caducous sporangium of *Phytophthora palmivora* (Cating *et al*., 2010). (B) Non-caducous sporangia of *Phytopythium delawarense* (Broders *et al*., 2009). Arrow: pedicel.

4) Chlamydospores

The presence of chlamydospores is a unique characteristic in various species in Pythiaceae. The presence or absence of chlamydospore may be a useful criterion for identification of species but it may be limited because the shape does not vary enough (Waterhouse *et al*., 1983; Erwin and Ribeiro, 1996c). Morphology of chlamydospore can be seen in globose, irregular, or oblong.Chlamydospore can be distinguished from sporangia by wall thickness.Chlamydospore wall is thicker than sporangia wall (Erwin and Ribeiro, 1996c). Typical chlamydospore morphology is shown in Figure 2.8.

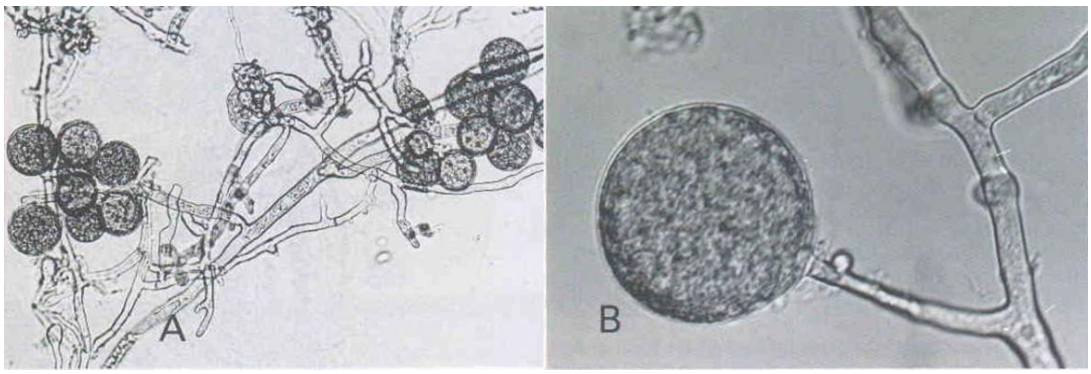

Figure 2.8 Chlamydospores of *Phytophthora cinnamomi*. (A) Chlamydospores and hyphal swelling. (B) Wall of chlamydospore. (Erwin and Ribeiro, 1996c)

5) Sex organs

Oogonium is a female structure whereas antheridium is a male structure (Erwin and Ribeiro, 1996c). The important appearance for species identification is the attach position of antheridium to oogonium. Amphigynous antheridium surrounds the oogonial stalk, and paragynous is the antheridium which attaches to the side of oogonium (Figure 2.9) (Ho, 1983). Homothallic or heterothallic is also used as the criterion for species identity (Drenth and Sendall, 2001).

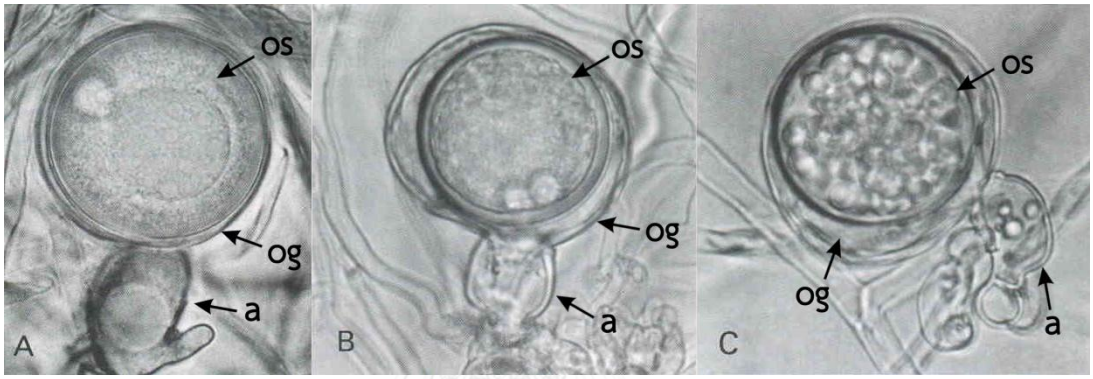

Figure 2.9 Morphology of antheridium (a), oogonium (og) and oospores (os). (A) and (B) Amphigynous antheridium on a mature oogonium. (C) Paragynous antheridium on oogonium. (Erwin and Ribeiro, 1996c)

6) Comparison of the characteristic of the genus in Pythiaceae

The two traditional genus, *Phytophthora* and *Pythium* are similar in that both produce aseptate hyphae, oospores, and are diploid in their vegetative stages but they can be differentiated by some characteristics as described in Table 2.1.

Table 2.1 Difference characteristics between *Phytophthora* and *Pythium* (adapted from Erwin and Riberio (1996c) and Bala and colleagues (2010))

Characteristic	*Phytophthora*	*Pythium*
Asexual spore		
Chlamydospore	Presence	Presence
Sporangium		
Shape	Usually ovoid or obpyriform; rarely globose.	Vary; filamentous, spherical, occasionally ovoid or pyrifrom.
Papilla	Presence	Often non-papillate.
Caducity	Caducous or non-caducous.	Non-caducous.
Zoospore discharge	Zoospores release through the apical opening without forming a discharge tube.	Zoospores discharge through a discharge tube and vesicle.
Sexual structure		
Antheridia	Amphigynous or paragynous.	Paragynous.
Oogonia	Pigmented; smooth or ornamented wall.	Colorless; smooth or spiny wall.
Hyphal swelling	Presence	Rare
Hymexazol resistance	Some species aren't inhibited.	Many species are inhibited.
Host specificity	Specific or wide host range.	Numerous host or rarely host specific.

The morphological types of the genus *Phytopythium* had not been clarified clearly. Only some mismatch characteristics between the genus and the traditional two genera were described. According to the first mention of the genus *Phytopythium* by Bala and colleagues (2010), with *Phy. sindhum* as a type species, mode of zoospores discharge of *Phytopythium* is *Pythium*-like, while sporangia papillation is *Phytophthora*-like.

2.1.2 Isolation and detection of Pythiaceae

Pythiaceae can be isolated from soil or plant tissue (Erwin and Ribeiro, 1996b; Baten *et al*., 2014). Oospores and chlamydospores are dormant spores in soil or plant host and germination occur in optimal conditions (Ayers and Lumsden, 1975; McCarren *et al*., 2005).The soil dilution plate technique has been used for many years (Johnson and Curl, 1972).Erwin and Riberiro (1996b) noted that *Phytophthora* isolation from recently infected plant tissue is the most successful method and most effective in an active stage of infection. Necrotic tissue is not suitable for *Phytophthora* isolation due to the absence of active mycelium and the suppressive effect of antagonistic bacteria. The diseased plant tissue should be surface sterile before plating on a selective medium (Drenth and Sendall, 2001). Surface sterile agents can be running water, 0.5% sodium hypochlorite, 2 ppm chlorine or fresh 70% ethanol with a proper contact time (Erwin and Ribeiro, 1996b). Baiting method has been also used both in soil and plant tissues as the original inoculums (Eden *et al*., 2000; Waller, 2001). This method was established before the arrival of selective media and was found to induce sporangia production by trapping zoospores with the bait (Cooke *et al*., 2007). Once *Phytopthora* produces sporangium, zoospores are released and swim to the baits by chemotactical attraction before the infected bait is then transfer to a medium for *Phytophthora* amplification (Erwin and Ribeiro, 1996b).

The use of chemical in the medium for selective isolation from cohabit bacteria and fungi has been developed. The commonly used amendments and their properties are described in Table 2.2.

Table 2.2 Example of selective chemicals for Pythiaceae isolation and their properties (adapted from Erwin and Riberiro (1996b))

Chemical	Properties
Ampicillin	Antibiotic with bactericidal activity; inhibit Gram-positive bacteria.
Rifampicin (Rifamycin)	Antibiotic; inhibit mycobacteria and Gram-negative bacteria; light sensitive.
Streptomycin sulfate	Antibiotic; inhibit many Gram-positive and some Gram-positive bacteria; toxic to some *Phytophthora* spp.
Rose bengal	Toxic to most bacteria and some fungi; reduce growth and toxic to some *Phytophthora* spp.
Pimaricin (Devocid)	Antibiotic; inhibit most fungi except Pythiaceae and *Mortierella*; light sensitive.
Pentachloronitrobenzene (PCNB, Terraclor)	Fungicide; inhibit growth of many fungi but not oomycetes.
Hymexazol (Tachigaren)	Fungicide; inhibit most *Pythium* and *Mortierella*; at concentration lower than 50 ug/ml does not toxic to many *Phytophthora* spp.; light sensitive.

2.1.3 Pythiaceae identification

Because of the similarity in morphological characteristics between the two genus, this makes it difficult to differentiate Pythiaceae in species level, making molecular approach using Polymerase Chain Reaction (PCR) technique and sequencing analysis becomes the important tools to improve the accuracy of identification (Erwin and Ribeiro, 1996c; Kong *et al*., 2003; Martin *et al*., 2004). Genetic markers have been developed in both genus and species levels. The genus and some species specific primer pairs and their target genes are listed in Table 2.3.

Table 2.3 Diagnostic markers and primers for identification of Pythiaceae by conventional PCR

Level of specificity	Target gene	Primer set (Forward/Reverse)	Reference
Genus			
Phytophthora	Internal transcribed spacer region of rDNA (ITS)	A2/I2,	Drenth *et al*., 2006
		FITS_15Ph/RITS_279Ph	Kox *et al*., 2007
	ITS1 - 28s rDNA	Phy1s/ Phy2a	Tsai *et al*., 2006
	Ras protein Ypt1 gene	Yph1F/Yph2R	Schena *et al*., 2008
	Cytochrome oxidase (COX)	Phy-8b/Phy-10b,	Martin *et al*., 2004
		FM75/FM78	Martin and Tooley., 2003
Pythium	COX	FM66/FM58	Martin, 2000
Species			
P. capsici	ITS1-5.8s	PC-1/PC-2	Zhang *et al*., 2006
P. citrophthora	ITS2	Pc2B/Pc7	Ippolito *et al*., 2002
P. nicotianae	Elicitin ParA1 gene	IL7/IL8,	Lacourt and Duncan, 1997
	ITS2	Pn5B/Pn6	Ippolito *et al*., 2002
P. palmivora	ITS1-5.8s-ITS2	Pal1s/Pal2a	Tsai *et al*., 2006

2.2 Para rubber tree and rubber diseases caused by Pythiaceae

2.2.1 Para rubber tree (*Hevea brasiliensis*)

According to statistic data recorded by Office of Agricultural Economics (OAE) (2015), Thailand was placed second on rubber harvested areas but was ranked first in the world's exporter, 37.2% of global production, followed by Indonesia, 28.4% of global production and Malaysia, 13.3%of total global production. In 2015, amounting to 4.46 million tons of natural rubber was exported by Thailand totaled 194 billion baht. This makes natural rubber the major agricultural product of Thailand in term of income, which is higher than rice and rice product's value of export since 2002 (OAE, 2015). China, USA and India are major natural rubber consumers (Malaysian Rubber Export Promotion Council, 2017). Demand in natural rubber consumes in China and India has been increased because of automotive industry and economic expansion (Warren-Thomas *et al*., 2015). The collected latex or field latex from rubber tree is a raw material for the manufacture of various products. The main use of natural rubber is the basic constituent of the tires used in automotive vehicles (Rose and Steinbuchel, 2005). Other latex products are medical or household products such as gloves, condoms, conveyor belts, door or window seals, hoses, etc (Nair, 2010).

The plant genus *Hevea* is one of the ten species of the family Euphorbiaceae, from all 10 species in this family, *H. brasiliensis* Muell. Arg. or rubber tree is the only one cultivated commercially (Webster and Paardekooper, 1989). Rubber tree is an important economic plant for natural rubber production. It was initially cultivated in Amazon basin and Peru, South America in sixteenth century before it was botanical described and classified in the plant genus *Hevea* in 1775 (Korwutthikulrangsri, 1997; Nair, 2010; Blagodatsky *et al*., 2016).Vulcanization of natural rubber developed in 1839, led to high demand in rubber tree cultivation for latex production (Nandris *et al*., 1987). The seedlings were introduced to England, India and Sri Lunka but planting in these regions were not successful. Seedlings from Sri Lanka were sent to Singapore and Malaysia in 1877 and the planting was accomplished, this was marked as the first rubber cultivation in South East Asia (Korwutthikulrangsri, 1997; Rivano *et al*., 2015). Rubber plantations have been

expanded in South East Asia, making this tropical area the major world natural rubber production due to the optimal conditions for rubber tree plantation, which is in the humid tropics (Raj *et al.*, 2005; Rivano *et al.*, 2015).Rubber tree requires warm temperature around 25-28°C, rainfall 100-150 days evenly distributed over the course of the year, 2000–4000 mm of annual precipitation and high humidity ranging between 67-82%for high rubber yield (Priyadarshan, 2003; Yu *et al.*, 2014).For physical factors, soil should be well-drained (Priyadarshan and Clement-Demange, 2004). Plant nutrients in soil are also considered. Nutrition status should be medium to high (Watson, 1989). Rubber tree needs macronutrients like other plants and trace elements for micronutrient (Oku *et al.*, 2012). The excess trace minerals is prohibited because some elements can be toxic to the plant (Kabata-Pendias, 1995). For good cultivation practice, the plantation area should be kept free of rotting plant debris (Mohammed *et al.*, 2014).

In Thailand, the first rubber plantation was established in Trang province, Southern region around 1899-1901, using seedlings from Malaysia (Rantala, 2006). After 10 years of the first cultivation, rubber plantations were expanded through other southern areas and were introduced to Chanthaburi province in the east (LDD, 2005). Nowadays, not only traditional orchards are in the south and the east, rubber farms also spread throughout the north and the northeast regions, the sub-optimal area for supplying an increased demand of natural rubber (Chantuma *et al.*, 2012). The largest planted area is approximately 2.35 million hectare (ha)in the southern region, followed by 754,000 ha in northeast, 427,000 ha in the central and eastern region, and 194,000 ha in the northern region but latex yield per ha was found largest form the southern, the central and eastern, the northeast and the northern, respectively (OAE, 2015). Since climatic factor is the most important key for high latex yield, the sub-optimal area affects the rubber yield due to high altitude, low temperature and dry seasons, making the sub-optimal plantations prone to lower latex production than the traditional areas (Rodigo, 2007; Clermont-Dauphin *et al.*, 2013). Moreover, field latex yield also depends on rubber bleeding (Yip, 1990). Rubber Research Institute of Thailand (RRIT)has been researched in rubber breeding and genetic improvement of rubber clones. Rubber clones recommended by RRIT for high latex yield

only are RRIT 226, RRIT 251, RRIT 408, BPM 24 and RRIM 600. These well characterized clones were approved for large scale planting and confirmed to produce high rubber yield in optimal environment (RAOT, 2011). Almost 75% of planted rubber clone in Thailand is RRIM 600 (Pethin *et al.*, 2015). Rubber clone RRIM 600 was reported to present low initial yield but this clone has long tapping period, and was found to produce highest latex yield over along tapping age (Department of Agriculture (Thailand),1986). Rubber tree lives normally for over 100 years and can be tapped for latex at the age of six years (Anekchai, 1986; Webster and Paardekooper, 1989). Economic life span of rubber tree in plantation is more than 30 years before the latex production becomes lower (Nair, 2010).

2.2.2 Rubber diseases caused by Pythiaceae

Due to the optimal climate for latex production is in the tropical lowland with high rainfall, this condition can lead to the development of various diseases and may be affected tapping process (Watson, 1989). The *Hevea* rubber is affected by number of diseases throughout its parts including leaf, stem, panel and leaf, mostly caused by fungi (Wastie, 1975; Sunpapao and Pornsuriya, 2014). Pythiaceae attack leaf and panel of rubber tree.*P. palmivora*, *P. botryosa*, *P. capsici*, *P. citrophthora*, *P. nicotianae* and *P. meadii* are the most important, causing abnormal leaf fall (ALF) disease. Panel diseases are caused by *P. palmivora*, *P. botryosa* and *P. meadii*, and *Py. vexans* (Holliday, 1980; Gupta and Gupta, 1992; Zeng *et al.*, 2005).

According to rubber clones recommended by RRIT for large scale planting, there is variation in susceptibility among rubber clones to various diseases as shown in Table 2.4.

Table 2.4 Disease susceptibility of different rubber clones (RRIT, 2012)

Rubber clone	Disease resistance[a]	
	ALF	Black stripe
RRIT 226	3	3
RRIT 251	3	2
RRIT 408	2	3
BPM 24	2	2
RRIM 600	5	4

[a] 1 = excellent, 2 = good, 3 = fair, 4 = poor, 5 = bad

Since the high latex yield producing clone has been RRIM 600, this clone is a major planted rubber in Thailand, about 75% of all farms. Moreover, this clone shows high susceptiblity to many diseases and has been informed that it should not be cultivated in high risk area (RAOT, 2011; Pethin *et al*., 2015). ALF and Black stripe diseases are abundantly spread in the south and the east; especially Chanthaburi and Trat province in the east, have been recorded severe *Phytophthora* infection (RAOT, 2016).

1) Abnormal leaf fall disease

Abnormal leaf fall in rubber reported worldwide is caused by 6 species of *Phytophthora*, including *P. palmivora*, *P. botryosa*, *P. capsici*, *P. citrophthora*, *P. nicotianae* and *P. meadii*. Of these, *P. palmivora* is the most common (Edathil and George, 1976; Johston, 1989). Geographical distribution of each species is showed in Table 2.5.

Table 2.5 Pythiaceae species associated with Abnormal leaf fall disease in rubber and geographical distribution (Johston, 1989; Erwin and Ribeiro, 1996d; Zeng *et al*., 2005; Laohasakul *et al*., 2017)

Species	Country
P. botryosa	Asia; Malaysia, Thailand and Vietnam
P. capsici	Asia; China
	South America; Brazil
P. citrophthora	Africa; Ivory Coast
	Asia; China, Indonesia and Thailand
	South America; Brazil
P. meadii	Africa; Cameroon, Congo, Ghana, Liberia and Nigeria
	Asia; Cambodia, India, Indonesia, Malaysia, Myanmar, Sri Lanka, Thailand and Vietnam
	North America; Costa Rica and Nicaragua
	South America; Brazil and Peru
P. nicotianae	Asia; China and India
P. palmivora	Africa; Cameroon, Congo, Liberia and Uganda
	Asia; China, India, Indonesia, Malaysia, Myanmar, Philippines, Thailand and Sri Lanka
	North America; Costa Rica
	South America; Brazil
	South Pacific Ocean; Fiji Papua and New Guinea

Leaf fall disease in rubber tree is closely linked with pod rot. Infection begins on young pods, when the infected pod turns mature, it is an ideal substrate for the pathogen to grow and produce spores (Liyanage, 1985). Once the pod is infected, it turns black and remains on the tree (Sdoodee, 2004). During rainy season, the dead pods are covered by germinated white mycelium and sporangium with zoospores inside which can be released and washed down to cause infection of leaf at petiole (Liyanage, 1985).

Infected young foliage and mature leaves were found remaining green and appear healthy while shedding and still attach to leaf stalks (Liyanage, 1985; Sdoodee, 2004). The typical symptoms of shed leaves caused by *Phytophthora* infection is the presence of dark-brown bruising lesion with coagulated latex on the lesion at the petoles, this symptom can be distinguished from wintering or annual shedding of rubber leaves in dry or winter weather which occurs in a short time and refoliation is rapidly completed. In area where refoliation is not completed before the arrival of rainfall period, leaf disease can invade the young foliage and cause secondary leaf fall (Webster and Paardekooper, 1989; Sunpapao and Pornsuriya, 2014). Defoliation of rubber tree may reduce field latex yield by up to 30-50% (Chee, 1969a). Girth expansion needs photosynthesis that takes place on the leaves. If the source of photosynthesis decreases, it leads to reduce in latex yield and wood production as well (Webster and Paardekooper, 1989). After infection, *Phytophthora* produces different types of spores, zoospores are produced in large scale in the wet season and spread to another parts of the tree, especially on the tapping bark and cause black stripe disease and also spread to neighboring trees by rain splash while chlamydospores and oospores are produced and remained in shoots, pods and barks as resting stage for long term survival in dry season (Johston, 1989).

2) Black stripe disease

Black stripe disease in tapped rubber tree has been also known as bark rot, cambium rot, black thread, black line or stripe canker (Holliday, 1980; Johston, 1989). Pathogen of this disease is the species of AFL with *P. palmivora* as the most common but in Thailand *P. botryosa* is the most common agent (Johston, 1989). There was a reported claimed that *Py. vexans* also causes strip canker disease in rubber tree in plantation in China (Zeng *et al*., 2005). The first sign of infection presents above the tapping cut at the bark (Liyanage, 1985). The vertical dark grey line is observed. In severe infection, pathogen invades vessel before the barks peel away and the black lines are seen. The dark lines also deeply embed into the wood and spread to untapped panel (Holliday, 1980; Sdoodee, 2004). If left untreated, it causes irregular regeneration of the

bark and becomes the dead tissue, and latex exudation was found, making difficult or impossible to tap again and tapping age of the tree is reduced for 8-16 years (Deetae, 1986; Johston, 1989).

To manage black stripe and abnormal leaf fall diseases in rubber tree, prophylactic fungicidal spraying on rubber plants is the normal practice (Priyadarshan, 2011). Management practices are the following;

• Planting the resistant seedling clones are recommended for cultivation in new plantation (RAOT, 2011).

• In rubber seedling younger than 2 years, Difolatan ($C_{10}H_9Cl_4NO_2S$), metalaxyl ($C_{15}H_{21}NO_4$) or fosetyl aluminium ($C_6H_{18}AlO_9P_3$) fungicides are used by spraying on the bush every week during the occurrence of leaf fall disease (Deetae, 1986; RRIT, 2012).

• For mature trees with severe leaf fall, aerial spraying with helicopters is developed in the large plantation (Johston, 1989). Bordeaux mixture ($CuSO_4$and $Ca(OH)_2$) is usually applied with addition of zinc sulfate (Priyadarshan, 2011). Copper oxychloride ($Cu_2(OH)_3Cl$) in mineral oil can be aerial or ground sprayed, it is an alternative to Bordeaux (Radhakrishna and George, 1973; Omorusi *et al*., 2014).

• To protect the healthy tapping cut from *Phytophthora* infection, always apply the tapping cut with the fungicide after tapping (Deetae, 1986).

• For black stripe infected tree, early detection of the disease is more effective. Treatment begins with removing of the infected bark by knife and then apply fungicide Difolatan, metalaxyl or fosetyl aluminium at the tapping cut within 24 hours after the bark is removed (Deetae, 1986).

Bordeaux fungicide adheres well to leaves but has some disadvantages. Bordeaux is easily washed off by heavy rainfall especially on monsoon period in the tropical areas. This can be improved by using oil-mixed fungicides. Both Bordeaux and

copper oxychloride have copper (Cu), an active ingredient. It serves as a necessary cofactor of enzyme for normal plant growth (Drenth and Guest, 2004b; Palma *et al*., 2013). However, using copper-based fungicides for a long period leads to long-term accumulation of copper in soils. If copper concentration in soils is higher than the amount of trace elements that the plant requires for healthy growth, it can worsen the growth and productivity of the plant (Brunetto *et al*., 2016). Plant roots can be the first target for copper-accumulated damage (De Vos *et al*., 1991). Sheldon and Menzies (2005) reported excess copper accumulation resulting in damage to the root cuticles, and affect to root proliferation by reduction in the length and numbers of root hairs.

Metalaxyl fungicide is widely used for controlling *Phytophthora* diseases (Drenth and Guest, 2004b). There are disadvantages of using metalaxyl. This fungicide is rapidly degraded by soil microorganisms (Guest *et al*., 2010). In case of non-degradation, metalaxyl is stable in soil and water. Continued use of metalaxyl in agriculture leads to groundwater contamination (Sukul and Spiteller, 2000). Moreover, various *Phytophthora* species showed resistance development to the fungicide (Cohen and Coffey, 1986).

Fosetyl aluminium has been classified as a Class C, possibly carcinogenic to humans by US Environmental Protection Agency with toxicity evidence in animal research but little or no human data (Cheremisinoff and Rosenfeld, 2011).

2.3 Mycovirus

Mycovirus is a virus that infects fungi and oomycetes (Ran *et al*., 2016). Mycoviruses require the living cells of other organisms to be their host for replication like animal and plant viruses but have some unique characteristics. Mycoviruses lack an essential movement protein to complete life cycle, and mode of transmission of most mycoviruses is generally limited to intracellular routes by vertically transferred via sporulation and cell division or by horizontally transferred by protoplasmic fusion but lack extracellular phase of transmission (Son *et al*., 2015). The origin of mycoviruses still remains a mystery. Two major hypotheses have been used to explain the origin of mycovirus. The first one is the ancient coevolution hypothesis. It is based on the proposals

that mycoviruses lack extracellular infectivity, take advantage of fungal protein to multiply and live quietly in their host (Ghabrial, 1998; Pearson *et al*., 2009). This indicates that asymptom relationship between mycovirus and host is long-term coevolution (Son *et al*., 2015). The latter one is plant virus hypothesis. Koonin and colleagues (1991) compared polypeptide sequences between double-stranded RNA (dsRNA)of the chestnut blight fungus *Cryphonectria parasitica* and the two plant viruses in genus *Potyvirus*, barley yellow mosaic virus and pea seed-borne mosaic virus. The sequence analysis demonstrated that RNA-dependent RNA polymerase (RdRp), RNA helicase and papain-like cysteine proteases encoded by *C. parasitic* dsRNA show significant similarity to conserved domains of protease from the plant potyviruses. This study may be the evidence of the exchange of viruses between fungi and plant hosts (Ghabrial, 1998).

First published fungal virus was found in the edible botton mushroom, *Agaricus bisporus* showing die-back disease (Hollings, 1962). Mycoviruses are common in all major taxonomic groups of fungi (Nuss, 2005). Eleven families of virus infecting fungi and oomycetes were assigned by International Committee on Taxonomy of Viruses (ICTV) (Table 2.6) (King *et al*., 2011). Most of mycoviruses are RNA genome, both double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) but dsRNA is the major by two-thirds of all RNA mycoviruses (Pearson *et al*., 2009). There are two DNA mycoviruses reported as dsDNA and ssDNA genome, infecting oomycetes *Rhizidiomyces* sp. and a fungus *Sclerotinia sclerotiorum*, respectively (Yu *et al*., 2010; Kraberger *et al*., 2013). The ssDNA mycovirus has not been included in ICTV (2011), *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1 infecting the fungus *Sclerotinia sclerotiorum* causing disease in rapeseed plant. The virus has non-envelope particle (Yu *et al*, 2010)

Table 2.6 Summary of recognized mycovirus (King *et al*, 2011)

Genome	Family	Genus	Species	Morphology
dsDNA		*Rhizidiovirus*	*Rhizidiomyces virus*	Isometric, non-enveloped
ss(+)RNA	*Alphaflexiviridae*	*Botrexvirus*	*Botrytis virus X*	Flexuous, non-enveloped
	Barnaviridae	*Barnavirus*	*Mushroom bacilliform virus*	Bacilliform, non-enveloped
	Gammaflexiviridae	*Mycoflexivirus*	*Botrytis virus F*	Flexuous, non-enveloped
	Hypoviridae	*Hypoovirus*	*Cryphonectria hypovirus 1-4*	Pleomorphic vesicles
	Narnaviridae	*Mitovirus*	*Cryphonectria mitovirus 1*	No true virions
			Ophiostoma mitovirus 3a	
			Ophiostoma mitovirus 4-6	
		Narnavirus	*Saccharomyces 20S RNA narnavirus*	No true particles, virions compose of ribonucleo-protein complex
			Saccharomyces 23S RNA narnavirus	
ss(+)RNA-RT	*Metaviridae*	*Metavirus*	*Cladosporium fulvum T-1 virus*	Spherical, enveloped
			Fusarium oxysporum Skippy virus	
			Saccharomyces cerevisiae Ty3 virus	
			Schizosaccharomyces pombe Tf virus	

Table 2.6 Summary of recognized mycovirus (continued) (King *et al*, 2011)

Genome	Family	Genus	Species	Morphology
ss(+)RNA-RT	*Metaviridae*	*Metavirus*	*Schizosaccharomyces pombe Tf2 virus*	Spherical, enveloped
	Pseudoviridae	*Hemivirus*	*Candica albicans Tca2 virus*	Icosahedral, non-enveloped
			Candica albicans Tca5 virus	
		Pseudovirus	*Saccharomyces paradoxux Ty5 virus*	
			Saccharomyces cerevisiae Ty1-2-4 virus	Icosahedral, non-enveloped
dsRNA	*Chrysoviridae*	*Chrysovirus*	*Helminthosporium victoriae virus 145S*	Icosahedral, non-enveloped
			Penicillium brevicompactum virus	
			Penicillium chrysogenum	
			Penicillium cyaneo-fulvam	
	Endornaviridae	*Endornavirus*	*Helicobasidium mompa endornavirus 1*	No virions
			Phytophthora endornavirus 1	
	Partitiviridae	*Partitivirus*	*Agaricus bisporus virus 4*	Symmetric icosahedral, non-enveloped
			Aspergillus ochraceous virus 1	
			Atkinsonella hypxylon virus	
			Ceratocystis resinifera virus 1	

Table 2.6 Summary of recognized mycovirus (continued) (King *et al*, 2011)

Genome	Family	Genus	Species	Morphology
dsRNA	*Partitiviridae*	*Partitivirus*	*Discula destructiva virus 1*	Symmetric icosahedral, non-enveloped
			Discula destructive virus 2	
			Fusarium poae virus 1	
			Fusarium solani virus 1	
			Gaeumannomyces graminis virus 019/6A	
			Gaeumannomyces graminis virus T1-A	
			Gremmeniella abietina RNA virus MS1	
			Helicobasidium mompa	
			Heterobasidion annosum	
			Ophiostoma partitivirus 1	
			Penicillium stoloniferum virus F	
			Penicillium stoloniferum virus S	
			Pleurotus ostreatus virus 1	
			Rhizoctonia solani virus 717	
			Rosellinia necatrix virus 1	

Table 2.6 Summary of recognized mycovirus (continued) (King *et al*, 2011)

Genome	Family	Genus	Species	Morphology
dsRNA	*Reoviridae*	*Mycoreovirus*	*Cryphonectria parasitica mycoreovirus-1*	Symmetric icosahedral, non-enveloped
			Cryphonectria parasitica mycoreovirus-2	
			Rosellinia necatrix mycoreovirus-3	
	Totiviridae	*Totivirus*	*Saccharomyces cerevisiae virus L-A*	Asymmetric icosahedral, non-enveloped
			Saccharomyces cerevisiae virus L-BC (La)	
			Ustilago maydis virus H1	
		Victorivirus	*Coniothyrium minitans RNA virus*	Symmetric icosahedral, non-enveloped
			Epichloe festucae virus 1	
			Gremmeniella abietina RNA virus L1	
			Helicobasidium mompa totivirus 1-17	
			Helminthosporium victoriae virus 190S	
			Magnaporthe oryzae virus 1	
			Sphaeropsis sapinea RNA virus 1-2	

2.3.1 Pythiaceae viruses

RNA viruses have been reported in several species of family Pythiaceae. Phytophthora endornavirus 1 (PEV1) was the first virus found infecting *Phytophthora* sp. from Douglas fir in USA (Hacker *et al*., 2005; Cai and Hillman, 2013). PEV1 has dsRNA genome of 13,883 base pairs (bp). Sequence analysis of PEV1 genome revealed a single open reading frame (ORF) that was predicted to encode a polyprotein with motifs characteristic of viral RdRP, RNA helicase and UDP glycosyltransferase (Hacker *et al*., 2005). Kozlakidis and colleagues (2010) generated primers to amplify specific regions of the three motifs of endornavirus genome for systemic detection of PEV1 in *P. ramorum*, the forest trees pathogen, causing the sudden oak death and found that *P. ramorum* populations from UK and Europe also contain PEV1. However, there was no phenotypic change of fungal host caused by PEV1. Cai and colleagues (2009) detected 4 patterns of dsRNA segments in 9 *P. infestans* isolates from USA and Mexico and named PiRVs 1-4. PiRV-1 consists of 2 segments with independent internal sequence in size of 2.9 and 3.3 kilobase (kb). RNA1 is the 3.3 kb fragment with a single ORF encoding RdRP. The second segment, RNA2 has 2 forms, RNA2A and RNA2B in which replacement of 19-nucleotide (nt) by a 9-nt fragment was found. Both RNA1 and RNA2 share similarities of 5' and 3' secondary structure untranslated regions (UTRs) and poly (A) tail on one strand which considered as positive strand. The resulting RNA structure and RdRP sequence analysis suggested that *PiRV1* has genome characteristic of a positive-sense ssRNA virus and may be the first member of a new family. PiRV-2 may enhance virulence of its host. *P. infestans* contained PiRV-2 produce abundant sporangia compared with the virus-cured strains. PiRV-2 RNA genome of 11,170 bp was predicted as having motifs of cysteine protease and RdRP (Cai and Hillman, 2013). The dsRNA genome of PiRV-3 is 8,112 bp. ORF1 and ORF2 in the genome are linked by frame shift sequence. RdRP is encoded by ORF2 (Cai *et al*., 2013). PiRV-4 has a small dsRNA segment with 2,984 nt. Genome of PiRV-4 consists of a single ORF which predicted to encode RdRP. Neighbor-joining tree based on RdRp sequences demonstrated that PiRV-4 belong to the virus family *Narnaviridae* (Cai *et al*., 2012). PiRV-4 was also detected in *P. infestans* from China (Zhan

et al., 2016). Nevertheless, out of these three *Phytophthra* species, none has been reported harboring viral nucleic acid.

Double-stranded RNA element was reported in 2 species of the genus *Pythium*, *Py. butleri* and *Py. irregulare* (Cai and Hillman, 2013). No virus-like particle (VLP) was found in *Py. butleri* but the dsRNA was detected using antisera against Polyinosinic:polycytidylic acid which is similar to dsRNA structure (Moffitt and Lister, 1975; Dantzer, 2017). *Py. irregulare* causing disease in cucumber was detected containing dsRNA both with or without VLP and the dsRNA segments were ranged 1.8-6.0 kb (Klassen *et al*., 1991). Other dsRNA elements were also found in *Py. irregulare* with 12 patterns of 6 different sizes of dsRNA between 1-6 kb (Gillings *et al*., 1993). The effect of dsRNAs to their *Pythium* hosts have not been discussed in any publications. The genome of mycoviruses and dsRNA elements found in Pythiaceae were summarized in Table 2.7.

Table 2.7 Summary of Pythiaceae viruses and infected dsRNA

Host	Viral species	Genome	Genome size	VLP	Effect to host	Reference
Phytophthora spp. Douglas fir	Phytophthora endornavirus1 (PEV1)	dsRNA	13.88 kb	-	Asymptom	Hacker *et al*., 2005
Phytophthora infestans	Phytophthora infestans RNA virus 1 (PiRV-1)	ss(+)RNA	2-9, 3.3 kb	-	N/A[a]	Cai *et al*., 2009
	Phytophthora infestans RNA virus 2 (PiRV-2)	dsRNA	11.17 kb	-	Hypervirulence[b]	Cai and Hillman, 2013
	Phytophthora infestans RNA virus 3 (PiRV-3)	dsRNA	8.11 kb	-	Asymptom	Cai *et al*., 2013
	Phytophthora infestans RNA virus 4 (PiRV-4)	dsRNA	2.98 kb	-	N/A[a]	Cai *et al*., 2012

[a]No available (failed to attempt the transmission of mycovirus/dsRNA element via hyphal fusion or making virus curing strain has not been successful).

[b]Abundant sporulation.

Table 2.7 Summary of Pythiaceae viruses and infected dsRNA (continued)

Host	Viral species	Genome	Genome size	VLP	Effect to host	Reference
Pythium butleri		dsRNA[c]		-		Moffitt and Lister, 1975
Pythium irregulare		dsRNA[c]	3.2, 4.7, 5.0, 5,8, 6.0 kb	+		Klassen *et al*., 1991
		dsRNA[c]	1.8, 3.2, 4.7, 5.0, 5.2, 5.7 kb	-		Klassen *et al*., 1991
		dsRNA[c]	1-6 kb	+	N/A[a]	Gillings *et al*., 1993

[a]No available (failed to attempt the transmission of mycovirus/dsRNA element via hyphal fusion or making mycovirus/dsRNA curing strain has not been successful).

[c]The structure of dsRNA has not been confirmed.

2.3.2 Effects of mycoviruses on host phenotype

Relationships between mycoviruses and hosts are commonly symptomless infection but certain mycovirus causes its host phenotypic changes (Pearson *et al*., 2009). Mycoviruses that reduce growth and virulence of their fungal hosts have been known as hypovirulence (Nuss, 2005). The impact of hypovirulence mycovirus to plant pathogenic fungi had attracted attention by the case of successful introduction of *Cryphonectria* hypoviruses in field condition to control chestnut blight in Europe (Nuss, 1992, 2005). Firstly, the *Cryphonectria* hypoviruses were recognized by French phytopathologist who observed the spontaneous healing of canker diseased on stumps of chestnut trees (Grente and Sauret, 1978). Atypical strains of *Cryphonectria parasitica* were isolated from nonlethal cankers showing lower pigmentation and sporulation and reduced virulence on inoculated bark compared to normal strains (Heiniger and Rigling, 1994). Normal strain could be converted to hypovirulence strain by hyphal fusion or anastomosis with atypical strain in laboratory observation. This experiment led to the hypothesis that transmissible hypovirulence is cytoplasmically controlled (Van Alfen *et al*., 1975; Nuss, 1992). The hypovirulence phenotype was later demonstrated causing by dsRNA elements (Day *et al*., 1977; Fulbright, 1984). *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1 (SsHADV-1) with a ssDNA genome was also reported causing debilitation of its fungal host (Yu *et al*., 2010). SsHADV-1 was marked as the first mycovirus that has an extracellular route of transmission. Purified particles of SsHADV-1 could infect hyphal fragment and intact hypha of *S. sclerotiorum* virus-free strain when applied extracellularly. Moreover, therapeutic activity of SsHADV-1 against naturally *S. sclecrotiorum* infected rapeseed plants in field trail showed that the resulting of spraying hypha of fungi virus-containing strain in diseased field could suppress the development of lesion, reduce disease severity and enhance rapeseed yield significantly under field conditions. The virus has a narrow host range between sister species of the genus *Sclerotinia* without biological safety concerns to other beneficial microorganisms to be used for biological control strategy (Yu *et al*., 2013). In other cases, hypervirulence means the fungus is more virulent when it contains mycovirus (Ghabrial and Suzuki, 2009).

There have been several mycoviruses reported as hypervirulence. The 6.0 kb dsRNA phylogenically related to plant cryptic viruses in the ascomycetous fungus, *Nectria radicicola* that causes root rot disease in ginseng was associated with asexual spore overproduction, interfered with signal-transduction pathways via up regulation of laccase activity, increased disease severity, and restored disease incident in plant host when the plants were test by infecting with *N. radicicola* dsRNA-recipient strain (Ahn and Lee, 2001). Two other hypervirulence mycoviruses were not in the plant fungus. The study of virulence of dsRNA elements in opportunistic fungal pathogen *Aspergillus fumigatus* that causes lung disease in humans and animals showed that the fungus infected with dsRNAs called profile A28 containing 4 segments ranged between 2.5-1.0 kb increased radial growth, and enhanced pathogenic virulence in moth (*Galleria mellonella*) larvae used as a model for mammalian immune system by monitoring and comparing fungal housekeeping gene expression between dsRNA-infected and dsRNA-free strain in infected larvae (Özkan and Coutts, 2015). The virulent of dsRNA mycovirus in the entomopathogenic fungus *Beauveria bassiana* were also studied in the moth, and the moth larvae infected with the dsRNA containing fungus strain showed significant decreasing in survival rate comparing with dsRNA-free strain (Kotta-Loizou and Coutts, 2017).

CHAPTER III
METHODS

3.1 Pythiaceae isolation

3.1.1 Pythiaceae isolation from soil

Soil samples were collected from para rubber tree plantation at 5 cm depth in plastic bags and stored at room temperature until processing. One gram of soil was suspended in 9 ml of sterile water, vigorously vortexed, sonicated for 10 minutes and left at room temperature to allow the soil to settle down. Clear suspensions were diluted to make a 10-fold dilution series. Subsequently, 100 µl of each dilution series from 10^{-4}-10^{-6} were spread on V8 selective medium (Jeffers and Martin, 1986) (Appendix 1), supplemented with 250 mg/l ampicillin, 10 mg/l rifampicin, 5 mg/l pentachloronitro-benzene and 50 mg/l hymexazol, and incubated at 25°C for 5-7 days in the dark. After incubation, mycelium and sporangium were observed under stereo microscope (SZ30, Olympus, Japan) and light microscope (CH30, Olympus, Japan).

3.1.2 Pythiaceae isolation from diseased para rubber petioles

3.1.2.1 Pythiaceae isolation on selective medium

Petioles were collected from para rubber trees showing ALF disease symptoms in plastic bags and stored at 4°C until processing. Petioles were cut to 1 cm in length, especially petioles that showed brownish lesions with droplets of latex. Cut petioles were surface sterilized by soaking in 0.5% hypochlorite for 45 seconds, washed two times with sterile water and dried by blotting on sterile gauze. Dried petioles were placed on V8 selective medium supplemented with antibiotics and incubated at 25°C for 5-7 days in the dark. After incubation, mycelium and sporangium were observed under stereo and light microscope.

3.1.2.2 Pythiaceae isolation by baiting technique

Diseased leaves were cut and surface sterilized with 0.5% hypochlorite. Five pieces of cut leaves were immersed in 20 ml of P3 water containing autoclaved sesame seeds in a sterile petri dish and incubated at 25°C until mycelium germinated around sesame seeds. Mycelia grown on sesame seeds were observed under light microscope and the infected sesame was transferred on to V8 selective medium containing antibiotics.

3.2 Mycovirus detection

3.2.1 DNA virus detection

3.2.1.1 Total nucleic acid extraction

Agar plug of Pythiaceae isolates were cultured on 5% V8 selective medium with antibiotics and incubated at 25°C for 7 days in the dark. Five to ten of mycelium plugs were cut from the agar plate using a sterile 5 mm diameter cork borer and then transferred to 50 ml of 20% V8 broth with antibiotics, and incubated for 7 days at 25°C on a rotary shaker (NewBrunswick Scientific, USA) at 150 rpm. The mycelium was harvested by filtration through sterile gauze, washed with sterile deionized water and stored at -80°C until use.

Total DNA was extracted from frozen mycelium using Cetyl-trimethyl ammonium bromide (CTAB) procedure adapted from Shen and colleagues (2005), 50 mg of frozen mycelium was ground with liquid nitrogen to fine powder using a mortar and pestle. 150 µl of extraction buffer (5 mM EDTA (pH7.5), 0.1 M Tris, 20 mM sodium bisulphate, 0.35 M sorbitol) were added and the mixture was transferred into a new 1.5 ml microcentrifuge tube and vortexed. 150 µl of nuclei lysis buffer (50 mM EDTA (pH 7.5), 2 mM sodium chloride, 0.2 M Tris, 2% CTAB) was added, followed by 60 µl of 5% sarkosyl, mixed by vortexing and incubated at 65°C for 30 minutes in a heat box (Bioer Technology, China). One volume of chloroform:isoamyl alcohol (24:1) was added, mixed vigorously and centrifuged at 13,800 xg for 15 minutes at room temperature (Force 1418, Select Bioproducts, USA). The aqueous phase was transferred into a new tube, and

chloroform extraction was repeated once. Genomic DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2), and 2 volumes of absolute ethanol at room temperature and invert mixed gently. The tube was centrifuged for 10 minutes at 11,750 xg at room temperature. The supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol. Air dried and resuspend in 50 µl of TE buffer (0.1 mM EDTA (pH 8.0), 10 mm Tris-HCl).

3.2.1.2 Removal of RNA and visualization of non-genomic DNA element

To remove RNA from the sample, total nucleic acid was treated with 1 µl of 10 mg/ml RNase A (RBC Bioscience, Taiwan) at 37°C for 30 minutes in a heat box. Non-genomic DNA band was detected by visualizing the RNase A-treated total nucleic acid on electrophoresis on 1% (w/v) agarose gel in TBE buffer using electrophoresis equipment (MUPID-2 plus, Advance, Japan) and visualized using Gel Documentation (Bio-Rad, Italy).

3.2.2 RNA virus detection

3.2.2.1 Optimized conditions of cellulose column for dsRNA isolation

3.2.2.1.1 dsRNA extraction from *E. coli* dsRNA expressing strain

The equivalent of 2 × OD_{600} of cells of *E. coli* dsRNA expressing strain was obtained from Assoc. Prof. Dr. Wanchai Assavalapsakul's laboratory. The *E. coli* strain was genetically modified and induced to express dsRNA of Yellow Head Virus's protease. The harvested cells were treated by boiling for 2 minutes with 100 µl of 0.1% SDS in PBS buffer and placed on ice for 5 minutes. The lysate was added with 5X RNase A buffer at 1X final concentration followed by 0.1 µg of RNase A (Bio Basic, Canada) and incubated at 37°C for 30 minutes. The dsRNA was extracted using 200 µl of RiboZol (AMRESCO, USA) according to the manufacturer's protocol. The dsRNA pellets were dissolved in 20 µl of 150 mM NaCl.

3.2.2.1.2 Cellulose column preparation

The methods for isolation of dsRNA using cellulose column were adapted from Das and colleagues (2014) and Chandavimol (2011). To prepare a column device, interlining was cut in circle with 0.86 cm in diameter, soak in

double deionized water containing 0.1% (v/v) Diethyl Pyrocarbonate (DEPC), shook vigorously, incubated overnight at room temperature and autoclaved. The DEPC-treated interlining was put at the bottom of a 3 ml syringe barrel. Cellulose fiber (C6288, Sigma, USA) was pretreated by mixing 0.5 g of cellulose with 2.5 ml of 1X STE buffer containing 15% (v/v) ethanol in a 15 ml centrifuge tube and mixed by vortex (Genie 2, Scientific Industries, USA). To bind the dsRNA with the pretreated cellulose, ethanol was added to the extracted dsRNA to a final concentration of 15% (v/v) and combined the mixture with the pretreated cellulose. Then the tube was invert mixed at room temperature for 5 minutes. The slurry was loaded into the column device. The gravity-flow liquid phase was collected and reloaded once into the column. After repeat loading, the flow-through was discarded. The column was washed with 50 ml of washing buffer (1X STE containing 15% (v/v) ethanol) to remove ssRNA and DNA. The dsRNA was then eluted by adding 2 ml of 1X. The eluent was collected in a 15 ml centrifuge tube. To precipitate dsRNA, 200 µl of 3 M sodium acetate was added, followed by 2.5 ml of ethanol, inverted mixing and incubated at -20°C overnight. The supernatant was centrifuged at 12,470 xg for 15 minutes at 4°C (mikro 22 R, Hettich, Germany). The pellets were washed twice with 80% (v/v) ethanol and centrifuged at 12,470 xg for 10 minutes at 4°C. The dsRNA pellets was air dried briefly and resuspend in 50 µl of 1X STE buffer. The dsRNA band was determined by electrophoresis on 1% agarose gel in TAE buffer.

3.2.2.2 dsRNA detection in Pythiaceae

3.2.2.2.1 Total nucleic extraction

Protocol of total nucleic extraction for dsRNA detection was adapted from Okada and colleagues (2015). 200 mg of frozen mycelium was ground with liquid nitrogen in a mortar and pestle into fine powder. 500 µl of 2X STE buffer containing 1% (w/v) SDS was added and transferred the mixture to a 1.5 ml microcentrifuge tube. 5 µl of β-mercaptoethanol was added, followed by 250 µl of saturated phenol and 250 µl of chloroform:isoamyl alcohol (24:1) (phenol:chloroform: isoamyl alcohol; 25:24:1)and shook vigorously for 1 minute. The crude extract was centrifuged at 10,625 xg for 15 minutes at 4°C and transferred the aqueous phase to a

new 1.5 microcentrifuge tube. Double-stranded RNA was isolated from the aqueous phase using cellulose column.

3.2.2.2 2 dsRNA isolation using cellulose column

Double-stranded RNA was isolated form total nucleic acid using the optimized cellulose column method from 3.2.2.1.2. Pretreated cellulose was mixed with the aqueous phase from 3.2.2.2.1 containing 15% ethanol before invert mixing and loaded to the column device. After elution and precipitation, the pellet was resuspend in 1x STE buffer. RNA was quantified using nanodrop spectrophotometer (nanodrop 200 UV-Vis Spectrophotometer, Thermo Scientific, USA). The presence of dsRNA was determined by electrophoresis on 1% agarose gel in TAE buffer.

3.2.2.2.3 Nuclease treatment of dsRNA

To determine whether the viral genome was dsRNA, DNase I, RNase A and S1 nuclease treatments were performed. For RNase A digestion, the eluent containing 600 ng of dsRNA from cellulose column was divided into three microcentrifuge tubes and adjusted volume to 300 µl by adding 1X STE. Each tube was added with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol, and incubated overnight at -20°C. The mixture was divided into 3 microcentrifuge tubes, each tube contained 200 ng of dsRNA and centrifuged at 12,470 xg for 15 minutes at 4°C. The pellets were washed twice with 80% ethanol, centrifuged at 12,470 xg for 10 minutes at 4°C and air dried briefly. The resulting pellets were resuspended with 20 µl of nuclease-free water, 20 µl of 0.1X SSC (15 mM NaCl, 1.5 mM sodium citrate) and 20 µl of 2X SSC (300 mM NaCl, 30 mM sodium citrate) for a control, low salt and high salt condition, respectively. The low salt and the high salt reactions were treated with RNase A (1 µg/ml final concentration) (Bio Basic, Canada) at room temperature for 10 minutes. The treated RNA was immediately determined by electrophoresis on 1% agarose gel in TAE buffer.

For DNase I treatment, 500 ng (per reaction) of dsRNA from cellulose column was reprecipitated using phenol:chloroform extraction method. After air dried the resulting pellets, the control tube was resuspended with 18 µl of nuclease-free water and 2 µl of 10X DNase I buffer (1X DNase buffer final concentration in 20 µl reaction), and the

treatment tube was resuspended with 18 µl of nuclease-free water, 2 µl of 10X DNase I buffer and 0.1 U of DNase I (Bio Basic, Canada). Both reactions were incubated at 37°C for 10 minutes in heat box. After incubation, the samples were checked by electrophoresis on 1% agarose in TAE buffer.

S1 nuclease treatment was conducted by using 500 ng of dsRNA (per reaction) from cellulose column. The dsRNA was reprecipitated using phenol:chloroform extraction method. The treatment reaction was resuspended in 16 µl of nuclease free water, 2 µl of 10X S1 buffer and 2 µl of 1 U/µl S1 nuclease (Promega, USA) for the enzyme final concentration at 0.1 U/µl. The control was resuspended in 20 µl of nuclease-free water containing 1X S1 buffer at final concentration. The treatment was carried out at 37ºC for 30 minutes before immediately checking the results by electrophoresis on 1% agarose gel in TAE buffer.

3.3 dsRNA identification

3.3.1 Detection of reported Pythiaceae viruses

3.3.1.1 Preparation of RNA sample

Total nucleic acid was extracted from frozen mycelium using the same method as the dsRNA detection protocol in 3.2.2.2.1. After centrifugation, the aqueous phase was collected. The total nucleic acid was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol and centrifuged. The pellet was washed with 80% ethanol. Then the pellets was resolved in 50 µl of nuclease-free water. Before reverse transcription PCR (RT-PCR) step, RNA was quantified using nanodrop spectrophotometer and treated with DNase I to remove contaminated DNA. The sample containing 10 µg of RNA was treated with DNase I at 37°C for 30 min in 1 ml reaction volume containing 100 U of RNase inhibitor (RBC bioscience, Taiwan), 1X DNase I buffer, 0.01 U of DNase I (Bio Basic, USA). After DNase I treatment, the reaction was stopped by adding 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), mixed by shaking vigorously, centrifuged at 12,470 xg for 15 minutes at 4°C. Then the aqueous phase was transferred into a new microcentrifuge tube. The resulting RNA was

precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol before centrifugation. The pellet was washed twice with 80% ethanol in RNase-free water followed by centrifugation. The RNA was resuspend in RNase-free water and quantified using nanodrop spectrophotometer.

3.3.1.2 Reverse transcription polymerase chain reaction (RT-PCR)

Two-step RT-PCR was performed to detect genes of reported Pythiaceae viruses which have genome sequences deposited in GenBank (NCBI) including PEV1, PiRV-1, PiRV-3 and PiRV-4. Pythiaceae housekeeping gene, β-tubulin, with or without adding reverse transcriptase was used as control reactions. The reaction contained approximately 500 ng of RNA from 3.3.1.1 and the first-strand synthesis was conducted by priming with random hexamer primers and SuperScript III reverse transcriptase according to the manufacturer's instructions of SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The first-strand product was used as a template for second-strand synthesis using conventional PCR with KOD Hot Start DNA Polymerase (Novagen, USA) and specific primers. The primers for detecting viruses were designed to target RdRp region by aligning with the published sequences of PEV1 (AJ877914.1), PiRV-1 (NC_013220.1), PiRV-3 (JN603241.1) and PiRV-4 (JN400241.1), including PEV1-FRd/PEV1-RRd primers for PEV1, PiRV1-FRd/PiRV1-RRd primers for PiRV1, PiRV3-FRd/PiRV3-RRd primers for PiRV3 and PiRV4-FRd/PiRV4-RRd primers for PiRV4, the length of amplicons are length between 400-800 bp. Another primer sets were designed or obtained from previously reports and products are approximately 900-3,000 bp, targeting the entire ORFs including PiRV1-F/PiRV1-R primers for PiRV1, PiRV3-F/PiRV3-R primers for PiRV3 and PiRV4-F/PiRV4-R primers for PiRV4 and udp-F2/udp-R2 primers targeting 5'- of the UGT gene towards the helicase-like region of PEV1 (Appendix 3). β-tubulin partial coding sequences of *Py. vexans* (GU133402.1, GU133454.1, GU931700.1, EU080484.1) and *Py. cucurbitacearum* (KJ595460.1) were used for designing the antisense primer (BT-Pyt) of β-tubulin gene. Reaction mixture of 25 µl comprised 2 µl of the first-strand synthesis,1X Buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTPs (each), 0.2 µM Sense primer, 0.2 µM Anti-Sense primer and 0.02 U/µl of KOD Hot Start

DNA Polymerase. For self-priming test, only Forward or Reverse primer was added to the second-strand reactions. Cycling conditions for all primer sets were 95°C for 2 minutes, 95°C for 20 seconds and 40 cycles of the lowest primer Tm°C shown in Appendix 3 for 15 seconds. The results were checked by visualization 5 µl of cDNA products on 1% agarose gel in TBE buffer.

3.3.2 Partial sequence of dsRNA

For cDNA synthesis, the dsRNA eluted from cellulose column was treated with S1 nuclease to reduce contaminated host's mRNA. The enzyme was inactivated and removed by phenol-chloroform extraction. The dsRNA was precipitated with 0.3 M sodium acetate, followed by washing twice with 80% ethanol and dissolved the pellet with nuclease-free water. 1 µg of S1 nuclease-treated dsRNA was used as a template for first-strand cDNA synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) with random hexamer primer according to the manufacturer's instructions. Second-strand cDNA was constructed from the first-strand synthesis product using protocol adapted from D'Alessio and Gerard (1988). The first-strand reaction mixture was 100 µl in nuclease-free water, 1X NEBuffer 2, 250 U/ml of DNA polymerase I (*E. coli*) (New England Biolabs, USA), 8.5 U/ml of *E. Coli* RNase H (Invitrogen, USA), 10 U/ml of T4 ligase (RBC Bioscience, Taiwan). The second-strand reaction was incubated at 16°C of 2 hours. The reaction was stopped and the resulting cDNA was purified by phenol:chloroform extraction method. After the pellets were air dried, 10µl of TE buffer was added to resuspend the cDNA pellets.

The cDNA was ligated into pMiniT plasmid from NEB PCR cloning kit (New England Biolabs, USA) according to the manufacturer's protocol. The amount of cDNA to be added in the reaction was used at the maximum volume recommended by the manufacturer. The ligation reaction was transformed into *E. coli* DH10B competent cells (NEB 10-beta Competent *E. coli*) provided in the cloning kit according to the manufacturer's instructions. The transformants were selected by spreading 100 µl of transformation mixture on LB agar containing 100 µg/ml ampicillin and incubated at 37°C for 15 hours.

Transformants with an insert fragment were screened using rapid size screening methods (Law and Crickmore, 1997). The transformant colony was put in 30 µl 55°C lysis buffer, ground and continuously incubated at 55°C for 5 minutes, and then left on ice for 5 minutes. The mixture was centrifuge at 13,800 xg for 5 minutes at room temperature. The approximate size of plasmid containing cDNA was estimated by running 25 µl of the lysis supernatant on 1% (w/v) agarose gel in TBE buffer. The clones that showed plasmid size larger than or similar to 3 kb by rapid size screening were selected for insert screening by colony PCR using L30350F (Cloning Analysis Forward Primer) and L71-1R (Cloning Analysis Reverse Primer) specific primers of the plasmid's cloning site (New England Biolabs, USA). The 25 µl reaction mixture composed of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer and 1.25 U *Taq* polymerase (Apsalagen, Thailand), and the transformant colony. The PCR condition was 94 ºC for 3 minutes followed by 30 cycles of 94 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 2 minutes and final extended at 72 ºC for 5 minutes. The size of PCR products was analyzed by electrophoresis on 1% (w/v) agarose gel in TBE buffer.

For cDNA sequencing, colony of transformants were inoculated in 5 ml of LB broth and incubated overnight at 37°C with shaking (220 rpm). Plasmids were extracted from the overnight cultures using HiYield™ Plasmid Mini Kit (RBC Bioscience, Taiwan) according to the manufacturer's protocol for high-copy plasmid DNA. The extracted plasmids were sequenced by Macrogen Inc., Korea by reading from the upstream regions of the plasmid. The sequencing results were blast with GenBank in NCBI (National Center for Biotechnology Information). Protein sequence was predicted using ExPASy-Translate tool (http://web.expasy.org/translate/) and compared the similar sequence in database using BLASTP (NCBI). Protein alignment was proceeded using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and GeneDoc software.

3.4 Identification of Pythiaceae harboring dsRNA elements

3.4.1 Morphological observation

Colony pattern of the dsRNA host was observed by transferring the agar plugs onto V8 agar with or without antibiotics, potato dextrose agar (PDA) and black bean agar (Sopee *et al*., 2012) (Appendix 1). All cultures were incubated at 25°C in the dark.

Sporangia, chlamydospore and sexual organs were observed from the various agar cultures. Sporangium production was induced in water culture via bating technique by co-incubation of culture agar plugs with sesame seeds and incubated at 25°C. The spore formation and morphology was observed under a stereo microscope and a light microscope.

Resistance to the hymexazol fungicide was examined by transferring the agar plug of V8 agar culture on V8 agar containing different concentrations of hymexazol; 0, 50, 100, 200, 300 and 400 mg/l final concentration and incubated at 25°C in the dark. Growth of the cultures were measured daily in diameter and reported in term of mycelial growth rate (mm/day).

3.4.2 Molecular identification

Nested polymerase chain reaction (Nested PCR) technique was used for *Phytophthora* genus identification. ITS region of 18s ribosomal gene was amplified using primer ITS1 and ITS4. The reaction mix composed of 50 ng of extracted DNA from 3.2.1.1, 1X PCR buffer, 0.2 mM dNTPs,1.5 mM $MgCl_2$, 1 µM of each primer and 1.25 U of *Taq* polymerase (Apsalagen, Thailand) in 25 µl total volume. The PCR condition was 94 ºC for 3 minutes followed by 30 cycles of 94 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 1 minutes, and 72 ºC for 5 minutes for final extension. The product was electrophoresis on 1% (w/v) agarose gel in TBE buffer.

The ITS1/ITS4 PCR product was diluted to 1:100 concentration with nuclease-free water and used as a template for nested PCR reaction. A2/I2 primers which are specific to the inner region of ITS1-5.8S-ITS2 ribosomal DNA gene designed by Drenth and colleagues (2006) were used in nested PCR. The reaction of 25 µl contained 1 µl of 1:100 ITS1/ITS4 product, 1X PCR buffer, 0.2 mM dNTPs, 1 µM of each primer and 1.25 U

of *Taq* polymerase (Apsalagen, Thailand). The PCR condition was 94 ºC for 3 minutes followed by 30 cycles of 94 ºC for 30 seconds, 56 ºC for 30 seconds and 72 ºC for 1 minutes, and final extended at 72 ºC for 5 minutes. The presence of ITS1/ITS4 and A2/I2 nested products were checked by electrophoresis on 1% (w/v) agarose gel in TBE buffer.

For species identification by sequencing, A-tails were added to the PCR product of ITS1/ITS4 primers by preparing 20 µl of the reaction containing ITS1/ITS4 product, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 M dNTPS and 1.25 U of *Taq* polymerase (Apsalagen, Thailand) and incubated 70°C for 30 minutes. The amplicons were ligated into TA cloning vector from RBC TA cloning kit (RBC Bioscience, Taiwan) according to the manufacturer's instructions. The transformation was performed as followed, 1 µg of ligation plasmid was mixed with 100 µl of *E. coli* DH5α competent cells and stood on ice for 30 minutes. The transformation mixture was heated at 42°C for 90 seconds and immediately placed on ice for at least 2 minutes. 900 µl of 2X YT medium was added to the mixture and incubated at 37°C for an hour in incubator shaker at 220 rpm. The resulting transformants were spread on LB agar containing 50 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal, and incubated at 37°C for 16 hr.

The transformants were screened by colony PCR using ITS1/ITS4 primers. Colony of transformants were picked up with sterile pipette tip and dipped into the PCR reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 0.5 µM of each primer and 1.25 U *Taq* polymerase (Apsalagen, Thailand) in 25 µl total volume. The PCR condition was 94 ºC for 3 minutes followed by 30 cycles of 94 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 1 minutes, and final extended at 72 ºC for 5 minutes. The size of approximately 900 base pairs of the PCR product was determined by electrophoresis on 1% (w/v) agarose gel in TBE buffer.

The transformants containing ITS fragment were inoculated in LB broth with 50 µg/ml ampicillin and incubated on a rotary shaker overnight at 37°C. After incubation, cells were harvested and plasmids were extracted using HiYield Plasmid Mini Kit (RBC Bioscience, Taiwan) according to the manufacturer's instructions. The extracted plasmids were sequenced by Macrogen Inc., Korea in direction from upstream forward

primer binding site (M13F-pUC universal primer). The sequencing results were blast with GenBank in NCBI multiple sequence alignment using Clustal Omega (https://www.ebi.ac.uk/ Tools/msa/clustalo/), and phylogenetic tree was constructed using Mega 6 program (http://www.megasoftware .net/).

CHAPTER IV
RESULTS & DISCUSSION

4.1 Methods for Pythiaceae isolation from environmental sample

Members of Pythiaceae in general produce 3 types of propagules including zoospore, chlamydosprore and oospore. The zoospore is short life infectious propagules, while chlamydospore and oospore are the long-term survival spores. In this study, 3 Pythiaceae isolation methods suitable for isolation of each spore types were used. Soil dilution technique was used to isolate the long-term survival spores. Baiting technique was used to isolated zoospores. The third method, using selective medium was useful for various types of environmental samples.

Soil dilution and baiting techniques failed to isolate any *Phytophthora* or *Pythium* from soil or plant tissues. Isolation from soil was based on the note that oospores and chlamydospores are dormant spores in soil and soil dilution plate method has been commonly used for bacteria and fungi isolation from soil (Johnson and Curl, 1972). In laboratory, germination of oospores occurs in ideal conditions and requires time for breaking dormancy form 2-30 days (Erwin and Ribeiro, 1996b). These make detection of *Phytophthora* in soil difficult because the oospores take a long time to germinate and cannot compete with fast-growing fungal population in soil (Erwin and Ribeiro, 1996b). Moreover, *Phytophthora* spp. causing disease in rubber tree are mostly heterothallic, and produce oospores on plant tissue (Jeffers and Martin, 1986; Rahimian and Mitchell, 1988; Erwin and Ribeiro, 1996c). The wide spread *Phytophthora* of rubber tree in Thailand are *P. botryosa* and *P. palmivora*. Both species are heterothallic. *P. botryosa* rarely produces chlamydospore, while *P. palmivora* produces chlamydospore in abundant (Erwin and Ribeiro, 1996c) Amount of inoculation is also important. The population of chlamydospores should be high enough for germination on selective medium.

Phytophthora and *Pythium* isolation by baiting technique was established before the development of selective media. Plant tissue is commonly used as bating material providing nutrient for swimming spores. Zoospores are produced and release from

zoosporangia, and chemotactically attracted to the bait before encystment (Erwin and Riberiro, 1996b). By this strategy, *Phytophthora* and *Pythium* isolation by baiting technique was decided to establish in this study but this method failed to recover any isolates. Competitive bacteria and other fungi are dominantly found even though the baits with Pythiaceae successfully colonized were transplanted onto selective medium.

Erwin and Riberiro (1996b) noted that direct transfer of surface sterile diseased plant tissue to a selective medium is the most successful method for *Phytophthora* isolation. Diseased plant tissue should be in an active stage of infection because necrotic tissue lacks active viable mycelium and is a source of antagonistic bacteria. Placing diseased tissue on the selective medium make *Phytophthora* isolation a lot easier because chemicals in the medium could inhibit the associative bacteria and fungi since the initial step of isolation. However, there has not been the best chemical mentioned for detection of *Phytophthora*. Using combination of various chemical in proper concentration is common in many reports but some antibiotics may have little effects on *Phytophthora* spp. (Erwin and Riberiro, 1996b).

Selective medium was the only successful method in this experiment. All 79 isolates of Pythiaceae from 15 plantations in Chanthaburi and Rayong province were recovered by placing diseased tissue directly on selective medium after surface sterilization. Coordinates and maps of sampling sites were listed in Table 4.1 and showed in Figure 4.1, respectively.

Table 4.1 List of Pythiaceae isolates and sampling sites

Isolate	Sampling site		
	No.	Address	Coordinate
R1, R2	1	475 Moo 8, Sam Phi Nong, Kaeng Hang Maeo District, Chanthaburi	12°56'24.5"N 101°51'14.6"E
R3	2	477 Moo 8, Sam Phi Nong, Kaeng Hang Maeo District, Chanthaburi	12°59'31.6"N 101°50'36.9"E
R4-R14	3	Cham Samo Village, Kong Din, Klaeng District, Rayong	12°48'38.2"N 101°46'03.1"E
R15-R18, R22-R26, R28-R29, R89-R91	4	Moo 6, Soi Rong Ka Nom Jeen, Wat Chak KhunWiset Road, Kong Din, Klaeng District, Rayong	12°47'10.2"N 101°46'56.4"E
R19-R21, R27	5	98 Moo 6, Soi Wat Chak Khun Wiset, Kong Din, Klaeng District, Rayong	12°46'39.4"N 101°47'00.4"E
R30-R40	6	3433 Sukhumvit Road, Kong Din, Klaeng District, Rayong	12°47'14.5"N 101°48'28.4"E
R41-R46	7	Khao Yai Phring Village, Soi 1, Moo 8, 0221 Road, Kong Din, Klaeng District, Rayong	12°47'51.9"N 101°48'34.7"E
R47-R59	8	Khao Noi - Thung Sao Thong 0209 Road, Phang Rat, Klaeng District, Rayong	12°45'02.0"N 101°46'55.2"E
R60-R64	9	Wat Khao Noi, Soi Ban KoLoi, Kong Din, Klaeng District, Rayong	12°45'29.7"N 101°46'33.1"E
R65-R70	10	Ban Ko Loi Road, Ban Chak Khun Wiset, Kong Din, Klaeng District, Rayong	12°46'23.0"N 101°46'53.4"E
R71-R83	11	Moo 4, Bon Noen-Ram Phan Road, Ramphan, Tha Mai District, Chanthaburi	12°38'59.3"N 101°56'14.8"E

Table 4.1 List of Pythiaceae isolates and sampling sites (continued).

Isolate	Sampling site		
	No.	Address	Coordinate
R84	12	20/1 Moo 9, Bon Noen-Ram Phan Road, Khamong, Tha Mai District, Chanthaburi	12°39'27.2"N 101°56'15.7"E
R85	13	Chaloem Burapha Chonlathit 31 Road, Krachae, Na Yai Am District, Chanthaburi	12°41'03.6"N 101°49'55.6"E
R86	14	Soi Huang Som, Chaloem Burapha Chonlathit 32 Road, Na Yai Am District, Chanthaburi	12°40'58.5"N 101°50'32.6"E
R87, R88	15	Ban Nong Si Nga, Moo 3, Wang Mai, Na Yai Am District, Chanthaburi	12°44'21.4"N 101°53'35.4"E

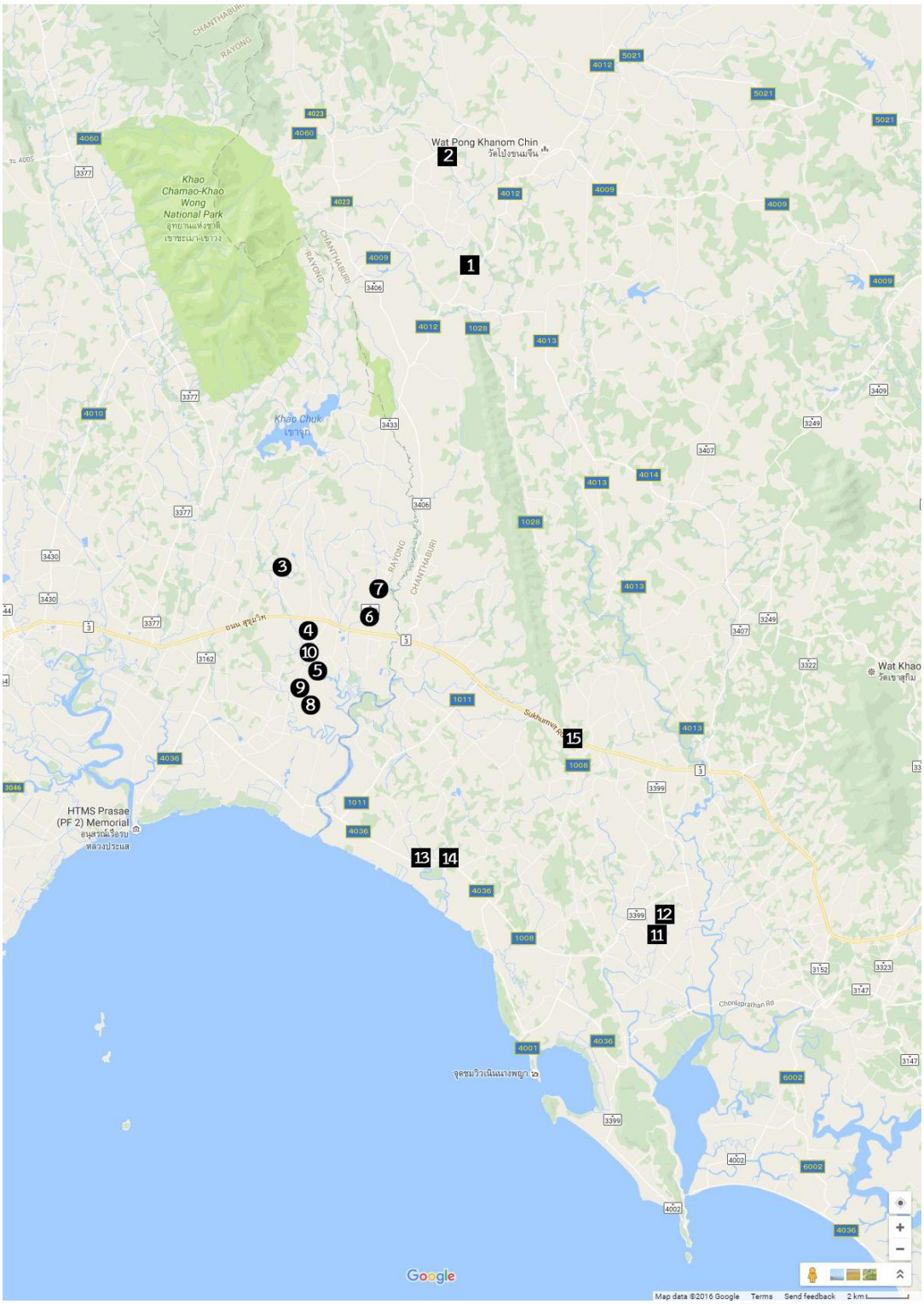

Figure 4.1 Sampling sites of rubber leaves showing ALF symptoms in eastern, Thailand. Square locate in Chanthaburi province. Circle locate in Rayong province.

The selective medium used in this study was formulated for selection of *Phytophthora*. According to Jeffers and Martin (1986), adding hymexazol at 50 mg/L in media should allow most *Phytophthora* including *P. palmivora* and *P. botryosa* to grow but inhibit most *Pythium* species. Nonetheless, not all *Pythium* was inhibited, and colony morphology of *Phytophthora* and *Pythium* are very similar on the agar medium if sporangium are not produced. As a result, some isolates recovered from diseased plant tissue in this study were confirmed later as *Pythium*, which are discussed later.

Phytophthora and *Pythium* colonies on selective medium showed colorless aerial mycelia at the center of the colony with flat hypha expanded from the agar plug and substrate mycelia appressed in agar surface (Figure 4.2). In early day of incubation, other competitive fungi and bacteria are clearly observed and then they were suppressed, and *Phytophthora* and *Pythium* colonies began to be recognized after a week of incubation. Hyphal tip of each isolate was transferred and maintained on V8 agar. According to the colony patterns discussed in Erwin and Ribeiro (1996c), a colony type of each isolate was listed in Table 4.2. There are 4 colony types found among all isolates including stellate, petallate, stellate-petaloid and rosaceous. Colony patterns of some samples are shown in Figure 4.3.

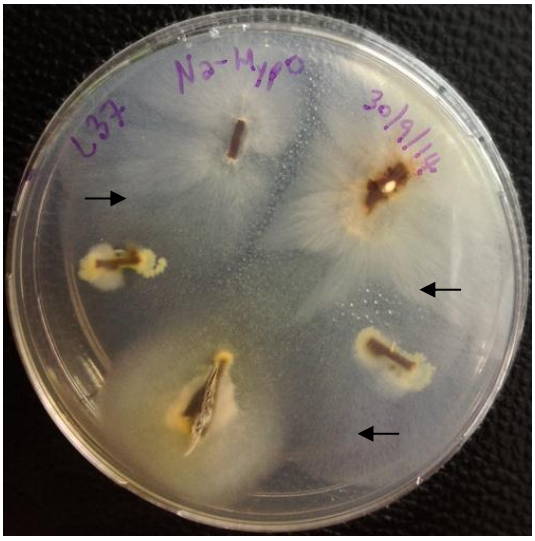

Figure 4.2 Pythiaceae isolation from petioles of para rubber leaf showing ALF symptoms on selective medium after 10 days of incubation. Arrow: Pythiaceae colonies.

Table 4.2 Colony patterns of isolated Pythiaceae on V8 agar

Colony pattern	Isolate
Stellate	R1, R2, R5, R6, R7, R8, R9, R10, R11, R12, R13, R20, R27, R29, R30, R31, R32, R33, R34, R35, R37, R38, R39, R40, R41, R42, R43, R44, R45, R46, R47, R48, R49, R51, R52, R53, R54, R55, R56, R59, R60, R63, R68, R69, R70, R72, R73, R75, R76, R77, R79, R81, R82, R84, R86, R87, R88, R89, R90
Petallate	R4, R15, R16, R17, R18, R19, R21, R22, R24, R25, R28, R36, R57, R61, R62, R65, R66, R67, R71
Stellate-petalloid	R3, R14, R26, R58, R91
Rosaceous	R23, R64, R74, R80, R83

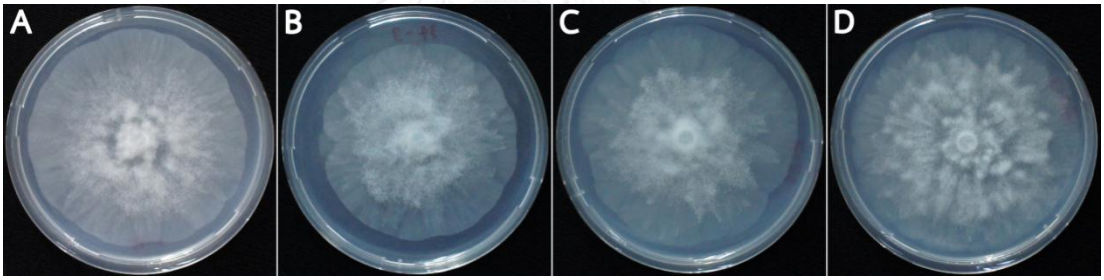

Figure 4.3 Colony patterns of isolated Pythiaceae on V8 agar after 5 days of incubation. (A) Stellate pattern of isolate R1. (B) Petallate pattern of isolate R4. (C) Stellate-petalloid of isolate R14. (D) Rosaceous pattern of isolate R23.

Each isolate was examined under the light microscope for the presence of non-septate hyphae. Some isolates produced sporangium on V8 agar after a week of incubation. Hyphal swelling can be seen in some isolates (Figure 4.4).

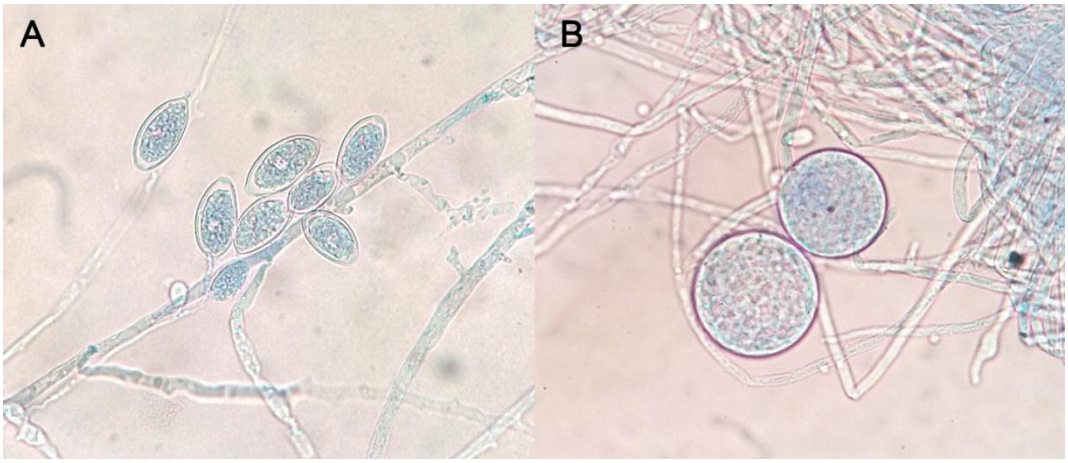

Figure 4.4 Typical vegetative structures produced by Pythiaceae isolated from rubber leaves showing ALF symptoms on V8 agar. (A) Aseptate hyphae and sporangium of isolate R1. (B) Hyphal swelling characteristic of isolate R31.

Some isolates did not produced sporangia on V8 agar even after a month of observation. Isolates with colony pattern of Pythiaceae and with aseptate hypha similar to typical colony morphology of Pythiaceae were expected as species of the family and were processed in further study together.

4.2 Mycovirus detection

The hosts of currently identified Pythiaceae viruses represent only a very small proportion of known Pythiaceae species. Clearly, none was from ALF agents of rubber tree. Scarce of knowledge in virus of Pythiaceae prompted me to search for DNA and RNA viruses in these pathogens. In the present study, total nucleic acid extraction was performed and searched for extrachromosomal elements focusing on DNA and dsRNA virus.

4.2.1 DNA mycovirus detection

To screen for DNA virus, the extracted total nucleic acid was treated with RNase A to remove contaminated RNA and visualized on agarose gel electrophoresis. From all isolates, none of them carried extrachromosomal DNA elements as shown in Figure 4.5.

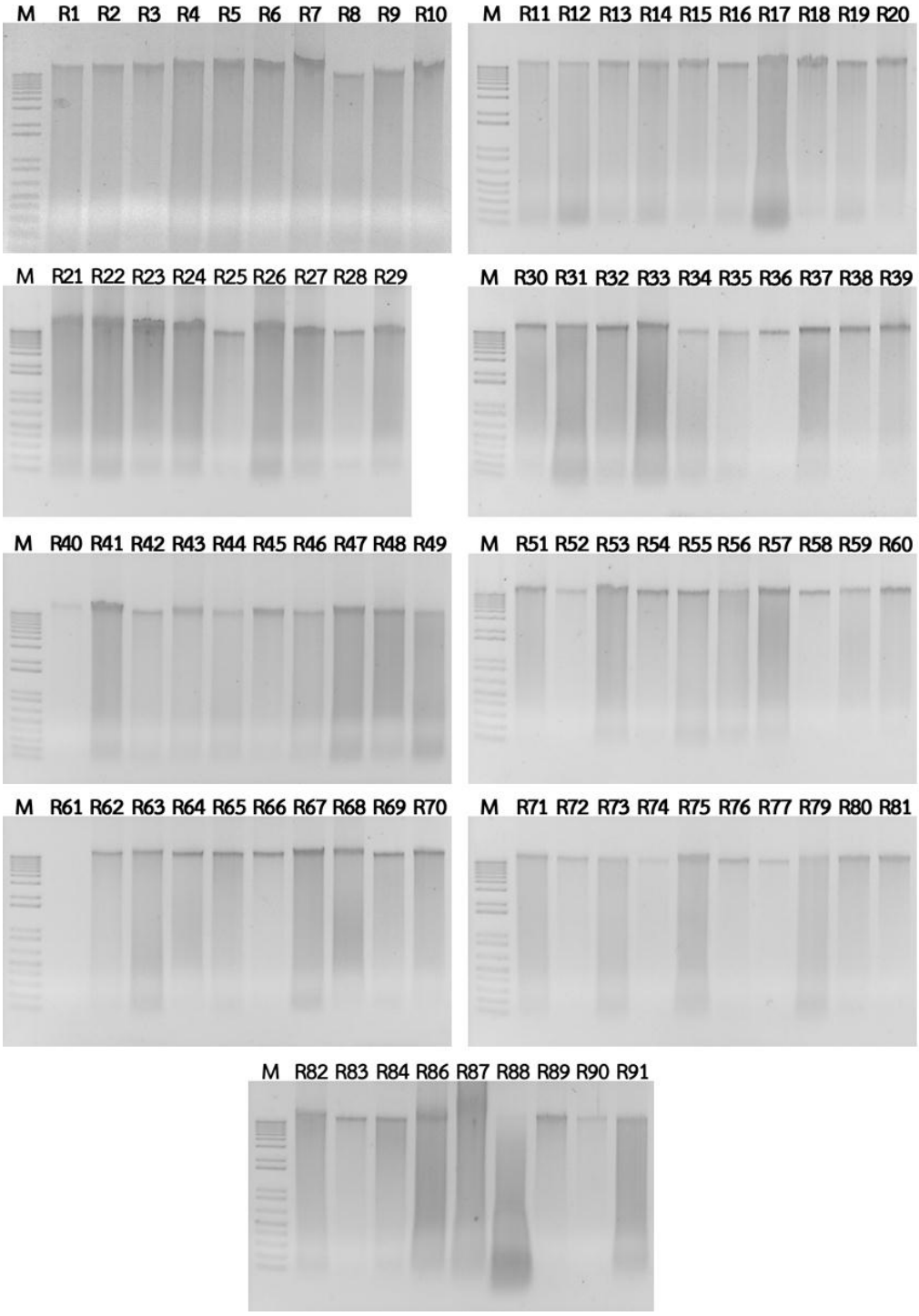

Figure 4.5 DNA mycovirus detection in Pythiaceae isolates. M: 1Kb Plus DNA Ladder (Invitrogen, USA).

DNA mycovirus was found less often than RNA mycovirus. The reason could relate to the transmission mode of most mycoviruses. Lacking extracellular transmission is general in RNA mycoviruses (Yu *et al*., 2013). Mycoviruses have to adapt for quiet long term living in their host (Ghabrial, 1998). Since oomycetes are eukaryotes, the DNA copying enzyme is located in nucleus where DNA replication takes place (Cooper, 2000; Zheng and Mackrill, 2016). Some DNA viruses can replicate their genomic materials only after their genome is integrated into host genome and taking advantage of host's DNA machineries but dsRNA viruses have some tricks by also using dsDNA replication enzyme such as topoisomerase I. Not only DNA viruses require host's topoisomerase to unwind their supercoil genomes to initiate viral replication, but also dsRNA viruses. For example, viruses infecting killer yeast also needs topoisomerase to maintenance their M dsRNA element encoding the killer toxin, making it would be too competitive for DNA viruses (Thrash *et al*., 1984; Wickner, 1996; Ghabrial, 1998). Moreover, some naked dsRNA viruses enclose their genomes in host vesicle throughout the life cycle and progeny is only packed with sense-stranded RNA genome in capsids (Hansen *et al*., 1985; Ghabrial, 1998).

Different mutation rates between DNA and RNA genome may be another possible reason that mycoviruses are mostly RNA virus. The coevolution hypothesis of the origin of mycoviruses is a coherent explanation especially for asymptom relationship between viruses and their fungal hosts. Mycoviruses and hosts have coadapted to a considerable degree for living together (Ghabrial, 1998). In this case, the viral nucleic acid mutation is considered. RNA virus has more mutation rate than DNA virus. RNA replication requires viral RNA-dependent RNA polymerase (RdRP), this enzyme lacks a proofreading activity (Choi, 2012) while DNA polymerase for DNA replication has 3'-5' proofreading activity to reverse its direction if the mismatch base is recognized and replace the mismatch with the correct base (Khare and Eckert, 2002). Sanjuan and colleagues (2010) studied mutation rate of 23 viruses. They collected statistical data of nucleotide substitutions per nucleotide per cell infection of each virus and revealed that DNA viruses have mutation rate between 10^{-8}-10^{-6} mutation per base pair per generation while that of RNA viruses are

10^{-6}-10^{-4}. Negative relationship between genome size and mutation rate was also pointed out. The larger genome size, the more mutation rate can be found. Genome of dsDNA viruses can be larger than 100 kb having high error rate than ssDNA and RNA viruses, these two viral genomes have smaller size which not larger than 32 kb (Holmes, 2011). These conclude may be related to DNA mycovirus which is uncommon in fungi.

4.2.2 RNA virus detection

4.2.2.1 Optimized conditions of cellulose column for dsRNA isolation

Cellulose column has been widely used for isolation of dsRNA from fungi since it was developed and discussed by Morris and Dodds (1979). The method based on the affinity of cellulose powder for nucleic acid in different concentration of ethanol. Frankin (1966) labeled RNA in viral ssRNA or dsRNA infected cell with isotope, extracted total nucleic acid, loaded into column containing cellulose slurry as a binding phase, and eluted nucleic acids with STE buffer containing different concentrations of ethanol. The eluents then were tested for RNase A resistance, and detected for radioactive signals. The author found that dsRNA could be eluted with buffer alone while ssRNA and denatured DNA were eluted by buffer containing 15% ethanol. An experiment by Levine and colleges (1963) revealed that ethanol gave 50% denaturation of DNA at 1.2 M or 7% (v/v) concentration.

To date, cellulose method has been change in many steps by various research groups to suit their works but all reports still based on the same principle. Cellulose powders with a difference in molecular weight and manufacturers have been used as a binding phase. The CF-11 cellulose (Whatman®) originally used by Morris and Dodds (1979) was discontinued. An alternative including a cellulose from Sigma (Catalog Number C6288) and Cellulose A (100-200 mesh) and D (40-100 mesh) (ADVANTEC, Japan) had been used (Das *et al*., 2014; Okada *et al*., 2015). In this study, cellulose from Sigma was available and was tested for dsRNA isolation with known dsRNA extracted from *E. coli*. The method was adapted from Das and colleagues (2014) and Chandavimol (2011). dsRNA produced by *E. coli* dsRNA-expressing strain was extracted and treated

with RNase A to remove mRNA and ssRNA. Then isolation of dsRNA was continued using cellulose column. To test the dsRNA affinity of the column, three fractions were collected for visualizing eluted dsRNA including loading fraction, wash fraction and elute fraction. The result are shown in Figure 4.6.

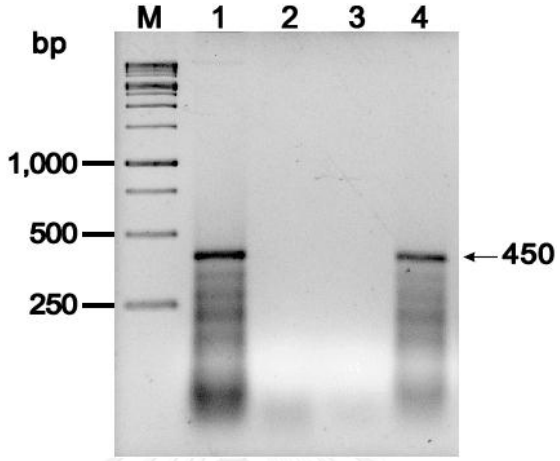

Figure 4.6 Affinity testing of cellulose column for isolation of dsRNA. M:1Kb DNA Ladder RTU (GeneDireX, Taiwan). Lane 1: control. Lane 2: loading fraction. Lane 3: wash fraction. Lane 4: elute fraction.

The extracted dsRNA from *E. coli* was mixed with pretreated cellulose in STE buffer containing 15% ethanol. At this ethanol concentration, dsRNA was assumed to bind with cellulose powder. In conventional protocol by Morris and Dodds (1979), cellulose was packed in the column first and then the extracted nucleic acid was loaded. In this study, the exposure time of cellulose and dsRNA was adjusted to be longer, referring to the study and protocol from Jacobson and Nishimura (1964) and Das and colleagues (2014) to increase the amount of dsRNA binding to cellulose by gently inverse mixing the slurry at room temperature for 5 minutes. To confirm, after the slurry was loaded into column device, the flow through was collected for checking if the dsRNA bound with cellulose. The flow through from washing fraction was also examined to ensure that dsRNA could not be eluted along with buffer containing 15% ethanol but could be eluted by the buffer alone. In Figure 4.6, control lane was the extracted dsRNA without cellulose column process. The amount of dsRNA that was loaded into the cellulose column was

equate to that in control, it was approximately 200 ng. There was no band detected in lane 2 and 3 from loading and wash fractions. This confirmed that dsRNA bound to cellulose in buffer containing 15% ethanol and could not be eluted with wash buffer at the same ethanol concentration. The band in lane 4 was eluted with buffer without ethanol. This eluted fraction contained dsRNA when elute with buffer alone but yield of the eluted dsRNA was found less than the initial amount used for cellulose column by half. The results showed that cellulose used in this study can be used instead of the traditional cellulose and the adapted protocol is usable for isolation of dsRNA from extracted nucleic acid in further study.

4.2.2.2 dsRNA detection in Pythiaceae

Double-stranded RNA was detected using the optimized cellulose column method explained above. From all 79 isolates, only one isolate, isolate R84, carried RNA extrachromosomal elements. The dsRNA containing strain was from a plantation in Chanthaburi province. The three dsRNA segments found in isolate R84 were approximately 2,320, 3,750 and 9,220 bp as shown in Figure 4.7 together with dsRNA-free strain on 1% agarose.

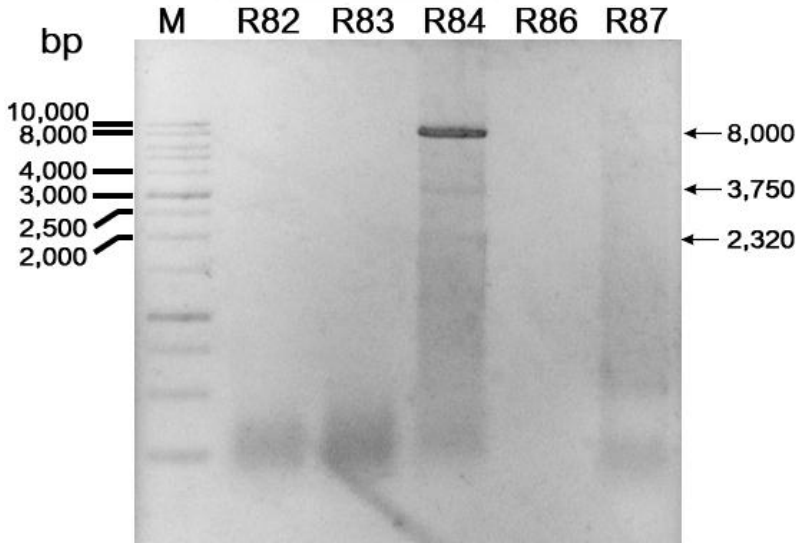

Figure 4.7 dsRNA fragments of isolate R84. M: 1Kb DNA Ladder RTU (GeneDireX, Taiwan)

4.2.2.3 Nuclease treatment of dsRNA

To determine whether the viral genome in isolate R84 is dsRNA. Treatments with 3 nuclease including DNase I, RNase A and S1 nuclease were performed. DNase I degrades single and double-stranded DNA (Holmfeldt *et al.*, 2012). RNase A digests different structure of nucleic acid in different conditions, it cleaves ssRNA, dsRNA and RNA-DNA hybrid in low salt buffer (0-100mM NaCl), while it cleaves only ssRNA in high salt buffer (0.3 M NaCl) (Cai *et al*., 2009). S1 nuclease hydrolyses ssRNA and ssDNA (Gite and Shankar, 1992).

dsRNA was treated with DNase I (0.1 U, 37°C, 10 minutes) and visualized on agarose gel. The results in Figure 4.8 showed that three dsRNAs elements from isolate R84 resisted to DNase I digestion. This suggests that the viral genome in isolate R84 are RNA. The band of 2.3 kb disappeared from the dsRNA control reaction. Appearance of this smallest fragment depended on different mycelium batches and was not always seen in multiple subcultures.

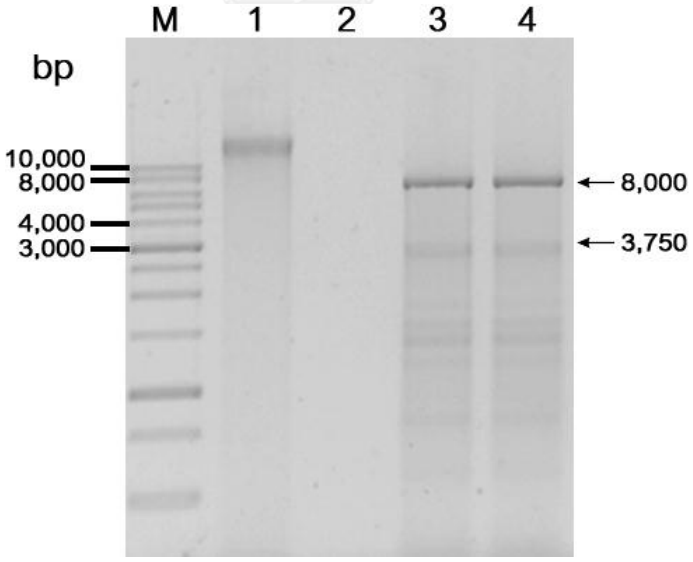

Figure 4.8 DNase I treatment of the viral genome from isolate R84. The viral genome was treated with DNase I (Bio Basic, Canada) 0.1 U at 37°C for 10 minutes. M: 1Kb DNA Ladder RTU (GeneDireX, Taiwan); Lane 1: DNA of *Phytophthora botryosa*; Lane 2: DNA of *Phytophthora botryosa* with DNase I treatment; Lane 3: dsRNA without DNase I; Lane 4: dsRNA with DNase I treatment.

To determine whether the viral genome is ssRNA or dsRNA, the viral genomes were treated with RNase A in different ionic strength and S1 nuclease. For RNase A digestion, the viral genome was treated in low salt (15 mM NaCl) and high salt buffer (300 mM NaCl). The results are shown in Figure 4.9A. With RNase A, there are 2 dsRNA segments with 8,000 and 3,750 bp found in high salt but disappeared in low salt condition, suggested that these 2 elements are dsRNA. The smaller bands with 2,320 disappeared in all condition including control fraction. This element could not be seen clearly since they were purified form the cellulose column due to the low amount and may be lost during reprecipitation process for salt removing before enzymatic treatment. S1 nuclease treatment was preceded but the dsRNA segments that was extracted successfully and resistant to S1 nuclease was the two largest bands (Figure 4.9B). Double-stranded RNA sample for S1 degradation was extracted from different lots of mycelia. Unfortunately, the smallest 2 segments were unstable and lost during repeated subculturing, only the largest one was permanently seen in multiple subcultures.

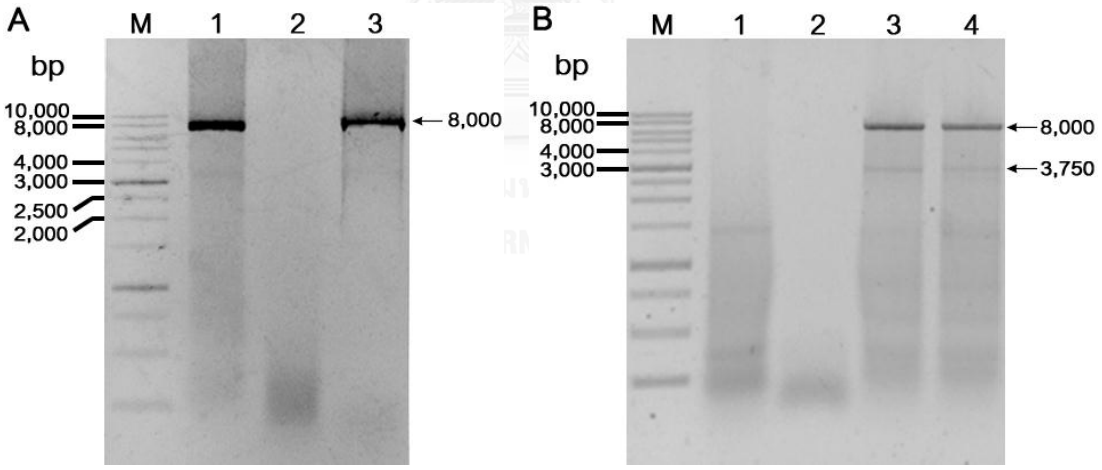

Figure 4.9 RNase A and S1 nuclease treatment of dsRNA from isolate R84. (A) dsRNA treatment with RNase A; Lane 1: control; Lane 2: dsRNA treatment with RNase A in low salt condition; Lane 3: dsRNA treatment with RNase A in high salt condition. (B) dsRNA treatment with S1 nuclease; Lane 1: Total RNA of *Phytophthora botryosa*; Lane 2: Total RNA of *Phytophthora botryosa* with S1 nuclease treatment; Lane 3: control dsRNA; Lane 4: dsRNA treatment with S1 nuclease. M: 1Kb DNA Ladder RTU (GeneDireX, Taiwan).

4.3 dsRNA identification

4.3.1 Detection of reported Pythiaceae viruses

To determine whether the dsRNA elements in isolate R84 are the same mycoviruses previously reported in Pythiaceae, two-step RT-PCR was performed. Random hexamer primer was used in first-strand synthesis and the following second-strand was proceeded using viral genome specific primers. Primer sets of 4 Pythiaceae viruses which have nucleotide sequences in GenBank were conducted in this study including PiRV-1, PiRV-3, PiRV-4 and PEV1. Primers for the first two viruses were designed in this study to target the entire open reading frame (ORF) of the viruses with RdRP gene located in the middle, while primers for the last two viruses were from Zhan and colleagues (2016) targeting the entire ORF of PiRV-4 genome and from Kozlakidis and colleagues (2010) for detecting UGT gene towards the helicase-like region of PEV1. The predicted amplicon lengths are approximately 940 base pairs for PEV1 and approximately 3,000 base pairs for PiRVs. Another primer sets for detecting Pythiaceae viruses were designed in this study to target only RdRP region. The expected size of products were shorter than the first primer sets, approximately 590 bp, 480 bp, 720 bp and 520 bp for PEV1, PiRV-1, PiRV-3 and PiRV-4, respectively. DNase I-treated total RNA was use as a template. Quality of RNA was confirmed with the presence of product form β-tubulin primers. The results are shown in Figure 4.10.

Since the dsRNA found in this study was unknown, procedure for detecting known viruses needed to be careful in case of the negative RT-PCR result. Use of intact RNA as a template for RT-PCR was checked. The reactions of housekeeping gene β-tubulin were conducted to determine the quality of RNA as a template for RT-PCR reaction and gave a product approximately 568 bp in a positive reverse transcriptase reaction (+RT) but showed no product in a negative control without reverse transcriptase (-RT). These indicated that there was no genomic DNA contaminated in total RNA sample to give a false positive result and the RNA template used for the reaction was in a good quality. The HeLa reaction was recommended by manufacturer of the SuperScript III First-

Strand Synthesis Kit (Invitrogen, USA) to confirm if the reverse transcription mixture was not mistaken.

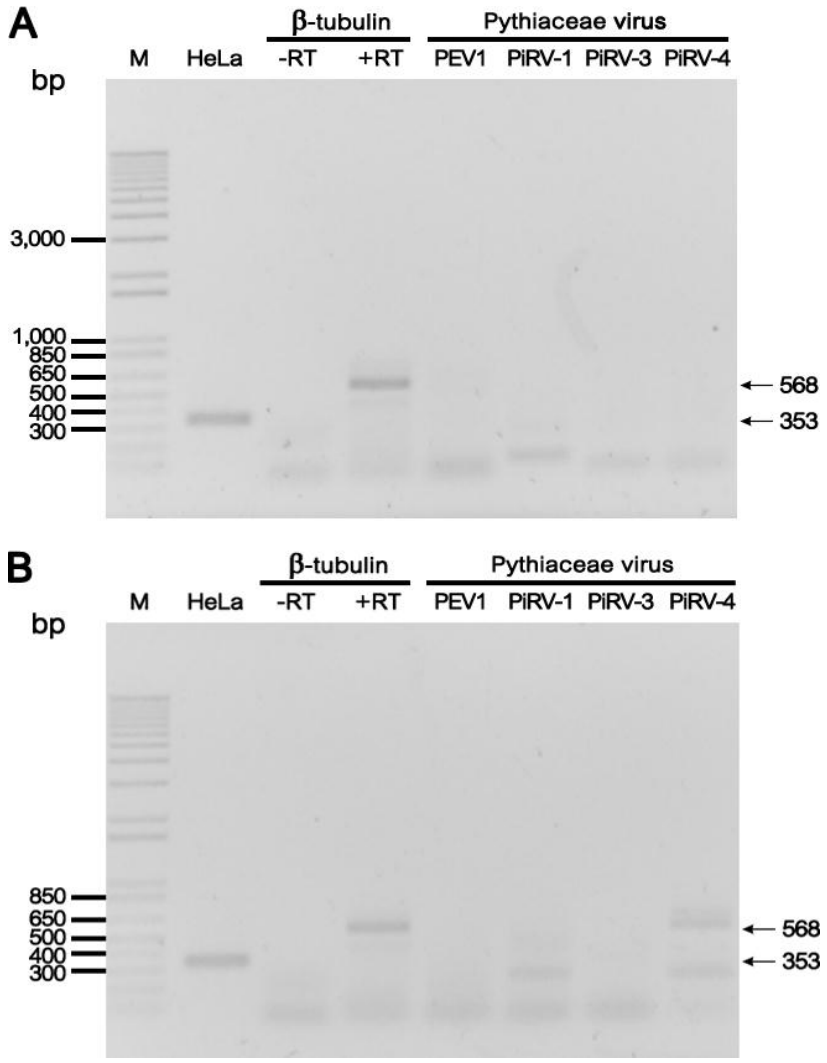

Figure 4.10 Detection of reported Pythiaceae viruses from total RNA of isolate R84. (A) Two-step RT-PCR using the primer sets for the large amplicons (UGT-helicase region of PEV1 and the entire ORF of PiRVs). (B) Two-step RT-PCR using primers targeting only RdRP region of the viruses. M; 1Kb Plus DNA Ladder (Invitrogen, USA).

To determine whether dsRNA virus of the isolate R84 is known Pythiaceae viruses using RT-PCR reactions, first-strand step was carried out using random hexamer primer and the virus specific primers were used separately for each virus species in second-

strand synthesis reaction. KOD hot start DNA polymerase was used in second-strand step instead of *Taq* DNA polymerase. The hot start DNA polymerase has reduced non-specific amplification and showed high extension rate with high fidelity than the conventional *Taq* enzyme (Ishino and Ishino, 2014; McInerney *et al*., 2014). The first primer sets were used for detecting the large RNA fragments which would give more sequence information for cDNA sequencing if the bands at the expected size are present. The result in Figure 4.10A showed no band in all Pythiaceae viruses reactions. However, because there was no coding sequence of *Pythium* housekeeping gene in GenBank available for designing primer to give the control product with similar size of the viral entire ORF reaction. No cDNA from the first primer sets could mean that the RT-PCR was not effective enough to amplify a long fragment. RT-PCR experiment later with designed primers targeting RdRP region with the fragment size closer the control was performed. There was no band with predicted size of viral targets presented in any primer sets (Figure 4.10B). Non-specific bands found in PiRV-4 were between 300-400 bp and 650-850 bp, and were not of the known viral's RdRP sizes. Self-priming test as described by Farrell (2005) were performed to confirm that the non-specific bands of PiRV-4 reaction were not from viral genome as shown in Figure 4.11.

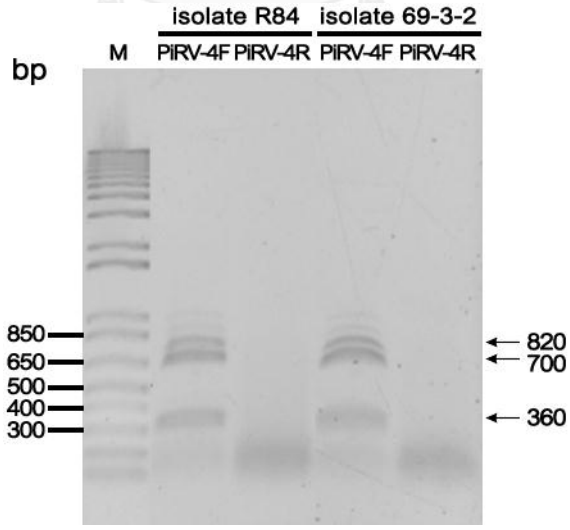

Figure 4.11 PiRV-4 self-priming test. Total RNA of isolate R84 (containing dsRNA) and isolate 69-3-2 (dsRNA-free) were used as a template for primers self-priming test. The tested primers were PiRV-4F (Forward primer) and PiRV-4R (Reverse primer). M; 1Kb Plus DNA Ladder (Invitrogen, USA).

DNase I-treated total RNA of isolate R84 containing dsRNA and isolate 69-3-2 which is dsRNA-free were used for PiRV-4 self-priming test. Isolate 69-3-2 was also isolated from rubber leaves showing ALF symptoms but was collected from different sampling sites, and was identified later as the same species of isolate R84 and contained no dsRNA element. There were cDNA bands presented in self-primer test of PiRV-4's RdRP Forward primer in both dsRNA-containg and dsRNA-free strain, approximately 360, 700 and 820 bp, while the predicted RdRP target was 520 bp. These confirmed that the non-specific bands were not the results from viral genome as a template. Moreover, the size of all 3 dsRNA segments found in isolate R84 including 8.0, 3.7 and 2.3 kb were in different sizes from those of reported Pythiaceae viral genomes (Table 2.7). These suggested that the dsRNA hidden in isolate R84 was not one of reported Pythiaceae viruses.

4.3.2 Partial sequence of dsRNA

The largest dsRNA segment with 8.0 kb was used to generate cDNA libraries in this study because it was stable in later subcultures. Double-stranded RNA isolated from the cellulose column was treated with S1 nuclease to remove ssRNA from host's mRNA before cDNA was synthesized. First-stranded synthesis was carried out using random hexamer primer due to the dsRNA was unknown and the procedure of second-strand synthesis was adapted from D'Alessio and Gerard (1998) via RNase H-DNA polymerase I-mediated second-strand cDNA synthesis. After the first-strand synthesis step, DNA-RNA hybrid was generated. Then RNase H and DNA polymerase I were combined in the reaction. RNase H help generating primer for synthesis of second-stranded cDNA by nicking RNA in DNA-RNA hybrid molecules (Pallan and Egli, 2008). DNA polymerase I with 5' to 3' polymerase activity uses nicked RNA as a template to extent the complementary strand, and the nicks are sealed by T4 ligase activity (Rittié and Perbal, 2008). After ligation and transformation, plasmids were screened, extracted and sequenced. There was a clone from all 28 clones found containing a fragment of 233 nt which could be predicted to encode amino acid (aa) sequence of 77 aa in direction of 3'

to 5' Frame 2. The clone was named clone 26. Nucleotide and amino acid sequences of the clong 26 are shown in Figure 4.12 and 4.13, respectively.

```
CGGCCACCACGCGCTCTCGTCCGCTCCAACCAAGGCCCAACGCCAGCAACCGCCGCCTCGCAAAA
GCAAGAACGACAATCACTGACTGAGGTGTCAAGAAGACATCCAGCATCTGCCACCACCAAAGATC
TGCCAACCCGAAGGCGACACACCTAAGGCAACGACAGACACCGAATACCCTCCATCTTCCACCCC
TGGAACCCAAGCCGAAACATGACGAGCGCACGCGTGGC
```

Figure 4.12 cDNA sequence of clone 26

```
PRVRSSCFGLGSRGGRWRVFGVCRCLRCVAFGLADLWWWQMLDVFLTPQSVIVVLAFARRRLLAL
GLGWSGRERVVA
```

Figure 4.13 Predicted amino acid sequence of cDNA from clone 26

Nucleotide of cDNA from clone 26 showed no significant similarity with data in GenBank. Amino acid sequence was predicted and compared to sequence databases BLASTP (NCBI). Amino acid sequences from database showing highly similar to cDNA of clone 26 and statistical significance are listed in Table 4.3.

Table 4.3 Amino acid sequences producing significant alignments to cDNA of clone 26

Description		Accession Number	Identity
1	PREDICTED: alpha-1,3/1,6mannosyl-transferase ALG2 [*Brachypodium distachyon*]	XP_010240336.2	15/38(39%)
2	Alpha-1,3-mannosyltransferase ALG2 [*Aegilops tauschii*]	EMT12485.1	15/38(39%)
3	PREDICTED: alpha-1,3/1,6-mannosyl-transferase ALG2-like [*Setaria italica*]	XP_004972818.1	15/38(39%)
4	PREDICTED: alpha-1,3/1,6-mannosyl-transferase ALG2-like [*Setaria italica*]	XP_004954295.1	15/38(39%)
5	alpha-1,3/1,6-mannosyltransferase ALG2 [*Aegilops tauschii* subsp. tauschii]	XP_020155914.1	15/38(39%)
6	predicted protein [*Hordeum vulgare* subsp. vulgare]	BAK02906.1	13/38(34%)
7	PREDICTED: alpha-1,3/1,6-mannosyl-transferase ALG2 [*Oryza brachyantha*]	XP_006652716.1	13/38(34%)
8	hypothetical protein AURDEDRAFT_ 112045 [*Auricularia subglabra* TFB-10046 SS5]	XP_007341101.1	16/40(40%)
9	PREDICTED: alpha-1,3/1,6-mannosyl-transferase ALG2-like [*Setaria italica*]	XP_004976635.1	14/34(41%)
10	PREDICTED: alpha-1,3/1,6-mannosyl-transferase ALG2 [*Oryza sativa* Japonica Group]	XP_015635731.1	12/38(32%)

Surprisingly, the amino acid sequences from database showing significant similarily to cDNA of clone 26 are all mannosyltransferase protein from plants except one

hypothetical protein from the jelly fungi, *Auricularia subglabra* but all identity scores were low. Alpha-1,3/1,6-mannosyltransferase or ALG2 protein is a member of glycosyltransferase enzyme, involved in the protein glycosylation pathway (Kampf *et al.*, 2009). ALG2 catalyses the formation of lipid-linked oligosaccharide precursor for *N*-linked glycosylation which is a common protein modification process in eukaryotes and archaea (Burda and Aebi, 1999). The study of ALG2's function revealed that this enzyme played important roles in cell wall integrity in fungi and cell cycle regulation in yeast and human (Motteram *et al.*, 2011). The substrate-specific mannosyltransferase protein family has not been reported in virus but there were glycosyltransferases reported in few DNA viruses; bacteriopharge, baculoviruses, herpesviruses, hycodnaviruses and poxviruses (Markine-Goriaynoff *et al.*, 2004). The glycosyltransferase protein in RNA virus has been only predicted in Pythiaceae virus. Polyprotein analysis of PEV1 showed that polyprotein encoded by this virus contained 524 aa of putative UDP glycosyltransferases (UGTs) which showed significant alignments with bacterial and fungi UDP-glucose: sterol glucosyltransferases and the highest similarity score belonged to the protein of the fungus, *Ustilago maydis*. Protein analogue sequence analysis of other species in endornavirues revealed two viruses in the genus, bacteriopharge and plant virus, also contained UGT motifs (Hacker *et al.*, 2005). The concept of 'glycovirology' was reviewed by Markine-Goriaynoff and colleagues (2004), one mechanism that viruses use to modify sugar molecules is by expressing their own glycosyltransferase for many advantages. In the review of glycosyltransferase produced by DNA viruses, the protein involves in protecting viral DNA form host's nuclease, conversion of host's antigen to prevent coinfection by other phages, post-translational modification of capsid protein, interfering with host's life cycle by inactivation of host's hormone (O'Reilly and Miller, 1989; Vrielink *et al.*, 1994; Byl and Kropinski, 2000; Graves *et al.*, 2001). Function of glycosyltransferases encoded by RNA viruses has not been studied. The motifs were predicted in silico and still remains as a putative protein (Hacker *et al.*, 2005).

Glycosyltransferases are general in both prokaryotes and eukaryotes (Breton *et al.*, 2006). For viruses, viral glycosyltransferase genes are believed to be obtained from

hosts based on the long-term coevolution hypothesis, especially for mycoviruses which the transmission mode is generally limited to intracellular routes and living quietly in their host is a normal (Markine-Goriaynoff *et al*., 2004; Son *et al*., 2015). Once acquired from host, it is possible for the viruses to maintain the gene or evolve with their host if the gene functions and gives benefit to them. As same as a putative mannosyltransferase found in this study that the coevolution may be the possible hypothesis, comparison between protein sequence of clone 26 and ALG2 mannosyltransferase produced by species in oomycetes which have amino acid sequences of ALG2-like protein in NCBI database was performed. Unfortunately, there was no sequence of ALG2 produced by *Pythium* and *Phytopythium* for making an alignment. Available sequences were from genus *Saprolegnia* (*S*.), *Albugo* (*A*.), *Aphanomyces* (*Ap*.) and *Phytophthora* (*P*.), the results are shown in Figure 4.14.

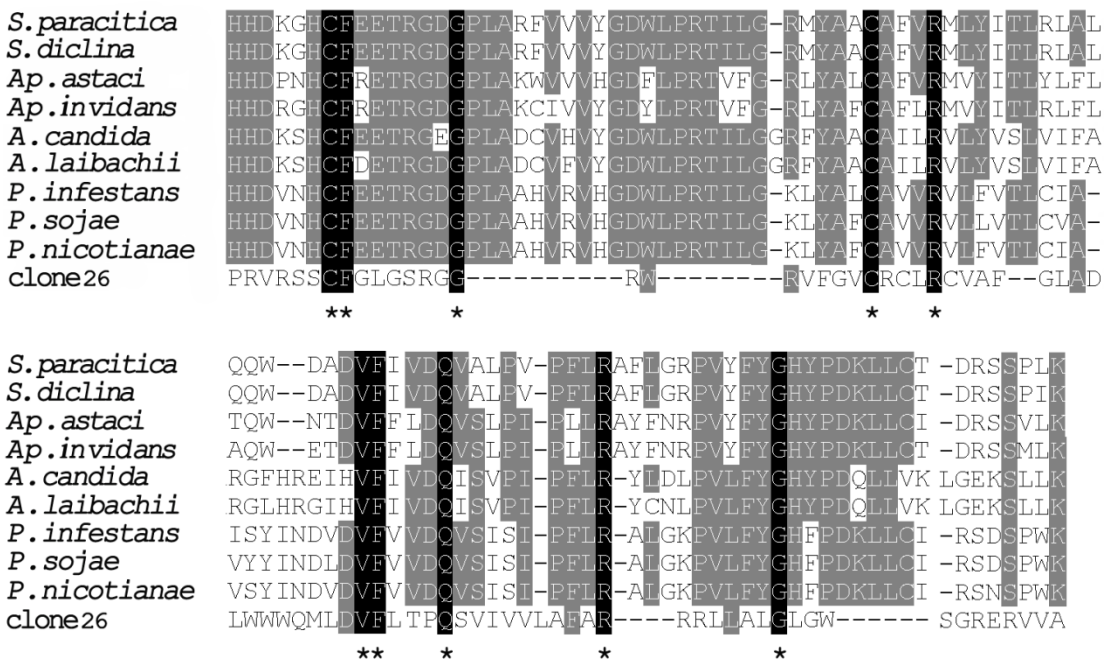

Figure 4.14 Multiple alignment of amino acid sequence of the ALG2-like regions of clone 26 and species in oomycetes. Residues identical in all sequences are presented in black background and asterisk below the sequences. Sequences in grey background are highly conserved among species in oomycetes.

Multiple alignment of amino acid sequence of the ALG2-like regions showed that sequence homology of clone 26 and species in oomycetes was very low, suggested that ALG2 gene of the dsRNA found in isolate R84 has not been obtained recently from the host genome.

Amino acid sequence of the ALG2-like protein of clone 26 was also aligned with mannosyltransferase from plants (Figure 4.15). The result showed that the protein of clone 26 had higher sequence homology to the protein from plants than oomycetes. The hypothesis of plant viruses as an origin of mycovirus might be involved. The viral mannosyltransferase gene may be obtained from plant once the virus originated and living in the plant before moving to fungus (Son *et al.*, 2015).

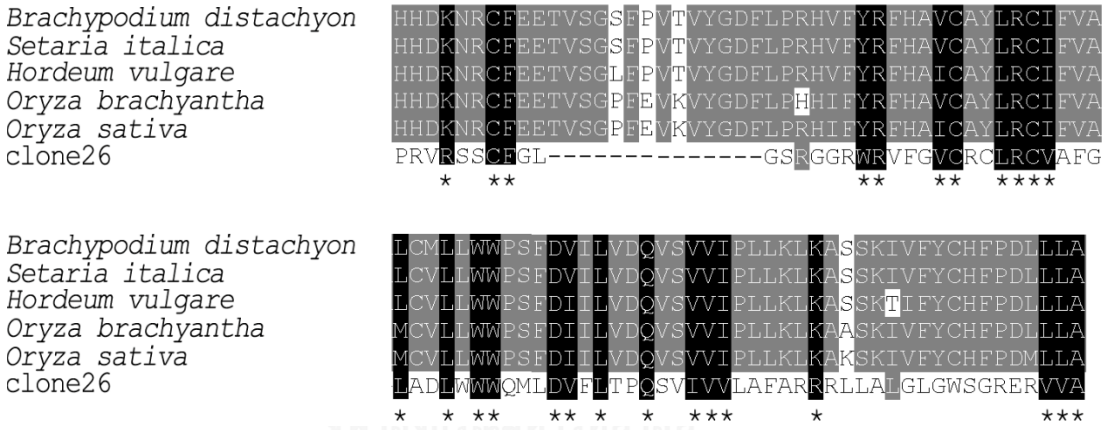

Figure 4.15 Multiple alignment of amino acid sequence of the ALG2-like regions of clone 26 and plants. Residues identical in all sequences are presented in black background and asterisk below the sequences. Sequences in grey background are highly conserved in plants.

Because the ALG2-like nucleotide sequence of the dsRNA is not a full length, making it difficult to identify the genus of dsRNA. Inaddition, there has not been reported viral genome containing ALG2 gene for comparison. Making cDNA library to detect other viral genes such as capsid protein of RdRP region or analysis of whole genome sequence should be conducted.

4.4 Identification of the dsRNA host

The DNA of dsRNA containing strain was extracted and amplified with conventional PCR using the fungi and oomycetes universal primers, ITS1 and ITS4, which the binding site located in conserved non-coding region between 18S, 5.8S and 28S ribosomal RNA genes (White *et al*., 1990) . The results shown in Figure 4.16A. *Aspergillus nidulans* gave a product of 530 bp while *P. botryosa* was 870 bp. Isolate R84 showed a product approximately 900 bp. Product size from ITS1/ITS4 primers of some *Phytophthora* and *Phytopythium* species were listed in Table 4.4. The ITS product of isolate R84 was larger than the range between 841-892 bp of *Phytophthora* and *Phytopythium* which have been reported causing disease in rubber tree. The ITS product was diluted to avoid a false positive result from ITS primers carry over and then amplified by nested-PCR using A2/I2 primers targeting the inner sequence of ITS product. A2/I2 primers are the genus *Phytophthora*-specific (Drenth *et al*., 2006). There was no band detected from isolate R84 (Figure 4.16B). The results suggested that isolate R84 was not the genus *Phytophthora* and may be one of other oomycetes that has not been reported associated with rubber tree.

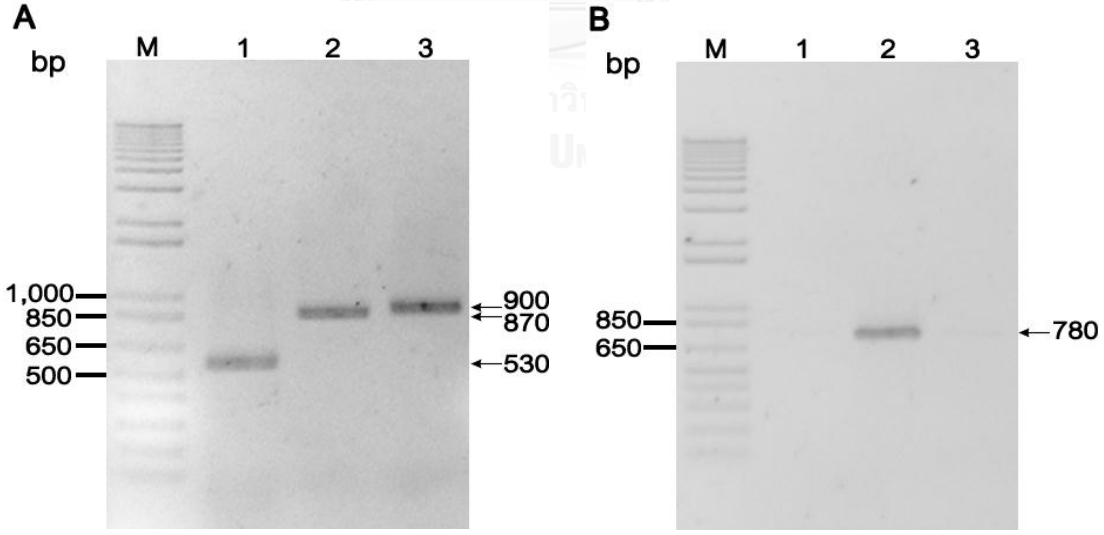

Figure 4.16 PCR products of fungi and oomycetes universal primers, and *Phytophthora* specific primers of isolate R84. (A) PCR product of ITS1/ITS4 primers. (B) Product from ITS-nested PCR with A2/I2 primers. M: 1Kb Plus DNA Ladder (Invitrogen, USA); Lane 1: *A. nidulans*; Lane 2: *P. botryosa*; Lane 3: isolate R84.

Table 4.4 Predicted product size of primer ITS1/ITS4 of Pythiaceae reported associated with rubber tree

Primers	Organism	Accession number	Product size (bp)
ITS1/ITS4	*Phytophthora botryosa*	KC247929.1, EU079939.1	870
	Phytophthora capsici	KJ576799.1	841
	Phytophthora citrophthora	GU133068.1	873
	Phytophthora meadii	KC247925.1, EU079892.1, LC076470.1	868
	Phytophthora nicotianae	KJ494920.1	892
	Phytophthora palmivora	KF263691.1	875
	Phytopythium vexans	KX098471.1	882

Table 4.5 Predicted product size of primer A2/I2 of *Phytophthora* reported associated with rubber tree

Primers	Organism	Accession number	Product size (bp)
A2/I2	*Phytophthora botryosa*	KC247929.1	782
	Phytophthora capsici	KJ576799.1	753
	Phytophthora citrophthora	GU133068.1	783
	Phytophthora meadii	LC076470.1	780
	Phytophthora nicotianae	KJ494920.1	804
	Phytophthora palmivora	KF263691.1	788

To identify genus and species of isolate R84, PCR product of ITS primers were ligated into a commercial vector and transformed into *E. coli* competent cells. The transformants were selected and plasmids of three ITS positive clones were extracted and

sequenced. The DNA sequence of ITS1/ITS4 primers product of isolate R84 was shown in Figure 4.17.

```
TCCGTAGGTGAACCTGCGGAAGGATCATTACCACACCTAAAAAACGCCCTTCCACGTGA
ACCGTTTTGTTTTGCTTTCGAGTGCTTTGTTGCGCTCGGAGCATGTTTTGGGCTTCGCT
GCTGGCGCTTGATTGTGCTGGCGGCTCGAGGCCATCAAGTGGCGTTTTGAGTGTGCTTT
GCGCAATTGAAACGTCGAAACCTTTTTTTTAAACCCATTTGATTGAAAACTGAAGTATA
CTGTGGGGACGAAAGTCCTCGCTTTGAAACTAGATAACAACTTTCAGCAGTGGATGTCT
AGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGAT
TCAGTGAGTCATCGAACTTTTGAACGCATATTGCACTTTCGGGTTACGCCTGGAAGTAT
GTCTGTATCAGTGTCCGTACACTAAACTTGCGTCTCTTCCGTCGTGTAGTCGTCGGTTG
TTTTGATTGCAGATGTGAGGTTGTCTCGCAGTCGACCACTTCTTTTGGATGGATAGGTT
GCGAGTCCCTTTAAAAGTCGGACGCGTGTTTTTTCCGTTTTGTGCTTGATGGGGGTGCG
GCTGCGGCCGTGTCTGCTGGCGGGTCCGGTGACCTTTGGCGATGGCATGAGAGTGGATT
GCTCGATTTGCGGTATGTTAGGCTTCGGCTTTGACAATGCAGCTTATTGGGTGTGTTCG
CTTGGCTGTTGCTGTATGGGGTGAGCTGGATGGTCGGTGGATGCGTTTGTTGCGTGTCG
TTTTTTCATGGAGTGCGTTGCGGTTGTCGTCGCCATTTGGGAATTTAATGTTTTGAGTC
TCGATTCAATACATCTCATTTGGACCTGATATCAGACAAGATTACCCGCTGAACTTAAG
CATATCAATAAGCGGAGGA
```

Figure 4.17 DNA sequence of PCR product of ITS1/ITS4 of isolate R84

Sequencing result of PCR product from ITS1/ITS4 primers of isolate R84 was 904 bp in length, not in the length of Pythiaceae that were previously reported causing disease in rubber tree. Sequence similarity searching in GenBank was conducted using nucleotide BLAST (NCBI), optimized program for highly similar sequences (megablast). Surprisingly, product of ITS1/ITS4 primers of isolate R84 showed high similarity to gene between 18S rRNA gene, ITS1, 5.8S rRNA gene, and ITS2, and 28S rRNA gene of *Py. cucurbitacearum*, not one of known rubber tree-associated *Phytophthora* or *Phytopythium* species (Table 4.6).

Table 4.6 DNA sequences ITS1/ITS4 producing significant alignments to isolate R84

Organism		Accession Number	Identity
1	*Pythium cucurbitacearum* isolate 1241Pc	HQ237483.1	902/904 (99%)
2	*Pythium cucurbitacearum* isolate Pp37-Wera 3	KP183959.1	901/905(99%)
3	*Pythium cucurbitacearum* isolate Pp09-Luwu Utara 2	KP183937.1	898/906 (99%)
4	*Pythium cucurbitacearum* isolate Pp35-Kaumrejo 1	KP183957.1	899/907 (99%)
5	*Pythium cucurbitacearum* isolate Pp18-Mekarsari	KP183946.1	861/868 (99%)
6	*Pythium cucurbitacearum* isolate Pp27-Amban	KP183954.1	881/899 (98%)
7	*Pythium cucurbitacearum* isolate PCTu238	HQ237482.1	889/908 (98%)
8	*Pythium cucurbitacearum* strain CBS 748.96	AY598667.1	864/876 (99%)
9	*Pythium* sp. P16681	GU983646.1	850/859 (99%)
10	*Pythium cucurbitacearum* isolate Pp33-Anjungan	KP183955.1	880/909 (97%)

Py. cucurbitacearum was first reported in 1941, causing damping-off disease in cucumber in Japan (Takimoto, 1941). This species of *Pythium* has been known as a mysterious species because it has rarely been reported. Moreover, the ex-type strains had died, no viable culture is existed, and not many internal transcribed spacer (ITS), cytochrome oxidase I (COXI or COI) and II (COXII), and β-tubulin sequences in GenBank are available (Spies *et al*., 2011). Since the first discovery in 1941, a few publications of *Py. cucurbitacearum* have been published, reported associated with point gourd (*Trichosanthes dioica*) in India, common bean in Rwanda, leaves submerged in sea water near mangrove habitat in China and durian in Indonesia (Chaudhuri, 1975; Nzungize *et*

al., 2011; Ho *et al*., 2012; Santoso *et al*., 2015). *Py. cucurbitacearum* has been mentioned closely related to *Pythium vexans* by sharing various identical characteristics (Santoso *et al*., 2015). Lévesque and De Cock (2004) grouped *Py. vexans* and *Py. cucurbitacearum* together in *Pythium* clade K along with *Py. boreale*, *Py. ostracodes*, *Py. oedochilum*, *Py. chamaehyphon*, *Py. helicoides*, and *Py. indigoferae* based on the similarity of ITS sequences. The common morphology of the clade K is producing globose sporangia. Lévesque and De Cock (2004) noted that many species in clade K have papilla but sporangia illustrated by Santoso and colleagues (2015) showed the most sporangia of *Py. cucurbitacearum* are non-papillate. Sporangial morphology of the clade K is varied; globose, ovoid, obpyriform or pyriform, and may be terminal, intercalary or filamentous inflated sporangia which only found in *Py. indigoferae* (Lévesque and de Cock, 2004; Tao *et al*., 2011; Yu *et al*., 2016). Classification of *Py. cucurbitacearum* has been unclear, Dick (1990) even suggested that it might be a member in the genus *Phytophthora*. In the field of *Phytophthora*'s protein study, the inducer of defense response in tobacco named elicitin are thought to be a protein family specifically secreted by *Phytophthora* species but later found to be produced by some *Pythium* species including *Py. vexans* and *Py. oedochilum* in clade K, and *Py. marsipium* in clade E. As a result, differentiation among the genus in Pythiaceae should be revisited (Yu, 1995; PanabiÈRes *et al*., 1997; Lévesque and de Cock, 2004). Nowadays, elicitin proteins is general in *Phytophthora* and *Pythium* because the proteins also reported in *Pythium* including *Py. oligandrum* in sugar beet and *Py. insidiosum* from clinical sample. These two are in clade D and C, respectively (Lévesque and de Cock, 2004; Takenaka *et al*., 2006; Lerksuthirat *et al*., 2015). The presence of elicitin proteins in *Py. vexans* is not enough to fill the question if species in *Pythium* clade K belong to the genus *Phytophthora*.

The doubtful in classification of *Py. vexan*s group was generally concluded in many reports due to the difference in phylogenetic analysis of various genes. *Py. vexans* was separated from *Phytophthora* and other *Pythium* species by the study of large-subunit (LSU) ribosomal DNA and ITS sequences carried out by Briard and colleagues (1995) and Cooke and colleagues (2000). An early article about phylogenetic relationship of

Pythium spp. was assessed by Martin (2000) using COXII mitrochondrial gene shown that the representative isolates can be divided into 3 clades from 27 species, however, this study did not include the *Py. vexans* group. Combination of morphological and phylogenic analysis using nucleotide data of ITS gene from GenBank among 116 strains of *Pythium* by Lévesque and De Cock (2004) was the first report sorting *Pythium* into 11 clades named A - K, this report included *Py. vexans* group (clade K), which located between *Phytophthora* spp. and other 10 clades of *Pythium*. The distinct group of *Pythium* clade K was confirmed in phylogenetic tree reconstructed by Villa and colleagues (2006) with adding analysis of partial COXII and β-tubulin sequences, and the clade was later established as a new genus and named *Phytopythium* by Bala and colleagues (2010) or a synonym as *Ovatisporangium* denominated by Uzuhashi and colleagues (2010).

Even though the *Pythium* clade K was named the genus *Phytopythium* but not all species in the group was renamed and *Py. cucurbitacearum* was that one. There are 21 species from former clade K and new established species associated in the genus *Phytopythium* up to now with *Phytopythium sindhum* as a type species (Mycobank; access on April 15, 2017). The data published by de Cock and colleagues (2015) described nomenclature changing from *Pythium* clade K to the genus *Phytopythium* including *Phytopythium vexans* (formerly *Py. vexans*) but *Py. cucurbitacearum* was excluded in the phylogenetic analyses because researchers could not obtain a viable culture of representative strain of *Py. cucurbitacearum* cbs 748.96 from fungal culture collection for morphological study. Moreover, organismal description of *Py. cucurbitacearum* in GenBank still uses the traditional name of the genus as *Pythium* (Nucleotide search for *Pythium cucurbitacearum*; access on April 15, 2017). Therefore, genus of isolate R84 containing dsRNA in this study has remained as *Pythium cucurbitacearum*.

Evolutionary analyses of isolate R84 with *Phytopythium*, *Pythium* clade A-J and *Phytophthora* was performed based on the nucleotide sequence of ITS1/ITS4 products. The phylogram was Figure 4.18.

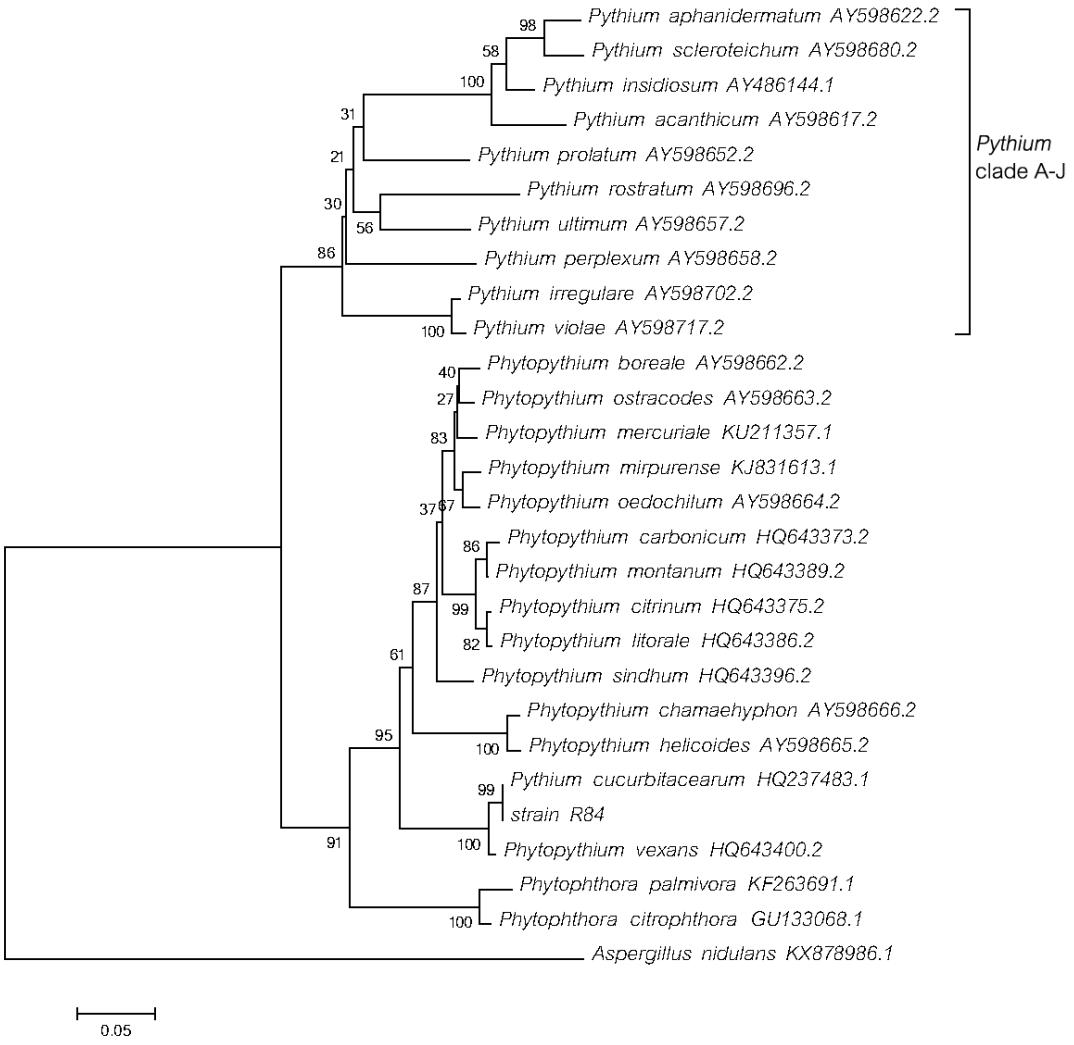

Figure 4.18 Phylogram of ITS1/ITS4 products which located between 18S rRNA gene, ITS1, 5.8S rRNA gene, and ITS2, and 28S rRNA gene of isolate R84, *Pythium* clade A-J, *Phytopythium* spp., *Phytophthora* spp. and *A. nidulans* as an out group. Multiple alignment was analyzed by Clustal Omega and phylogenetic tree was conducted by Mega 6 using the Neighbor-joining method with 1000 bootstrap replications.

Isolate R84 is classified closest to *Py. cucurbitacearum* isolate 1241Pc (HQ237483.1) and clustered in the same group with *Phy. vexans* which is more closely related to the genus *Phytophthora* than *Pythium*, similar to the previously report by Lévesque and De Cock (2004).

Morphological characteristics of *Py. cucurtbitacearum* isolate R84 was observed on various agar medium. Strain 84 showed variability in colony patterns on different media as shown in Figure 4.19.

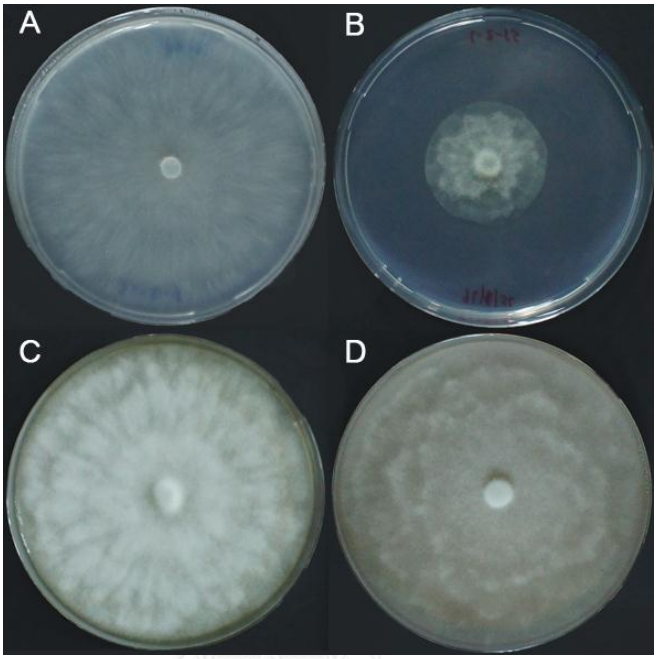

Figure 4.19 Colony morphology of isolate R84 on various media. (A) Stellate pattern on V8 agar. (B) Petallate pattern on V8 agar supplemented with antibiotics. (C) Cotton-stellate pattern on PDA. (D) Petalloid-stellate pattern on black bean agar.

Sporangium morphological observation of isolate R84 was carried out by bating technique. Sporangium failed to form on various agar media. Strain 84 did not produced sporangium on agar media after a month of observation even on V8 agar supplemented with antibiotics or black bean agar which are recommended for sporulation (Jeffers and Martin, 1986; Sopee *et al.*, 2012). Only thick-wall chlamydospores were observed from growth culture on black bean agar, showing globose shape with average size of 14.2 µm in diameter (n=100) (Figure 4.20).

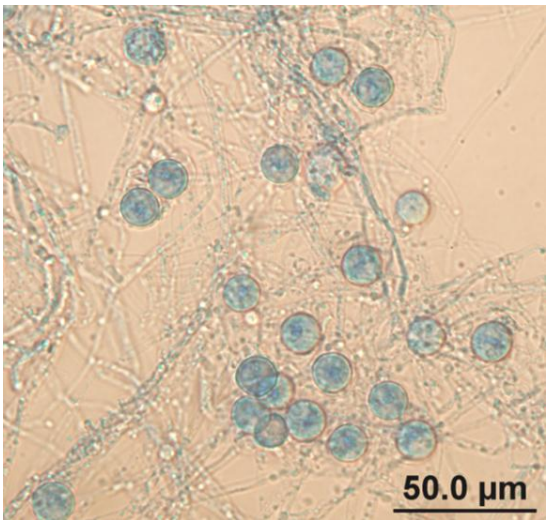

Figure 4.20 Chlamydospores of isolate R84 produced on black bean agar after 8 days of incubation at 25°C.

Baiting technique by inoculation of agar plugs with sesame seeds in P3 water was successfully employed to induce sporangium production. Sporangia are formed abundantly in water after a week of incubation. The resulting spores are shown in Figure 4.19. Sporangia produced by isolate R84 were found vary in shape and papillation. Globose with nonpapillate is the majority. Other minor shapes could also be found such as subglobose, ovoid, obturbinate, ellipsoid and limoniform, with or without papillae. Some sporangia were bipapillate. Zoospore discharge can be seen (Figure 4.21M). Zoospores released into vesicle through a short discharge tube at the side of sporangia.

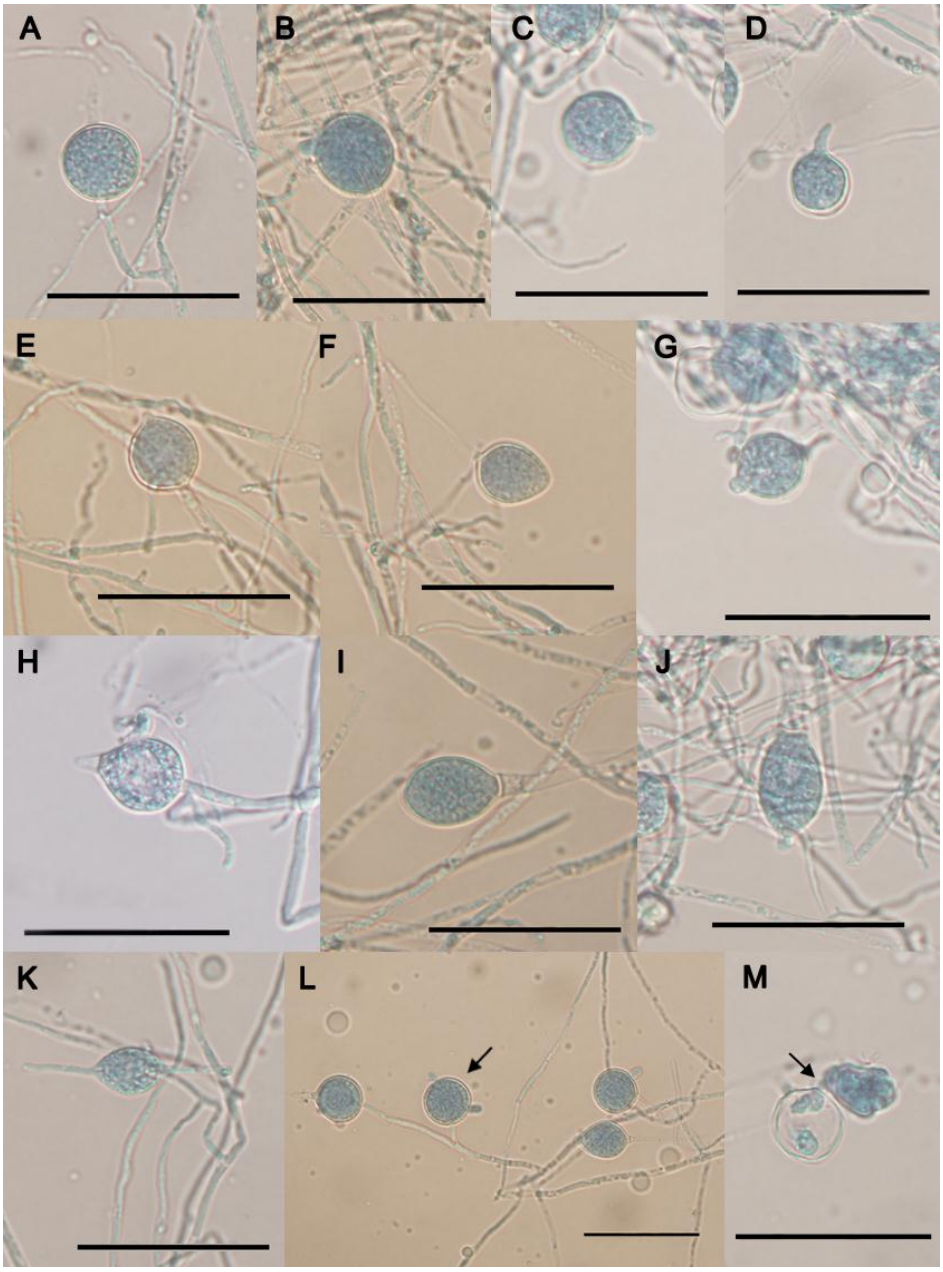

Figure 4.21 Typical shape and papillate of sporangia produced by isolate R84 by baiting technique after 4 days of incubation at 25°C. (A) nonpapillate globose. (B) semipapillate globose. (C) papillate globose. (D) germinate globose. (E) semipapillate subglobose. (F) nonpapillate ovoid. (G) papillate ovoid. (H) papillate obturbinate. (I) nonpapillate ellipsoid. (J) papillate ellipsoid. (K) limoniform with outgrowing papilla. (L) different sporangia shapes in cluster; Arrow: bipapillate sporangia. (M) discharged sporangia; Arrow; discharge tube. Bar = 50 µm.

Isolate R84 was noted as homothallic species because sexual structures were found in the same culture, showing smooth-walled oogonium with antheridium encircles the oogonial stalk (Figure 4.22).

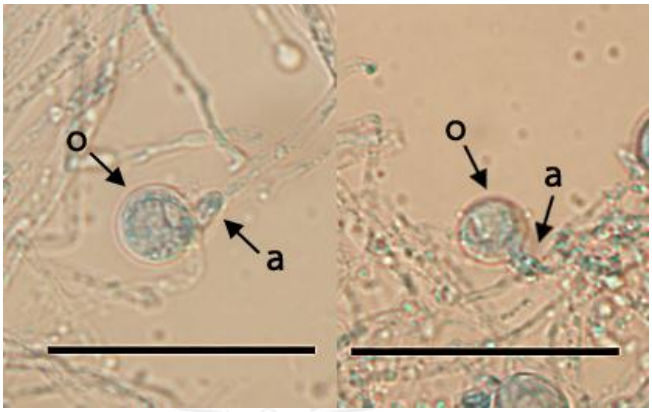

Figure 4.22 Sexual structures of isolate R84 produced on black bean agar after 8 days of incubation at 25°C. Arrow; (a) antheridium, (o) oogonium. Bar = 50 µm.

According to the data published by Bala and colleagues (2010), the common morphological characteristic of the genus *Phytopythium* was described by using *Phytopythium sindhum* as a type species. *Phytophthora*-like features are sporangia morphology of globose to ovoid shape, internal proliferation and mostly papillate. Mode of zoospore discharge is similar to *Pythium* by moving the undifferentiated mass of protoplasm which is differentiate later into zoospores through formed discharge tube and vesicle at the tip of sporangium. Sporangia characteristics of *Py. cucurbitacearum* isolate R84 is between *Phytophthora* and *Pythium* but some are not in the description of the *Phytopythium*. Internal proliferation likes *Phytophthora* was not observed and a few sporangia have papilla. Position of zoospore discharge tube is on the side of sporangium not at the tip like *Pythium*. Unfortunately, there are limited publications recorded about morphological characteristic of this mysterious species. Baten and colleagues (2014) mentioned that *Py. cucurbitacearum* produces nonpapillate and nonproliferation sporangia but sporangium characteristics of isolate R84 are not uniform. However, it seems that the heterogeneous sporangia shapes of isolate R84 resembles to what Santoso and colleagues (2015) recorded in their work. They isolated *Py. cucurbitacearum*

from durian showing tree-decline disease in Indonesia and the species was identified by ITS nucleotide sequencing. The isolates were found producing different shapes of sporangium including globose, ovoid, ellipsoid, limoniform, oblong and irregular, with or without papilla. Globose without papilla is dominant.

For sexual structure, *Py. cucurbitacearum* was described as homothallic, produced smooth oogonia, and forms bell-shaped antheridia on the same hyphae (monoclinous) as oogonium with oospores inside and left the space between oogonium wall and oospores for sexual reproduction (Lévesque and De Cock, 2004; Baten *et al*., 2014). Oogonium morphology of isolate R84 showed similarity to the previous reports.

Although *Py. cucurbitacearum* has been known as a mysterious species but nucleotide sequence of ITS region of isolate R84 showed highly identity and similarity to the species, together with morphology features of isolate R84 resemble to the previous reports. By using combination of morphological and molecular techniques, the dsRNA host isolate R84 in this study was identified as *Py. cucurbitacearum*.

4.5 Effect of the dsRNA on host phenotype

The resistance to hymexazol of *Py. cucurbitacearum* isolate R84 was also determined. There are suggestions that adding hymexazol at 50 mg/l allows most *Phytophthora* to grow and could inhibit most *Pythium* species (Jeffers and Martin, 1986; Erwin and Riberiro, 1996b). Even though the resulting dsRNA containing strain in this study was first isolated successfully on the selective medium containing hymexazol and it was first though as *Phytophthora* spp. but molecular analysis later revealed that isolate R84 was *Py. cucurbitacearum*. These suggested that isolate R84 may resist to hymexazol at the concentration generally used for growth inhibition of *Pythium*. According to information summarized by Erwin and Riberiro (1996b), *Py. cucurbitacearum* was not included in the list of reported *Pythium* species to be affect by hymexazol, the only mentioned species in the same group with *Py. cucurbitacearum* was *Py. vexans* but the sensitivity was found vary in different strains. Use of 50 ppm (mg/l) of hymexazol gave 100% radial growth inhibition of *Py. vexans* spp. in data recorded by Masago and

colleagues (1977) while isolates of Tsao and Gut (1977) and Kato and colleagues (1990) showed moderately resistant by 60% inhibition at 100 ppm. Santoso and colleagues (2015) used PDA supplemented with 50 mg/l hymexazol to isolate pathogen of tree-decline disease from durian and recovered both *Phytophthora* and *Pythium* spp. including *Py. vexans* and *Py. cucurbitacearum*. This could be marked that the use of hymexzol to distinct between *Phytophthora* and *Pythium* should be proceeded with more caution. In this study, the growth rate of the dsRNA containing *Py. cucurbitacearum* isolate R84 was observed and compared with isolate 69-3-2 which is the dsRNA-free strain on agar based medium supplemented with hymexazol up to 400 mg/l. The results are shown in Figure 4.23.

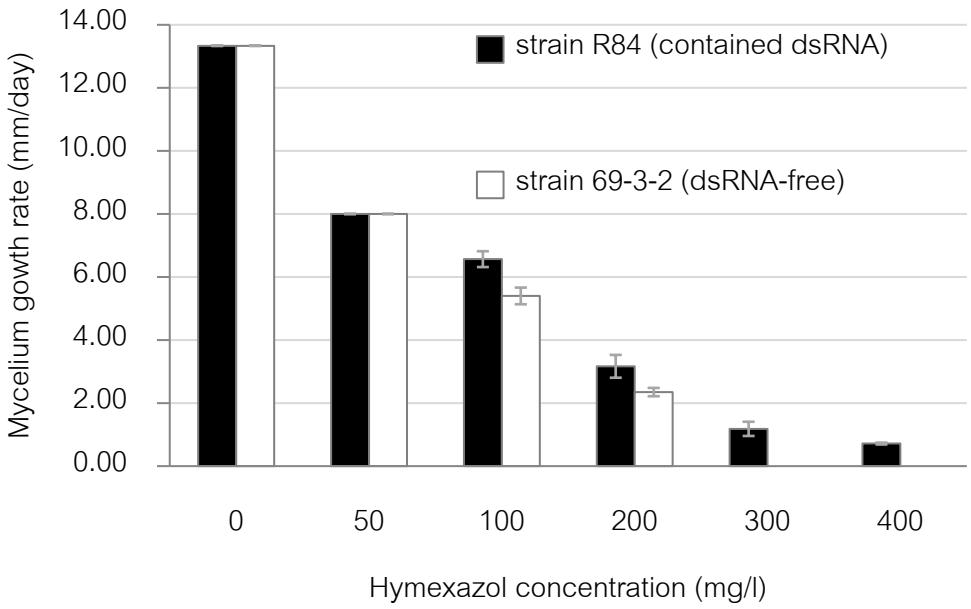

Figure 4.23 Comparison of mycelium growth rate of *Pythium cucurbitacearum* dsRNA-free and dsRNA containing strain on V8 agar supplemented with different hymexazol concentrations.

At 50 mg/l as a normal concentration for separating *Phytophthora* from *Pythium*, both strains could not be inhibited and the growth rates were indifferent. This indicates that *Py. cucurbitacearum* is insensitive or moderately resistant to hymexazol. Difference between growth of dsRNA containing and dsRNA-free strain began to recognize at 100

mg/l and still be seen at 200 mg/l by the growth rate of dsRNA containing strain was higher. Surprisingly, the maximum concentration for the dsRNA-free strain to grow was at 200 mg/l while the dsRNA containing strain resisted to hymexazol at the maximum concentration tested in the study, up to 400 mg/l. This indicated that the dsRNA may be responsible for the hyper-resistant to hymexazol of isolate R84.

There are several researches relating to hypervirulence mycovirus but resistant to fungicide of pathogenic fungi caused by dsRNA element or mycovirus infection has not been reported. Double-stranded RNA found in this study may be the first dsRNA element related to fungicide resistance trait of its host. However, to conclude exactly that the phenotypic change of host is caused by mycovirus, the study should be done in virus curing strain. In Pythiaceae viruses, PiRV-1, PiRV-4 and dsRNA infecting *Pythium irregulare*, attempts to determine effect of these viruses to hosts by viral transfer to a virus-free strain via hyphal fusion or making virus-cured strain had not been successful (Gillings *et al*., 1993; Cai *et al*., 2009; Cai *et al*., 2012). Hyphal fusion or anastomosis in fungi is the way to exchange biomolecules but inability to fuse hypha called vegetative incompatibility (VI) also has been found. VI is based on genetic non-self recognition. Once VI occurs, program cell death triggered and lysis of two hyphal compartments can be seen. This phenomenon is considered as a barrier for viral transmission in fungi (Ikeda *et al*., 2013). VI in the species of Pythiaceae was found in *P. infestans*, the host for PiRV-1 which researcher failed to attain viral transmission via anastomosis (Cherepennikova-Anikina *et al*., 2002; Cai *et al*., 2009). Although VI in *Pythium* has not been reported, there was also no evident of hyphal fusion in viral transmission experiment of the dsRNA infecting *Pythium irregulare* (Gillings *et al*., 1993). VI may be the reason that makes it difficult to study the effect of viruses to their hosts. Curing virus in fungi and lower eukaryotes is often difficult (Cai *et al*., 2012). Making virus-cured strain of Pythiaceae virus was failed in PiRV-4. Cai and colleagues (2012) used other method for curing oomycetes virus by using transplantation of hyphal tips onto media containing the anti-viral drug ribavirin but during six months of trying, they could not obtain any cured isolates free of PiRV-4. Extracellular transmission may be an option. Infection of intact hypha with extracted virus particles and

observation of host phenotypic changing would confirm the hyper- or hypo-virulence of the virus if the virus has a particle. Yu and colleagues (2013) inoculated virus particles on intact hyphae of *Scerotinia sclerotiorum* grown on PDA and found that the virus successfully infected the hyphae and changed the morphology of the fungus by reducing growth and hyphal density. However, out of this study has not been reported attempting an extracellular transmission experiment. As a last choice, if all suggestions mentioned above could not successfully transferred or cured the virus, increasing the population of the wide type strains that do not contain the virus to compare the phenotype with virus-infected strain may be the most practical method.

Even though *Py. cucurbitacearum* isolate R84 in this study may be the first incidence of hypervirulence dsRNA elements related to fungicide resistant but it is only a suggestive conclusion. For further study, transferring virus to fungus virus-free isolate or making virus curing strain could make the experiment more complicated but it is required for identifying biological effect of the dsRNA element to the host.

CHAPTER V
CONCLUSIONS

1. All 79 Pythiaceae could be isolated from rubber leaves showing ALF disease symptoms from 15 plantations in Chanthaburi and Rayong province, Thailand by directly transplanting the plant tissues on selective medium.
2. All 79 Pythiaceae isolates were screened for viral DNA elements but none of the extra-genomic DNA elements were found in all isolates.
3. The cellulose column method for isolation of dsRNA was optimized. Using the Sigma cellulose fiber (C6288) instead of the traditional CF11 Whatman cellulose powder for dsRNA isolation was successful.
4. Using the optimized cellulose column, Pythiaceae isolate R84 was the only one isolate found containing 3 dsRNA elements with 8.0, 3.7 and 2.3 kb in length.
5. The genome type of dsRNAs were confirmed using nucleases treatment including DNase I, S1 nuclease and RNase A in different ionic strength. The largest dsRNA element with 8.0 kb was confirmed as dsRNA and was found permanently in multiple subcultures.
6. The dsRNA of isolate R84 was not the one of reported Pythiaceae viruses.
7. Partial cDNA sequence of the dsRNA of isolate R84 indicated that dsRNA contained a gene encoding ALG2 mannosyltrasferase-like protein but the genus of the dsRNA could not be identified.
8. Using combination of morphological observation and molecular technique, the dsRNA host, isolate R84 was identified as *Pythium cucurbitacearum*.
9. The 8.0 kb dsRNA element might confer hyper-resistance of hymexazol fungicide of the *Py. cucurbitacearum* isolate R84.

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APPENDIX

APPENDIX 1

Media

1. V8 selective medium (Jeffers and Martin, 1986)

Ingredient	Amount per 1 l		Final concentration	
Basal medium				
5% V8				
Clarified V8[a]	50	ml		
Deionized water	950	ml		
Agar	15	g		
20% V8				
Clarified V8[a]	200	ml		
Deionized water	800	ml		
Agar	15	g		
Amendment				
Sodium ampicillin (0.1 g/ml)[b]	2.5	ml	250	mg/l
Rifampicin (20 mg/ml)[c]	0.5	ml	10	mg/l
Pentachloronitrobenzene (24% w/v) (Terraclor, Crompton Crop, USA)	208	µl	50	mg/l
Hymexazol (36% w/v) (Tachigaren, Mitsui Chemicals Agro, Japan)	139	µl	50	mg/l

Mix the ingredients for basal medium and autoclave at 121°C at 15 psi for 15 minutes. Cool to 55°C. Add the amendments. Distribute into petri dished and let agar solidify. Store the plates at 4°C in the dark until use. Broth has the same formula but with the agar omitted.

[a]Clarified V8

V8 Vegetable Juice Original (Campbell's, USA)	340	ml
$CaCO_3$ (Univar, Australia)	3.4	g

Add $CaCO_3$ to the V8 juice and stir until mix well. Centrifuge the mixture at 7,000 rpm for 10 minutes at 4°C. Collect the supernatant and store at -20°C in 50 ml aliquots.

[b]Sodium ampicillin (0.1 g/ml)

Sodium ampicillin (T.P. Drug Laboratories, Thailand)	1	g
Sterile deionized water	10	ml

Dissolve sodium ampicillin with sterile deionized water in sterile vial and store at 4°C until use.

[c]Rifampicin (20 mg/ml)

Rifampicin (Siam Bheasach, Thailand)	300	mg
Sterile deionized water	15	ml

Dissolve rifampicin in sterile deionized water and store at 4°C in the dark.

2. Black bean agar (Sopee*et al*., 2012) (1 l)

Black beans	60	g
Glucose (Merck, Germany)	20	g
Agar	15	g

Soak black beans in 200 ml of deionized water with rotary shaker at room temperature for 24 hours. Retain the liquid. Grind the swollen beans in deionized water and incubate at 65°C for 1 hour. Collect the liquid by filter the slurry through cheesecloth. Combine the filtered liquid with the retained liquid. Adjust volume to 1 l by adding deionized water. Add glucose and agar. Autoclave at 121°C at 15 psi for 15 minutes.

3. Potato dextrose agar (PDA) (1 l)

Potato dextrose broth powder (Himedia, India)	24	g
Agar	15	g
Deionized water	1	L

Mix all components and autoclave at 121°C at 15 psi for 15 minutes.

4. LB medium (1 l)

Tryptone (Himedia, India)	10	g
Yeast extract (Springer, France)	5	g
NaCl (MERCK, USA)	10	g
Agar	15	g

Dissolve the components without agar in 800 ml of deionized water. Adjust pH to 7.0 with 1 M NaOH. Adjust volume to 1 l with deionized water. Add agar if necessary. Autoclave at 121°C at 15 psi for 15 minutes.

5. 2X YT medium (1 l)

Tryptone (Himedia, India)	16	g
Yeast extract (Springer, France)	10	g
NaCl (MERCK, USA)	5	g

Dissolve all ingredients in 800 ml of deionized water. Adjust pH to 7.0 with 1 M NaOH. Add deionized water to 1 l final volume and autoclave at 121°C at 15 psi for 15 minutes.

6. P3 water (1 l)

Pond water	250	ml
Deionized water	750	ml

Collect water from a natural clean pond and filter through cheesecloth. Mix filtered pond water with deionized water and autoclave at 121°C at 15 psi for 15 minutes

APPENDIX 2

Chemicals

1. Chemicals for DNA extraction (CTAB method)

1.1) Extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA, 0.02 M sodium bisulfite) (150 ml)

Sorbitol (MERCK, Germany)	9.56	g
Tris (Research organic, USA)	1.82	g
EDTA (Bio Basic, Canada)	0.22	g
Sodium bisulfate (Sigma-Aldrich, USA)	0.31	g

Dissolve all components with 100 ml of sterile double deionized water and adjust pH to 7.5. Add sterile deionized water to 150 ml final volume. Do not autoclave. Store the buffer at 4°C.

1.2) Nuclei lysis buffer (2% w/v CTAB, 0.2 M Tris, 0.05 M EDTA, 2 M NaCl) (150 ml)

Cetrimonium bromide (CTAB) (Sigma, USA)	3.00	g
Tris (Research organic, USA)	3.64	g
EDTA (Bio Basic, Canada)	2.19	g
NaCl (MERCK, USA)	17.53	g

Dissolve all components in 100 ml of deionized water. Adjust pH to 7.5. Adjust volume to 150 ml with deionized water. Autoclave at 121°C at 15 psi for 15 minutes. Store the buffer at room temperature.

1.3) 5% w/v Sarkosyl (100 ml)

N-lauryl sarcosine (Sigma, USA)	5	g

Dissolve N-lauryl sarkosine in 100 ml of deionized water and autoclave at 121°C at 15 psi for 15 minutes. Store at room temperature.

1.4) Chloroform:isoamyl alcohol (24:1) (200 ml)

Chloroform (MERCK, Germany)	192	ml
Isoamyl alcohol (Ajax Finechem, New Zealand)	8	ml

Mix all compositions in darkglass bottle and store at room temperature.

1.5) 3 M Sodium acetate (pH 5.2) (100 ml)

Sodium acetate (MERCK, Germany) 24.61 g

Dissolve sodium acetate in 80 ml of deionized water. Adjust pH to 5.2 by adding acetic acid glacial (Scharlan, Spain). Add deionized water to 100 ml final volume. Autoclave at 121°C at 15 psi for 15 minutes. Store at room temperature.

1.6) TE buffer (10 mM Tris, 0.1 mM EDTA) (50 ml)

1 M Tris (pH 8.0) (Stock solution)

Tris (Research organic, USA) 7.82 g

Dissolve Tris in 40 ml of deionized water. Adjust pH to 8.0 with NaOH pellets. Adjust volume to 50 ml by adding deionized water. Autoclave at 121°C at 15 psi for 15 minutes.

0.5 M EDTA (pH 8.0) (Stock solution)

EDTA (Bio Basic, Canada) 9.3 g

Dissolve EDTA in 40 ml of deionized water. Adjust pH to 8.0 by adding NaOH pellets. Add deionized water to 50 ml final volume. Autoclave at 121°C at 15 psi for 15 minutes.

Mix 0.5 ml of 1 M Tris (pH 8.0) with 0.1 ml of 0.5 M EDTA (pH 8.0). Add deionized water to 50 ml final volume. Autoclave at 121°C at 15 psi for 15 minutes. Store the buffer at room temperature.

2. Chemicals for dsRNA extraction from *E. coli* dsRNA expressing strain

2.1) 0.1% SDS in PBS buffer (1% w/v SDS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) (50 ml)

SDS (Sigma, USA)	0.10	g
NaCl (MERCK, Germany)	0.80	g
KCl (MERCK, Germany)	20	mg
Na_2HPO_4 (MERCK, Germany)	0.14	g
KH_2PO_4 (MERCK, Germany)	24	mg

Dissolve all components in 80 ml of deionized water. Adjust pH to 7.4 with 1 M HCl and then add deionized water to 50 ml final volume. Autoclave at 121°C at 15 psi for 15 minutes. Store the solution at room temperature.

2.2) 5X RNase A buffer (50 mM Tris (pH 7.5), 25 mM EDTA, 1.5 M NaCl)

1 M Tris (pH 7.5) (Stock solution) (50 ml)

Tris (Research organic, USA)	7.82	g
0.5 M EDTA	2.5	ml
NaCl	4.39	g

Dissolve Tris in 40 ml of deionized water. Adjust pH to 7.5 with concentrated HCl. Adjust volume to 50 ml by adding deionized water. Autoclave at 121°C at 15 psi for 15 minutes and then cool down the Tris solution. Mix 2.5 ml of 1 M Tris (pH 7.5) with 2.5 ml of 0.5 M EDTA and 4.39 g of NaCl. Add deionized water to 50 ml final volume and autoclave at 121°C at 15 psi for 15 minutes.

2.3) DEPC-treated (RNase-free) water (1 l)

Deionized water	1	L
Diethyl pyrocarbonate (DEPC) (AMRESCO, USA)	1	ml

Mix all components in glass bottle, shake the bottle vigorously and keep the bottle overnight at room temperature. Autoclave at 121°C at 15 psi for 15 minutes.

2.4) 150 mM NaCl (50 ml)

NaCl (MERCK, USA)	0.44	g

Dissolve NaCl in 50 ml of DEPC-treated water and autoclave at 121°C at 15 psi for 15 minutes. Store the solution at room temperature.

3. Chemicals for total nucleic acid for dsRNA detection

3.1) 2X Sodium Chloride-Tris-EDTA (STE) buffer containing 1% (w/v) SDS

4X STE buffer (0.2 M Tris, 0.4 M NaCl, 4 mM EDTA) (1 l)

Tris (Research organic, USA)	24.23	g
NaCl (MERCK, Germany)	23.38	g
EDTA (Bio Basic, Canada)	1.49	g

Dissolve Tris and NaCl in 800 ml of DEPC-treated water. Adjust pH to 8.0 with concentrated HCl. Add EDTA and stir until the solution is homogeneous. Adjust pH to 7.0 with concentrated HCl. Add DEPC-treated water to 1 l final volume and autoclave at 121°C at 15 psi for 15 minutes and then cool down the solution. Dilute 4X to 2X STE by mixing 500 ml of 4X STE with 500 ml of deionized water. Add 10 g of SDS and stir until the mixture is evenly mixed. Autoclave at 121°C at 15 psi for 15 minutes. Store the buffer at room temperature.

3.2) Phenol:chloroform:isoamyl alcohol (25:24:1)

Freshly prepared fresh before use by mixing 1 volume of saturated phenol (AMRESCO, USA) with 1 volume of chloroform:isoamyl alcohol (24:1).

4. Chemicals for dsRNA isolation by cellulose column

4.1) 1X STE buffer (1 l)

4X STE buffer	250	ml
DEPC-treated water	750	ml

Mix all solutions and store at room temperature.

4.2) 1X STE buffer containing 15% (v/v) ethanol (1 l)

4X STE buffer	250	ml
DEPC-treated water	600	ml
Ethanol (RCI labscan, Thailand)	150	ml

Prepare fresh before use by mixing all solutions at room temperature.

5. Chemicals for dsRNA enzymatic treatment

5.1) 2X Saline Sodium Citrate (SSC) (300 mM NaCl, 30 mM sodium citrate) (100 ml)

NaCl (MERCK, USA)	1.75	g
Sodium citrate (MERCK, Germany)	0.88	g

Dissolve all components in 80 ml of DEPC-treated water. Adjust pH to 7.0 with 1 M HCl. Autoclave at 121°C at 15 psi for 15 minutes. Store the solution at room temperature.

5.2) 0.1X SSC (15 mM NaCl, 1.5 mM Sodium citrate) (100 ml)

2X SSC	5	ml
DEPC-treat water	95	ml

Mix all components and store at room temperature.

5.3) 10X DNA I buffer (100 mMTris (pH 7.5), 25 mM $MgCl_2$, 5mM $CaCl_2$) (50 ml)

Tris (Research organic, USA)	7.88	g
$MgCl_2$ (MERCK, Germanny)	119	mg
$CaCl_2 \cdot 2H_2O$ (MERCK, Germany)	36.76	mg

Dissolve Tris in 30 ml of nuclease-free water (Apsalagen, Thailand) and adjust pH to 7.5 with 1 M NaOH. Add $MgCl_2$ and $CaCl_2 \cdot 2H_2O$ to the solution and stir until the mixture is evenly mixed. Do not autoclave. Store the buffer at -20°C.

6. Chemicals for competent cells preparation

6.1) 1 M $CaCl_2$(50 ml)

$CaCl_2 \cdot 2H_2O$ (MERCK, Germany)	7.35	g

Dissolve $CaCl_2 \cdot 2H_2O$ in 50 ml of deionized water. Sterile by filter through 0.45 µm disposable syringe filter (Merck Millipore, Ireland). Store at 4°C.

6.2) 1 M $MgCl_2$(50 ml)

$MgCl_2 \cdot 6H_2O$ (MERCK, Germany)	10.17	g

Dissolve $MgCl_2 \cdot 6H_2O$ in 50 ml of deionized water. Sterile by filter through 0.45 µm disposable syringe filter (Merck Millipore, Ireland). Store at 4°C.

6.3) $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$) (100 ml)

1 M $CaCl_2$	2	ml
1 M $MgCl_2$	8	ml
Deionized water	90	ml

Prepare fresh before use. Mix all components and filter the solution through 0.45 µm disposable syringe filter (Merck Millipore, Ireland). Keep on ice until use.

7. Chemicals for transformants screening

7.1) 100 mM IPTG (5 ml)

isopropylthio-β-D-1-galactoside (IPTG) (Sigma, USA)	119	mg

Dissolve IPTG in 10 ml of deionized water. Sterile by filtration through 0.45 µm disposable syringe filter (Merck Millipore, Ireland). Store the solution at -20°C in 1 ml aliquots.

7.2) X-gal (50 mg/ml) (1 ml)

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma, USA)	50	mg
N,*N*–dimethylformamide (DMF) (Fluka, Switzerland)	1	ml

Dissolve X-gal in DMF in 1.5 ml microcentrifuge tube. Wrap the tube with aluminum foil to prevent light damaging and store at -20°C.

7.3) Lysis buffer (100 mM NaOH, 60 mM KCl, 5 mM EDTA, 0.25% SDS, 10% Sucrose, 0.05% Bromophenol blue) (50 ml)

NaOH (MERCK, Germany)	0.2	g
KCl (MERCK, Germany)	0.23	g
0.5 M EDTA	0.5	ml
SDS (Sigma, USA)	125	mg
Sucrose (MERCK, USA)	5	g
Bromophenol blue (APS Ajax Finechem, Australia)	25	mg

Mix all components and adjust volume to 50 ml with deionized water. Autoclave at 121°C at 15 psi for 15 minutes. Store the buffer at -20°C.

8. Chemicals for agarose gel electrophoresis and gel documentation

8.1) 10X TAE buffer (stock solution, 1 l)

Tris (Research organic, USA)	48.4	g
Acetic acid glacial (Scharlan, Spain)	11.4	ml
EDTA (Bio Basic, Canada)	3.7	g
DEPC-treated water	1	L

Mix all components and autoclave at 121°C at 15 psi for 15 minutes. Store at room temperature.

8.2) 1X TAE buffer (1 l)

10X TAE	100	ml
DEPC-treated water	900	ml

Mix all solutions and store at room temperature.

8.3) 10X TBE buffer (stock solution, 1 l)

Tris (Research organic, USA)	48.4	g
Boric acid (Bio Basic, Canada)	11.4	g
EDTA (Bio Basic, Canada)	3.7	g
Deionized water	1	L

Mix all components and autoclave at 121°C at 15 psi for 15 minutes. Store at room temperature.

8.4) 0.8X TBE (1 l)

10X TBE buffer	80	ml
Sterile deionized water	920	ml

Mix all solutions and store at room temperature.

8.5) 0.5X TBE running buffer (1 l)

10X TBE buffer	50	ml
Sterile deionized water	950	ml

Mix all solutions and store at room temperature.

8.6) 1% agarose gel (50 ml)

Agarose (AMRESCO, USA)	0.5	g
1X TAE or 0.8X TAE	50	ml

Mix all components and melt by microwave heating at 800 W for 1 minute. Cool down the evenly mixed solution at room temperature and pour the agarose solution into the gel mould.

8.7) Ethidium bromide solution (0.5 µl/ml for agarose gel staining) (200 ml)

10 mg/µl Ethidium bromide (AMRESCO, USA)	10	µl
Sterile deionized water	190	ml

Mix all components in close-lid container and keep in the dark.

APPENDIX 3

Primers and PCR mixtures

1. Primer used in this study

Primer	Sequence (5' → 3')	Tm (°C)
ITS1[a]	TCCGTAGGTGAACCTGCGG	61
ITS4[a]	TCCTCCGCTTATTGATATGC	56
A2[b]	ACTTTCCACGTGAACCGTTTCAA	61
I2[b]	GATATCAGGTCCAATTGAGATGC	61
BT5[c]	GTATCATGTGCACGTACTCGG	55
BT-Pyt[d]	GCCTTGATGTTGTTCGGGAT	55
PEV1-FRd[d]	GATGGGAACTGGAGACTGCC	58
PEV1-RRd[d]	ATACCCTTAGCCTGCCACAC	57
PiRV1-FRd[d]	ACTCTCAGGTGTGGACTG	53
PiRV1-RRd[d]	CTCAGATTGTCATCGCCAT	52
PiRV3-FRd[d]	ACAGGCGTCGTTTTTATGGG	55
PiRV3-RRd[d]	CCGTTCTACCAATATCTCACC	52
PiRV4-FRd[d]	ACTGAGGTAAAGGCGAAGGC	57
PiRV4-RRd[d]	CTCAGGCACCGTAGAAGCTC	58
udp-F2[e]	GTCAGAGCCACTTTCTCGCG	63
udp-R2[e]	CTCCTGCGTAGGTGGAGTAGG	63
PiRV1-F[d]	ATGCAGATAAACGTCGGAGGG	61
PiRV1-R[d]	TCAAAACATGACTTCAGTCGGG	58
PiRV3-F[d]	TCTAGCTTTCCAGGTTGCGG	61
PiRV3-R[d]	ATCCGACAAATGGGTGATACC	58
PiRV4-F[f]	GTGGAAAGCACGTTTATGCAGG	60
PiRV4-R[f]	GTATCTACGCCTCACCCTAC	58

1. Primer used in this study (continued)

Primer	Sequence (5' → 3')	Tm (°C)
L30350F (Cloning Analysis Forward Primer)[g]	ACCTGCCAACCAAAGCGAGAAC	55
L71-1R (Cloning Analysis Reverse Primer)[g]	TCAGGGTTATTGTCTCATGAGCG	55

[a]White *et al*., 1990, [b]Drenth *et al*., 2006, [c]Villa *et al*., 2006, [d]This study,

[e]Kozlakidis *et al*., 2010, [f]Zhan and Zhu, 2016,

[g]Cloning Analysis Primer provided in NEB PCR cloning kit (New England Biolabs, USA).

2. PCR mixtures

2.1) Reaction mixture for *Taq* polymerase (Apsalagen, Thailand)

Component	Volume (µl/reaction)	Final concentration
10X Reaction buffer	2.50	1X
50 mM $MgCl_2$	0.75	1.5 mM
10 mM dNTP Mix	0.50	0.2 mM
10 µM Forward primer	1.25	0.5 µM
10 µM Reverse primer	1.25	0.5 µM
Template DNA - DNA 50 ng/µl - Colony PCR	 1 Bacterial colony	
Taq DNA Polymerase (5U/µl)	0.125	0.05 U/µl
Nuclease-free water	to 25 µl total volume	

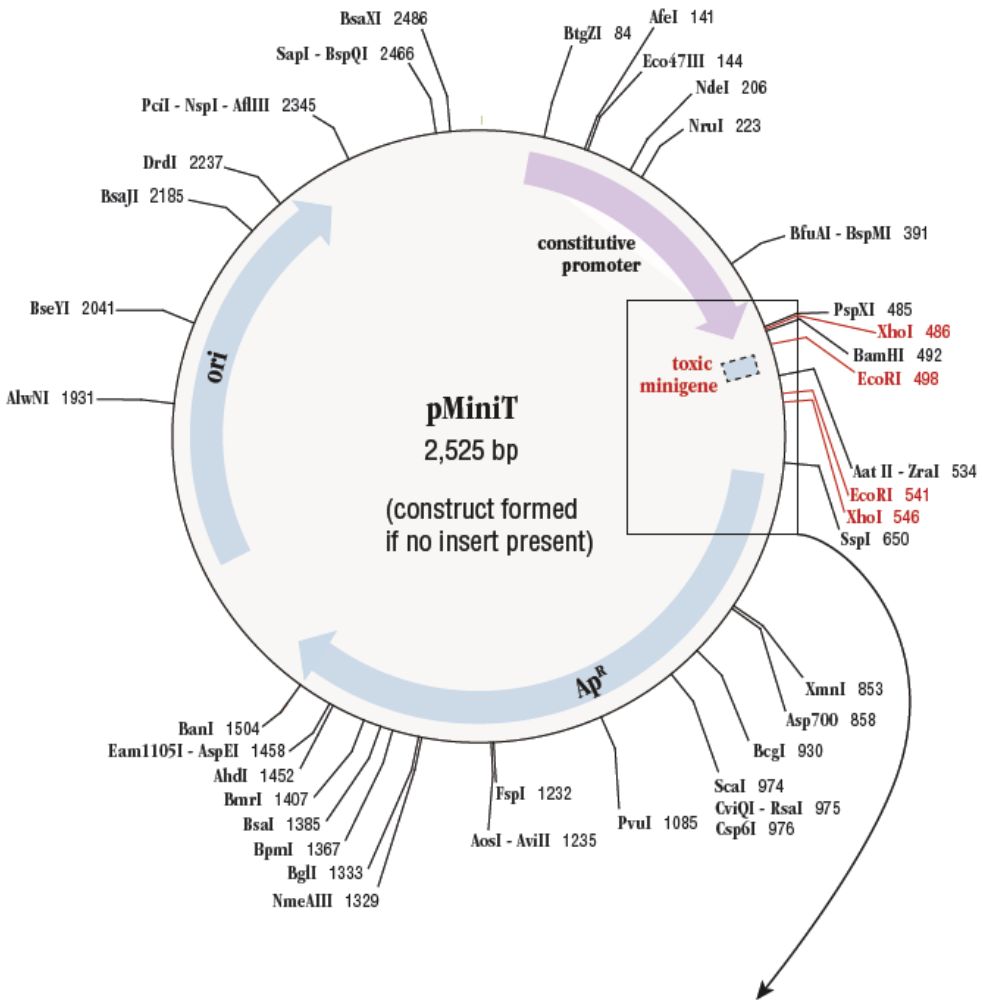
2.2) Reaction mixture for KOD Hot Start DNA polymerase (Novagen, USA)

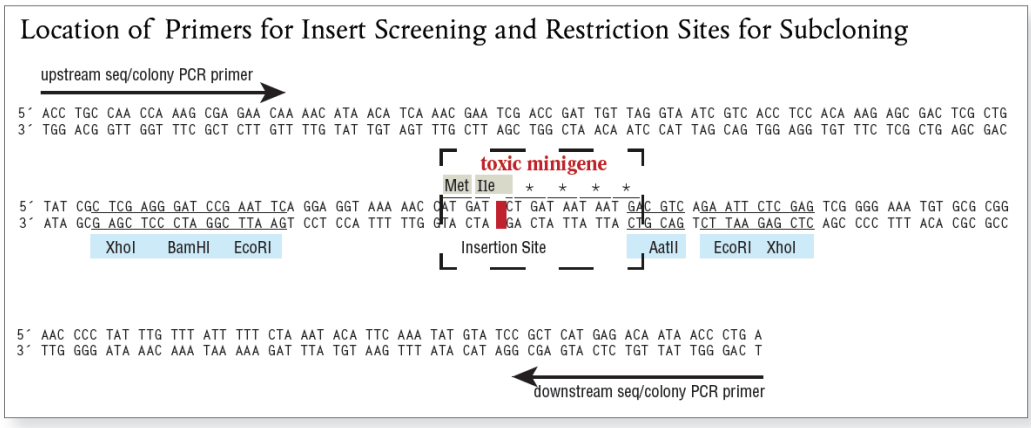
Component	Volume (µl/reaction)	Final concentration
10X Reaction buffer	2.50	1X
25mM $MgSO_4$	1.50	1.5 mM
2mM dNTP each	2.50	0.2 mM
10 µM Forward primer	0.75	0.3 µM
10 µM Reverse primer	0.75	0.3 µM
Template cDNA	2.00	
KOD Hot Start DNA Polymerase (1U/µl)	0.50	0.02 U/µl
Nuclease-free water	to 25 µl total volume	

APPENDIX 4

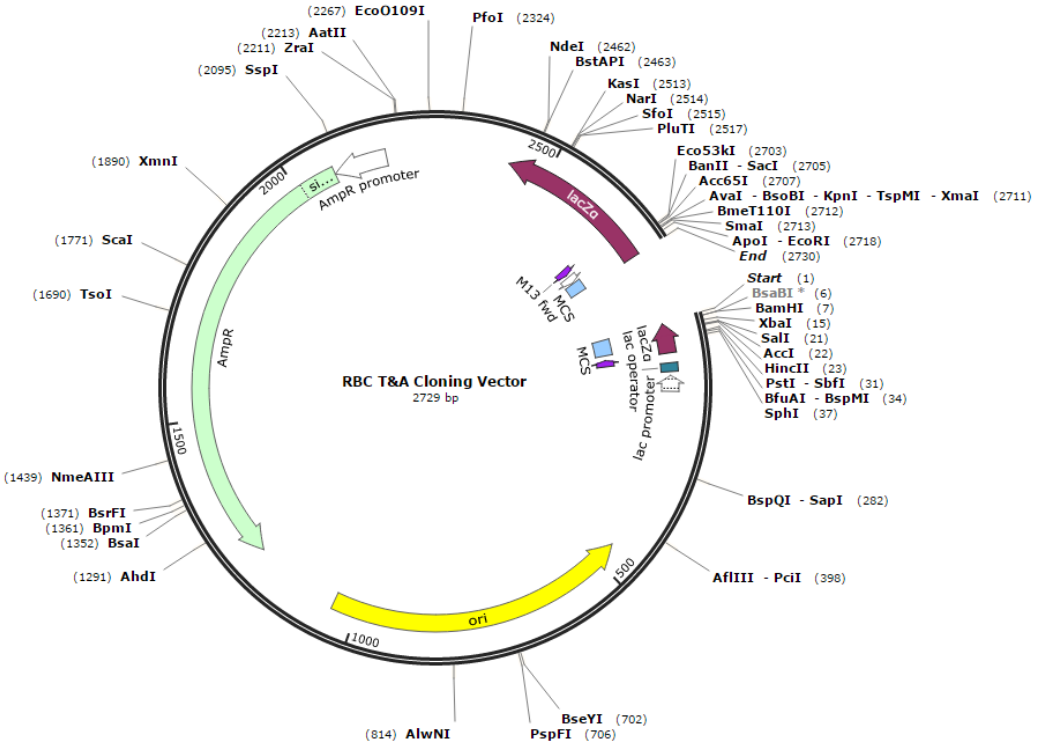
Plasmids

1. pMiniT Vector (NEB PCR Cloning Kit, New England BioLabs, USA)





2. RBC TA Cloning Vector (TA Cloning kit, RBC Bioscience, Taiwan)



APPENDIX 5

Competent cells preparation

The protocol for was adapted form (Sambrook and Russell, 2011). A single colony of *E. coli* DH5α from 16-20 hours culture on LB plates were inoculated into 10 ml of LB broth and incubated overnight at 37°C in rotary shaker at 220 rpm. The culture was transferred to 90 ml of LB broth and incubated at 37°C with vigorous agitation for 3-4 hours until the OD_{600} reached 0.35-0.40. To make sure that the culture does not overgrowth, measure the OD_{600} after an hour of incubation and every 15-20 minutes after the OD_{600} is higher than 2.5-3.0. The culture was stored on ice for 10 minutes and then centrifuged at 4,000 rpm at 4°C for 10 minutes. The supernatant was discarded. 60 ml of cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$) was added to resuspend the pellet. The cells were collected by centrifugation at 4,000 rpm for 10 minutes at 4 °C and the supernatant was discarded. The pellet was resuspended by adding 4 ml of cold 0.1 M $CaCl_2$. To prepare frozen stock of competent cells, 140 µl of DMSO (MERCK, Germany) was added and mixed gently. The suspension was kept on ice for 15 minutes. DMSO was added again to the suspension and stored on ice. The suspension was aliquot by taking 150 µl into 1.5 microcentrifuge tubes and immediately snap-freeze the competent cells in liquid nitrogen. The tubes were stored at -80°C until use.

VITA

Chanoknan Hattapanichaporn was born in 1991 in Chanthaburi province, Thailand. She obtained a Bachelor degree of Science in Microbiology from Chulalongkorn University in 2013 before attended a Master's Degree in Microbiology and Microbial Technology, Department of Microbiology, Faculty of Science, Chulalongkorn University. Some parts of this research were published in proceeding of The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference at The Empress Hotel Chiang Mai, Thailand.