MORPHOLOGICAL AND RIBOSOMAL DNA- BASED CHARACTERIZATION OF COMMERCIAL *CORDYCEPS MILITARIS* STRAINS IN THAILAND

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ABSTRACT

Cordyceps militaris, an entomopathogenic fungus, has been extensively utilized for many years as functional food and for medicinal remedies in Thailand. The fungus has numerous strains which are commonly sold in herbal markets and not all have been identified. Seven commercial *C. militaris* isolates (*Cmi*1, *Cmi*2, *Cmi*3, *Cmi*4, *Cmi*5, *Cmi*6 and *Cmi*7) were studied using macroscopic studies. The stromatal colour of seven isolates were golden yellow. Of these, six isolates had a cylindrical stipe of stromata (approximately 0.3-0.4 cm wide x 4-6 cm long) with a round cap, except the *Cmi*4, stroma had the widest stipe (approximately 1.5-2.5 cm wide and 2-4 cm long) with a shape cap. Moreover, the mycelium had the highest mycelial growth rate, but only the *Cmi*4 had the lowest. To determine the stromatal characteristics using cross section technique and observing under a microscope, the result revealed that all isolates were golden yellow at the edge, and colourless at hyphae. To investigate the spore characteristics using the spore shooting technique, and observing under a microscope, the result revealed that most spores were globular shape. To study the conidial formations and hyphae characteristics using the slide culture and SEM techniques, the result showed that most spores had globose and oval shapes, moreover these germinated and produced long hyphae. The conidia formed mostly single globe heads and rare group globe heads in short chains and subtending conidia. Some conidia had pyriform or cylindrical shapes. Genetic variation among

the seven isolates gave 100% nucleotide-sequence homology at the ITS and 28S rDNA regions. These data suggested that the macroscopic method was a highly powerful tool for strain identification of *C. militaris*.

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CHAPTER I

INTRODUCTION

1.1 General introduction

Cordyceps militaris is an entomopathogenic fungus whose spore invades and grows in a specific insect host in caterpillar stage which hibernates underground during the winter season. Later, during the summer season, the spore develops into a fruiting-body formation on the surface of the insect's cadaver (Guo et al., 2017). In Chinese traditional medicine, the stroma (fruiting body) of *C. militaris* is credited with several pharmacological properties, and widely used as a herb, functional food, for improvement of immunity (Izgi et al., 2015; J.-y. Liu et al., 2016; Song et al., 2016), for bacteriostasis (Avtonomova, Krasnopolskaya, Shuktueva, Isakova, & Bukhman, 2015; Chiu et al., 2016), antitumor (P. Zhang et al., 2015), antiasthmatic effects (Tianzhu, Shihai, & Juan, 2015), and as antihyperuricemic (Yong et al., 2016) and antihypoglycemic (L. Ma, Zhang, & Du, 2015). Main abundant bioactive compounds produced by various *Cordyceps* species were adenosine (Tatani et al., 2016), ergosterol, mannitol and exopolysaccharide (Raethong, Laoteng, & Vongsangnak, 2018), and cordycepin (Yong et al., 2016). Of these, cordycepin is an extremely valuable bioactive compound produced only by *C. militaris*. Artificial cultivation gives higher production of cordycepin compared to nature; however, its quality and quantity depend on fungal culture media (Lin, Lai, Wu, Hsu, & Tai, 2018), culture conditions (G.-H. Sung, Shrestha, Han, & Sung, 2011), and isolates (T. Wen, Long, Kang, Wang, & Zeng, 2017). Recently, *C. militaris* has been isolated and estimated to many isolates in Asian counties (Kang et al., 2017) including in Thailand (Tapingkae, 2012), where isolates contained various amounts of cordycepin (T. Wen et al., 2017). Therefore, classification among isolates of *C. militaris* was beneficial to screen for isolates that were able to produce high cordycepin content. During the past decade, two methods were widely used for morphological classification of *C. militaris* isolates consisting of macroscopic such as morphological characteristics (such as shape, size, and colour of stroma) (H.-j. Liu, Hu, Chu, Li, & Li, 2011) and mycelium

growth (Dang, Wang, & Lay, 2018). Addition, microscopic such as cross section (Guo et al., 2017), spore shooting technique (Choi, Hyde, & Ho, 1999), slide culture technique (Leck, 1999) and SEM techniques (Albini, 2017). Recently, molecular markers corresponding to internal transcribed spacer (ITS) (Lam et al., 2015) and 28S-ribosomal DNAs (rDNAs) regions (Y. Li, Jiao, & Yao, 2013) has gained prominence to classify among *Cordyceps* isolates. Therefore, the aim of this study was to identify Thai commercial isolates of *C. militaris* using both morphological characteristics and molecular markers. Results will be beneficial by providing the accurate method for identifying commercial isolates of Thai medicinal fungus *C. militaris*.

1.2 Objectives of the study

1) To study morphological characteristic of *C. militaris* isolates from Thai commercial sources.

2) To study nucleotide variation at rDNA regions using ITS- and 28S-markers.

1.3 Research Significance

The morphological characteristic and genetic variation among *C. militaris* isolates are highly powerful methods to identify among isolates of *C. militaris* in Thai commercial strains.

1.4 Research Scope

C. militaris were collected from seven different commercial farms in Thailand. The methods used for identifying and studying *C. militaris* isolates were morphological characteristics (such as shape, size, and colour of stroma), growth development on media, cross section of stroma, spore shooting technique, slide culture technique, and SEM technique. Genomic DNA was isolated using InstaGene Matrix (Bio-Rad, United States) kit, quantified using gel electrophoresis system and spectrophotometric system. ITS and 28S rDNA markers were used for DNA finger printing using PCR techniques. Purified DNA samples were performed using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster

City, Canada) and then sequenced and analyzed using an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, Canada).

1.5 Keywords

Cordyceps militaris, Morphology, Ribosomal DNA

1.6 Research Hypothesis

Macroscopic, microscopic and molecular marker methods would be proficient tools for morphological classification of *C. militaris* isolates in Thailand.

CHAPTER II

LITERATURE REVIEW

Fungi, eukaryotic organisms, have one or more nucleus enclosed within membrane without chlorophyll. Therefore, they cannot photosynthesize their cells. Heterotrophic fungi abort any organic maters from external sources and environment. Moreover, they may exist as parasites obtaining nutrients from a living host. Fungal cell wall consists of cellulose, hemicellulose and chitin compound (Suwanpinit & Suwanpinit, 1998).

Generally, the structures of fungi are made up of thread like filaments and clourless hyphae. Its functions are associated with growth development, and reproduction by forming spores. The hyphal tip region is the highest growth rate, and many hyphae are formed to mycelium (5-10 μm diameter) (Sangtian, 2009). Their cell structures of hyphae are different characteristics which can be divided into 2 types, septate hyphae and non-septate hyphae (Figure 1).

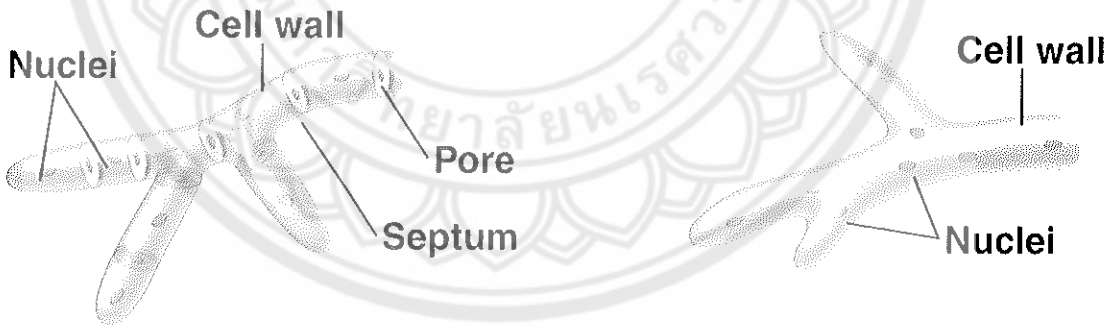


Figure 1 Two types of hyphae characteristics, septate hyphae (left) and non-septate hyphae (right) (Alexopoulos, Mims, & Blackwell, 1996)

2.1 Kingdom fungi

Dube (2013) reported that the fungus belongs to the Kingdom Fungi which can be divided of various phylum (Sylvia S. Mader et al., 2007) as following:

1) Phylum Chytridiomycota

Most fungi in this phylum have small thallus formations, producing sporangiums, and have root like structures, called rhizoids. The general characteristic of this fungus has a single flagellum, which is using for movement. In addition, the flagellum is able to differentiate to a zoospore with one nucleus, used as sexual-reproduction systems such as planogametic copulation, gametangial copulation and somatogamy.

2) Phylum Zygomycota

The fungi in this phylum are often referred to lower fungi with having a single cell and non-septate. Most fungi can grow and live in the water, soil, and fossil (decaying plant or animal material). Some fungi are parasites to plants, insects, and small animals, while others are symbiotic relationships with plants. In an asexual reproductive stage, a hundreds of haploid spores are developed from a sporangium at the tip of upright hyphae.

3) Phylum Basidiomycota

The fungi in this phylum are often referred to higher fungi with having septate hyphae, packing to a stick like a stem such as fruiting body. As an asexual reproductive stage, the conidia are formed at the conidiophore. As a sexual reproductive stage, the spore is formed at the basidium, called basidiospore.

4) Phylum Ascomycota

Its members are commonly, known as the sac fungi or ascomycetes, and characterized by a saclike structure. The ascus contains four to eight ascospores in the sexual stage. For an asexual reproductive stage, the conidia are formed at the end of hyphae.

2.2 Phylum Ascomycota

Phylum Ascomycota (sac fungi) is classified as higher fungus, consisting of approximately 60,000 species, which are extensively found in all land ecosystems. Their spores and hyphal fragments are found in the atmosphere, in fresh-water, and in salt-water environments. In this phylum, the largest class is ascomycetes, including

the morel (cup fungi) and the truffle (mushroom). Some fungi in class ascomycetes (in phylum Ascomycota) are plant pathogens, animal pathogens, edible mushrooms, and living on dead organic matter (as saprobes). The unique characteristics of ascomycetes are spore formation, which is in a sac-like structure called an ascus. During sexual reproduction, thousands of asci are located in a large stroma called the ascocarp (Figure 2). The ascus contains four to eight ascospores in the sexual stage. For an asexual reproductive stage, the conidia are formed at the end of hyphae. Mycelia are composed of septate hyphae. The hyphae of ascomycetes consits of homokaryotic hypha or heterokaryotic hypha and pseudomycelium (Sangtian, 2009). The economic importance of ascomycetes is used in food production, such as yeast, which is used to produce alcohol and make bread rise. Ascomycetes are used in medicine as well, as it can see with the antibiotic penicillin. Some ascomycete fungi exist as parasites with obtaining nutrients from a living host such as insects. Insect's fungi in this class are considered important to the economy. It has been a highly valued traditional medicines such as the genus of *Ophiocordyceps*, *Isaria* and especially *Cordyceps* (Tapingkae, 2012).

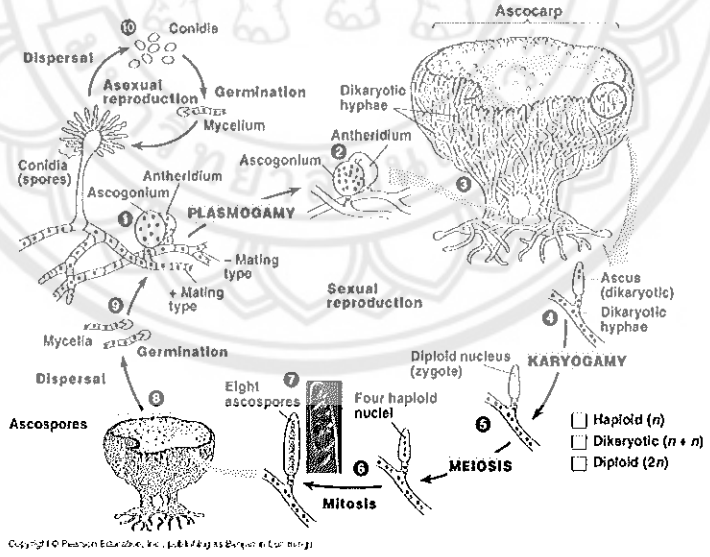


Figure 2 Life cycle of fungi in phylum Ascomycota

Note: This illustration shows fungal life cycle either asexual (leaf) or sexual (right) reproduction (Sangtian, 2009)

2.3 *Cordyceps* group

Cordyceps group is commonly referred to as Chinese caterpillar fungus or winter-worm and summer-grass, which is a literal translation from its Chinese name, Dong Chong Xia Cao. *Cordyceps* is an entomopathogenic fungus whose spore invades and grows in a specific insect host in a caterpillar stage which hibernates underground covered with snow during the winter season. Later, during the summer season, the spores grow and absorb nutrient and mineral from the insect host, and develops into a stroma formation on the surface of the insect's cadaver. The stroma has been developed to mushroom, shooting out many spores for sexual reproduction (Guo et al., 2017) (Figure 3). In nature, *Cordyceps* is consist of head of stroma, stalk (look like mushroom) and larva (Buenz, Bauer, Osmundson, & Motley, 2005; Shrestha, Zhang, Zhang, & Liu, 2010) (Figure 4).

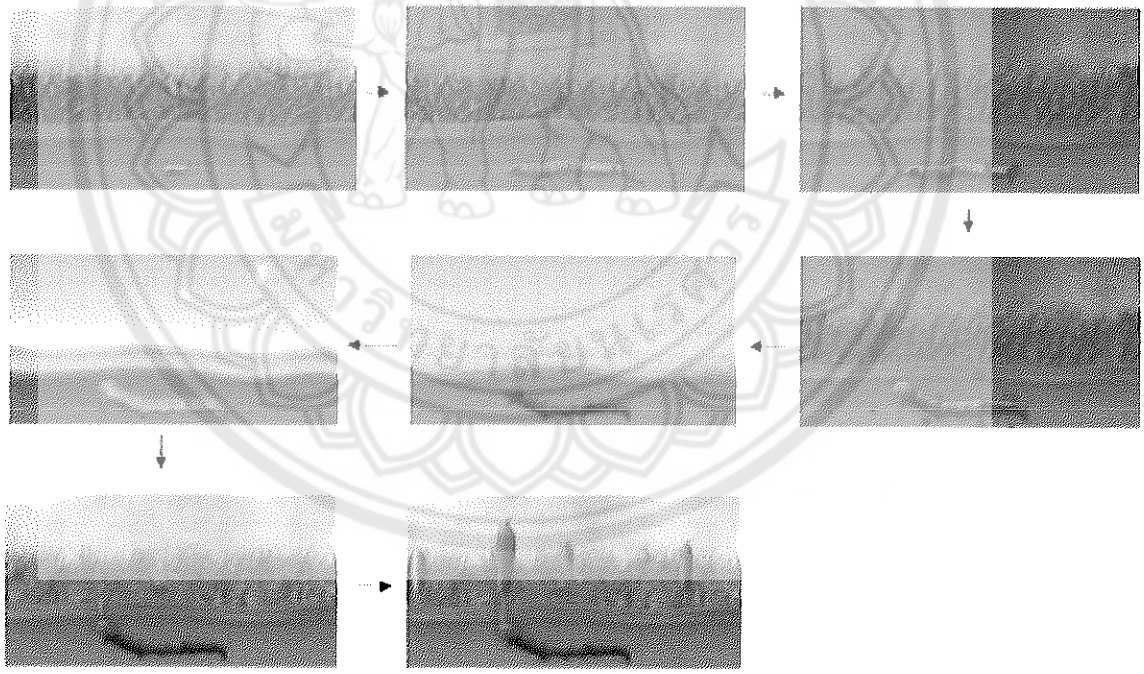


Figure 3 Life cycle of *Cordyceps*

Note: This illustration shows Life cycle of *Cordyceps* (Tapingkae, 2014)

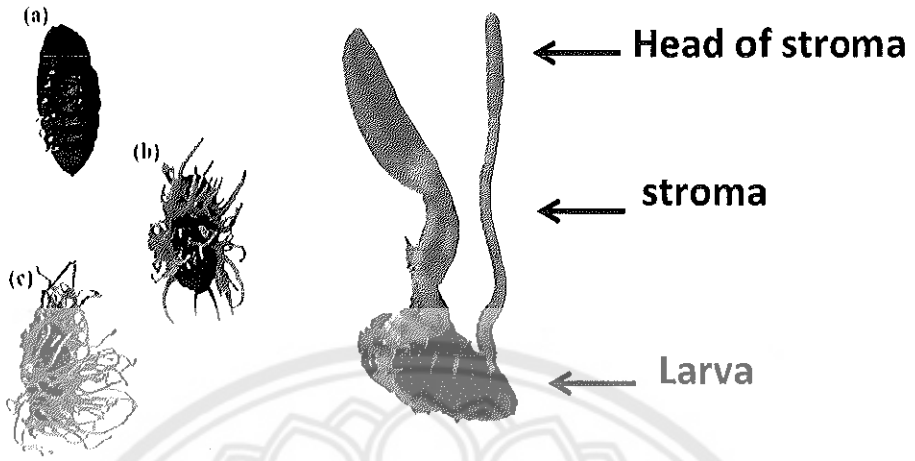


Figure 4 Morphology of *Cordyceps*

Note: This illustration shows morphology of *Cordyceps* in the nature (Tapingkae, 2014)

2.4 Source of *Cordyceps*

C. Li et al. (2006) reported that *Cordyceps* were classified more than 750 species around the world. Out of these, the most species (approximately 400 species) have been found in Asia (Nepali, China, Japan, Korea, Vietnam and Thailand), because its majority regions are various humid climate regions and tropical forest (G.-H. Sung et al., 2007). In Thailand, Sivichai (2001) found more than 80 *Codyceps* species. Tapingkae (2012) studied diversity of *Codyceps* at Doi Suthep-Pui National Park in Chiang Mai, found that genus of *Codyceps* belongings to family Clavicipitaceae, consisting of many *Codyceps* species which were parasites in various insect varieties. The various hosts of *Cordyceps* consist of ant, bee, wasp, hornet, spider, aphis, grub, dragonfly, butterfly and worm etc. Generally, insect fungi are specific to particular host species (Sung et al., 2007). Insect fungi in genus of *Cordyceps* was used to pharmaceutical industry as food for humans and not allergic (G.-H. Sung et al., 2007). The important *Cordyceps* in this genera are *Ophiocordyceps sinensis*, *Isaria tenuipes*, *Isaria sinclairii* and *C. militaris* (Tapingkae, 2012).

2.5 *C. militaris*

C. militaris can be classified according to taxonomy as follows (Das, Masuda, Sakurai, & Sakakibara, 2010).

Kingdom	Fungi
Phylum	Ascomycota
Sub-phylum	Ascomycotina
Class	Ascomycetes/ Pyrenomycetes
Order	Hypocreales
Family	Clavicipataceae
Genus	*Cordyceps*
Species	*Cordyceps militaris*

C. militaris, as an entomopathogenic fungus, infects many insects, especially most Lepidoptera larvae of different host species as shown more detail in Table 1 (Tapingkae, 2012).

Table 1 Host species of *C. militaris* belonging to different order and families of insects

Order	Family	Species	References
Coleoptera	Scarabaeidae	*Lachnosterna quercina*	(Farlow & Seymour, 1888)
	Scolytidae	*Ips sexdentatus*	(Kryukov, Yaroslavtseva, Lednev, & Borisov, 2011)
	Tenebrionidae	*Tenebrio molitor*	(De Bary, 1867)
Diptera	Tipulidae	*Tipula paludosa*	(Müller-Kögler, 1964)
Hymenoptera	Cimbicidae	*Cimbex similis*	(Y. Kobayasi, 1941)
Lepidoptera	Arctiidae	*Euprepia caja*	(De Bary, 1867)
	Bombycidae	*Andraca bipunctata*	(Panigrahi, 1995)
		Bombyx caja	(Cesati, 1861)
		B. mori	(Gu & Liang, 1987)
		B. pythiocampa	(Y. Kobayasi, 1941)
		B. rubi	(Tulasne, 1865)
	Drepanidae	*Cymatophora duplaris*	(Lagerberg, 1922)
		C. flavicornis	(Lagerberg, 1922)
		Palimpsestis duplaris	(Ulvinen, 1969)
		Polyploca flavicornis	(Ulvinen, 1969)
	Geometridae	*Biston 10alcate*	(T. Ma et al., 2007)
		Culcula panterinaria	(Qiwu, 1997)
		Lycia hirtaria	(Ulvinen, 1969)
	Hepialidae	*Hepialus sp.*	(Petch, 1948)
	Lasiocampidae	*Dendrolimus latipennis*	(Jing, 1987)
		D. pini	(Sopp, 1911)
		D. sibiricus and Macrothylacia rubi	(Kryukov et al., 2011)
		Gastropacha quercus	(De Bary, 1867)
	Lymantriidae	*Dasychira pudibunda*	(Ulvinen, 1969)
		Leucoma salicis	Kryukov et al. 2011
	Notodontidae	*Fentonia ocypete, Lampronadata cristata, Phalera assimilis and Phalerodonta albibasis*	(Qiwu, 1997)
		Phalera bucephala	(Gray, 1858)
		Quadricalcarifera punctatella	(Sato, Shimazu, & Kamata, 1994)
		punctatella	

Table 1 (Cont.)

Order	Family	Species	References
	Saturniidae	*Anisota senatoria*	(Hitchcock, 1961)
	Sphingidae	*Callambulyx tatarinovi*	(Y. Kobayasi, 1941)
		Laothë populi	(Ulvinen, 1969)
		Marumba sperchius	(Qiwu, 1997)
		Mimas tiliae	(Ulvinen, 1969)
		Sphinx euphorbiae	(De Bary, 1867)
		S. pinastri	(Ulvinen, 1969)
	Thyatiridae	*Tetheella fluctuosa*	(Kryukov et al., 2011)
		Ochropacha duplaris	
		Tethea ocularis	

2.6 Benefits of *C. militaris*

Nowadays, the *Cordyceps* was publically used as safely herbal products, which has gained popular increase in the United States, Japan, Australia, China, Switzerland, Korea, Taiwan, Malaysia, Canada, Hong Kong and Thailand (Das et al., 2010). It had previous reported that the *C. militaris* and the *C. sinensis*, now known as *O. sinensis* (G.-H. Sung et al., 2007), had high Cordycepin, which was major bioactive compound with higher than other types of spices (Dong, Lei, Ai, & Wang, 2012). Minor bioactive compounds, found in *C. militaris*, consist of monosaccharide, disaccharide, polysaccharide, beta-glucan, mannitol, galactose, adenosine, cordycepic, amino acid and vitamin had been found in *C. militaris* (Dong et al., 2012), including organic matters of protein, lipid, vitamin, mineral and amino acid (Tapingkae, 2013). The *Cordyceps* had various mineral such as potassium, sodium, calcium, magnesium, iron, copper, manganese, zinc, phosphorus and selenium (Bhandari et al., 2010). The digested enzymes related in monogastric simple nonruminants *C. militaris* found in *C. militaris* are amylase, pectinase, β-glucanase, endoglucanase, cellulose, protease and phytase (Tapingkae, 2013). In Chinese traditional medicine, the stroma of *C. militaris* is credited with several pharmacological properties, and widely used as a herb, functional food, for improvement of immunity (Izgi et al., 2015; J.-y. Liu et al., 2016; Song et al., 2016), for bacteriostasis (Avtonomova et al., 2015; Chiu et al., 2016), antitumor (P. Zhang et al., 2015), antiasthmatic effects (Tianzhu et al., 2015), and as a antihyperuricemic (Yong et al., 2016)and antihypoglycemic (L. Ma et al., 2015).

2.8 Effect of parameters on *C. militaris* cultivation

The *C. militaris* cultivation in *in vitro* conditions were depending on various parameters such as culture medium, light-dark, temperature, pH, and humidity. Many previous studies have been reported as shown below:

Holliday, Cleaver, Loomis-Powers, and Patel (2004) have been developed the unique method for improving hybridized strains of *Cordyceps*, from wild collected *Cordyceps*, by using rattlesnake venom to enhance somatic fusion of two mycelial strains. These artificially hybridized strains were able to produce a higher and more consistent quality levels of adenocine and cordycepin compound.

Shrestha, Lee, Han, and Sung (2006) studied the growth of *C. militaris* on various media under light conditions. The result found that the light is most important for growth and pigmentation of mycelium. The hyphae had the highest growth under darkness condition, compared to light condition. Addition, Shrestha studied the effect of 22 culture media on growth of *C. militaris*. Of these, only three media which were Sabouraud Dextrose agar plus Yeast Extract (SDAY), Sabouraud Maltose agar plus Yeast Extract (SMAY) and Czapek Yeast Extract agar (CZYA) gave the highest growth development of *C. militaris*.

J.-M. Sung et al. (2006) and Pathania, Joshi, and Sagar (2015) reported that the suitable temperature at 25°C under darkness condition for 7 days gave the best mycelium growth of *C. militaris*.

Hong et al. (2010) reported that the appropriate condition for growing mycelial colonies and forming stroma of *C. militaris* was that mycelium infected on silkworm, incubated at 20°C under photoperiod at 1,000 lux and relative humidity 90% 9-10 days.

P.-D. Kang et al. (2010) referred to the popular method of culture *Isaria tennipes* in Korea using by silkworm (*Bombyx mori*). The larva of silkworm was feed in a tray in the evaporative cooling system. The starter of *Isaria tennipes* mixed with starch syrup and spray at 0, 12 and 24 hours on larva, which it was on 5 states of molting process (weakest moments). The larva was feed by leaf, and would go into pupa for 11 days. After that, the cocoon was cut and took the pupa out. The pupa (infected starter of *Isaria tennipes*) was incubated 20-22°C humidity 95% and would go into the stroma.

Kim, Shrestha, Sung, Han, and Sung (2010) studied optimum condition for regeneration of fruiting state and develop to the stroma of *Cordyceps*. The result showed that the optimum culture was incubated 20 ± 1°C under the light condition and relative humidity 70- 80% for 60 days.

Pathania et al. (2015) studied the effect of pH in medium on mycelium growth of *Cordyceps* found that the mycelium had the highest growth on the medium at pH 7.

G.-H. Sung, Shrestha, Han, Kim, and Sung (2010) studied the effect of various carbon, nitrogen or mineral sources on mycelium growth of *Cordyceps*. The result showed carbon sources (fructose and xylose) gave the highest growth in comparison with other carbon sources (maltose, starch, dextrose, dextrin, sucrose, lactose and water agar), followed by nitrogen source (peptone) and mineral sources (K_2HPO_4, $MgSO_4 \cdot 7H_2O$ and NaCl). Similarly, G.-H. Sung et al. (2007) mention culture of *Cordyceps* genus can regenerate easily on liquid and solid medium supplement different concentration of carbon source and nitrogen source.

T. Wen, Li, Kang, Kang, and Hyde (2014) reported that various pH (5.0-8.0) conditions promoted stroma growth of *C. militaris*. The result revealed that the maximum stroma growth (1.81 ± 0.08 g/bottle) and the maximum cordycepin production (7.40 ± 0.01 mg/g dry weight) were accomplished at low pH ranged from 5.5 to 6.0, cultured on 40 g/L glucose, 5 g/L peptone, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L K_2HPO_4 and 1.0 mg/L NAA for optimal stroma growth and 10 g/L glucose, 10 g/L peptone, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L K_2HPO_4 and 1.0 mg/L NAA.

2.9 Morphological characteristics study of *Cordyceps*

The mycelium of anamorph stage, some can identify by observation of conidia formation on culture medium (asexual reproduction). Some cannot describe character of fungus through culture medium. Therefore, it is the reason that makes the morphology study quite difficult and has restriction. Generally, insect fungi in genus *Cordyceps* found sexual stage, which is period of time to produce stroma. Therefore, identification of this genus should be used the teleomorph state, which this state is produce ascospores inside of asci. The teleomorph state clearly to see a difference between species in *Cordyceps* such as shape, size, length and diameter of stroma. The

previous researches, since 1982-2013, have been successfully used to the identification of *Cordyceps* species using morphology techniques.

Y Kobayasi (1982) and Eriksson et al. (2003) identified *Cordyceps* species using morphological characteristics such as colour, shape of stroma and perithecia. The result found that these characteristics can be used to classify four species such as *C.* subg. *bola cordyceps*, *C.* subg. *cordyceps*, *C.* subg. *neocordyceps*, and *Cordyceps* subg. *Ophiocordyceps*

A. Liu (1997) and Shrestha, Han, Yoon, and Sung (2005) reported the approaches of different ascospore germination and conidial formation of *C. militaris* under the light microscope and electron microscope were utilized to determine *C. militaris* species. Generally, conidial formation was either solitary or whorl, subsequently the succeeding conidia was cylindrical and nearly globose which germinated to produced long hyphae.

Shrestha et al. (2005) studied the ascospore pigmentation of seven *C. militaris* strains infected on Lepidopteran pupae, collected from natural habitats. They were different colours such as yellow, orange and white, while the white colour (albino) was a mutant strain caused by gene mutation. The heritability of pigmentation characteristic is controlled by gene in parental strains, which this gene was transferred from parents and had segregation in F_1 or F_2 progenies

H.-j. Liu et al. (2011) studied shape and inner structure of stroma in *Cordyceps* using by cross section technique. The result showed that *C. sinensis* and *C. gunnii* found perithecia had ranged from elliptical to oval, embedded at the surface of the fertile portion of a stroma. In contract, *C. barnesii*, *C. liangshanesis* and *C. militaris* not found perithecia. The *C. barnesii* had outer cortex brown and inner cortex colourless. The *C. liangshanesis* had outer cortex brownish-yellow and inner cortex colourless. The *C. militaris* had colourless hyphae only.

T.-C. Wen et al. (2013) discovered new species of fungus called *C. sensu* lato. This species was identified by using by morphological technique, The *C. sensu* lato had cylindrical stroma, yellow with brown and the inner structure of mycelium sometimes abundantly and sometime negligent. The mycelium had gray and was found growing on Hepialidae host. This indicated that it can be described the difference among *Cordyceps* species.

2.10 Molecular markers used for genotyping fungi

A molecular marker is defined as a segment of DNA sequence, associated with a part of genome either linkage to gene of interest or target gene encoding a certain trait. The marker was widely used to evaluate DNA polymorphisms, patterns of heredity, genomic variations, evolutions, allele–allele linkages, and genetic map position in living organism(Joshi, Ranjekar, & Gupta, 1999). In *Cordyceps* species, most common markers used to determine genetic diversity were the random amplified polymorphic DNA (RAPD), developed to verify different species (Lam et al., 2015). The restriction fragment length polymorphism (RFLP) was also used to analyze the genetic relationship between two fungal species, such as *C. brongniartii* and *Beauveria brongniartii* (Sasaki, Miyamoto, Tamai, & Yajima, 2007). The inter-simple sequence repeat (ISSR) was evaluated the genetic diversity among eighteen *C. sisnensis* species in China (Liang et al., 2008). The internal transcribed spacer (ITS) sequence analysis was exploited to identify genetic difference between *C. sisensis* from other related *Corrdyceps* species, such as *C. militaris* and *C. gunnii* (Chen et al., 2004). Additionally, the 28S rDNA were also used to study the genetic relationship and diversity among *Cordyceps* species (Artjariyasripong, Mitchell, HywelJones, & Jones, 2001; G.-H. Sung, Spatafora, Zare, Hodge, & Gams, 2001). Of these markers, the ITS and 28S rDNA markers have been successfully used to identify nucleotide-sequence divergence in medicinal fungi, as previous publications in *Cordyceps* (Lam et al., 2015; Y. Li et al., 2013). For these, both markers were beneficially useful for describing genetic variation and structure of *C. militaris* populations. Therefore, the aim of this study was to classify genetic difference of *C. militaris* among species by using the ITS and 28S rDNA markers.

2.9 Definition of Internal transcribed spacer (ITS) and 28S ribosomal DNA

Internal transcribed spacer (ITS) refers to nucleotide sequences at the spacer DNA segments, located between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding to transcribed region in the polycistronic rDNA precursor (Peay, Kennedy, & Bruns, 2008). The ITS is especially useful for elucidating genetic relationships among species and closely related genus (Hao, Chen, Xiao, & Peng, 2010).

The 28S rDNA is the sequence of DNA that codes for ribosomal RNA, it was the first to be targeted for in detail studies of genetic variation in fungi (Simon, Binder, Adam, Hartig, & Ruis, 1992). The 28S have been useful to clarify the taxonomy among fungal phylum, Glomeromycota (Da Silva, Lumini, Maia, Bonfante, & Bianciotto, 2006). Sequence data from ribosomal sequences has represented an additional tool for the exact identification of fungal taxa, having low morphological divergence (Redecker, 2002). The 28S rDNA provides the genetic coding from which rDNA molecules are constructed.

The small subunits contain ITS1, 5.8S, ITS4, and 28S rDNA. The ITS1 is close to the 18S-rDNA border, and ITS 4 anneals to 28S rDNA near the ITS 4 border as shown in the Figure 5 (Lord, Kaplan, Shank, Kitts, & Elrod, 2002).

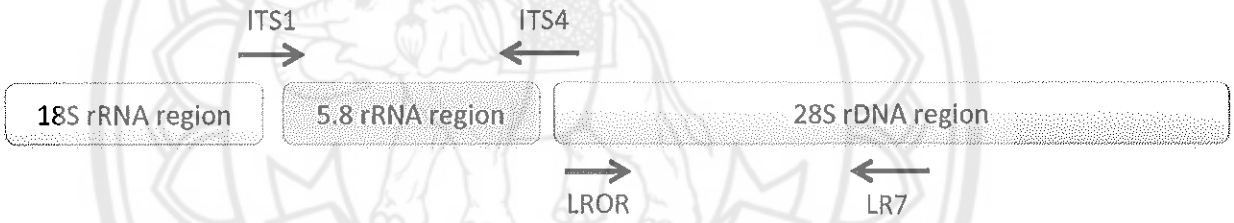


Figure 5 Map positions of primer pairs (ITS1-ITS4 and LROR-LR7) used to amplify PCR products.

Note: Primer ITS 1 is close to the 18S-rDNA border, and primer ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') anneals to 28S rDNA near the ITS 4 border.

2.10 Identification of *Cordyceps* using ITS and 28S rDNA markers

The previous researches, since 2000-2015, have been successfully used to the identification of *Cordyceps* species using ITS and 28S rDNA markers.

J. Kang, Liang, Liu, and Kong (2000) reported analysis of fungal DNA sequences, amplified by the 5.8S-rDNA and partial ITS2 markers. The result indicated that *C. gansuensis* and *C. sinensis* were conspecific (belonging to the same species).

Yue-Qin, Ning, Hui, and Liang-Hu (2002) studied genetic relationship between *C. sinensis* species, collected from different geographical regions of China.

The result found that the ITS sequences within *C. sinensis* have been high homologs, regardless of geographical origin. In contrast, *C. sinensis* was highly divergent from *C. militaris* and *C. gunnii*.

Z. Liu, Liang, Whalley, Liu, and Yao (2001) reported that DNA fragment of *C. sobolifera* was generated using a marker pair of ITS4 and ITS5. Resulting of the aligned DNA sequence between *C. sobolifera* and *Beauveria sobolifera*, collected from China, were not different.

Kinjo and Zang (2001) analyzed the phylogenetic relationships among the *C. sinensis* species from 11 geographic regions in Southwestern China, based on the sequences of ITS1-ITS2 and 28S rDNA regions. Both of markers indicated that the variations among different geographic regions were rather small.

Bae et al. (2002) reported that *C. militaris*, collected from Korea, Japan and China, were determined the genetic diversity by using ITS1 and ITS2 markers. All sequence analysis was low nucleotide divergence, ranged from 0.2% (1 bp) to 0.4% (2 bp). The genetic distance value was calculated by lower than 0.03. It was clearly shown that the nucleotide sequences at ITS region within *C. sinensis* were highly homologous, regardless of geographical origin.

Chen et al. (2004) report that three *Cordyceps* species (*C. sinensis* (synonym *O. sinensis*), *Hirsutella sinensis* and *Paecilomyces sinensis*) collected from Qingzang plateau, was analyze genetic variation by using the ITS1, ITS2, and 5.8S rDNA markers. The result show that the sequence comparison of *C. sinensis* was most closely related to *Hirsutella sinensis*, but clearly divergent from *Paecilomyces sinensis*.

(Kuo, Su, Yang, & Chen, 2005) reported that the ITS1, 5.8S rRNA, and ITS2 markers were used to construct a phylogenetic tree of 17 *Cordyceps* isolates. The result revealed that *C. sinensis* was less closely related to studied 15 *Cordyceps* species, but shared a closer relationship with *C. agriota*.

Wang, Zhang, Hu, Chen, and Qu (2008) investigated the genetic variation of *C. militaris* from different regions (Britain, China, Japan, Korea and Norway), and a PCR product was generated by using the ITS marker. The phylogenetic tree indicated that *C. militaris* was extremely small genetic variation (≤ 0.01, K2P model), which did not correlate to geographical origins. Moreover, this study indicated *C. militaris*

had the same distance level as *C. kyushuensis* and *C. roseostromata* collected from different regions.

Lam et al. (2015)reported that the ITS marker was developed to authenticate different genetic among *Cordyceps* species. The result found that twelve *C. sinensis* collected from geographical origin were highly homologous. In contrast, *C. sinensis* was high divergence from *C. gracilis*, *C. hawkesii* and *C. gunnii*.

CHAPTER III

MATERIALS AND METHODS

3.1 Macroscopic studies

1) Macroscopic characters of *C. militaris*

All *Cordyceps* sp. samples were collected from seven commercial farms in Thailand. The stromata of all samples was studied determination of morphological characteristics such as shape, size, colour and stromata.

2) Mycelium growth

Individual fruiting bodies of *C. militaris* were collected from seven different commercial farms in Thailand. They were cut and plated on a potato dextrose agar (PDA) plate (containing 200 g/l potato, 20 g/l dextrose and 20 g/l agar), and cultured at 25°C in the dark for 7 days. The end of each mycelium was cut and plated on a new PDA plate. Colony diameter (cm) was measured at 3, 6, 9, 12, 15, 18 and 21 days of culture. Mean comparison between *C. militaris* strains were calculated by were compared Duncan's Multiple Range Test (DMRT).

3.2 Microscopic studies

1) Cross section of stromata

The stroma was cut using cross section techniques and stained with lactophenol cotton blue. Morphological characteristics such as surface color, mycelium color and perithecium formation were observed under a microscope (Olympus CH30RF200, Japan) and photographed.

2) Spore shooting

The spores from the stromata were allowed to shoot upwards onto a cover-slip, placed on the middle PDA medium plate, and incubated at room temperature for 24 hours in dark condition. The cover-slip was removed, and then placed upside down on a new slide, containing a drop of lactophenol cotton blue. Morphology of spores was assessed under the microscope.

3) Slide culture technique

Slide culture technique is a valuable technique to study fungal morphology in hyphae and conidia state. Firstly, the mycelium of *C. militaris* was cultivated on PDA at 25°C under dark condition for 7 days. Meanwhile, the slide culture technique was set up as following steps the V shaped glass rod was placed in sterilized petri dishes, the slide was put on the V shaped glass rod, a small agar piece (0.5x0.5x0.5 mm3) was placed on the coverslip, the mycelium (cultivated on PDA) was picked up using a needle to inoculate on the four side of the face-centered square of agar. The coverslip was carefully placed over the agar block; sterile distilled water (5 ml) was poured into the petri dishes. Finally, the slide culture set was incubated at 25°C under dark conditions for 14 days. At the end of experiment, the agar piece was removed, and the mycelia were grown on the slide and coverslip, and then produced conidia. It was stained by lactophenol cotton blue and observed using a compound microscope.

4) SEM Study

Mycelium was individually cultured on PDA medium at 25°C under dark condition for 14 days. The growing mycelium was cut with sterile scalpels into small pieces (1x1 cm) and fixed in 2.5% (v/v) glutaraldehyde. It was washed in 0.1 M phosphate buffer for 15 minutes repeated twice, and washed with 1% osmium tetroxide for 1 hour. The mycelium was then dehydrated through a series of 30, 50, 70, 90 and 100% ethyl alcohol for 30 minutes, respectively. Finally, the mycelium was dehydrated in acetone for 30 minutes before mounting on an aluminum stub and coating with gold-palladium alloy. Spore structures were examined under a scanning electron microscope (JEOL LV 5910, Japan).

3.3 Genomic DNA (gDNA) extraction and PCR amplification

The medium, grown on PDA for 3-7 days, was collected for genomic DNA (gDNA) extraction using InstaGene Matrix (Bio-Rad, United States) according to the manufacturer's instructions.

Two primer pairs as ITS primer pair (ITS1 and ITS4) and 28S rDNA primer pair (LR0R and LR7) were used in this study; their sequences are shown in Table 1. The primers of ITS1 and ITS4 were located on the 3′ end of 18S-rDNA border and the end of 28S rDNA border, respectively (Figure. 5). The ITS primer pair was used to amplify the DNA fragment to flank the entire ITS region and intervening 5.8S subunit (Figure. 5). The PCR reaction was carried out with 20 ng of gDNA as the template in a 30 µl reaction mixture using an EF-*Taq* (SolGent, Korea). The PCR condition was performed with the initial denaturation as follows: activation of *Taq* polymerase at 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C and 72°C for 1 minute each, and a final extension at 72°C for 10 minutes.

Meanwhile, the LR0R and LR7 primers were used for the DNA fragment at the partial 28S rDNA region. The amplified products were purified using a multiscreen filter plate (Millipore Corp., Bedford, MA, United States). Purified DNA samples were performed using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Canada) containing the extension products added to Hi-Di formamide. The mixture was incubated at 95°C for 5 minutes, followed by 5 minutes on ice, and then sequenced and analyzed using an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, Canada).

3.4 DNA sequence alignment

Partial nucleotide-sequence reads were approximately 548 and 1223 nucleotides, amplified by ITS and 28S rDNA primer pairs, respectively. Both sequence reads were assembled using Bioedit Analysis software (Hall, 1999) and compared to known sequences of ITS and 28S rDNA regions of fungi published from the NCBI database using CLUSTAL W (European Bioinformatics Institute (EMBL-EBI), United Kingdom).

Table 2 Primers used to amplify fungal 28S rDNA genes and ITS region sequences

Primer	Sequence (5'→3')	Reference
28S rDNA region		
LROR	ACCCGCTGAACTTAAGC	(Bunyard, Nicholson, & Royse, 1994)
LR7	TACTACCACCAAGATCT	(Bunyard et al., 1994)
ITS region		
ITS1	TCCGTAGGTGAACCTGCGG	(White, Bruns, Lee, & Taylor, 1990)
ITS4	TCCTCCGCTTATTGATATGC	(White et al., 1990)

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Macroscopic studies

1) Macroscopic characters of *C. militaris*

In this study, specimens of *C. militaris* have been collected from seven commercial farms in Thailand. Morphological characteristics (shape, size and colour of the stromata, including growth rate and colour of mycelium) of *C. militaris* (*Cmi*1-*Cmi*7). Seven isolates were examined through the macroscopic (naked eyes) and microscopic techniques. All studied isolates had only a single golden yellow stroma (Figure 6). The stroma of *Cmi*4 had the shortest and widest stipe, compared to other isolates, width 1.5-2.5 cm length 2-4 cm. The *Cmi*7 had the longest stroma stipe (width 0.3-0.4 cm length 5.2-5.5 cm), and its head was indented. Moreover, the stromata of *Cmi*1, *Cmi*2 and *Cmi*3 had cylindrical stipes (width 0.3-0.8 cm and length 4-6 cm), and head of stoma were spherical (Figure 6). Resulting of different stromatal characters among studied *C. militaris* would be caused by various culture condition effects, which were supported by many published reports that morphological changes of *C. militaris* relied on pH (J.-P. Park, Kim, Hwang, & Yun, 2001) and (G.-H. Sung et al., 2011), temperature (J.-M. Sung, Choi, Shrestha, & Park, 2002), humidity (Tapingkae, 2012), light intensity(Sato & Shimazu, 2002) and (G.-H. Sung et al., 2011), carbon, nitrogen and mineral sources culture media (G.-H. Sung et al., 2011), strains and culture methods (Lee et al., 2013). Moreover, different cultivation times may affect morphological changes, which had been reported by G. Zhang and Liang (2013) and J.-M. Sung et al. (2002) who respectively found that the suitable harvesting times for *C. militaris* 45 days and 50 days.

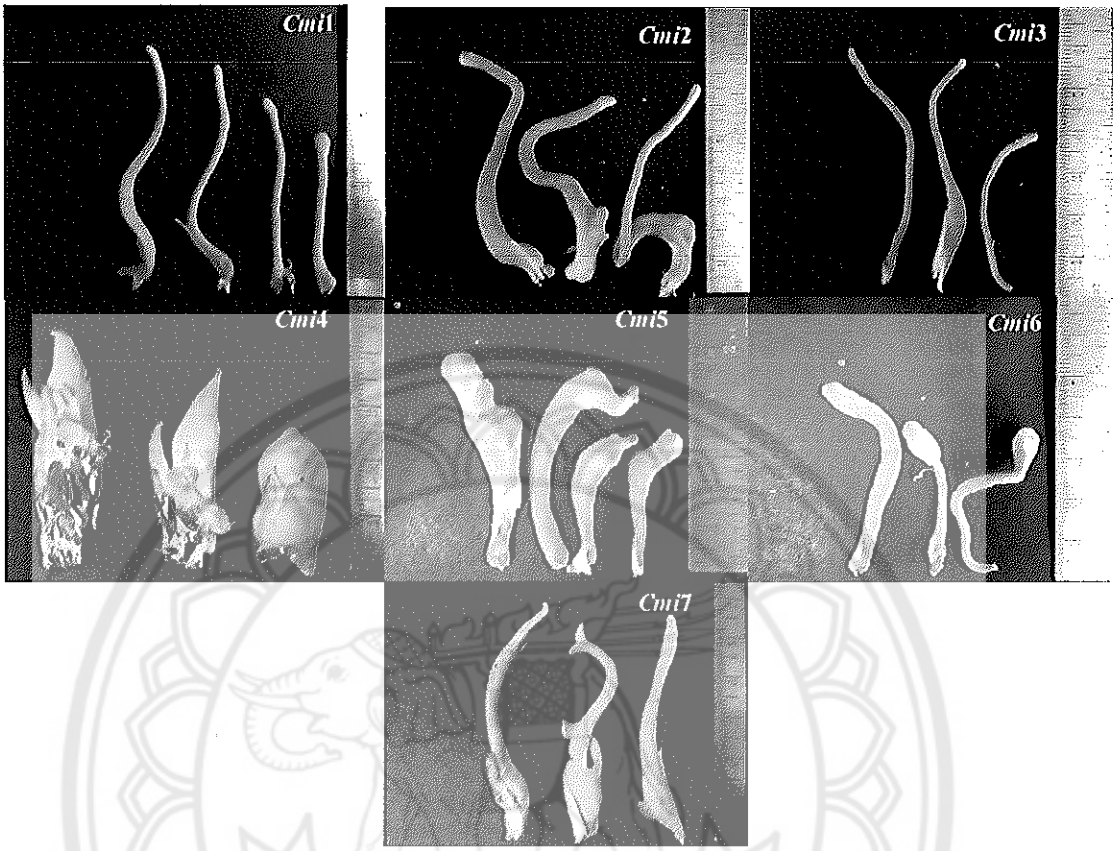


Figure 6 Morphological characteristic of stromata among *C. militaris* isolates *(Cmi*1- *Cmi*7)

2) Mycelium growth

Pathania et al. (2015) reported that mycelium growth of *C. militaris* cultured on PDA (Potato dextrose agar) medium was not significantly different from a PDA medium. In this experiment, the PDA medium was used to study mycelium growth of seven isolates (*C. militaris*) because it was a simple and inexpensive preparation. All isolates of *C. militaris* were cultured on the PDA medium in a modified incubator at 25°C in the dark for 21 days, and mycelium-growth rates were examined every three days. The result revealed that the mycelial growth rates were divided into two groups. Firstly, highest growth rate (*Cmi*1, *Cmi*2, *Cmi*3, *Cmi*5, *Cmi*6, and *Cmi*7) had a colony diameter ranged from 1.90 ± 0.17 to 8.50 ± 0.00 cm. Secondly, *Cmi*4 had the lowest growth rate (Figure 7) and gave colony diameter ranged from 1.78 ± 0.09 to 6.94 ± 0.14 cm) (Table 4). Meanwhile, seven isolates of *C. militaris* were cultured on the

PDA medium in modified incubator at 25°C under dark conditions for 21 days, the growth characteristics were determined every 7 days until 21 days. The results found that the mycelial characteristics of six isolates (*Cmi*1, *Cmi*2, *Cmi*3, *Cmi*5, *Cmi*6, and *Cmi*7) were abundant mycelial density, rough and semi-cottony mycelial texture, and yellowish colony (Table 3 and Table 5). Only the mycelial characteristic of *Cmi*1 was formed stroma on the PDA medium (Figure 7). Moreover, *Cmi*4 mycelium was compact formed densely layers, and dark yellow in middle and white at the edges on PDA (Figure 7). These results indicate that mycelial growth rates and the mycelial characteristics of individually studied isolates were significantly varied, which may depend on different cultivation times (Lee et al., 2013; Shrestha et al., 2006), and different strains (Lee et al., 2013).

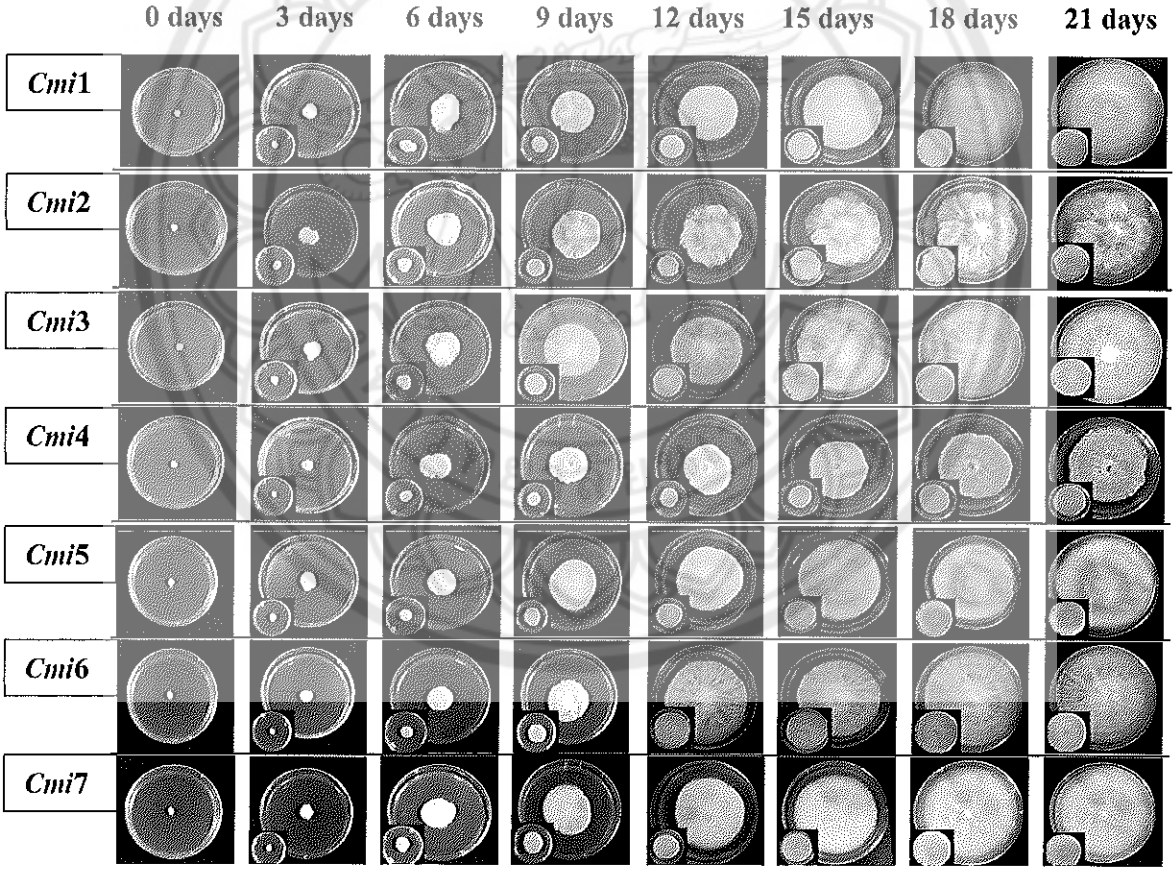


Figure 7 Mycelial growth rates and characteristics among seven *C. militaris* isolates (*Cmi*1 to *Cmi*7) cultured on PDA medium for 3, 6, 9, 12, 15, 18 and 21 days

Table 3 Mycelial growth characteristics of seven *C. militaris* isolates (*Cmi*1-*Cmi*7) on PDA

isolates	Mycelial colony					
	7 days		14 days		21 days	
	Mycelial density	Mycelial texture	Mycelial density	Mycelial texture	Mycelial density	Mycelial texture
*Cmi*1	+ to ++	R to SC	++	F to C	++	F to C
*Cmi*2	+, ++	R to SC	++	R to SC	++	R to SC
*Cmi*3	+, ++	R to SC	++	R to SC	++	R to SC
*Cmi*4	+, ++	R to SC	++	R to SC	++	R to SC
*Cmi*5	+, ++	F to SC	++	F to C	++	F to C
*Cmi*6	+, ++	R to SC	++	R to SC	++	R to SC
*Cmi*7	+, ++	R to SC	++	R to C	++	R to C

Note: + indicates moderate density; ++ indicates abundant density, F indicates flat mycelial texture, R indicates rough mycelial texture; SC indicates semi-cottony mycelial texture; and C indicates cottony mycelial texture

Table 4 Colony diameter of seven *C. militaris* (*Cmi*1- *Cmi*7) isolates on PDA medium

strain	Colony diameter measured of *C. militaris* (cm)						
	3 days	6 days	9 days	12 days	15 days	18 days	21 days
*Cmi*1	1.39±0.11^{c}	2.46±0.05^{d}	4.14±0.07^{b}	5.93±0.52^{bc}	5.86±0.40^{bc}	7.96±0.03^{ab}	8.50±0.00^{a}
*Cmi*2	1.90±0.05^{a}	3.25±0.15^{a}	4.30±0.18^{b}	5.48±0.24^{c}	7.13±0.85^{ab}	7.05±0.40^{c}	8.50±0.00^{a}
*Cmi*3	1.58±0.08^{c}	3.31±0.07^{a}	5.08±0.10^{a}	6.45±0.22^{ab}	7.23±0.49^{ab}	8.50±0.00^{a}	8.50±0.00^{a}
*Cmi*4	1.78±0.09^{ab}	2.73±0.06^{cd}	3.61±0.07^{c}	4.59±0.24^{d}	5.50±0.28^{c}	6.33±0.12^{d}	6.94±0.14^{b}
*Cmi*5	1.86±0.01^{ab}	3.08±0.36^{bc}	4.36±0.19^{b}	5.70±0.20^{bc}	6.88±0.15^{ab}	7.81±0.20^{b}	8.50±0.00^{a}
*Cmi*6	1.58±0.08^{bc}	2.78±0.37^{cd}	4.33±0.07^{b}	6.26±0.10^{bc}	7.36±0.06^{a}	8.45±0.05^{a}	8.50±0.00^{a}
*Cmi*7	1.97±0.12^{a}	2.46±0.16^{ab}	4.86±0.88^{a}	6.85±0.07^{a}	7.60±0.15^{a}	8.20±0.15^{b}	8.50±0.00^{a}

Note: colony diameter value (mm) represents by mean ± se, was measured from all *C. militaris* isolates (*Cmi*1 -*Cmi*7) with triplicates. The same letters in each column indicates non-significant difference of mean values, calculated by using DMRT method.

Table 5 Colony pigmentation of *C. militaris* isolates (*Cmi*1- *Cmi*7) on PDA medium

Strain	Colony pigmentation during					
	6 days		12 days		18 days	
	Margin	Center	Margin	Center	Margin	Center
*Cmi*1	Y	YW	W	YW to Y	W	Y
*Cmi*2	W to PY	YW	W to YW	Y	W to YW	Y
*Cmi*3	W to PY	PY	W to YW	W to PY	W to YW	W to YW
*Cmi*4	W to YW	MY	W to MY	MY	W	W to MY
*Cmi*5	W	W	W to YW	YW	W	W to Y
*Cmi*6	W	W	W to YW	YW	W to YW	W to YW
*Cmi*7	W to PY	W	W to Y	W	W to YW	W to YW

Note: W indicates white; Y indicates yellow; YW indicates yellowish white; PY indicates pale yellow; and MY indicates mustard yellow.

4.2 Microscopic characteristics studies

1) Microscopic characteristics of stromata among *C. militaris*

The outer surface of the seven *C. militaris* isolates (Figure 8) found that most studied isolates had long, smooth, golden yellow stipe and round-surface stromata, except for the *Cmi*4 had a short stipe and sharp cap. These were agreed with Zhang et al., 2013 reported that the cultivated stromata of *C. militaris* was clavate with smooth shaft and orange stipe. Moreover, the *Cmi*5 and *Cmi*6 had a rugged surface. These may be possible that they were different strains, ages of stroma and condition of culture media (Lee et al., 2013).

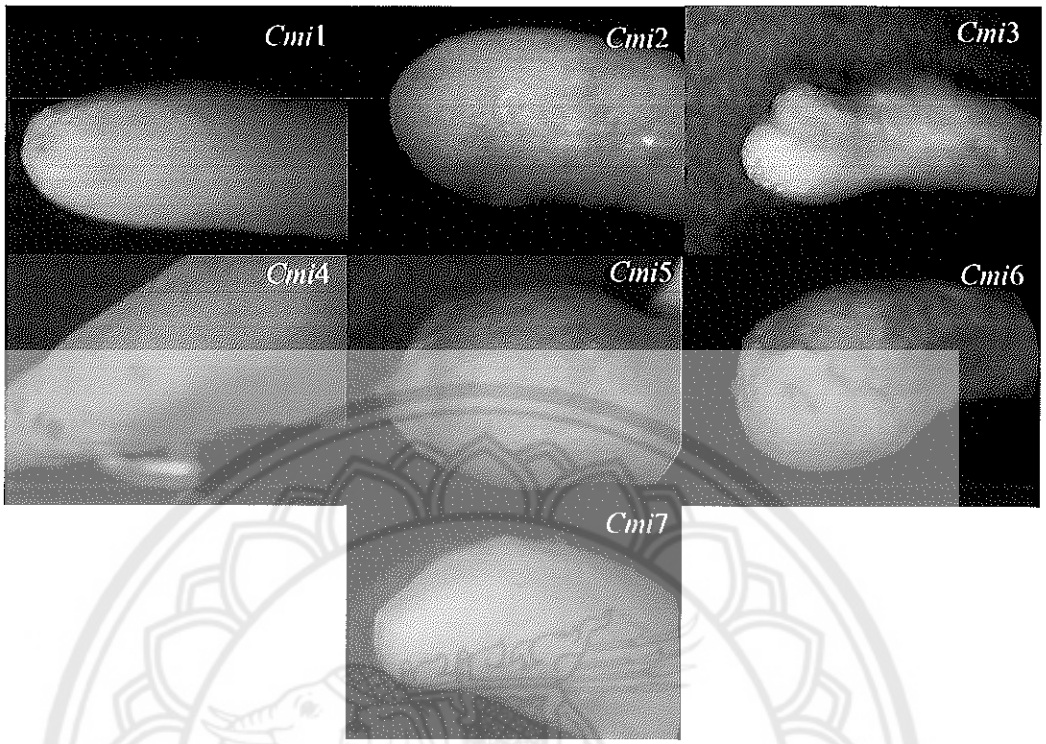


Figure 8 The outer surface characteristics of stromata among seven *C. militaris* isolates observed under Stereo microscope

2) Cross section of stromata among *C. militaris*

Cross sections of individual stroma morphologies of the seven *C. militaris* isolates were examined by staining with lactophenol cotton blue. All isolates showed similar stroma characteristics with smooth yellow gold color outer cortex surfaces, and inner cortexes containing colorless hyphae without a perithecium (Figure 9). These results indicated that the presence or absence of perithecia might be beneficially used to evaluate the morphological diversity of *C. militaris*. As supported by previous publications, some *C. militaris* species showed the presence of perithecia (Xiong, Xia, Zheng, Shi, & Wang, 2010) but other species did not have perithecia (H.-j. Liu et al., 2011) in the cortex region of the stroma. In contrast, all *C. militaris* species had a smooth outer cortex surface and colorless hyphae at the inner cortex (H.-j. Liu et al., 2011).

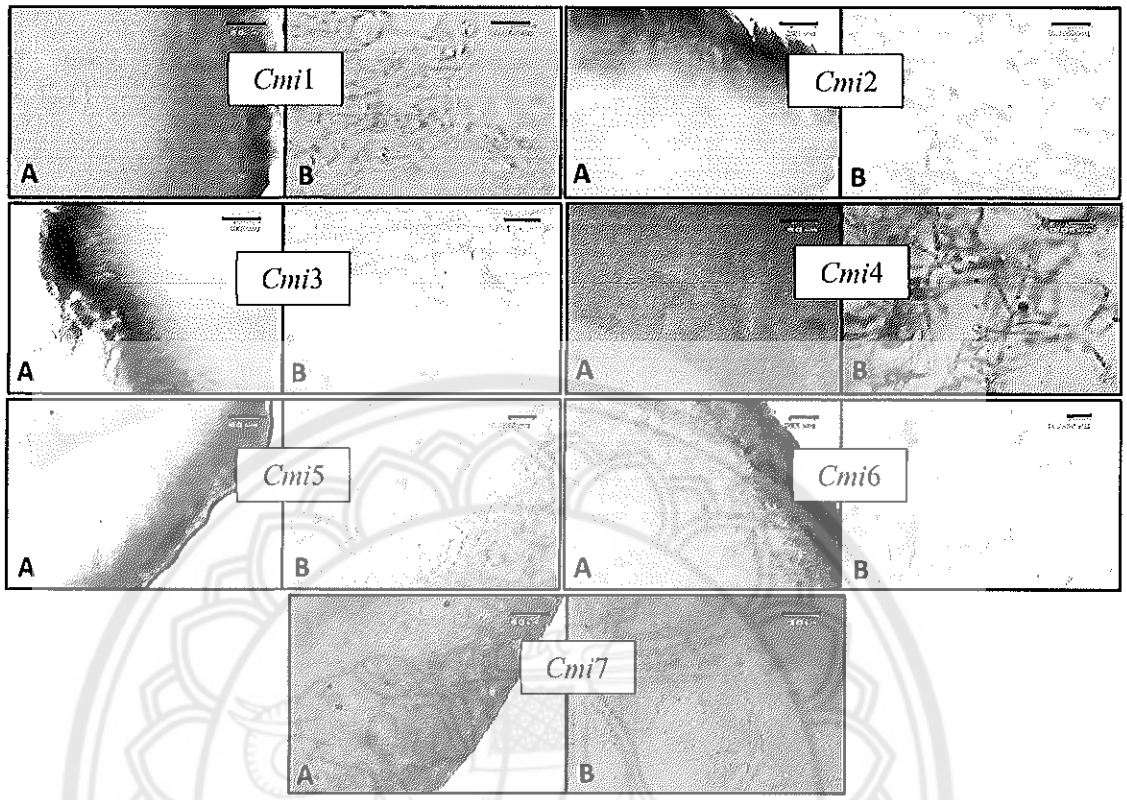


Figure 9 Evaluation of stromal characteristics among seven *C. militaris* isolates (*Cmi*1 to *Cmi*7) by cross section technique under a compound microscope.
Note: random stroma (A) were cross sectioned to examine microscopic characteristics (B); the scale bar indicates 10X (=60 μm)

3) Spore shooting technique

Spore shooting technique is basically used to study spore characteristics among fungal species, based on a single spore (Strobel et al., 1996). In this research, study of spores from stromata *C. militaris* isolates were discharged on cover slip and their morphology was determined under a microscope. The result revealed that all isolates had cylindrical, globose and formed oval spores (Figure 10). These spores were similar to result from the study of Shrestha et al. (2005) who reported that freshly discharged ascospore of *C. militaris* in sterile water showed spores oval and oblong in shape.

Moreover, it was found that the discharged spore produced germ tube after 24 hours, in agreement with Tulasne (1865) found that the spore of *C. militaris* usually germinate in a short time period.

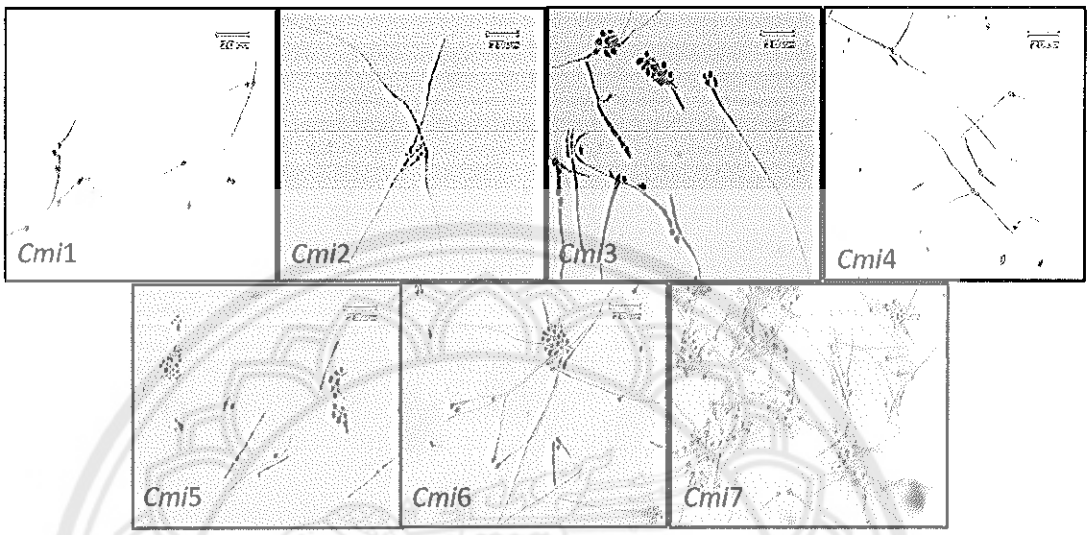


Figure 10 Spore production of *C. militaris Cmi*1-*Cmi*7 isolates by spore shooting
Note: bar indicates 40X (=20 µm)

4) Slide culture technique

To examine the hyphae germination and conidial formation of seven different *C. militaris* isolates (*Cmi*1- *Cmi*7), an individual mycelium was cultivated on PDA medium at 25°C under dark conditions for 14 days by using slide culture technique. The result found that all isolates had similar hyphae morphology and conidial formation. Most isolates produced long hyphae, formed several branches and first-formed conidia as ovoid, pyriform or cylindrical and slim heads. However, some conidia formed short chains, and had a globose head (Figure 11). It has been reported that specimens of different *C. militaris* strains formed branched germinating hyphae, produced conidia in short chains, globose head and slimy head, first-formed conidia were ovoid, pyriform or cylindrical, with the succeeding conidia being globose, nearly subglobose or ellipsoid (Shrestha et al., 2005).

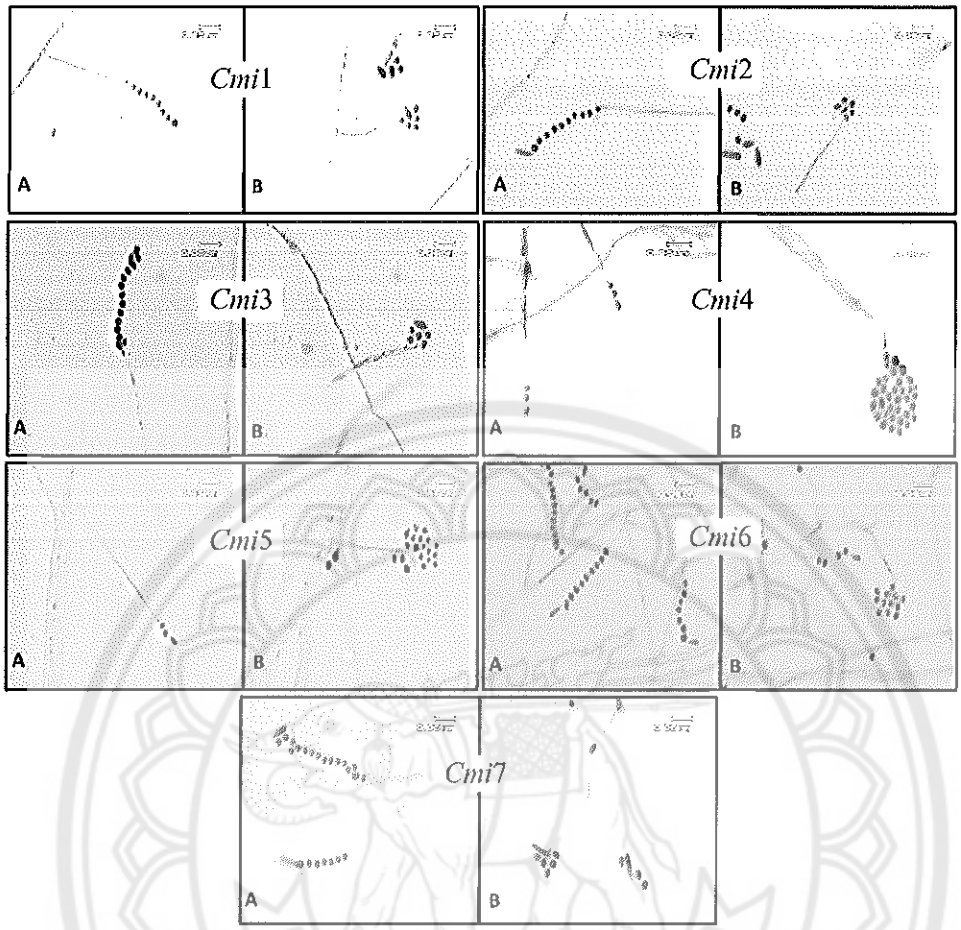


Figure 11 Spore germination of different isolates of *C. militaris Cmi*1-*Cmi*7 by slide culture technique for 14 days, (A) Conidia formed in short chains (B) Conidia formed in globose head

Note: bar indicates A = 100X, B = 100X (=0.06 µm)

5) Observation of conidial formation by Scanning Electron Microscopy (SEM)

Morphologies of the conidia of seven *C. militaris* isolates (*Cmi*1 to *Cmi*7) were determined using SEM. Results showed that most conidia were globose and oval shaped which germinated to produce long hyphae. Most conidia were formed at the end of the hyphae as single globe heads but rare groups were globose in short chains. A few conidia formed as oval shapes (Figure 12).

As previously reported, hyphae of *C. militaris* (EFCC 11255) produce various forms of conidia, either as a single globose head or groups of globose short chains

(Shrestha et al., 2005). Moreover, conidia of *C. militaris* were presented as various shapes including globose (Tulasne, 1865) cylindrical and spherical (Brown & Smith, 1957; De Bary, 1867), almost spherical (Pettit, 1895), and oval (Petch, 1936).

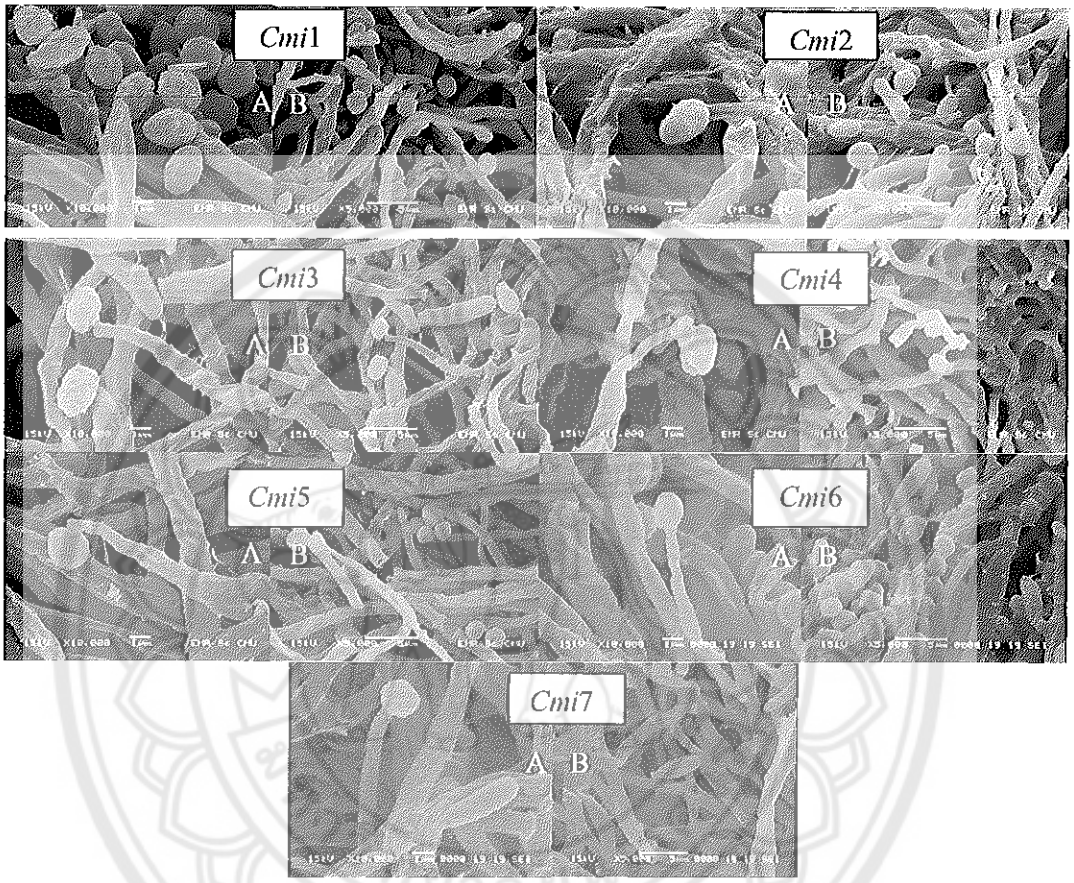


Figure 12 Scanning electron micrographs of conidial hyphae of *C. militaris* *Cmi*1-*Cmi*7 14 days after culture on PDA (Scales: A = 1 μm, B = 5 μm)

4.3 DNA sequencing

Both the internal transcribed spacer (ITS) and 28S rDNA regions are highly divergent in nucleotide sequences and play a vital role in evolution among fungal species (Lam et al., 2015). Therefore, these areas were used to assess genetic variation among the seven isolates of *C. militaris* based on PCR assay. Two ITS primer pairs (ITS1 and ITS4) were used to amplify the DNA fragment flanking the entire ITS region and intervening 5.8S subunit, giving an ITS-PCR-fragment (548 bp long). Nucleotide sequences from all the studied isolates gave 100% similarity, indicating

that the seven isolates of *C. militaris* did not have genetic distance at the ITS. This distance values of the ITS region among the seven isolates were slightly different when compared to *C. militaris* isolates NBRC100741 (Figure 13) (Schoch et al., 2012). Only one nucleotide (adenine, A) at position 93 of the ITS region of each studied strain was substituted by guanine (G) of NBRC100741 strain (Schoch et al., 2012) (Figure 13). The nucleotide substitution might be caused by evolutionary effect (Y.-J. Park & Min, 2005), and provide information for phylogenetic reconstruction (Watanabe et al., 2011) fungal species.

Meanwhile, two 28S rDNA primer pairs (LROR and LR7) were used to amplify the DNA fragment at the 28S rDNA region, giving a 28S rDNA-PCR-fragment (1223 bp long). Nucleotide sequence reads of both the ITS-PCR-fragment and the 28S rDNA-PCR-fragment from individual isolates were aligned using the ClustalW program and then compared to their known fungal sequences published from the NCBI database. Also, nucleotide sequences from all the studied isolates gave 100% 28S rDNA (Figure 16). This distance values of the 28S rDNA regions among the seven isolates were slightly different when compared to *C. militaris* strains JM0807 (Zhong, Peng, Qi, Lei, & Liu, 2010). Furthermore, a nucleotide at position 136 in the 28S rDNA region of *C. militaris* JM0807 strain was inserted by a thymine base (T) compared to all studied strains, as previously reported by Zhong et al. (2010) (Figure 14). The nucleotide insertions can be correlated with the evolutionary distance among fungal species (Y.-J. Park & Min, 2005).

However, nucleotide sequenes, located on ITS and 28S region of all studied *C. militaris* isolates were compared to other *C. militaris* strains by using Blast data program. The results found that these comparisons had 99-100% and 99% the percentages of identity at ITS (Table 6) and 28S (Table 7), respectively. These confirmed that all studied isolates were truly *C. militaris* species.

In summary, comparative analysis of nucleotide sequences both ITS and 28SrDNA were clearly indicated that there were not genetic variation among all studied *C. militaris* isolates. However, they exhibited only one nucleotide substation in ITS and one nucleotide insertion in 28SrDNA, compared to *C. militaris* strains, NBRC100741 or JM0807, respectively.

```
Samples   1  AAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACGTTCAGAGTTGGGCGTTTTACGG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     785  AAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACGTTCAGAGTTGGGCGTTTTACGG  726

Samples  61  CGTGGCCACGTCGGGTTCCCGGTGCGAGTTGGAGTACTACGCAGAGGTCGCCGCGGACGG  120
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Ref     725  CGTGGCCACGTCGGGTTCCCGGTGCGAGTTGGGGTACTACGCAGAGGTCGCCGCGGACGG  666

Samples 121  GCCGCCACTTCATTTCGGGGGCGGCGGTGTGCTGCCGGTCCCCAACGCCGACATCCCCCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     665  GCCGCCACTTCATTTCGGGGGCGGCGGTGTGCTGCCGGTCCCCAACGCCGACATCCCCCA  606

Samples 181  GGGGACGTCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     605  GGGGACGTCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGC  546

Samples 241  GCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     545  GCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC  486

Samples 301  ATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTGATT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     485  ATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTGATT  426

Samples 361  CATTTGTTTTGCCTTGCGGCGGATTCAGAAAAACTGGTAGATACAGTGTTTGGGGCCCCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     425  CATTTGTTTTGCCTTGCGGCGGATTCAGAAAAACTGGTAGATACAGTGTTTGGGGCCCCC  366

Samples 421  GACGGCCGCCGCCCAGGCCCGCGTCCAGGCGCTGGGCGAGTCCGCCGAAGCAACGATAGG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     365  GACGGCCGCCGCCCAGGCCCGCGTCCAGGCGCTGGGCGAGTCCGCCGAAGCAACGATAGG  306

Samples 481  TATGTTCACAAAGGGTTGGGAGTTGGAAAACTCGTTAATGATCCCTCCGCTGGTTCACCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     305  TATGTTCACAAAGGGTTGGGAGTTGGAAAACTCGTTAATGATCCCTCCGCTGGTTCACCA  246

Samples 541  ACGGAGAC  548
             ||||||||
Ref     245  ACGGAGAC  238
```

Figure 13 Comparative analysis of nucleotide sequences between seven studied *C. militaris* isolates and *C. militaris* NBRC100741 strain using ClastaW program

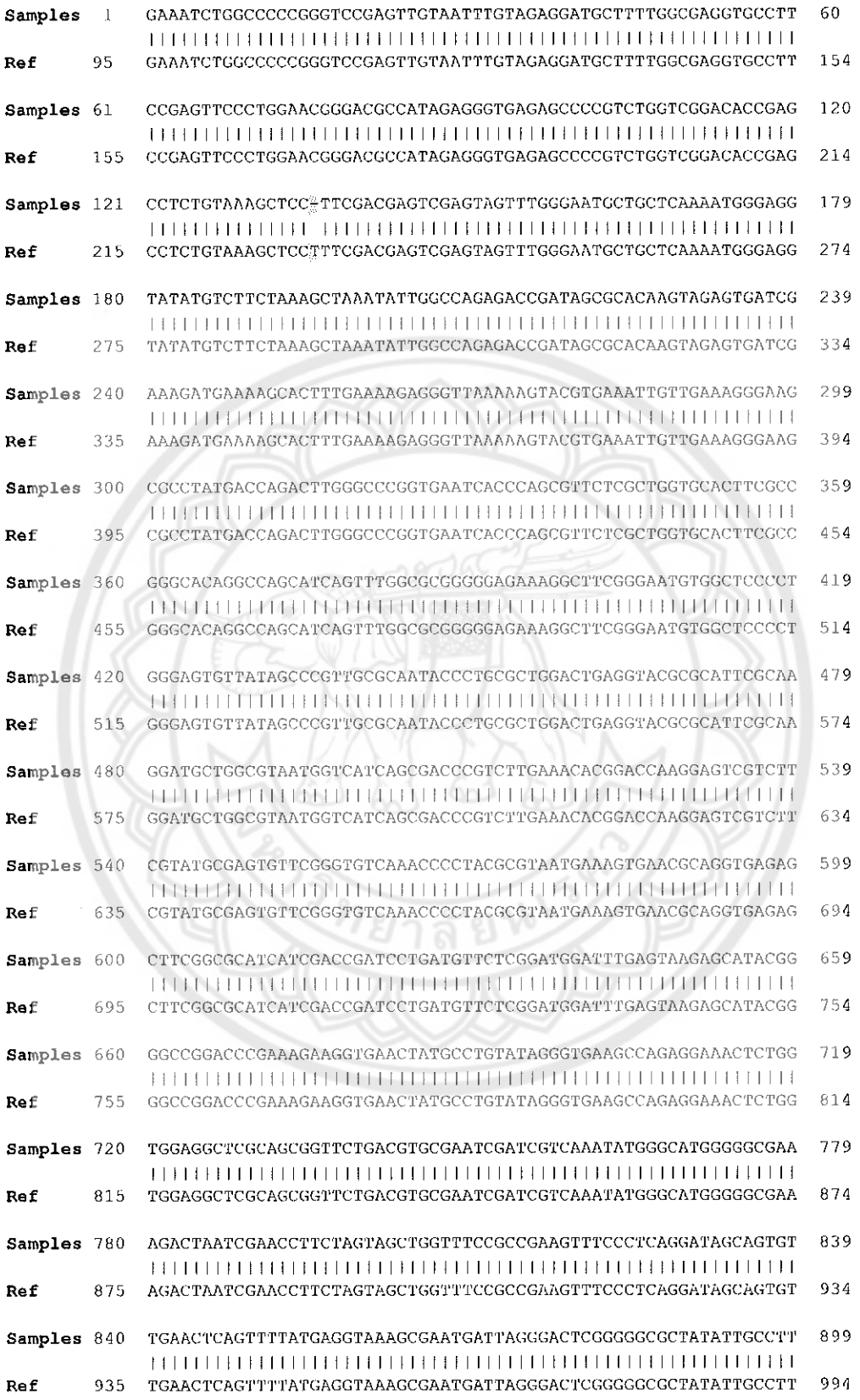
Note: samples indicate seven studied *C. militaris* isolates. Ref indicates *C. militaris* NBRC100741 strain, yellow lighted letter indicates the nucleotide substitution in ITS region, and number indicates position of nucleotide sequences.

Table 6 Blast results of the ITS nucleotide sequences generated from studied *C. militaris* isolates using nucleotide blast (NCBI)

Description	Query cover	Identities	Accession
C. militaris isolate WU-CMH	100%	100%	KM197165.1
C. militaris strain NBRC 30377	99%	100%	JN943300.1
C. militaris strain NBRC 100741	100%	99%	JN943437.1
C. militaris strain NBRC 9787	100%	99%	JN943433.1
C. militaris strain BCRC33736	100%	99%	HQ591387.1
C. militaris strain SPNU1002	96%	99%	KY407774.1
C. militaris strain KACC50001	96%	99%	KY407771.1
C. militaris strain *KACC44454*	96%	99%	KY407766.1
C. militaris strain *KACC40226*	96%	99%	KY407760.1

Note: Query cover is the percentage of sequences from seven *C. militaris* isolates, aligned to a sequence in each accession of *C. militaris* strain.

Identity is the percentage of sequences from seven *C. militaris* isolates which is highly significant match to reference sequences.



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Samples 900   CATCCATTCTCAAACTTTAAATATGTAAGAAGCCCTTGTTACTTGATTGAACGTGGGCAT  959
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref     995   CATCCATTCTCAAACTTTAAATATGTAAGAAGCCCTTGTTACTTGATTGAACGTGGGCAT  1054

Samples 960   TCGAATGTATCAACACTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAA  1019
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref    1055   TCGAATGTATCAACACTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAA  1114

Samples1020   CCGAACGCGAGGTTAAGGTGCCGGAGTGGACGCTCATCAGACACCACAAAAGGTGTTAGT  1079
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref    1115   CCGAACGCCAGGTTAAGGTGCCGGAGTGGACGCTCATCAGACACCACAAAAGGTGTTAGT  1174

Samples1080   ACATCTTGACAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGACTGTGTAACAA  1139
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref    1175   ACATCTTGACAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGACTGTGTAACAA  1234

Samples1140   CTCACCTGCCGAATGTACTAGCCCTGAAAATGGATGGCGCTCAAGCGTCCCACCCATACC  1199
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ref    1235   CTCACCTGCCGAATGTACTAGCCCTGAAAATGGATGGCGCTCAAGCGTCCCACCCATACC  1294

Samples1200   TCGCCCTCAGGGTAGAAACGAAGC  1223
              ||||||||||||||||||||||||
Ref    1295   TCGCCCTCAGGGTAGAAACGAAGC  1318
```

Figure 14 Comparative analysis of nucleotide sequences between seven studied *C. militaris* isolates and *C. militaris* JM0807 strain using ClastaW program

Note: samples indicate seven studied *C. militaris* strains, Ref indicates *C. militaris* JM0807 strain, yellow lighted letter indicates the nucleotide insertion in 28S rDNA region, and number indicates position of nucleotide sequences

Table 7 Blast results of the 28S rDNA nucleotide sequences generated from studied *C. militaris* isolates using nucleotide blast (NCBI)

Description	Query cover	Identities	Accession
C. militaris strain ATCC 34164	100%	99%	CP023322.1
C. militaris strain JM0807	100%	99%	JN411084.1

Note: Query cover is the percentage of sequences from seven *C. militaris* isolates, aligned to a sequence in each accession of *C. militaris* strain.

Identity is the percentage of sequences from seven *C. militaris* isolates which is highly significant match to reference sequences.

CHAPTER V

CONCLUSION

Seven commercial isolates of *C. militaris* (*Cmi*1, *Cmi*2, *Cmi*3, *Cmi*4, *Cmi*5, *Cmi*6 and *Cmi*7) collected in Thailand were investigated for morphological study using various macroscopic and microscopic methods.

In macroscopic studies, the results concluded that most isolates had cylindrical shapes and golden yellow of stromata. Moreover, they had the highest mycelial growth rate and abundant mycelial density with rough and semi-cottony mycelial texture, and yellowish colony. Of these, *Cmi*4 had the lowest growth rate, and its mycelium was compact, formed of dense layers, and dark yellow on PDA.

In microscopic studies using a cross section technique, the stromal characteristics revealed that all isolates were golden yellow at the edge, and colourless hyphae. The spore characteristics, investigated by using the spore shooting technique, revealed that most conidiospores were globular shape. Conidial formation was studied using slide culture and SEM techniques. Results determined that most spores were globose and oval shaped; moreover, these germinated and produced long hyphae. At the ends of the hyphae, most conidia were formed as single globe heads but rarely as global groups in short chains. A few conidia were formed with an oval shape.

Meanwhile, all studied *C. militaris* isolates were evaluated genetic variation by using nucleotide sequence analysis of ITS and 28S rDNA regions. Results concluded that all studied isolates showed no genetic variation. However, they had one nucleotide substitution in the ITS region and one nucleotide insertion in the 28S rDNA region, compared to *C. militaris* NBRC100741 or JM0807 strains, respectively.

Suggestion

These results were clearly elucidated that the macroscopic characteristics (such as stroma characteristic, mycelial growth rate, and mycelial pigmentation) were basically significant tools for fungal classification, including *C. militaris* isolates of this studied.

However, the microscopic characteristics and molecular markers (in case ITS and 28S rDNA) have been still not found differences. Therefore, further research is necessary to collect more samples (*C. militaris*) from different commercial sources in Thailand to examine morphological techniques and other molecular markers such as random amplification of polymorphic (RAPD), terminal restriction fragment (TRF) and amplified ribosomal DNA restriction analysis (ARDRA). Furthermore, it should be study quantity and quality of bioactive compound (cordycepin, adenosine) in *C. militaris*.

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APPENDIX

Appendix 1 Groups aligned by sequence fragments (approximately 548 and 1223) generated from ITS and 28S gene respectively, amplified by using DNA template from seven strains *C. militaris*

ITS gene

>Cmi1

AAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACGTTCAGAGTTGGG
CGTTTTACGGCGTGGCCACGTCGGGTTCCCGGTGCGAGTTGGAGTACTAC
GCAGAGGTCGCCGCGGACGGGCCGCCACTTCATTTCGGGGGCGGCGGTGT
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TGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTGCG
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TTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAG
TTTTGATTCATTTGTTTTGCCTTGCGGCGGATTCAGAAAAACTGGTAGATA
CAGTGTTTGGGGCCCCCGACGGCCGCCGCCCAGGCCCGCGTCCAGGCGCT
GGGCGAGTCCGCCGAAGCAACGATAGGTATGTTCACAAAGGGTTGGGAGT
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>Cmi2

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GCAGAGGTCGCCGCGGACGGGCCGCCACTTCATTTCGGGGGCGGCGGTGT
GCTGCCGGTCCCCAACGCCGACATCCCCCAGGGGACGTCGAGGGTTGAAA
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>Cmi3

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>Cmi4

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>Cmi5

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>Cmi7

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28S gene

>Cmi1

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>Cmi2

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>Cmi6

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