ความหลากหลายของราแมลง *Ophiocordyceps nutans* ในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทย

DIVERSITY OF AN INSECT FUNGUS *OPHIOCORDYCEPS NUTANS* COLLECTED FROM CENTRAL AND NORTH-EASTERN PARTS OF THAILAND

ศรีคุณ ขาวงาม

โครงงานพิเศษเล่มนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร

ปริญญาวิทยาศาสตรบัณฑิต (จุลชีววิทยาอุตสาหกรรม)

ภาควิชาชีววิทยา คณะวิทยาศาสตร์

สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

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DIVERSITY OF AN INSECT FUNGUS *OPHIOCORDYCEPS NUTANS* COLLECTED FROM CENTRAL AND NORTH-EASTERN PARTS OF THAILAND

SRIKHUN KHAO-NGAM

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Name	Srikhun Khao-ngam
Student ID	57050897
Degree	Bachelor of Science (Industrial Microbiology)
Department	Biology
Academic Year	2017
Supervisor	Nattawut Rungjindamai Ph.D.
Co-supervisor	Janet Jennifer Luangsa-ard Ph.D.

Committee	Signature
Suchada Mongkolsamrit Chairperson	Suchada Mongkolsamrit
Nattawut Rungjindamai Supervisor	Nattawut Rungjindamai
Janet Jennifer Luangsa-ard Co-supervisor	Luangsa-ard

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หัวข้อโครงงานพิเศษ ความหลากหลายของราแมลง *Ophiocordyceps nutans* ในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทย

ชื่อนักศึกษา ศรีคุณ ขาวงาม รหัสนักศึกษา 57050897

ปริญญา วิทยาศาสตรบัณฑิต (จุลชีววิทยาอุตสาหกรรม)

ภาควิชา ชีววิทยา

คณะ วิทยาศาสตร์

ปีการศึกษา 2560

อาจารย์ที่ปรึกษา ดร.ณัฐวุฒิ รุ่งจินดามัย

อาจารย์ที่ปรึกษาร่วม ดร.เจเน็ท เจนนิเฟอร์ เหลืองสอาด

บทคัดย่อ

ราก่อโรคในแมลงหรือราแมลงมีรายงานการพบตามพื้นที่ต่างๆ โดยในประเทศไทยมีการค้นคว้าศึกษาวิจัยราแมลงหลากหลายสกุล การศึกษานี้มุ่งเน้นศึกษาอนุกรมวิธานและความหลากหลายของรา *Ophiocordyceps nutans* (Hypocreales, Ophiocordycipitaceae) ก่อโรคกับแมลงกลุ่มมวน อันดับ Hemiptera สามารถพบราชนิดนี้ได้ในกองใบไม้ทับถมกัน หรือบนดิน ลักษณะก้านราเป็นสีดำหรือน้ำตาล ส่วนปลายมีสีส้มอมเหลืองหรือสีแดง เก็บตัวอย่างรา *O. nutans* จากพื้นที่ป่าได้แก่ (1) ป่าชุมชนบ้านเผ่าไทย จังหวัดพิษณุโลก (2) น้ำตกเจ็ดคต อุทยานแห่งชาติเขาใหญ่ จังหวัดสระบุรี (3)น้ำตกตาดฟ้า อุทยานแห่งชาติภูเวียง จังหวัดขอนแก่น จำนวน 18, 11 และ 6 ตัวอย่าง ตามลำดับ ศึกษาสัณฐานวิทยาได้แก่ ก้านชูสปอร์ ถุงหุ้มสปอร์ สปอร์ รวมทั้งศึกษาความสัมพันธ์เชิงวิวัฒนาการระดับโมเลกุลทั้งหมด 4 ยีนส์ ได้แก่ Internal Transcribed Spacers (ITS), Large Subunit Ribosomal RNA (LSU), Elongation Factor 1-alpha (EF1) และ RNA Polymerase II Subunit B (RPB1)เมื่อวิเคราะห์ของเชื้อ *O. nutans* ทั้งหมด 26 isolates โดยวิเคราะห์แบบแบบรวมทุกยีนส์พบว่า แยกกลุ่มออกได้เป็น 2 กลุ่มย่อย ได้แก่ กลุ่มที่ (1) กลุ่มที่มีความใกล้เคียงกับ *O. nutans* ที่ได้รับการศึกษามาก่อนหน้า (23 isolates) และ กลุ่มที่ (2) กลุ่มที่คาดว่าจะเป็นชนิดใหม่ (3 isolates) ผลการศึกษาทั้งด้านสัณฐานวิทยาและชีวโมเลกุลสอดคล้องกัน และพบว่าเชื้อราที่แยกได้กลุ่มที่(1) และ (2) มีแนวโน้มเป็นราแมลงชนิดใหม่ จากการศึกษาจึงตั้งเป็นราแมลงชนิดใหม่ ได้แก่ *Ophiocordyceps thailandensis* และ *Ophiocordyceps phukhiaoensis*

คำสำคัญ : ราก่อโรคในแมลง, ความสัมพันธ์เชิงวิวัฒนาการ, อนุกรมวิธาน

Title	Diversity of An Insect Fungus, *Ophiocordyceps nutans* Collected From Central and North-Eastern Parts of Thailand
Students	Srikhun Khao-ngam Student ID 57050897
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Supervisor	Nattawut Rungjindamai Ph.D.
Co-supervisor	Janet Jennifer Luangsa-ard Ph.D.

Abstract

Entomopathogenic fungi or insect fungi has been reported worldwide especially in South-East Asia. Thailand has one of the largest collections of insect fungi in the world due to a huge geographic diversity. There are hundreds species of insect fungi recorded in Thailand. In this study, taxonomy and diversity of *Ophiocordyceps nutans* (Hypocrales, Ophiocordycipitaceae) are focused. This fungus is a specific parasite in stink bugs (Order Hemiptera). Specimens are usually found in leaf litter and soil. Traditional classification of *O. nutans* is mainly based on two morphological characters (1) Stroma is brown to blackish brown (2) Color of fertile head is red or orange-yellow. The fungal specimens were collected from 3 locations (1) Ban Phao Thai Community Forest (Phitsanulok), (2) Chet Kot Waterfall, Khao Yai National Park (Saraburi) and (3) Tard Fah Waterfall, Phu Wiang National Park (Khonkaen). Eighteen, 11 and 6 specimens were collected, respectively. Morphological study was done at microscopic level and 3 morphological features including perithecia, asci and ascospores were photographed and measured. Phylogenetic relationship of 26 isolates of *O. nutans* was studied using a combined dataset based on 4 genes consisting of Internal Transcribed Spacers (ITS), Large Subunit Ribosomal RNA (LSU), Elongation Factor 1-alpha (EF1) and RNA polymerase II Subunit B (RPB1). The results show that our 26 isolates of *O. nutans* were separated into 2 subgroups; (1) subgroup A – 23 isolates are closely related to other *O. nutans* and (2) subgroup B – 3 isolates emerge as a new lineage and they should be assigned into two new species including *Ophiocordyceps thailandensis* and *Ophiocordyceps phukhiaoensis*.

Keywords: Invertebrate-pathogenic fungi, molecular phylogeny, taxonomy

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Finally, I would like to dedicate this dissertation to my parents and all members of my family who always support and encourage me in every way that they could.

Srikhun Khao-ngam
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Abbreviations/Symbols

Abbreviations/Symbols	Description
BBH	BIOTEC Bangkok Herbarium
BCC	BIOTEC Culture Collection
°C	degree Celsius
DNA	Deoxyribonucleic acid
EF1-α	Elongation factor 1-alpha
ITS regions	Internal transcribed spacer
LSU	Large subunit of the ribosomal DNA
Min	Minute
μ	Micro
RPB1	RNA polymerase subunit 1
rpm	Rounds per minute
O. nutans	*Ophiocordyceps nutans*
PDA	Potato dextrose agar
PCR	Polymerase chain reaction

Chapter 1
Introduction

1.1 Biological role of fungi in ecological system

Fungi, best known as the biggest organism in the world and having a high diversity in nature, can be found in a wide range of natural environments ranging from terrestrial to aquatic habitats because they are capable to adapt themselves to live in those diverse habitats. Fungi play an important role in ecological system by acting as a saprophyte. They decompose animal remains and plant debris therefore organic substances in those decaying matters can be recycled and released into surroundings. Then those nutrients can be utilised by other organisms in the nature.

Fungi are frequently categorised according to their modes of living or their bioactivity for example marine fungi, thermotolerant fungi, soil fungi, endophytic fungi, medicinal mushroom and edible mushroom.. In this study, a fungal group called insect fungi is focused. This group of fungi is best known for their capability to produce a diverse group of useful chemical compounds used in medical and pharmaceutical industries. Furthermore, a huge diversity of insect fungi has been reported from Thailand due to richness of natural resources.

1.2 Objective of this study

1. To collect and study morphologically of *Ophiocordyceps nutans* collected from the forests in different parts of Thailand
2. To analyse phylogenetically of *O. nutans* based on DNA sequence of the internal transcribed spacer (ITS) regions, Large subunit of the ribosomal DNA (LSU), Elongation factor 1-alpha (EF1-∝) and RNA polymerase subunit 1 (RPB1)

1.3 Experimental scope of this study

1. To collect 30-40 specimens of *O. nutans*
2. To isolate 10-15 axenic cultures of *O. nutans* from these specimens
3. To compare morphological characteristics of these newly collected isolates with those previously collected
4. To analyse all isolates of *O. nutans* collected in this study using phylogenetic methods

1.4 Expected outcomes

1. Better understanding of the relationship of an insect fungus, *O. nutans* and its insect hosts.
2. Knowledge of genetic diversity of *O. nutans* at species level based on phylogenetic analyses.

Chapter 2
Literature Review

2.1 Taxonomy of An Insect Fungus – *Ophiocordyceps nutans*

Insect fungi can be classified into three families, the Clavicipitaceae, the Cordycipitaceae and the Ophiocordycipitaceae (Ascomycota, Sordariomycetes, Hypocreales). The classification of insect fungi is traditionally based on morphological characteristics of the fungi. Molecular approaches and phylogenetic analyses are used to confirm new genera and species. However there are some species of insect fungi which are difficult to identify due to their close similarity and ambiguity of among species. These groups of organisms are called cryptic species. Identification is one of the most important steps for studying biodiversity because it provides crucial information of the identity of the organisms and leads to other areas of research.

The fungus was firstly described as *Cordyceps nutans* Pat. (Petch 1931) which was isolated from Japan by Kobayashi in 1941 and was later collected from other counties including New Guinea, China and Thailand (Hywel-Jones 1995). The genera name was changed from *Cordyceps* to *Ophiocordyceps* by Sung *et al.* (2007). An insect fungus – *Ophiocordyceps nutans* – which is one of the cryptic species was selected and studied morphologically and phylogenetically. The genus *Ophiocordycpes* is certainly placed in the Ophiocordycipitaceae (Sung *et al.* 2007) but the taxonomy of a species of *O. nutans* is highly doubtful. Several studies showed that *O. nutans* is species complex (Sasaki *et al.* 2012). With the advent of phylogenetic analysis, Friedrich *et al.* (2018), who studied taxonomic placements of various isolates of *O. nutans* collected from different geographical locations in Brazil, their results showed that several lineages within the *O. nutans* species complex have been found. So a novel species of *Ophiocordyceps, O. neonutans,* is proposed.

2.2 The Host – Fungus Relationship

Ophiocordyceps nutans is a specific parasite on stink bugs (Hemiptera) (ตัวมวน in Thai) but there are few reports host specificity of the *O. nutans*. Some insect fungi have a wide host range which can be used to control diverse groups of insect families but some clades show a high degree of insect-host specificity which can only be used to attack a certain group of insects.

So that understanding of the host-fungus relationship is extremely essential for further commercial development of agricultural products. In this study, specificity of *O. nutans* and its insect hosts are focused to solve this issue.

A comprehensive study of the impact of the fungal parasites on insect host population dynamics and host–parasite co-evolution requires an understanding of the ecology and life cycle of the parasite itself. However, little is known about the parasite-invertebrate host relationship in terms of population dynamics (Mongkolsamrit *et al.* 2012).

Ophiocordyceps nutans was collected on forest ground or on leaf litter. In addition, *O. nutans* was revealed host as pentatomid stink bugs. The previous report from Sasaki *et al.* (2004) of *O. nutans* showed molecular study of this species. The first evidence of the fungus and its host was reported by Sasaki *et al.* (2008) and also was confirm the host-specificity by work published by Sasaki *et al.* (2012) which found that *O. nutans* can classified into two types according to the family of insect hosts. Recently, Friedrich *et al.* (2018) reported new species – *Ophiocordyceps neonutans* were found parasitizing stink bug belonging to the subfamilies including the Discocephalinae, Edessinae and Pentatominae of the Pentatomidae, they were visibly different and larger than the Japanese host of *O. nutans* complex. Moreover, the host subfamilies are known to be endermic to Neotropical regions, showing the host specificity of *O. neonutans.*

2.3 Application of insect fungi

Insect fungi are well known for their various applications particularly in medical and pharmaceutical industries. Studies on bioactivity show that these group of fungi are able to produce numerous groups of chemicals with diverse bioactivities for example antibacterial (Prathumpai & Kocharin 2016) antimalarial (Isaka *et al.* 2011), antimicrobial (Isaka *et al.* 2011).

Insect fungi are also used as biological control agents (BCAs) which are beneficial organisms that suppress other pathogenic organisms into the level that is no longer danger to man and environment. Because of their capability of producing toxic substances against insects, entomopathogenic fungi are widely and successfully used for controlling insect pests in organic farming and agricultural business. Numerous genera of insect fungi such as *Beauvaria*, *Isaria, Metarhizium, Paecilomyces* and *Verticillium* have been studied as BCAs.

Beauvaria basiana and *Metarhizium anisopliae* was used as broad spectrum BCAs. The study about *B. bassiana* by Fancelli *et al.* (2013) showed the efficiency of the three strains of *B. bassiana* was compared to a synthetic chemical substance (carbofuran, 4g/trap) and absence of control. Carbofuran caused 90% of mortality in the population of the insect pest, *Cosmopolites sordidus* (Coleoptera: Curculionidae). Aw and Hue (2017) found that *M. anisopliae* was a potential BCA of termites, mosquitoes and cattle ticks. This fungus has the capability to be developed into a commercial product.

In this study *Ophiocordyceps nutans* is studied in order to prove the host specificity on stink bugs. Insect fungi make many important benefit to our life, they are well known for producing various groups of secondary metabolites which can be either used as medicine for cure and treatment some illness such as *Ophiocordyceps sinensis* or used as pest control agents to suppress population of insect pests that destroy agricultural product.

Table 2.1 Examples of *Ophiocordyceps* species and their hosts

Common name of insect	Order of insect host	*Ophiocordyceps* species	Reference
Ant	Hymenoptera	*Ophiocordyceps camponoti-leonardi*	Kobmoo *et al.* (2015)
		Ophiocordyceps camponoti-saundersi	Kobmoo *et al.* (2015)
		Ophiocordyceps halabalaensis	Luangsa-ard *et al.* (2011)
		Ophiocordyceps myrmecophila	Sung *et al.* (2007), Hywel-Jones (1996)
		Ophiocordyceps polyrhachis-furcata	Kobmoo *et al.* (2015)
		Ophiocordyceps pseudolloydii	Evans and Samson (1984), Sung *et al.* (2007)
		Ophiocordyceps unilateralis	Petch (1931), Evans and Samson (1984), Sung *et al.* (2007)
Beetle larva	Coleoptera	*Ophiocordyceps barnesii*	Sung *et al.* (2007)
		Ophiocordyceps brunneipunctata	Sung *et al.* (2007), Hywel-Jones (1995)
Butterfly larva	Lepidoptera	*Ophiocordyceps cochlidiicola*	Kobayasi (1980), Sung *et al.* (2007)
Cicada	Hemiptera	*Ophiocordyceps longissima*	Kobayasi (1963), Sung *et al.* (2007)
Cockroach	Blattodea	*Ophiocordyceps blattae*	Petch (1931), Hywel-Jones (1995), Sung *et al.* (2007)
Dragonfly	Odonata	*Ophiocordyceps odonatae*	Kobayasi (1981), Sung *et al.* (2007)
Stink bug	Hemiptera	*Ophiocordyceps nutans*	Hywel-Jones (1995), Sung *et al.* (2007)
Termite	Blattodea	*Ophiocordyceps communis*	Sung *et al.* (2007)
True fly	Diptera	*Ophiocordyceps dipterigena*	Berk and Broome (1873)

Chapter 3
Material and Methods

3.1 Survey and collection

3.1.1 Sampling sites

The systematic collections of an insect fungus, *Ophiocordyceps nutans* were made in three sampling sites which are situated in different parts of Thailand. The surveys were conducted during rainy season during June to November 2017. The details of each site were given below.

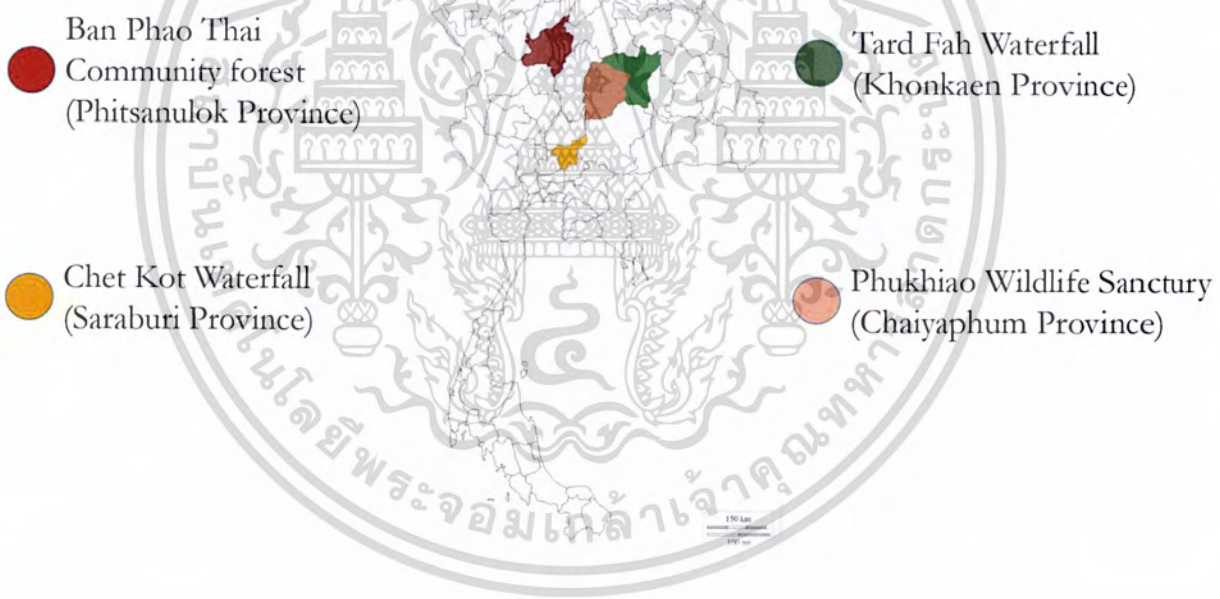

Fig 3.1 A map of geographical locations of four collecting sites used in this study including (1) Ban Phao Thai Community forest, (2) Chet Kot Waterfall, (3) Tard Fah Waterfall and (4) Phukhiao Wildlife Sanctuary (highlighted in red, yellow, green and cream, respectively).

(1) The Ban Phao Thai Community Forest (ป่าชุมชนบ้านเผ่าไทย)

The community forest is located in Phitsanulok Province situated in the lower northern part of Thailand. This site was a former military operation unit which was set up during the war against the communists. These days, this site is a community forest which villagers play a major role in making decision on land use and forest management in the support of government.

(2) The Chet Kot Waterfall (น้ำตกเจ็ดคต)

The Chet Khot Waterfall, which is a part of Khao Yai National Yark in Saraburee in the central part of Thailand, is in the middle of a dense tropical forest. It is best known for ecotourism due to its picturesque and biodiversity of flora and fauna.

(3) The Tard Fah Waterfall (น้ำตกตาดฟ้า)

This waterfall is located in the Phu Wiang National Park, Khonkaen Province in the north-eastern part of Thailand. The Tad Fah Waterfall which is surrounded by tropical rain forests is a popular destination for tourists during rainy season.

3.1.2 Collection of fungal specimens

Survey took place during June 2017 to October 2017. The fungal specimens of *Ophiocordyceps nutans* was collected by involving careful examination of leaf litter, fallen plant structures, for examples leaves and branches on the forest floor in order to detect the emergence of stroma from insect hosts. Digging is needed if the samples were found in soil and then they were carefully excavated from soil. The specimens were kept in plastic boxes and brought back to the Microbe Interaction and Ecology Lab (BMIE) at BIOTEC for further identification and isolation for pure culture. The numbers of samples collected from three locations are listed in Table 3.1

3.2 Isolation of pure cultures

The fungal specimens collected in the field were brought back to the BMIE Lab at BIOTEC and the isolation was done as soon as possible in order to avoid the contamination and decomposition of specimens. The spore discharge method was used for isolation of pure cultures of *O. nutans*. The perithecial head of specimen was put above Potato Dextrose Agar (PDA) petri plates. This method was performed in a moist chamber and left overnight allowing spores to be discharged fully and freely onto the medium. After fungal spores were discharged from the specimens, the isolation of PDA plates was carefully checked and remove contaminants under the stereo microscope. The germinated spores were picked and transferred to fresh PDA plates. The cultures of *O. nutans* were incubated at room temperature for 3-4 weeks. When diameters of fungal colonies reach 1-2 cm, they were deposited to the BIOTEC Culture Collection (BCC), Thailand and the BCC codes were given.

Table 3.1 Detail of survey and collection of *Ophiocordyceps* spp. including collecting sites, its host of collection and number of isolates.

Province	No.	Original Code	BBH	Insect Host (Family)
Saraburi	1	MY11779	43627	*Dalapada clavata* Fabr. (Pentatomidae)
	2	MY11780	43628	*Dalapada clavata* Fabr. (Pentatomidae)
	3	MY11781	43629	*Dalapada oculata* Fabr. (Pentatomidae)
	4	MY11782	43630	*Dalapada* sp. (Pentatomidae)
	5	MY11783	43631	*Dalapada clavata* Fabr. (Pentatomidae)
	6	MY11784	43632	*Dalapada oculata* Fabr. (Pentatomidae)
	7	MY11785	43633	*Dalapada clavata* Fabr. (Pentatomidae)
	8	MY11786	43634	*Dalapada* sp. (Pentatomidae)
	9	MY11787	43635	*Dalapada clavata* Fabr. (Pentatomidae)
	10	MY11788	43636	*Dalapada clavata* Fabr. (Pentatomidae)
	11	MY11789	43637	Unidentified Host (Coreidae)

Table 3.1 (cont.) Detail of survey and collection of *Ophiocordyceps* spp. including collecting sites, its host of collection and number of isolates.

Province	No.	Original Code	BBH	Insect Host (Family)
Phitsanulok	12	MY11793	43638	Nymph *Tessaratoma* sp. (Pentatomidae)
	13	MY11794	43639	*Dalader* sp. (Coreidae)
	14	MY11795	43640	*Dalader* sp. (Coreidae)
	15	MY11796	43641	*Dalader* sp. (Coreidae)
	16	MY11797	43642	*Dalapada* sp. (Pentatomidae)
	17	MY11798	43643	*Dalader* sp. (Coreidae)
	18	MY11799	43644	*Dalader* sp. (Coreidae)
	19	MY11800	43645	*Dalader* sp. (Coreidae)
	20	MY11870	43646	Unidentified Host (Coreidae)
	21	MY11871	43647	*Tessaratoma javanica* Thunb. (Pentatomidae)
	22	MY11872	43648	*Tessaratoma javanica* Thunb. (Pentatomidae)
	23	MY11873	43649	Unidentified Host (Coreidae)
	24	MY11874	43650	*Dalader* sp. (Coreidae)
	25	MY11875	43651	*Dalader* sp. (Coreidae)
	26	MY11876	43652	*Dalader* sp. (Coreidae)
	27	MY11877	43653	*Dalader* sp. (Coreidae)
	28	MY11878	43654	*Dalapada* sp. (Pentatomidae)
	29	MY11879	43655	*Dalader* sp. (Coreidae)
Khonkaen	30	MY11880	43656	*Dalapada* sp. (Pentatomidae)
	31	MY11881	43657	*Dalapada* sp. (Pentatomidae)
	32	MY11882	43658	Unidentified Host
	33	MY11883	43659	*Dalapada* sp. (Pentatomidae)
	34	MY11884	43660	*Dalapada* sp. (Pentatomidae)
	35	MY11885	43661	Unidentified Host (Coreidae)

3.3 Morphological observation

The twenty-five specimens were studied macroscopically and microscopically. The lengths and breadths of fertile head, stipe, fruit body, perithecia, asci and partspores were measured and recorded.

3.3.1 Macroscopic observation

Three macroscopic features of *O. nutans* including (1) sizes of perithecial heads, (2) sizes of fruit bodies and (3) their colours were observed with a naked eye. Lengths and wide of the perithecial head and the fruit body of *O. nutans* specimens growing on insect host were measured using a ruler and the data were recorded in millimeter (mm). The colours of the perithecial heads were compared to the Colour Chart of Naturalist (Smithe 1975).

3.3.2 Microscopic observation

The perithecial heads of *O. nutans* attached on insect hosts were cross-sectioned, mounted in a glass slide and stained with lactophenol cotton blue before covered with a cover slip. Three morphological characters of *O. nutans* consisting of (1) perithecia, (2) asci and (3) partspores were observed under a light microscope and photographed. Detailed examination of the shape and size of these three characteristics were done by measuring with 10, 20, 40 replicates, respectively.

3.3.3 Specimen preservation

The leftover fungal specimens used in this study were deposited in the BIOTEC Bangkok Herbarium (BBH). Samples were dried using an electric food dryer and then stored in a labelled plastic boxes. Plastic boxes were sealed with cello tape to prevent contamination from phyloplanes and mites before depositing at the BBH and the BBH codes were given.

3.4 Molecular phylogeny

3.4.1 DNA Extraction

The cultures of *O. nutans* were grown on PDA and incubated for four weeks. The mycelium was harvested *en masse* by scratching aerial mycelium on the PDA using a sterile spatula. The genomic DNA of *O. nutans* was extracted using E. Z. N. A. Forensic DNA kit (Omega bio-tek) and the standard protocol according to manufacturer's recommendation was followed. The genomic DNA was stored at -20°C for further use.

Moreover, The genomic DNA was extracted using CTAB method. The mycelium was added into 1.5 Eppendorf tube containing 600 µl of CTAB buffer. The buffer was mixed and incubated at 65°C for 15 minutes on heat box. Six hundred µl of CIAA: Isoamyl were added into the tube and inverted 50 times. The tube was centrifuged at 13,000 rpm for 5 minutes and the supernatant was separated.

Collecting the supernatant and added into new 1.5 Eppendorf tube. A volume of 300 µl of 100% isopropanol was added, the tube was mixed and inverted 20 times softly. Freezing at -20°C for 20 minutes then was centrifuged at 4°C, 13,000 rpm for 15 minutes. The supernatant was discarded and washed with 100 µl 70% cold ethanol. The tube was left at room temperature until pellet was dried. Fifty µl of TE buffer was added and the genomic DNA was stored at -20°C for further use.

3.4.2 PCR Amplification

The four regions of DNA including (1) Internal Transcribed Spacer (ITS), (2) Large Subunit (LSU), (3) Elongation Factor 1 (EF1) and (4) RNA Polymerase Subunit 1 (RPB1) used for amplified in this study. The names and sequences of forward and reverse primers corresponding each gene are listed in the Table 3.2. The formulae of PCR mixture for PCR amplification (final volume 50 µl) is given in the Table 3. 3 The PCR conditions used for each gene are listed in Table 3.4. The PCR amplification was performed using a T-100 Thermal Cycler (Bio-Rad). PCR Product was purified using QIA quick PCR purification kit by following the manufacturer's recommended protocols.

Table 3.2 List of forward and reverse primers for four genes used in this study.

Gene	Primers		Sequence	Reference
ITS	Forward	ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	White *et al.* (1990)
	Reverse	ITS4	5'TCCTCCGCTTATTGATATGC-3'	White *et al.* (1990)
LSU	Forward	LROR	5'- GTACCCGCTGAACTTAAGC-3'	Rehner and Samuels (1994)
	Reverse	LR7	5'-TACTACCACCAAGATCT-3'	Vilgalys and Hester (1990)
RPB1	Forward	CRPB1	5'-CCWGGYTTYATCAAGAARGT-3'	Castlebury et al (2004)
	Reverse	RPB1CR	5'-CCNGCDATNTCRTTRTCCATRTA-3'	Matheny *et al.* (2002)
EF1	Forward	983F	5'-GCYCCYGGHCAYCGTGAYTTY AT-3'	Rehner and Buckley (2005)
	Reverse	2218R	5'-ATGACACCRACRGCRACRGTYTG-3'	Rehner and Buckley (2005)

Table 3. 3 PCR components and the concentrations of chemical reagents

Mixture	Initial concentration	Final concentration	Volume (µl)
Nano pure water	-	-	13.4
Betaine	4 M	0.4 M	2.5
Taq buffer	10x	1x	2.5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs mix	10 mM	0.4 mM	1
Forward primer	10 mM	0.2 µM	0.5
Reverse primer	10 mM	0.2 µM	0.5
Taq DNA polymerase	5 U/µl , 500 U	1 U/µl	0.1
DNA template	-	40-100 ng/µl	2
Total			25

Table 3.4 PCR Profiles for four genes

Step	Temperature and time			
Locus	ITS	LSU	EF1	RPB1
Reference	Sasaki *et. al* (2008)	LSU profile in BMIE Lab	5 genes Profile in BMIE Lab	5 genes Profile in BMIE Lab
Initialization	94 °C / 5 min	94 °C / 3 min	94 °C / 3 min	94 °C / 3 min
Denaturation	94 °C / 1 min	94 °C / 1 min	94 °C / 1 min	94 °C / 1 min
Annealing	55 °C / 1 min	55 °C / 1 min	50 °C / 1 min	50 °C / 1 min
Extension	72 °C / 3 min	72 °C / 3 min	72 °C /1.30 min	72 °C /1.30 min
Cycle	25	35	35	35
Final extension	72 °C / 7 min	72 °C / 10 min	72 °C / 8 min	72 °C / 8 min
Final hold	15 °C	15 °C	15 °C	15 °C

3.4.3 Purification of PCR products

PCR Products were purified using the QIA Quick PCR purification kit following the manufacturer' s standard protocols. The PCR product was eluted to a final volume of 25-35 µl. The PCR products were checked before sending out for DNA sequencing. Two microliters of purified PCR product was mixed with 2 µl of loading dye, electrophoresed and photographed.

3.4.4 DNA Sequencing

The purified PCR Products were sent out to Macrogen (Korea) for DNA sequencing. The sequencing reactions were performed using the same set of primers used for PCR amplification. The forward and reverse DNA sequences were trimmed on each end to remove ambiguous sequences. The pairs were aligned and consensus sequences for each gene were made.

3.4.5 Sequence Alignment

1) Combined Gene Dataset

To examine the phylogenetic relationship and taxonomic position of *O. nutans* at the familial and ordinal levels, the combined gene dataset of *O. nutans* was constructed based on four genes including ITS, LSU, EF1 and RPB1. DNA Sequences of the four genes of *O. nutans* generated in this study were compared with the other sequences of numerous species of *Ophiocordyceps* previously published by Sanjuan *et al.* (2015) and freely available at the GenBank Database. The combined dataset was multiple aligned using Clustal W 1.6 (Thompson *et al.* 1994) and manually adjusted where necessary using BioEdit 7.2.5 (Hall 1999). Gap adjustments were made to improve quality of the dataset.

2) Single Gene Dataset

ITS Regions as a universal DNA barcode for fungi were analysed as a single gene in order to solve and clarify species complex of *O. nutans*. The dataset was constructed based on sequences previously published by Sazaki et al. (2012) and a recent paper from Brazil (Shrestha *et al.* 2018). ITS sequences of 25 isolates of *Ophiocordyceps* cf. *nutans* were compared with sequences deposited in GenBank using the BLASTn Search tool to obtain the closest matched sequences. The ITS dataset was multiple aligned and manually adjusted where necessary using the same software metioned for the combined dataset.

3.4.6 Phylogenetic analyses

The two datasets were phylogenetically analysed using three different algorithms consisting of (1) Maximum parsimony analysis (MP), (2) Maximum likelihood analysis (ML) and (3) Bayesian analyses (BY). Analyses based on MP and ML were conducted by using PAUP 4.0b10 while analyses based on BY were conducted using Mr. Bayes 3.1.2. Statistical supports of tree branches for each analysis were calculated and expressed as MP Bootstrap (MPBS), ML Bootstrap (MLBS) and BY posterior probabilities (BYPP),respectively. The phylogenetic trees were visualized using TreeView.

Chapter 4
Results

4.1 Isolates of *Ophiocordyceps* cf. *nutans* used in this study

The fungal specimens were collected from four sampling sites including (1) Ban Phao Thai Community Forest, Phitsanulok province, (2) Chet Kot Waterfall, Saraburi province and 3) Tard Fah Waterfall, Khonkaen province and 4) Phukhiao Wildlife Sanctuary, Chaiyaphum province. Three specimens form Phukhiao Wildlife Sanctuary were identified their insect host as *Papillosa* sp. (Tessaratomidae). The details of all specimens used in this study are shown in the Table 4.1.

After the fungal isolation was made, the specimens were sent out for identification of insect hosts which was carried out by an entomologist, Miss Jomsurang Duangthisan at the Plant Protection Research and Development Office, the Department of Agriculture, Bangkok. The results show that various species of insect in the Pentatomidae are the hosts of *Ophiocordyceps* cf. *nutans* which *Dalapada* is the common genus of insect found in this study. However, there were few samples which could not be identified at the generic level due to the deterioration of specimens.

Table 4.1 List of isolates *Ophiocordyceps* spp. used to study including collecting sites, its hosts, of collection and number of isolates.

Province	Original Code	BCC	Insect Host	Total
Chaiyaphum	MY10827	-	*Papillosa* sp. (Tessaratomidae)	3
	MY10829	79226		
	MY10830	-		
Saraburi	MY11779	86875	*Dalapada clavata* Fabr., *Dalapada oculata* Fabr. (Pentatomidae)	6
	MY11780	86876		
	MY11781	86877		
	MY11782	86878		
	MY11784	86879		
	MY11785	-		
	MY11786	86880		
Phitsanulok	MY11340	82789	Unidentified genus (Pentatomidae)	11
	MY11625	84230		
	MY11631	84236		
	MY11624	84229	*Dalader acuticosta* (Coreidae)	
	MY11628	84233		
	MY11629	84234		
	MY11630	84235		
	MY11633	84238		
	MY11800	86440		
	MY11793	86436	Nymph *Tessaratoma* sp. (Pentatomidae)	
	MY11878	-	*Dalapada* sp. (Pentatomidae)	
Khonkaen	MY11880	86883	*Dalapada* sp. (Pentatomidae)	4
	MY11881	-		
	MY11883	86884		
	MY11884	-		
All				24

4.2 Morphology study

Ophiocordyceps* cf. *nutans

Insect host: stink bug (Pentatomidae) (Figure 4.1)

Location: Chet Kot Waterfall, Saraburi Province

Specimens were found in the leaf litter in soil. The fungus produces one or two black stromata arising from the abdominal regions of the insect host (body size 14–20 mm). These stromata grow straight up through the soil, 55–114 mm in length, 1.2–2.0 mm in breadth. The perithecial head is terminal and orange-rufous (color 132C), 11.0–15.0 mm in length, 1.2–2.0 mm in breadth. The region is below the perithecial head is chamois (color 123D). Perithecia are immersed, hyaline-walled and oblique with a curved neck, (400.0–) 437.0–605.8 (–700.0) μm in length, (100–) 139.0–223.0 (–300) μm in breadth. Asci are cylindrical, (195–) 230.8–345.4 (–425) μm in length, (5.0–) 4.8–7.3 (–7.5) μm in breadth. Ascospores break into 64 part-ascospores. Partspores are cylindrical or slightly barrel-shaped and the end blunt, (8-) 9–13 (–17) μm in length, 2–4 μm in breadth.

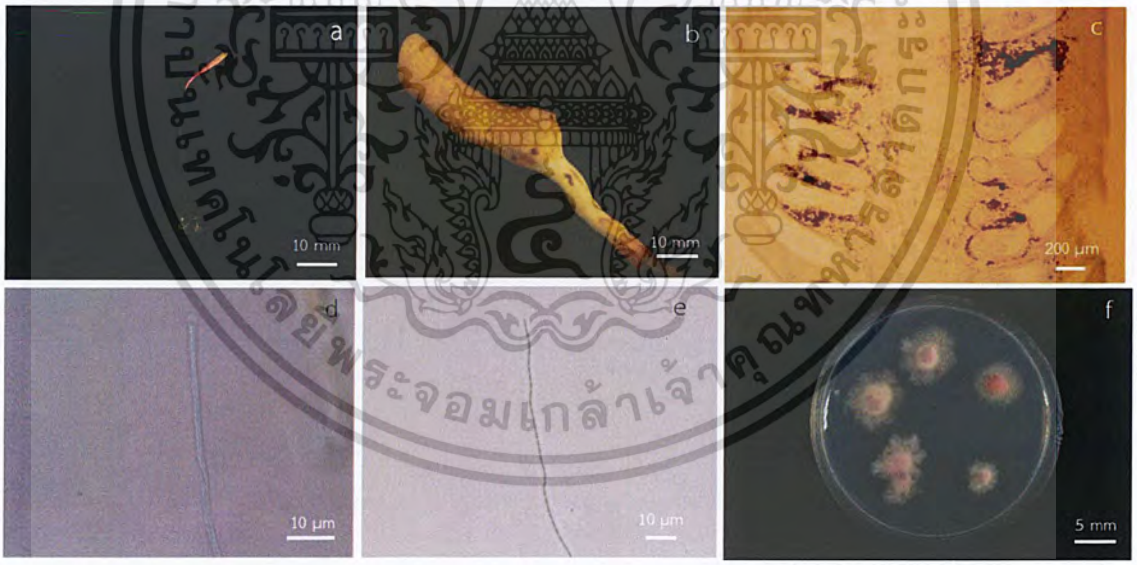

Figure 4.1 *Ophiocordyceps* cf. *nutans* (MY11779) **a** stromata arose from stink bug (Tessaratomidae); **b** perithecial head; **c** cross section of perithecial head showing perithecia; **d** ascus & ascus tip; **e** partspores ; **f** colonies on PDA

Anamorph state: *Hymenostilbe nutans* Samson & H.C. Evans

Other specimens examined: MY11780, MY11781, MY11782, MY11784, MY11785, MY11786

Ophiocordyceps* cf. *nutans

Insect host: stink bug (Pentatomidae) (Figure 4.2)

Location: Tard Fah Waterfall, Khonkaen Province

Specimens were found in the leaf litter in soil. The fungus produces one or two black stromata arising from the abdominal regions of the insect host (body size 14–20 mm). These stromata grow straight up through the soil, 28–130 mm in length, 1.2–1.8 mm in breadth. The perithecial head is terminal and chamois (color 123D), 10–22 mm in length, 1.2–2.0 mm in breadth. The region is below the perithecial head is chamois (color 123D). Perithecia are immersed, hyaline-walled and oblique with a curved neck, (370.0–) 425.0–576.0 (–650.0) μm in length, (110–) 154–224 (–250) μm in breadth. Asci are cylindrical, (182.5–) 233–356 (–435.0) μm in length, (5.0–) 5.8–8.3 (–10.0)μm in breadth. Ascospores break into 64 part-ascospores. Partspores are cylindrical or slightly barrel-shaped and the end blunt, (6-) 8–11 (–15) μm in length, 2–3 μm in breadth.

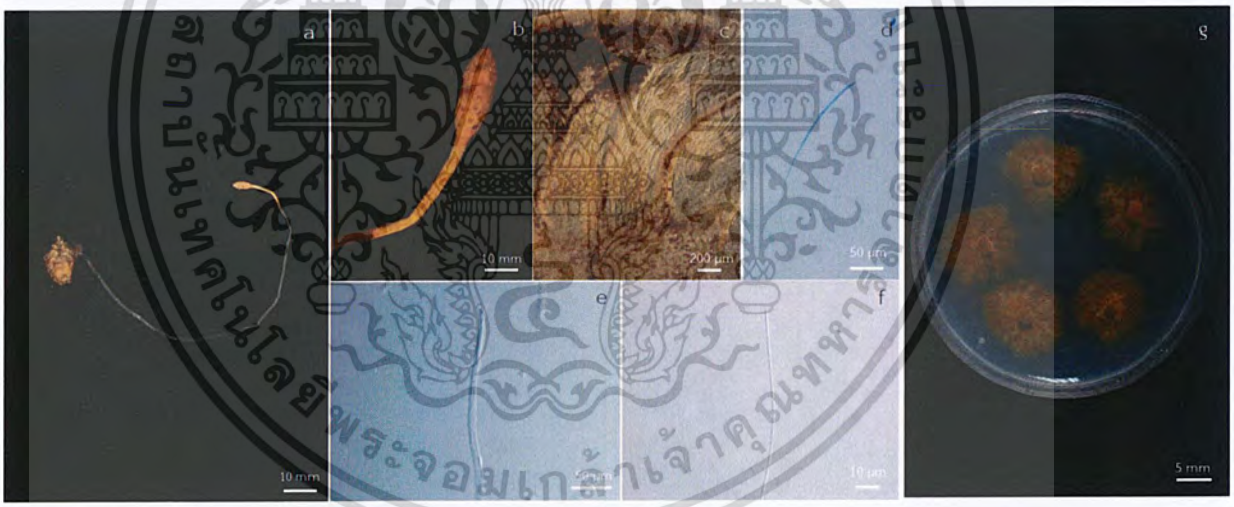

Figure 4.2 ***Ophiocordyceps* cf. *nutans* (MY11884)** **a** stroma arose from stink bug (Pentatomidae); **b** perithecial head; **c** cross section of perithecial head showing perithecia; **d** ascus; **e** ascus tip; **f** partspores; **g** colonies on PDA

Anamorph state: *Hymenostilbe nutans* Samson & H.C. Evans

Other specimens examined: MY11880, MY11881, MY11883

4.3 Comparison of morphological characters of *Ophiocordyceps* cf. *nutans*

Twenty-five isolates of *Ophiocordyceps* cf. *nutans* were morphologically studied. Three fungal characteristics including (1) fertile head, (2) stipe and (3) fruit body of these isolates were compared at the macroscopic level. The detail of the length and breadth of their structures are shown in Table 4.2

Table 4.2 Comparison of fungal structure of *Ophiocordyceps spp.* from four locations at macroscopic level

Province	Strain	Perithecial head		Stipe	Fruit body
		Length (mm)	Breadth (mm)	Length (mm)	Length (mm)
Chaiyaphum	MY10827	34	2	98	132
	MY10829	21.5	2	80	101.5
	MY10830	17.5	2	77.5	95.5
Phitsanulok	MY11340	6	1.8	74	80
	MY11624	8	1.8	75	83
	MY11625	4.5	1.2	55.5	60
	MY11628	19.5	1.9	85.5	105
	MY11629	15	2	100	115
	MY11630	10	2	82	92
	MY11631	7	1.8	83	90
	MY11633	9.5	1.9	80	89.5
	MY11793	N/A	N/A	N/A	70
	MY11800	N/A	N/A	N/A	130
	MY11878	20	1.8	75	95

Table 4.2 (Cont.) Comparison of fungal structure of *Ophiocordyceps spp.* from four locations at macroscopic level

Province	Strain	Perithecial head		Stipe	Fruit body
		Length (mm)	Breadth (mm)	Length (mm)	Length (mm)
Saraburi	MY11779	11	1.6	64	75
	MY11780	15	2	55	70
	MY11781	12	1.4	58	70
	MY11782	15	2	97	102
	MY11784	11	1.2	114	125
	MY11785	13	1.6	92	105
	MY11786	15	2	77	92
Khonkaen	MY11880	12	1.6	28	40
	MY11881	20	1.5	48	68
	MY11883	22	2	70	92
	MY11884	10	1.2	130	140

Moreover, the comparison of between *Ophiocordyceps* spp. and *Ophiocordyceps neonutans* – a new recently described species – was made. The morphological data of *O. nutans* from six different sources including one from the previous study by Hywel-Jones (1995), one from Sasaki *et al.* (2008) and four sources from Thailand collected in this study. While the data of *O. neonutans* published by Shrestha *et al.* (2018) were included. Our isolates isolated from Thailand were compared macroscopically (Table 4.3) and microscopically (Table 4.4) with those isolates.

Size of stroma of *Ophiocordycep* cf. *nutans* collected in Thailand were generally similar with other isolates previously reported except isolates of *Ophiocordyceps* cf. *nutans* collected from Chaiyaphum. Their stromas were larger than others which ranging from 95.5 – 132.0 mm compared with 50.0 – 90.0 collected by Hywel-Jones (1995) and 32.0-112.0 mm collected by Sasaki et al (2008). But stromas of *O. neonutans* were considerably different from the rest because it ranged from 23.0-170.0 mm. Similar results were observed, the fertile head and size of insect host of *Ophiocordyceps* cf. *nutans* from Chaiyaphum were larger than the others which they ranged between 17.5-34.0 x 2.0 mm^2 and 23.0-30.0 mm respectively. Moreover, the fertile head and fruit body from Phitsanulok, Saraburi and Khonkaen were larger than *O. nutans* from Japan.

Three microscopic characteristics consisting of (1) perithecia, (2) asci and (3) partspores of *Ophiocordyceps* cf. *nutans* isolated in this study were compared with *O. nutans* isolated from Japan and *O. neonutans* isolated from Brazil. But the measurement of perithecia, asci and partspores that was compared in this study wasn't significantly different from Japan or Brazil.

Table 4.3 Comparison between *Ophiocordyceps* spp. and *O. neonutans* at the macroscopic level.

Species	Stromata (mm)	Fertile head (mm)	Host (mm)
O. nutans (Hywel-Jones 1995)	50.0-90.0	6.0-17.0 × 3.0-5.0	10.0-30.0
O. nutans (Sasaki et. al 2008)	32.0-112.0	2.5-14.0 × 1.5-3.7	10.0-16.0 × 5.0-8.0
O. neonutans (Shrestha et. al 2018)	23.0-170.0	6.0-19.0 × 0.9-2.0	12.0-25.0 × 8.0-15.0
Ophiocordyceps cf. *nutans* from Chaiyaphum (this study)	95.5-132.0	17.5-34.0 × 2.0	23.0-30.0
Ophiocordyceps cf. *nutans* from Phitsanulok (this study)	60.0-130.0	4.5-19.5 × 1.2-2.0	15.0-35.0 × 2.0-4.0
Ophiocordyceps cf. *nutans* from Saraburi (this study)	70.0-125.0	11.0-15.0 × 1.2-2.0	14.0-20.0 × 1.5-3.0
Ophiocordyceps cf. *nutans* from Khonkaen (this study)	40.0-140.0	10.0-22.0 × 1.2-2.0	14.0-20.0 × 1.5-2.0

Table 4.4 Comparison between *Ophiocordyceps* spp. and *O. neonutans* at the microscopic level.

Host families	Perithecia (μm)	Asci (μm)	Partspores (μm)
O. nutans (Type II) - Japan Pentatomidae	610-1150 × 190-560	225-850 × 5-9	4.0-20.0 × 1.5-2.0
O. nutans (Type II) - Japan Acanthosomatidae	610-1170 × 200-500	200-875 × 5-9	3.5-14.5 × 1.5-2.5
O. nutans (Type I) - Japan Coreidae	950-970 × 250-260	N/A	N/A
O. neonutans - Brazil Pentatomidae	630-1200 × 130-360	220-900 × 3-8	6.0-15.0 × 1.2-3.0
Ophiocordyceps cf. *nutans* - Thailand Tessaratomidae (this study)	650-1030 × 200-280	240-790 × 5-6	9.0-14.0 × 1.5-2.5
Ophiocordyceps cf. *nutans* - Thailand Pentatomidae (this study)	370-710 × 100-300	182.5-435.0 × 5-10	6.0-17.0 × 2.0-4.0
Ophiocordyceps cf. *nutans* - Thailand Coreidae (this study)	800-1050 × 180-250	250-560 × 5.0-7.5	10.0-14.0 × 1.5-2.0

4.4 Molecular study of *Ophiocordyceps* cf. *nutans*

Twenty fours isolates of *Ophiocordyceps* cf. *nutans* were phylogenetically studied. One regions, three genes including ITS, LSU, EF1 and RPB1 were chosen . List of genes and DNA sequences generated in this study are shown in the Table 4.4. All DNA sequences of ITS regions and most of the other three regions were retrieved. Only few isolates of are yet to be sequenced.

Table 4.5 Summary of four genes of *Ophiocordyceps* spp. used in this study

Sample Code	ITS	LSU	EF1	RPB1
MY11340	✓	✓	✓	✓
MY11624	✓	✓	✓	–
MY11625	✓	✓	✓	✓
MY11628	✓	–	✓	✓
MY11629	✓	✓	✓	–
MY11630	✓	✓	✓	✓
MY11631	✓	–	✓	✓
MY11633	✓	–	–	–
MY11634	✓	–	–	–
MY11779	✓	✓	✓	✓
MY11780	✓	✓	✓	✓
MY11781	✓	✓	✓	✓
MY11782	✓	✓	✓	✓
MY11784	✓	✓	✓	✓
MY11785	✓	✓	✓	–
MY11786	✓	✓	✓	✓
MY11793	✓	✓	✓	✓
MY11800	✓	✓	✓	✓
MY11878	✓	✓	✓	✓
MY11880	✓	✓	✓	–
MY11881	✓	✓	✓	✓
MY11883	✓	–	–	–
MY11884	✓	✓	✓	✓
Outstanding reaction	0	5	7	3

The data are as of May 2018

Remark

✓ : The genes have been sequenced and the consensus DNA sequences are ready for molecular analyses.

– : PCR Amplification of these genes is in progress.

4.5 Molecular phylogeny of *Ophiocordyceps* cf. *nutans*

(1) Phylogenetic relationship of *Ophiocordyceps* cf. *nutans* at the familial level

Taxonomic placement of *O. nutans* at the familial level was examined using a combined dataset constructed from four gene regions (ITS, LSU, EF1 and RPB1). The dataset was based on Sasaki *et al.* (2012) related taxa of *Ophiocordyceps* spp. previously published by Sanjuan *et al.* (2015) which two taxa of *Cordyceps* (*C. militaris* OSC93623 and *C. kyushuensis* EFCC5886) were used as the outgroup (Figure 4.5). The result shows that *Ophiocordyceps* cf. *nutans* was firmly placed within the Ophiocordycipitaceae. Furthermore, there were four lineages within the genus *Ophiocordyceps* i.e. (1) Hymenostilbe, (2) Ravenelii, (3) Unilateralis and (4) Sobolifera. A total of 26 isolates of *Ophiocordyceps* cf. *nutans* isolated from Thailand formed a strong relationship with *Ophiocordyceps* in the Hymenostilbe Clade with high support (100% MPBS). Our isolates formed a sister clade with two *Ophiocordyceps* species (*O. australis* and *O. evansii*). This confirms the its taxonomic position within the Ophiocordycipitaceae.

(2) Phylogenetic relationship of *Ophiocordyceps* cf. *nutans* at the species level

ITS Regions of *Ophiocordyceps* cf. *nutans* collected in Thailand were analysed phylogenetically in order to clarify its identity and solve complexity of the species. They were compared with *O. nutans* from other geographical regions and some more related species of *Ophiocordyceps*. The dataset was constructed based on taxa published by Sazaki et al 2008 and Shesthra et al 2018 with *C. mililaris* OSC93623 used as the outgroup. Three species of *Ophiocordyceps* (*O. evansii, O. irangiensis* and *O. sphecodephala*) form as a basal clade of the ITS phylogenetic tree. A group of six isolates of *O. neonutans* shows the closest relationship with *O. nutans* but it forms a separate clade from *O. nutans* with a long branch length.

With the species complex of *O. nutans*, five distinct lineages of *O. nutans* which is based on their geographical locations and insect hosts have been revealed based on the ITS analysis. According to sites of locations, a group of *O. nutans* isolates can be divided into five clades including two, two and one sites from Thailand, Japan and China, respectively. In Thailand, three isolates of *Ophiocordyceps* cf. *nutans* (THA2) isolated from Phukhiao Chaiyaphum (MY10827, MY10829 and MY10830) were placed distantly from other isolates with a long branch length and high statistical support (100%MPBS). This emphasises the novelty of these three isolates. On the other hand, 21 isolates of *Ophiocordyceps* cf. *nutans* (THA1) were closely related with each other, and they had a relationship with two groups of *O. nutans* isolated from Japan and China.

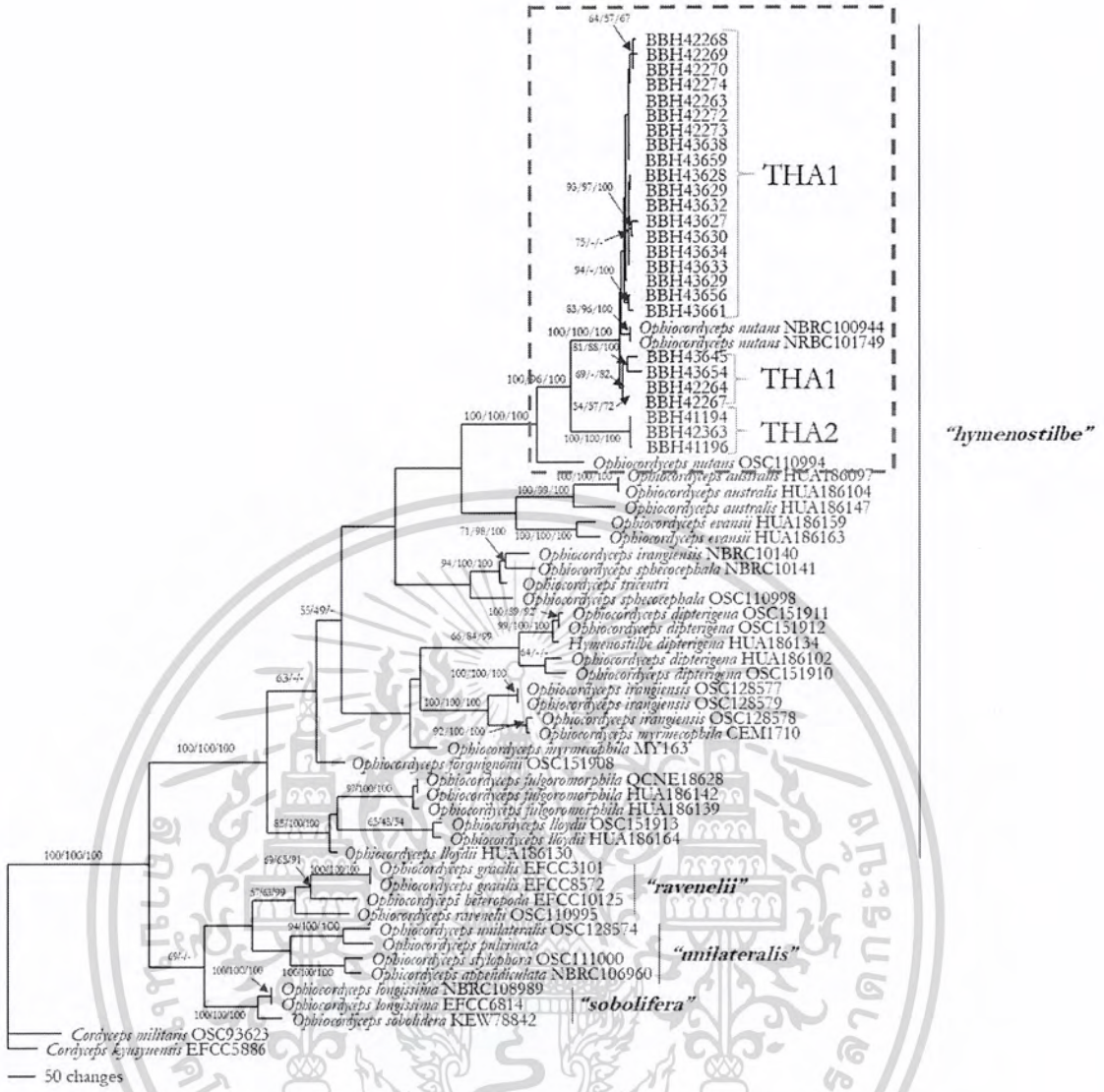

Figure 4.3 The phylogenetic tree of *Ophiocordyceps spp.* with in the Ophiocordycipitaceae. The analysis is based on combined dataset of 4 genes (ITS, LSU, EF1 and RPB1). This tree was generated by the Maximum Parsimony, Maximum Likelihood and Bayesian analysis, respectively.

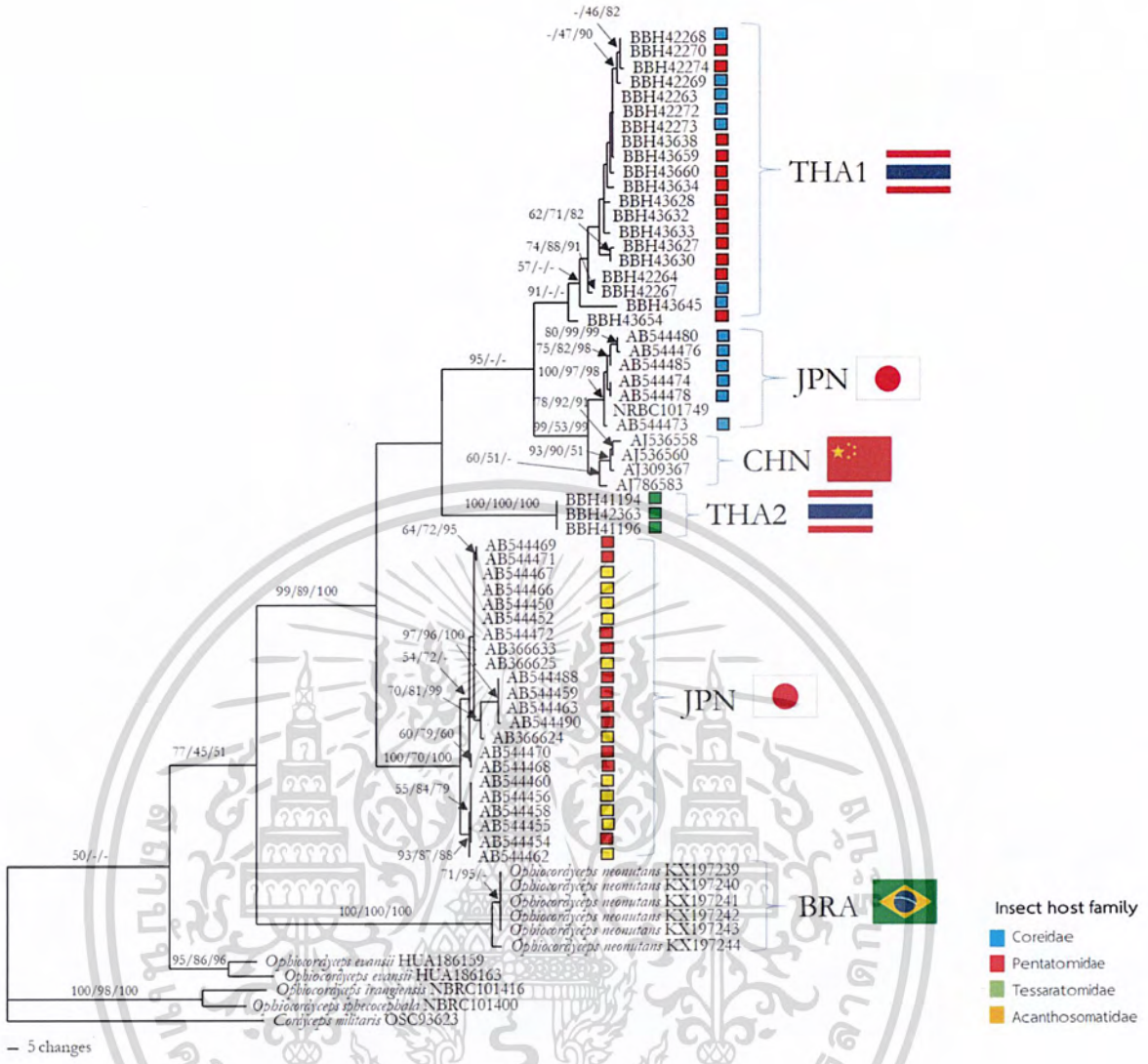

Figure 4.4 The phylogenetic tree of *Ophiocordyceps* spp. based on DNA sequences of ITS regions. This tree was generated by the Maximum Pasimony, Maximum Likelihood and Bayesian Analysis, respectively. Twenty-four isolates of *O. nutans* from Thailand are labelled as THA1 and THA2.

Chapter 5
Discussion

5.1 Collecting samples

Insect fungi can be found under forest soil or leaf litter scattering on the forest floors so there was a significant problem on contamination from plant debris and dirt. In the deep forests with high and dense canopy, the sunlight shaded very little into many parts of forest floor, and it was difficult to spot and distinguish the fungal specimens from leaf litter. Therefore, the collectors should have prior knowledge of insect fungi especially colours and shapes of fungal specimens. If the samples are found under the soil, digging must be done carefully. Otherwise the insect fungi and their hosts will be permanently damaged so isolation of the fungi and identification of the host cannot be done.

Collecting fungal samples in rainy reason is absolutely recommended. Friedrich *et al.* (2018), who surveyed insect fungi in Latin America, reported that more than 100 specimens of *O. nutans* and *O. neonutans* were collected from Brazil during rainy seasons from 2011 – 2015. In this study, all field trips were made during the rainy season in Thailand which usually last from June to November.

5.2 Isolation methods

Cleaning the fungal specimens using a surface sterilisation method plays a critical role in isolation of insect fungi. Getting rid of all dirt from the specimens and then they were wiped with 70% ethanol before conducting the single spore isolation. If the surface sterilisation method is omitted, this will increase the chance of getting a chance of severe contamination from phylloplanes and air-borne fungi, which are fast growing. As a result, there is little chance of getting insect fungi, which are slow growing.

Thirty percent of sodium hypochlorite was suggested for isolation of *O. nutans* (Sasaki *et al.* 2004). In this study 70% ethanol was used as a disinfectant and the isolation of *Ophiocordyceps* cf. *nutans* was succeed. So ethanol is also recommended for isolation of insect fungi.

5.3 Morphological study of *Ophiocordyceps* cf. *nutans*

In this study, colony colours of *Ophiocordyceps* cf. *nutans* grown on potato dextrose agar (PDA) at room temperature for four weeks were pink to darkish brown. Its colours and growth characteristics on other types of mycological media should be investigated. Most of studies related to isolation of *O. nutans* were performed on PDA – a standard and general purpose medium for fungal isolation (Sasaki *et al.* 2008, 2012; Friedrich *et al.* 2018). This fungus, however, grew slowly on PDA, so enrichment media for example malt extract agar (MEA), yeast malt extract agar (YM agar), V8 agar should be tested for growth of *O. nutans* or *Ophiocordyceps* spp.. Because if the fungus grows faster, the study on its morphology and taxonomy can be speeded up and more result will be gained.

5.4 Phylogeny of *Ophiocordyceps* cf. *nutans*

Phylogenetic analyses based on single and multiple gene datasets firmly indicates that there are two novel species of *Ophiocordyceps* which are emerging from species complex of *O. nutans*. Friedrich *et al.* (2018) pointed out that some morphological characteristics of *Ophiocordyceps* species are sometimes ambiguous and identification at species level can be confused. So the ITS regions – the universal DNA barcode for Fungi – were used to confirm the taxonomy of a newly described species, *O. neonutans*.

Likewise, ITS regions were used in this study in order to confirm the novelty of our two species. These two species of *Ophiocordyceps* cf. nutans (THA1 and THA2) clearly separated from other species of *Ophiocordyceps*. This emphasises the importance of molecular evidence to support morphological study.

Chapter 6
Conclusion and Suggestions

6.1 Conclusion

A total of twenty-four isolates of *Ophiocordyceps nutans* were collected from four locations in Thailand in this study, two novel species – *Ophiocordyceps thailandensis* (THA1), *Ophiocordyceps phukhiaoensis* (THA2) are proposed based on morphological and molecular evidence.

6.2 Suggestions

1) Collecting specimens of *Ophiocordyceps* spp. from other geographical locations particularly southern and eastern parts of Thailand.

2) Optimisation of *Ophiocordyceps* spp. on different types of mycological media such as SDYA, MEA, OA, V-8.

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Appendices

Appendix A
Mycological Media

Potato-dextrose agar (PDA)

Potato extract	200 grams
Glucose	20 grams
Agar	15 grams
Distilled water	1 litre
PH 5.6 ± 0.1 @ 25°C	

Directions

Add 200 grams of scrubbed and diced potatoes to 1 liter of water and boil for 1 hour. Let it pass through a fine sieve, add 15 grams of agar and 20 grams of glucose and boil until dissolved.

Appendix B

Chemical Reagents for Molecular Study

Item	Purpose	Company
Extraction DNA use E.Z.N.A.® Forensic DNA Kit	For genomic DNA extraction	E.Z.N.A.
Extraction DNA use CTAB method (cetyltrimethylammounium bromide)	For genomic DNA extraction	-
Taq DNA polymerase and PCR Amplification Kit	PCR Amplification	Thermo Fisher Scientific
Qia Quick PCR Purification Kit	PCR Product Purification	Qiagen

Appendix C
Resume

Name Srikhun Khao-ngam Student ID 57050897
Contact srikhun1121@hotmail.com

Scholarship
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Khao-ngam, S., Rungjindamai, N., Mongkolsamrit, S. and Luangsa-ard, J.J. 2017. Diversity of An Insect Fungus, *Ophiocordyceps nutans* Collected From Central and North-Eastern Parts of Thailand. YSTP Progress Report Meeting 2018, 26 January 2018, NSTDA Convention Center, Pathum Thani, Thailand.

งานทะเบียนคณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
คำรับรองเล่มโครงงานพิเศษ

วันที่ 9 เดือน กรกฎาคม พ.ศ 2561

ข้าพเจ้า นายศรีคุณ ขาวงาม รหัสประจำตัว 57050897
นักศึกษาหลักสูตรวิทยาศาสตรบัณฑิต สาขาวิชา จุลชีววิทยาอุตสาหกรรม ภาควิชา ชีววิทยา
ขอรับรองว่าโครงงานพิเศษ เรื่อง
ชื่อภาษาไทย: ความหลากหลายของราแมลง *Ophiocordyceps nutans* ในภาคกลางและภาคตะวันออกเฉียง-เหนือของประเทศไทย
ชื่อภาษาอังกฤษ: Diversity of An Insect Fungus *Ophiocordyceps nutans* Collected from Central and North-Eastern Parts of Thailand
ปีการศึกษา 2560
เป็นผลงานวิจัยที่มิได้คัดลอกหรือละเมิดลิขสิทธิ์ของผู้อื่นและได้ผ่านการตรวจสอบความซ้ำซ้อนเรียบร้อยแล้ว และได้แนบเอกสารการตรวจสอบการลอกเลียนงานวรรณกรรมที่ตรวจสอบจากเล่มโครงงานพิเศษฉบับสมบูรณ์แล้ว

โปรแกรม Turnitin 3%

ลงชื่อ.....ศรีคุณ ขาวงาม.....
(นายศรีคุณ ขาวงาม)
นักศึกษา

ข้าพเจ้า ผศ.ดร.ณัฐวุฒิ รุ่งจินดามัย อาจารย์ที่ปรึกษาโครงงานพิเศษได้ตรวจสอบโครงงานพิเศษของนักศึกษาข้างต้น แล้ว ขอรับรองว่าเป็นผลงานวิจัยของนักศึกษาจริงและมีเนื้อหาสมบูรณ์ จึงลงชื่อไว้เป็นหลักฐาน

ลงชื่อ.....ณัฐวุฒิ รุ่งจินดามัย.....	ลงชื่อ.....Jhuangca-ard.....	ลงชื่อ.....สุชาดา มงคลสัมฤทธิ์.....
ผศ.ดร.ณัฐวุฒิ รุ่งจินดามัย	ดร.เจเน็ท เจนนิเฟอร์ เหลืองสอาด	นางสุชาดา มงคลสัมฤทธิ์
อาจารย์ที่ปรึกษา	อาจารย์ที่ปรึกษาร่วม	อาจารย์ที่ปรึกษาร่วม