DETECTION OF FLAGELLATED PROTOZOA BELONGING TO THE TRYPANOSOMATIDAE FAMILY IN SAND FLIES COLLECTED FROM SONGKHLA PROVINCE, SOUTHERN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Parasitology
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การตรวจหาเชื้อโปรโตซัวในวงศ์ Trypanosomatidae ที่อาศัยในริ้นฝอยทรายจากจังหวัดสงขลา ประเทศไทย

นายคมสัน ฉินวิรุฬห์ศิริทรัพย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คมสัน ฉินวิรุฬห์ศิริทรัพย์ : การตรวจหาเชื้อโปรโตซัวในวงศ์ Trypanosomatidae ที่อาศัยในริ้นฝอยทรายจากจังหวัดสงขลา ประเทศไทย. (DETECTION OF FLAGELLATED PROTOZOA BELONGING TO THE TRYPANOSOMATIDAE FAMILY IN SAND FLIES COLLECTED FROM SONGKHLA PROVINCE, SOUTHERN THAILAND) อ.ที่ปรึกษาหลัก : ศ. ดร. นพ.เผด็จ สิริยะเสถียร, อ.ที่ปรึกษาร่วม : อ. ดร. นพ.กนก พฤฒิวทัญญู

ผู้ป่วยโรคลิชมาเนียในประเทศไทยมีรายงานเพิ่มจำนวนขึ้นในช่วงที่ผ่านมา โดยเกิดจากการได้รับเชื้อ *Leishmania สายพันธุ์ L. orientalis* และ *L. martiniquensis* ซึ่งโรคลิชมาเนียสามารถติดต่อได้จากการถูกกัดโดยแมลงริ้นฝอยทรายเพศเมียที่มีเชื้อ นอกจากนี้ยังมีรายงานการตรวจพบเชื้อโปรโตซัวอื่นๆ ในกลุ่ม trypanosomatids จากแมลงพาหะนำเชื้อกลุ่มนี้ โดยในปี 2559 ประเทศไทยมีรายงานการตรวจพบเชื้อ *Trypanosoma sp.* ในริ้นฝอยทรายจากจังหวัดสงขลา ซึ่งพื้นที่นี้เคยมีรายงานพบผู้ป่วยโรคลิชมาเนียมาก่อน ในวิจัยนี้มีวัตถุประสงค์เพื่อค้นหาสายพันธุ์ของแมลงพาหะนำเชื้อกลุ่ม trypanosomatids โดยอาศัยการวิเคราะห์จากข้อมูลสัณฐานวิทยาและลักษณะทางพันธุกรรมของยีน *Cytb* เพื่อระบุสายพันธุ์ของริ้นฝอยทรายที่เก็บจากพื้นที่ดังกล่าว พร้อมทั้งตรวจหาเชื้อปรสิตด้วยเทคนิคพีซีอาร์โดยใช้ตำแหน่งของยีน *ITS1* และ *SSU rRNA* สำหรับใช้ในการระบุสายพันธุ์ของเชื้อลิชมาเนียและเชื้อทริพพาโนโซม จากจำนวนทั้งสิ้น 349 ตัวอย่าง พบริ้นฝอยทรายทั้งหมด 5 สายพันธุ์ และตรวจพบเชื้อ *Trypanosoma sp.* จำนวน 3 ตัวอย่าง (0.9%) จากริ้นฝอยทราย *Sergentomyia* (*Parrotomyia*) *barraudi* จำนวน 5 ตัวอย่าง (1.4%) จาก *Se.* (*Grassomyia*) *indica* จำนวน 13 ตัวอย่าง (3.7%) จาก *Se. khawi* จำนวน 4 ตัวอย่าง (1.1%) จาก *Phlebotomus stantoni* และหนึ่งตัวอย่าง (0.3%) จากแมลงที่ไม่สามารถระบุสายพันธุ์ได้ ผลการวิเคราะห์จากแผนภูมิวิวัฒนาการของยีน *SSU rRNA* พบว่า 23 ตัวอย่างของเชื้อ *Trypanosoma sp.* จัดอยู่ในกลุ่ม Frog 1 ของเชื้อทริพพาโนโซมที่พบในสัตว์จำพวกกบและคางคก และอีก 2 ตัวอย่าง ถูกจัดอยู่ในกลุ่ม Frog 2 และในการศึกษาวิจัยนี้ได้ตรวจพบสารพันธุกรรมของเชื้อ *L. Infantum* จากยีน *ITS1 ในริ้นฝอยทรายสายพันธุ์ Ph. stantoni* จำนวน 1 ตัวอย่าง นี้จึงเป็นรายงานการตรวจพบเชื้อ *L. infantum* ในริ้นฝอยทรายสายพันธุ์ดังกล่าวครั้งแรกของประเทศไทย นับตั้งแต่ที่ได้มีรายงานการพบผู้ป่วยโรคลิชมาเนียจากการติดเชื้อ *L. infantum* ในปี 2551

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KEYWORD: Leishmania infantum, Thailand, Songkhla, Anuran trypanosome, Sergentomyia, Phlebotomus, Sand fly, Cytb, ITS1, SSU rRNA

Komson Chinwirunsirisup : DETECTION OF FLAGELLATED PROTOZOA BELONGING TO THE TRYPANOSOMATIDAE FAMILY IN SAND FLIES COLLECTED FROM SONGKHLA PROVINCE, SOUTHERN THAILAND. Advisor: Prof. PADET SIRIYASATIEN, M.D., Ph.D. Co-advisor: Kanok Preativatanyou, M.D., Ph.D.

Autochthonous leishmaniasis cases in Thailand are increasing dramatically, the disease is caused by two major of Leishmania species; *L. orientalis* and *L. martiniquensis*. Leishmaniasis is transmitted to the vertebrate hosts through the bite of the infected female sand fly. Moreover, other trypanosomatid protozoa have also been reported in this insect vector. In 2016, *Trypanosoma sp.* has been detected in sand fly from Songkhla province, where the leishmaniasis case has been reported. The aims of this study are to investigate the potential vectors of trypanosomatids in this area using morphological and molecular identification based on the *Cytb* gene. The parasites were screened by using *ITS1* and *SSU rRNA*-PCR based methods. Five species of female sand flies were found in this study. Among 349 samples tested, DNA of trypanosomatids was detected in *Sergentomyia* (*Parrotomyia*) *barraudi* (0.9%), *Se*. (*Grassomyia*) *indica* (1.4%), *Se*. *khawi* (3.7%), *Phlebotomus stantoni* (1.1%), and non-sand fly (0.3%). Based on retrieved *SSU rRNA* sequences, phylogenetic analysis reveals that 96.2% of the detected parasites belong to anuran trypanosomes. Twenty-three were the anuran *Trypanosoma spp.* of clade Frog 1, and the other two were novel species of clade Frog 2. Moreover, *Leishmania infantum* DNA was detected in a *Ph. stantoni* (3.8%) based on *ITS1*-PCR. Since 2008, there had never been reported the detection of *L. infantum* in any leishmaniasis cases from Thailand or in sand flies. Therefore, this present study demonstrates for the first time that *L. infantum* DNA can be detected in *Ph. stantoni* collected from Songkhla, Thailand.

Field of Study:	Medical Parasitology	Student's Signature ....
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		Co-advisor's Signature ....

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Chulalongkorn University

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER I INTRODUCTION 1

1.1 Background and Rationale 1

1.2 Research Questions 2

1.3 Objectives of the Study 3

1.4 Hypothesis 3

1.5 Definitions of Key Terms 3

1.6 Expected Benefits and Application of This Study 4

CHAPTER II LITERATURE REVIEW 6

2.1 Medical Importance of Pathogenic Protozoa belonging to Family: *Trypanosomatidae* 6

2.2 *Leishmania* Protozoa 7

2.3 Life Cycle of *Leishmania* Protozoa 8

2.4 Leishmaniasis and Clinical Manifestations 10

2.4.1 Cutaneous Leishmaniasis 10

2.4.2 Visceral Leishmaniasis 10

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4.3 Mucocutaneous Leishmaniasis 10

2.5 Leishmaniasis in Thailand 11

2.6 *Trypanosoma* Protozoa 13

2.7 The Emerging of Atypical Human Trypanosomiasis 15

2.8 Atypical Human Trypanosomiasis in Thailand 18

2.9 Trypanosomatid Detection Methods 18

2.10 Sand Fly 23

2.11 Life Cycle of Sand Fly 24

2.11.1 Egg Stage 25

2.11.2 Larvae Stage 25

2.11.3 Pupae Stage 27

2.11.4 Adult Stage 27

2.12 The Vector Sand Fly Species of Trypanosomatid Parasites 28

2.13 Sand Flies in Thailand 29

2.14 Morphological Characters of Sand Fly Species in Thailand 30

2.14.1 *Phlebotomus stantoni* 31

2.14.2 *Phlebotomus argentipes* 32

2.14.3 *Sergentomyia barraudi* 33

2.14.4 *Sergentomyia iyengari* 35

2.14.5 *Sergentomyia indica* 36

2.14.6 *Sergentomyia hivernus* 38

2.14.7 *Sergentomyia perturbans* 39

2.14.8 *Sergentomyia gemmea* 40

2.14.9 *Sergentomyia hodgsoni* 42

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.14.10 *Sergentomyia khawi* 44
2.15 Species Identifications of Sand Flies 44
CHAPTER III MATERIALS AND METHODS 48
3.1 Sources of Samples 48
3.2 Sample Size 48
3.3 Collection of Sand Flies 49
3.4 Identification of Sand Fly Species 50
3.4.1 Morphological Identification of Sand Fly Species 50
3.4.2 Molecular Genetic Detection of Sand Fly Species 50
3.4.2.1 Amplification of the *Cytb* Gene Sequences 50
3.5 Detection of Trypanosomatid Parasites 51
3.5.1 Molecular Genetic Detection of Trypanosomatid Parasites 51
3.5.1.1 Amplification of the *SSU rRNA* Gene Sequences 51
3.5.1.2 Amplification of the *ITS1* Gene Sequences 52
3.6 Sand Fly DNA Extraction 53
3.7 Gel Electrophoresis and DNA Visualization 53
3.8 Gene Cloning 54
3.8.1 DNA Ligation 54
3.8.2 Transformation 54
3.9 Colony PCR 54
3.10 Plasmids Extraction 55
3.11 DNA Sequencing 56
3.12 Sequence Alignment and Phylogenetic Analysis 56
3.13 Ethical 63

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER IV RESULTS 64

4.1 Morphological Identification and Classification 64

4.1.1 Phlebotomine Sand Fly Species Collected from Songkhla Province 64

4.2 Molecular Identification and Phylogenetic Analysis 68

4.2.1 Species Identification Results of Trypanosomatid Parasites Detected in Female Sand Flies Collected from Songkhla Province..................................69

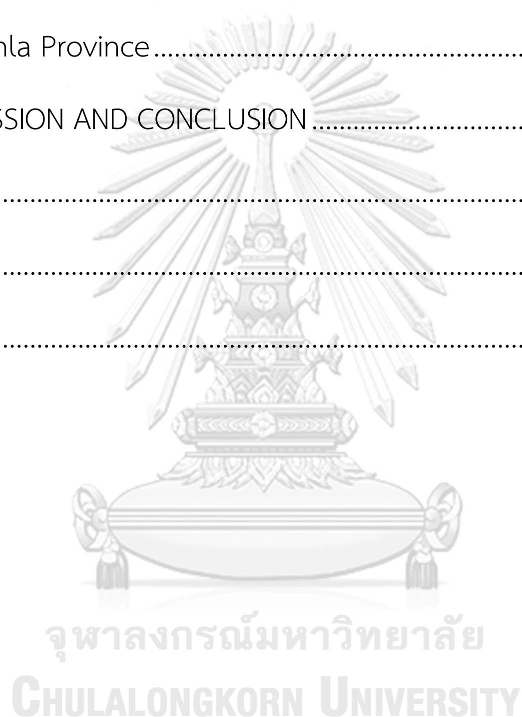
4.2.2 Species Identification Result of Female Sand Flies Collected from Songkhla Province..74

CHAPTER V DISCUSSION AND CONCLUSION 78

REFERENCES...83

APPENDIX A...98

VITA 100


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF TABLES

Page

Table 1: The predicted PCR amplicon length related to Leishmania species derived from LeF and LeR primers. 20

Table 2: The predicted PCR amplicon length related to Trypanosoma species derived from TRY927F and TRY927R primers....22

Table 3: Molecular gene markers used for molecular identification of sand flies. 45

Table 4: Trypanosomatid species, host, and GenBank accession numbers of SSU rRNA and ITS1 sequences included in the phylogenetic analyses. 57

Table 5: The species of female sand flies collected from Songkhla province of southern Thailand....66

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Page

Figure 1: Life cycle of Leishmania protozoa 9

Figure 2: The autochthonous leishmaniasis in Thailand 12

Figure 3: The distribution of atypical human trypanosomiasis 17

Figure 4: Life cycle of sand fly 24

Figure 5: The recorded sand fly species from Southern Thailand 29

Figure 6: Phlebotomus stantoni 31

Figure 7: Phlebotomus argentipes 33

Figure 8: Sergentomyia barraudi 34

Figure 9: Sergentomyia iyengari 36

Figure 10: Sergentomyia indica 37

Figure 11: Sergentomyia hivernus 38

Figure 12: Sergentomyia perturbans 40

Figure 13: Sergentomyia gemmea 42

Figure 14: Sergentomyia hodgsoni 43

Figure 15: Representative morphological features of five sand fly species in this study 67

Figure 16: The living flagellated parasites observed in Se. (Gra.) indica sand fly (SK57) 68

Figure 17: Representative images of ITS1-PCR amplicons detected in this study 70

Figure 18: Phylogenetic tree of ITS1 (18S rRNA-ITS1-5.8S rRNA) sequences 70

Figure 19: Representative images of SSU rRNA-PCR amplicons detected in this study 72

Figure 20: Phylogenetic tree of SSU rRNA (18S rRNA) sequences 73

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 21: Representative images of Cytb-PCR amplicons detected in this study. 75

Figure 22: Phylogenetic tree of Cytb (Cytb-ND1) sequences 76

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Leishmaniasis in Thailand has been emerged since the last half-century in many regions of this country (1-13), while trypanosomiasis among Thai populations is just being reported (14, 15). In 2005, trypomastigote has been observed in 45-day-old Thai infant (14). This flagellated DNA was detected and genetically identified as *Trypanosoma lewisi*-like species belonging to subgenus *Herpetosoma*, also known as the animal protozoa (14, 15). However, an atypical human trypanosomiasis due to the animal trypanosomes is not only reported in Thailand but also founded in several countries (15-17). Nowadays, there are nineteen cases caused by these animal trypanosomes such as *T. b. brucei* (15, 16), *T. vivax* (15), *T. evansi* (15, 17), *T. congolence* and *T. lewisi* (15), among which the last species has been studying the defensive ability against trypanolysis induced by human apolipoprotein L1 (*ApoL*-1), which implies that *T. lewisi* is potentially become a neglected human pathogen (18). Additionally, *T. evansi* and the other animal trypanosomes like *T. lewisi* have frequently been detected in numerous Thai rodents and fleas (14, 19-24), that particularly known as natural hosts of these protozoa. Nevertheless, although phlebotomine sand flies are generally known as the vector of the *Leishmania* parasites, they are also able to carry and circulate the other genera of Trypanosomatid protozoa (e.g., *Trypanosoma* (25-31), *Blastocrithidia* (25), *Endotrypanum* (32, 33), *Leptomonas* (34, 35) and *Herpetomonas* (25)). Recently, a novel unnamed trypanosome has been detected in *Phlebotomus stantoni* collected

from southern area of Thailand (31); this area also has been surveyed and detected the *L. siamensis* from *Sergentomyia (Neophlebotomus) gemmea* and *Se. (Parrotomyia) barraudi* (36, 37), which these kinetoplastid parasites induced CL and VL leishmaniasis in Thai reported cases (4).

Therefore, aims of this study are to survey and collect sand flies in the trypanosomatid-reported area of Songkhla province, southern Thailand, where the autochthonous leishmaniasis case associated with both CL and VL clinical manifestations has previously been reported (4). Among the collected female sand flies were firstly be processed for species identification based on morphological characteristics (31, 38-44); the trypanosomatid protozoa were also be screened and detected in those sand flies by microscopic and molecular characterization using the internal transcribed spacer 1 (*ITS1*) and small subunit ribosomal RNA (*SSU rRNA*) genes to taxonomically identify the parasite species, and using *Cytb* marker for detection, differentiation and identification of the vector species which modified from the previously published (31, 45-53). The interesting consequence obtained from this study provided more information about the currently prevalence of trypanosomatid protozoa among sand fly populations, number and kind of discovered species, including the diversity based on mitochondrial cytochrome b gene of their vectors in Songkhla province.

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Chulalongkorn University

1.2 Research Questions

1. How many and what species of the trypanosomatids are detected in sand flies collected from Songkhla province?

2. How many and what species of sand flies are collected from Songkhla province?

1.3 Objectives of the Study

1. To detect and identify species of the trypanosomatid protozoa in sand flies collected from Songkhla province based on *ITS1* and *SSU rRNA* sequences analysis.

2. To explore species diversity of sand flies collected from Songkhla province using both morphological and molecular identification.

1.4 Hypothesis

1. Sand flies in southern Thailand are able to carry the trypanosomatid protozoa.

2. Both methods of morphological and molecular identification base on *Cytb* sequences analysis can be applied to distinguish species diversity of sand flies collected from Songkhla province.

1.5 Definitions of Key Terms

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1. Atypical human trypanosomiasis is human infectious diseases caused by protozoa belonging to genus *Trypanosoma* which typically infect animals but recently have been reported in several human cases, mostly found in Asia and the Middle East, especially in India comprised eight cases and also one case from Thailand. The species which have been reported in atypical trypanosome infections are *T. evansi*, *T. lewisi, T. vivax*, *T. b. brucei* and *T. congolense*.

2. *Phlebotominae* are a subfamily of the family *Psychodidae* comprised many genera of blood-feeding flies. These hematophagous insects are the primary vectors of *Leishmania* protozoa and other pathogenic microorganisms. Genus *Phlebotomus*

and *Sergentomyia* are the main vectors caused leishmaniasis from the Old World while *Lutzomyia spp*. commonly found in New World.

3. *Leishmania* is a genus of protozoa which these flagellated parasites infect in several animals and human caused leishmaniasis, comprised three clinical manifestations e.g. visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis. These protozoa are dixenous, two hosts in the life cycle, which one develops into promastigotes stage in the sand fly gut and transmits by the bite of the female sand fly during blood feeding and the other one stage replicates intracellularly in the macrophage of vertebrate hosts, called amastigotes.

4. *Trypanosoma* is genus of protozoa which these parasites infect many vertebrates, avians, amphibians, reptiles, and including human caused trypanosomiasis classified into two major clinical manifestations according to species of infection, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* caused sleeping sickness or Human African trypanosomiasis transmitted by tsetse fly, and *Trypanosoma cruzi* caused Chagas disease transmitted by triatomine bugs.

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Chulalongkorn University

1.6 Expected Benefits and Application of This Study

1. Information about the current situation of the prevalence of trypanosomatid protozoa infection in sand flies collected from Songkhla Province, Thailand.

2. The insight of which species can play a role as the potential vectors of these trypanosomatid protozoa in order to further intensively study those vector species for finding the ways to control and develop the prevention plans of monitoring and controlling disease outbreaks in this area.

3. The *Cytb* gene sequence data of sand flies collected from Songkhla province, including the *ITS1* and *SSU rRNA* gene sequence of detected protozoa for submitting to GenBank database.

CHAPTER II

LITERATURE REVIEW

2.1 Medical Importance of Pathogenic Protozoa belonging to Family: *Trypanosomatidae*

All species belonging to family *Trypanosomatidae* possess a single nucleus and can be found in the elongated body with a single forwardly extending flagellum or spheroid shape with a very short, nonprotruding or slightly projecting flagellum. The *Trypanosomatidae* is a single family of the order *Trypanosomatida*, which is composed of several monoxenous and heteroxenous organisms. Some genera of family *Trypanosomatidae* such as *Herpetomonas, Crithidia, Blastocrithidia, Leptomonas,* are monoxenous insect parasites, which the last mention species can be found in molluscs, nematodes, and even other protozoa. In addition, the other genus like *Phytomonas* is considered the only *kinetoplastid* genus specifically adapted to infect plants. However, this family also contains the most important genera involved in several severe human diseases e.g. leishmaniasis, Chagas disease, sleeping sickness, which are many species belonging to genus *Leishmania* and *Trypanosoma* (51, 54-57).

Both genera *Leishmania* and *Trypanosoma* cause many even acute or chronic severe clinical manifestations leading to the mortality and morbidity and the increasing infection among a million people around the world. Recently, the information from Centers for Disease Control and Prevention (CDC) (58, 59) and World Health Organization (WHO) (60-62) show that leishmaniasis is found in more than 90 countries from the tropics areas, subtropics, and southern Europe, and approximately six to seven million people worldwide, mainly in Latin America

countries, infected with *Trypanosoma cruzi* caused Chagas disease, and including sleeping sickness, that occurs in 36 sub-Saharan Africa countries, in people who are infected with *T. b. gambiense* from 24 countries in west and central Africa and infected with *T. b. rhodesiense* from 13 countries in eastern and southern Africa, which currently there are 2804 recorded cases of both species.

2.2 *Leishmania* Protozoa

Several members belong to genus *Leishmania* known as causing leishmaniasis in human. These parasites are heteroxenous which one part of their life cycle is spent in a sand fly gut, where they become promastigotes, and another past is developed inside vertebrate tissues, where only amastigotes are found (56). Leishmaniasis is a parasitic disease or known as zoonosis caused by infection of *Leishmania* parasites through the bite of phlebotomine sand flies, which are the main vectors of this protozoan belonging to the *Phlebotomus* genus from Old World and *Lutzomyia* genus from New World (63). This disease is classified as a Neglected Tropical Disease (NTD) that is spread in many parts of the tropics, subtropics, and southern Europe (57). It is found among several animals such as canine, feline, cattle, sheep, treeshrews, rodents, horses, and absolutely in humans which are considered as accidental host (63). There are many different clinical manifestations of leishmaniasis in people. The most common disease conditions are cutaneous leishmaniasis, which causes skin sores, and visceral leishmaniasis, which affects several internal organs e.g. usually spleen, liver, and bone marrow, and including mucocutaneous leishmaniasis, which leads to ulcerative destruction of the nasooropharyngeal mucosa (56, 57).

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2.3 Life Cycle of *Leishmania* Protozoa

These protozoa characterized by 2 developmental stages are amastigotes, which this form can be found in humans or in other vertebrates, and promastigotes form which be found in Phlebotomine sand flies. *Leishmania* infection occurs when infected female sand fly bites a human or animal during sucking blood and injects these promastigotes. These protozoa enter the macrophage of host cells by phagocytosis or phagolysosome and then develop into promastigotes form that later reproduces by binary fission causing macrophage shattering and then seeks for another macrophage. Normally, these parasites infection can be found in many organs such as bone marrow, liver, and spleen. Moreover, the next transmission occurs when female sand fly bites an infected human or animal and uptakes these amastigotes that pass through digestive tract then transform to the procyclical promastigotes within 18-24 hours and become the nectomonads using around 3-4 days and after that eventually develop into the metacyclics form which is the infective stage (63).

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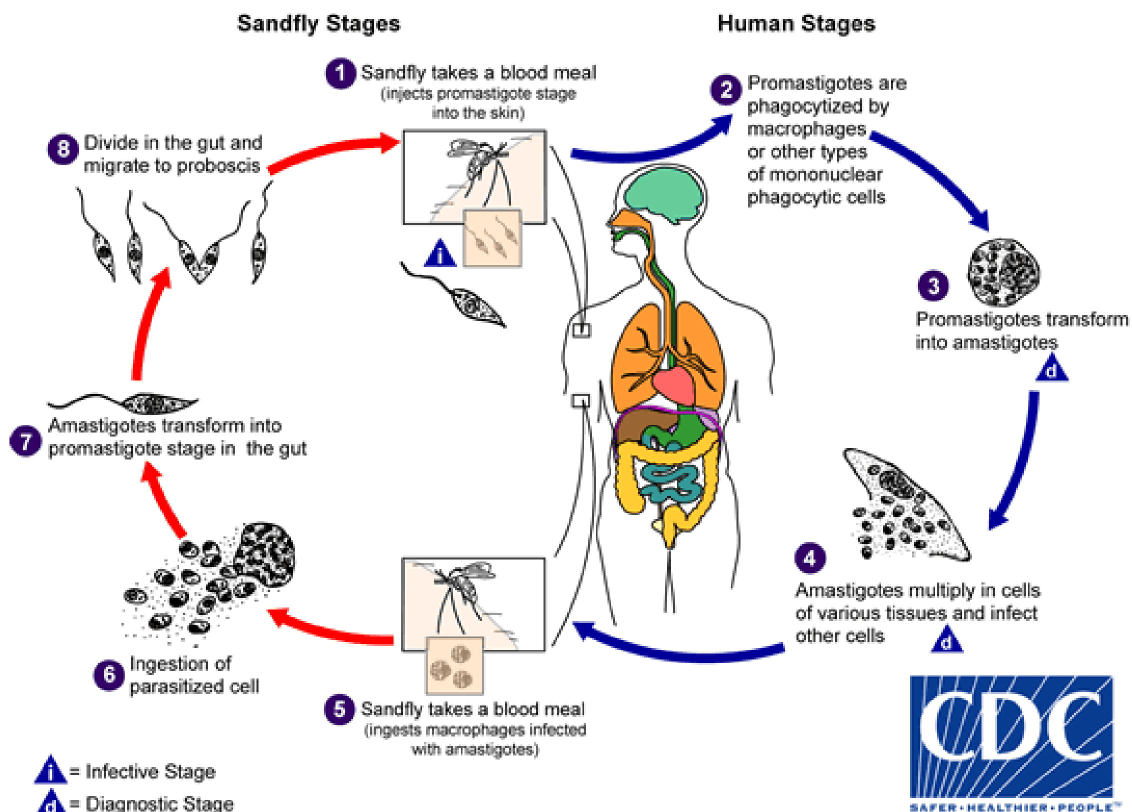

Figure 1: Life cycle of *Leishmania* protozoa

Figure 1: 1-2: promastigotes that reach the puncture wound are phagocytized by macrophages and other types of mononuclear phagocytic cells, 3: promastigotes transform into the amastigotes and then multiply by binary fission, 4: amastigotes proceed to infect other mononuclear phagocytic cells which consequence of macrophage rupture, 5-6: sand flies become infected by ingesting infected cells during blood meals, 7: amastigotes transform into promastigotes developing in the hindgut or midgut which depend on subgenus of those protozoa, 8: the metacyclic promastigotes migrate to the proboscis. The figure is created by CDC, The Centers for Disease Control and Prevention website (CDC; 2013).

2.4 Leishmaniasis and Clinical Manifestations

Leishmaniasis manifestations generally occurred in 3 forms including cutaneous, mucocutaneous, and visceral leishmaniasis, which depends on the parasite species and the immune status of patients.

2.4.1 Cutaneous Leishmaniasis

Cutaneous leishmaniasis is the most common form affecting humans. The manifestation occurs in skin cells caused by the parasite's infection that is transmitted by the bite of a phlebotomine sand fly, which begins as a red papule to nodular plaques, that eventually becomes open sores with a raised border and central crater called ulcer, that may take several years to be completely healed and usually causes morbidity in immunocompetent individuals (9).

2.4.2 Visceral Leishmaniasis

Visceral leishmaniasis has known as Kala Azar, typically cause by these protozoa such as *L. donovani*, *L. infantum* and *L. chagasi* (64). Most of the patients manifest with tiredness, nausea, vomiting, dry skin, hepatosplenomegaly which is specific signs of this clinical condition, and some patients come up with lymphadenopathy, usually pancytopenia, which leads to the paleness of skin, lymphocytes lower than 4,000 cells per cubic millimeter (mm^3), thrombocytopenia, and hypoalbuminemia. If untreated, these severe conditions of infestations can lead to mortality which is mainly associated with visceral infection (9).

2.4.3 Mucocutaneous Leishmaniasis

The clinical condition of mucocutaneous leishmaniasis is similarly to cutaneous manifestations but the cutaneous lesions occur at nasooropharyngeal

mucosa such as a nasal septum or mouth. Patients present with fever, pale, exhausted, lose weight. If untreated, the disease can progress to ulcerative destruction of the nasooropharyngeal mucosa or deaths (63).

2.5 Leishmaniasis in Thailand

Leishmaniasis is an emerging disease that has been appeared and documented in Thai populations for approximately fifty years since 1960. Currently, two species of this parasite that has found and reported in Thailand are *L. martiniquensis* named after the Caribbean island of Martinique (9), and the other one species which has molecular differences compared to the genomes of the other submitted species declared as *L. siamensis* (3). The clinical manifestations of leishmaniasis in Thailand are found in two forms, which one is cutaneous leishmaniasis (3, 4, 7-9) and the other is visceral leishmaniasis (1-5, 9-13). Moreover, the similar infections of reported species from Thailand in cows and horses have been reported in these countries e.g. America, Switzerland, and Germany (63).

In the past, several patients diagnosed as leishmaniasis are beginning reported in Thailand. Since 1960 to 1987, most of the reported patients are imported leishmaniasis cases who have a working history or traveling in the countries of the Middle East where *L. donovani* is prevalent (6, 7). However, after 1996 until now, Thai infected patients who neither work nor travel outside their country have been reported about sixteen cases of the autochthonous leishmaniasis including seven cases of visceral leishmaniasis (1, 2, 5, 10-13), five cases of cutaneous leishmaniasis (4, 7, 8), and four cases with both cutaneous and visceral leishmaniasis (3, 4, 9). Additionally, several cases of autochthonous leishmaniasis are reported from Southern region of Thailand and this infection has been continuously reported in both immunocompromised (3, 4, 9, 11) and immunocompetent cases, non-HIV

infected patient (1, 2, 5, 7, 10, 12, 13). Surprisingly, the autochthonous leishmaniasis caused by *L. donovani* (12) and *L. Infantum* (10) have also been found and reported in this country.

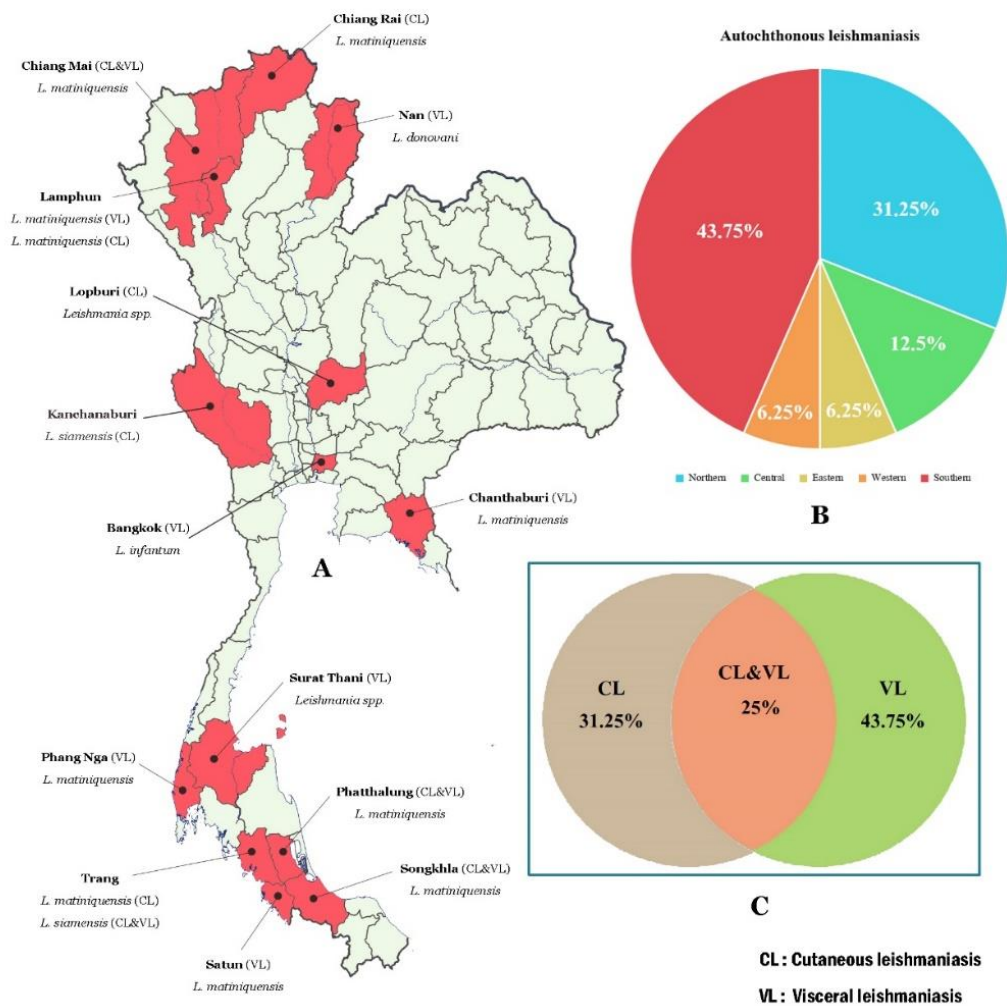

Figure 2: The autochthonous leishmaniasis in Thailand

Figure 2: A: the sixteen autochthonous leishmaniasis cases refer to their provinces which have already been reported combining with the information of the clinical manifestations and types of species, B: a percentage of the number of autochthonous leishmaniasis cases in Thailand, which the most is the Southern region, Northern, Central, Western and Eastern region, respectively. C: a Venn diagram displays a percentage of the types of the clinical manifestations found in this

country, which the most is VL following by CL and both CL&VL co-conditions. The figure is modified from the files of Thailand location map.svg on Wikipedia, The Free Encyclopedia website (Wikipedia; 2009) and Siriyasatien (2016).

The first autochthonous leishmaniasis case diagnosed as visceral leishmaniasis in Thailand was reported from Surat Thani province without species confirmation (1). And later, several provinces have been continuously reported in this infection e.g. Nan (12), Phang Nga (2), Bangkok (10), Chanthaburi (11), Trang (3, 4), Songkhla (4), Chiang Rai (8), Lopburi (7), Lamphun (9, 13), Satun (5), Phatthalung, Chiang Mai (9), and recently Kanchanaburi. In addition, 10 of the autochthonous leishmaniasis cases (62.5%) are infected by *L. martiniquensis*, excepts three cases are individually infected by *L. donovani* (12), *L. infantum* (10), and *L. siamensis (3)* and two cases are not identified species (1, 7).

2.6 *Trypanosoma* Protozoa

Trypanosome protozoa belong to family *Trypanosomatidae* in genus *Trypanosoma*. Many biting insects are vectors of this flagellated protozoon and transmit these parasites to several vertebrate species (18, 21-23). Generally, the tsetse flies are known as the transmitted vectors of *Trypanosoma brucei rhodesiense* caused the acute manifestation or *Trypanosoma brucei gambiense* caused the chronic manifestation of Human African Trypanosomiasis (HAT) or sleeping sickness in Africa and also the triatomine bugs are known as the transmitted vectors of *Trypanosoma cruzi* caused American trypanosomiasis or Chagas disease in human located in Latin America (16, 21-23, 65) where most cases are chronic and asymptomatic (22). Additionally, trypomastigotes of African trypanosomes actively divide in the blood circulation and lymphatic system, where they cause progressive somnolence leading to coma or death depended on an invasion of the central

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nervous system, whereas trypomastigotes of *T. cruzi* typically develop to amastigotes within muscle cells of their host without dividing along with the bloodstream (18). Moreover, the other biting insects such as sand flies (25-31), stomoxys, tabanids (23) carrying these *trypanosomatid* protozoa have recently been reported.

In addition, the genus *Trypanosoma* is comprised of two categories based on the assembling site of infective parasites living inside the vector. The first is Salivarian trypanosomes which undergo anterior station development (foregut) of their vectors, mostly transmitted by inoculation (21, 23). These protozoa are considered to be pathogenic to humans, and even domestic and wild animals, such as *T. evansi*, *T. brucei brucei*, *T. congolense* and *T. vivax* (16, 21-23, 55) which the last three species usually cause animal trypanosomiasis in Africa, called nagana, but *T. evansi* infection is called surra that is found in Asia, Europe, Africa, and including South America (22, 55). The second one is Stercorarian trypanosomes which develop in the posterior part of the digestive tract of their vectors, typically transmitted by contamination. Conversely, most of the Stercorarian protozoa are considered to be non-pathogenic such as *T. lewisi* and *T. lewisi*-like species abundantly distributed in rodents, which these species belonging to the subgenus *Herpetosoma*, *T. melophagium* found in sheep, *T. theileri* found in antelopes and bovines, which the last two species belonging to the subgenus *Megatrypanum*, except the protozoa of subgenus *Schizotrypanum* like *T. cruzi* can cause chagas disease (18, 21, 23). However, although the human trypanosomiasis caused by *T. b. rhodesiense*, *T. b. gambiense* or *T. cruzi* infections have never been reported from Thailand, the incidents of atypical human infections by these considered non-pathogenic protozoa e.g. *T. b. brucei*, *T. vivax, T. congolense, T. evansi*, and *T. lewisi* have been reported in this country and widely distributed in other regions, especially in Asia (14-18, 21-24, 47).

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2.7 The Emerging of Atypical Human Trypanosomiasis

Currently, the outbreak of some atypical human infections caused by other *Trypanosoma* species have continuously been reported, mainly due to *T. evansi* and *T. lewisi* which both species infect several reported cases and the other species such as *T. b. brucei*, *T. vivax, T. congolense* also have been found (14-17, 21-24, 47, 66).

First of all, *Trypanosoma evansi* derived from *T. brucei* lineage (18, 21) is known as a livestock parasite normally caused a subclinical disease among wild and domestic animals, mostly found in cattle (21), caused animal trypanosomiasis, called surra (17, 22). This species relies on the biting flies as a vector such as tabanids and stomoxys to mechanically transmit these protozoa to vertebrates or even humans (23). Nevertheless, there have few reported cases of atypical human infections due to *T. evansi* such as three cases from India, and two cases from Sri Lanka and Egypt (15, 22). Normally, human has an innate immune response against most of the trypanosome protozoa including this species, which can be lysed by normal human serum (NHS) (21, 22), the human apolipoprotein L1 (*ApoL*-1) (18). However, Hawking (1978) has been tested and found that two strains of *T. evansi* can also resist to human plasma on his experiments. As previously mentioned, most of all recorded cases who are infected by *T. evansi* are recovery, except one case is death due to that patient is lack of the trypanolytic *ApoL*-1 protein in serum (16) resulted from genetic mutations, which means the expression of *ApoL*-1 protein cannot occur in this patient (18). In addition, the transmission of this disease has been hypothetically described which occurs by peroral or transcutaneous penetration of the parasite (21). In addition, trypanocidal drugs used against African trypanosomiasis can be applied for the treatment of this infection (21).

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And the next species, *Trypanosoma lewisi* is previously considered non-pathogenic to the natural hosts (18). This species is a worldly parasite of rets, transmitted by fleas, and caused animal trypanosomiasis in many species of rats when ingesting fleas or their feces (21). *T. lewisi* infection in humans is mainly found in Asia including five cases from India, and a case from Malaysia (15, 67). Moreover, the other species belonging to subgenus *Herpetosoma*, such as *T. lewisi*-like (15, 67) is also found from two cases, one from Gambia and the other one from Thailand. On the other hand, *T. lewisi* naturally can resist to trypanolysis by NHS unlike *T. evansi*, thus, this species has a natural potential for causing human infection (21, 67). Additionally, the motility of this infection has been reported from one case in India who has renal complications when treated with suramin, caused toxic effects due to this drug, and died eventually (21). In addition, Desquesnes et al. (2016) have recently been tested the trypanocidal drugs e.g. suramin, pentamidine, eflornitine, nifurtimox, benznidazole and fexinidazole against *T. Lewisi* infection of experimentally infected rats, which all of the results are clearly unaffected to these *T. lewisi* protozoa.

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Moreover, the last three tsetse-transmitted trypanosomes e.g. *T. b. brucei*, *T. vivax* and *T. congolense*, generally affects animals, which are well known as causes of African Animal Trypanosomiasis (AAT). Additionally, although *T. b. brucei* usually can be lysed by NHS, these two species of *T. vivax* and *T. congolense* can come up with the capacity to resist the synthesize proteins of human innate immune responses (15). Moreover, *T. congolense* can be categorized into three distinguishable phenotypes according to its resistance to NHS, which some strains of these protozoa are totally resistant to NHS, as similar to *T. vivax* (15, 21, 68), *T. b. gambiense* and *T. b. rhodesiense* (69). Consequently, these two flagellated protozoa, *T. congolense* and *T. vivax*, might be able to infect humans or animals (68, 69). Since 1917 to 2003,

there are only six cases of atypical human infections from these species, that have officially been reported including one case infected by *T. vivax* from Ghana, four cases infected by *T. b. brucei* from these countries (e.g. France, Congo, Ethiopia, and Ghana), and the last one infected by *T. congolense* from Côte d'Ivoire. In addition, only two cases of *T. b. brucei* and *T. congolense* infection from Ghana, and Côte d'Ivoire, respectively, are using the PCR method to confirm species identity, while the rest of cases are using morphology to identify those organisms (15, 22).

As previously mentioned, the fact that is some of trypanosomes species (e.g. *T. lewisi*, *T. vivax, T. congolense*, and *T. evansi*) presented with the capacity to resist the human innate immune responses, that mean they might be considered being one of the naturally infective species to human (15, 21, 68, 69).

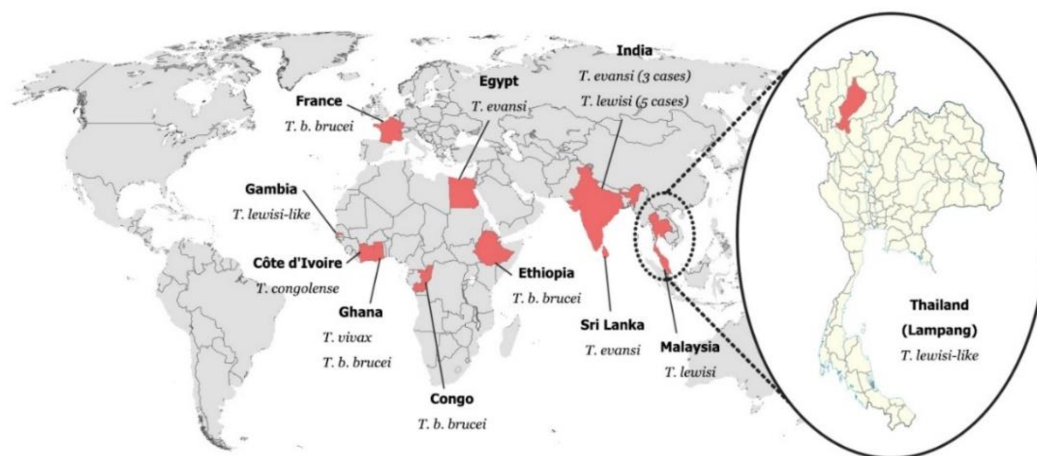

Figure 3: The distribution of atypical human trypanosomiasis

Figure 3: this figure demonstrates the numbers of reported cases with species individually related to atypical human trypanosomiasis, which widely distribute in many regions e.g one case in Europe, seven cases in Africa, and especially in Asia comprised of eleven cases, one cases infected with *T. lewisi-like* species reported from Lampang province, Thailand. The figure is modified from the files of Thailand

location map.svg and A large blank world map with oceans marked in blue.svg on Wikipedia, The Free Encyclopedia website (Wikipedia; 2011).

2.8 Atypical Human Trypanosomiasis in Thailand

Generally, Thailand is also known as a non-endemic area for typical human trypanosomiasis infected by *T. b. rhodesiense* or *T. b. gambiense* caused sleeping sickness found in Africa, even though, *T. cruzi* caused Chagas disease located in Latin America (18, 21, 22). Formerly, although several Thai people are gone for working in a country of the Middle East and then returned to Thailand which carried some parasitic diseases such as leishmaniasis, those returned laborers have never been reported in human trypanosome infection caused trypanosomiasis (66).

However, there also has reported a case of atypical human trypanosomiasis in this country (14, 66). In 2005, the first admitted case of *T. lewisi* atypical infection in Thai infant lived in Lampang province has been found and a blood sample from this child has been molecularly diagnosed as a T. lewisi-like species by Sarataphan et al (2007). So that, the existence of the atypical human trypanosomiasis in Thailand might provoke awareness of the importance of this infection to the public health officer and medical scientists (66) for understanding pathogenesis and finding the vectors which can efficiently carry those parasites in order to discover and develop the effective prevention.

2.9 Trypanosomatid Detection Methods

Currently, there are several methods for detecting the parasites belonging to family *Trypanosomatidae*, particularly many members of genus *Leishmania* and *Trypanosoma*. Laboratory identifications of those parasites can process by using the direct microscopic examination of fresh or stained specimens, and even performing

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detection of parasite antigens or antibodies by using immunological methods (e.g. agglutination test, indirect fluorescent antibody, direct agglutination test, ELISA, immunochomatic strip test). Alternatively, molecular techniques such as the polymerase chain reaction (PCR) technique is considered to be the worthwhile methods with high sensitivity and specificity for detecting and identifying species compared to the gold standard technique like isoenzyme electrophoresis, which is an arduous, time-consuming and costly method (45, 63).

Therefore, characterization of *Leishmania* species by PCR-base method generally focuses on using these genes e.g. the Kinetoplastid DNA (*kDNA*), the small subunit ribosomal RNA (*SSU rRNA*), miniexon, tubulin, *gp63* and the internal transcribed spacer (*ITS*) region for analysis the genetic variation and identification of those detected species (45). In Thailand, those previously mentioned genes can be successfully applied to both stains, which are *L. siamensis* and *L. martiniquensis,* reported in many infectious cases of leishmaniasis from this country. Moreover, it's been over decade, since the *ITS1* gene has been used to extensively study the organisms belonging to genus *Leishmania* by performing analysis of polymorphic sequences within species and categorization (45). For this reason, Spanakos et. al. (2007) has been considered, that *ITS1* gene is sufficiently polymorphic to distinguish strains of *Leishmania* parasites, and because there have many of *ITS1* gene sequences derived from *Leishmania* species, including those pathogenic strains, which are available on NCBI. Spanakos et al. (2007) have been designed the primers for amplifying the *Leishmania*-specific *ITS1* gene, which comprises of the twenty-two nucleotides forward and reward primers, named LeF and LeR.

LeF5'-TCC GCC CGA AAG TTC ACC GAT A-3'

LeR5'-CCA AGT CAT CCA TCG CGA CAC G-3'

These primers can detect and amplify the *ITS1* amplicon from the samples, which contains just a single one parasite, that the sensitivity is 0.1 parasites per reaction in a final volume of 50 microliters. And the specificity of the primers, the primer LeR is not only binding to the specific gene of *Leishmania* but also recognizes other trypanosomatid sequences, except the members of genus *Endotrypanum*. On the other hand, The LeF primer is completely complementary only to Leishmania (45). Furthermore, the amplicons of the *ITS1* of *L. martiniquensis* and *L. siamensis,* that reported from Thailand*,* gives a size 379 bp (4) and 371 bp (31), respectively.

Table 1: The predicted PCR amplicon length related to Leishmania species derived from LeF and LeR primers.

The predicted PCR amplicon length related to *Leishmania* species derived from LeF and LeR primers.			
Species	PCR amplicon length (bp)	Accession numbers	References
Leishmania aethiopica	350	FN677356	(70)
Leishmania amazonensis	362	DQ182536	(71)
Leishmania braziliensis	326, 327	DQ182537, JQ397604	(71)
Leishmania donovani	347, 350	FN677358, FR799614	(70), (72)
Leishmania guyanensis	328	DQ182539	(71)
Leishmania lainsoni	361	DQ182542	(71)
Leishmania major	369, 371	FR796423, KF981802	(73), (74)
Leishmania naiffi	449	DQ182543	(71)
Leishmania panamensis	329	CP009396	(75)
Leishmania tropica	359	FN677341	(70)

This table demonstrates the sizes of predicted amplified PCR fragments correlated with species of *Leishmania* protozoa, which are available on NCBI

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nucleotide database website with GenBank accession numbers, derived from both LeF and LeR primers.

The characterization of these kinetoplastid flagellates has been used many genes for molecular phylogenetic analysis and description such as glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*), spliced leader (*SL*) RNA gene, and 18S rRNA gene, which are the most frequently used to identify species (55).

Noyes et al. (1999) have been designed primers for detecting and screening trypanosomes protozoa in a comprehensive range of terrestrial vertebrates or even avian hosts. These primers can amplify the *SSU rRNA* sequences of entirely Stercorarian trypanosomes, which this groups combined with several pathogenic protozoa caused humans or animal trypanosomiasis, such as *T. cruzi,* that causes Chagas disease transmitted by kissing bugs, *T. brucei,* that currently reported in many cases of atypical human trypanosomiasis (15, 21, 68, 69), *T. avian, T. rotatorium, T. pestanai, T. triglae,* and even the other *Kinetoplastid* parasites like *Endotrypanum monterogeii*, *Crithidia fasciculata*, *Phytomonas serpens* known as a tomato parasite, and *L. donovani* . In addition, the amplicons are produced approximately 927 bp (799-1726) of *SSU rRNA* and can be differentiated by restriction fragment length polymorphism or direct sequencing. The primers comprise of nineteen nucleotides of forwarding primer, named TRY927F, and eighteen nucleotides reverse primer, TRY927R.

TRY927F 5’-GAA ACA AGA AAC ACG GGA G-3’

TRY927R 5’-CTA CTG GGC AGC TTG GA-3’

Table 2: The predicted PCR amplicon length related to Trypanosoma species derived from TRY927F and TRY927R primers

The predicted PCR amplicon length related to *Trypanosoma* species derived from TRY927F and TRY927R primers.			
Species	PCR amplicon length (bp)	Accession numbers	References
Trypanosoma avium	935	KT728401	(76)
Trypanosoma b. rhodesiense	927	AJ009142	(77)
Trypanosoma b. Gambiense	927	AJ009141	(77)
Trypanosoma evansi	927	AJ009154, KT023565	(78)
Trypanosoma fallisi	978	AF119806	(79)
Trypanosoma grayi	931	AJ223565	(80)
Trypanosoma lewisi	941	AJ009156, GU252209	(78), (81)
Trypanosoma melophaguim	933	HQ664912	(82)
Trypanosoma mega	1012	AJ009157	(78)
Trypanosoma microti	942	AJ009158	(78)
Trypanosoma minasense	931	AB362411	(83)
Trypanosoma ralphi	931	KF546521	(84)
Trypanosoma vespertilionis	969	AJ009157	(78)
Trypanosoma rotatorium	964	AJ009161, U39583	(78), (85)
Trypanosoma terrestris	930	KF586848	(86)
Trypanosoma theileri	932	AB569250, JX178182	(87), (88)
Trypanosoma therezieni	991	AJ223571	(80)
Trypanosoma thomasbancrofti	940	KT728392	(76)

This table demonstrates the sizes of predicted amplified PCR fragments correlated with species of *Leishmania* protozoa, which are available on NCBI

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nucleotide database website with GenBank accession numbers, derived from both TRY927F and TRY927R primers.

2.10 Sand Fly

The phlebotomine sand flies belonging to the family *Psychodidae* in the order Diptera (28, 89). These flies are bloodsucking arthropods which able to transmit several various pathogens like Arbovirus, Bartonellosis (89). Less than 10 percent of over 800 species known as the potential vectors carried such as flagellate protozoa of the genus *Leishmania* caused leishmaniasis in human and invertebrates (28, 90, 91). In addition, some flagellate protozoa that infect many mammals, reptiles or amphibians belonging to the genus *Trypanosoma* are also transmitted by phlebotomine sand flies (25-29, 31, 32). Furthermore, there have been documented the species of phlebotomine sand flies which served as vectors of the human pathogenic *Leishmania* species such as *Phlebotomus* and *Sergentomyia spp.* conveyed these protozoa caused leishmaniasis from the Old World, while Lutzomyia spp. found in New World (89, 90). In Asia, some species of sand flies like *Phlebotomus argentipes* are described as natural vectors of such as *L. donovani* reported from Bangladesh, India, Nepal, and Thailand and *Ph. major major* and *Ph. papatasi* the proven vector of *L. infantum* commonly found in the Mediterranean and many places, also including Thailand (64, 92-94). Whereas, *Sergentomyia* in Thailand is the most prevalent genus found in throughout the country (37). Likewise, *Se. (Neophlebotomus) gemmea* is mostly observed in Southern Thailand (37) that *Se. gemmea* and *Se. (Par.) barraudi* might be potential vectors of *L. siamensis*, which currently being confirmed as *L. martiniquensis* (92, 95), the causative agent of autochthonous visceral leishmaniasis in southern Thailand (92).

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Sand flies are small, with slender legs and covered by a hair over the entire body. Usually, they hold their wings in V shape above their abdomen when resting, whereas hopping around on the host before come down to bite during their blood meal and they also travel not more than 1 km from their breeding sites. Additionally, either female or male sand flies consume sugar from natural sources like plant juices and honeydew secreted by aphids, while only the females feed on blood for producing eggs that required nutrition. Moreover, some species of sand flies have anautogeny, the oviposition strategy in female insects that they produce eggs without consuming blood (91). Furthermore, the saliva of sand flies contains with potent pharmacologically active components that are necessary for helping them to successfully feed blood and it also helps the parasite to establish in their vertebrate hosts, that the infection begins transmitted through the bite of phlebotomine sand flies (64, 91). remarkably, there have currently been studied the role of the cellular immune response of mice against protein from the sand fly saliva, which shows it's enough to control *L. major* infection in mice, that means salivary proteins are potential candidates for a vector-based vaccine (64).

2.11 Life Cycle of Sand Fly

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Sand fly is characterized by completed metamorphosis including 4 developmental stages, egg, larva, pupa and adult.

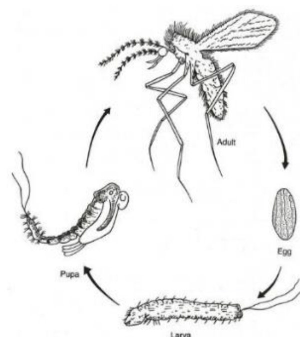

Figure 4: Life cycle of sand fly

Figure 4: the complete-metamorphosis life cycle combining with 4 stages; adult (top), egg (right), larva (bottom), and pupa (left). The figure is created and posted on YourArticleLibrary website by Samiksha (2014)

2.11.1 Egg Stage

The sand fly egg has a brownish ellipse shape and egg sizes about 0.3 - 0.4 mm in length which has a variety of size depend on spices. The surface of eggshell showing a mosaic-like pattern by inside the egg has two dark parallel lines called the caudal bristles which can be seen through the eggshell. The female generally lays single eggs separately in the damp dark places combined with organic matter, like the animal stables or livestock shelters, stones, loose bricks, the crack lines in brick, the gaps or animal burrows inside and outside house, caves, termite hills and even toilets. The eggs hatch in 1-2 weeks based on the humidity and temperature (96).

2.11.2 Larvae Stage

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The larvae grow through four developmental stages called instars. Body parts of larva composed of 12 segments resembles butterfly larva, white long body, dark hypognathous head covered with branching bristles or commonly called matchstick hairs on head and entire body, the mouth part composed of the obvious mandible, measuring approximately 5 mm in length in 4th instar larvae, and the tip of the abdomen combined with long hairs are called caudal bristles which can be used to distinguish the first instar from the others following instars using the pairs number of caudal bristles that larva possessed. The 2nd – 4th instars have a progressively larger size than first instar larvae and come up with two pairs of caudal bristles (96).

1. First instar larvae possess the body segments with pale cream color, dark brown head measured 0.06 - 0.08 mm in width and between 0.04 - 0.05 mm in length, the caudal lobule including caudal setae are light brown. The 1st instar larvae come up with only single pairs of caudal bristles and these larvae body length around 0.40 - 0.68 mm. The antennal tubercle is larger than the complete antenna. The second antennal segment is oval form but the first segment is long and thin form contains with an arista (96).

2. Second instar larvae have a similar color to the first instar but these larvae have a larger size approximately twice the body length, with two pairs of caudal bristles (96).

3. Third instar larvae are increasingly larger than the second instar, but the color is similar to both previous instars. These larvae also have two pairs of caudal bristles like the second instar and the head capsule presents with a Y-form dorsal suture (96).

4. Fourth instar larvae are progressively larger than previous instars. The body length is between 2.2 - 2.5 mm as approximately the same as the length of the caudal. These larvae possess four well-defined stemmata and two setae with a round and outstanding base, which present on each side of the head and in the mandibles. The tergum area of the eighth abdominal segment including the caudal lobule also is a dark brownish color. Locomotory organs on the thorax have not found (96).

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The larvae absorb water and their food through the skin, particularly in younger instar larvae. The breeding site for larvae growing contains with abundant of soil moisture and organic chemistry which can be found in tree litter, forest soil,

and gerbil borrows, termite hills, significantly correlated to the proximal river valley. In the laboratory, the larvae diapause and transform to the pupal stage in three weeks to two mounts, depending on the species (63).

2.11.3 Pupae Stage

The pupae begin to turn into the obtect after the mature of 4th instar larvae stop consuming and search for a drier place to pupate, which they have glued the terminal abdominal segments to a stone or dead leaf. The pupae sizes are approximately 2.6 mm in length looking closely like a shape of the butterfly chrysalis. The pupa contains with contracted larval skin can be noticed at the posterior end of the pupa. In addition, the pupae stage develops into adult sand flies within 7 – 10 days (96).

2.11.4 Adult Stage

Sand fly is fairly small arthropods, larger size in male flies rather than females, pale yellow or light brownish body covered with woolly hairs, spacious dark eyes, and long-legged flies with narrow-bodied. The wings length is less than 3 mm, and those wings are held erect above the body. Typically, sand flies are nocturnally active insects, but they have no potentiality to fly for long direct distances, no more than a kilometer, usually, they are used jumping (91). The mouthparts are well-developed with cutting teeth on elongated mandibles inside the proboscis which in females are piercing-sucking proboscis for veins cutting and blood taking whereas simplified sucking type in male flies without mandibles. Adult flies dwell in moist, dark places during a day such as cracks in trees, beneath rocks or animal burrows. The phenotypic differences or sexual dimorphism between males and female sand flies are distinctly divergent from one another, which males have the obviously

noticeable external terminalia and a slender abdomen compared to the female flies (96).

2.12 The Vector Sand Fly Species of Trypanosomatid Parasites

Phlebotomine sand flies are mainly known as the vectors of *Leishmania sp.* parasites which these protozoa have been epidemiological explored, detected and reported from many species of sand flies such as *L. siamensis* in *Sergentomyia.gemmea* from Thailand (92, 95, 97), *L. infantum* in *Se. dubia* (95), *Phlebotomus papatasi* (98), and in *Lutzomyia longipalpis* sand flies (99), *L. major* in *Sa darling* (95), *L. (Viannia) braziliensis* in *Lu. Spinicrassa*, in addition, *Nyssomyia intermedia* (99, 100) considered as the vector of *L. Braziliensis*. Moreover, many of sand flies also carry and circulate the other *Trypanosomatid* protozoa such as *Trypanosoma, Blastocrithidia*, *Leptomonas, Endotrypanum,* and *Herpetomonas*, including bacteria and arbovirus. Currently, the detection of *Trypanosomatid* DNA in sand flies has been explored and recorded. For instance, *Blastocrithidia sp.* in *Nyssomyia whitmani* (25) and *Trypanosoma sp.* in *Evandromyia evandroi* (25) from Brazil, *Trypanosoma sp.* in *Sergentomyia africana africana* (26) from Ghana, *Trypanosoma (Megatrypanum) freitasi* in *Psychodopygus claustrei* (27) from the Amazon Rainforest, Brazil, *Trypanosoma sp.* in *Phlebotomus kazeruni* (28) from Pakistan. *Endotrypanum monterogeii* in *Nyssomyia trapidoi* (32) from Ecuador, *Trypanosoma rangeli* (29) and *Endotrypanum schaudinni* (33) in *Lutzomyia sp.* (*shannoni* group), *Endotrypanum schaudinni* in *Psychodopygus ayrozai (33)*, *Trypanosoma sp.* in *Viannamyia tuberculata* from Brazil, *Herpetomonas pessoai* (30) in antropophilic sand flies from Southeast Brazil, *Herpetomonas samuelpessoai* in *Pintomyia fischeri* (25) sand flies, *Leptomonas sp.* in wild Colombian *Lutzomyia spp* (35). In addition, scientists have been successfully experimental propagating *Leptomonas seymouri* in *Phlebotomus argentipes (34)* and *Ph. orientalis* (34). And

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also, *Trypanosoma spp.* can be found within the other insects belong to the order *Diptera*, such as *Simulium rugglesi,* black fly which has been experimental infected *Trypanosoma avium* (101). Recently, unknown *Trypanosoma sp.* also has been reported in *Phlebotomus stantoni* sand fly (31) from Southern Thailand.

2.13 Sand Flies in Thailand

The species of sand flies in Thailand have approximately around 27 species which already have been recorded, consist of *Phlebotomus stantoni*, *Ph. argentipes*, *Ph. teshi, Ph. hoepplii, Ph. asperulus, Ph. philippinensis gouldi, Ph. barquesae, Ph. (Euphlebotomus) mascomai, Ph. major major, Sergentomyia punjabensis, Se. (Neophlebotomus) iyengari, Se. indica, Se. (Neophlebotomus) gemmea, Se. (Parrotomyia) barraudi, Se. perturbans, Se. hivernus, Se. silvatica, Se. dentata, Se. bailyi, Se. anodontis, Se. mahadevani, Se. quatei, Se. hodgsoni, Se. (Neophlebotomus) khawi, Nemopalpus vietnamensis, Idiophlebotomus longiforceps* and *Chinius barbazani.*

Figure 5: The recorded sand fly species from Southern Thailand

Figure 5: The sand fly discovered species that have been surveyed and recorded of four provinces including Chumphon, Nakhon Si Thammarat, Satun, and Songkhla from the Southern region of Thailand consist of ten *Phlebotomine* sand flies, *Phlebotomus stantoni*, *Ph. argentipes*, *Sergentomyia perturbans*, *Se. (Par.) barraudi, Se. iyengari, Se. hodgsoni, Se. indica, Se. hivernus, Se. gemmea* and *Se. khawi.* The figure is modified from Thailand location map.svg on Wikipedia, The Free Encyclopedia website (Wikipedia; 2009).

The Southern Thailand has reported detection of *Leishmania DNA* from *Se. gemmea* and *Se. (Par.) barraudi* (36). In 2016, the *Ph. stantoni* (31) from Southern Thailand which remains *Trypanosoma sp.* DNA currently has been detected.

2.14 Morphological Characters of Sand Fly Species in Thailand

Generally, for the sand fly species identifying and taxonomic characterization can use many organs including the physical appearance, shapes, sizes, pigment patch, hair or sockets and appendages of those flies to distinguish and identify among all of the Phlebotomine sand flies (Diptera: *Psychodidae*). Normally, the head and the terminal abdominal segments of each sand fly are separately dissected and mounted on a glass slide for furthermore microscopic morphological characterization. The numerous organs and body parts of individual sand flies which usually can be used for species identifying are the physical appearances of male phlebotomine sand flies terminalia such as the paramere, style, aedeagus, and for female sand flies normally using shape of the spermatheca, character of the cibarium and also appearance of the pharynx for characterizing those sand flies' species.

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2.14.1 *Phlebotomus stantoni*

The *Phlebotomus stantoni* is commonly known as a potential vector, which has circulated many important pathogens, including *Leishmania spp.*(89) and also other *Trypanosomatid* microorganisms (31). This species can be found in many Asian countries like Sri Lanka (41, 44, 102, 103), India (41, 102, 104), Vietnam (31, 41, 102, 105), Cambodia (95), Laos (41, 102), Malaya (31, 41, 102, 105, 106), China (41, 102, 107), and these flies are the main genera that presented in Thailand such as Trang (37) Chumphon (89), Phuket (89), Saraburi (108), Uthai Thani (109), Phitsanulok (110), Lamphun (111), Uttaradit (90), Chiangmai (105), Satun (92), and Songkhla province, which had been detected *Trypanosoma sp.* DNA from *Ph. stantoni* (31). In Thailand, *Ph. stantoni* are commonly found in cave dwellers (89).

The morphological taxonomic keys of *Ph. stantoni* were used to identify species such as the male style with 4 spines, 2 apical, 2 basal, and female with antennal segment III (A3) extending to tip of proboscis and Cibarium with 2 teeth considerably larger than others, and the cibarium with trapezoidal pigment patch (39, 41, 102). Moreover, for the female genitalia, the spermatheca is tubular, with more than 10 segments; obviously thin spermatheca ducts which shorter than spermatheca, long common ducts.

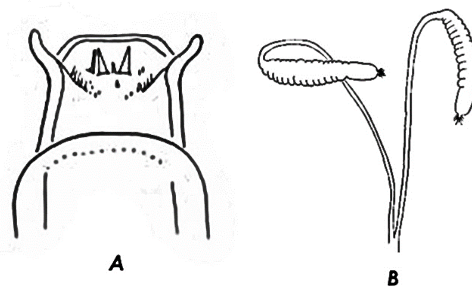

Figure 6: *Phlebotomus stantoni*

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Figure 6: A: cibarium with trapezoidal pigment patch, B: tubular spermatheca with more than 10 segments, thin spermatheca ducts. The figure is created by Lewis (1978) and Quate (1962).

2.14.2 *Phlebotomus argentipes*

The *Phlebotomus argentipes* is the vector, also known as natural host of *Leishmania spp.* (106, 109, 110) which cause kala-azar or visceral leishmaniasis caused by *Leishmania donovani* in India (44, 93, 94). *Ph. argentipes* is widely distributed in India to Borneo island, Laos, Viet Nam and spreads across a lot of area in Thailand including Chiang Rai, Chiang Mai, Kanchanaburi, Nakhon Si Thammarat, Nong Khai, Nakhon Ratchasima, Nakhon Nayok, Prachinburi, Lopburi, Loei, Lampang, Saraburi, Satun and Songkhla (63).

The morphological identifying of *Ph. argentipes*, moderate sized with erect hairs on all abdominal tergites, thickest on posterior border of tergites. In female, wing is 2.1 mm in length same as male except eyes separated by 4 or 5 facets. The cibarium armed with few scattered and weak teeth which chitinous arch can be visible, but quite weak, and pharynx combines with cluster of spines at apex. Spermatheca is ovoid shaped, strongly annulated, with long slender ducts, no pigment patch, and furca with serrations along lateriobasal margins (102). For male fly, wing length is 1.8 - 1.9 mm, and eyes separated by 6 facets. Cibarium is without teeth and chitinous arch very weak. Pharynx comprises with smaller spines than in females. Antenna has pair of the ascoids on segments 4 to 15 and segment 3 much shorter than proboscis. The male style with 5 spines, 2 terminals, 2 apical and 3 median spines, combined with forked parameres, slender spine on each side of aedeagus (41, 102).

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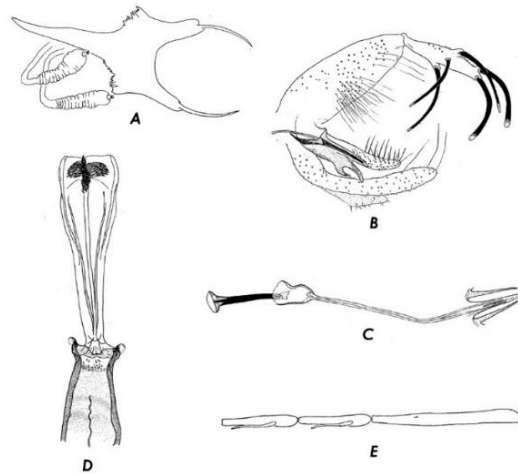

Figure 7: *Phlebotomus argentipes*

Figure 7: A: female ovoid shaped spermathecae and furca, B: male genitalia, C: the male genital filaments, D: female cibarium and pharynx, E: female antennal segments with the ascoids. The figure is created by Quate and Fairchild (1961)

2.14.3 *Sergentomyia barraudi*

Leishmania DNA amplicon also has been identified in *Se. (Par.) barraudi* from Thailand (36, 46). The *Se. (Parrotomyia) barraudi* was first morphological described in 1929 by Sinton, and in 1934 to 1935, Raynal & Gaschen have been redescribed this species again (112). Geographical distribution of this species can be found in Southeast Asia such as Thailand, Laos, Cambodia, Viet Nam, and also the parts of oriental region, particularly in India and China (41, 112).

Morphological identifying from the dissected head of this flies, Alves-Pires et al. (1996) have described the morphological of the female *Se. (Par.) barraudi* cibarium, comprising several cibarial teeth, which are approximately 40 to 60 pieces, correlated to the numbers that referred by Lewis in 1978, but those have a little difference in the quantity of cibarial teeth which usually counted around 40 to 50 pieces that described by Sinton, Raynal & Gaschen in 1934, Parrot & Clastrier in 1952

and Quate (1962) described the cibarium of this species combines with 45-70 cibarial teeth. In the female flies, Alves-Pires et al. (1996) have been observed and described the pigmented patch of cibarium that apparently come up with the bifid shape as same as the characteristic of this species which was described in 1929 by Sinton. And the *Se. (Par.) barraudi* pharynx has the shape of lamp glass-like (112), covered with spines which may also vary in size and number (41). And the male flies' cibarium has 16 teeth which less than in females (112). In addition, for the morphological identifying from the dissected terminal abdominal segments of male flies, Alves-Pires and teams have described the style which consists of four apical spines, come along with the length normally longer than its width around four times, and presented with the hooked paramere, that are related to characteristic of *Se. (Par.) barraudi* as described by Sinton, Raynal & Gaschen and Cates & Lien, in the year 1929, 1934 and 1970 respectively. In females, the organ which generally used for identifying is the characteristic of spermathecae, plump elliptical spermatheca, smooth, without setae (41, 112), with a mean length 67.55 µm and 25.4 ± 1.3 µm in width (112).

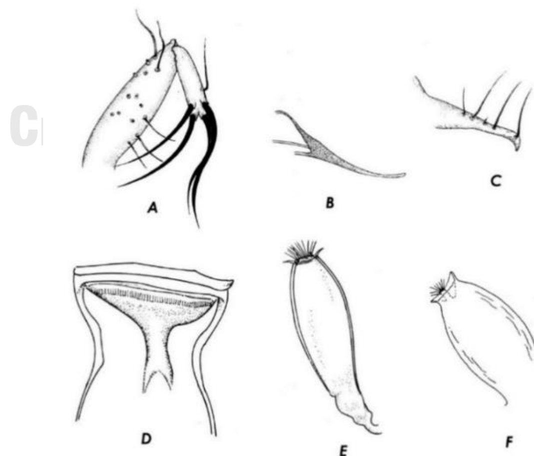

Figure 8: *Sergentomyia barraudi*

Figure 8: A – E; A: the male style with four apical spines, B: the male aedeagus, C: the male hooked paramere, D: the lamp-glass shaped pharynx, E – F:

the female elliptical spermatheca. The figure is created by Alves-Pires et al. (1996) and Quate (1962)

2.14.4 *Sergentomyia iyengari*

Sergentomyia iyengari distributes in India, Malaysia, Laos, Hainan, the smallest and southernmost province of China and also Thailand, where the place can be discovered this species in most of all regions of Thailand such as Chiang Mai (41), Phitsanulok (110), Nong Khai, Loei, Udon Thani, Sara Buri, Bangkok (41), Satun (92), and Songkhla (31).

The morphological identification from the dissected female head of *Se. iyengari*, Quate (1962) have been described the characteristic of this cibarium, combines with 14 to 17 vertical sharp teeth (41, 102), and come up with 4 central teeth of ½ reduced size, the erect teeth which can be seen entirely absent or variable number from 4 - 20 pieces in single row to double rows in each row, and the appearance of pigment patch look like top-shaped with anterior projection reaching arch or apparently not present at all, which leaves the patch hemispherical (41). In addition, for the morphological identifying from the dissected terminal abdominal segments of this flies, using the characteristic of spermatheca, tubular shaped with a deep-set apical knob surrounded by a high collar and with long setae (41).

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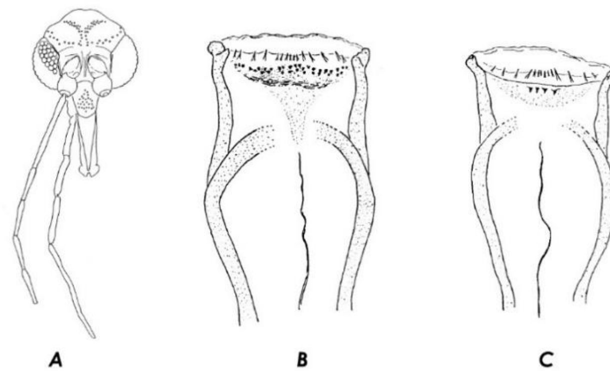

Figure 9: *Sergentomyia iyengari*

Figure 9: A: head, B: cibarium with approximately 20 pieces in each row, top-shaped pigment patch, C: cibarium with approximately 4 pieces in single row, hemispherical pigment patch. The figure is created by Quate (1962).

2.14.5 *Sergentomyia indica*

The *Sergentomyia (Grassomyia) indica* is also first known as *Phlebotomus squamipleuris* (102, 113) and this species found in Pakistan, Cambodia (95), China, Taiwan, Hong Kong (112), India, Indonesia, Laos, Malaysia, Nepal, and also including many provinces of Thailand (31, 102, 107), such as Lamphun (111), Phitsanulok, Kanchanaburi, Saraburi, Satun, Songkhla (31), Bangkok, Buri Ram, Chiang Mai, Chiang Rai, Loei, Nakhon Nayok, Nakhon Si Thammarat, Nonthaburi, Phang Nga, Phrae, Suphan Buri, Surat Thani, Ubon Ratchathani (90).

The morphological identification of *Se. (Gra.) indica* (113) can be using the external parts of dissected female head such as palps 0.6 mm long, antennal segment III (A3) 0.15 mm long with ascoids, 0.1 mm long, and 0.08 mm of each the fourth and fifth antennal segments (A4, A5 respectively) combined with 0.08 mm ascoid, 0.16 mm of proboscis. For morphological identifying of internal parts such as cibarium is 0.07 mm in width, with 35-48 horizontal teeth arranged in a convex row,

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triangular inward projections at lateral walls, dark and slightly concave pigment patch with 0.06 mm and 0.01 mm in length and width respectively without anterior process. A large chitinous structure-Pharynx, large bottle shaped is measured approximately 0.13 mm in length which more than 1.63 times of its greatest breadth lying at the posterior to the cibarium, and a patch of coarse pigmented teeth present at the base, patch come up with 0.05 long and 0.03 broad. In addition, female genitalia like spermatheca is 0.68 mm in length and 0.56 in greatest width, 0.2 basal width, with smooth anterior part and posterior part with transverse striations arranged in slight curved lines, and comprised with 1.4 mm of furca whereas male genitalia come up with 0.2 mm long, 0.06 mm wide of coxite, style is 0.09 mm long and 0.03 mm wide, with two spines terminal and 2 sub-terminal, thin and short aedeagus, 0.08 mm in length, paramere 0.15 long, with blunt end, its apex quite forwarding ahead than of the surstyle which is 0.17 long.

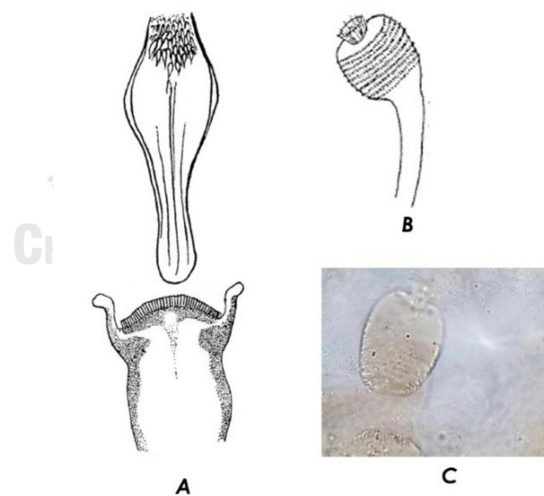

Figure 10: *Sergentomyia indica*

Figure 10: A: cibarium with 35-48 horizontal teeth like a convex shaped, and a large chitinous structure-Pharynx, B-C, quite-rounded shape spermatheca with smooth anterior part and posterior part with transverse striations arranged in slight

curved lines, and furca. The figure is created by Abonnenc (1969) and Kakarsulemankhel (2008)

2.14.6 *Sergentomyia hivernus*

The *Se. hivernus* is also known as *Ph. hivernus* which has been described by Raynal and Gaschen in year 1935 from Vietnam. Quate (1962) considered this species is synonym of *Se. iyengari*, whereas Phumee et al. (2016) have the opposite season because the characteristic of spermathecae and cibarium from these two species are different. In Southern Thailand, *Se. hivernus* recently has been reported in Songkhla province.

Morphological characterization of *Se. hivernus* has described by Phumee et al. (2016). *Se. hivernus* spermatheca is wide tubular shaped, and also longer when compared to *Se. iyengari*. In addition, this species spermatheca isn't having limits between the body and duct whereas *Se. iyengari* is easily observed limit between both previous mentioned parts. Cibarium of this species shows many denticles whereas *Se. iyengari* without any denticles which has been described by Sinton in 1933 (31).

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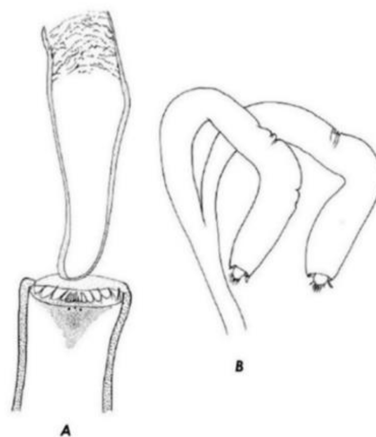

Figure 11: *Sergentomyia hivernus*

Figure 11: A: female cibarium and pharynx, B: long and wide, tubular-shaped spermatheca without limits between the body and duct. The figure is created by Phumee et al. (2016).

2.14.7 *Sergentomyia perturbans*

The *Sergentomyia perturbans* has been surveyed and reported in Malaysia (106), and especially in many provinces of Thailand (90, 92, 108, 110, 114) such as Kanchanaburi, Nakhon Nayok (90), Saraburi (90, 108, 114), Lamphun (111), Phrae, Nakhon Si Thammarat, Surat Thani (90) and Satun (92).

Morphological characterization of *Se. perturbans* has described by Quate (1967) classification criteria. For the internal parts such as female cibarium is 8 mm long, vertical teeth with bases apparently embedded in dark nail-shaped pigment patch, no horizontal teeth evident, and pharynx unarmed, slightly tilted and apex not clear. The external parts such as antenna segments III (A3) extends to tip of proboscis, shorter than A4 and A5 combined, come up with pair of ascoids on segments A3-16, ascoids shorter than segment bearing them, and palpal formula=1, 4, 2, 3, 5; Newstead's scales on inner surface of segment 3, but scattered along surface from about basal 1/5 to center and not in differentiated pocket. In addition, Quate (1967) describing the cibarium of this species is distinctive and unlike any other Asian species member of *Sergentomyia* by the armed cibarium, the recumbent hairs on the abdomen. Moreover, the female genitalia of this species such as the spermatheca is simple sec-like shaped.

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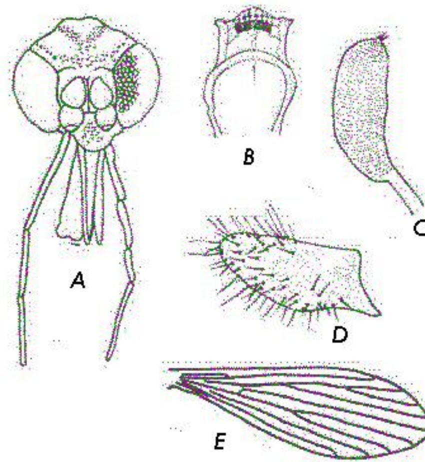

Figure 12: *Sergentomyia perturbans*

Figure 12: A: head, B: cibarium with dark nail-shaped pigment patch, without horizontal teeth, C: sec-like spermatheca, D: male cercus, E: wing, scale line 0.5 mm. The figure is created by Quate (1967).

2.14.8 *Sergentomyia gemmea*

The *Se. (Neophlebotomus) gemmea* has been considered as potential vector of *Leishmania* parasites (26, 36, 95, 109) which detected *L. siamensis* DNA from this species (36, 37, 46). The *Se. gemmea* has been reported in Malaysia (106), and almost entirely distributed in many provinces of Thailand regions such as, Nan, Phitsanulok (90, 110), Phrae (90), Lamphun (111), Uthai Thani (109), Nakhon Nayok, Nonthaburi, Suphan Buri, Kanchanaburi, Buri Ram, Ubon Ratchathani, ,Saraburi (90, 108, 114), Surat Thani, Phang Nga, Nakhon Si Thammarat (31, 90), Trang (37, 90), Satun (90, 92), and Songkhla (36).

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Lewis (1978) has been described and demonstrated the morphological characterization of *Se. gemmea* which the characteristic of female head combines with labrum 0.29-0.34 mm long. Cibarium with ten hind teeth with broad bases narrowing abruptly to fine points, with one row of the eight cibarial fore teeth of this

species have a large size, two rows of small teeth in front of them, and a patch of small fore teeth at each side; pigment patch pale; arch strong. Pharynx with linear and with finely speculate ridges. Hypopharynx has distinct teeth wider than high. Antenna segments III (A3) 0.34-0.39 mm long, 1.22-1.31 length of A4+5, two ascoids on segments A3-15, that on A4 is 0.87 length of segment and reaching next one, with spur; no papilla on 5. Mandible pointed. Maxilla has about eight lateral teeth and about 41 ventral, a few of them very small, dental depth 0.12 mm, palpal ratio 10: 16: 31: 34: 61. 1.08-1.19 length of labrum. Wing lengths are 1.98-2.00 mm, 2.7 times width. Abdominal tergites 3-6 with a few erect hairs on hind margins. In addition, spermatheca narrow with some wrinkles proximally, with knob in deep narrow pit, delicate ducts uniting into short common one Whereas, male labrum is 0.22 mm long. Cibarium with about six irregular hind teeth and about 20 irregular fore teeth of which a few posterior ones are slightly larger than the others: pigment patch indefinite. Pharynx has faint ridges. Antenna 3 = 0.35 mm long, 0.21 length of wing, 1.21 length of 4+5, 1.59 length of labrum, one ascoid on segments 3-15, that on 4, 0.64 length of segment: no papilla on 5. Wing length is 1.64 mm, 3 times width. Genital filament 3.1 times length of pump. Coxite broad with patch of about 43 narrow hairs merging into large meso-dorsal hairs: style with seta at 0.65 and two of stout spines at about 0.68.

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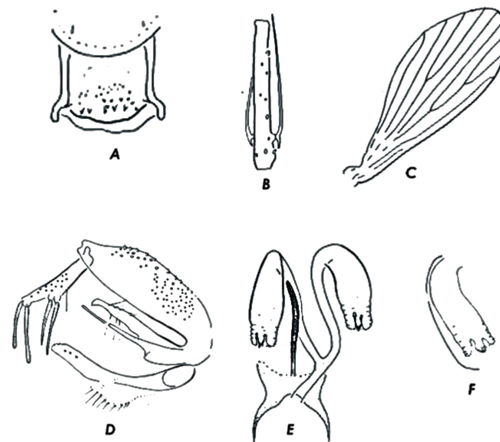

Figure 13: *Sergentomyia gemmea*

Figure 13: A: cibarium with 10 hind teeth and 8 large cibarial fore teeth, B: antenna IV with 2 ascoids on segments, C: wing, D: male terminalia, E-F, spermatheca with knob in deep narrow pit, and some wrinkles proximally. The figure is created by Lewis (1978).

2.14.9 *Sergentomyia hodgsoni*

Sergentomyia hodgsoni has been found and reported in Pakistan (115, 116), Iraq (116), Iran (117), India (104), Malaysia (106), and including Thailand (89, 110) which has been discovered in Saraburi (108), Kanchanaburi (90), and Chumphon (89). Most of *Se. hodgsoni* has been reported from Thailand commonly found in cave dwellers (89).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Kakarsulemankhel (2004) has morphological redescribed the female parts of the *Se. hodgsoni* head from Pakistan, which consist of palp, 0.622 mm long, 0.2 mm of proboscis, 0.136-0.14 mm of antennal segment III (A3) with ascoid 0.036 long on A3, antennal segment IV (A4) 0.08-0.09 mm long combined with ascoid in same length as A3, antennal segment V (A5) 0.076-0.08 mm long with 0.035 mm of ascoid. And for internal organs such as cibarium is 0.058-0.06 mm in broad with a

armature consisting of a single and an almost straight row of teeth around 45-49 in number and a line of 10-15 punctiform denticles at bases of teeth which these anterior denticles are difficult to count as pigment patch, a triangular dark pigment patch with a pale forward extension, and this female species come up with 0.15-0.16 mm of pharynx which the length is more than 3.75-4.0 times the greatest breadth with the narrow anterior portion , width about 0.022-0.024 mm, and the armature 0.032-0.04 mm height comprising of several fine, short and transverse lines occupying the posterior portion of pharynx. Moreover, female flies' genitalia like spermatheca is 0.032 mm long and 0.028 mm wide, without collar but combined with a distinct anterior knob at the apex, individual duct laying behind the main body of spermathecal capsule which is quite short and comparatively less broader about 0.056 mm in long and 0.02 mm wide with some faint irregular striations, a common duct about 0.096 mm long and 0.056 mm broad, genital atrium 0.032 mm broad and furca 0.088 mm in length (116).

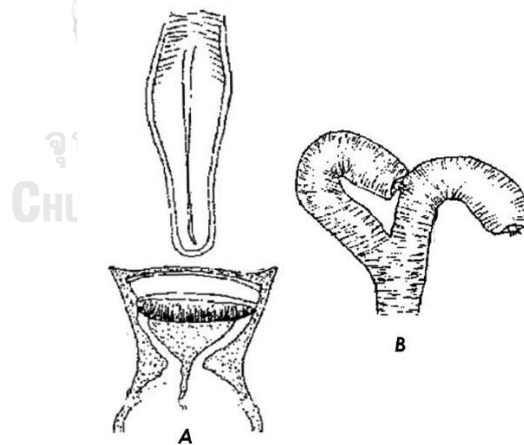

Figure 14: *Sergentomyia hodgsoni*

Figure 14: A: cibarium with 45-49 mostly straight teeth, and pharynx with several fine, short and transverse lines at the posterior portion, B: spermatheca with

a distinct anterior knob at the apex, without collar. The figure is created by Kakarsulemankhel (2004).

2.14.10 *Sergentomyia khawi*

The *Sergentomyia khawi* has been distributed in China (31, 41) like Beijing (107), Tianjin, Hebei, Liaoning, Shanxi, Shandong (107), Cambodia, Phnom Penh (41), Kampot (95), Malaysia (31), and also including Thailand, Songkhla (31).

Generally, the subgenus Sergentomyia has the reclined abdominal hairs on tergites 2-6, sockets much smaller than on tergites 1. The male dististyle with 2 spines at apical one quarter, and palpal formula =1, 2, 3, 4, 5, which palpal segment 4 longer than half-length of segment 3. The cibarium of this male species combines with 20 equal-sized teeth (41) but 21-30 in female (43), 2 rows of 20 erect teeth in each row whereas the third row is incomplete with 4 teeth on either side, with beet-shaped pigment patch and anterior projection not reaching arch (41), well developed pharynx cover with scales (43). And the female genitalia such as spermatheca is a tubiform structure, small spermatheca head without neck, with distinctly thin spermatheca ducts that's shorter than twice of spermatheca (43).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.15 Species Identifications of Sand Flies

Generally, many scientists usually use the morphological technique to identify species of sand flies, which required experts who have the knowledge and experience particularly in this field because some flies have a few morphological differences among species. In addition, the specific molecular identification techniques also have been used to differentiate genetically between closely related species by comparing nucleotide sequences of variable regions from expected gene markers which amplify by polymerase chain reaction method. Nevertheless, only the

molecular identification technique cannot be performing phylogenetic characteristics of sand flies' species because this method need to simultaneously consider and analyze with the morphological technique for correctly taxonomical classification. Normally, the reported gene markers for sand flies' species identification that frequently have been analyzed for phylogenetic grouping are comprised of mitochondrial DNA genes which are the most commonly used such as Cytochrome b (*Cytb*), with complete sequences or not, with or without partial NADH1 sequences (97), and also nuclear DNA genes.

Table 3: Molecular gene markers used for molecular identification of sand flies.

Molecular gene markers used for molecular identification of sand flies	
Gene type	Gene markers
Mitochondrial DNA (mtDNA)	Cytochrome b
	COI
	NADH1
	NADH4
	12S
Ribosomal DNA (rDNA)	*ITS1*
	ITS2
	18S
	28S
Other genes	EF-alpha
	Period
	Cacophony
	copulatory courtship songs
	Reverse transcriptase
	para genes

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Molecular gene markers used for molecular identification of sand flies	
Gene type	Gene markers
	Ca1D
	Rp49
	Maxadilan
	SP15-Saliva protein

This table combined with the available gene markers which have been used for Phlebotomine sand flies' species (Diptera, Psychodidae) identification including mitochondrial DNA, ribosomal DNA and others, The Table is modified from Table 1 of Depaquit (2014).

Depaquit J (2014) has been reviewed and reported the frequency of Phlebotomine gene-markers studying of 137 reported papers between 1991 to 2014, at least 54% of total articles using the mitochondrial DNA genes for molecular detection and species-specific identification, including Cytochrome b (*Cytb*) which has been used approximately 36% of species-identifying, 11% of Cytochrome c oxidase subunit I (*COI*), and nearly 7% of the rest gene markers like *NADH1*, *NADH4* and *12S* gene. Moreover, the other location gene markers which have been used to identify the species of Phlebotomine sand flies are *ITS2* (14%), 18S (6%) and also 4% of Cacophony gene.

Recently, a lot of the Old-World species belong to the genus *Phlebotomus* have been molecular phylogenetic characteristics whereas a few of the genus *Sergentomyia*, *Grassomyia*, *Chinius,* and *Idiophlebotomus* have not been sequenced yet (97). In Southern of Thailand, Phlebotomine sand flies (Diptera: *Psychodidae, Phlebotominae*) such as *Ph. stantoni* already has been species identified and submitted in NCBI by using the *COI* gene (90, 111), *Cytb* (31, 105, 118), *28S* (118), and

ITS2 (105, 118), *Ph. argentipes* also has been used the *Cytb* gene, *ITS2* (105, 119),18S (103, 119-121), 28S, *COI* (119, 122), salivary protein *SP01 mRNA* (64), and including bromodomain and *PHD* finger-containing protein gene (123) for processing the species-specific identification, *Se. gemmea* has been molecularly identified by applying the *COI* gene (111), *Cytb* and 18S (37)*, Se. (Par.) barraudi COI* (111) (51)*, Cytb* , 18S (124), and for available species identifying of *Se. iyengari, Se. perturbans* and *Se. (Gra.) indica* has been used the *COI* gene (111) whereas *Se. hodgsoni, Se. hivernus,* and *Se. khawi* have not been available on GenBank.

In this study, the main reason is to detected trypanosomatids in sand flies collected from Songkhla province, southern Thailand for processing the species identification based on standard dideoxynucleotide (DNA) sequencing technology to explain the molecular variation of nucleotides differences among these investigated sand flies from amplified cytochrome b gene using the conventional polymerase chain reaction (PCR) method which modified from the previously published (31, 45-52, 57, 125-128), concurrently with the microscopic morphological characterization following the criteria and keys of previous reports (31, 39, 41, 44, 46, 89, 102, 112, 113, 116). Furthermore, the trypanosomatid protozoa of all the collected sand flies are also detected by using the Internal transcribed spacer 1 (*ITS1*) and the small subunit ribosomal RNA (*SSU rRNA*) genes to taxonomically identify the species of *Leishmania* and the other genera belonging to the order *Trypanosomatida*, respectively, by performing modified conditions PCR accorded with the previous reported experimental studies (31, 45, 53). In order that this investigation of the epidemiological survey of sand fly populations naturally infected by trypanosomatid protozoa in this area can provide us understanding and information about the prevalence of parasites among these fly’s populations, including number and kind of discovered species of these flagellate parasites and their vectors even more.

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Chulalongkorn University

CHAPTER III

MATERIALS AND METHODS

3.1 Sources of Samples

Tha Pradoo sub-district, Na Thawi district, Songkhla province was selected as the site for epidemiological study and surveillance on the endemic area of one reported autochthonous leishmaniasis case with both cutaneous and visceral manifestation (4, 13, 129) who never leave Thailand for working or traveling and no history of blood transfusion, or needle sharing before the onset of the disease. Moreover, Phumee et al. (2016) have recently been reported *Trypanosoma sp*. in sand fly like Chusri et al. (2012) also have been detected *L. siamensis*, which has recently been proved and described as *L. martiniquensis* (4) in sand fly from this area.

3.2 Sample Size

The numbers of samples were used in this study to detect trypanosomatid protozoa calculated from the proportion of the positive samples in sand fly's populations of the previous study (31) which is 2.22%. The sample size required will be calculated according to the following formula:

$$n = \frac{\left(Z{\alpha/2}^{2}\ \mathrm{P}(1-\mathrm{P})\right)}{\mathrm{e}^2}$$

$$n = \frac{(1.96)^2 \times (0.0222) \times (0.9778)}{(0.03)^2}$$

$$n = 92.65$$

Description: n = required sample size

$Z_{\alpha/2}$ = confidence level at 95% (standard Z value of 1.96 (two-tail))

P = Proportion of the sand fly's populations infected with Trypanosomatid protozoa estimated from the previous study

e = acceptable margin of error at 3% (standard value of 0.03)

According to the calculation above, the sufficient sample size in this study was approximately 93 samples. However, in this study, 349 female sand flies were collected from this area. The specimens were divided into two groups, 229 samples of dissected sand flies and 120 samples of non-dissected sand flies.

3.3 Collection of Sand Flies

Sand flies were collected from the house of the leishmaniasis patient, located at Tha Pradoo sub-district, Na Thawi district of Songkhla province, southern Thailand. Center for Disease Control (CDC) light traps were deployed six different places outside of the house (two traps under the basement, two traps around termite mounds, and the other two traps were set under basement and cooking, dishwashing area of his parent's house). The light traps were set at 1 to 1.5 m above the ground and simultaneously operated for over 12 hours from 06:00 p.m. to 06:00 a.m. After overnight trapping, all insects were anesthetized by keeping a collector bags in the freezer for 30 minutes, and then female sand flies were separated from other insects and males using the morphological feature of genitalia. In this study, the female flies were divided into two groups; one was dissected by cutting off the head and last three abdominal segments of each fly and the rest of body parts (thorax, upper abdominal segments, wings, and legs) were preserved in lysis buffer G for further DNA extraction, as well as the other group but without dissection. For taxonomic identification, the dissected pieces of each specimen were mounted on

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

microscope slides in Hoyer's medium and then identified using the morphological characteristics of cibarium, pharynx and spermatheca were used as criteria for species identification.

3.4 Identification of Sand Fly Species

3.4.1 Morphological Identification of Sand Fly Species

Individual dissected female sand fly was cut off in a drop of normal saline under stereomicroscope by using 26G x 1/2" sterile needles, and take the head and abdomen part to another slide which contain a drop of Hoyer's mounting medium by placing the head of sand fly facing up on a coverslip and then left the slide to dry at 37 °C in incubator around 48-72 hours for further species identification, following by morphological key references and criteria of those articles (31, 38-44, 46, 89, 102, 112, 113, 116).

3.4.2 Molecular Genetic Detection of Sand Fly Species

The rest of sand fly body parts, thorax, legs, wing and the upper abdominal segments (229 samples) and non-dissected sand flies (120) were stored individually in 50 µl of lysis buffer G in a 1.5 ml sterile tube for further DNA extraction and PCR amplification.

3.4.2.1 Amplification of the *Cytb* Gene Sequences

Primers N1N-PDR: 5’- CAY-ATT-CAACCW- GAA-TGA-TA -3’ and C3B-PDR: 5’- GGT-AYW-TTG-CCTCGA- WTT-CGW-TAT-GA -3’ which have been used for *Cytb* gene amplification of sand fly following previously published articles (31, 46-52, 125-128), these primers were used to amplify *Cytb* gene in a 25 µl of PCR reaction mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM of MgCl2, 0.24 mM of

dNTPs, 1.25 unit of Taq DNA polymerase (Thermo scientific), 0.5 μM of each primer. Amplification was performed using a PCR Mastercycler Pro (Eppendorf, Germany) under the following conditions: total 40 cycles divided to 5 cycles of first step combining with 3 min at 95 °C of initial denaturation, 30 sec at 95 °C of denaturation, 45 sec at 48 °C of annealing, 45 sec at 72 °C of extension, and the rest 35 cycles combining with 30 sec at 95 °C of denaturation, 30 sec at 48 °C of annealing, 45 sec at 72 °C of extension, and a final extension at 72 °C for 7 min and cooling of the reaction mixture. The PCR products were run on 1.5% agarose gel electrophoresis for 40 min at 100 volts and visualized under ultraviolet light.

3.5 Detection of Trypanosomatid Parasites

3.5.1 Molecular Genetic Detection of Trypanosomatid Parasites

The Extracted DNA of sand fly was amplified to identify trypanosomatid parasite species by using the *SSU rRNA* and the *ITS1* region as markers following previously articles (31, 45, 53) and the rest of DNA were kept for further species identification of sand fly. Although the primers TRY927F and TRY927R could amplify the *SSU rRNA* gene of genus *Trypanosoma*, these primers can also bind to another species belonging to family *Trypanosomatidae* (53). Moreover, the primers LeF and LeR also can be used to detect *Leishmania spp.* and *Trypanosoma* DNA (31).

3.5.1.1 Amplification of the *SSU rRNA* Gene Sequences

Primers TRY927F: 5’ - GAA-ACA-AGA-AAC-ACG-GGA-G- 3’ and TRY927R: 5’ - CTA-CTG-GGC-AGC-TTG-GA- 3’ which have been used for the small subunit ribosomal DNA (*SSU rRNA*) gene of trypanosomatid parasites following previously published articles (31, 53), these primers were used to amplify *SSU rRNA*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

gene in a 25 μl of PCR reaction mixture which contained approximately 30 ng of extracted sand fly DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.16 mM of dNTPs, 1.25 mM of MgCl2, 1.0 unit of Taq DNA polymerase (Thermo scientific, Waltham, MA, USA), 0.24 μM of each primer and the remaining volume made up by nuclease-free water. Amplification was performed using a PCR Mastercycler Pro (Eppendorf, Germany) under the following conditions: total 40 cycles of 5 min at 95 °C of initial denaturation, 30 sec at 95 °C of denaturation, 60 sec at 53 °C of annealing, 90 sec at 72 °C of extension and 7 min at 72 °C of final extension. The PCR products were run on 1.5% agarose gel electrophoresis for 40 min at 100 volts and visualized under ultraviolet light.

3.5.1.2 Amplification of the *ITS1* Gene Sequences

Primers LeR: 5'- CCA-AGT-CAT-CCA-TCG-CGA-CAC-G- 3' and LeF: 5' -TCC-GCC-CGA-AAG-TTC-ACC-GAT-A- 3' which have been used for the internal transcribed spacer 1(*ITS1*) gene amplification of Leishmania parasites following previously published articles (31, 45), these primers were used to amplify *ITS1* gene in a 25 μl of PCR reaction mixture which contained approximately 30 ng of extracted sand fly DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.16 mM of dNTPs, 1.25 mM of MgCl2, 1.0 unit of Taq DNA polymerase (Thermo scientific, Waltham, MA, USA), 0.24 μM of each primer and the remaining volume made up by nuclease-free water. Amplification was performed using a PCR Mastercycler Pro (Eppendorf, Germany) under the following conditions: total 40 cycles of 5 min at 95 °C of initial denaturation, 60 sec at 95 °C of denaturation, 60 sec at 50 °C of annealing, 60 sec at 72 °C of extension and 7 min at 72 °C of final extension. The PCR products were detected by agarose gel electrophoresis as mentioned above.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.6 Sand Fly DNA Extraction

DNA extraction was performed using an Invisorb Spin Tissue Mini Kit (STRATEC Molecular, Germany). The sand fly DNA was isolated from a 1.5 ml sterile tube which already contained with 100 ml of lysis buffer G and the other parts of female sand fly, thorax, legs, wing, and the rest of abdomen part, following DNA isolation protocol as described in the Invisorb spin tissue mini kit by added more 300 µl of lysis buffer and 40 µl of proteinase K, then crushed sample into little pieces with a pestle. Incubated at 52 °C until lysis was completed and added 200 µl of binding buffer A then vortex. Placed a spin filter into a 2.0 ml receiver tube and transferred lysate onto the spin filter then incubated at room temperature for 1 min. Centrifuged at 11,000 rpm for 2 min and discard filtrate then added 550 µl of wash buffer onto spin filter and centrifuged at 11,000 rpm for 1 min. Discarded filtrate and centrifuged for 4 min at 13,000 rpm for ethanol removal. Placed the spin filter into 1.5 ml receiver tube then add 40 µl of prewarmed elution buffer and incubated at room temperature for 3 min. Centrifuged at 11,000 rpm for 1 min and discarded the spin filter. The concentration of DNA was determined by measuring the absorption at 260 nm in a UV spectrophotometer, Nanodrop 2000c (Thermo-scientific, USA), and then stored at −20 °C.

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Chulalongkorn University

3.7 Gel Electrophoresis and DNA Visualization

After amplifying process, Twenty percent of each PCR reaction was mixed with 1x of loading dye and separated by 1.5% agarose gel electrophoresis at 100 volts for 30 - 40 min. after electrophoresis, the DNA bands were stained with ethidium bromide (EtBr) for 5 min and then destained for 15 min. The target amplicons were visualized under UV light using a Quantity One Quantification Analysis Software version 4.5.2 (Gel Doc EQ System; Bio-Rad, CA).

3.8 Gene Cloning

3.8.1 DNA Ligation

The positive amplicons of *ITS1*, *SSU rRNA* and *Cytb* were cloned by ligating into a pGEM®-T Easy Vector (Promega, USA). Total 5 µl of the ligation reaction was performed by mixing 0.5 µl of pGEM®-T Easy Vector, 2.5 µl of 2x Rapid Ligation Buffer, 0.5 µl of T4 DNA Ligase, and 1.5 µl of PCR products as a template in 1.5 ml sterile tube then incubated at 4 °C overnight.

3.8.2 Transformation

Ligated products were transformed into *Escherichia coli* DH5α competent cells using heat shock method. The transformation was performed by combining 5 µl of the ligation reaction with 30 µl of thawed competent cells (DH5α) and incubated the suspension on ice for 30 min. Heat the tube containing the suspension in a water bath at exactly 42 °C for 50 sec and immediately return the tube on ice for 2 min and then add 970 µl of SOC medium. Bring the tube to incubate and shake at 37 °C and 170 rpm for 90 min. After that, centrifuge the tube to spin cells down and remove 800 µl of the medium then resuspend the pellet by pipette and plate 120 µl of the suspension onto LB plates supplemented with 100 µg/ml of ampicillin, 0.1 M of IPTG and 20mg/ml of X-Gal. Incubate the plates overnight at 37 °C and then select white colonies to perform colony PCR for detection of colonies which contain the vector with inserted DNA of interest and culture the selected colonies.

3.9 Colony PCR

The white *E. coli* colonies were picked up with a sterilized toothpick and transferred into a 0.2 ml PCR tube contained with PCR master mix and mixed

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

colonies with solutions. And a sterilized toothpick that uses for pick each colony was transferred into 15 mL tube contained with 6 ml of LB broth, and 100 µg/ml of ampicillin and incubated at 37 °C overnight (16-18 hours) for further extraction of plasmid DNA. For colony PCR conditions were performed following the procedure that related to *ITS1, SSU rRNA*, and *Cytb* specific genes from the same previously mentioned steps of the conventional PCR. The PCR products were detected by 1.5% agarose gel electrophoresis as mentioned above.

3.10 Plasmids Extraction

Plasmids extraction was performed by following the protocol of Invisorb® Spin Plasmid Mini Two kit. Centrifuge 15 mL falcon tube of suspension cells at 7000 rpm for 5 min and remove the medium until liquid remained at 1.0-1.5 mL then the pelleted cells were resuspended by vortex. Aliquot the suspension into 1.5 mL sterile tube and centrifuged at maximum speed (11,000-13,000 rpm) for 60 sec then remove the supernatant completely. Resuspend the bacterial pellet in 250 µl of solution A and added 250 µl of solution B then mixed gently, but thoroughly 4-6 times following by added 250 µl of solution C and mix gently by inverting the tube then centrifuged at maximum speed for 5 min. Transfer the clarified supernatant onto the spin filter and incubated for 60 sec at room temperature then centrifuged at 11,000 rpm for 1 min. Discard the filtrate and added 750 µl of wash solution then centrifuged at 11,000 rpm for 1 min, followed by discarding the filtrate. And continuously centrifuged at full speed for 3 min to remove ethanol then place the spin filter into a new 1.5 mL receiver tube following by added 60 µl of nuclease-free water and left the tube for 60 sec at room temperature for incubation. Centrifuge at 11,000 rpm for 1 min to elute plasmids DNA off the filter.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.11 DNA Sequencing

The Purified plasmids were subjected to sequencing by AITbiotech Pte. Ltd., Singapore, using universal primer T7 and SP6. The chromatogram data were validated in SnapGene v4.0.6 and edited in BioEdit version 7.2.6. All sequences generated in this study were deposited in GenBank.

3.12 Sequence Alignment and Phylogenetic Analysis

In this study, the trypanosomatid *SSU rRNA* gene sequences (*18S rRNA*) and *ITS1* (*18S rRNA-ITS1-5.8S rRNA*) were phylogenetically analyzed with other protozoan species belonging to the family *Trypanosomatidae*, obtained from GenBank as listed in Table 4. In addition, sand fly *Cytb* gene sequences (*Cytb-ND1*) used for the analysis consisted of *Ph. stantoni* (MG770904, MG770928), *Se. (Par.) barraudi* (MG770902, MG770911), *Se. gemmea* (JX852706, MG770898, MG770920, MG770933), *Se. iyengari* (MG770924, MG770926-7), and other insect sequences used as out-group (*Euthalia irrubescens*, KF590527; *Allancastria cerisyi*, LS974636; *Archon apollinus*, LT999971). These input sequence data sets were generated by using MEGA X (version 10.0.4). Multiple sequence alignment (MSA) was carried out by using the Probabilistic Alignment Kit (PRANK) v170427. The ambiguously aligned positions were excluded from subsequent phylogenetic analyses using Gblocks version 0.91b. After nucleotide alignment, the presence of indels was treated by coding gaps as a binary partition using FastGap v1.2 following the simple methods described in Simmons and Ochoterena. For model selection, jModelTest version 2.1.10 was executed to estimate the optimal substitution models for each alignment based on the Akaike information criterion (AIC), which be employed for further Bayesian analysis.

The phylogenetic trees were constructed using MrBayes v3.2.6 with a suitable substitution model selected by using AIC criterion for each DNA sequence data, while

the restriction site model was applied by setting as “coding=variable” to this partition. Four simultaneous Markov Chain Monte Carlo (MCMC) chains were run for 1 x 10^7 generations, with tree sampling every 1000 generations. Subsequently, the initial 25% of generations were discord as the burn-in. The convergence of independent runs was assessed by determining the values of the average standard deviation of split frequencies, which should be below 0.01. To ensure a sufficient number of sampling, the effective sampling size (ESS) was checked with program Tracer version 1.7.1 using log files from each runs. The phylogenetic tree was visualized and edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and iTOL version 4.4.2 (Interactive Tree Of Life; http://itol.embl.de).

Table 4: Trypanosomatid species, host, and GenBank accession numbers of *SSU rRNA* and *ITS1* sequences included in the phylogenetic analyses.

Species (Isolate)	Host species	Accession number
SSU rRNA sequences		
Trypanosoma cobitis (LUMP 1243)	*Noemacheilus barbatulus*	AJ009143
Trypanosoma granulosum (UK)	*Anguilla anguilla*	AJ620551
Trypanosoma siniperca	*Siniperca chuatsi*	DQ494415
Trypanosoma ophiocephali	*Channa argus*	EU185634
Trypanosoma sp. carpio	*Cyprinus carpio*	EF375882
Trypanosoma sp. fulvidraco	*Pseudobagras fulvidraco*	EF375883
Trypanosoma pleuronectidium (TMA)	*Melanogrammus aeglefinus*	DQ016618
Trypanosoma murmanensis (THH-5)	*Hippoglossus hippoglossus*	DQ016616
Trypanosoma epinepheli	*Lates calcarifer*	MG598625
Trypanosoma triglae	*Trigla lineata*	U39584
Trypanosoma boissoni	*Zanobatus atlanticus*	U39580

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Species (Isolate)	Host species	Accession number
Trypanosoma binneyi	*Platypus*	AJ132351
Trypanosoma sp. (RrS3)	*Pelophylax ridibundus*	MH424286, MH424290, MH424288, MH424289, MH424287
Trypanosoma sp. (444)	*Leptodactylus chaquensis*	EU021225
Trypanosoma sp. (646)	*Aplastodiscus leucopygius*	EU021234
Trypanosoma sp. (660)	*Scinax hayii*	EU267075
Trypanosoma sp. (RrS2)	*Pelophylax ridibundus*	MH424280, MH424279
Trypanosoma sp. (RrS1)	*Pelophylax ridibundus*	MH424293, MH424292, MH424272
Trypanosoma sp. (103)	*Evandromyia infraspinosa*	EU021237
Trypanosoma sp. (887)	*Sciopemyia sp.*	EU021245
Trypanosoma sp. (888)	*Sciopemyia servulolimai*	EU021241
Trypanosoma grosi (HANTO)	*Apodemus peninsulae*	AB175623
Trypanosoma lewisi (TryBiIDN203)	*Bandicota indica*	AB242273
Trypanosoma niviventerae (TryNcCHN503)	*Niviventer confucianus*	AB242274
Trypanosoma rabinowitschae	*Cricetus cricetus*	AY491765
Trypanosoma musculi LUM 343	*Mus musculus*	AJ223568
Trypanosoma blanchardi	*Eliomys quercinus*	AY491764
Trypanosoma lewisi (Af)	*Alouatta fusca*	GU252209
Trypanosoma otospermophili	*Spermophilus richardsonii*	AB175625

จุฬาลงกรณ์มหาวิทยาลัย CHULALONGKORN UNIVERSITY

Species (Isolate)	Host species	Accession number
Trypanosoma kuseli	*Pteromys volans*	AB175626
Trypanosoma sp. (TryEaNG/HP405)	*Eothenomys andersoni*	AB242276
Trypanosoma microti (TRL 132)	*Microtus agrestis*	AJ009158
Trypanosoma sapaensis (TryCdVNM301)	*Crocidura Dracula*	AB242822
Trypanosoma anourosoricis (TryAsTWN301)	*Anourosorex squamipes yamashinai*	AB242823
Trypanosoma wauwau (TCC1022)	*Pteronotus parnellii*	KT030830
Trypanosoma cruzi (TryCC 793)	*Myotis levis*	FJ900241
Trypanosoma erneyi (TCC1946)	*Mops condylurus*	JN040989
Trypanosoma dionisii	*Carollia perspicillata*	FJ001667
Trypanosoma livingstonei (TCC1953)	*Hipposideros caffer*	KF192984
Trypanosoma cruzi NRcl3	*Homo sapiens*	AF228685
Trypanosoma sp. (H25)	*Kangaroo*	AJ009168
Trypanosoma sp. noyesi	*Bettongia penicillata*	KU354263
Trypanosoma irwini	*Phascolarctos cinereus*	FJ649479
Trypanosoma culicavium (PAS109)	*Ficedula albicollis*	HQ107966
Trypanosoma avium (09-cab)	*Lutzomyia caballeroi*	AB566384
Trypanosoma avium (2015W_B20239)	*Xanthomyza Phrygia*	KT728401
Trypanosoma sp. (BUT19)	*Buteo buteo*	JN006828
Trypanosoma sp. (CUL15)	*Culex pipiens*	JN006830
Trypanosoma thomasbancrofti (2015WB40442)	*Xanthomyza Phrygia*	KT728394
Trypanosoma bennetti (AP07)	*Aquila pomarina*	JF778738
Trypanosoma minasense (LSTM)	*Saimiri boliviensis*	AJ012413
Trypanosoma sp. AP-2014	*Sand fly*	KJ467217
Trypanosoma grayi	*Glossina gambiensis*	AJ223565

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Species (Isolate)	Host species	Accession number
Trypanosoma grayi (Crocamp1)	*Crocodylus niloticus*	KF546526
Trypanosoma sp. (624)	*Caiman yacare*	EU596253
Trypanosoma ralphi (TCC2218)	*Caiman crocodilus*	KF546523
Trypanosoma sp. (TCC1611)	*Caiman yacare*	KF546517
Trypanosoma vespertilionis	*Pipistrellus pipistrellus*	AJ009166
Trypanosoma therezieni	*Chamaeleo brevicornis*	AJ223571
Trypanosoma theileri	*Bos Taurus*	JX178182
Trypanosoma sp. IKAZ/PK/04/SKF32	*Phlebotomus kazeruni*	AB520638
Trypanosoma sp. ZCZR2	*Pelophylax esculentus*	MH424313, MH424311, MH424310, MH424307
Trypanosoma sp. (TSD1)	*Cervus nippon yesoensis*	AB569248
Trypanosoma sp. (PJH2013b)	*Cervus elaphus Canadensis*	JX178178
Trypanosoma sp. (KrSl7)	*Chrysops divaricatus*	MK156793
Trypanosoma rotatorium B2-II	*Rana catesbeiana*	AJ009161
Trypanosoma rotatorium B21	*Rana catesbeiana*	U39583
Trypanosoma rangeli	*Rhodnius prolixus*	AJ012414
Trypanosoma ranarum	*Rana pipiens*	AF119810
Trypanosoma melophagium	*Melophagus ovinus*	HQ664912
Trypanosoma mega ATCC30038	*Bufo regularis*	AJ009157, AJ223567
Trypanosoma fallisi	*Bufo americanus*	AF119806
Trypanosoma conorhini	*Rattus rattus*	AJ012411
Trypanosoma chattoni	*Rana pipiens*	AF119807
Trypanosoma cf. cervi	*Odocoileus virginianus*	JX178196

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Species (Isolate)	Host species	Accession number
Leishmania tropica	_	GQ332363
Leishmania sp. siamensis	*Homo sapiens*	KJ467218
Leishmania Mexicana	*Homo sapiens*	FR799580
Leishmania martiniquensis	*Homo sapiens*	AF303938
Leishmania major	_	XR002460812
Leishmania infantum	_	XR001203206
Leishmania donovani	*Homo sapiens*	CP022642
Leishmania amazonensis	_	GQ332354
Leishmania aethiopica	_	GQ920678
Herpetomonas ztiplika	*Culicoides cubitalis*	AF416560
Herpetomonas puellarum	*Coenosia tigrina*	KC205995
Endotrypanum sp. (102)	*Psathyromyia dendrophyla*	EU021239
Endotrypanum monterogeii	_	X53911
Crithidia luciliae	_	KY364901
Crithidia fasciculate	_	Y00055
Leptomonas tenua	*Monopsyllus sciurorum*	KF054114
Leptomonas pyrrhocoris	*Dysdercus fasciatus*	JQ658808
Bodo saltans (JC02)	_	AY490227
Blastocrithidia miridarum	_	EU079128
ITS1 sequences		
Trypanosoma evansi evansi	*Camelus dromedaries*	AB551922
Trypanosoma brucei (Suzena)	*Homo sapiens*	AF306775
Trypanosoma brucei gambiense	*Homo sapiens*	FN554966
Trypanosoma brucei (TH2)	*Homo sapiens*	AF306777
Trypanosoma sp. (AP-2014)	*Sand fly*	KJ467211

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Species (Isolate)	Host species	Accession number
Trypanosoma theileri	*Bos Taurus*	AB569250
Trypanosoma cruzi cruzi	*Homo sapiens*	CP015675
Trypanosoma grayi	*Glossina tachinoides*	MG255201
Trypanosoma lewisi	*Rattus norvegicus*	FJ011094
Trypanosoma grosi	*Apodemus peninsulae*	AB175623
Trypanosoma kuseli	*Pteromys volans*	AB175626
Trypanosoma otospermophili	*Spermophilus richardsonii*	AB175625
Crithidia expoeki	*Bumblebee*	GU321178
Crithidia mellificae	*Vespula squamosal*	KJ722757
Leishmania lainsoni	*Homo sapiens*	DQ182542
Leishmania naiffi	–	DQ182543
Leishmania sp. siamensis	*Rattus sp.*	JQ866906
Leishmania sp. siamensis	*Sand fly*	JQ866907
Leishmania guyanensis	*Homo sapiens*	DQ182539, DQ182540
Leishmania panamensis	*Homo sapiens*	CP009396
Leishmania braziliensis	*Homo sapiens*	DQ182537, JQ397604
Leishmania major	*Homo sapiens*	FN677357, KY882278
Leishmania aethiopica	*Homo sapiens*	FN677354, FN677344
Leishmania tropica	*Homo sapiens*	FN677341
Leishmania amazonensis	*Homo sapiens*	DQ182536
Leishmania donovani	*Homo sapiens*	CP022642, FR799614,

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Species (Isolate)	Host species	Accession number
		FN677363
Leishmania infantum (RH2, RCP9)	*Vuples vulpes*	MK675908, MK675907

3.13 Ethical

This study was ethical approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The approval number was 303/60.

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CHAPTER IV

RESULTS

The 349 female sand flies had been collected around the house of the leishmaniasis patient, located at Songkhla province of southern Thailand. The samples were divided into two groups, one for both morphological and molecular identification and the other one for only molecular identification. The first group was composed of 229 dissected sand flies (65.6%) and the second group was composed of 120 non-dissected sand flies (34.4%).

4.1 Morphological Identification and Classification

4.1.1 Phlebotomine Sand Fly Species Collected from Songkhla Province

All dissected sand flies were identified according to the structures of the spermatheca, cibarium, and pharynx using the morphological taxonomic keys based on oriental genera (31, 38-44, 46, 89, 102, 112, 113, 116). Among 229 dissected sand flies, we found 5 species in 2 genera including *Ph. stantoni* (29.7%), *Se. (Par.) barraudi* (30.6%), *Se. (Gra.) indica* (7.4%), *Se. khawi* (31.9%), and *Se. hivernus* (0.4%) as shown as Table 5. Furthermore, living flagellated organisms were observed in 1 specimen of *Se. (Gra.) indica* sand flies (Figure 16).

1 *Phlebotomus stantoni.* Our survey reveals that 29.7% of female sand flies are *Ph. stantoni.* This species has the cibarium with large sharp teeth with neither denticles nor a pigmented patch. The pharynx appears to be with faint transverse zig-zag lines and small pointed pharyngeal teeth. The spermatheca has a

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fusiform body with several segments in which apical piece is a narrow cylinder. Moreover, the spermatheca body is shorter compared to its individual ducts.

2 *Sergentomyia hivernus.* In the present study, we discovered only female fly of this species. The posterior teeth of *Se. hivernus* cibarium are unequally arranged between the central teeth, which are obviously shorter and more contiguous than the peripheral left and right teeth and the numbers of posterior teeth are 12 pieces. In addition, the cibarium clearly presents in a single row of 3 denticles and triangle shape of a pigmented patch. The *Se. hivernus* pharynx has club-shaped with a narrower space at the posterior part, where its almost invisible pharyngeal teeth are obliquely lined across. In part of the spermatheca, this species has long tubular with smooth sides and a wide duct without limits between the spermathecal body and duct. The terminal knob has a small cluster of short hair-like.

3 *Sergentomyia (Parrotomyia) barraudi.* In the 70 female sand flies, we observed that the cibarium is composed of ~50 parallel equidistant teeth and a pigmented patch with bifurcated tip and with or without Denticles (fore teeth). The pharynx of these species has appeared like a lamp-glass shape. Moreover, among all the spermathecae of *Se. (Par.) barraudi* is presented with an elliptical barrel shape, smooth and thick wall. Additionally, at the terminal of spermatheca has a narrow collar with a hair-like cluster.

4 *Sergentomyia (Grassomyia) indica.* Among discovered species collected from Songkhla, 7.9 % is *Se. (Gra.) indica.* The cibarium of this species has a convex arrangement of 32-39 teeth with a pigmented patch which is slightly pointing to the labium. The pharynx has a bulge shape at the posterior end with a cluster of pharyngeal teeth. Moreover, *Se. (Gra.) indica* spermatheca presents with a distinctive

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rounded shape with a wrinkled surface including ducts and shows genital filaments at the terminal knob.

5 *Sergentomyia khawi.* The most species which has been found in this study is *Se. khawi* (31.4%). For the important features under the head, the *Se. khawi* cibarium presents with 13-19 teeth and numerous denticles in 3-4 irregular rows including a semicircular pigmented patch with central projection reaching to the sclerotized arch or known as chitinous arch. The pharynx has several scales thus formed by the small pharyngeal teeth which are almost invisible. Moreover, the spermatheca of *Se. khawi* is the tubular appearance with a conspicuous collar of terminal knob enclosed by long filaments.

Table 5: The species of female sand flies collected from Songkhla province of southern Thailand

Sand fly species	Number of samples
Phlebotomus stantoni	68
Sergentomyia khawi	72
Sergentomyia (Parrotomyia) barraudi	70
Sergentomyia (Grassomyia) indica	18
Sergentomyia hivernus	1
Total	229

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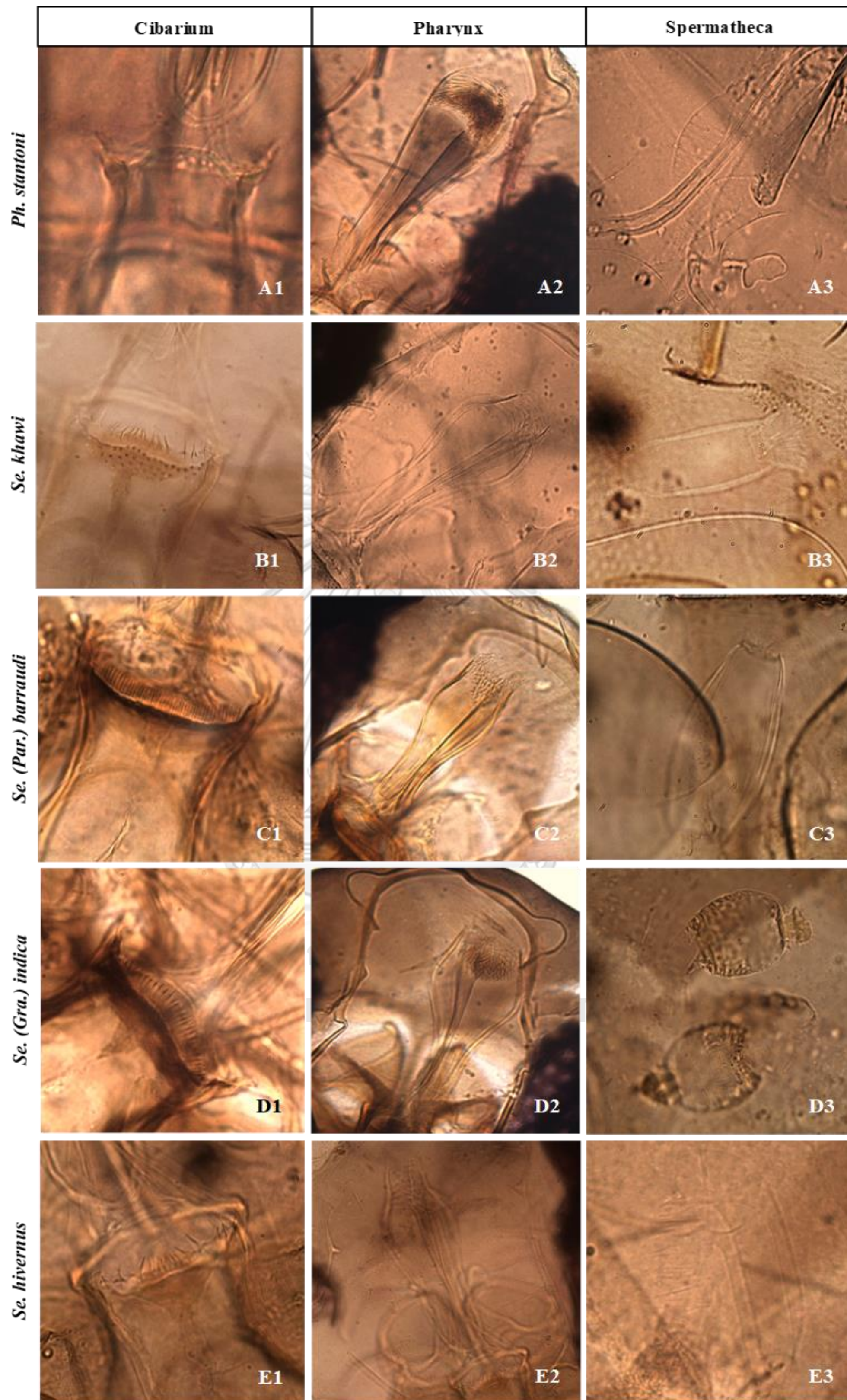

Figure 15: Representative morphological features of five sand fly species in this study

Figure 15: (**A1**) Cibarium of *Ph. stantoni* (SK82). (**A2**) Pharynx of *Ph. stantoni* (SK48). (**A3**) Spermatheca of *Ph. stantoni* (SK82). (**B1**) Cibarium of *Se. khawi* (SK90). (**B2**) Pharynx of *Se. khawi* (SK223). (**B3**) Spermatheca of *Se. khawi* (SK164). (**C1**) Cibarium of *Se. (Par.) barraudi* (SK203). (**C2**) Pharynx of *Se. (Par.) barraudi* (SK203). (**C3**) Spermatheca of *Se. (Par.) barraudi* (SK188). (**D1**) Cibarium of *Se. (Gra.) indica* (SK57). (**D2**) Pharynx of *Se. (Gra.) indica* (SK153). (**D3**) Spermatheca of *Se. (Gra.) indica* (SK220). (**E1-3**) Cibarium, Pharynx, and Spermatheca of *Se. hivernus* (SK108), respectively.

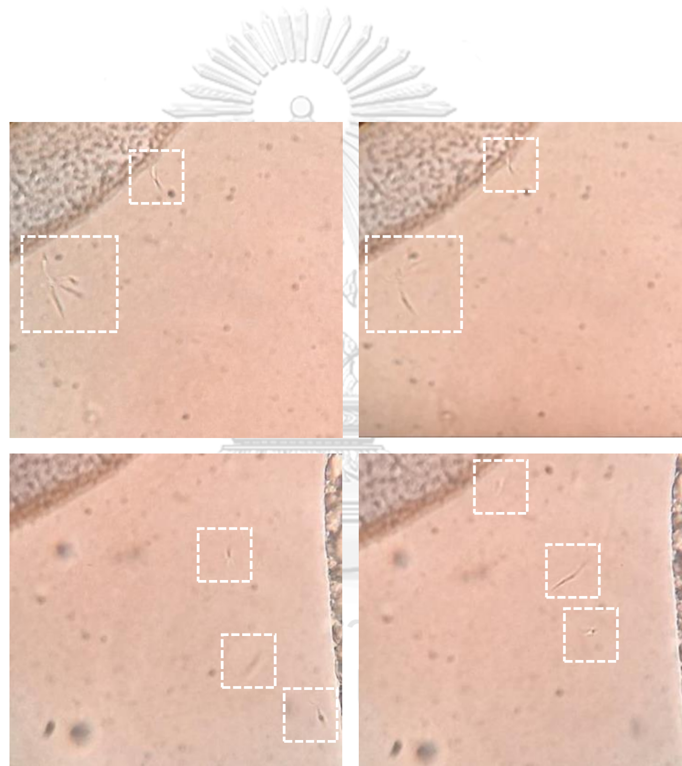

Figure 16: The living flagellated parasites observed in *Se.* (*Gra.*) *indica* sand fly (SK57)

4.2 Molecular Identification and Phylogenetic Analysis

Briefly, the extracted DNA from all 349 female sand flies collected from this study were used as a template for targeted locus amplification. To detect parasites in samples, two sets of specific primers were applied for amplifying the *ITS1* and *SSU rRNA* regions, and one set for classifying phlebotomine sand fly species based on the *Cytb* gene. Thereafter, the positive amplicons were sent to DNA sequencing. These

sequences were BLASTn-searched for the closest match species in the GenBank database (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi) and were included for the phylogenetic analysis.

4.2.1 Species Identification Results of Trypanosomatid Parasites Detected in Female Sand Flies Collected from Songkhla Province

We used *ITS1* and *SSU rRNA* gene-specific primers to detect the trypanosomatid parasites. In this investigation, *ITS1*-PCR positives were detected in one of *Ph. stantoni* and two *Se. khawi*. The *ITS1* amplicons were generated in two sizes, which consisted of 347 bp from *Se. khawi*, and 345 bp form *Ph. stantoni* (Figure 17). Based on BLASTn analysis, only *ITS1* sequence detected in *Ph. stantoni* sand fly was identical to *L. infantum* isolated from red fox (with 100% coverage, 100% identity: accession number MK675908). The BLAST result showed that sequence also matches the other *L. donovani* complex with 97-100% identity and full 100% coverage. Nevertheless, another two of *ITS1* amplified products were shared similarity on the initial (~118 bp) and final part of sequences (~54 bp) to genus *Trypanosoma* rather than *Leishmania* parasites, whereas the middle part showed no significant similarity to any related sequences in the GenBank database. Using phylogenetic analysis, we confirmed that our *ITS1* sequence isolated from *Ph. stantoni* was closely related to *L. donovani* complex species, including *L. infantum* (MK675907- MK675908) and *L. donovani* (FN677363, FR799614, CP022642), with strong Bayesian posterior probability support (BPPs) (Figure 18).

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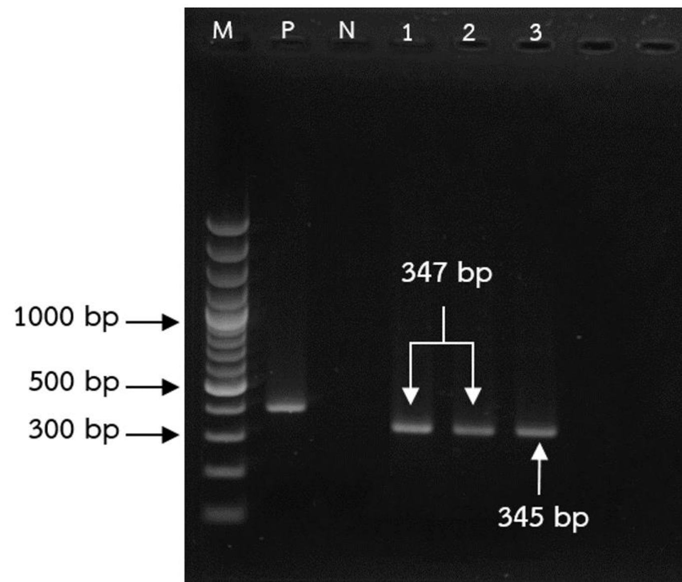

Figure 17: Representative images of *ITS1*-PCR amplicons detected in this study

Figure 17: *ITS1* bands were separated and imaged after electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. **(M)** DNA molecular weight marker. **(P)** Positive control. **(N)** Negative control. **(1-2)** *ITS1* amplicons (374 bp) detected in *Se. khawi* from isolated CODE SKF31 and SKF103, respectively. **(3)** *ITS1* amplicons (345 bp) detected in *Ph. stantoni* from isolated CODE SKF82.

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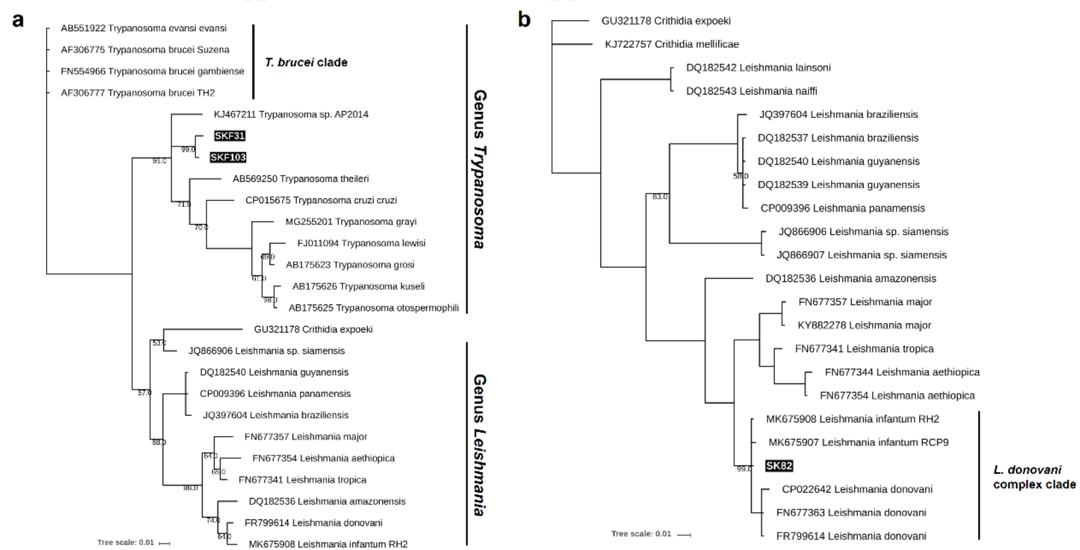

Figure 18: Phylogenetic tree of *ITS1* (*18S rRNA-ITS1-5.8S rRNA*) sequences

Figure 18. Phylogenetic tree inferred by Bayesian inference analysis of *ITS1* sequences using GTR+G model. **(a).** The molecular phylogenetic comparison of 23 sequences from trypanosomatids (*Trypanosoma, Leishmania, Crithidia*), including two isolates from *Se. khawi* sand flies (279 characters, including indels matrix). **(b).** the phylogenetic tree of 22 sequences from genus *Leishmania* and *Crithidia*, including one isolates from *Ph. stantoni* (431 characters + indels matrix). Numbers below branches indicate the percentage of Bayesian posterior probability support (BPPs) < 100. Black shading under the isolated code indicates the detection of trypanosomatid parasites.

Based on *SSU rRNA* gene sequences, we detected *Trypanosoma* DNA within four species of 24 female sand flies, *Ph. stantoni* (12.5%), *Se. (Gra.) indica* (20.8%), *Se. khawi*, (54.2%), and *Se. (Par.) barraudi* (12.5%). Furthermore, the trypanosome DNA was unexpectedly detected in one of non-dissected group, which *Cytb* sequence of this sample was closely related to a butterfly species on NCBI database as shown in the phylogenetic tree (Figure 22). The lengths of *SSU rRNA*-PCR sequences obtained in this study were 931 bp (8%), 948 bp (12%), 972 bp (40%) and 978 bp (40%) as illustrated in Figure 19. Phylogenetic analysis revealed two of the anuran trypanosomes clusters that found in separate amphibian clades (Figure 20). Twenty-three sequences were clustered into clade An04 or Frog 1 as described by Spodareva, V.V. et al (130), which including *T. mega* (AJ009157, AJ223567), *T. ranarum* (AF119810, MH424272, MH424307, MH424311), *T. fallisi* (AF119806), *T. rotatorium* (B2-I, U39583, B2-II, AJ009161), *T. therezieni* (AJ223571), *T. sp. ring* (MH424310, MH424313), and *Trypanosoma sp.* IKAZ/PK04/SKF32 from *Ph. kazeruni* sand fly (28) (AB520638) (Figure 20). In this clade, three samples of 931 bp fragments and *Trypanosoma sp*. IKAZ/PK04/SKF32, were grouped together with the maximum branch support score, while the main group from this investigation (972-978 bp) was

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separated into another cluster with moderate probability support (BPPs=61). Additionally, Two out of 25 *SSU rRNA* sequences (948 bp fragments) were sub-clustered from the An03 clade or Frog 3 (BPPs=96), distinctively, and placed into the An01+An02 clade (Frog 2) with high BPPs support (BPPs=98). This clade consisting of *Trypanosoma spp*. isolated from Amazon frogs in Brazil (131) (EU021225, EU021234, EU267075), and two species of *T. sp. nautilus* (MH424280, MH424292, MH424293) and *T. rotatorium* (MH424279, MH424287, MH424289) isolated from marsh frog in Ukraine (130).

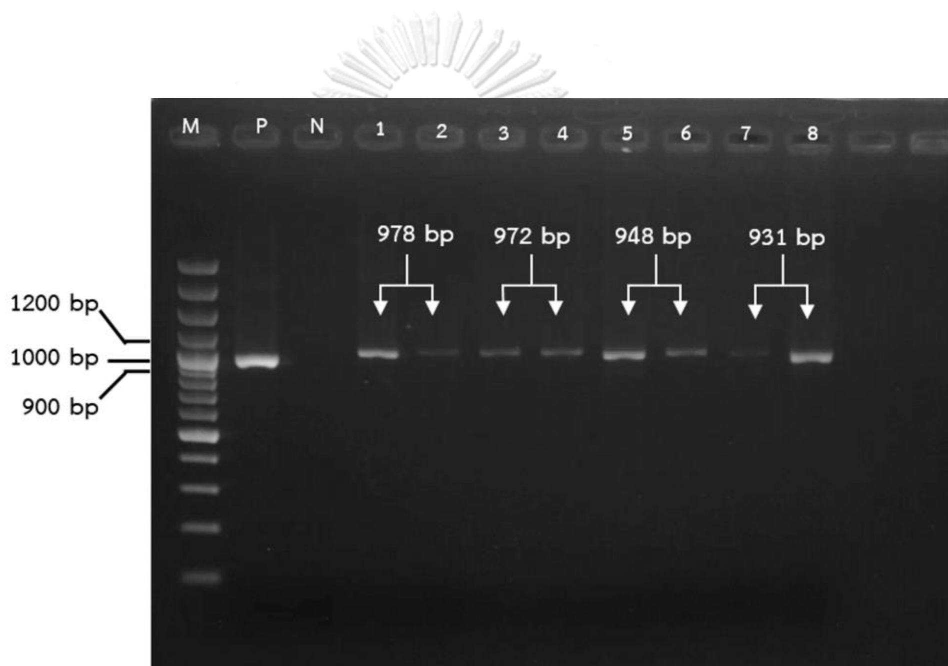

Figure 19: Representative images of SSU rRNA-PCR amplicons detected in this study

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Figure 19: *SSU rRNA* bands were separated and imaged after electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. **(M)** DNA molecular weight marker. **(P)** Positive control. **(N)** Negative control. **(1-2)** *SSU rRNA* amplicons (978 bp) detected in *Ph. stantoni* (SKF102) and *Se. (Gra.) indica* (SKF110), respectively. **(3-4)** *SSU rRNA* amplicons (972 bp) detected in *Se. khawi* (SK210 and SKF31). **(5-6)** *SSU rRNA* amplicons (948 bp) detected in *Se. (Gra.) indica* (SK153) and *Se. khawi* (SKF103). **(7-8)** *SSU rRNA* amplicons (931 bp) detected in *Se. khawi* (SK207) and unknown insect (SKF115).

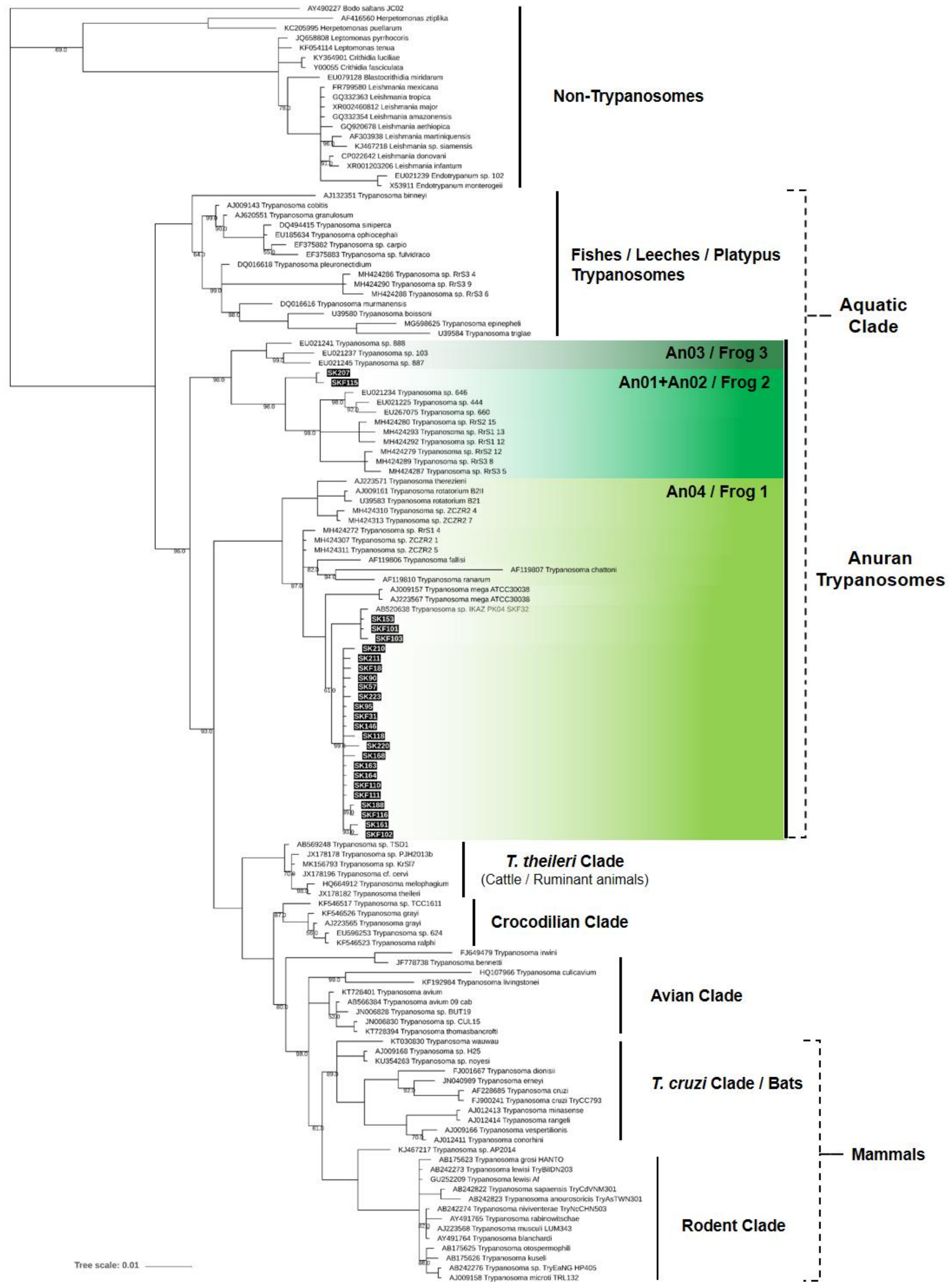

Figure 20: Phylogenetic tree of *SSU rRNA (18S rRNA)* sequences

Figure 20. Phylogenetic tree inferred by Bayesian inference analysis of *SSU rRNA* (18S rRNA) sequences using GTR+I+G model. The molecular phylogenetic comparison of 104 sequences from trypanosomatids, including 25 isolates from sand flies (1106

characters, including indels matrix). Numbers below branches indicate percentage of Bayesian posterior probability support (BPPs) < 100. Black shading under the isolated code indicate the detection of trypanosomatid parasites.

4.2.2 Species Identification Result of Female Sand Flies Collected from Songkhla Province

After detecting for parasites, twenty-six positives for trypanosomatid DNA (17 dissected flies, 8 non-dissected flies, 1 non-sand fly), were subjected to amplify *Cytb* sequences for species identification. The sizes of *Cytb* amplicons were 543 bp (*Ph. stantoni*), 536 bp (*Se. (Gra.) indica*), 534 bp (*Se. hivernus*), 545 bp (*Se. (Par.) barraudi*), 537 bp (*Se. khawi*), and 540 bp (non-sand fly) as shown in Figure 21. In addition, five species of non-infected flies that morphologically identified as *Ph. stantoni* (SK48, SK51), *Se. (Gra.) indica* (SK49 SK120, SK149), *Se. khawi* (SK36, SK208, SK211), *Se. (Par.) barraudi* (SK169, SK172, SK182, SK203), *Se. hivernus* (SK108), and including three specimens of the non-dissected group (SKF105, SKF112, SKF120), were included for phylogenetic analysis. The *Cytb* tree revealed that about half of positive specimens were clustered within the *Se. gemmea* clade (accession no. JX852706, MG770898, MG770920, MG770933), which previously reported by Siripattanapipong et al. (2018) (132). However, this species was recently re-identified as *Se. khawi* by Depaquit et al. (38) (Figure 22). Similarly to *Se. hivernus* classification, the only one species that detected in this study was placed into *Se. iyengari* cluster (accession no. MG770924, MG770926, MG770927), which latterly re-described as *Se. hivernus* (38). The posterior probability support of this clustering pattern was 100%. Three of the infected *Se. (Par.) barraudi* sequences had 93-99% identical to the available submitted sequences of *Se. (Par.) barraudi* (MG770911, MG770902) on the NCBI database, corresponding with our morphological result. Another clade of genus *Sergentomyia*, the sequences of *Se. (Gra.) indica* were

clearly clustered into one group separated from other species in this genus, with strongly support branch values (BPPs=100). Moreover, 19.2% of trypanosomatid DNA were detected in this species. For the genus *Phlebotomus*, *Cytb* gene sequences from infected flies were BLAST homology to available *Ph. stantoni* species with 98-100% identity, which concordance to the phylogenetic result (Figure 22).

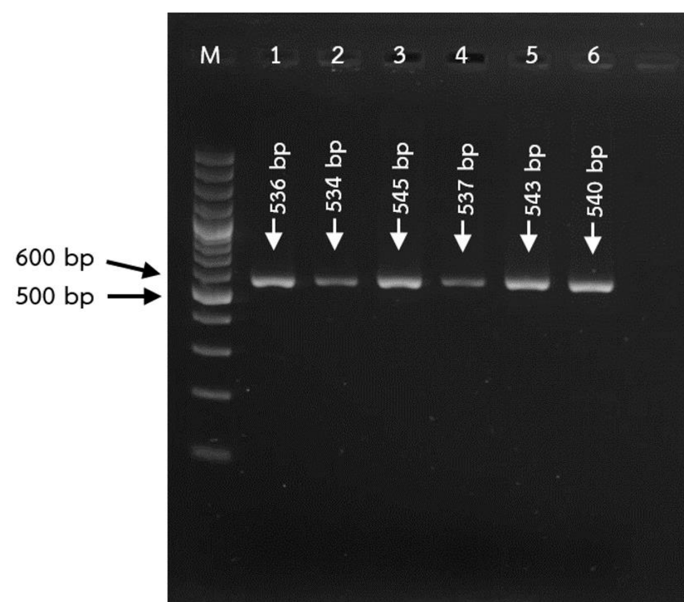

Figure 21: Representative images of *Cytb-PCR* amplicons detected in this study.

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Figure 21: *Cytb* bands were separated and imaged after electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. **(1)** *Cytb* amplicons (536 bp) of *Se. (Gra.) indica* (SK153). **(2)** *Cytb* amplicons (534 bp) of *Se. hivernus* (SK108). **(3)** *Cytb* amplicons (545 bp) of *Se. (Par.) barraudi* (SK188). **(4)** *Cytb* amplicons (537 bp) of *Se. (Par.) barraudi* (SK223). **(5)** *Cytb* amplicons (543 bp) of *Ph. stantoni* (SK82). **(6)** *Cytb* amplicons (540 bp) of a *non*-sand fly (SKF115).

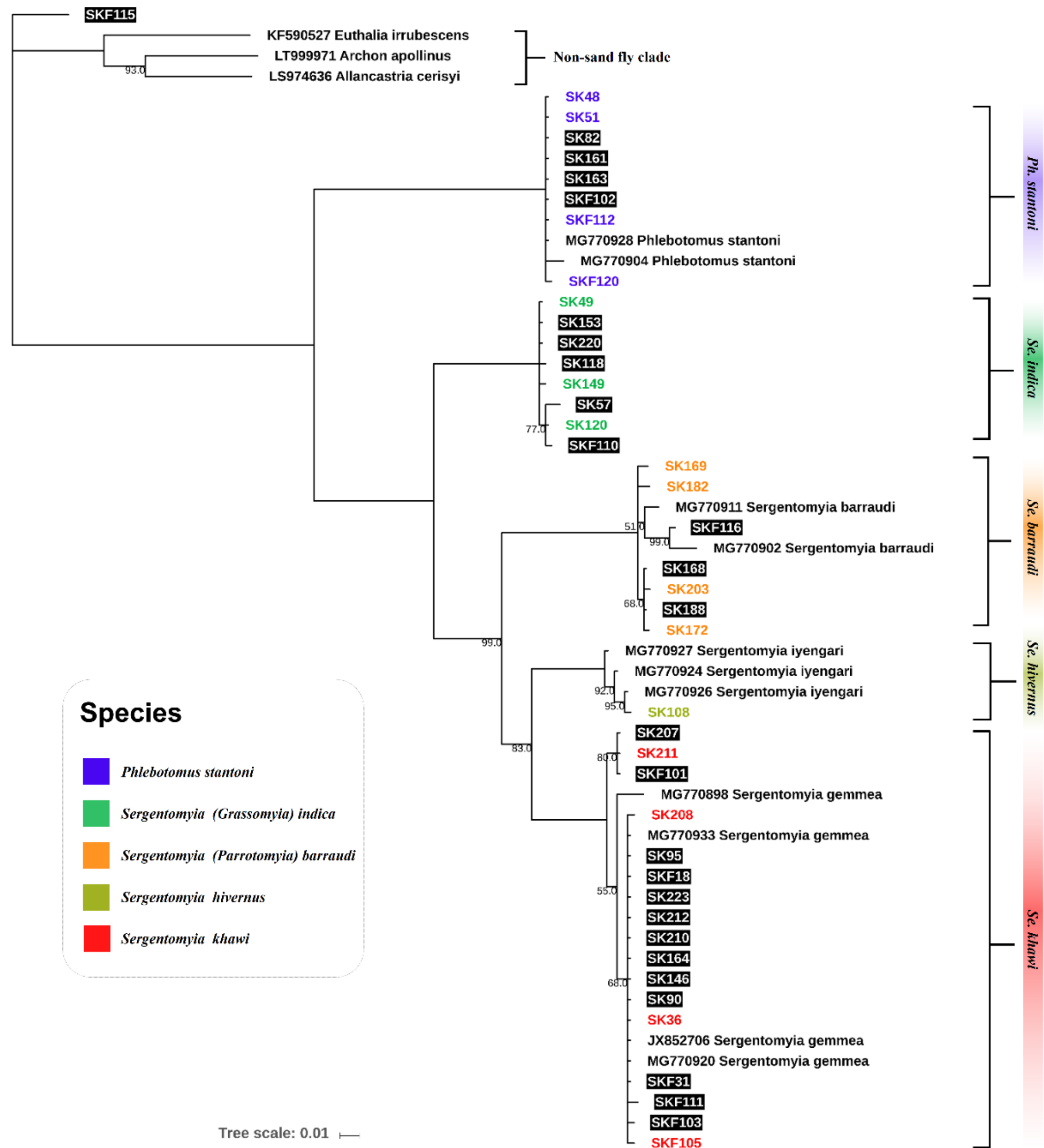

Figure 22: Phylogenetic tree of *Cytb (Cytb-ND1)* sequences

Figure 22: Phylogenetic analysis of phlebotomine sand fly species collected in this study based on the mitochondrial *Cytb* sequences (455 characters, including indels matrix), referring to the Bayesian inference (BI) method. The BI tree was constructed under Hasegawa-Kishino-Yano model with a gamma-distributed rate of variation among sites (HKY+G). Numbers below branches indicate percentage of Bayesian

posterior probability support (BPPs) < 100. Black shading under the isolated code indicate the detection of trypanosomatid parasites.

CHAPTER V

DISCUSSION AND CONCLUSION

A total of 349 female sand flies were captured in this survey studies. The morphological characterization was applied to identify species of 229 female sand flies (65.6%). The morphology result shows that five species were found in this area, Songkhla province, including *Se. khawi* (31.4%), *Se. (Parrotomyia) barraudi* (30.6%), *Ph. stantoni* (29.7%), *Se. (Grassomyia) indica* (7.9%), and *Se. hivernus* (0.4%). In present study, all female sand flies were tested for trypanosomatid parasites using *ITS1* and *SSU rRNA* gene locus. Among the morphologically assigned species of 229 sand flies, we molecularly detected *Trypanosoma* DNA in 8 *Se. khawi* (3.5%), 4 *Se. (Gra.) indica* (1.8%), 2 *Se. (Par.) barraudi* (0.9%), and 2 *Ph. stantoni* (0.9%), and also detected *Leishmania* DNA in one of *Ph. stantoni* (0.4%). For another group of 120 non-dissected sand flies, nine samples were positive for *Trypanosoma* DNA based on *SSU rRNA* gene. Of the nine sand flies samples tested, only two were positive for both *ITS1* and *SSU rRNA* gene. Furthermore, we applied the molecular-based taxonomy for species identification among twenty-six trypanosomatid-positive samples and sixteen non-positive samples using mitochondrial *Cytb* gene. For species classification of *Ph. stantoni* and *Se. (Par.) barraudi,* the result of the *Cytb* phylogeny of these sand flies agreed with the morphological identification of both species. The phylogenetic analysis revealed that *Ph. stantoni* captured in this study was closely related to the same species collected from Trang province, which recently reported by Siripattanapipong et al. (132). Similarly, the *Se. (Par.) barraudi* captured in this survey was clustered into the same clade of this species from previously mentioned area (132) with high scores of Bayesian posterior probability support (99%).

Nevertheless, two species that found in this investigation including *Se. khawi* and *Se. hivernus* were clustered into different species on the GenBank's nucleotide database. For *Se. khawi* species, our sequences were phylogenetically placed within *Se. gemmea* cluster (37, 132) with strong posterior probability support. However, the *Cytb* sequences of *Se. gemmea* sand fly (MG770898, MG770920, MG700920, and JX852706) deposited in NCBI database, was phylogenetically re-identified as *Se. khawi* (38). Depaquit et al. (38) have been described that *Se. khawi* is presented without ascoidal spurs. Unlike *Se. gemmea,* they elucidated that species has the spurs on ascoids (38). Moreover, only one sample of *Se. hivernus* was clustered into *Se. iyengari* branch (132) (MG770924, MG770926, and MG770927). Nonetheless, this species has also been re-identified as *Se. hivernus* by Depaquit et al. (38) based on both phylogenetic and morphological considerations. The cibarium teeth of *Se. hivernus* trapped in this study, are 12 in number with a single row of 3 denticles. The spermatheca structure is long, smooth tubular without limit between the spermathecal body and duct, and a small cluster of short hair-like at the terminal knob. The morphology was corresponding to previous studies by Sor-suwan et al. and Phumee et al. (31, 38, 133). They differentiated both *Se. hivernus* and *Se. iyengari* using the present of denticles which can be found in *Se. hivernus* species (31) based on Sinton's described appearance of two female *Se. iyengari* sand flies from India (134). Furthermore, although there are no sequences available of *Se. (Gra.) indica* on NCBI database for phylogenetic comparison, all the *Cytb* sequences of this species were grouped together with high probability support. According to the morphological characteristics, this species consistently related with *Se. (Gra.) indica* sand flies collected from Chiang Mai province as shown and described by Sor-suwan et al. (133). Among five species of sand flies reported in this survey, three out of five were found that species feed on human blood including *Ph. stantoni, Se. (Par.) barraudi,* and *Se. iyengari* (132), which the last mentioned species was re-described

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

as *Se. hivernus* (38). For this reason, it has been suggested that three species are capable of transmitting parasites during blood feeding.

Based on retrieved *SSU rRNA* sequences, our result shows that parasites belong to anuran trypanosomes within the aquatic clade as shown in Figure 20. The phylogenetic analysis reveals that 23 sequences identified as anuran *Trypanosoma spp.* in this study were clustered into clade An04 that was recently known as Frog 1 (130). This clade contains several species of frog trypanosomes including *T. mega*, *T. ranarum*, *T. fallisi*, *T. rotatorium*, *T. therezieni, T. sp. ring* (130); and *Trypanosoma sp.* IKAZ/PK04/SKF32 from *Ph. kazeruni* sand fly (28). Interestingly, we also found that our two samples of anuran *Trypanosoma sp.* were grouped into clade Frog 2 (An01+An02), implying these sequences belong to the novel *Trypanosoma* species in this amphibian clade. Contrastedly, the unknown *Trypanosoma* DNA sequence isolated from *Ph. stantoni* sand fly (Accession no. KJ467217) from the previous study by Phumee et al. (2016) was clustered closely to the rodent clade. Altogether, the result suggests that *Ph. stantoni* is likely to have capability to transmit a variety of *Trypanosoma* spp. among the amphibian and rodent hosts.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

In addition, our result shows that *Leishmania infantum* DNA can be detected in a *Ph. stantoni* sand fly sample using *ITS1*-PCR. The obtained *ITS1*-PCR sequence was highly identical to several species of *L. donovani* complex deposited in GenBank database. Furthermore, the phylogenetic analysis reveals that *ITS1* sequence in this study is closely related to the previously reported sequences of *L. donovani* complex species, including *L. infantum* (MK675907- MK675908) and *L. donovani* (FN677363, FR799614, CP022642), with strong Bayesian posterior probability support as depicted in Figure 18b. In Thailand, we found the first reported case of visceral leishmaniasis (VL) caused by *L. infantum* in a Thai-lumber truck driver (10). The patient was presumed to have this infection before returning from Middle East

countries (10). Since 2008, there had never been reported the detection of *L. infantum* in any leishmaniasis cases from Thailand or in sand flies.

Therefore, this present study demonstrates for the first time that *L. infantum* DNA can be detected in *Ph. stantoni* sand fly collected from Songkhla, Thailand. In previous studies, the DNA of *Leishmania* parasites has also been detected in sand flies collected from this endemic area. For instance, *L. martiniquensis* DNA was detected in both *Se. barraudi* (36) and *Se. gemmea* (37, 135) while *L. orientalis* was found in *Se. hivernus* (38, 132). However, detection of *L. infantum* in *Phlebotomus* sand flies has already been reported from several countries in North Africa (136, 137), Europe (64, 138) and Southwest Asia (98, 139). In endemic areas of North Africa, *Ph. perniciosus* was known as the principal vectors of *L. infantum* parasites across the Mediterranean basin countries (136-138). Also, other subgenera *Larroussius* including *Ph. ariasi, Ph. mascittii, Ph. kandelaki, Ph. neglectus, Ph. perfiliewi, Ph. major* and *Ph. tobbi* could be the vectors of *L. infantum* in such countries (64, 136-139). For Thailand, the information concerning the origin, evolution, and distribution of *L. infantum* in sand flies still needs to be fulfilled.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Intriguingly, there are two unknown *ITS1* parasite sequences that were detected in *Se. khawi*, showing significant alignments (query coverage of 27% and E-value of 7e-28) with *Trypanosoma* species in the GenBank database. As illustrated in Figure 17a, phylogenetic evidence suggested that these two parasite sequences were likely to be the genus *Trypanosoma* rather than the genus *Leishmania.* According to low query coverage, it not enough to conclude that two *ITS1* sequences detected in *Se. khawi* were an amphibian trypanosomes or other *Trypanosoma* species, which co-infected in these samples.

Additionally, we also demonstrated the living flagellated parasites under the microscope in one sample of *Se. (Gra.) indica* (CODE number SK57), which showed positive *SSU rRNA*-PCR result, as shown in Figure 16. This finding can ensure us that *Se. (Gra.) indica* is a potential vector of anuran trypanosome parasites. For other sand fly species, the detection of amphibian *Trypanosoma* sequences has also been reported in *Ph. kazeruni* sand fly from Pakistan, *Evandromyia infraspiosa* and *Sciopemyia servulolimai* collected from Brazil's Amazon forest (25, 28, 140). According to previous reports and our study showing, there are more than one genus of sand fly that be able to carry these anuran parasites. Thus, four species of sand flies collected from Songkhla including *Se. khawi*, *Se. (Par.) barraudi*, *Se. (Gra.) indica*, and *Ph. stantoni* could be potential vectors of anuran trypanosomes. Interestingly, the previous studies has been reported that *Ph. stantoni* sand fly is able to feed on human blood (106, 132). Moreover, the detection of human DNA has also been reported in genus *Sergentomyia* from southern Thailand including *Se. (Par.) barraudi* and *Se. khawi* sand flies (132). Accordingly, the result implied that three species found in this endemic area are capable of transmitting the parasites to humans.

However, even though there are never found an atypical human trypanosomiasis caused by anuran trypanosomes or a second case of leishmaniasis caused by *L. infantum* in Thailand, these blood-feeding sand flies are distributed throughout many regions of this country. Therefore, an extensive survey of the trypanosomatid protozoa in sand flies across the country is required to monitor the possible outbreak of emerging diseases caused by *L. infantum* and other trypanosomatid pathogens, and to develop the effective prevention and control strategies.

จุฬาลงกรณ์มหาวิทยาลัย
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Chulalongkorn University

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Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
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APPENDIX A

The accession number of *Cytb*, *SSU rRNA*, and *ITS1* sequences generated in this study.

Isolated code	Host origin		Trypanosomatids isolate	
	Species	Accession number	Accession number	
		Cytb	*SSU rRNA*	*ITS1*
SK36	*Sergentomyia khawi*	MK460569	-	-
SK48	*Phlebotomus stantoni*	MK431377	-	-
SK49	*Sergentomyia (Gra.) indica*	MK460561	-	-
SK51	*Phlebotomus stantoni*	MK431378	-	-
SK57	*Sergentomyia (Gra.) indica*	MK460562	MG844520	-
SK82	*Phlebotomus stantoni*	MK431379	-	MH989566
SK90	*Sergentomyia khawi*	MK460570	MH989542	-
SK95	*Sergentomyia khawi*	MK460571	MH989543	-
SK108	*Sergentomyia hivernus*	MK460586	-	-
SK118	*Sergentomyia (Gra.) indica*	MK460563	MH989544	-
SK120	*Sergentomyia (Gra.) indica*	MK460564	-	-
SK146	*Sergentomyia khawi*	MK460572	MH989545	-
SK149	*Sergentomyia (Gra.) indica*	MK460565	-	-
SK153	*Sergentomyia (Gra.) indica*	MK460566	MH989546	-
SK161	*Phlebotomus stantoni*	MK431380	MH989547	-
SK163	*Phlebotomus stantoni*	MK431381	MH989548	-
SK164	*Sergentomyia khawi*	MK460573	MH989549	-
SK168	*Sergentomyia (Par.) barraudi*	MK442494	MH989550	-
SK169	*Sergentomyia (Par.) barraudi*	MK442495	-	-
SK172	*Sergentomyia (Par.) barraudi*	MK442496	-	-
SK182	*Sergentomyia (Par.) barraudi*	MK442497	-	-
SK188	*Sergentomyia (Par.) barraudi*	MK442498	MH989551	-
SK203	*Sergentomyia (Par.) barraudi*	MK442499	-	-
SK207	*Sergentomyia khawi*	MK460574	MH989552	-
SK208	*Sergentomyia khawi*	MK460575	-	-
SK210	*Sergentomyia khawi*	MK460576	MH989553	-

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The accession number of *Cytb*, *SSU rRNA*, and *ITS1* sequences generated in this study. (Continued)

Isolated code	Host origin		Trypanosomatids isolate	
	Species	Accession number	Accession number	
		Cytb	*SSU rRNA*	*ITS1*
SK211	*Sergentomyia khawi*	MK460577	MH989554	-
SK212	*Sergentomyia khawi*	MK460578	-	-
SK220	*Sergentomyia (Gra.) indica*	MK460567	MH989555	-
SK223	*Sergentomyia khawi*	MK460579	MH989556	-
SKF18	*Sergentomyia khawi*	MK460580	MH989557	-
SKF31	*Sergentomyia khawi*	MK460581	MH989558	MH989567
SKF101	*Sergentomyia khawi*	MK460582	MH989559	-
SKF102	*Phlebotomus stantoni*	MK431382	MH989560	-
SKF103	*Sergentomyia khawi*	MK460583	MH989561	MH989568
SKF105	*Sergentomyia khawi*	MK460584	-	-
SKF110	*Sergentomyia (Gra.) indica*	MK460568	MH989562	-
SKF111	*Sergentomyia khawi*	MK460585	MH989563	-
SKF112	*Phlebotomus stantoni*	MK431383	-	-
SKF115	Unknown spp.	-	MH989564	-
SKF116	*Sergentomyia (Par.) barraudi*	MK442500	MH989565	-
SKF120	*Phlebotomus stantoni*	MK431384	-	-

จุฬาลงกรณ์มหาวิทยาลัย
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