รายงานวิจัยฉบับสมบูรณ์

โครงการ การประยุกต์ใช้ดีเอ็นเอบาร์โค้ด สำหรับจำแนกชนิดเหลือบ (Diptera: Tabanidae) ในประเทศไทย

โดย

ผู้ช่วยศาสตราจารย์ ดร.นายสัตวแพทย์ธนศักดิ์ ช่างบรรจง

ธันวาคม 2560

สัญญาเลขที่ TRG5880179

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

บทคัดย่อ

รหัสโครงการ: TRG5880179

ชื่อโครงการ: การประยุกต์ใช้ดีเอ็นเอบาร์โค้ด สำหรับจำแนกชนิดเหลือบ (Diptera: Tabanidae) ในประเทศไทย

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เหลือบ (Diptera: Tabanidae) เป็นกลุ่มของแมลงที่มีความสำคัญทางด้านการแพทย์และการสัตวแพทย์ ประเทศไทยมีรายงานชนิดของเหลือบประมาณ 80 ชนิด การสำรวจถึงชนิดและการกระจายตัวของเหลือบมีความสำคัญในการป้องกันและควบคุมแมลง รวมทั้งโรคที่สามารถถ่ายทอดโดยแมลงดังกล่าว ปัจจุบันการจำแนกชนิดเหลือบใช้ลักษณะทางสัณฐานวิทยา ซึ่งวิธีนี้ต้องอาศัยผู้ที่มีทักษะและความชำนาญทางด้านลักษณะทางสัณฐานวิทยา นอกจากนี้เหลือบบางชนิดที่มีลักษณะทางสัณฐานวิทยาคล้ายกันอาจยากต่อการจำแนกชนิด วิธีทางอณูชีววิทยาได้ถูกนำมาใช้ประโยชน์อย่างกว้างขวาง สำหรับการระบุชนิดได้อย่างรวดเร็วและแม่นยำในแมลงชนิดต่างๆ การศึกษานี้เราใช้ดีเอ็นเอบาร์โค้ดของยีน mitochondrial cytochrome oxidase I (COI) เพื่อประเมินประสิทธิภาพในการจำแนกชนิดเหลือบในประเทศไทย โดยทำการวิเคราะห์ลำดับนิวคลีโอไทด์ที่ความยาว 658 คู่เบสของ COI บาร์โค้ดจากจำนวนตัวอย่างทั้งหมด 145 ตัวอย่างประกอบด้วย 48 ชนิด มีค่าความแตกต่างของนิวคลีโอไทด์ภายในชนิดเดียวกันอยู่ในช่วงร้อยละ 0 ถึงร้อยละ 4.4 ขณะที่ค่าความแตกต่างของนิวคลีโอไทด์ระหว่างชนิดอยู่ในช่วงร้อยละ 0 ถึงร้อยละ 16.2 จากผลการศึกษาชี้ให้เห็นว่า COI บาร์โค้ดมีประสิทธิภาพในการจำแนกชนิดของเหลือบส่วนใหญ่ในประเทศไทยบนหลักการของ barcoding gap และการวิเคราะห์ความสัมพันธ์ทางวงศ์วานวิวัฒนาการ นอกจากนี้ยังพบว่า COI บาร์โค้ดไม่มีความสามารถในการจำแนกชนิดความแตกต่างของเหลือบในกลุ่ม *T. striatus* complex และในบางชนิดของกลุ่ม *T. ceylonicus*

คำหลัก: Cytochrome oxidase I, ดีเอ็นเอบาร์โค้ด, เหลือบ, Tabanidae, พาหะ

Abstract

Project Code: TRG5880179

Project Title: Applying DNA barcodes for identification of horse flies, *Tabanus* spp. (Diptera: Tabanidae) in Thailand

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Project Period: 2 years (1 July 2015-30 June 2017)

Horse flies (Diptera: Tabanidae) include a group of insects that are of medical and veterinary importance. Approximately 80 species of horse flies have been reported in Thailand. Surveillance of the presence of horse fly species and their distribution are important for prevention and control of these flies and also diseases that horse flies can transmit. Currently, the species identification of horse flies is based on morphological method. This method requires considerable skills and taxonomic expertise. Additionally the morphologically closed species may cause difficulties during the identification process. DNA-based identification methods have become increasingly useful for rapid and accurate identification of various insect species. The present study, we used mitochondrial cytochrome oxidase I (COI) barcodes to assess the efficiency for the identification of horse flies in Thailand. We sequenced 658 bp of COI barcodes from 145 adult specimens belonging to 48 morphologically identified species. The levels of intraspecific divergence ranged from 0.0% to 4.4%, whereas interspecific divergence among species ranged from 0.0% to 16.2%. Our results indicated that the COI barcodes have the effectiveness in discriminating the majority of horse flies in Thailand based on barcoding gap and phylogenetic analyses. Moreover, we revealed that COI barcodes were not able to distinguish members of the *T. striatus* complex and some species of the *T. ceylonicus* group.

Keywords : Cytochrome oxidase I, DNA barcode, Horse flies, Tabanidae, Vector

Introduction

Tabanus spp. or horse flies (Diptera: Tabanidae) belonging to the infraorder Tabanomorpha, represents over 1,300 described species worldwide, of which approximately 80 species are found in Thailand. Females are haematophagous flies of considerable medical and veterinary importance. They are involved in the mechanical transmission of several pathogens such as *Trypanosoma evansi* causing severe disease (surra) in horses, camels and dogs and less severe illness in several other mammals, *T. equinum* the causative the causative agent of mal de Caderas in equines, cattle, sheep and goats, *T. vivax* the causative agent of nagana in cattle, sheep, goats and equines. Moreover, they also mechanically transmit other infectious diseases, including anthrax, tularemia, equine infectious anemia and anaplasmosis.

The specific identification of adult *Tabanus* is based largely on head structures (particularly the shape and proportions of the frons, antennae and maxillary palps) and the color and patterns of the body and wings. Most body patterns are produced by a subtle combination of hairs overlying minute surface structures (usually referred to as pollinosity or tomentum). They are often somewhat variable, however, even in the same species and this combined with the general lack of structural characters, is the commonest cause of difficulty with specific identification. So, this method requires great skill and considerable time. Moreover, some stage of life, incomplete or fragile specimens and the morphologically-closed species are difficult to identify. Accurate species identification is a gold standard for any taxonomic system. Correct identification not only allows critical access to the broad body of literature available on a particular taxon but also permits the implementation of adequate control measures to contend with species of medical or veterinary importance. A DNA-based species identification system, DNA barcoding "658-bp portion of the mitochondrial gene cytochrome oxidase I (COI)" offers a promising supplemental tool for identification of many species of insects, where morphology-based identifications are problematic, and for enabling those investigators lacking the morphology-based skills to identify taxa reliably. For these reasons, the aim of this study was to test the efficacy of COI barcodes for identifying horse fly species in Thailand.

Literature review

The Tabanidae are a cosmopolitan family belonging to the suborder Brachycera, infraorder Tabanomorpha and include approximately 4,300 species within 133 genera (Mullen, 2009). Members of this family are commonly known as horse flies (*Tabanus*), deer flies (*Chrysops*) and clegs (*Haematopota*). They are recognized vectors or potential vectors of many diseases that affect humans and animals (Lehane, 2005; Mullens, 2009).

The genus *Tabanus* (horse flies) comprises more than 1,300 described species worldwide (Abu El-Hassan et al., 2013). The specific identification of adult *Tabanus* is based largely on head structures (particularly the shape and proportions of the frons, antennae and maxillary palps) and the color and patterns of the body and wings. Most body patterns are produced by a subtle combination of hairs overlying minute surface structures (usually referred to as pollinosity or tomentum) (Chainey, 1993). In Thailand, the family Tabanidae was studied early by Stone (1975), who recorded 46 species of genus *Tabanus*. After 1975 even more species were newly recorded from Thailand. Burton (1978) summarized and keyed 79 species of *Tabanus* in the Thailand fauna. Subsequently, Tumrasvin (1989) listed 20 species of *Tabanus* and their distribution in different regions of the country. Females of *Tabanus* are haematophagous flies, as in other biting flies. They require blood as a protein source to complete egg development. Most of them are active diurnally. Biting activity varies between species, peaking once or twice during the day depending on environmental conditions (particularly humidity, light levels and temperature) (Chainey, 1993). The hosts are located by sight (color and movement), odor, carbon dioxide emission and body heat. They are attracted to large objects such as cars and traps can be used to exploit this tendency.

Horse flies are significant pests of livestock, particularly cattle, horses and many wildlife species (Mullens, 2009; Desquesnes et al., 2013). Their painful bite results in the flies often being disturbed while feeding, but they are persistent and will commonly move directly to another animal to recommence feeding. Heavy attack of flies can cause direct reductions in weight gains of beef cattle, reduced feed utilization efficiencies, and hide damage from the feeding punctures. Cattle protected from flies attack in screened enclosures have been shown to gain up to 0.1 kg/day. Such direct

losses can be increased by a concomitant reduction in feed utilization efficiencies of up to 17 %. A daily loss of 200 ml of blood per animal may be common during periods of intense tabanid attack (Mullens, 2009). Horse flies serve as vectors of a number of disease agents of animals. They are involved in the mechanical transmission of several pathogens such as *Trypanosoma evansi* causing severe disease in horses, camels and dogs and less severe illness in several other mammals, *T. equinum* the causative the causative agent of mal de Caderas in equines, cattle, sheep and goats in South America, *T. vivax* the causative agent of nagana in cattle, sheep, goats and equines in Africa. Moreover, they also mechanically transmit other infectious diseases, including anthrax, tularemia, equine infectious anemia and anaplasmosis (Lehane, 2005; Desquesnes et al., 2013).

Trypanosoma evansi is an important haemoprotozoan disease of domesticated animals, livestock and wild animals. It is commonly termed as Surra in all animal species. The infected animals may be asymptomatic or exhibit clinical sign such as stiffness, fever, lameness, chronic emaciation, limb edema, reduced milk yield, nervous signs, abortion and death (Desquesnes et al., 2013). The disease can cause immune suppression which resulted in inadequate immunity after vaccination and sensitive to other bacterial or viral infections. In Thailand, *T. evansi* infection was first reported in 1916 through the imported mules from Algeria, but the disease was first reported in the northeastern region in buffaloes in 1981 (Indrakamhang, 1998). The previous study from 2008 to 2012 in the northeastern region revealed that there were 66 confirmed cases. The prevalence of disease in beef cattle, buffaloes, dairy cattle and pig was 51.5%, 39.4%, 6.1% and 3.0%, respectively. (Pholpark and Pholpark, 2013). A study on seroprevalence carried out in dairy cattle demonstrated the presence of the parasite in most parts of the country. The mean seroprevalence was 8%, ranging from 0 to 100% at farm level and 25% of dairy cattle are exposed to the infection (Desquesnes, 2009).

DNA based methods aiming to modernize taxonomy were proposed by Hebert et al. (2003). They encouraged the study of molecular diversity as a means to recognize and identify organisms by bringing up the inherent limitations of morphology and the steady decrease in the number of specialists available for the task of uncovering our yet unknown diversity. Four significant limitations of morphology-based taxonomy were mentioned by them. First, phenotypic plasticity to environmental factor of given diagnostic characters employed for species recognition

lead to incorrect identifications. Second, morphologically cryptic species are often overlooked. Third, there is a lack of taxonomic keys to identify immature specimens of many species. Finally, traditional taxonomy requires high levels of expertise in any given group and is therefore restricted to specialists. According to those authors, because DNA sequences are unique for each species, they can be viewed as genetic "barcodes" and have the potential to solve the problems inherent to the kind of taxonomy practiced so far (Pires and Marinoni, 2010).

DNA barcoding is a taxonomic system structured on sequence information from a short gene sequence, a barcode, from a standardized region of the genome as a universal and unique identification marker for animal species (Hebert et al., 2003). An approximately 658 bp from the 5'end of the mitochondrial gene cytochrome c oxidase I (COI) was initially proposed as the barcode source to identify and define all animal species. The core idea of DNA barcoding is the existence of "barcoding gap" that means that the variation of the nucleotide sequences within species is much less than the differences among species. DNA barcoding aims to provide a rapid and reliable tool for species-level identification. The methodology involves the sequencing of that portion of DNA, followed by a comparison with other sequences previously deposited in a database. Species are identified by matching the obtained sequence with sequences of known identity already in the database (Hebert et al., 2003). The two essential components for an effective DNA barcode system are the standardization on an uniform barcode sequence, such as cox1 gene, and a database of sequences linked to named voucher specimens (Hebert et al., 2004a). DNA Barcode of Life project aims to develop a standardized, rapid and inexpensive species identification method accessible to non-specialists (i.e. non-taxonomists). The two main ambitions of DNA barcoding are to (i) assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible morphology (Frezal and Leblois, 2008). Several projects have demonstrated the effectiveness of this approach, based on cox1 gene, in many groups of animals, such as birds (Hebert et al., 2004a), fish (Ward et al., 2005), gastropods (Remigio and Hebert, 2003), crustacea (Costa et al., 2007) and ants (Smith et al., 2005) etc. Currently, DNA barcoding has been applied in pest monitoring and quarantine (Armstrong and Ball, 2005; Floyd et al., 2010), and its usefulness has been confirmed in many arthropods of medical and veterinary importance such as mosquitoes (Cywinska et al., 2006; Kumar et al., 2007;

Ashfag et al., 2014), blow flies (Nelson et al., 2007), flesh flies (Meiklejohn et al., 2011; Meiklejohn et al., 2013), sand flies (Kumar et al., 2012; Contreras Gutierres et al., 2014), black flies (Rivera and Currie, 2009; Pramual and Kuvangkadilok, 2012; Pramual and Adler, 2014), tabanid flies (Cywinska et al., 2010; Banerjee et al., 2015) and stomoxyini flies (Changbunjong et al., 2016).

Objective

1. To determine geographic distribution of horse flies in Thailand by using morphological-based identification
2. To apply DNA barcoding technique for identification of horse flies
3. To determine phylogentic and genetic relationship of horse flies in Thailand

Materials and Methods

Collection sites

Horse flies were collected at 20 localities from the different geographical regions of Thailand (Fig. 1). The collection sites were classified into 3 habitat types: primary forest, secondary forest and village (Table 1) following these definitions: a primary forest is a forest that has never been logged and has developed following natural disturbances and under natural processes; a secondary forest is a forest that has been logged and has recovered naturally or artificially (CBD, 2007) and a village is a clustered human settlement or community.

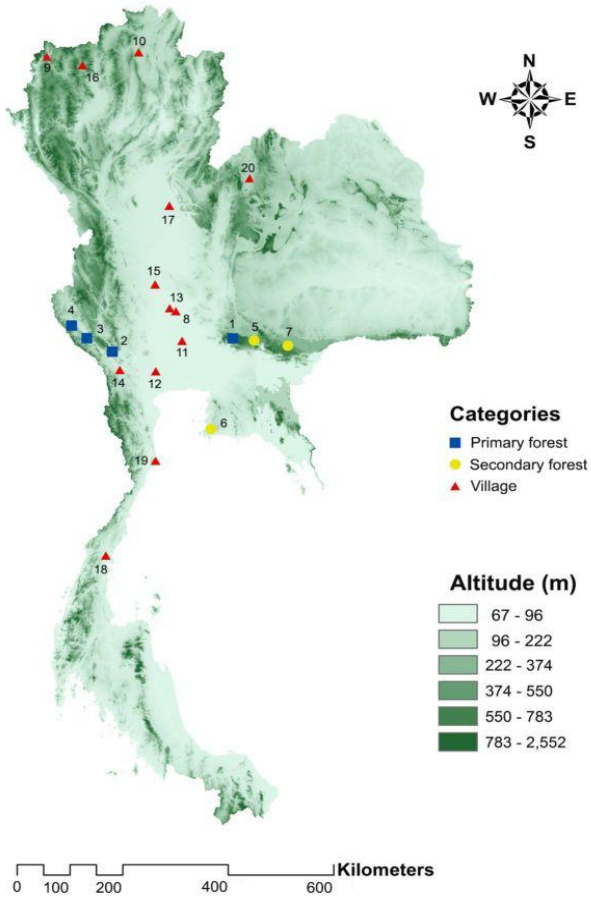

Fig. 1. Map of horse fly collection sites in Thailand: Nakhon Ratchasima (1, 5, 7), Kanchanaburi (2, 3, 4, 14), Chon Buri (6), Singburi (8), Mae Hong Son (9), Chiang Rai (10), Phra Nakhon Si Ayutthaya (11), Nakhon Prathom (12), Chainat (13), Uthai Thani (15), Chiang Mai (16), Phitsanulok (17), Chumphon (18), Prachuap Khiri Khan (19), Loei (20).

Table 1 Horse fly collection sites and dates in Thailand.

Habitat	No	Date	Characteristic of collection sites	District/Province	Altitude	Coordinates (Lat/Long)
Primary forest	1	Apr 2012	Wildlife species in Khao Yai National Park	Pak Chong, Nakhon Ratchasima	774	N14°24'55.1", E101°22'33.4"
	2	Feb 2015	Buffalo farm in Salakpra Wildlife Sanctuary	Mueang, Kanchanaburi	54	N14°11'06.8", E099°15'23.4"
	3	Apr 2015	Beef cattle farm in Sai Yok National Park	Sai Yok, Kanchanaburi	102	N14°25'52.8", E098°48'32.3"
	4	Jun 2016	Beef cattle farm in natural forest	Thong Pha Phum, Kanchanaburi	174	N14°39'28.0", E098°32'19.7"
Secondary forest	5	Feb 2015	Buffalo farm around Khao Phaeng Ma No Hunting Wildlife Area	Wang Nam Khiao, Nakhon Ratchasima	498	N14°22'23.4", E101°44'51.9"
	6	May 2015	Wildlife species in Khao Chi On No Hunting Wildlife Area	Sattahip, Chon Buri	93	N12°46'34.1", E100°58'30.8"
	7	Feb 2016	Beef cattle and buffalo farm around Thap Lan National Park	Soeng Sang, Nakhon Ratchasima	263	N14°16'54.3", E102°28'16.7"
Village	8	Aug 2013	Beef cattle farm	Mueang, Singburi	18	N14°54'59.5", E100°22'38.5"
	9	Jul 2014	Beef cattle farm	Mueang, Mae Hong Son	452	N19°31'45.5", E098°04'48.6"
	10	Jul 2014	Beef cattle farm	Mueang, Chiang Rai	415	N19°36'39.9", E099°44'18.1"
	11	Jul 2015	Buffalo farm	Bang Ban, Phra Nakhon Si Ayutthaya	8	N14°22'43.9", E100°28'57.1"
	12	Jul 2015	Beef cattle farm	Mueang, Nakhon Prathom	7	N13°49'50.0", E100°01'04.9"
	13	Aug 2015	Beef cattle and pig farm	Sankhaburi, Chainat	19	N14°58'12.4", E100°16'01.9"
	14	Aug 2015	Beef cattle and buffalo farm	Dan Makham Tia, Kanchanaburi	52	N13°51'18.5", E099°23'09.7"
	15	Nov 2015	Buffalo farm	Mueang, Uthai Thani	38	N15°24'13.7", E100°00'49.6"
	16	Jan 2016	Beef cattle and buffalo farm	Chiang Dao, Chiang Mai	624	N19°22'42.2", E098°43'25.7"
	17	Jan 2016	Beef cattle, buffalo and horse farm	Mueang, Phitsanulok	50	N16°49'41.8", E100°16'28.2"
	18	Apr 2016	Beef cattle and buffalo farm	Mueng, Chumphon	14	N10°29'33.5", E099°08'28.1"
	19	Apr 2016	Beef cattle farm	Sam Roi Yot, Prachuap Khiri Khan	2	N12°12'28.1", E100°00'25.2"
	20	Dec 2016	Buffalo farm	Wang Saphung, Loei	277	N17°18'36.8", E101°42'35.6"

Table 2 Climatic data at the collection sites.

No	District/Province	Temperature (°C)		Humidity (%)
		Max	Min	
1	Pak Chong, Nakhon Ratchasima	32	23	81
2	Mueang, Kanchanaburi	33	22	68
3	Sai Yok, Kanchanaburi	30	26	74
4	Thong Pha Phum, Kanchanaburi	34	25	76
5	Wang Nam Khiao, Nakhon Ratchasima	35	22	69
6	Sattahip, Chon Buri	35	27	71
7	Soeng Sang, Nakhon Ratchasima	34	21	70
8	Mueang, Singburi	33	25	70
9	Mueang, Mae Hong Son	32	24	75
10	Mueang, Chiang Rai	31	23	76
11	Bang Ban, Phra Nakhon Si Ayutthaya	33	24	70
12	Mueang, Nakhon Prathom	32	24	74
13	Sankhaburi, Chainat	33	25	71
14	Dan Makham Tia, Kanchanaburi	32	24	68
15	Mueang, Uthai Thani	32	22	71
16	Chiang Dao, Chiang Mai	30	14	67
17	Mueang, Phitsanulok	33	20	74
18	Mueng, Chumphon	34	24	70
19	Sam Roi Yot, Prachuap Khiri Khan	35	27	76
20	Wang Saphung, Loei	29	15	73

Horse fly collection

Adult horse flies were collected at various times using 10 Nzi traps between April 2012 and December 2016. This trap is highly specific for tabanid flies (Mihok, 2002). The traps were made locally, using blue and black fabric named Solon® (Thailand Supplier) being 100% polyester. Each trap was randomly placed at the collection sites from 6.00 a.m. to 6.00 p.m. over a 2 day period. The temperature and relative humidity at collection sites were recorded (Table 2). The captive flies were collected at 2 or 3 hour intervals to prevent specimen damage for morphological identification. All flies were euthanized in the freezer (-10 ˚C) and transported to the

Vector-Borne Diseases Research Unit (VBRU), Faculty of Veterinary Science, Mahidol University for species identification.

Morphology identification

The specimens were identified to species level by descriptions and keys (Burton, 1978) under a stereomicroscope. They were separated by species, sex, date and collection site.

DNA extraction

The legs of each specimen were used in the experiment. Genomic DNA was extracted from individual fly specimens using reagents in the DNeasy® Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions and adding some additional step. The DNA extraction protocol was described as follow:

1. Place 2-3 legs of deer flies in a 1.5 ml microcentrifuge tube contained 180 µl of Buffer ATL.

2. Add 20 µl Proteinase K mixed by vertexing and incubate at 56 °C by place in a water bath overnight.

3. After briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid, add 200 µl Buffer AL to the sample, mixed by vortexing for 15 sec, incubate at 56 °C C for 10, briefly centrifuge again.

4. Add 200 µl absolute ethanol to the sample, mixed by vortexing for 15 sec. This step was done for DNA precipitation.

5. Carefully apply the mixture from previous step to the DNeasy Mini spin column (without wetting the rim) placed in a 2 ml collection tube, and centrifuge at 8,000 rpm for 1 min. Discard the flow-through and collection tube.

6. Place the Spin Column in a clean 2 ml collection tube, add 500 µl Buffer AW1 without wetting the rim, centrifuge at 8,000 rpm for 1 min. Discard the flow-through and collection tube.

7. Add 500 µl Buffer AW2 without wetting the rim, centrifuge at 14,000 rpm for 3 min. Discard the flow-through and collection tube.

8. Place the spin column in clean 1.5 ml microcentrifuge tube.

9. Elute the DNA by adding 200 µl Buffer AE, incubate at room temperature for 1 min and then centrifuge at 8,000 rpm for 1 min.

10. Repeat step 7.4.9 for increased DNA yield. Then the DNA extraction product was keep at -20 °C for perform the next laboratory experiment.

COI amplification and sequencing

COI amplification was performed by using Polymerase Chain Reaction technique (PCR).

The primer pair LepF1 (5′-ATTCAACCAATCATAAAGATATTGG-3′) and Lep R1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) was subsequently used to amplify a 658 bp fragment of COI gene (Hebert et al., 2004b).

Each PCR master mix, following Canadian Centre for DNA Barcoding (CCDB) protocol, contained 5 µl of 10x PCR Buffer, 2.5 µl of 50 mM of MgCl2, 36.01 µl of distilled water, 0.25 µl of 10 mM of dNTPs, 0.24 µl of 1.2 unit of Taq polymerase (InvitrogenTM, USA), 0.5 µl of 10 mM of each primer and 5 µl of DNA template. To check the effectiveness of the PCR reagent, positive and negative control, were included in the test.

The PCR reaction was consisted of initial denaturation at 94 °C for 1 min; 5 cycles of 94 °C for 30 sec, annealing at 45 °C for 40 sec and extension at 72 °C for 1 min; followed by 30-35 cycles 94 °C for 30 sec, 55 °C for 40 sec and 72 °C for 1 min with a final extension at 72 °C for 10 min, followed by indefinite hold at 4 °C. Amplified PCR products were separated in 1.5% agarose gel electrophoresis and GeneRulerTM 100 bp DNA ladder as size marker to visualize the amount and size of DNA fragments present in the sample.

DNA sequencing of PCR products was performed by BIONEER (Korea). DNA sequences will be analyzed using ABI 3730 XL sequencer, fluorescent dyeterminator sequencing.

DNA analysis

The results of COI sequences were evaluated for data analysis by using several bioinformatics programs. MEGA 6.0 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2013) will be used for editing DNA sequences. Multiple alignments of all sequences will be aligned using CLUSTAL W. The COI sequence results will be compared with the available sequences in GenBank database by using BLAST (Basic Local Alignment Search Tool). All sequences from this study will be submitted to GenBank database.

Nucleotide sequence divergences within and between species were calculated using the Kimura-two-parameter (K2P) distance model, available within MEGA 6.0 software. The Neighbor-joining (NJ) and Maximum likelihood (ML) phylogenetic tree were performed with bootstrapping (1,000 replicates) in MEGA 6.0. Mr. Bayes 3.2.2 (Ronquist et al., 2012) was implemented for Bayesian analysis with best fit model and the Markov Chain Monte Carlo (MCMC) method. Bayesian analysis was run for 4,000,000 generations, with a sampling frequency of 100 generations. The tree generated through this process was visualized using FigTree v1.4.3. Haplotyping was carried out in DnaSP v5 (Librado et al., 2009) to calculate the number of haplotypes and polymorphic sites.

Results

Species distribution and abundance of horse flies

A total of 1,835 horse flies representing 45 species were collected (Table 3). Only female horse flies were collected in this study. The five most abundant species were *Tabanus striatus* (25.45%), followed by *T. megalops* (21.36%), *T. rubidus* (14.82%), *T. tamthaiorum* (7.90%) and *T. oxybeles* (6.38%). The less abundant species were *T. borealoriens*, *T. caduceus*, *T. discor*s, *T. equicinctus*, *T. lentis*, *T. salvazai* and *T. virgulatus*, with each species having a relative abundance of 0.05%. The highest proportion of horse flies was collected in villages (39.13%), followed by primary forests (34%) and secondary forests (26.87%) (Table 4).

In the primary forest, 37 species were collected; *T. tamthaiorum* was the most abundant species with 23.2%, followed by *T. oxybeles* (18.8%) and *T. rubicundus* (10.4%). Twenty-seven species collected in the primary forest were absent in the secondary forest and the village. In the secondary forest, 10 species were collected: *T. striatus* was the most abundant species with 23.2%, followed by *T. minimus* (1.83%) and *T. megalops* (1.62%). *T. fontinalis* was presented only in this habitat. In the village, 13 species were collected; *T. megalops* was the most abundant species with 52.23%, followed by *T. rubidus* (36.07%) and *T. minimus* (5.71%). *T. megalops*, *T. rubidus* and *T. striatus* were presented in the three habitats. *T. nigrotectus*, *T. systenus*, *T. thermarum* and *T. virgulatus* were abundant in the village but were absent from both other habitats (Table 4).

Molecular identification of horse flies using DNA barcodes

A total of 145 specimens of horse flies, belonging to 48 morphologically classified species (45 species of genus *Tabanus* and 3 species of genus *Atylotus*) were subjected to barcode analysis (Table 5). For molecular analyses, *Atylotus* species were also included in this study.

Table 3 Total number of horse flies collected at 20 collection sites in Thailand**.**

Species	Collection sites																				Total	Percent
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
T. admelanopygus	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. agnoscibilis	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	57	3.11
T. anabates	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0.27
T. aurilineatus	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. ballmeri	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. birmanicus	1	0	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	1.47
T. borealoriens	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. caduceus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. cepuricus	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0.49
T. ceylonicus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	5	0.27
T. crassus	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. discors	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. diversifrons	0	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	44	2.40
T. dorsilinea	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. equicinctus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. firmus	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. fontinalis	0	0	0	0	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0.27
T. griseilineis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. hypomacros	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. jucundus	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. kakhyenensis	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. konis	0	0	3	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	11	0.60

Species	Collection sites																				Total	Percent
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
T. lentis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. megalops	0	0	0	9	2	5	1	0	0	0	41	12	36	32	13	0	117	79	45	0	392	21.36
T. mesogaeus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	3	0.16
T. minimus	0	0	0	0	0	9	0	0	0	0	3	0	0	0	0	0	0	38	0	0	50	2.72
T. nigrotectus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.11
T. oknos	0	0	4	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	37	2.02
T. oxybeles	0	0	42	75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	117	6.38
T. pugiunculus	0	30	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	34	1.85
T. rhinargus	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. rubicundus	4	0	61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65	3.54
T. rubidus	0	0	0	6	5	0	2	50	0	20	9	0	20	20	59	20	0	60	0	1	272	14.82
T. rufiscutellatus	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.11
T. salvazai	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. striatus	1	0	0	0	5	0	452	0	0	0	0	0	0	4	0	5	0	0	0	0	467	25.45
T. symmetrus	0	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.44
T. systenus	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	3	5	0.27
T. tamthaiorum	1	0	6	138	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	145	7.90
T. thermarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	0.11
T. thurmani	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0.98
T. tonglai	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. virgulatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.05
T. xanthochrus	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. zodiacus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.11
Total	22	32	251	319	19	18	456	50	4	20	53	13	56	65	72	27	117	182	48	11	1,835	100

Table 4 Total number of horse flies in three habitats of collection sites.

Species	Habitat			Total
	Primary forest (4)	Secondary forest (3)	Village (13)	
T. admelanopygus	1	2	0	3
T. agnoscibilis	50	0	7	57
T. anabates	5	0	0	5
T. aurilineatus	4	0	0	4
T. ballmeri	4	0	0	4
T. birmanicus	27	0	0	27
T. borealoriens	1	0	0	1
T. caduceus	1	0	0	1
T. cepuricus	9	0	0	9
T. ceylonicus	2	0	3	5
T. crassus	2	1	0	3
T. discors	1	0	0	1
T. diversifrons	44	0	0	44
T. dorsilinea	2	1	0	3
T. equicinctus	1	0	0	1
T. firmus	3	0	0	3
T. fontinalis	0	5	0	5
T. griseilineis	4	0	0	4
T. hypomacros	3	0	0	3
T. jucundus	3	0	0	3
T. kakhyenensis	4	0	0	4
T. konis	3	0	8	11
T. lentis	1	0	0	1
T. megalops	9	8	375	392
T. mesogaeus	0	1	2	3
T. minimus	0	9	41	50
T. nigrotectus	0	0	2	2
T. oknos	37	0	0	37
T. oxybeles	117	0	0	117
T. pugiunculus	30	0	4	34
T. rhinargus	3	0	0	3
T. rubicundus	65	0	0	65
T. rubidus	6	7	259	272
T. rufiscutellatus	0	2	0	2
T. salvazai	1	0	0	1
T. striatus	1	457	9	467
T. symmetrus	8	0	0	8

Species	Habitat			Total
	Primary forest (4)	Secondary forest (3)	Village (13)	
T. systenus	0	0	5	5
T. tamthaiorum	145	0	0	145
T. thermarum	0	0	2	2
T. thurmani	18	0	0	18
T. tonglai	4	0	0	4
T. virgulatus	0	0	1	1
T. xanthochrus	3	0	0	3
T. zodiacus	2	0	0	2
Total	624	493	718	1,835

DNA sequence

A 658 bp length of the mitochondrial COI gene was successfully amplified from all 145 specimens. The sequences were submitted to the GenBank database under the accession numbers MG426043-MG426187. No insertions, deletions, or stop codons were observed in these sequences. The COI sequences from 45 species had high AT content (68.3%), with an average nucleotide composition of A = 39%, T = 29.3%, C = 15.3% and G = 16.3%. The polymorphic COI sequences included 428 conserved sites, 230 variable sites, 212 parsimony informative sites and 18 singleton sites. A total of 127 haplotypes were identified with a polymorphic site of 255. The number of haplotypes varied from 1 to 12 for each species of horse flies.

Sequence divergence

Pairwise analysis of COI sequences from 48 species of horse flies in Thailand was conducted in MEGA version 6.06. Intraspecific divergence based on Kimura 2-Parameter values of horse flies ranged from 0.0% to 4.4% (average intraspecific divergence = 1.1%). Average intraspecific divergence ranged from 0% in *T. ballmeri*, *T. diversifrons*, *T. firmus*, *T. konis*, *T. symmetrus* and *T. tamthaiorum* to 2.3% in *T. megalops* and *T. minimus*. The maximum intraspecific divergence was observed in *T. megalops* and *T. minimus* with 4.4% and 3.5%, respectively.

Interspecific divergence of horse flies ranged from 0.0% to 16.3% (average interspecific divergence = 8.5%). Average interspecific divergence between horse flies varied from 1.4% (*T. kakhyenensis* and *T. salvazai*) to 15.9% (*T. caduceus* and *T. dorsilinea*). The intraspecific and interspecific divergence values showed overlap

from 0% to 5% (Fig. 2). As a result, there wasn't distinct between intraspecific and interspecific divergences in some horse fly species.

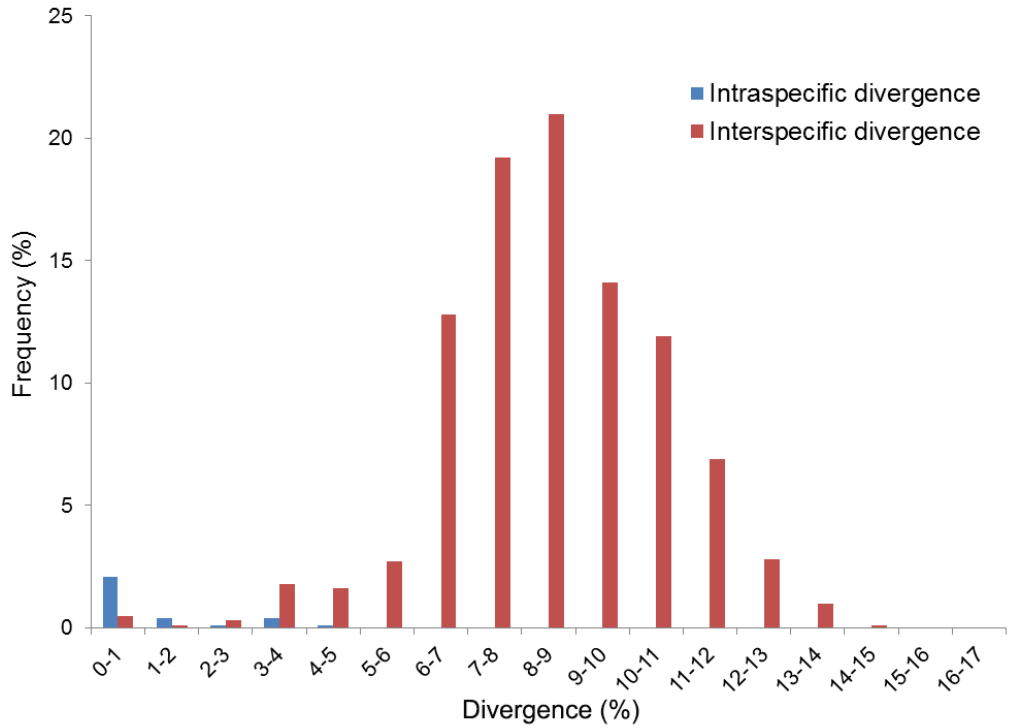

Fig. 2. Frequency distribution of intra- and interspecific divergence of 658 bp COI barcodes of horse flies in Thailand.

Phylogenetic analyses

Phylogenetic relationships among COI sequences of 48 species of Thai horse flies were reconstructed by NJ, ML, and Bayesian analyses (Fig. 3-5). All phylogenetic analyses revealed quite similar results. All species horse flies in Thailand formed a nonmonophyletic group. The sequences of the most species were clearly separated into distinct clusters, except for *T. megalops* and *T. minimus*. *T. megalops* was separated into three groups, one of which was included within *T. striatus* group. In the *T. striatus* group, four sequences of *T. megalops* (accession number MG426104, MG426106, MG426112 and MG426113) were represented among *T. striatus* species. For *T. minimus*, this species was separated into two groups, one of which (accession number MG426122) was clustered with *T. mesogaeus* species. In addition, *T. rubidus* was clustered with *T. fontinalis* in NJ and BA tree whereas this species was clustered with a group of *T. firmus* and *T. rubicundus* in ML tree.

Table 5 List of horse flies included in the study with their locations, accession numbers, haplotypes, and K2P intraspecific divergences.

Species	No. of sample	Location	Accession number	Haplotype	Polymorphic site	K2P distance (mean)
A. cryptotaxis	1	Nakhon Ratchasima	MG426043	1	n/c	-
A. gilvellus	1	Phitsanulok	MG426044	1	n/c	-
A. lotus	1	Nakhon Ratchasima	MG426045	1	n/c	-
T. admelanopygus	2	Nakhon Ratchasima	MG426046-47	3	9	0.2-1.4 (0.9)
	1	Kanchanaburi	MG426048			
T. agnoscibilis	2	Kanchanaburi	MG426049,51	3	15	0.2-2.3 (1.6)
	1	Loei	MG426050			
T. anabates	4	Kanchanaburi	MG426052-55	2	1	0-0.2 (0.1)
T. aurilineatus	3	Kanchanaburi	MG426056-58	3	2	0.2-0.3 (0.2)
T. ballmeri	2	Kanchanaburi	MG426059-60	1	0	0
T. birmanicus	1	Nakhon Ratchasima	MG426061	3	14	0.3-2.2 (1.4)
	2	Kanchanaburi	MG426062-63			
T. borealoriens	1	Nakhon Ratchasima	MG426064	1	n/c	-
T. caduceus	1	Kanchanaburi	MG426065	1	n/c	-
T. cepuricus	3	Kanchanaburi	MG426066-68	2	5	0-0.8 (0.5)
T. ceylonicus	3	Chumphon	MG426069-71	3	6	0.5-0.9 (0.6)
T. crassus	1	Chonburi	MG426072	3	11	0.6-1.4 (1.1)
	1	Nakhon Ratchasima	MG426073			
	1	Kanchanaburi	MG426074			
T. discors	1	Kanchanaburi	MG426075	1	n/c	-
T. diversifrons	3	Kanchanaburi	MG426076-78	1	0	0
T. dorsilinea	3	Kanchanaburi	MG426079-81	3	5	0.2-0.8 (0.5)
T. equicinctus	1	Kanchanaburi	MG426082	1	n/c	-
T. firmus	2	Nakhon Ratchasima	MG426083-84	1	0	0
T. fontinalis	1	Kanchanaburi	MG426085	3	7	0.5-1.1 (0.8)
	2	Chonburi	MG426086-87			

Species	No. of sample	Location	Accession number	Haplotype	Polymorphic site	K2P distance (mean)
T. griseilineis	2	Nakhon Ratchasima	MG426088-89	3	11	0.6-1.4 (1.1)
	1	Chumphon	MG426090			
T. hypomacros	3	Nakhon Ratchasima	MG426091-93	3	16	0.2-2.5 (1.7)
T. jucundus	2	Kanchanaburi	MG426094-95	2	1	0.2-0.2 (0.2)
T. kakhyenensis	3	Kanchanaburi	MG426096-98	3	2	0.2-0.3 (0.2)
T. konis	3	Kanchanaburi	MG426099-101	1	0	0
T. lentis	1	Kanchanaburi	MG426102	1	n/c	-
T. megalops	2	Phitsanulok	MG426103-104	12	33	0.2-4.4 (2.3)
	3	Phra NaKhon Si Ayutthaya	MG426105-106			
	2	Kanchanaburi	MG426107-108			
	1	Nakhon Ratchasima	MG426109			
	2	UthaiThani	MG426110-111			
	3	Chainat	MG426112-113, MG426116			
	1	Songkhla	MG426114			
T. mesogaeus	2	Chumphon	MG426117-118	3	11	0.8-4.4 (1.1)
	1	Nakhon Ratchasima	MG426119			
T. minimus	2	Chonburi	MG426120-21	3	22	0.2-3.5 (2.3)
	1	Phra NaKhon Si Ayutthaya	MG426122			
T. nigrotectus	1	PrachuapKhiriKhan	MG426123	1	n/c	-
T. oknos	3	Kanchanaburi	MG426124-26	3	2	0.2-0.3 (0.2)
T. oxybeles	3	Kanchanaburi	MG426127-29	2	1	0-0.2 (0.1)
T. pugiunculus	3	MaeHongSon	MG426130, MG426134-35	6	8	0.2-0.9 (0.5)
	3	Kanchanaburi	MG426131-33			
T. rhinargus	3	Kanchanaburi	MG426136-38	3	3	0.2-0.5 (0.3)
T. rubicundus	3	Kanchanaburi	MG426139-41	3	9	0.2-1.5 (1.0)
T. rubidus	2	ChiangMai	MG426142-43	11	22	0-1.9 (0.9)
	2	UthaiThani	MG426144-45			
	2	Nakhon Ratchasima	MG426146-47			

Species	No. of sample	Location	Accession number	Haplotype	Polymorphic site	K2P distance (mean)
	2	Chumphon	MG426148-49			
	2	Kanchanaburi	MG426150-51			
	2	Chainat	MG426152-53			
T. rufiscutellatus	2	Nakhon Ratchasima	MG426154-55	3	3	0.2-0.5 (0.3)
	1	Kanchanaburi	MG426156			
T. salvazai	1	Kanchanaburi	MG426157	1	n/c	
T. striatus	2	ChiangMai	MG426158-59	11	15	0-1.1 (0.5)
	3	Nakhon Ratchasima	MG426160-61, MG426168			
	2	Chonburi	MG426162-63			
	1	SuphanBuri	MG426164			
	3	Kanchanaburi	MG426165-67			
	1	UthaiThani	MG426169			
T. symmetrus	2	Kanchanaburi	MG426170-71	1	0	0
T. systenus	1	NakhonPathom	MG426172	2	2	0-0.3 (0.2)
	2	Kanchanaburi	MG426173-74			
T. tamthaiorum	2	Kanchanaburi	MG426175-76	1	0	0
T. thermarum	2	ChiangMai	MG426177-78	2	2	0.3-0.3 (0.3)
T. thurmani	2	Kanchanaburi	MG426179-80	2	2	0.3-0.3 (0.3)
T. tonglai	2	Kanchanaburi	MG426181-82	2	12	1.9-1.9 (1.9)
T. virgulatus	1	Prachuap Khiri Khan	MG426183	1	n/c	-
T. xanthochrus	3	Kanchanaburi	MG426184-86	2	3	0-0.5 (0.3)
T. zodiacus	1	Nakhon Ratchasima	MG426187	1	n/c	-

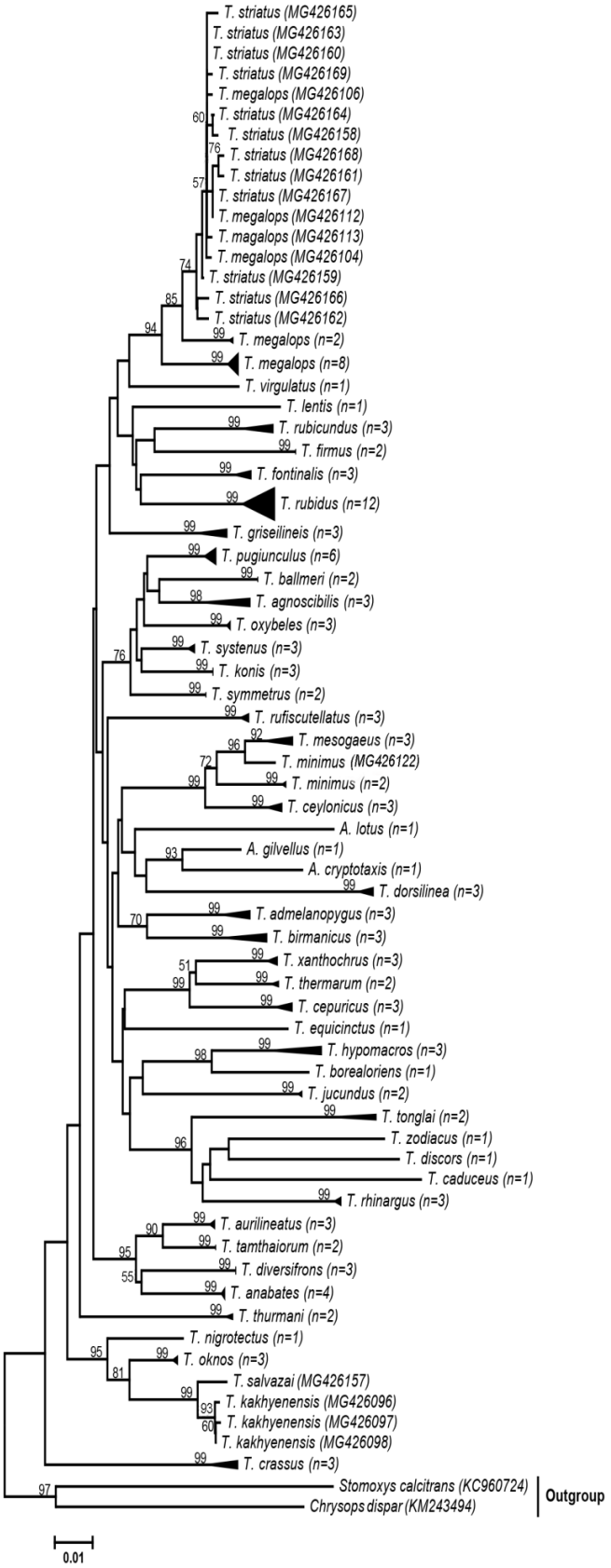

Fig. 3. Neighbor-joining tree under Kimura 2-parameter distance of COI sequences obtained from 48 species of horse flies in Thailand. Only bootstrap values ≥ 50% are shown.

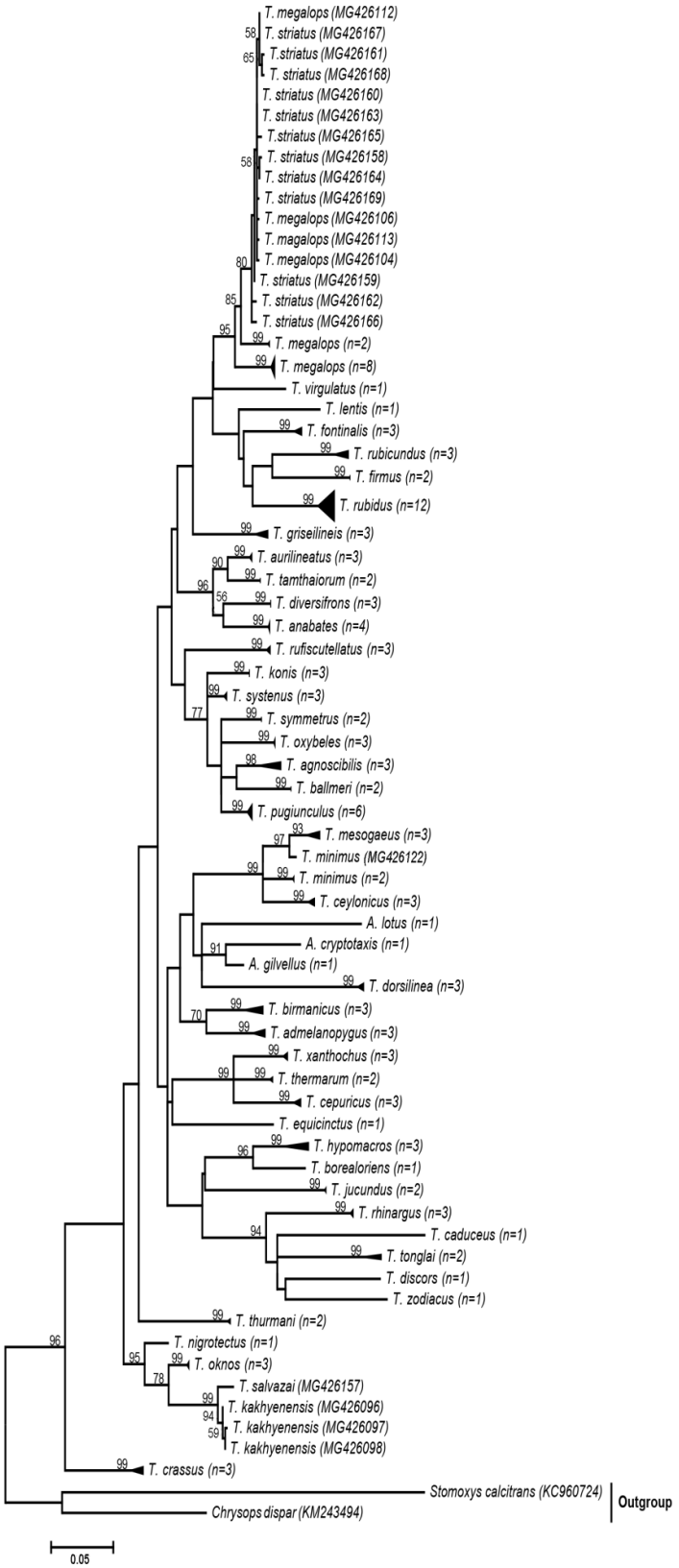

Fig. 4. Maximum likelihood tree of COI sequences under GTR+G+I model obtained from 48 species of horse flies in Thailand. Only bootstrap values ≥ 50% are shown.

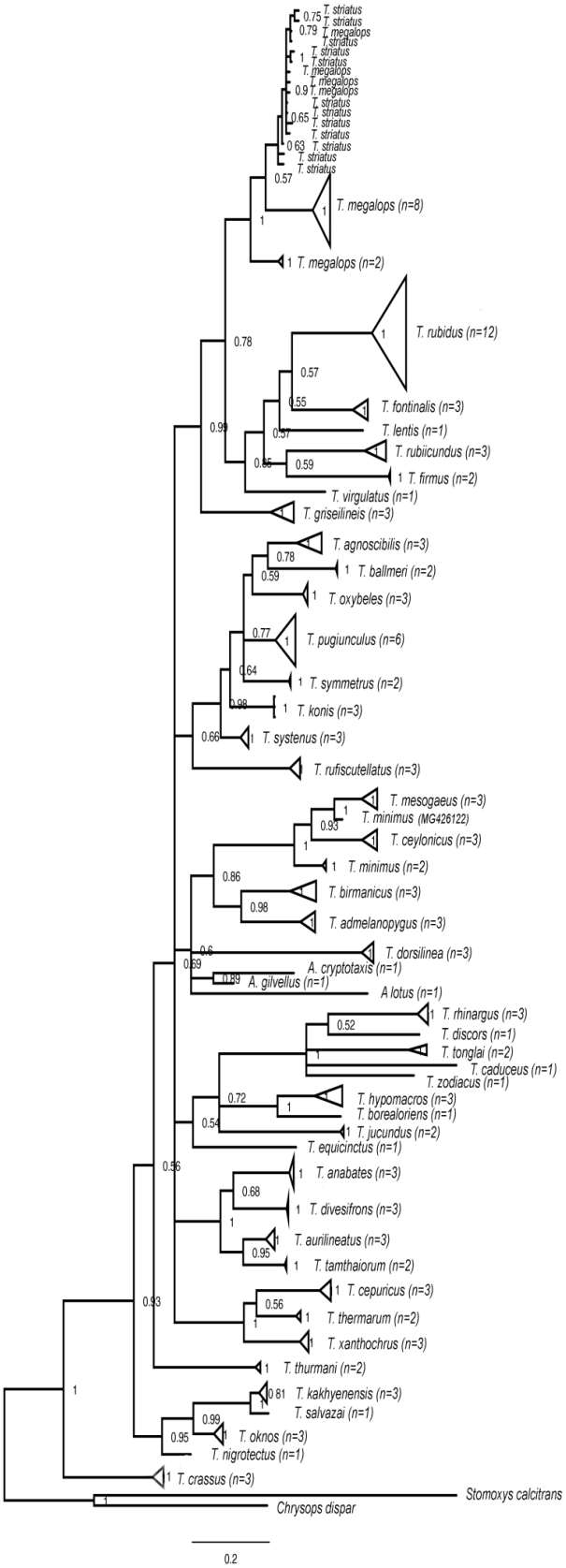

Fig. 5. Beyesian tree of COI sequences obtained from 48 species of horse flies in Thailand. Support values correspond to posterior probabilities.

Discussion

Species distribution

There have been few studies that describe the *Tabanus* fauna in Thailand. Our study revealed updated data about the species diversity and abundance of *Tabanus* spp. in three habitat types in Thailand. All 45 species found in this study were known to be present in Thailand (Burton, 1978). Many species were newly recorded in the areas studied. The number of species was less than that previously recorded by Burton (1978), who recorded 80 species of genus *Tabanus*, while subsequent studies found only 20 species in Thailand (Tumrasvin,1989). The most abundant species were *T. striatus*, *T. megalops* and *T. rubidus*. All these three species have been reported as the most common species in Thailand (Burton, 1978; Boonchit et al., 1996; Klinsri an, 1999). Burton (1978) found that *T. striatus, T. megalops, T. rubidus* were present in the plains. They also were found in the epidemic area of trypanosomosis in central Thailand (Boonchit et al., 1996). In the present study, those species were collected in three habitats. However, *T. rubidus* and *T. megalops* were more frequently found in villages than in primary and secondary forests, while *T. striatus* was most frequently found in secondary forest. Therefore, *T. rubidus* and *T. megalops* may be associated with human activities or human settlements, while *T. striatus* prefers forests rather than anthropized environments. The less abundant species in our study were *T. borealoriens*, *T. caduceus*, *T. discor*s, *T. equicinctus*, *T. lentis*, *T. salvazai* and *T. virgulatus.* All these species (except *T. virgulatus*) were only collected in the primary forest. In Thailand, Burton (1978) recorded *T. borealoriens* and *T. discor*s in zoological gardens, *T. caduceus* and *T. equicinctus* in hilly areas, *T. lentis* in forests and *T. salvazai* in wide geographical ranges. For *T. virgulatus*, we collected this species from the village close to a coastal area. These results were supported by Burton (1978) who revealed *T. virgulatus* as a coastal breeder species.

Tabanid flies are known to be abundant in different habitats (Harley, 1965; Mavoungou et al., 2012; Bitome Essono et al., 2015; Suh et al., 2015). In the present study, the highest numbers of *Tabanus* were collected from villages, followed by primary and secondary forests. These results show a difference with other studies. In Gabon, Mavoungou et al. (2012) found that the tabanid species were most abundant in secondary forests followed by villages and primary forests, while Bitome Essono et

al. (2015) found that tabanids did not show a clear habitat preference. In Uganda, Harley (1965) found that the tabanids were more abundant in open habitats. In Korea, tabanids were most abundant in the habitats near forested areas (Suh et al., 2015). However, the abundance of tabanids can vary by season and climate. In Gabon, Bitome Essono et al. (2015) found an increase in abundance during the rainy season, while the different species can be collected throughout the year at lower abundance. In our study, horse flies were collected at various times and each site was not collected simultaneously. Hence, the seasonal and climate differences among sites may have greatly affected the number of flies. In Thailand, Phasuk et al. (2011) showed the number of tabanids increased at the beginning of the rainy season; there were high numbers during the rainy season and these declined in the dry season. Additionally, the differences in abundance may also be related to other factors such as trap types and the effects of sunlight on traps (Mavoungou et al., 2012; et al., 2014).

Molecular identification

We used DNA barcoding to discriminate between 48 morphological distinct species of horse flies. (DNA barcode provided 92% corrected identification) DNA barcodes correctly identified approximately 92% of the sample taxa (44 from 48 morphologically distinct species). The nucleotide sequences showed a high AT content (68.3%), which is consistent with previous report on Tabanid flies (Cywinska et al., 2010; Banerjee et al., 2015) and other insect mitochondrial genes (Nelson et al., 2007; Ashfag et at al., 2014; Pramual and Adler, 2014; Changbunjong et al., 2016; Gajapathy et al., 2016).

The species discrimination of DNA barcoding method is based on the present of a barcoding gap which the intraspecific divergence is lower than interspecific divergence (Hebert et al., 2003; Meyer and Paulay, 2005). The K2P model has been widely used in DNA barcoding to calculate the intra- and interspecific divergences (Nei and Kumar, 2000). In the present study, the average intra- and interspecific divergences of horse flies were calculated as 1.1% (range 0.0-2.3%) and 8.5% (1.4-15.9%), respectively. The average intraspecific divergences of horse flies was slightly less than the 1.31% for Indian tabanids but higher than the 0.49% for Canadian tabanids, while the average interspecific divergence was higher than both the Indian (6.17%) and Canadian (5.96%) tabanid species (Cywinska et al., 2010; Banerjee et al., 2015). When compared with Indian tabanid flies, the lower average intraspecific

divergence may be the effect of sample size (Zhang et al., 2010). The sample size per species in our study ranged from 1 to 12 specimens but only 3 species had more than 10 specimens. However, many species of *Tabanus* can be collected in small numbers in Thailand (Changbunjong et al., 2018). In contrast, the higher average intraspecific divergence compared with Canadian tabanid flies may be due to the present of cryptic species of some Thai horse flies. In the present study, the high number of haplotypes and high intraspecific divergence of *T. megalops* may suggest presence of cryptic species. Additionally, we observed that *T. minimus* may also presence of cryptic species because this species also showed high the intraspecific divergence especially *T. minimus* (accession number MG426121) and *T. minimus* (accession number MG426122) with 3.5%. However, the high value of intraspecific divergence was also related to geographical distribution of horse fly species. Our study, *T. megalops* was collected from 5 different geographical regions of Thailand. The average interspecific divergence was high in Thai horse flies which indicated that the DNA barcode sequences are highly efficient for discriminating horse fly species. Low average interspecific divergence was found between the closely related species under *T. nephodes* complex (*T. kakhyenensis* and *T. salvazai*) ranged from 1.2%-1.5%. Although the results of our study showed that the average sequence divergence among species was higher than the average divergence within species. The overlap between intra- and inter specific divergence was observed in some horse fly species. These overlaps were due to the low interspecific divergence in *T. megalops* and *T. striatus* (range 0.0-3.9%), and *T. mesogaeus* and *T. minimus* (range 1.7%-2.5%) compared with intraspecific divergence of *T. megalops* (range 0.2%-4.4%), *T. striatus* (0.0%-1.1%), and *T. mesogaeus* (0.8%-4.4%) and *T. minimus* (0.2%-3.5%), respectively. Therefore, the results indicated that COI barcodes cannot discriminate some Thai horse flies (with overlap of barcoding gap), especially in *T. striatus* complex. While three species (*T. dorsiger*, *T. tenens* and *T. striatus*) under *T. striatus* species complex in India can be discriminated using DNA barcodes (Banerjee et al., 2015).

The results from phylogenetic analyses supported as nonmonophyletic relationships of the genus *Tabanus* (Morita et al., 2016) and also revealed a nonmonophyly of the genus *Atylotus*. Most species of horse flies were clustered within a distinct group that corresponded to the species previously identified by using morphological characters, while some species, *T. megalops* and *T. minimus*, were not clustered with the same morphological species. In the present study, the sequences of

T. megalops were included within *T. striatus* clade whereas a sequence of *T. minimus* was clustered with *T. mesogaeus*. The results confirmed that these four species cannot clearly discriminate using COI barcodes. Furthermore, our results provided support for *T. biannularis* group of *T. caduceus, T. discors, T. rhinargus, T. tonglai* and *T. zodiacus*, and *T. ceylonicus* group of *T. ceylonicus, T. mesogaeus* and *T. minimus* whereas were not support *T. basalis* group of *T. admelanopygus* and *T. thurmani*. For *T. rubidus* group, although all phylogenetic trees were supported *T. rubidus* and *T. fontinalis* within this group, but another species, *T. virgulatus* was placed in this group only from Bayesian tree. However, the using of COI gene together with other molecular markers in mitochondrial and/or nuclear genes for further study can be provided the deep genetic relationships and evolution of horse flies in Thailand.

Based on the morphological characters of *T. striatus* complex, *T. megalops* and *T. striatus*, are distinguished by the midline of 2nd tergite crossed by a stripe of pale tomentum and hairs in *T. megalops*, but does not have a stripe in *T. striatus*. Additionally, the dark pattern on the abdominal dorsum generally has a quite black in *T. striatus* than *T. megalops* (Burton, 1978). In the present study, almost all specimens of *T. striatus* complex species are very clear when we used these two characters together. Only single specimen of *T. megalops* (accession number MG426112) showed unclear of a strip of pale tomentum and hairs on 2nd tergite, but the abdominal dorsum has a distinctly brown pattern. For the *T. ceylonicus* group, *T. mesogaeus* can be separated from *T. miminus* by a combination of the blackened beard, lacking pale hairs on thorax and abdomen, and facial tomentum brown (Burton, 1978). All specimens of *T. mesogaeus* and *T. minimus* in our study are clearly identified from the beard color.

The molecular technique, DNA barcoding showed their effectiveness in discriminating the majority of horse flies in Thailand. Our study was specific to Thai horse fly species and was not showed the results compared with aboard species. However, when we tested our sequences of *T. megalops* with nucleotide blast in NCBI, the result showed 99% identity with *T. striatus* in India. Therefore, the other DNA markers and/or other methods such as geometric morphometric should be conducted to clarify the species status of *T. striatus* complex in Thailand. Moreover, more specimens from various sites will be provided additional information on the genetic diversity of horse fly species.

In conclusion, this study reveals the first of the standardized COI barcodes for identification of Thai horse fly species. The data derived from the COI sequences were found to be helpful for species identification and may be applied in future for identifying *Tabanus* species in Asian country, and in the discovery of potential cryptic species. Our study also revealed that COI barcodes were not able to distinguish members of the *T. sriatus* complex and some species of the *T. ceylonicus* group in Thailand.

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Appendices

Publication

Changbunjong T. Sedwisi P, Weluwanarak T, Nitiyamatawat E, Sariwongchan R, Chareonviriyaphap T. Species diversity and abundance of Tabanus spp. (Diptera: Tabanidae) in different habitats of Thailand. . J Asia-Pac Entomol. 2018;21:134-39. **ISI impact factor 2016: 1.046, Scimago journal rank 2016: 2** (Appendix A)

Poster presentation

Changbunjong T, Sedwisai P, Ruangsittichai J, Chareonviriyaphap T. Distribution and abundance of *Tabanus* spp. (Diptera: Tabanidae) in Thailand. Poster Presented at the meeting of Higher Education Commission and Thailand Research fund. The Regent Beach Chaam, Petchaburi, Thailand. 11-12 January 2017. (Appendix B)

กิจกรรมที่เกี่ยวข้องกับการนำผลโครงการไปใช้ประโยชน์อื่น ๆ

- ตัวอย่างเหลือบที่ได้จากการศึกษานี้นำไปใช้ประกอบกิจกรรมฝึกอบรมหลักสูตรสัตว์ป่า: โรคปรสิตที่ติดต่อระหว่างสัตว์และคน หัวข้อ การจำแนกชนิดแมลงดูดเลือดที่สำคัญในสัตว์ป่า: เหลือบและแมลงวันคอกสัตว์ระหว่างวันที่ 3 พฤษภาคม 2559 ถึง 1 มิถุนายน 2559 ณ สถาบันสุขภาพสัตว์แห่งชาติ
- ตัวอย่างเหลือบที่ได้จากการศึกษานี้บางส่วนนำมาใช้ในการเรียนการสอนภาคปฏิบัติการรายวิชาปรสิตวิทยาทางการสัตวแพทย์ (สพปส 333) สำหรับนักศึกษาชั้นปีที่ 3 คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล และการเรียนการสอนรายวิชาการวินิจฉัยทางจุลชีววิทยา และปรสิตวิทยาทางการสัตวแพทย์ (ทนสพ 304) สำหรับนักศึกษาชั้นปีที่ 3 คณะเทคนิคการแพทย์มหาวิทยาลัยมหิดล ประจำปีการศึกษา 2559-2560
- ตัวอย่างเหลือบที่ได้จากการศึกษานี้ได้ถูกนำมาแสดงเพื่อให้ความรู้แก่นักเรียนนักศึกษา นักวิจัยและผู้ที่สนใจทั่วไป.ในโอกาสต่างๆ เช่น กิจกรรมงานวันมหิดลคนรักสัตว์และงาน open house ของคณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ประจำปี 2559-2560

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Species diversity and abundance of *Tabanus* spp. (Diptera: Tabanidae) in different habitats of Thailand

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ABSTRACT

Tabanus spp. or horse flies (Diptera: Tabanidae) are haematophagous flies of medical and veterinary importance. They are known to cause trypanosomosis or surra in domestic and wild animals in Thailand. This study conducted an entomological survey of horse flies from different sites in Thailand. Horse flies were collected from three different habitats: primary forests, secondary forests and villages using Nzi traps between April 2012 and December 2016. A total of 1835 female horse flies were collected and 45 species were identified. The five most abundant species were *T. striatus* (25.45%), followed by *T. megalops* (21.36%), *T. rubidus* (14.82%), *T. tamthaiorum* (7.90%) and *T. oxybeles* (6.38%). The highest proportion of horse flies was collected in villages (39.13%), followed by primary forests (34%) and secondary forests (26.87%). The species diversity of horse flies in primary forests was higher than in other habitats. The results of this study may be used for a horse fly control program.

Introduction

Tabanus spp. or horse flies are classified into the suborder Brachycera, infraorder Tabanomorpha and family Tabanidae. This family includes approximately 4500 species worldwide and over 1300 species are in the genus *Tabanus* (Morita et al., 2016). The adult female of the horse fly is haematophagous, feeding on domestic or wild animals and occasionally attacking humans. Horse flies can transmit several disease pathogens including protozoa, bacteria and viruses (Foil, 1989; Mullens, 2009; Baldacchino et al., 2014). They are mechanical vectors not only of *Trypanosoma evansi* but other trypanosomes as well (*T. brucei*, *T. congolense* and *T. vivax*) (Desquesnes et al., 2013; Baldacchino et al., 2014). They can also mechanically transmit *Besnoitia besnoiti*, various bacteria such as *Anaplasma marginale*, *Francisella tularensis* and *Bacillus anthracis*, and retroviruses such as equine infectious anemia virus and bovine leucosis virus. Additionally, they are considered as a biological vector of *T. theileri* in cattle (Baldacchino et al., 2014).

Numerous studies have investigated the species distribution and/or the abundance of horse flies within countries or specific areas (Barros, 2001; Krcmar, 2005; Sasaki, 2005; Mikuska et al., 2008; Al Dhafer et al., 2009; Hackenberger et al., 2009; Krcmar, 2011; Altunsoy and Kilic, 2012; Mavoungou et al., 2012; Müller et al., 2012; Chandra et al., 2015; Bitome Essono et al., 2015; Suh et al., 2015; Maity et al., 2016; Al Talafha et al., 2016; Lydie et al., 2017). Some studies also showed the species diversity and habitat preference of these flies (Mavoungou et al., 2012). In Thailand, previous reports on horse flies were those of Stone (1975), followed by Berton (1978) and Tumrasvin (1989). They established a list of species and their distribution in different geographical regions of the country. However, little is known about the species diversity in different habitats in Thailand. Thus the objective of this study was to determine the species and abundance of horse flies in the three main habitats including primary forests, secondary forests and villages in different geographical regions of Thailand.

Materials and methods

Collection sites

Horse flies were collected at 20 localities from the different geographical regions of Thailand (Fig. 1). The collection sites were

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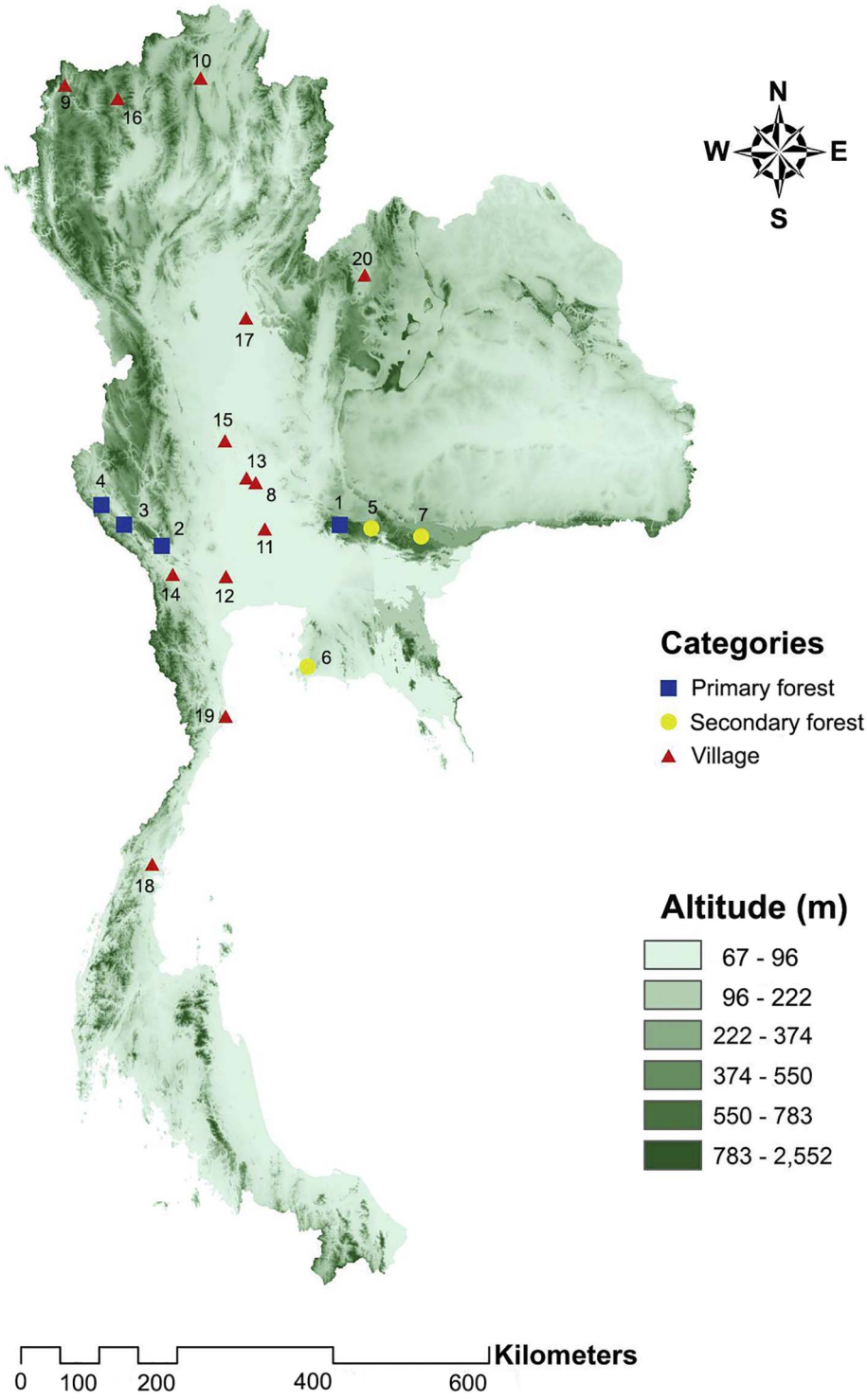

Fig. 1. Map of horse fly collection sites in Thailand: Nakhon Ratchasima (1, 5, 7), Kanchanaburi (2, 3, 4, 14), Chon Buri (6), Singburi (8), Mae Hong Son (9), Chiang Rai (10), Phra Nakhon Si Ayutthaya (11), Nakhon Prathom (12), Chainat (13), Uthai Thani (15), Chiang Mai (16), Phitsanulok (17), Chumphon (18), Prachuap Khiri Khan (19), Loei (20).

classified into 3 habitat types: primary forest, secondary forest and village (Table 1) following these definitions: a primary forest is a forest that has never been logged and has developed following natural disturbances and under natural processes; a secondary forest is a forest that has been logged and has recovered naturally or artificially (CBD, 2017) and a village is a clustered human settlement or community.

Horse fly collection

Adult horse flies were collected at various times using 10 Nzi traps between April 2012 and December 2016. This trap is highly specific for tabanid flies (Mihok, 2002). The traps were made locally, using blue and black fabric named Solon® (Thailand Supplier) being 100% polyester. Each trap was randomly placed at the collection sites from 6.00 a.m. to 6.00 p.m. over a 2 day period. The temperature and relative humidity at collection sites were recorded (Table 2). The captive flies were collected at 2 or 3 h intervals to prevent specimen damage for morphological identification. All flies were euthanized in the freezer (− 10 °C) and transported to the Vector-Borne Diseases Research Unit (VBRU), Faculty of Veterinary Science, Mahidol University for species identification.

Table 1
Horse fly collection sites and dates in Thailand.

Habitat	No	Date	Characteristic of collection sites	District/Province	Altitude	Coordinates (Lat/Long)
Primary forest	1	Apr 2012	Wildlife species in Khao Yai National Park	Pak Chong, Nakhon Ratchasima	774	N14°24′55.1″, E101°22′33.4″
	2	Feb 2015	Buffalo farm in Salakpra Wildlife Sanctuary	Mueang, Kanchanaburi	54	N14°11′06.8″, E099°15′23.4″
	3	Apr 2015	Beef cattle farm in Sai Yok National Park	Sai Yok, Kanchanaburi	102	N14°25′52.8″, E098°48′32.3″
	4	Jun 2016	Beef cattle farm in natural forest	Thong Pha Phum, Kanchanaburi	174	N14°39′28.0″, E098°32′19.7″
Secondary forest	5	Feb 2015	Buffalo farm around Khao Phaeng Ma No Hunting Wildlife Area	Wang Nam Khiao, Nakhon Ratchasima	498	N14°22′23.4″, E101°44′51.9″
	6	May 2015	Wildlife species in Khao Chi On No Hunting Wildlife Area	Sattahip, Chon Buri	93	N12°46′34.1″, E100°58′30.8″
	7	Feb 2016	Beef cattle and buffalo farm around Thap Lan National Park	Soeng Sang, Nakhon Ratchasima	263	N14°16′54.3″, E102°28′16.7″
Village	8	Aug 2013	Beef cattle farm	Mueang, Singburi	18	N14°54′59.5″, E100°22′38.5″
	9	Jul 2014	Beef cattle farm	Mueang, Mae Hong Son	452	N19°31′45.5″, E098°04′48.6″
	10	Jul 2014	Beef cattle farm	Mueang, Chiang Rai	415	N19°36′39.9″, E099°44′18.1″
	11	Jul 2015	Buffalo farm	Bang Ban, Phra Nakhon Si Ayutthaya	8	N14°22′43.9″, E100°28′57.1″
	12	Jul 2015	Beef cattle farm	Mueang, Nakhon Prathom	7	N13°49′50.0″, E100°01′04.9″
	13	Aug 2015	Beef cattle and pig farm	Sankhaburi, Chainat	19	N14°58′12.4″, E100°16′01.9″
	14	Aug 2015	Beef cattle and buffalo farm	Dan Makham Tia, Kanchanaburi	52	N13°51′18.5″, E099°23′09.7″
	15	Nov 2015	Buffalo farm	Mueang, Uthai Thani	38	N15°24′13.7″, E100°00′49.6″
	16	Jan 2016	Beef cattle and buffalo farm	Chiang Dao, Chiang Mai	624	N19°22′42.2″, E098°43′25.7″
	17	Jan 2016	Beef cattle, buffalo and horse farm	Mueang, Phitsanulok	50	N16°49′41.8″, E100°16′28.2″
	18	Apr 2016	Beef cattle and buffalo farm	Mueng, Chumphon	14	N10°29′33.5″, E099°08′28.1″
	19	Apr 2016	Beef cattle farm	Sam Roi Yot, Prachuap Khiri Khan	2	N12°12′28.1″, E100°00′25.2″
	20	Dec 2016	Buffalo farm	Wang Saphung, Loei	277	N17°18′36.8″, E101°42′35.6″

Table 2
Climatic data at the collection sites.

No	District/Province	Temperature (°C)		Humidity (%)
		Max	Min	
1	Pak Chong, Nakhon Ratchasima	32	23	81
2	Mueang, Kanchanaburi	33	22	68
3	Sai Yok, Kanchanaburi	30	26	74
4	Thong Pha Phum, Kanchanaburi	34	25	76
5	Wang Nam Khiao, Nakhon Ratchasima	35	22	69
6	Sattahip, Chon Buri	35	27	71
7	Soeng Sang, Nakhon Ratchasima	34	21	70
8	Mueang, Singburi	33	25	70
9	Mueang, Mae Hong Son	32	24	75
10	Mueang, Chiang Rai	31	23	76
11	Bang Ban, Phra Nakhon Si Ayutthaya	33	24	70
12	Mueang, Nakhon Prathom	32	24	74
13	Sankhaburi, Chainat	33	25	71
14	Dan Makham Tia, Kanchanaburi	32	24	68
15	Mueang, Uthai Thani	32	22	71
16	Chiang Dao, Chiang Mai	30	14	67
17	Mueang, Phitsanulok	33	20	74
18	Mueng, Chumphon	34	24	70
19	Sam Roi Yot, Prachuap Khiri Khan	35	27	76
20	Wang Saphung, Loei	29	15	73

Morphology identification

The specimens were identified to species level by descriptions and keys (Berton, 1978) under a stereomicroscope. They were separated by species, sex, date and collection site.

Data analysis

The Shannon-Wiener diversity index (H) was used to analyze species diversity in the three habitat types.

$$H = -\sum_{i=1}^{s} P_i \ln P_i$$

The proportion of species (i) relative to the total number of species (P_i) is calculated and then multiplied by the natural logarithm of this proportion ($\ln P_i$). The result is summed across species and multiplied by −1 (Magurran, 2004). The evenness index (E) was used to count the homogeneity or pattern of distribution of species in relation to other species.

$$E = H/\ln S$$

where H is the number derived from the Shannon-Wiener diversity index and S is the total number of species. This index varies between 0 and 1, with 1 showing complete evenness.

Results

A total of 1835 horse flies representing 45 species were collected (Table 3). Only female horse flies were collected in this study. The five most abundant species were *Tabanus striatus* (25.45%), followed by *T. megalops* (21.36%), *T. rubidus* (14.82%), *T. tamthaiorum* (7.90%) and *T. oxybeles* (6.38%). The less abundant species were *T. borealoriens*, *T. caduceus*, *T. discors*, *T. equicinctus*, *T. lentis*, *T. salvazai* and *T. virgulatus*, with each species having a relative abundance of 0.05%.

The highest proportion of horse flies was collected in villages (39.13%), followed by primary forests (34%) and secondary forests (26.87%) (Table 4). The species diversity of horse flies in primary forests (H = 2.56) was much higher than in villages (H = 1.17) and secondary forests (H = 0.34). In the primary forest, 37 species were collected; *T. tamthaiorum* was the most abundant species with 23.2%, followed by *T. oxybeles* (18.8%) and *T. rubicundus* (10.4%). Twenty-seven species collected in the primary forest were absent in the secondary forest and the village. In the secondary forest, 10 species were collected: *T. striatus* was the most abundant species with 23.2%, followed by *T. minimus* (1.83%) and *T. megalops* (1.62%). *T. fontinalis* was presented only in this habitat. In the village, 13 species were collected; *T. megalops* was the most abundant species with 52.23%, followed by *T. rubidus* (36.07%) and *T. minimus* (5.71%). *T. megalops*, *T. rubidus* and *T. striatus* were presented in the three habitats. *T. nigrotectus*, *T. systenus*, *T. thermarum* and *T. virgulatus* were abundant in the village but were absent from both other habitats. The evenness indices of horse flies in the primary forest, the secondary forest and the village were 0.71, 0.15 and 0.45, respectively (Table 4).

Discussion

There have been few studies that describe the *Tabanus* fauna in

Table 3
Total number of horse flies collected at 20 collection sites in Thailand.

Species	Collection sites																				Total	Percent
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
T. admelanopygus	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. agnoscibilis	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	57	3.11
T. anabates	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0.27
T. aurilineatus	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. ballmeri	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. birmanicus	1	0	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	1.47
T. borealoriens	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. caduceus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. cepuricus	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0.49
T. ceylonicus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	5	0.27
T. crassus	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. discors	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. diversifrons	0	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	44	2.40
T. dorsilinea	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. equicinctus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. firmus	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. fontinalis	0	0	0	0	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0.27
T. griseilineis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. hypomacros	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. jucundus	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. kakhyenensis	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. konis	0	0	3	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	11	0.60
T. lentis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. megalops	0	0	0	9	2	5	1	0	0	0	41	12	36	32	13	0	117	79	45	0	392	21.36
T. mesogaeus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	3	0.16
T. minimus	0	0	0	0	0	9	0	0	0	0	3	0	0	0	0	0	0	38	0	0	50	2.72
T. nigrotectus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.11
T. oknos	0	0	4	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	37	2.02
T. oxybeles	0	0	42	75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	117	6.38
T. pugiunculus	0	30	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	34	1.85
T. rhinargus	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. rubicundus	4	0	61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65	3.54
T. rubidus	0	0	0	6	5	0	2	50	0	20	9	0	20	20	59	20	0	60	0	1	272	14.82
T. rufiscutellatus	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.11
T. salvazai	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.05
T. striatus	1	0	0	0	5	0	452	0	0	0	0	0	0	4	0	5	0	0	0	0	467	25.45
T. symmetrus	0	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.44
T. systenus	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	3	5	0.27
T. tamthaiorum	1	0	6	138	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	145	7.90
T. thermarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	0.11
T. thurmani	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0.98
T. tonglai	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.22
T. virgulatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.05
T. xanthochrus	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0.16
T. zodiacus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.11
Total	22	32	251	319	19	18	456	50	4	20	53	13	56	65	72	27	117	182	48	11	1835	100

Thailand. Our study revealed updated data about the species diversity and abundance of *Tabanus* spp. in three habitat types in Thailand. All 45 species found in this study were known to be present in Thailand (Berton, 1978). Many species were newly recorded in the areas studied. The number of species was less than that previously recorded by Berton (1978), who recorded 80 species of genus *Tabanus*, while subsequent studies found only 20 species in Thailand (Tumrasvin, 1989). The most abundant species were *T. striatus*, *T. megalops* and *T. rubidus*. All these three species have been reported as the most common species in Thailand (Berton, 1978; Boonchit et al., 1996; Klinsri and Leksawasdi, 1999). Berton (1978) found that *T. striatus, T. megalops, T. rubidus* were present in the plains. They also were found in the epidemic area of trypanosomosis in central Thailand (Boonchit et al., 1996). In the present study, those species were collected in three habitats. However, *T. rubidus* and *T. megalops* were more frequently found in villages than in primary and secondary forests, while *T. striatus* was most frequently found in secondary forest. Therefore, *T. rubidus* and *T. megalops* may be associated with human activities or human settlements, while *T. striatus* prefers forests rather than anthropized environments. The less abundant species in our study were *T. borealoriens*, *T. caduceus*, *T. discors*, *T. equicinctus*, *T. lentis*, *T. salvazai* and *T. virgulatus*. All these species (except *T. virgulatus*) were only collected in the primary forest. In Thailand, Berton (1978) recorded *T. borealoriens* and *T. discors* in zoological gardens, *T. caduceus* and *T. equicinctus* in hilly areas, *T. lentis* in forests and *T. salvazai* in wide geographical ranges. For *T. virgulatus*, we collected this species from the village close to a coastal area. These results were supported by Berton (1978) who revealed *T. virgulatus* as a coastal breeder species.

Tabanid flies are known to be abundant in different habitats (Harley, 1965; Mavoungou et al., 2012; Bitome Essono et al., 2015; Suh et al., 2015). In the present study, the highest numbers of *Tabanus* were collected from villages, followed by primary and secondary forests. These results show a difference with other studies. In Gabon, Mavoungou et al. (2012) found that the tabanid species were most abundant in secondary forests followed by villages and primary forests, while Bitome Essono et al. (2015) found that tabanids did not show a clear habitat preference. In Uganda, Harley (1965) found that the tabanids were more abundant in open habitats. In Korea, tabanids were most abundant in the habitats near forested areas (Suh et al., 2015). However, the abundance of tabanids can vary by season and climate. In

Table 4
Total number and species diversity of horse flies in three habitats of collection sites.

Species	Habitat			Total
	Primary forest (4)	Secondary forest (3)	Village (13)	
T. admelanopygus	1	2	0	3
T. agnoscibilis	50	0	7	57
T. anabates	5	0	0	5
T. aurilineatus	4	0	0	4
T. ballmeri	4	0	0	4
T. birmanicus	27	0	0	27
T. borealoriens	1	0	0	1
T. caduceus	1	0	0	1
T. cepuricus	9	0	0	9
T. ceylonicus	2	0	3	5
T. crassus	2	1	0	3
T. discors	1	0	0	1
T. diversifrons	44	0	0	44
T. dorsilinea	2	1	0	3
T. equicinctus	1	0	0	1
T. firmus	3	0	0	3
T. fontinalis	0	5	0	5
T. griseilineis	4	0	0	4
T. hypomacros	3	0	0	3
T. jucundus	3	0	0	3
T. kakhyenensis	4	0	0	4
T. konis	3	0	8	11
T. lentis	1	0	0	1
T. megalops	9	8	375	392
T. mesogaeus	0	1	2	3
T. minimus	0	9	41	50
T. nigrotectus	0	0	2	2
T. oknos	37	0	0	37
T. oxybeles	117	0	0	117
T. pugiunculus	30	0	4	34
T. rhinargus	3	0	0	3
T. rubicundus	65	0	0	65
T. rubidus	6	7	259	272
T. rufiscutellatus	0	2	0	2
T. salvazai	1	0	0	1
T. striatus	1	457	9	467
T. symmetrus	8	0	0	8
T. systenus	0	0	5	5
T. tamthaiorum	145	0	0	145
T. thermarum	0	0	2	2
T. thurmani	18	0	0	18
T. tonglai	4	0	0	4
T. virgulatus	0	0	1	1
T. xanthochrus	3	0	0	3
T. zodiacus	2	0	0	2
Total	624	493	718	1835
Diversity index (H)	2.56	0.34	1.17	
Evenness index (E)	0.71	0.15	0.45	

Gabon, Bitome Essono et al. (2015) found an increase in abundance during the rainy season, while the different species can be collected throughout the year at lower abundance. In our study, horse flies were collected at various times and each site was not collected simultaneously. Hence, the seasonal and climate differences among sites may have greatly affected the number of flies. In Thailand, Phasuk et al. (2011) showed the number of tabanids increased at the beginning of the rainy season; there were high numbers during the rainy season and these declined in the dry season. Additionally, the differences in abundance may also be related to other factors such as trap types and the effects of sunlight on traps (Mavoungou et al., 2012; Baldacchino et al., 2014).

Species diversity of horse flies was highest in primary forest habitat (37 *Tabanus* species) followed by villages and secondary forests. These results differ from the previous study conducted by Mavoungou et al. (2012) in Gabon. They found that the species diversity of tabanids was highest in secondary forests, followed by primary forests and villages. In Thailand, Berton (1978) revealed the habitats of horse flies and animal hosts in different geographical areas. Most *Tabanus* species were collected from northern Thailand (especially in Chiang Mai Province) with their habitats including mountains, hilly areas and jungle areas, while the remaining species were collected from other sites with habitats including plains, valley areas, coastal lowland situations, agricultural flatland and coastal areas. In our study, the most *Tabanus* species were collected from western Thailand in Kanchanaburi Province with 31 species included. The primary forests in our study were national parks, wildlife sanctuary and natural forests. These areas include mountains and hills covered with mixed deciduous or evergreen forests. In some forested areas, villagers bring animals, especially cattle, to graze in the forest and this can be a major feeding source of the horse flies. The lower species diversity of horse flies was found in villages and secondary forests. The village is an anthropized environment and some of the *Tabanus* species may not be able to adapt to the human environment. Therefore, there are only some species that can adapt and multiply in this environment (Klinsri and Leksawasdi, 1999). In the secondary forests, some areas have been degraded, mostly though loss of dry forest clearing for agriculture and tree plantations, and this can cause low diversity habitats. Besides the habitat types, the diversity of species captured can also be influenced by other factors such as collection period and season (Berton, 1978). Our collections have been made at different years and different seasons, and it is very difficult to compare one site to another in these conditions.

In conclusion, this study provides a data update about species diversity and abundance of horse flies in Thailand. Our results confirm the presence of species of the genus *Tabanus* among three habitat types; primary forest, secondary forest and village. The populations of horse flies in villages are high compared to other habitats. The three habitat types were different in species diversity. Further studies about species diversity and abundance of horse flies related to animal diseases in the areas can provide epidemiological data to develop control strategies.

Conflicts of interest

None.

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Distibution and Abundance of *Tabanus* spp. (Diptera: Tabanidae) in Thailand

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Abstract

Tabanus spp. or horse flies (Diptera: Tabanidae) are haematophagous flies of considerable medical and veterinary importance. The females of flies feed on the blood of mammals causing nuisance to humans, major irritant pests of livestock and wildlife and also transmit of certain parasites and pathogens. The aim of the present study was to determine the distribution and abundance of horse flies from different sites in Thailand. Horse flies were collected at three important sites: livestock farms, national parks and a wildlife conservation area using Nzi traps between July 2015 and August 2016. Species were identified based on morphological characteristics. A total of 1,490 individuals of 29 species were collected. The most abundant species was *Tabanus striatus* (31.34%), followed by *T. megalops* (24.1%), *T. rubidus* (12.35%), *T. agnoscibilis* (7.25%) and *T. tamthaiorum* (6.51%). This study provides information that may be useful for horse flies control programs

Keywords: Tabanus, horse flies, Tabanidae, Morphology

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