The Thai Journal of Veterinary Medicine

Volume 42
Issue 3 *September, 2012*

Article 12

9-1-2012

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Available at: https://digital.car.chula.ac.th/tjvm/vol42/iss3/12

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Original Article

Molecular Analysis of Medically and Veterinary Important Muscid Flies (Diptera: Muscidae) in Thailand

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Abstract

We demonstrated the using of the internal transcribed spacer (ITS2) of ribosomal DNA as a tool for identification of medically and veterinary important Muscidae flies in Thailand. A total of 27 fly samples were collected from various regions of Thailand. Six fly species in three subfamilies including Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) and Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* and *S. sitiens*) were identified base on morphological taxonomy. PCR amplicons of the ITS2 gene of these flies varied between 312-377 bp with A+T content of 76.6%. ITS2 sequences of the flies in this study were 93-100% identity to sequences in database and 21 samples were compatible with morphological identification, while sequences of 6 samples did not match any sequences in the database. The intra- and inter-specific divergence analysis results showed that the maximum of intra-specific (within species) variation (6.9%) was found in *M. domestica* while the minimum inter-specific (between species) variation (11.9%) was found in the sister grouped couple of *S. sitiens* and *S. indicus*. No overlapping between intra- and inter-specific divergences was found in all species of this study. The bootstrapped NJ tree constructed showed ability to classify each subfamily in to monophyletic clades. PCR-RFLP using *Xap*I restriction enzyme digestion was able to differentiate between the three *Stomoxys* species. Data obtained from this study would be valuable for both medical and veterinary entomologists for more accurate identification of important fly species. Therefore, it could be used for population dynamics studies and enrolled in integrated pest management control program.

Keywords: ITS2, molecular identification, Muscidae fly, PCR-RFLP, Thailand

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บทคัดย่อ

การวิเคราะห์ทางชีวโมเลกุลของแมลงวันในวงศ์ Muscidae ที่มีความสำคัญทางการแพทย์และสัตวแพทย์ในประเทศไทย

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ผู้วิจัยรายงานการใช้บริเวณ internal transcribed spacer (ITS2) ของ ribosomal DNA ในการจำแนกสายพันธุ์ของแมลงวันที่มีความสำคัญทางการแพทย์และสัตวแพทย์ในวงศ์ Muscidae ในประเทศไทย พบว่าแมลงวัน 27 ตัวอย่างที่เก็บจากพื้นที่ต่างๆ ในประเทศไทยถูกจำแนกด้วยลักษณะทางสัณฐานวิทยาจำแนกเป็น 6 สปีชีส์ ใน 3 วงศ์ย่อย ประกอบด้วยวงศ์ Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) และ Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* และ *S. sitiens*) ผลิตภัณฑ์พีซีอาร์ของ ITS2 มีขนาดตั้งแต่ 312-377 bp และมี A+T content เท่ากับร้อยละ 76.6 ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของ ITS2 ในการทดลองนี้กับฐานข้อมูลพบว่าตรงกับฐานข้อมูลร้อยละ 93-100 โดยที่แมลงวัน 21 ตัวอย่างมีผลการเปรียบเทียบตรงกับการจำแนกด้วยสัณฐานวิทยา ในขณะที่อีก 6 ตัวอย่างไม่มีข้อมูลลำดับนิวคลีโอไทด์ในฐานข้อมูล การวิเคราะห์ค่าความผันแปรภายในและระหว่างสปีชีส์พบว่าค่าความผันแปรภายในสปีชีส์สูงสุดพบในแมลงวัน *M. domestica* เท่ากับร้อยละ 6.9 ในขณะที่ค่าความผันแปรระหว่างสปีชีส์ต่ำสุดพบในคู่ของแมลงวัน *S. sitiens* กับ *S. indicus* เท่ากับร้อยละ 11.9 ทั้งนี้พบว่าไม่มีค่าความผันแปรภายในสปีชีส์มากกว่าค่าความผันแปรระหว่างสปีชีส์ของทุกสปีชีส์ในการทดลองนี้ แผนภูมิต้นไม้พันธุกรรมแบบ NJ method สามารถแบ่งวงศ์ย่อยของแมลงวันให้มีลักษณะเป็น monophyletic clade ได้ ผล PCR-RFLP โดยใช้เอ็นไซม์ *Xap*I สามารถบอกความแตกต่างระหว่างแมลงวัน *Stomoxys* ทั้ง 3 สปีชีส์ได้ ข้อมูลที่ได้จากการทดลองนี้จะเป็นประโยชน์สำหรับนักกีฏวิทยาทางการแพทย์และทางสัตวแพทย์ในการจำแนกสายพันธุ์แมลงวันที่มีความสำคัญทางการแพทย์และสัตวแพทย์ รวมถึงใช้ในการศึกษาความชุกของแต่ละสายพันธุ์ซึ่งจะเป็นข้อมูลสำคัญในการใช้ควบคุมแมลงวันแบบบูรณาการต่อไป

คำสำคัญ: ITS2 การจำแนกสายพันธุ์ด้วยอณูชีววิทยา แมลงวัน Muscidae, PCR-RFLP ประเทศไทย

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Introduction

Flies belong to the family Muscidae. They are of crucial importance to medicine and veterinary medicine because of their ability to transmit diseases to humans and animals. For example, stable flies (*Stomoxys* spp.), transmit diseases by biting. Larvae of some species cause myiasis in man and animals (Lane and Crosskey, 1993). Moreover, fly larvae can be found in corpses and, therefore, can be used as a forensic indicator for estimating Post-Mortem Interval (PMI) (Sukontason, 2007, Preativatanyou et al., 2010). Some adult flies of this family, especially house flies, are synanthropic which can cause annoyance and act as carriers of mechanical transmitting pathogens to human (Lane and Crosskey, 1993) and also transmit some pathogens biologically to animals (Iwasa, 1999).

Stomoxys flies (Musidae: Stomoxynae), known as stable flies, are blood sucking flies found worldwide especially in tropical zone, including

Thailand. These flies are the veterinary, medically and economically important flies due to both sexes feed on blood of large mammals, sometimes human. Their painful biting can be irritating and stressful to livestock resulting in significant decrease in weight and milk yield (Lane and Crosskey, 1993). Moreover, these species have been reported as mechanical vectors of pathogenic bacteria, *Mycobacterium* sp. (Fischer et al., 2001), *Bacillus anthracis* (Zumpt, 1973), and *Enterobacter sakazakii* (Mramba et al., 2007); and various virus, Surra disease and equine infectious anemia (EIA) virus (Veer et al., 2002) and Rift Valley fever virus (Turell and Knudson, 1987). The way to reduce problems from these flies is fly control. The most efficient of stable fly control is an integrated pest management (Williams et al., 1981) where surveillance is included. Results from surveys stable fly populations are not only data for prediction of the origins of outbreak and population dynamics but also for geographical patterns of insecticide resistance analysis. However, the important key for surveillance is accurate identification of stable fly species.

As described previously, identification of fly is essential for both epidemiological study and for control strategies. Accurate identification of fly larvae collected from corpses is can also help estimate the PMI more precisely. Although morphological identification of fly using taxonomic keys can identify flies at any stage (Tumrasvin et al., 1979, Greenberg and Kunich, 2002, Siriwattanarungsee et al., 2005, Sukontason et al., 2004, 2008[a,b]), this procedure is elaborate and, therefore, requires highly experienced. It also requires complete fly samples. However, the problem of morphological identification is there is no key covering all stages of fly species at present, especially in larval stage (Wells and Sperling, 2001, Wells et al., 2001). In order to solve the problems of taxonomic identification as previously described, molecular techniques such as nucleotide sequence analysis and PCR-RFLP are mostly chosen (Stevens and Wall, 2001). These techniques are fast, accurate and highly sensitive. Moreover, they can be performed even though the collected samples are damaged (Hajibabaei et al., 2007; Kress and Erickson, 2008).

Aims of this study were to analyze the nucleotide sequences of Muscid flies in Thailand. The internal transcribed spacer 2 (ITS2) between 5.8S and 28S ribosomal RNA (rRNA) gene region in the rRNA transcription unit of nuclear DNA was chosen for this study because this region is markedly different in length and sequence in each species of insect (Ratcliffe et al., 2003; Young and Coleman, 2004; Song et al., 2008[a]). Furthermore, it has been used as a DNA barcode for differentiation of some insect species such as mosquitoes (Torres et al., 2000; Van Bortel et al., 2000; Wilkerson et al., 2004; Li and Wilkerson, 2005), flies (Nelson et al., 2008; Ferreira et al., 2011) and black flies (Thanwisai et al., 2006). This study is the first molecular study in Muscid species in Thailand. Therefore, the nucleotide sequences from this study were accumulated to worldwide online database and will be valuable data for closely related fly specie identification in future study and useful for integrated pest management program of fly control.

Materials and Methods

Fly sampling and collection sites: Six muscid fly species in three subfamilies including Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) and Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* and *S. sitiens*) were included in the study. *Stomoxys* flies were collected near a dairy farm in Nakhon Ratchasima province (North-eastern), Thailand by using carbon dioxide bait trapping. Muscidae flies were collected from various regions of Thailand including Chiang Mai (Northern), Nong Khai (North-eastern), Bangkok (Central), Ranong (Southern) and Phuket (Southern) (Fig 1) by using ground pig liver bait trapping. Species of all specimens were identified by using a morphologically taxonomic key (Zumpt, 1973, Crosskey and Lane, 1993; Masmeatathip et al., 2006) under a stereomicroscope (SZX9, Olympus, Japan) and then individual specimens were coded (Table 1) before being kept in a 1.5 ml microcentrifuge tube and preserved with 70% ethanol and stored at 4^{0}C until next step.

DNA extraction: Genomic DNA was extracted from thorax of individual adult by using DNA extraction kits (Invisorb® Spin tissue mini Kit, Invitek, Berlin, Germany), following the manufacturer's instructions. The extracted DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo-scientific, DE, USA) and stored at -20^{0}C until used.

Figure 1 Map of Thailand, showing the collecting sites in this study

PCR amplification: The primer set anneal specific to the ITS2 region used in this study was designed by Song et al. (2008[a,b]). This primer was designed by using a sequence of 3′-ends of 5.8S and 5′-ends of 28S rRNA gene region (Tautz et al., 1988). The forward primer sequences were 5′TGCTTGGACTACATA TGGTTGA3′ and the reverse primer sequences were 5′GTAGTCCCATATGAGTTGAGGTT 3′.

PCR reaction was composed of 2 µl of 10X *Taq* buffer, 2 µl of 200 µM dNTP, 2 µl of 2.5 mM $MgCl_2$, 0.8 µl of 0.4 µM of each primer, 0.2 µl of *Taq* DNA polymerase (5U/µl; Invitrogen®, CA, USA) and 100 ng of DNA template, and DNase-free water to final volume of 20 µl. The PCR reactions were performed in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems®, Forster city, CA, USA) by using conditions as follows: initial denaturation step at 94^{0}C for 5 min, followed by 30 cycles of the amplification steps including 94^{0}C for 60 sec, 47^{0}C for 60 sec and 72^{0}C for 45 sec and finally extension at 72^{0}C for 10 min. The PCR amplicons were detected on a 1% agarose gel electrophoresis.

Cloning and Sequencing: PCR products were cloned into TA cloning vector, pTZ57R/T (InsTAclone™ PCR cloning kit; Fermentas, MD, USA) according to the manufacturer's protocol. The recombinant plasmid DNA was extracted by using FastPlasmid™ Mini kit (Eppendorf, Hamburg, Germany), following the manufacturer's instructions. Plasmid DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo-scientific, Wilmington, DE, USA). DNA sequencing was done by 1st BASE DNA sequencing services, Malaysia, using M13F (-20) primer (5' GTAAAACGACG GCCAGT 3'). Two recombinant colonies per specimens were chosen for DNA sequencing. Consensus sequence was then used for further analysis.

Sequence editing, Molecular identification and Accession numbers submission

BioEdit Sequence Alignment Editor Program Version 7.0.5.3 (Hall, 1999) was used to confirm the sequence electropherograms of both clones from each individual samples and to edit the ITS2 sequence where the 5′-ends and 3′-ends of each sequences were determined according to the sequences submitted by Tautz et al. (1988) and Song et al. (2008[b]).

The ITS2 sequences were compared with nucleotide sequence database collected in NCBI by optimizing highly similar sequences (megablast) using BLASTN 2.2.26+ (Basic Local Alignment Search Tool; http://www.ncbi.nlm.gov/ BLAST) (Zehner et al., 2004). Species were identified by considering the maximum percentage identities. All completed sequences obtained from this step were submitted to the NCBI database to be assigned the accession numbers.

Sequence variation and Phylogenetic Analysis

The consensus sequences of each species from each region were aligned with reference sequence and the percentage of intra-specific and inter-specific divergences were computerized using Clustal W version 2.0 (Thompson et al., 1994) implemented in the BioEdit Sequence Alignment Editor Program Version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Program MEGA version 4.0.2 (http://www.megasoftware.net/; Tamura et al., 2007) was chosen to construct phylogenetic trees using the Neighbor-Joining method with the Kimura 2-parameter model (Tamura et al., 2007). The reliability of an inferred tree was tested by 1000 bootstrap and tree was rooted with *Drosophila melanogaster* (Song et al., 2008[b]).

Polymerase chain reaction-Restriction fragment length polymorphism; PCR-RFLP

The NEBcutter V2.0 web-based program (available at http://tools.neb.com/ NEBcutter2/index.php) was used to determine restriction enzymes sites with the sequences from each fly species. With the most appropriate patterns of digestion from the result of prediction (Table 3), *Xap*I restriction enzyme was chosen for RFLP in the reaction containing a final volume of 10 µl, which contained 1µl of 10X fast digest buffer (Fermentas® CA, USA), 1 µl of *Xap*I enzyme, 400 ng of PCR product and sterilized distilled water to final volume of 10 µl. The digested products were demonstrated on an 8% native polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized on a Gel Doc EQ system (Bio-Rad, CA, USA).

Results

Species identification and distribution of sarcophigid flies in Thailand

Among the 27 specimens collected from this study, 15 Muscid fly specimens consisted of 7 specimens of *M. domestica* (3 specimens from Chiang Mai, 2 specimens from Ranong and one specimen from Nong Khai and Phuket), 8 specimens of *M. sorbens* (4 specimens from Phuket, 2 specimens from Nong Khai and one specimen from Chiang Mai and Bangkok). There was a total of 9 specimens of Stomoxys flies, 3 specimens for *S. calcitrans* and *S. indicus* from Nakhon Ratchasima, 3 specimens of *S. sitiens* from Nakhon Ratchasima and 3 specimens of *H. spinigera* from Chiang Mai (Table 1).

PCR amplification

Genomic DNA extracted from thorax of fly samples in this study were appropriate for using to amplify by the primers. Size of PCR amplicons were different between species. There were 406-418 bp for *M. domestica*, 405 bp for *M. sorbens*, 437 bp for *H. spinigera*, 376-377 bp for *S. calcitrans*, 367-373 bp for *S. indicus* and 371 bp for *S. sitiens*. However, the sequence of PCR product in this step still contained a surplus of 5.8S and 28S rRNA gene which need to be trimmed out before the ITS2 analysis and comparison.

ITS2 Sequences analysis

DNA sequences length of ITS2 region, consistent with the length of amplified PCR products excluding 5.8S and 28S partial regions, from

individual fly samples in this study varied from 348 bp to 360 bp (mean 342 bp) for *Musca* species, 377 bp for *H. spinigera* and from 312 bp to 322 bp (mean 317 bp) for *Stomoxys* species. The average A+T content from all sequences was up to 76.6% (T= 37.9%, C= 10.3%, A= 38.7% and G= 13.1%). All completed sequences were submitted to the NCBI database and assigned the accession numbers as listed in Table 1

The results of the comparison of intra and inter-specific divergences showed that the percentage of intra-specific divergences ranged from 0.3-6.9 for *M. domestica*, 0.7-2.2 for *S.calcitrans* and 1.0-3.5 for *S. indicus* whereas no variation in *H. spinigera*, *M. sorben*s and *S. sitiens* was found (Table 2). When comparing each species to the same species from different countries, the percentage of sequence divergences were 0.6-6.4 and 2.8-3.7 for *M. domestica* and *S.calcitrans*, respectively, but no reference sequences of *H. spinigera*, *S. indicus* and *S. sitiens* in database for comparison were found.

Furthermore, the between species sequence comparison showed that the percentages of inter-specific divergences between *M. domestica* and *M. sorbens*, *H. spinigera*, *S. calcitrans*, *S. indicus*, *S. sitiens* were 19.2-21.2% (average 20.3%), 43.1-45.3% (average 44.3%), 31.3-34.5% (average 33.0%), 30.0-33.3% (average 31.5%) and 32.9-35.4% (average 34.1%), respectively. Percentages of the inter-specific divergences between *M. sorben*s and *H. spinigera*, *S. calcitrans, S. indicus, S. sitiens* were 46.4%, 32.5-33.0% (average 32.7%), 30.9-31.9% (average 31.3%) and 34.7%, in the order. The percentages of inter-specific variation between *H. spinigera* and *S. calcitrans*, *S. indicus*, *S. sitiens* were 41.9-42.2% (average 42.0%), 42.6-43.1% (average 42.8%) and 44.1%, respectively. When comparing *S. calcitrans* to *S. indicus* and *S. sitiens* the inter-specific divergences were 23.2-24.4% (average 23.8%) and 25.3-25.8 (average 25.6%), respectively. In the same way, the percentage of inter-specific variation between *S. indicus and S. sitiens* was 10.9-12.7% (average 11.9%).

Table 1 Muscid fly species in this study, their analyzed individual codes, source localities, sex, number of base pairs of ITS2 in each isolates, maximum identity (%) and accession number of their ITS2 sequences

Species based on Morphology with isolated codes	Sampling sites	Sex	Accession No. in each clones	ITS2 length (bp)	Species based on ITS2 sequence	% identity	References
Musca domestica							
Md1	Chiang Mai	♀	JQ811271-72	348	*M. domestica*	99	EU555401
Md2	Chiang Mai	♀	JQ811274-75	348	*M. domestica*	99	EU555401
Md3	Chiang Mai	♂	JQ811276-77	351	*M. domestica*	99	EU555401
Md4	Nong Khai	♀	JQ811278-79	359	*M. domestica*	99	EU555399
Md5	Phuket	♀	JQ811280-81	359	*M. domestica*	99	EU555399
Md6	Ranong	♀	JQ811282-83	348	*M. domestica*	99	EU555401
Md7	Ranong	♀	JQ811284-85	360	*M. domestica*	99	EF061807
Musca sorbens							
Ms1	Bangkok	♀	JQ811252-53	347	*M. sorbens*	100	EF061810
Ms2	Chiang Mai	♀	JQ811254-55	347	*M. sorbens*	100	EF061810
Ms3	Nong Khai	♀	JQ811258-59	347	*M. sorbens*	100	EF061810
Ms4	Nong Khai	♀	JQ811260-61	347	*M. sorbens*	100	EF061810
Ms5	Phuket	♀	JQ811263-64	347	*M. sorbens*	100	EF061810
Ms6	Phuket	♀	JQ811265-66	347	*M. sorbens*	100	EF061810
Ms7	Phuket	♀	JQ811267-68	347	*M. sorbens*	100	EF061810
MS8	Phuket	♀	JQ811269-70	347	*M. sorbens*	100	EF061810
Hydrotaea spinigera							
Hs1	Chiang Mai	♀	JQ811245-46	377	*S. calicitrans*	72	EU851201
Hs2	Chiang Mai	♀	JQ811248-49	377	*S. calicitrans*	72	EU851201
Hs3	Chiang Mai	♂	JQ811250-51	377	*S. calicitrans*	72	EU851201
Stomoxys calicitrans							
Sc1	Nakhon Ratchasima	♀	JQ811238-39	321	*S. calicitrans*	97	EF560191
Sc2	Nakhon Ratchasima	♀	JQ811240-41	322	*S. calicitrans*	98	EF560191
Sc3	Nakhon Ratchasima	♀	JQ811243-44	321	*S. calicitrans*	97	EF560191
Stomoxys indicus							
Si1	Nakhon Ratchasima	♀	JQ811229-30	318	*S. indicus*	93	EU851209
Si2	Nakhon Ratchasima	♂	JQ811233-34	313	*S. indicus*	95	EU851209
Si3	Nakhon Ratchasima	♂	JQ811236-37	312	*S. indicus*	95	EU851209
Stomoxys sitiens							
Ss1	Nakhon Ratchasima	♂	JQ811222-23	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213
Ss2	Nakhon Ratchasima	♂	JQ811225-26	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213
Ss3	Nakhon Ratchasima	♂	JQ811227-28	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213

Table 2 Intra and inter-specific divergences of ITS2 sequence between different isolates of Muscid flies from each regions (%)

	Md1	Md2	Md3	Md4	Md5	Md6	Md7	MdA	MdB	MdC	Ms1	Ms2	Ms3	Ms5	MsA	Hs1	Sc1	Sc2	Sc3	ScA	Si1	Si2	Si3	Ss1
Md1	-																							
Md2	1.8	-																						
Md3	0.9	0.9	-																					
Md4	4.5	6.1	5.3	-																				
Md5	4.2	5.9	5.0	0.3	-																			
Md6	0.3	2.0	1.2	4.8	4.5	-																		
Md7	5.3	6.9	6.1	1.4	1.2	5.6	-																	
MdA	4.8	6.4	5.6	0.9	0.6	5.1	1.7	-																
MdB	1.8	3.5	2.6	5.6	5.3	2.1	4.8	5.9	-															
MdC	5.3	5.3	4.5	2.5	2.3	5.6	3.3	2.8	6.4	-														
Ms1	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	-													
Ms2	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	-												
Ms3	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	-											
Ms5	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	0	-										
MsA	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	0	0	-									
Hs1	43.8	43.1	43.5	45.3	45.0	44.0	45.3	45.0	43.8	44.8	46.4	46.4	46.4	46.4	46.4	-								
Sc1	32.2	31.3	31.9	34.2	33.9	32.2	34.1	33.9	32.2	33.7	32.7	32.7	32.7	32.7	32.7	41.9	-							
Sc2	32.5	31.6	32.2	34.5	34.2	32.5	34.4	34.2	32.5	33.9	33.0	33.0	33.0	33.0	33.0	41.9	0.7	-						
Sc3	32.3	31.4	32.0	34.3	34.0	32.3	34.2	34.0	32.3	33.7	32.5	32.5	32.5	32.5	32.5	42.2	2.2	2.2	-					
ScA	33.1	32.5	33.1	35.1	34.8	33.1	35.0	34.8	33.1	34.8	33.7	33.7	33.7	33.7	33.7	41.1	3.4	2.8	3.7	-				
Si1	31.2	30.7	31.2	33.3	33.0	31.2	33.0	33.0	31.0	33.0	31.9	31.9	31.9	31.9	31.9	43.1	23.7	23.9	23.2	24.1	-			
Si2	30.6	30.0	30.6	32.7	32.4	30.6	32.2	32.4	30.1	32.4	30.9	30.9	30.9	30.9	30.9	42.6	23.5	23.8	23.3	24.2	2.6	-		
Si3	30.6	30.0	30.6	32.7	32.4	30.9	32.2	32.4	30.1	32.4	31.2	31.2	31.2	31.2	31.2	42.8	24.1	24.4	23.9	24.8	3.5	1.0	-	
Ss1	33.5	32.9	33.5	35.4	35.2	33.5	34.9	35.2	33.2	35.2	34.7	34.7	34.7	34.7	34.7	44.1	25.6	25.8	25.3	26.2	10.9	12.1	12.7	-

Codes in this table are corresponding with Table 1

Md1-7, *Musca domestica*; MdA, *Musca domestica* from China 1 (EF061808); MdB, *Musca domestica* from China 2 (EU555394); MdC, *Musca domestica* from Brazil (EF560189); Ms1-5, *Musca sorbens*; MsA, *Musca sorbens* from China (EF061810); Hs1,*Hydrotaea spinigera*; Sc1-3, *Stomoxys calcitrans*; ScA, *Stomoxys calcitrans* from Brazil (EF560191); Si1-3*Stomoxys indicus*; Ss1, *Stomoxys sitiens*

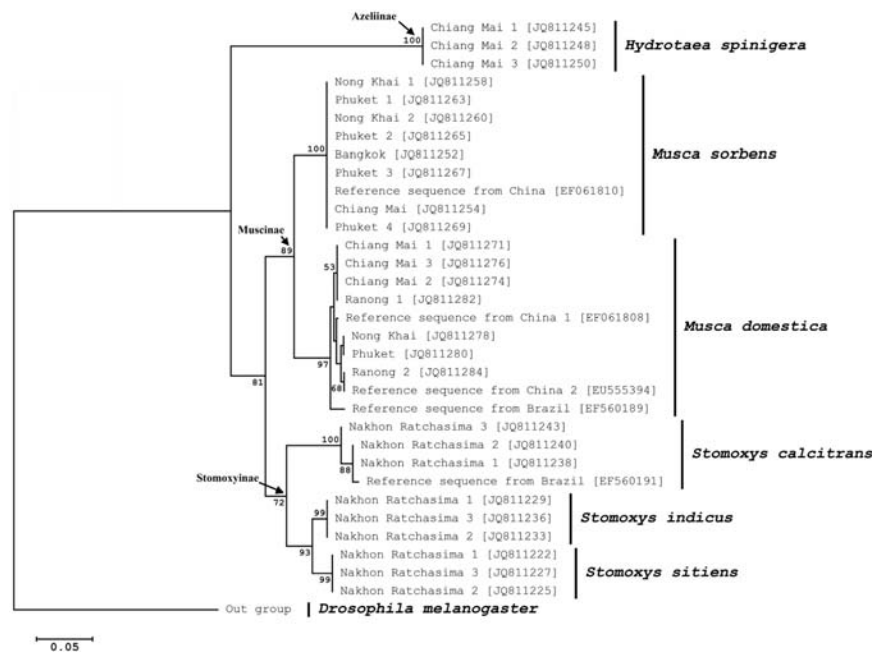

Figure 2 The 1000 bootstrapped Neighbor-Joining tree under the Kimura's 2-parmeter model based on ITS2 region sequence of four species of Muscidae flies and rooted with *D. melanogaster*.

Phylogenetic analysis

Consensus sequences of ITS2 region of Muscid flies obtained from this study together with sequences available from NCBI were used for constructing phylogenetic tree by using a 1,000 bootstraped Neighbor-Joining method under the Kimura 2-parameter and rooted with *D. melanogaster* (Song et al., 2008[b]) (Fig 2). The constructed phylogenetic tree demonstrated that ITS2 sequences could not only clearly separate Muscid flies in this study at subfamily level which consisted of clade of subfamily Azeliinae (bootstrap test 100%) consisting of *H. spinigera*, clade of subfamily Muscinae (bootstrap test 89%) consisting of *M. domestica* and *M. sorbens* and clade of subfamily Stomoxyinae (bootstrap test 72%) consisting of *S. calcitrans*, *S. indicus* and *S. sitiens*, but also separate Muscid flies deep to species level. Moreover, the same species from various regions in Thailand and other countries were clustered as individual clades with 97-100% bootstrap test. Although the clade of *M. domestica* and *S. calcitrans* were polyphyletic group, each was grouped together as well with 97-100% bootstrap supporting and clearly separated from their sister group (Fig 2).

PCR-RFLP

Based on the prediction of restriction site on ITS2 sequence using the NEBcutter program, *Xap*I restriction enzymes were appropriate for differentiation of Stomoxinae fly species in this study. The expected patterns of digested sequence in each species were summarized in Table 3. PCR-RFLP patterns of ITS2 fragment digested by enzyme *Xap*I were shown on an 8% native polyacrylamide gel electrophoresis (Fig 3-4).

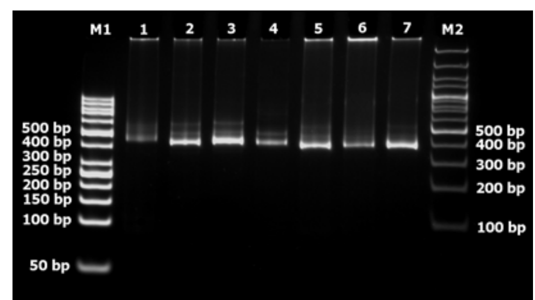

Figure 3 8% native polyacrylamide gel showed the undigested PCR products of ITS2 fragment. From left to right: lane M1 is 50 bp DNA standard marker; lanes 1–3 are PCR products from *S. indicus* (Si1, Si2 and Si3); lane 4 is PCR products from *S. sitiens* (Ss1); lanes 5-7 are PCR products from *S. calcitrans* (Sc1, Sc2 and Sc3); Lane M2 is 100 bp DNA standard marker.

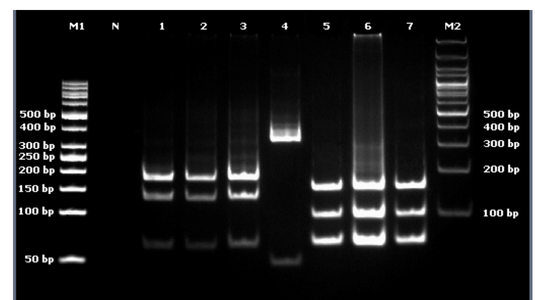

Figure 4 8% native polyacrylamide gel showed the different PCR-RFLP patterns of ITS2 fragment digested by enzyme *Xap*I. From left to right: lane M1 is 50 bp DNA standard marker; lane N is negative control; lanes 1–3 are digested PCR products from *S. indicus* (Si1, Si2 and Si3); lane 4 is digested PCR products from *S. sitiens* (Ss1); lanes 5-7 are digested PCR products from *S. calcitrans* (Sc1, Sc2 and Sc3); Lane M2 is 100 bp DNA standard marker.

Table 3 PCR-RFLP patterns among 3 species of Stomoxys flies in this study.

Flies species	Sample codes	*Xap*I digested patterns (bp)
Stomoxys indicus	Si1	179, 132, 62
	Si2	179, 127, 62
	Si3	179, 126, 62
Stomoxys sitiens	Ss1	322, 49
Stomoxys calcitrans	Sc1	154, 95, 65, 62
	Sc2	154, 95, 66, 62
	Sc3	154, 95, 65, 62

Discussion

This study determined the use of the ITS2 of rDNA region as a genetic marker to identify Muscid fly species. Twenty-seven fly samples collected from various regions of Thailand were identified based on morphology into 6 species consisting of *H. spinigera*, *M. domestica, M. sorbens*, *S. calcitrans*, *S. indicus* and *S. sitiens*. The ITS2 sequences of these 6 species were varies in length from 312 bp (*S. indicus*) to 377 bp (*H. spinigera*) average 341.45 bp. Moreover, these sequences had a high average of A+T content up to 76.6% (T= 37.9%, C= 10.3%, A= 38.7% and G= 13.1%). These high A+T content results are closely similar to recent reports on other flies such as *D. melanogaster* (80%) (Tautz et al., 1988) and Simuliidae (71-83.8%) (Thanwisai et al., 2006), but are quite different to some mosquito species which had a high G+C content such as *Anopheles crucians* (56.12%) (Wilkerson et al.,2004) and *Culex quinquefasciatus* (58%) (Severini et al.,1996). The high A+T content found in this study was caused by a repetitive region of A or T bases.

The results of comparison of ITS2 with reference sequences in NCBI indicated that the 21 morphology based identified samples of *M. domestica, M. sorbens*, *S. calcitrans* and *S. indicus* were matched with morphology identification with 93-100% identity. However, mismatching was found in 3 samples of *S. sitiens*, showing higher identity with *S. indicus* (90%) than *S. sitiens* (82%), because ITS2 sequence of *S. sitiens* in NCBI are partial sequences but *S. indicus* are completed sequences. Due to no reference sequence in *H. spinigera*, 3 sequences of these species were matched with a close species (*S. calcitrans*) with 72% identity.

Referring to a 1,000 bootstraped Neighbor-Joining with Kimura 2-parameter tree constructed based on ITS2 sequences, 27 samples of Muscid flies were separated into individual clade of each species with bootstrap supporting 97-100% as shown in Fig 2. In addition, this NJ tree could group flies species into 3 subfamilies consisting of Azeliine, Muscinae and Stomoxyinae with 72-100% bootstrap test. In species-level, ITS2 sequences from various regions in Thailand and other countries of *H. spinigera*, *M. sorbens*, *S. indicus* and *S. sitiens*, were each grouped into monophyletic clade. The paraphyly occurred in the clade of *M. domestica* and *S. calcitrans,* but no significance of variation pattern was found because it occur even in the same region. Although the paraphyly occurred in both clades, the NJ tree showed that all isolates of each species were grouped together into monophyletic clade by the high percentage of bootstrap (97% and 100%) and well separated from the sister group. These results indicated that NJ tree based on ITS2 could be used to identify 6 Muscid flies in this study at species level, but had low ability to differ same species from different regions.

According to intra-species variation analysis, the results showed no intra-species variations in *M. sorbens*, *H. spinigera* and *S. sitiens*. In contrast to *M. domestica, S. calcitrans* and *S. indicus*, intra-specific variations between Thai strains were 0.3-6.9%, 0.7-2.2% and1-3.5%, respectively. A maximum intra-species variation (6.9%) was found in *M. domestica* caused by indeling of short sequences (ATATTC, ATAATA, ACT). These results were similar when comparing to sequences from China (Accession no. EF061808) and Brazil (Accession no. EF560189). The results were also similar to recent report from Song et al. (2008[b]) which found that intra-specific divergence between *M. domestica* was high up to 5.34% and was caused by indeling of short sequences. Moreover, the intra-specific divergence between Thai and other country species (China and Brazil) ranges from 0-6.4% which causes a paraphyly in clade of *M. domestica* and divides Brazilian species out from Asian species. Although the intra-species variation was observed, each species was well grouped by the NJ tree into monophyletic clade and clearly separated from sister group.

The opportunity of species misidentification occurred when two species were similar or was a close species (Song et al., 2008[b]). Referring to phylogenetic tree and inter-specific divergence analysis as shown in Table 2, the closest species in this study was found in couple of *S. sitiens* and *S. indicus* with average inter-species divergence of 11.9%. Although the average inter-species divergence of this couple indicated that the ITS2 sequences were closely similar, the NJ tree showed that the monophyletic clade of each was clearly separated with 99% bootstrap supported.

According to a report from Wells and Stevens (2008), the intra- and inter-specific divergence are the important key for the differentiation of closely related species because if the inter-specific divergences are less than the intra-specific divergence, each clade of the sister group will be overlapped by phylogenetic tree construction. The results in this study showed no percentages of inter-specific divergence that were less than intra-specific divergence.

Identification of stable fly' species based on morphological characters by using taxonomic keys is based on body color, dorsal abdominal pattern, even in male genitalia (Masmeatathip et al., 2006). However, this procedure does not only require highly experienced person but also a complete fly samples. Moreover, some morphology still remains unclear for identification (Masmeatathip et al., 2006). The PCR-RFLP was used to demonstrate the use of molecular tool to differentiate three *Stomoxys* species in Thailand. The prediction of appropriate restriction

enzyme to differentiate these three species by using the NEB cutter web-based program resulted in the digestion patterns by *Xap*I (

Table), which can clearly discriminate these three species by the good resolution of different amount and length of DNA fragment as shown in Fig 4.

In conclusion, ITS2 gene region can be used as genetic marker to differentiate veterinary and medically important Muscid fly species in this study. The sequences of this region showed the ability to identify flies in this study deep to species level. PCR-RFLP can be used for screening of three *Stomoxys* species in this study. Although molecular tools were likely to be the effective method for differentiation between the fly species from this study, small sample size may not represent closely related species. Therefore, extensive survey for medically and veterinary important Muscids fly species in Thailand need to be investigated.

Acknowledgements

This work was supported by The Thailand Research Fund to W. Choochote (TRF Senior Research Scholar: RAT 5480006); Ratchdapisak Sompoj Grant (RA51/53), Faculty of Medicine, Chulalongkorn University and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1160A-55).

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