CHARACTERIZATION OF *STOMOXYS* SPP. (DIPTERA: MUSCIDAE) POPULATION IN CENTRAL THAILAND

INTRODUCTION

Two most important flies that develop on confined animal operations, in terms of their impact on human and animal welfare are the house fly, *Musca domestica* L. and the stable fly, *Stomoxys calcitrans* (L.). The stable fly tends to have different preferences for developmental habitats on shelters and dairies than the house fly (Meyer and Petersen, 1983). Annual losses caused by stable flies to the dairy industry alone are estimated at $59 million (Anonymous, 1979).

The biting fly *Stomoxys* spp. is an economically important pest of cattle and several studies have attempted to estimate their economic impact on cattle production. High level density of flies can lead to significant reductions in weight gains of livestock and milk production (Bruce and Decker, 1958; Miller *et al.*, 1973; Campbell *et al*., 1977, 2001; Wieman *et al.* 1992). They represent a serious nuisance not only because of their painful bites and the blood predation, but they can also be vector of pathogens (Zumpt, 1973; Foil *et al*., 1983; Skidmore, 1985; Freitas and Romero, 1991; D'Amico *et al.*, 1996; Foil and Gorham, 2000; Melo *et al*., 2001). *Stomoxys calcitrans* has been found to be a vector of *Trypanosoma evansi* (agent of surra), *Trypanosoma brucei* and *Trypanosoma vivax* (agent of nagana). Equine infectious anaemia may also be transmitted by these flies. *Stomoxys* can also serve as an intermediate host for *Habronema* spp. worm larvae. Hosts of these blood-feeding flies include cattle, horses, people, dog and swine. When the number of flies is too large, these is a nuisance for human and animal, thus a public health problem is possible.

At present, knowledge about stable fly as well as other *Stomoxys* spp. is very limited, eventhough they are cosmopolitan livestock pests and attacking up to 30 different species including mammals, birds, reptiles and even amphibians (Bishop, 1913; Surcouf, 1923; Hoskins, 1933; Hafez and Gammal-Eddin, 1959; Greenberg, 1971).

No study on the dynamics of *Stomoxys* spp. populations has ever been conducted in Thailand, eventhough they are suspected to be an important vector of animal trypanosomoses by the local authorities. Due to a favourable hot and humid climate, stable flies and other Stomoxyine species are present in all livestock farms. Yet the knowledge on their morphology and biology is a rarity, only few information on biology and effects of some juvenile hormone on *S. calcitrans* was first studied and reported in Thailand by Ketavan and Kanjanamungsuk (1987).

This will be the first investigation on *Stomoxys* spp. started in Thailand with the objectives of making the inventory of different species presented in the country. It is anticipated that a better knowledge of their biology and ecology will help to an effective control of populations and to reduce their pathogenic and economic impacts. Therefore, this study was designed to reveal on the morphological characteristics as well as population dynamics of *Stomoxys* spp. found in Thailand especially in central Thailand where livestock is one of the most important careers for people.

OBJECTIVES

1. Inventory of Stomoxyine flies found in central Thailand

2. Comparative morphological studies of Stomoxyine flies found at Nakhonpathom, Kanchanaburi and Saraburi provinces

3. Study on seasonal abundance of Stomoxyine flies found in central Thailand at Nakhonpathom province

4. Preliminary study on molecular characteristics of *Stomoxys calcitrans* (L.)

LITERATURE REVIEWS

General Characteristics of Stable Fly

Taxa of *Stomoxys calcitrans* (L.)

According to Zumpt (1973), the taxa of *Stomoxys calcitrans* (L.) is as follow:

Phylum Arthropoda
Subphylum Mandibulata
Class Insecta
Subclass Pterygota
Order Diptera
Suborder Cyclorrhapha
Division Schizophora
Section Calypterate
Family Muscidae
Subfamily Stomoxyinae
Tribe Stomoxini
Genus *Stomoxys* Geoffroy 1762
Species *calcitrans* Linnaeus 1758

The stable fly, *Stomoxys calcitrans* (L.) is the most familiar and widely distributed member of its genus and the only one to occur in the New World and can be recognized by their mouthparts which are modified into a piercing organ.

It is known by several common names including stable fly, biting house fly, dog fly, barn fly and power mower fly (Hall and Smith, 1986). In general appearance the flies of this genus all resemble house flies except that the mouthparts form a rigid, shiny black proboscis protruding forward from the lower side of the head.

Stomoxys spp. (Diptera: Muscidae) are about the same size as the house fly, but can be easily distinguished by the mouthparts, black proboscis which is used to pierce the skin and imbibe blood. Both males and females are blood-sucking muscid flies that attack domestic and wild animals throughout the world and sometimes human beings. The proboscis protrudes bayonet like in front of the head.
The antennal arista, unlike that of the house fly, bears hairs on the upper side only (James and Harwood, 1969). The thorax is grey with 4 longitudinal dark stripes. Owing to similarity in color and size, the stable fly is often mistaken by the house fly, *Musca domestica* L. However, the former is more robust and has a broader abdomen. The stable fly has a broader abdomen than the house fly and there is a checkerboard of dark spots on the second and third segments of the abdomen. In color, the stable fly is brownish gray with a greenish-yellow sheen, the outer of the four longitudinal thoracic stripes are broken and the abdomen is more or less checkered.
The wings when at rest are widely spread apart at the tips and are distinctly iridescent, the apical cell is open.

Feeding Habits

Although *S. calcitrans* is commonly called the “stable fly,” it occurs much less abundantly around stables, at least in many areas, than does the house fly. It is sometimes called the “biting house fly” because it may occur indoors, especially in the autumn and during rainy weather and at such times it bites human beings viciously. It is often very annoying along the sandy, vegetation-strewn shores of lakes and along the seashore where seaweed is windrowed by the tides. The name “dog fly” is sometimes applied to this species. The stable fly is typically an out-of-doors, day-biting fly and is usually to be found in abundance during summer and autumn where large numbers of domestic animals occur, horses and cattle affording an abundant food supply. Sunny fences, walls and light-colored surfaces in general, when in the proximity of animals are abundantly frequented by stable flies.

Populations are largest where wet plant debris has accumulated in compact mounds. When piles of plant parts have repeated additions of new material, an excellent source of these flies is maintained. If frequent rains and high temperatures prevail, the climate is especially favorable.

Adult stable flies rest in the shade near their larval sites on all sorts of surfaces. Poles, posts, slatted fences, wire fences, trunks of trees and sides of buildings are particularly favored perches. They are very sluggish and are not readily dislodged from resting sites unless they are ready for feeding. Several observers have reported intense activity at dawn during warm weather with a second period of activity in late afternoon.

The flies feed on all sorts of mammals and the choice is in proportion to availability of feeding sites on the body. An active animal is more likely to be visited than a sedentary one, the ears and lower legs of domestic animals are sites for feeding. Humans are often attacked about the ankles, but any exposed part of the body may become a feeding site. Both sexes of flies feed on blood, with little other food except succulent fruit or moisture on fresh manure. Mitzmain (1913) has recorded the act of feeding on human skin as follows: The fly punctures the epidermis and the capillary region of the dermis. It does so by the action of heavy, blade-like rows of denticles located along the inner face of the labella. The flexible labella at the end of a rigid labium are everted by hydrostatic pressure to expose the cutting teeth so they may be applied to the skin. By rapid eversion and inversion of the labella, the teeth cut into the skin and the labium is forced farther into the wound. A rapid twisting of the labella first one way then the other aids in the cutting. Within a short time the slender, anterior part of the labium is completely inserted. An undisturbed fly was seen to aspirate blood for over 12 minutes, though much of it passed on through the body and was immediately voided as a watery fluid. Many flies make preliminary punctures in the skin on one or more animals in a herd before beginning to feed and for this reason their mouthparts have potentiality as carriers of contaminants. After a feeding site is vacated,

the puncture exudes blood for some time and other muscids often feed on this surface blood.

The stable fly is a vicious biter that draws blood quickly and fills to full capacity in 3 to 4 minutes if undisturbed, but ordinarily, even when undisturbed, it changes position frequently or flies from one animal to another, where the meal is continued. Characteristic interrupted feeding makes it an excellent mechanical vector of pathogens. The fly feeds readily on many species of mammals such as rats, guinea pigs, rabbits, monkeys, cattle, horses and man. The flight of the stable fly is direct and swift and of long range, the fly sometimes traveling many miles (James and Harwood, 1969).

Biology of Stable Fly

In Thailand, Ketavan and Kanjanamungsuk (1987) reported that under laboratory condition of 25 ± 1°C, 88 ± 1% RH, stable fly adults were fed on cow blood while the larvae were reared with cow dung and cow blood. The developmental period in one generation of *S. calcitrans* showed no difference when using 2 different larval media, i.e. 27 days in both diet. It was also recorded that the longevity of stable fly female was slightly longer than those of male, 1 to 31 and 2 to 27 days for female and male, respectively. The percentage of survival of *S. calcitrans* from egg to adult was 57.25% when the stable fly larvae were fed on cow dung at 25 ± 1°C. Average egg laid per female obtained was 204.

Although the stable fly can be successfully reared in the manure of horses, cattle and sheep, it may be safely said that it does not breed commonly in excrement under field conditions unless this is mixed with decaying vegetable matter, straw or hay. Very good breeding places are afforded by the leftover soggy hay, alfalfa or grain in the bottoms of or underneath out-of-doors feed racks in connection with dairy shelters. This material becomes soggy and ferments and here virtually pure cultures of stable fly larvae may be found.

Flies begin to oviposit after taking about three blood meals and they continue to do so at intervals for 30 to 60 days. Gravid flies move to oviposition sites and then crawl into a pile of straw where odors of the wet, decaying materials are strong. Sometimes a female will go 2 to 3 inches below the surface and several may use the same tunnel into the fetid mass. Eggs may be deposited within 9 days after the adult emergence. An entire generation can be completed within a month or less (Horsfall, 1962).

The eggs of the stable fly are about 1 mm long, curved on one side and straight and grooved on the opposite side. In depositing her eggs, the female fly often crawls far into the loose material, placing them usually in little pockets in small numbers, often in pairs. Eggs depositions range in number from 23 to 100 (Parr, 1962; Hall and Smith, 1986). Females can deposit up to 600 eggs during their lifetime (Killough and McKinstry, 1965).

The incubation period varies from 2 to 5 days, at 26°C. Higher temperature result in a shorter incubation period, 2 days at 31°C. At temperatures of 25°C to 31°C the larvae reached their full growth stage in about 10 days (Ketavan and Kanjanamungsuk, 1987).

Larvae are whitish-yellow maggots, with 12 visible segments and a single mouth hook, measure from 1.5 mm (first instar) to 11.5 mm (third instar) in length. The larvae of the stable fly can readily be recognized by the form of posterior spiracles which is roughly triangular, widely separated from each other.

Before pupation the larvae usually crawl into the drier parts of the breeding medium, where the chestnut-colored puparia may be found in enormous numbers. The puparia are 6 to 7 mm long and may be recognized by the posterior spiracles as in the larvae (Harwood and James, 1979). The pupal period including the last larval stage that begins with the formation of the puparium varies depending on temperature. At 21°C to 26°C, this period varies from 5 to 26 days, with the greatest frequency 9 to 13 days (James and Harwood, 1969). Pupae are reddish to blackish brown

and retain the morphological features of the third instar larvae (Zumpt, 1973).

The total time for development of the stable fly from egg laying to emergence of adults was determined by Herms and James (1961) to be 33 to 36 days at 21°C and 27 days at 25 ± 1°C (Ketavan and Kanjanamungsuk, 1987).

As reported by Hoffman in 1968, adults are attracted to blood sources 1 day post-emergence. Both male and female stable flies are obligate blood feeders. Males consuming blood meals of 10.7 mg per fly and females 14.6 mg per fly. Females are anautogenous and males require a blood meal to attain sexual maturity (Anderson, 1978). Stable fly adults feed to capacity in 3 to 4 minutes. Blood meals may be up to 3 times the flies body weight. Adults reach sexual maturity 32 to 40 hours post-emergence and most are mated by 5 days. Males are polygamous, inseminating an average of 6 females and a maximum of 23 females in laboratory studies. Females are normally monogamous (Harris *et al*., 1966). The first egg batch is mature about 2 days after copulation. Oviposition sites include a range of decaying organic matter (Hall and Smith, 1986).

Longevity

Stable flies, like all Diptera, have holometabolous development. Larvae hatch from the eggs in 28 (24 to 28) hours at 22°C and 50-80% RH. First instar larvae molt to the second instar in less than 24 hours. Second instar larvae molt to the third instar 1 day later under optimal conditions.

Larval development ranges from 8 days (26°C and 80% RH) during the summer months to several months during the winter.

At 21 to 26°C, the pupal stage lasts from 6 to 26 days (Parr, 1962; Harwood and James, 1979). Once emerged, adults move rapidly for up to 15 minutes and then remain stationary for a period while the integument hardens.

Developmental period from egg to adult ranges from 12 days to 25 days. This was measured with approximately 4,000 flies under continuous daily observation in laboratory (Parr, 1962; Hoffman, 1968; Harwood and James, 1979).

Distribution and Abundance

Stomoxys are native to the Old World. However, specific information on the distribution and dispersal pattern of the *Stomoxys* fly, an important step in developing a pest management strategy is generally lacking (Gersabeck and Merritt, 1985). Hogsette and Ruff (1985) documented a wind assisted flight range of 225 km in Florida. Eddy *et al*. (1962) recovered marked adults 5 miles from the release point within 2 hours after release. Gersabeck and Merritt (1985) calculated a potential adult lifetime migration radius of 140 km. This was based on movement of 7 km per day (Bailey *et al*., 1973). Movement of stable flies in time and space is probably a function of host activity patterns, duration of feeding and potential of the insect to fly (Gersabeck and Merritt, 1985).

Problem and Economic Importance

Stomoxys calcitrans (L.) is an important economic pest of beef and dairy cattle. They stress hosts by blood feeding activity and reduce weight gain (Estienne *et al*., 1991; Weiman *et al*., 1992). The importance of this insect as a pest is not restricted to livestock. The stable fly is a human pest at recreational areas (Jones *et al*., 1987) and its pest status is increasing with encroachment of urban developments into rural areas (Hall and Smith, 1986; Thomas, 1993). Further, the haematophagous behavior of the stable fly may facilitate transmission of some pathogenic organisms (Harwood and James, 1979).

The Stable Fly as a Cattle Pest

The stable fly is an economically important pest. Biting activity reduces weight gain and feed efficiency in cattle (Estienne *et a*l., 1991; Weiman *et al*., 1992). Bishopp (1913) regards this fly as one of the most important sources of annoyance to livestock. Injury is brought about in various ways, for example worry caused by the mass attack of flies, loss of blood and loss of flesh. Freeborn *et al*. (1925) have shown that the reduction in milk production caused by the stable fly amounted to 9.2 percent, which, for a 5 month period means a loss of 50 gallons of milk per lactating animal.

Though the role of the stable fly as a vector of surra is purely a mechanical one, its transmission by this and other blood-sucking flies, particularly *Tabanidae*, is quite important. Mechanical transmission of *Trypanosoma brucei*, *T. rhodesiense* and *T. gambiense* also occurs. Eventhough, the fly is infective for only a brief period of time, the restless, intermittent type of feeding characteristic of the stable fly is very conducive to this type of transmission. On rare occasions, the stable fly becomes involved in accidental traumatic myiasis or enteric pseudomyiasis in man.
Adequate methods of control require thorough knowledge of the biology and ecology of this fly (Batra, 1982).

Control Strategies

Control programs developed for stable flies on confined livestock installations stressed the importance of sanitation in eliminating developmental sites (Rogoff, 1978). Sanitation, by reducing oviposition and larval developmental sites can be an effective means of control (Gilbertson, 1986). However, insecticides in most cases became the first line of defense. Residual insecticides are often used on stable fly resting areas to provide control (Campbell and Hermanussen, 1971).
It was therefore, not surprising to learn of the rapid development of insecticide resistance to the commercially available insecticides (Georghiou and Bowen, 1966; MacDonald *et al*., 1983).

In light of the existing problems with chemical control of stable flies, entomologists began to investigate the role of biological control agents, particularly the use of hymenopterous pupal parasites belonging to the family Pteromalidae, as a supplement to environmental and chemical control. Geetha-Bai and Sankaran (1977) conducted a survey for natural enemies of house fly and stable fly developing in dairy manure heaps in India. They reared three species of pteromalid pupal parasites, *Spalangia cameroni* Perkins, *Spalangia endius* Walker and *Spalangia nigroaenea* Curtis and one chalcid parasite, *Dibrinus trichiophthalmus* Masi. *Spalangia cameroni*, *S. endius* and *S. nigroaenea* were the only species which parasitized both house fly and the stable fly, supporting the worldwide pattern established for these species of parasites.

Ketavan and Kanjanamungsuk (1987) found 2 hymenopteran pupal parasites from the stable fly colony reared in the laboratory, i.e. *S. endius* Walker and *Pachycrepoideus vindemiae* (Rondani) as well as predator mite: *Macrocheles muscaedomesticae* (Scop.). Petersen and Meyer (1983) conducted an intensive study of the host preference and seasonal distribution of parasites associated with the stable fly complex on confined livestock installations in eastern Nebraska. Seven species of parasites were recovered including *Muscidifurax* spp. (*M. raptor* and *M. zaraptor* Kogan and Legner), *S. nigroaenea*, *S. cameroni*, *S. nigra*, *S. endius* and *P. vindemiae* (Rondani).

The natural enemies of muscoid flies breeding in the dung of domestic animals have been surveyed worldwide by E.F. Legner and co-workers (Legner *et al*., 1967; Legner and Olton, 1968). The successful biological control of *Stomoxys* spp. in Mauritius supports the belief that puparial parasitoids of muscoid flies show strong habitat preferences and indicates opportunities for finding parasitoids for the control of these flies in dispersed breeding sites (Greathead and Monty, 1982).

Genetic control strategies offer an environmentally safe alternative to insecticides for insect control. One strategy, sterile insect release method (SIRM), is based on the introduction of sterilized insects into a population to suppress or eradicate by reducing population fertility. However, stable fly control technology including sanitation and insecticides is generally unsatisfactory (Hoffman, 1968).

Stable Fly Genetics

Advances in biochemical and molecular methods have been increasingly given opportunity to visualize variability among individuals for gene products and for genes themselves. Firstly, since the protein electrophoresis technique was developed in 1968 and became the first widely used genetic survey technique for detecting molecular level variants. Then, DNA markers were developed and widely used. They revealed more powerful than protein electrophoresis and they have greatly facilitated research in genetic mapping, marker assisted selection, genome fingerprinting and for investigating genetic relatedness in plants and other organisms. Elucidation of population structure with protein and nucleic acid markers from geographical and temporal population samples may provide information for future control strategies.

Stable flies have 5 pairs of homomorphic chromosomes (Boyes, 1967; Joslyn *et al*., 1979; LaChance, 1964). Chromosomes 1 and 3 are metacentric while 2, 4 and 5 are submetacentric. Sex is controlled by loci located on chromosome 1 (Willis *et al*., 1981). Crossing over is reduced or absent in males (Joslyn *et al*., 1979).

Species Diversity

Biodiversity

Biodiversity involves the number of species on earth including plants, animals and microorganisms. But biodiversity exists at many different levels from the genetic diversity within individual local populations of a species or between geographically distinct populations of the same species and all the way up to communities or ecosystems (Hawksworth, 1995).

Morphological Diversity

Morphological divergence between populations and species are continuous and differs quantitatively rather than qualitatively. Differences among organisms are mostly depended on morphology and thus the similar characters known as homologies are commonly used to classify organisms especially for phylogeny studies (Gaston, 1996). However, there are limitation when comes to the species level especially among sibling species groups. Consequently other means for identification are employed for example, a molecular approach that greatly improves the accuracy of identification. Molecular methods for species identification have received great attention in recent years.

Genetic Diversity

Genetic diversity is a very important factor for species evolution and adaptation to the environmental changes. Species, in their most basic sense, are evolving lineages and maintain their capacity to evolve by acquiring genetic diversity. Their array of adaptations and ecological requirements are the products of this part of genetic diversity and subsequently species diversity.
Among the adaptations of a species are its habitat needs and its responses

to other species and these adaptations in turn are the basis for much of community structure (Hawksworth, 1995). Diversity of both within and between species can be examined by a variety of genetic methods. Recently, molecular genetic techniques were developed such as isozymes and DNA marker technologies which are widely used as tools for polymorphism detection, thus enable genetic mapping, marker assisted selection, genome fingerprinting and genetic relatedness (Kirby, 1990; Li and Graur, 1991; Phillips *et al*., 1994; Gaston, 1996; Powell *et al*., 1996).

The primary cause of evolution is the mutational change of genes. Evolution is dependent on 3 components: a source of genetic variation (mutation); forces that change the frequency of alleles (selection, genetic drift); and historical components in time and space (Dobzhansky, 1937). Mutations are "errors" that occur during DNA replication resulting in changes in nucleotide sequences (Futumya, 1986). Examples include nucleotide substitutions, duplications, insertion/deletions, recombination, translocations, inversions and gene conversion and so forth. These spread through the populations by genetic drift and/or natural selection. Frequently, mutations are lethal and the probability of their establishment in a population is very low (Hartl, 1988). However, over millions of years, mutations are incorporated into the species, resulting in genetic variations (Avise, 1994). Determination of the relative importance of mutation, natural selection, genetic drift, recombination and so forth is an important subject in population genetics.

Partitioning of genetic variation within and among populations is dependent on several factors including environmental, genetic drift and population bottlenecks. Population bottlenecks are important during the establishment of new populations. Excessive contributions to the gene pool by a few individuals during this period (genetic drift) reduces the genetic variability in subsequent generations (founders effect). Localized environmental forces can reduce migration (gene flow) among populations (Hartl, 1988). These environmental forces include geographical barriers or selection pressures (i.e., temperature tolerance, predators and mate preference etc.). Genetic drift can partition variation if immigration is absent.

Finally, bottlenecks during colonization can reduce genetic variation or change gene frequencies significantly (Hartl, 1988). Population genetic research is facilitated by methods that reveal detectable genetic variations. Among which, DNA analysis can be used to estimate genetic variation within and among populations.

Evolutionists are often interested in a phylogenetic tree that represents the evolutionary history of a group of species or populations. This type of tree is called a species or population tree. In a species tree, the time of divergence between 2 species refers to the time when the 2 species were reproductively isolated.

DNA

History of Population Genetics

The evolutionary change of morphological and physiological characters is so complex that this approach does not produce a clear-cut picture of evolutionary history and the details of the phylogenetic trees reconstructed have almost always been controversial. To study the evolutionary relationships of organisms by comparing their DNA has several advantages over the classical approach in which morphological and physiological characters are used. First, DNA consists of the 4 types of nucleotides, adenine (A), thymine (T), cytosine (C) and guanine (G), it can be used for comparing any group of organisms. Second, the evolutionary change of DNA follows a more or less regular pattern, it is possible to use a mathematical model to formulate the change and compare DNAs from distantly related organisms. Third, the genome has larger amount of phylogenetic information than the morphological characters.

Two kinds of DNA that are found in a cell; nuclear and mitochondrial. Nuclear DNA (nDNA), found within the nucleus of the cell, is composed of 2 sources of DNA. One source is the egg (from the mother) and the other is the sperm (from the father). This is the type of DNA that define us as individuals and is most often used in forensic cases and or paternity identification.

The mitochondria, organelles located outside the nucleus in the cytoplasm of the cell, are responsible for energy transfer and are basically known as the "powerhouses" of the cells. This form of DNA is in circles containing non coding sequence and hence does not mutate or change form very quickly. It is relatively stable and can be compared across several generations.

The mitochondrial genome has some important characteristics. First, this genome is inherited as a single entity, its genes represent a single genetic locus. Second, the effective population size of mtDNA is smaller than that of nuclear DNA because of its haploid nature. Within a population, the frequency of a single mtDNA haplotype should fluctuate more rapidly than the frequencies of nuclear DNA alleles. Mitochondrial DNA is only passed along the maternal line.

DNA replication

DNA replication in natural systems requires the following components: 1. All four of the activated precursors (dATP, dGTP, dCTP and dTTP); 2. The DNA polymerase enzyme; 3. A short RNA molecule (primer); 4. A template DNA strand and 5. The proper condition for the reaction to occur (pH, temperature, etc.). For in vitro synthesis, DNA primers are used instead of the RNA primer utilized in natural systems and the reaction buffer is used to establish the correct condition for optimal functioning of the DNA polymerase. Magnesium ion is the cofactor for the DNA polymerase. An important feature of PCR is that the length of the product sequence is defined by the position of the 2 primers.

Isolation of Genomic DNA

High quality genomic DNA is a fundamental requirement for most genome characterization and molecular mapping. The degree of purity and quantity requirement varies from application to application. Generally, a good DNA extraction procedure should fulfill major criteria of purity, high molecular weight, simple, high throughput, cost effective and safety (Clark, 1996).

For any tissues, the cell membrane must be disrupted in order to release the cellular constituents including DNA into the extraction buffer. This step is accomplished by using a detergent such as sodium dodesyl sulfate (SDS) or cetyl trimethylammonium bromide (CTAB). Then, the mixture containing DNA is purified by a number of organic extraction step using phenol and chloroform and the DNA finally recovered by ethanol precipitation (McPherson *et al*., 1991; Clark, 1996).

Most extraction buffers contain tris buffer to maintain the pH at around 8.0, salts such as NaCl, NaOAc and NH_4Cl to aid in dissociating proteins from the DNA, detergents such as SDS or sarkosyl to solubilize the membranes and some means of rapidly inactivating DNAse. Because DNAse requires magnesium ions for activity, EDTA is often added to sequester the magnesium ions. The initial DNA extracts often contain large amounts of RNA, proteins and polysaccharides which in some cases are difficult to separate from the DNA. RNA can be removed by using RNAse. Most proteins can be removed by denaturation and precipitation from the extract, for example using chloroform and/or phenol. Polysaccharides are often difficult to be removed, when present in high concentration they may cause the DNA even at relatively low concentration may interfere in subsequent procedures. Furthermore polysaccharides can inhibit the activity of DNA - modifying enzymes such as restriction enzymes, however they may be minimized in the precipitation step (Kirby, 1990; Clark, 1996).

Fresh tissues provide the best sources of DNA for biological analyses, but their availability is often limited. Although cryopreservation (freezing) has proved to be the most effective method for preserving various tissues, the most common fixatives are formaldehyde, ethanol, paraffin, haematoxylin and eosin. These tissues could be extracted DNA by using proteinase K incubation method (Kirby, 1990; McPherson *et al*., 1991).

Separating and Sizing DNA Fragments

The technique of gel electrophoresis, in which samples are made to migrate in an electric field through a gel, usually made from the complex polysaccharide agarose buffered at a somewhat alkaline pH. The DNA fragments, which carry a virtually uniform negative charge per unit mass, migrate towards the positive end of the gradient at a rate determined by the degree to which they are retarded by the pores of the gel. The larger the size of the fragments in relation to the pore size of the gel the more slowly they will move. Increasing the agarose concentration and thus decreasing the pore size, retards larger fragments more drastically than smaller ones. Within a certain size range, which will depend on the agarose concentration, there is an approximately logarithmic relationship between fragment size and distance moved. Mixtures of fragments of known sizes are run in parallel with the experimental samples to provide points for the construction of a standard curve for size estimation.

Gel electrophoresis then resolves the mixture into discrete bands that can be easily visualized and photographed after staining with the acridine drug ethidium bromide. This reagent binds into the stack of DNA base-pairs to form a complex that fluoresces intensely in ultraviolet light.

DNA Markers

DNA is containing all genetic information. DNA markers, whose development is based on polymorphism found at DNA level, have greatly facilitated research in taxonomy, phylogeny, ecology and genetic. There are many DNA markers, which have been used, however all are divided to 2 classes of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA).

Nuclear DNA

Nuclear DNA (nDNA) has been used to distinguish between members of species populations and complexes using the polymerase chain reaction. Studies in agriculture (Black, 1993; Birch *et al*., 1994; Fenton *et al*., 1994) and forestry pests (Pfeifer *et al*., 1995) and medically-important vector of disease (Paskewitz *et al*., 1993; Townson and Onapa, 1994) have shown variation between populations and biotypes using this method.

Mitochondrial DNA

When sperm and egg fuse to form a diploid zygote, the new individual gets half of its nuclear genetic information from each parent. That 50/50 split is the basis of Mendelian inheritance. However, due to the sheer size of the egg cell all (or nearly all) of the mitochondria in the embryo come from the mother. In other words, mitochondrial inheritance is maternal.

Mitochondrial DNA (mtDNA) has been used extensively in population studies of many insect species because it evolves rapidly. But many regions are conserved enough so that primers can be used in different insect taxa. Variation in mtDNA can be examined by sequencing procedures.

Animal Mitochondrial DNA

The animal mitochondrial genome consists of a circular DNA molecular 16 kb long that encodes for 13 messenger RNAs, 22 transfer RNAs and two ribosomal RNAs (Avise and Lansman, 1983). Since these products remain in the mitochondria, selection pressures and evolution occur often independent of the nuclear genome (Moritz *et al*., 1987). The small size of the mtDNA genome has facilitated sequencing it in its entirety. Complete sequences exist for several insect species including *Apis mellifera* (Crozier and Crozier, 1993), *Anopheles gambiae* (Beard *et al*., 1993) and *Drosophila yacuba* (Clary and Wolstenholme, 1985). Besides size, the structure

and genetic basis of inheritance (maternal) of mtDNA have made it better understood than any similarly sized region of the nuclear genome (Avise, 1994).

Technique Applied

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987) has revolutionized molecular systematic and population genetic studies (Avise, 1994). This technique uses a thermostable bacterial polymerase, Taq to replicate DNA. Repeated cycling of the replication event produces millions of copies of a specific genomic region.

PCR reduces the amount of DNA required and specific primers permit amplification of specific regions of the genome.

DNA Sequencing

Nucleotides are used as character states in DNA sequencing analysis. Silent and neutral substitutions undetected by allowzyme analysis are detectable by sequencing (Hillis *et al*., 1990). Selected regions of the genome are sequenced for each individual and the sequences are aligned and compared. The nucleotide polymorphism is evaluated and used to quantify the proportion of polymorphic loci, the average number of alleles per locus and measure the genetic distances between individuals or populations.

MATERIALS AND METHODS

Morphological Characteristic Study of Adult Stomoxyine Flies

Specimen Collection Area

Stomoxys flies were collected from the cattle shelters at three locations in central Thailand, i.e. Suwanvajokkasikit Animal Research and Development Institute (SARDI), Kasetsart University, Kamphaengsaen campus, Ban Yang district, Nakhonpathom province; Thai-Denish dairy cattle farm, Moak Lek district, Saraburi province and private dairy farm, Kanchanaburi province (Fig.1).

Trapping and Collecting Method

Adult flies were captured using Vavoua traps (Laveissiere and Grebaut, 1990; Mihok, 1995) placed near the cattle shelters. Specimens were also obtained by sweep net around the shelters of cattle. The number of flies, sex, species as well as the location of capture were recorded.

Morphological Characteristics Study

Samples of *Stomoxys* flies were identified to species level using the key for identification developed by Zumpt (1973). Specimens were examined with the aid of a dissecting microscope. The principles characteristics of each species, i.e. thoracic pattern, wings, dorso-abdominal pattern and legs were described and illustrated. The frontal-index of fly species was observed as well. Certain characteristics of adult flies of each species including heads and wings were photographed by the digital camera 3X attached to microscope with 2X eye piece and 10X objective lens.

Figure 1 Three localities in central of Thailand where S*tomoxys* flies were collected; Nakhonpathom, Saraburi and Kanchanaburi province

According to Rozendaal (1997), the Vavoua trap (Fig. 2) was adapted from the model used to trap the tsetse flies by Laveissière and Grébaut (1990).
The Vavoua trap was assembled as follow:

1. Cut out 3 pieces of black and 3 pieces of blue material with the dimensions shown in figure 1 (a) and mark points A, B, C and D on the black pieces.

2. Put one black piece on the top of another and stitch along line AB.

3. Fold the upper piece along line AB and put the third piece on top. Stitch along lines CD.

4. Stitch pieces 2 and 3 together along line CD on piece 2 and line AB on piece 3 (b).

5. Stitch the 3 blue pieces to the 3 black pieces as shown in figure1 (c). Allow a seam of 1 cm. Make a seam on the lower edge of each of the 3 black-blue parts.

6. Cut out 3 pieces of mosquito netting with the dimensions shown in figure 1 (d). Join the 3 pieces together in the shape of a cone and attach the cone to the black-blue material by stitching lines EF on the cone to lines GH on the blue material (e).

7. Take a piece of metal wire measuring 250 cm in length, bend it to from a circle with a diameter of 80 cm and twist or solder the ends together. Fold the edge of the netting cone over the wire hoop, pin in place and stitch a hem around the wire (e).

8. The trap can be put up in the field by inserting a metal rod 150 cm long and 1 cm in diameter in the tube of material in the middle of the black screens (e). Place a metalic cone and a cage on top to collect the flies (see Fig. 3).

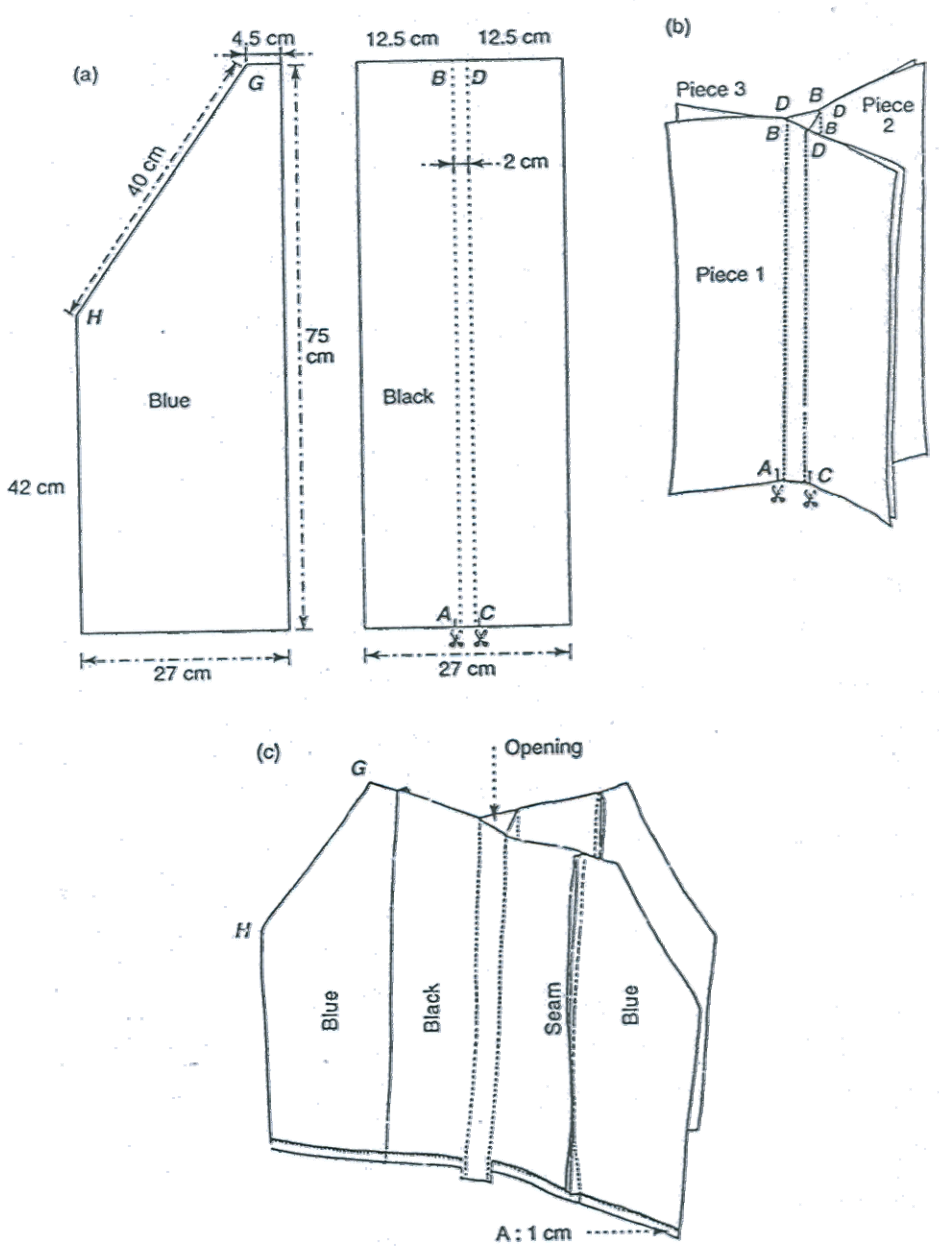


Figure 2 Assembly of the Vavoua trap

Source: Rozendaal (1997)

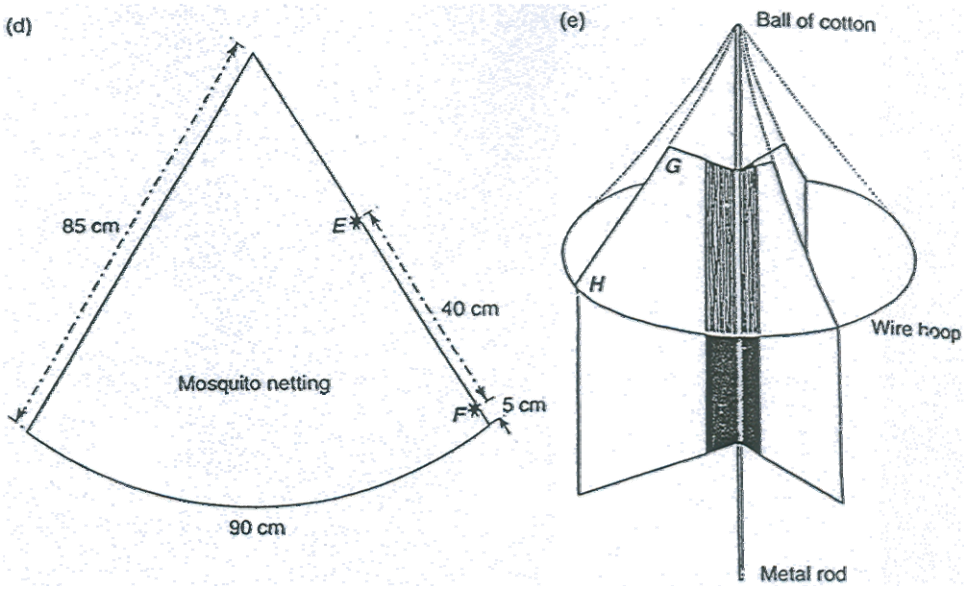


Figure 2 (Continued)

Figure 3 The Vavoua trap used for collecting S*tomoxys* flies during July 2004 to June 2005

Comparative Study of Male Genitalia of Adult Stomoxyine Flies

Comparative study of male genitalia of Stomoxyine flies collected was conducted in the laboratory. The species studied including *S. calcitrans* (L.), *S. sitiens* Rondani and *S. indica* Picard were collected from Kanchanaburi, Nakhonpathom and Saraburi provinces respectively.

The male genitalia were dissected from adult flies preserved in 95% ethyl alcohol. The samples were mounted for further study. Genitalia of each species was described and photographed by digital camera attached to microscope. The program Studio Quick Start Version 8 was used for photography. Average length and width of genitalalia was also measured by Motic Images Plus 2.0.

The male genitalia of Stomoxyine flies observed were mounted with Hoyer's medium (Distilled water 50 grams, Gum arabic (crystals) 30 grams, Chloral hydrate 200 grams and Glycerine 20 grams). The mounting procedures as described by Krantz (1975) are as follows:

1. After dissecting, keep the male genitalia in lactic acid for 24 hours.
2. Place a drop of Hoyer's medium in the center of a clean 1 x 3 micro-slide.
3. Remove the male genitalia from lactic acid and place specimen to the bottom of the droplet, arrange it on a vertical axis with a minuten pin probe. (If the specimen is on the surface of the droplet when the coverslip is applied, it will roll to the edge of the coverslip)
4. Using a clean pair of forceps, pick up a coverslip at its edge, apply the opposite edge to the edge of the Hoyer's droplet and allow coverslip to fall into place. Last second orientation of the specimen may be accomplished under the stereoscopic microscope with gentle pressure of a probe on the coverslip surface.
5. Mark the right side of the preparation with identifying number or letter.

6. Keep slide in an oven with approximate 45°C for 4 days-1 week. Temperatures above 55°C cause the medium to bubble at the edges of the coverslip, while those between 46 to 54°C may bring about excessive contraction and breakage of coverslips.

7. Heat-treated slides should be held for 1 week at room temperature to allow the extra-thin coverslip to return to its normal flat state.

8. Non-soluble material should be applied so as to seal the edge of the coverslip to the slide surface. A second coating applied after the first has dried is recommended to assure an impervious seal.

9. A locality label and an identification label should be placed where the side originally was marked. The locality label should include date, collector and locality. The mounting medium used in making the slide should be noted at the bottom of the identification label.

Seasonal Abundance and Daily Activity of Adult Stomoxyine Flies

Study Area

This experiment on seasonal abundance and daily activity of *Stomoxys* spp. was conducted in a beef and dairy cattle farm in Suwanvajokkasikit Animal Research and Development Institute (SARDI) in Kasetsart University, Kamphaengsaen Campus, Ban Yang district, Nakornpathom province (14° 01' N., 99° 58' E.), about 90 km West from the city of Bangkok (Thailand). The site is located in a plain surrounded by grassland, perennial plants and rice fields. The beef and dairy cattle farms housed 380 and 123 cattle, respectively. In this area the year is usually divided in three different seasons: wet season from June to October, dry season from November to February and hot season from March to May. Temperature, relative humidity, rainfall and radiated sunshine data were obtained from Kamphaengsaen Meteorological station.

Trapping method

The Vavoua traps (Laveissière & Grébaut, 1990) was placed near the beef and dairy cattle shelters (Fig. 3).

Daily activity and seasonal abundance

Adult flies were captured with Vavoua traps during 24 hours every 2 weeks from July 2004 to June 2005. Fly specimens were collected every 2 hours at 06:00, 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hrs (local time) respectively. The traps were left operational during the night until 06:00 am. The number of flies were recorded by collection hour, killed in a freezer and preserved in 95% ethyl alcohol. They were identified to species level in the lab in Bangkok according to Zumpt (1973). The number of flies was then recorded by date, species, sex and hour of capture.

Genetically Characteristic Study

Stable Fly Collection

Wild samples of adult *Stomoxys calcitrans* (L.) used in the study originated from 7 countries and 3 islands in 5 continents including Thailand (Asia), Canada and Columbia (America), Ethiopia, Senegal, Madagascar, Morocco, Uganda and Comoro island (Africa), France (Europe) and Nouvelle Caledonie island (Australia) (Table 1). After field capture, all stable fly were first identified on the basis of their morphology using to the key developed by Zumpt (1973). All adult specimens were preserved in ethanol at room temperature.

Molecular Markers

Mitochrondrial genes for Cytochrome oxidase I (Cox I) and Cytochrome B (Cyt-B) enzymes selected as molecular markers.

Table 1 The geographic origins collection of the stable fly, *Stomoxys calcitrans* (L.)

Country (locality)	Continent
Thailand (Nakhonpathom Province)	Asia
Thailand (Saraburi Province)	Asia
Thailand (Kanchanaburi Province)	Asia
Canada	North America
Columbia	South America
France (St. Martin de Londres)	Europe
France (La Vialasse)	Europe
Senegal	West Africa
Comoro Island (Anjouan)	Indian Ocean
Morocco	North Africa
Madagascar	Indian Ocean
Uganda	East Africa
Nouvelle Caledonie	Pacific Ocean

Extraction of DNA

Genomic DNA was extracted from single individual adult stable fly, whole specimens or fly parts such as legs and wings according to the procedure of QIAGEN Dneasy tissue kit handbook (2003).

Procedure (QIAGEN Dneasy tissue kit, 2003)

1. For specimen preserved in alcohol, remove the specimen from the alcohol and allow it to dry thoroughly either in a vacuum drier or covered on the bench at room temperature for about 5 minutes.

2. Adult of stable fly was ground with mortar and pestle in liquid nitrogen to be fine powdered.

3. The powdered stable fly was placed in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL (Tissue lysis buffer).

4. Add 20 µl Proteinase K, mix by vortexing and incubate at 55°C until the tissue is completely lysed. Lysis is usually complete in 1-3 hours or can be lysed overnight. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath and then vortex for 15 seconds.

5. Add 200 µl Buffer AL (Lysis buffer) to the sample, mix thoroughly by vortexing and incubate at 70°C for 10 minutes. (A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during the incubation at 70°C).

6. Add 200 ul ethanol (96-100%) to the sample and mix thoroughly by vortexing. (A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the Dneasy Mini Spin Column).

7. Pipet the mixture from step 6 into the Dneasy Mini Spin Column placed in a 2 ml collection tube. Centrifuge at 6,000 x g for 1 minute. Discard flow-through and collection tube.

8. Place the Dneasy Mini Spin Column in a new 2 ml collection tube, add 500 µl Buffer AW1 (wash buffer 1). Centrifuge at 6,000 x g for 1 minute. Discard flow-through and collection tube.

9. Place the Dneasy Mini Spin Column in a new 2 ml collection tube (provided), add 500 ul Buffer AW2 (wash buffer 2). Centrifuge at 20,000 x g for 3 minutes to dry the Dneasy membrane. Discard flow-through and collection tube.

10. Place the Dneasy Mini Spin Column in a clean 2 ml microcentrifuge tube and pipet 200 µl Buffer AE (Elution buffer) directly onto the Dneasy membrane. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 6,000 x g to elute.

11. Repeat elution once as described in step 10. Discard the Dneasy Mini Spin Column and then incubation the DNA extract at –20°C for keep.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR), this technique can amplify DNA sequence in between the two oligonucleotide primers complementary to the template in antiparallel orientation even starting from a single DNA molecule, detectable amounts of target DNA are generated in a few hours (McPherson *et al*., 1991; Sandifer, 1991; Karp *et al*., 1998).

PCR cycling consist of three basic steps required for any DNA synthesis reaction (Fig. 5): 1. Denaturation, to melt double stranded DNA template into single strands DNA and to eliminate secondary structure, 2. Annealing, the primers binds to the template and 3. Extension, to synthesize the new DNA strands from the primers by the polymerase adding dNTPs from 5' to 3’; bases are added complementary to the template. This step is usually carried out at 72°C, which is optimal for *Taq* polymerase (heat-stable DNA polymerase from a thermophilic bacterium, *Thermus aquaticus*). The amplification time is determined by the length of the sequence to be amplified.

The PCR conditions for Cytochrome oxidase I (Cox I) were composed of an initial hot start at 92°C for 5 minutes, followed by 5 cycles of denaturation at 92°C for 1 minute, annealing at 49°C for 30 seconds and extension at 70°C for1 minute, continues with 25 cycles of denaturation at 92°C for 30 seconds, annealing at 49°C for 30 seconds and extension at 70°C for1 minute. An additional final extension at 70°C for 7 minutes was included at the end one of last cycle.
The PCR conditions for Cytochrome B (Cyt B) were composed of an initial hot start at 94°C for 1.30 minutes, followed by 37 cycles were programmed of denaturation at 94°C for 25 seconds, annealing at 55°C for 35 seconds and extension at 70°C for 1.30 minute. An additional final extension at 72°C for 12 minutes was included at the end of last cycle. One negative control was included. To confirm amplifications, the PCR products were resolved by electrophoresis on 1.2% agarose gel stained with ethidium bromide in Tris-Borate-EDTA buffer (TBE buffer) and photographed under ultraviolet illumination.

Use of controls can provide important information regarding the efficiency and specificity of PCR. Controls should include a negative control that contains all the reaction components except the desired template. Any product obtained in this tube would indicate a problem with contamination by other template DNA.

Primers

Oligonucleotide primers were kindly provided by the Département "Ecologie des Arthropodes dans Les Agroécosystèmes méditerraneéns", Centre D'Ecologie Fonctionnelle et Evolutive, Laboratoire de Zoogéographie, Université Paul Valéry - Montpellier III, France. Primer names, sequences and size of the PCR product are shown in Table 2.

Materials Required (per one DNA extracted sample) (Lajoinie, 2004)

1. 2 µl PCR Reaction Buffer
2. 0.2 µl Deoxyribonucleotide triphosphate (dNTP)
3. 2 µl $MgCl_2$ (25 ppm)
4. 1 µl Reverse Primer (R primer)
5. 1 µl Forward Primer (F primer)
6. 11.7µl Pure water
7. 0.1 µl Taq Polymerase (Eurogentec)
8. The Programmable Thermal Controller (PTC)

Table 2 Polymerase chain reaction primers used for mtDNA amplication

Primer name	Sequence (5' to 3')	Primer size (bp)	Product size (bp)
CoxI-L (C1-J-2183)	5-CAA CAT TTA TTT TGA TTT TTT GG-3'	23	**831**
CoxI-D (TL2-N-3014)	5-TCC ATT GCA CTA ATC TGC CAT ATT A-3'	25	
CytB-L (CB-J-10933)	5-TAT GTA CTA CCA TGA GGA CAA ATA TC-3'	26	**434**
CytB-D (CB-N-11367)	5-ATT ACA CCT CCT AAT TTA TTA GGA AT-3'	26	

Polymerase Chain Reaction Procedure

1. Sterilize filter-containing micropipette tip and eppendorf tubes with UV light for 5 minutes.

2. Mix all of materials as stated into 2 ml eppendorf tubes, gentle shake.

3. Pipette 18 µl of mixture solution place into 0.5 ml eppendorf tubes.

4. Add 2 µl DNA extract samples mix well by pipetting up and down, then centrifuge for a minute at 1,000 x g.

5. Place the eppendorf tubes in Programmable Thermal Controller (PTC) for the PCR of each selected molecular marker.

6. Prepare 3 µl gel-loading buffer (40% saccharose and bromophenol) and 4 µl standard DNA (SmartLadder SF), mix to homogenize and gentle loading to first and last sample well of 1.2% agarose gel.

7. Prepare 3 µl gel-loading buffer and 4 µl the mixture solution of step 5 mix to homogenize and gentle loading to the next sample well.

8. After loading the samples completely, cover the lid of electrophoresis field chamber and insert an electric wire to the electrical battery with 40mA.
(Check for the anode and cathode were correctly positionned)

9. Turn off the electrical battery when bromophenol blue remove to proper space. To check DNA bands on gel by UV light and take photograph for analyse.

Smart Ladder SF

The SmartLadder SF (Small Fragments) produces a pattern of nine regularly spaced bands, ranging from 100 to 1000 basepairs (bp). The 100 and 1000 bp bands have a higher intensity than the others to allow quick and eas identification.
To make sizing as smart as possible, the size of each band is an exact multiple of 100 bp. Using a standard loading of 5 µl, the 1000, 800, 600, 400 and 200 bp bands orrespond to an exact quantity of DNA.

Agarose Gel Electrophoresis

Agarose gel was used for separate the DNA fragments by difference of structure and molecular weight. The pace of the DNA movement in agarose gel was depended of two factors: agarose concentration and the structure and molecular size of the DNA. DNA molecules migrate through the gel at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. The agarose gel contains ethidium bromide staining to visualize DNA bands. This method was used to check the purity of a PCR product before using it in other applications and estimate size by comparison with a size standard.

Preparation of 1.2% Agarose Gel

1. Prepare the materials of gel electrophoresis field such as the gel apparatus, gel plate support, a well-forming comb and a power supply (capable of at least 100 V and currents of up to 100mA).

2. To weigh carefully the agarose 0.48 g into a conical flask.

3. Add 40 ml of 0.5 x TBE Buffer (Tris-Borate electrophoresis buffer). Gentle swirling for homogenize the agarose and buffer, then place in a microwave for 30 seconds. (Composition of 0.5 x TBE buffer: Tris base 5.4 g, Boric acid 2.75 g, EDTA 2 ml, followed pure water to adjust the final volume to 1 litre)

4. Cool the solution to approximately 50°C by incubate at room temperature with magnetic stirrer for 15 minutes, add 40 µl BET (ethidium bromide) into the agarose gel solution.

5. To pour slightly the agarose gel solution into the gel plate support (This step ensures there are no air bubbles in the gel). A comb is inserted into the gel apparatus to form the wells.

6. Allow about 30-45 minutes at room temperature for left the gel to solidify.

7. When the gel has set, the comb can be carefully removed and the solidified gel, still on its gel plate support, placed into the running apparatus. Fill enough running buffer to just cover the wells.

8. Prepare 10 µl DNA extract samples and 3 µl gel-loading buffer, mix to homogenize on paraffin film. Load the samples into the wells in agarose gel. (Usually load the DNA ladder in first and last wells. The DNA ladder is a mixture of fragments with known sizes to estimate the size of DNA fragments in samples)

9. After completely loaded, the lid to the apparatus is closed and inserts an electric wire to the electrical battery with 1-5 V/cm. (Check for the anode and cathode are in correct position)

10. Turn off the electrical battery when the lower dye front will be one-half to two-thirds of the way down the gel. Check DNA bands on gel by UV light and take a photograph.

Place and Duration

Place

1. Suwanvajokkasikit Animal Research and Development Institute, Kasetsart University, Kamphaengsaen campus, Ban Yang district, Nakhonpathom province, Thailand

2. Laboratory of Medical Entomology, Department of Entomology, Faculty of Agriculture, Kasetsart University Bangkok, Thailand

3. Département Ecologie des Arthropodes dans Les Agroécosystèmes méditerraneéns, UMR 5175, Centre D'Ecologie Fonctionnelle et Evolutive, Université Paul Valéry - Montpellier III, France

Duration

February 2004 to September 2006

RESULTS AND DISCUSSION

Experiment 1

Morphological Characteristics of some *Stomoxy* spp. found in Central Thailand

Species Inventory

Morphology of *Stomoxys* species was studied for the first time in Thailand. During this investigation, Stomoxyine flies collected from three localities at Nakhonpathom, Kanchanaburi and Saraburi provinces by Vavoua traps placed near the beef and dairy cattle shelters. Adult flies were identified using the key developed by Zumpt (1973). Four *Stomoxys* species were observed including *Stomoxys calcitrans* (L.), *Stomoxys sitiens* Rondani, *Stomoxys indica* Picard and *Stomoxys bengalensis* Picard (Table 3). The flies trapped at Kasetsart University, Kamphaengsaen campus, Nakhonpathom province are shown in table 4 as well as sex of each species. Among the three localities, it was found that *S. calcitrans* was the most abundant species and commonly found in every locality, followed by *S. indica* and *S. sitiens* respectively. During this experiment only one *S. bengalensis* female was obtained from Nakhonpathom province (Table 3).

Table 3 Distribution of *Stomoxys* spp. observed at Nakhonpathom, Kanchanaburi and Saraburi provinces during July 2004 to June 2005

Province	*S. calcitrans* (L.)	*S. sitiens* Rondani	*S. indica* Picard	*S. bengalensis* Picard
Nakhonpathom	+	+	+	+
Kanchanaburi	+	-	-	-
Saraburi	+	-	+	-

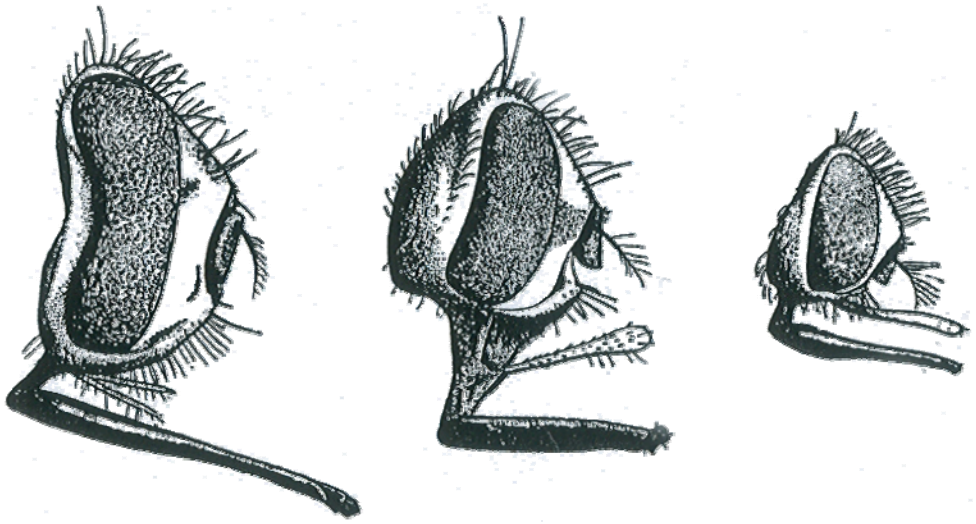
Table 4 Stomoxyine flies collected by Vavoua traps from beef and dairy cattle farms, Kasetsart University, Kamphaengsaen campus, Nakhonpathom province during July 2004 to June 2005

Species	Number of flies		
	Male	Female	Total
Stomoxys calcitrans (L.)	**68**	**64**	132
Stomoxys sitiens Rondani	23	36	59
Stomoxys indica Picard	8	14	22
Stomoxys bengalensis Picard	0	**1**	1

External Morphology of Four *Stomoxys* spp.

Samples of flies obtained by Vavoua traps and sweep net at Nakhonpathom, Kanchanaburi and Saraburi provinces were examined with stereomicroscope. Stomoxyine flies could be easily distinguished from other blood-sucking flies like *Haematobia* spp. by their mouthparts (Fig. 4) and size which are bigger.

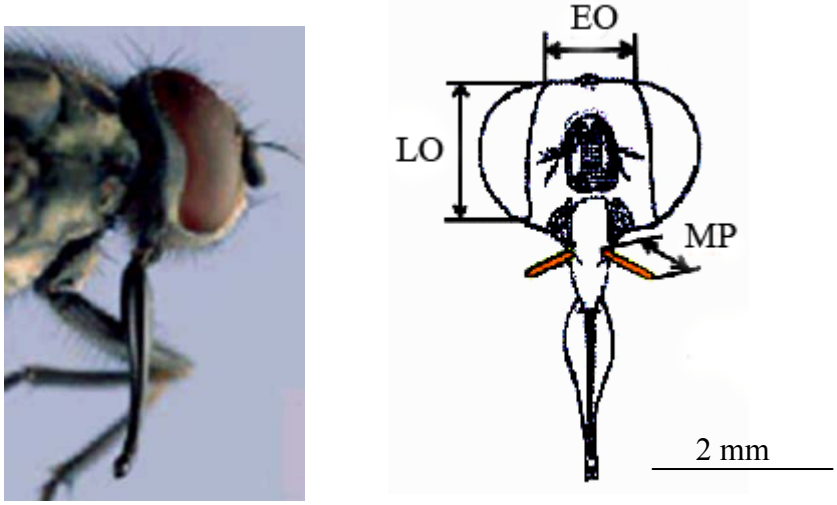
The external morphology of four *Stomoxys* species was described and illustrated. The main characteristics such as head and wing were shown in figures 5 and 6. Measurement of frontal index of each species as well as some major structures are presented in table 5.



A B C

Figure 4 Heads of A) *Stomoxys calcitrans* (Linnaeus), B) *Haematobosca stimulans* (Meigen), C) *Haematobia irritans* (Linnaeus)

Source: Edwards *et al*. in Zumpt (1973)



Figur 5 Head of *Stomoxys calcitrans* (L.): Left, lateral view showing mouthpart; Right, frontal view (diagram) showing measurement of the frontal index (frontal index = EO/LO with EO = frontal space of vertex, LO = eye length), MP = Maxillary Palpi

Source: Gilles (2005)

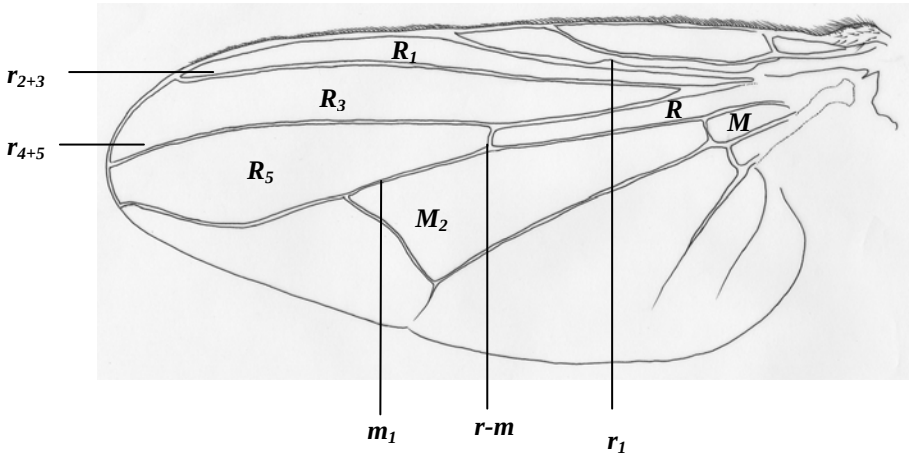


Figure 6 Wing of *Stomoxys calcitran*s (L.); *R* = first basal cell, R_1 = marginal cell, R_3 = submarginal cell, R_5 = first posterior cell, *M* = second basal cell, M_2 = second posterior or discal cell, r_1 = first longitudinal vein, r_{2+3} = second longitudinal vein, r_{4+5} = third longitudinal vein, m_1 = fourth longitudinal vein (media), *r-m* = discal cross-vein (text after Zumpt, 1973)

Table 5 Some morphological characteristics of *Stomoxys* spp. found in central Thailand at Nakhonpathom, Kanchanaburi and Saraburi provinces

Morphological characteristics	*S. calcitrans* L.		*S. sitiens* Rondani		*S. indica* Picard	
	Male (n = 49)	Female (n = 43)	Male (n = 12)	Female (n = 11)	Male (n = 3)	Female (n = 3)
Frontal index (mm)	0.33 ± 0.03	0.55 ± 0.04	0.19 ± 0.02	0.45 ± 0.02	0.13 ± 0.02	0.44 ± 0.03
Eye length (mm)	1.51 ± 0.10	1.50 ± 0.10	1.59 ± 0.10	1.48 ± 0.13	1.30 ± 0.00	1.22 ± 0.08
Frontal space (mm)	0.49 ± 0.04	0.82 ± 0.08	0.30 ± 0.04	0.66 ± 0.06	0.17 ± 0.03	0.53 ± 0.03
Number of longitudinal stripe on thorax	4 lines		4 lines		4 lines	
Abdominal pattern	1 marked median spot and 2 lateral dark round spots on the 2nd and 3rd segment		2 lateral transversely elongate dark spots on the 2nd and 3rd segment		dark transverse band on the 2nd and 3rd segment	
Body length (mm)	4 - 7		4 - 6		4 - 6	

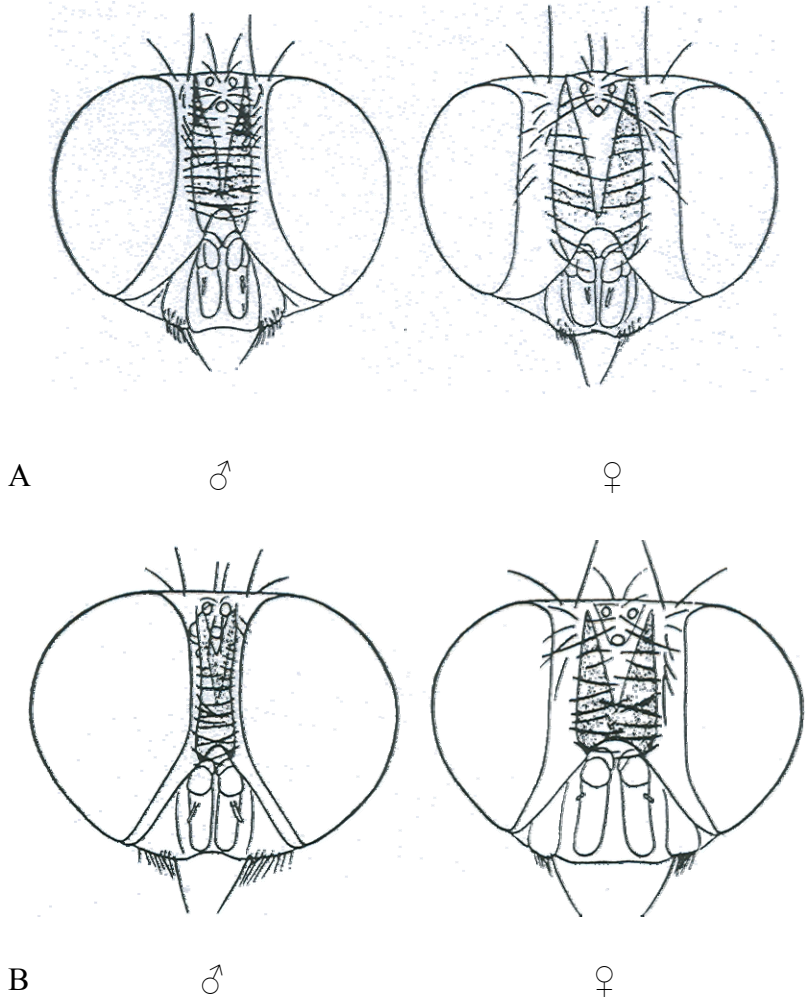


Figure 7 Frontal views of male and female heads; A) *Stomoxys calcitrans* (L.), B) *Stomoxys indica* Picard

Source: Hennig in Zumpt (1973)

***Stomoxys calcitrans* (L.)**

Body length: 4-7 mm.; easily recognized by 1 marked median spot and 2 lateral dark round spots presented on the second and third abdominal segment (Fig. 8).

Originated in the old world and cosmopolitan species, commonly found in many areas, mainly in temperate zones is to be regarded as one of a synanthropic fly (Zumpt, 1973).

Width of frons at its narrowest point measuring $^1/_3$ or more of eye length. Thorax and abdomen dark grey and olive-brown, with distinct pattern. Wing hyaline, terminal part of r_1 not setulose. Legs dark, only bases of tibiae more or less extensively pale.

Male – The average frontal index was 0.33 ± 0.03 mm (Table 8). Frons widened slightly from the vertex. Frontal stripe black. Antennae black-brown to nearly black, third segment about $2^1/_2$ times as long as the second, arista with long dorsal hairs. Palpi yellow, proboscis dark reddish-brown. Thorax black with a whitish-grey and dark olive pollinosity. Mesonotum with 2 pairs of dark and broad longitudinal stripes, which may be partly united laterally. Wing hyaline, r_1 with a few dorsal setae at base, r_{4+5} with dorsal and ventral setae restricted to the first half or more or less reaching *r-m*, R_5 at its broadest point $2^1/_2$ times as wide as at apex, *m* terminally sinuous, squamae hyaline, halter yellow. Legs dark-brown to blackish, tips of femur and basal parts of tibiae yellow. Abdomen with a grey and dark-brown pollinosity which is characteristic, but varies in extent (Zumpt, 1973).

Female – In the female the width of frons at vertex measures about half the eye-length, frontal index was 0.55 ± 0.04 mm (Table 5). Frontal stripe black. Ovipositor with slender cerci (Fig. 9).

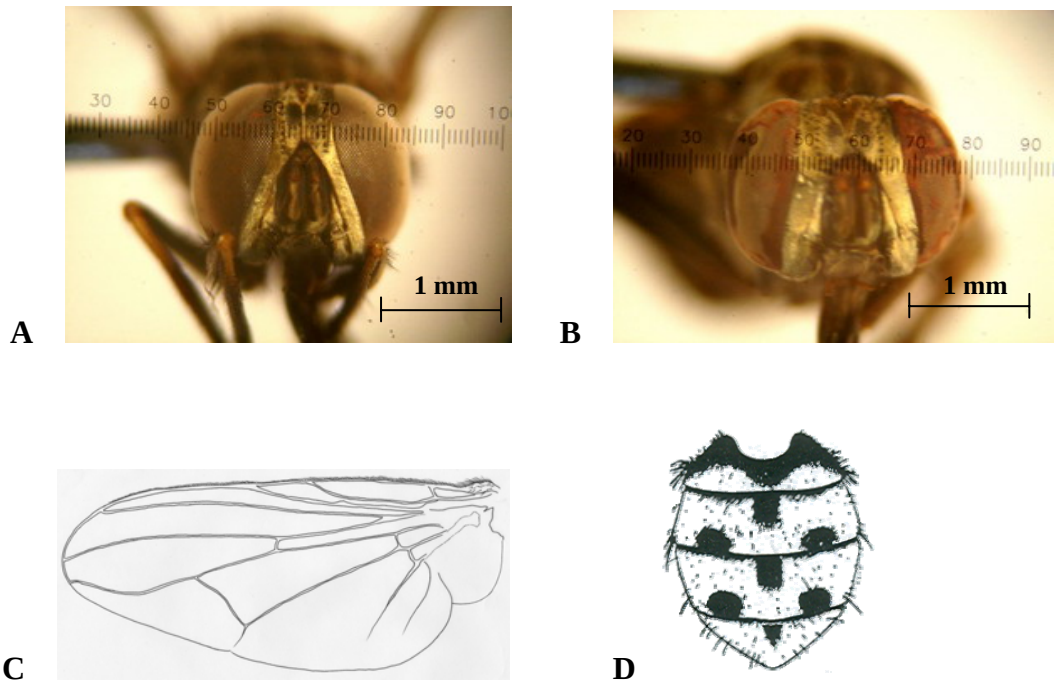


Figure 8 *Stomoxys calcitrans* (L.); A-B) Male and female head, anterior view, C) Wing, D) Dorsal abdominal pattern

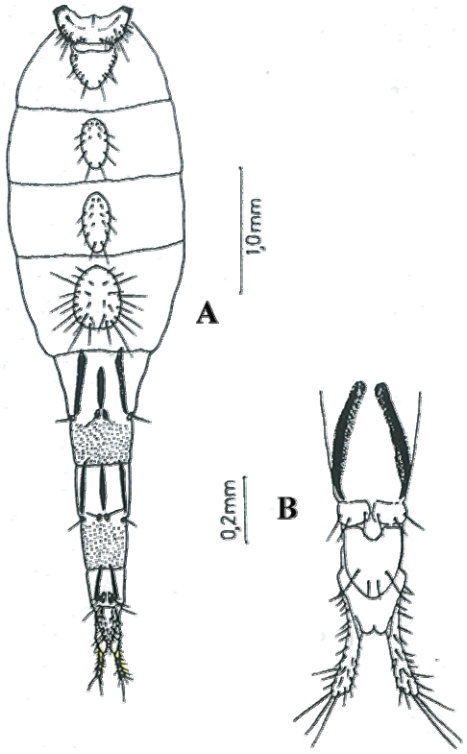


Figure 9 *Stomoxys calcitrans* (L.); A) Female abdomen in ventral view, B) Terminal part of ovipositor dorsally

Source: Zumpt (1973)

***Stomoxys sitiens* Rondani**

Body length: 4-6 mm. Adult with 2 distinct lateral dark spots, more transversely elongate on the second and third abdominal segment (Fig. 10).

Width of frons at the narrowest point measuring ¼ or less of eye length. Thorax and abdomen with dense grayish or brownish, not shiny. First posterior cell of wing (R_5) at apex less than one half as wide as at widest point, media more strongly curved upward and sinuous terminally. Wing with the apex of media slightly proximal to apex of r_4+$_5$. Legs dark colored, tibiae more or less extensively yellow, tarsi dark-brown or blackish. Hind-femur with relatively short ventral hairs.
Abdomen with distinct sports or transverse bands.

Male – Frons at the narrowest point measuring about $^1/_5$ of eye-length, the average frontal index was 0.19 ± 0.02 mm (Table 5). Frontal stripe black. Antennae dark-brown to blackish, third segment about 3 times as long as the second, arista with long dorsal hairs. Palpi yellow, proboscis dark reddish-brown.
Thorax black with a dense grey and olive pollinosity. Mesonotum with 4 longitudinal black stripes. Wing with r_1 dorsally bare, except for a few setae near the humeral cross-vein, r_4+$_5$ with dorsal and ventral setae along up to half of its length or more to *r-m*, apex of media slightly proximad to apex of r_4+$_5$. Squamae hyaline, halter yellow. Legs dark-brown to blackish, tips of femora and basal parts of tibiae yellow.
Abdomen with a grey and olive-brown pattern reminiscent of *S. calcitrans*, but the lateral spots are more transversely elongate (Zumpt, 1973).

Female – Head with a broad frons, frontal index was 0.45 ± 0.02 mm (Table 5). Frontal stripe black.

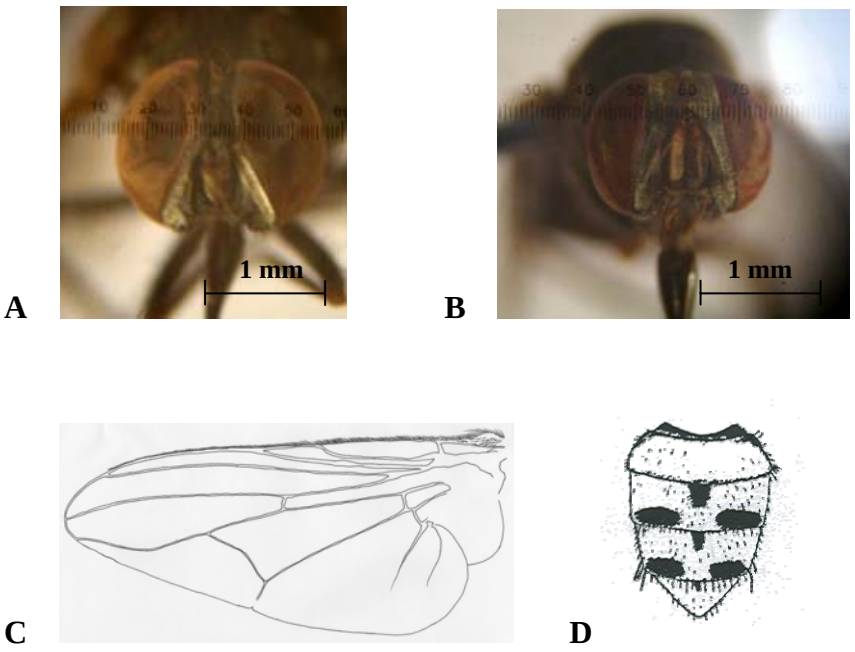


Figure 10 *Stomoxys sitiens* Rondani; A-B) Male and female head, anterior view, C) Wing, D) Dorsal abdominal pattern

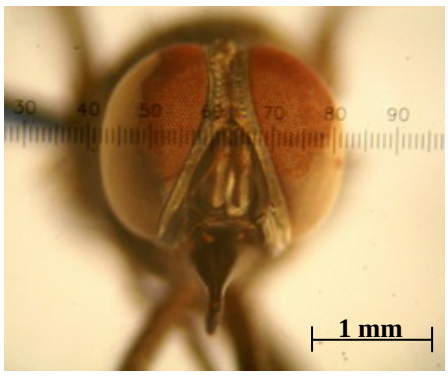
***Stomoxys indica* Picard**

Body length: was 4-6 mm. Abdomen grey to olive-brown with distinct spots or transverse stretch bands on the second and third abdominal segment (Fig. 11).

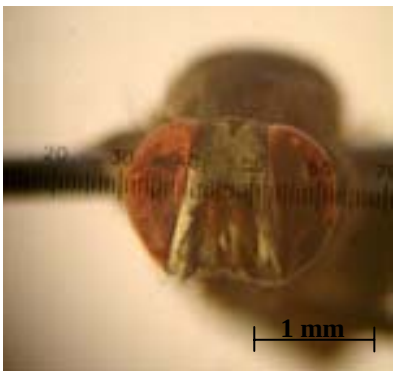
Width of frons at the narrowest point measuring ¼ or less of eye length. First posterior cell of wing (R_5) at apex one half as wide as at widest point, media only slightly curved upward. Legs with femur, tibiae and tarsi yellow-brown to dark-brown. Fore-metatarsus simple, without rows of erect hairs.

Male – The male frontal index was 0.13 ± 0.02 mm (Table 5). Third antennal segment light colored. Wing-vein $r_4{+}_5$ with dorsal and ventral setae reaching more or less to *r-m* (Zumpt, 1973). The majority of specimens have the femur blackish or dark-brown only the apices being yellowish.

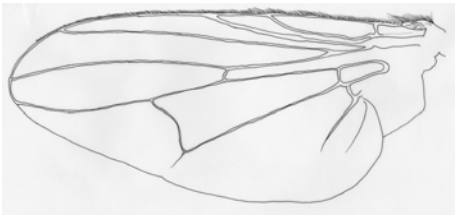
Female – The female frontal index was 0.44 ± 0.03 mm (Table 5).



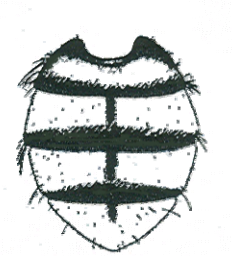
A



B



C



D

Figure 11 *Stomoxys indica* Picard; A-B) Male and female head, anterior view, C) Wing, D) Dorsal abdominal pattern

***Stomoxys bengalensis* Picard**

Body length: 6.5 mm.

Since the specimen obtained during the experiment was very small in number, only one female fly was observed. The morphology of *S. bengalensis* described by Zumpt (1973) is as follows:

Width of frons at the narrowest point measuring ¼ or less of eye length. Wing with the apex of media almost directly under apex of r_4+$_5$. First posterior cell of wing (R_5) at apex less than one half as wide as at widest point, media more strongly curved upward and sinuous terminally. Legs dark colored, tibiae more or less extensively yellow, tarsi dark-brown or blackish. Fore-metatarsus simple, without rows of erect hairs. The hind-femur with long ventral hairs compare to *S. sitiens* (Fig. 13). Thorax and abdomen with dense grayish or brownish, not shiny. Abdomen with distinct spots or dark transverse bands and narrow median vittae, this pattern is more or less reduced.

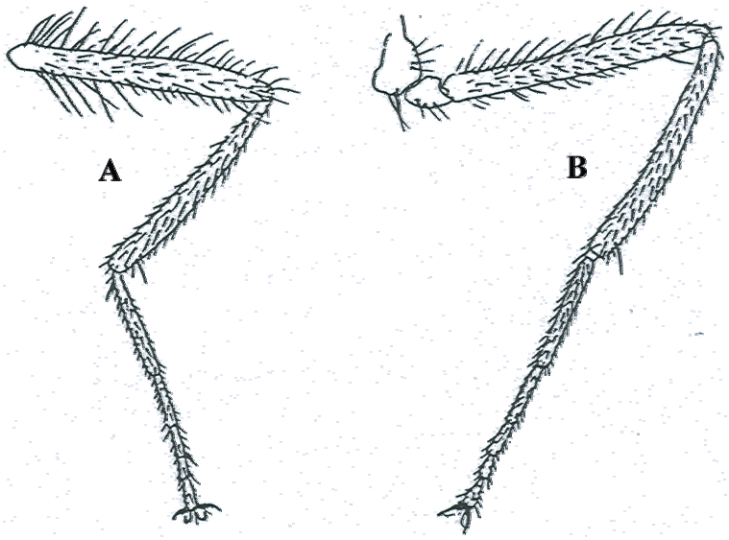


Figure 12 Hind legs of A) *Stomoxys bengalensis* Picard, B) *Stomoxys sitiens* Rondani
Source: Zumpt (1973)

Experiment 2

Comparative Study of Male Genitalia of Three *Stomoxys* spp. found in Central Thailand

Male genitalia of 3 *Stomoxys* spp. including *S. calcitrans* (L.), *S. sitiens* Rondani and *S. indica* Picard were studied in the laboratory. Adult flies were obtained from Nakhonpathom, Kanchanaburi and Saraburi provinces during 2004.

The different sclerites of genitalia or hypopygium of each species were studied and illustrated. According to Zumpt (1973) the lateral sclerite was divided into 2 parts, pregonite and postgonite (Fig. 14). It was found that the horned sclerites were clearly distinguished among the 3 species. The various sclerotized plates were also different in shape between *S. calcitrans*, *S. sitiens* and *S. indica* (Fig. 15).

The average sizes of male genitalia of flies were presented in table 6. It seemed that the size of male genitalia taken from *S. calcitrans* was the biggest, followed by *S. sitiens* and *S. indica*, i.e., 360 ± 5.32, 342 ± 34.81 and 282 ± 7.66 μm, respectively. The hypopygium (L) varies from 282 ± 7.66 to 360 ± 5.32 μm. The width of horned sclerite (W) observed was also highest in *S. calcitrans* (171 ± 7.42 μm) while that of *S. indica* is smallest (143 ± 2.16 μm).

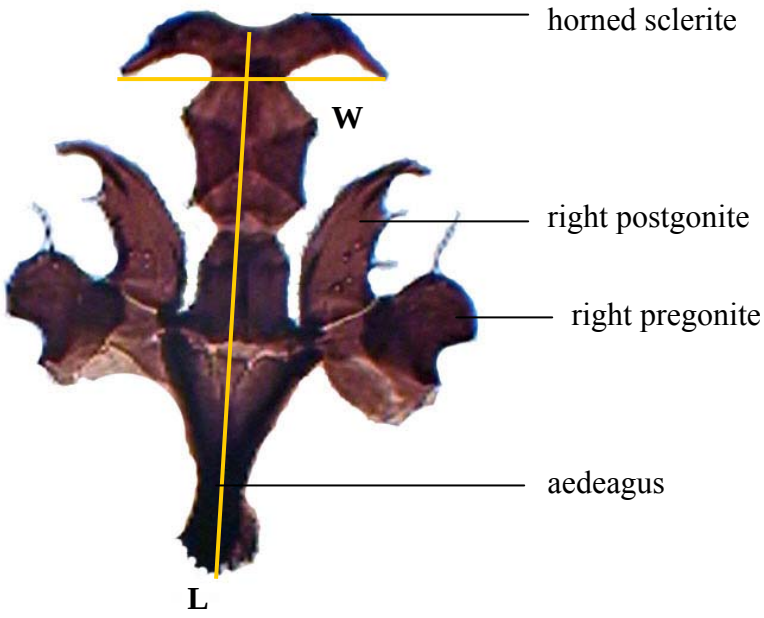


Figure 13 The male genitalia of *Stomoxys calcitrans* (L.);
W = width of horned sclerite, L = the length of genitalia

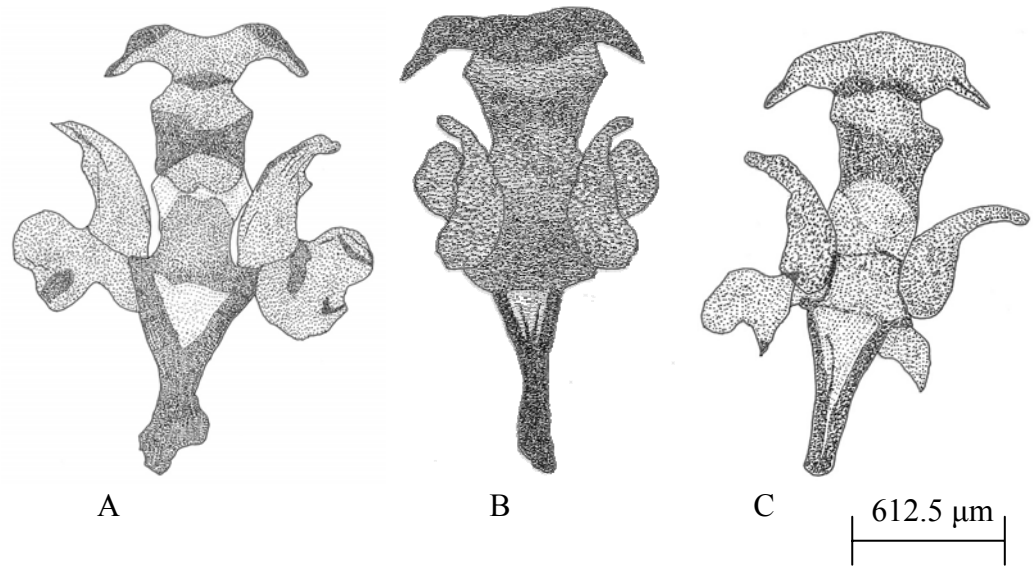


Figure 14 The male genitalia of A) *S. calcitrans* (L.); B) *S. sitiens* Rondani and C) *S. indica* Picard collected in central Thailand at Kanchanaburi, Nakhonpathom and Saraburi provinces respectively (40X)

Table 6 Average size of male genitalia of *Stomoxys* spp. collected in central Thailand at Nakhonpathom, Kanchanaburi and Saraburi provinces during July 2004 to June 2005

	S. calcitrans (L.) (n = 4)	*S. sitiens* Rondani (n = 4)	*S. indica* Picard (n = 4)
Average length of hypopygium (μm)	360 ± 5.32	342 ± 34.81	282 ± 7.66
Average width of horned sclerite (μm)	171 ± 7.42	166 ± 11.39	143 ± 2.16

Discussion

This paper was the first report on morphological characteristics of Stomoxyine flies in Thailand. The result from this study revealed that in Stomoxyine flies some species are relatively similar in external morphological characteristics eventhough adult can be distinguished by dorsal abdominal pattern.

During this experiment, *S. calcitrans* was the most commonly collected fly species of the total *Stomoxys* spp. As a synanthropic fly, *S. calcitrans* has a world wide distribution, not only in the tropical parts of the Old World and in the Oriental region. *Stomoxys calcitrans* was the only one Stomoxyine fly ever recorded to be found in Thailand as pest of cattle and swine (Ketavan and Kanjanamungsuk, 1987). Distribution of *S. sitiens* has been found in high number in the Ethiopian geographical region, especially in Egypt and in the Oriental region. *Stomoxys indica* is also quite common *Stomoxys* species in the Oriental region (Zumpt, 1973). However, during this investigation the number of *S. sitiens* was significantly greater than *S. indica* at the three localities of central Thailand. *Stomoxys sitiens* is separated from *S. calcitrans* by the wing-venation and dorsal abdominal pattern. However both species, *S. sitiens* and *S. indica* were reported to be found for the first time in Thailand during this investigation.

Stomoxys bengalensis is related to *S. calcitrans* and can be distinguished by having a narrower frons in both sexes and the abdominal pattern. *Stomoxys bengalensis* is evidently a rare species in the Oriental region with few records even by Zumpt (1973). During this experiment, only one female fly of *S. bengalensis* was trapped at Nakhonpathom province and was reported for the first time in Thailand. The morphological study of this species was very limited with only one specimen, further study should be conducted.

The frontal index of Stomoxyine flies of our investigation was slighty different from the previous studied. According to Zumpt (1973), the frontal index of *S. calcitrans*, *S. sitiens*, *S. indica* and *S. bengalensis* was 0.37-0.40, 0.5-0.6; 0.18-0.23, 0.42-0.45; 0.11-0.16, 0.37, 0.43; 0.20-0.26 and 0.44 mm for male and female, respectively.

At present, although entomologists have turned their attention to increase their knowledge of male genitalia to reassure the insect identification. Various sclerotized plates of hypopygium of Stomoxyine flies should be carefully studied for taxonomic purposes. This experiment attempted to demonstrate the presence of some *Stomoxys* spp. for the first time in Thailand. However, the comparative morphological study as well as male genitalia of Stomoxyine flies should be further conducted. This knowledge will be useful for fly identification, which will raise awareness for future investigation on the control of diseases transmitted by these flies.

Experiment 3

Seasonal abundance and Daily Activity of *Stomoxys* spp. (Diptera: Muscidae) in Kamphaengsaen Campus, Nakhonpathom Province, Thailand

The site of samples collection is located in a plain surrounded by grassland, perennial plants and rice fields. In this area the year is usually divided in 3 different seasons: wet season from June to October, dry season from November to February and hot season from March to May.

Three different species of *Stomoxys* were identified during this investigation: *Stomoxys calcitrans* (L.),1758, *Stomoxys sitiens* Rondani 1873 and *Stomoxys indica* Picard 1908.

Climatic Parameters

The main climatic parameters recorded at Kamphaengsaen meteorological station are presented in figure 16. Few variations were observed all along the year, which is typical of a tropical climate. During the period of this field survey, wet rainy season was observed from July to October 2004, while dry season was from November 2004 to April 2005 with a rain in March 2005 and a new rainy season beginning in May 2005.

Daily Activity

The daily activity of the different species of Stomoxyine flies was quite similar in the beef and dairy farms (Fig. 17). The figure 17 shows the number of flies captured per period of 2 hours during all the year under survey. No difference was observed between males and females and the results have been grouped for both sexes. For *S. calcitrans*, a major peak was observed between 08:00 am to 10:00 am and a smaller one around 04:00 pm. For *S. sitiens* and *S. indica*, there was a peak of activity in the evening, 06:00 pm to 06:00am (next day) and another one

in the morning, 06:00 am to 08:00 am.

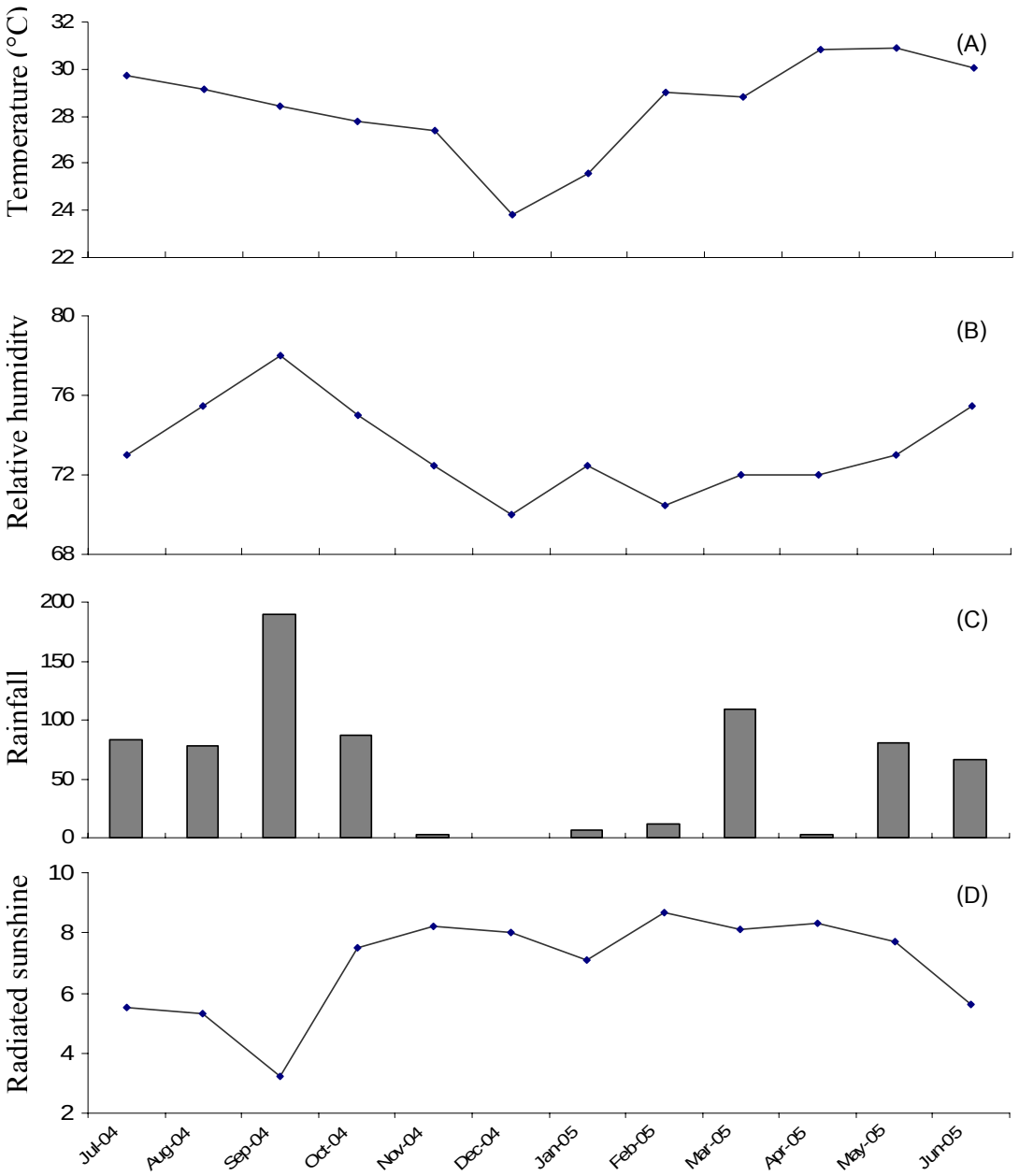


Figure 15 Monthly mean ambient temperature (A), relative humidity (B), rainfall (C) and radiated sunshine (D). Data obtained from Kamphaengsaen meteorological station

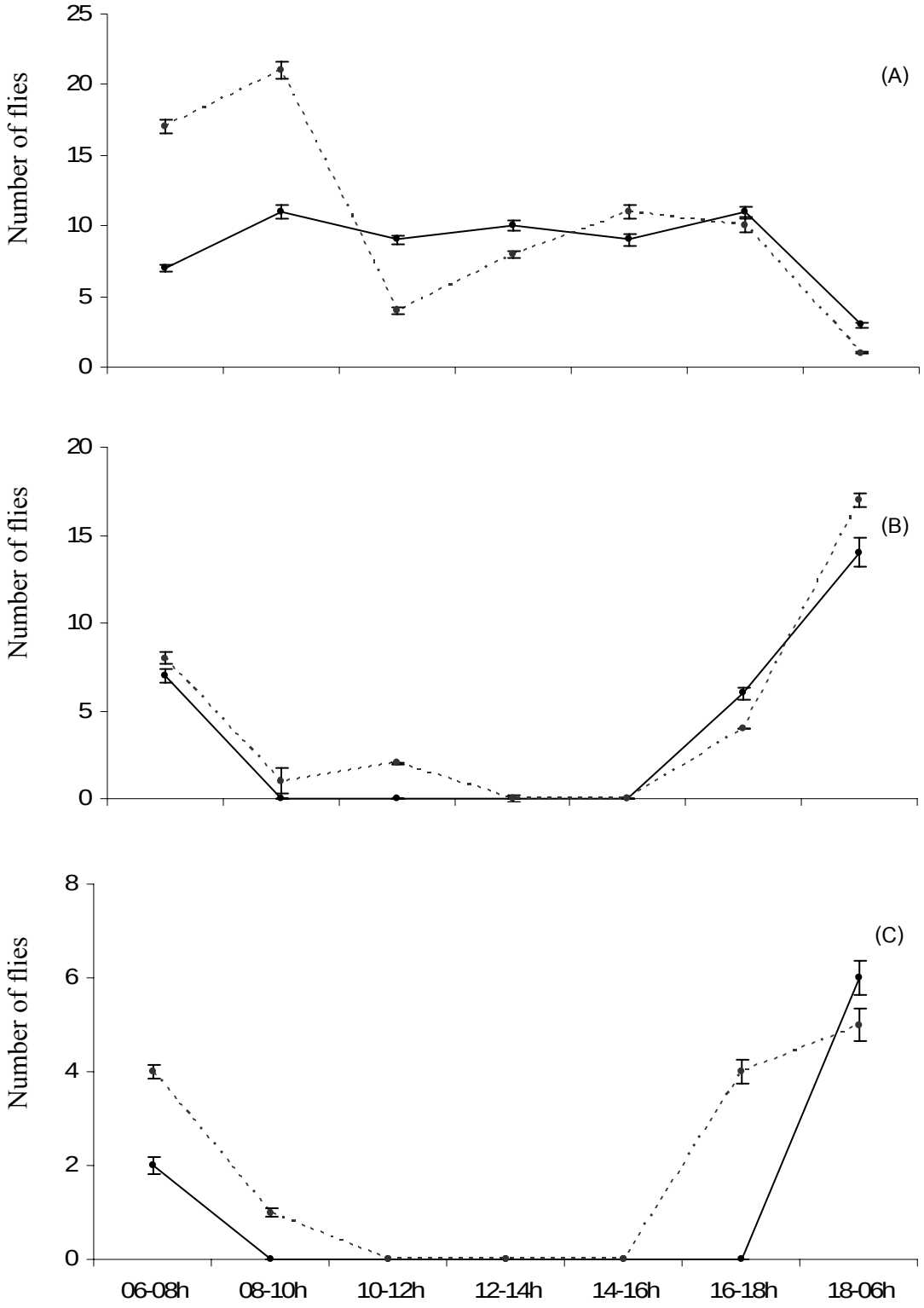


Figure 16 Daily activity of *S. calcitrans* (L.)(A), *S. sitiens* Rondani (B) and *S. indica* Picard (C) in a beef farm (plain line) and a dairy farm (dotted line), observed at Kamphaengsaen campus, Nakhonpathom province, Thailand. The number of flies is the total number capture during the servey period

Population Fluctuations

The monthly captures are shown in table 7 where the numbers indicate the total of flies captured in two days per month at each farm. For the total number of flies trapped during this period from both beef and dairy cattle farm, it was found that 80% of flies were captured during the rainy season (July to October 2004 and May to June 2005) and 20% during the dry season (November 2004 to April 2005). Only two *S. calcitrans* were captured in March from dairy cattle farm, but after the first rain during this month, the total number of flies increased to 17 in April, and only 9 in May. However, at the beginning of rainy season in June, the number of flies observed in dairy cattle farm increased notably (Table 7). During all the survey period, only the number of males *S. calcitrans* was slightly greater than those of females (Table 8). For the other species, the number of females was obviously exceeded the number of males. *S. calcitrans* was the most common species with 62% of captures, followed by *S. sitiens* (28%) and *S. indica* (10%).

Table 7 Monthly catches of *Stomoxys* spp. by Vavoua trap in a beef and a dairy cattle farm, Kasetsart University, Kamphaengsaen campus, Nakhonpathom province, Thailand from July 2004 to June 2005

Sites	Year	Months	*S. calcitrans* (L.)	*S. sitiens* Rondani	*S. indica* Picard	Total
Beef cattle farm	2004	July	17	1	0	**18**
		August	16	0	0	**16**
		September	17	11	4	**32**
		October	4	5	1	**10**
		November	0	0	0	0
		December	1	0	0	1
	2005	January	1	0	0	1
		February	1	1	3	5
		March	0	0	0	0
		April	0	7	0	7
		May	0	1	0	1
		June	3	1	0	4
Total			60	27	8	95
Dairy cattle farm	2004	July	7	2	0	**9**
		August	4	1	0	**5**
		September	2	4	0	**6**
		October	16	1	4	**21**
		November	0	1	0	1
		December	6	1	0	7
	2005	January	1	0	0	1
		February	4	2	1	7
		March	2	0	0	**2**
		April	1	8	1	10
		May	5	3	0	8
		June	24	9	8	**41**
Total			72	32	14	118

Table 8 Number of males and females flies observed during the survey period (July 2004 to June 2005) at Kasetsart University, Kamphaengsaen campus, Nakhonpathom province, Thailand

Sex	*S. calcitrans* (L.)	*S. sitiens* Rondani	*S. indica* Picard
Male	68	23	8
Female	64	36	14

Discussion

Stomoxys calcitrans, *S. sitiens* and *S. indica* have been identified during this survey *S. calcitrans* was found in great number while *S. indica* was reported to be found for the first time in Thailand.

Stomoxys calcitrans is a cosmopolitan species, which has followed human beings during their peregrinations everywhere in the world, from the tropical to the temperate and even cold areas. Its origin was probably the Oriental region (Zumpt, 1973) and not in the Ethiopian region, as usually thought. In support of these findings, a biogeographical study of this species started in our laboratory to know its cradle and the way it has invaded the world and phylogeographic studies performed on 37 different populations from all the biogeographical regions of the world seem to confirm this hypothesis (Porco *et al.*, unpublished data).

Stomoxys sitiens has been recorded from many places in the Ethiopian region from the Gambia to South Africa and Egypt, but this species is very rare in collections. It occurs also in the Oriental region from India to the Philippines, but the material is as rare as that from Africa (Zumpt, 1973). And few data on its biology are available (D'Amico *et al.*, 1996).

Stomoxys indica is a variable Oriental species, with many synonyms in the entomological literature. It has been recorded from many countries in this region, from India and Sri Lanka in the West to Samoa Island in the East (Zumpt, 1973). This paper is the first report on the occurrence of *S. indica* in Thailand. *Stomoxys indica* is described to be the most common *Stomoxys* species in the Oriental region after *S. calcitrans*, but during this survey only 22 *S. indica* were captured, against 59 *S. sitiens* and 132 *S. calcitrans*. It seemed that during the period of the survey using the Vavoua trap, *S. sitiens* was found more abundant than *S. indica* in Nakhonpathom province.

Daily Activity

Concerning the daily activity, despite the low number of captured flies, their activity could be easily observed among the 3 species. For *S. sitiens* and *S. indica*, the peaks observed showed that the activity was highest at sunset and dawn. On the opposite, *S. calcitrans* showed an activity all along the day with a peak between 08:00 am to 10:00 am. It was already reported (Kano, 1953 in Zumpt 1973) that *S. indica* seemed "to be active in the evenings, as they are readily collected by the light-trap". It seems that the crepuscular activity of some *Stomoxys* species could have limited their capture, explaining their scarcity in the collections. Most of the authors who have published about the activity of those flies worked on *S. calcitrans* only. Bimodal feeding activity patterns for stable flies were reported by Mitzmain (1913), Simmonds (1944), LaBrecque *et al.* (1975), Kunz and Monty (1976) and Charlwood and Lopes (1980). In contrast, Coaker and Passmore (1958) and Harley (1965) observed uniform or unimodal feeding activity patterns through the daylight hours. Berry and Campbell (1985) found that the pattern of daily activity of *S. calcitrans* was affected by temperature, humidity and the level of solar radiation, while Patterson *et al.* (1981) reported that direct solar radiation increased the internal temperature of resting stable flies up to 14.8°C above ambient temperatures and that the flies preferred shaded resting areas when their internal temperatures reached 31 to 34°C.

Abundance of Flies

During the entire survey, the total abundances recorded were quite similar in the beef and the dairy cattle farms with 95 and 118 *Stomoxys* spp. respectively. Globally, the most important captures were made during the warm and rainy season for all the species in both farms but these values were relatively low in comparison with others studies (Lysyk, 1993; Guo *et al*., 1998; Heath, 2002; Guglielmone *et al*., 2004; Gilles, 2005). It was rather difficult to explain these low number of flies in relation to the climatic parameters. Indeed, during this season, with i) monthly rainfalls between 80 and 180mm, ii) monthly relative humidity between 72 and 80 %

and iii) monthly mean temperatures ranged from 27 to 30 °C, we expected to observe a positive rate of increase of the Stomoxyine populations (Lysyk,1998; Gilles *et al.*,2005b, 2005c). Otherwise, the potential use of insecticides or anti-parasite drugs by the farmers during the survey could explain the low densities of flies, but it was impossible to get information about animal treatments. Another possibility could be an interspecific competition for the larval resources between the *Stomoxys* populations and the high densities of *Haematobia irritans* observed during the survey or maybe the presence of enemies like parasitoids or entomopathogenic fungi in these environments. Furthermore, the quasi disparition of the Stomoxyines, from November 2004 to February 2005 could be explained by the very low rainfalls and still relatively high temperatures (ranging from 24 to 29°C), resulting in a drying of larval developmental sites.

However, it can be concluded that during this study, *S. calcitrans* was the predominant species but in regard to the results of the daily activity, *S. sitiens* and *S. indica* seem to have a crepuscular activity so it will be necessary to find an adequate trapping method for these two species. It could be also explained that the different behaviour of male and female flies could result the number of sex ratio in *S. sitiens* and *S. indica*. These observations are really interested for future investigation.

A sex ratio distorsion has been observed (Table 8), mainly in *S. indica* species. But the low number of individuals and the bias due to the trap preclude any conclusion.

Experiment 4

Genetically Study of *Stomoxys calcitrans* (L.)

The main objective of this study is to construct a plylogenetic relationships among different geographic population of *S. calcitrans*, thus it provides an answer the questions of the origin of this species and the way it has invaded all the continents. To do that, two sequence of genes Cytochrome oxidase I (Cox I) and Cytochrome B (Cyt B) were used to compare several population all around the world.

The PCR products were obtained using primers designed for Cytochrome oxidase I (Cox I) and Cytochrome B (Cyt B) region of mtDNA. The amplified products were eletrophoresed on 1.2% agarose gel containing ethidium bromide under UV illumination. The sizes in basepairs (bp) of the fragments were given with comparison to a DNA ladder (lane 1 and 15). Lane 2, Negative control, without DNA; lanes 3, 4 and 5 samples from Thailand; lane 6 sample from Columbia; lanes 7 and 8 samples from France; lane 9 sample from Senegal; lane 10 sample from Comoro Island; lane 11 sample from Morocco; lane 12 sample from Madagascar Island; lane 13 sample from Uganda and lane 14 sample from Nouvelle Caledonie Island.

The Cytochrome oxidase I (Cox I) and Cytochrome B (Cyt B) fragment length were rough estimate 830 and 430 basepairs (bp) respectively (Fig. 18 and 19).

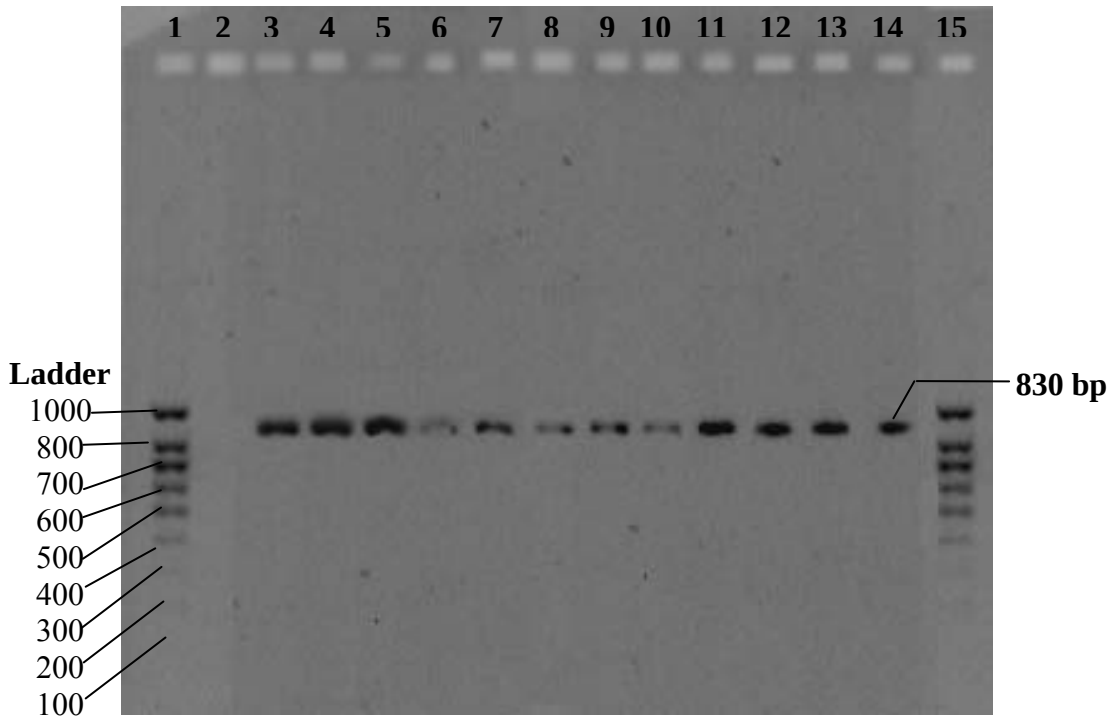


Figure 17 PCR products of *S. calcitrans* (L.) obtained by the primer designed for Cytochrome oxidase I (Cox I)

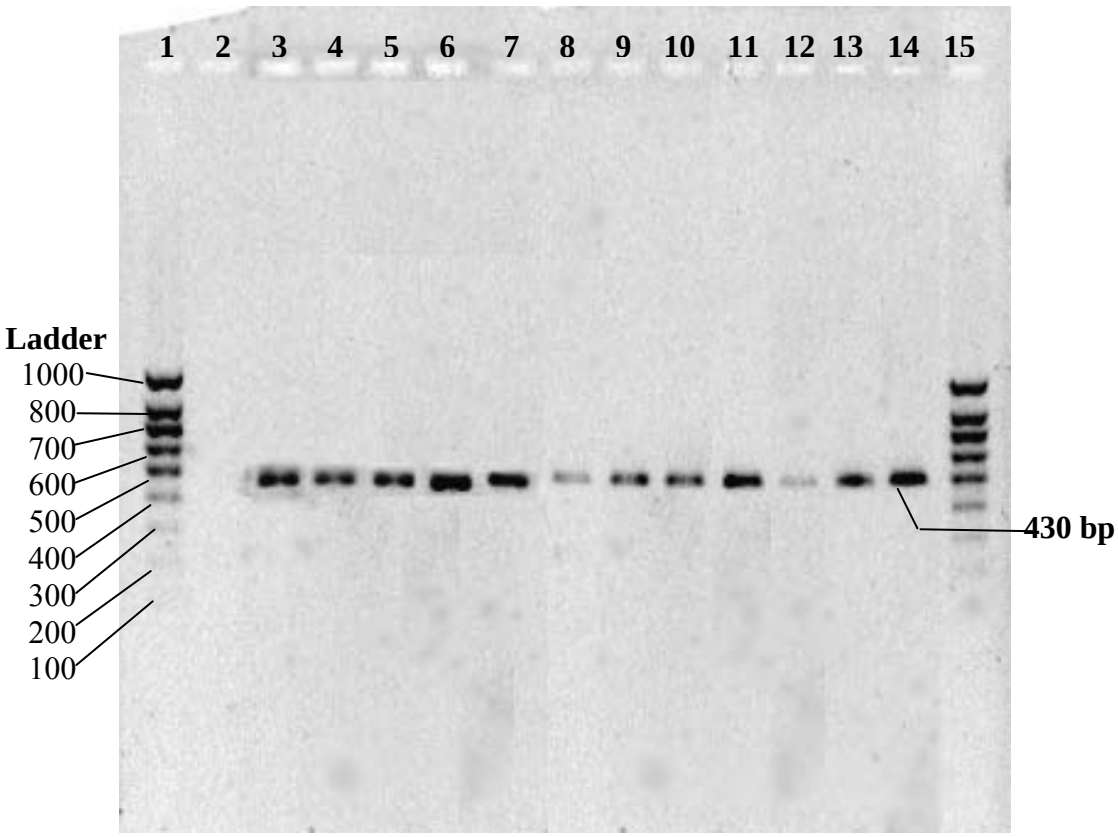


Figure 18 PCR products of *S. calcitrans* (L.) obtained by the primer designed for Cytochrome B (Cyt B)

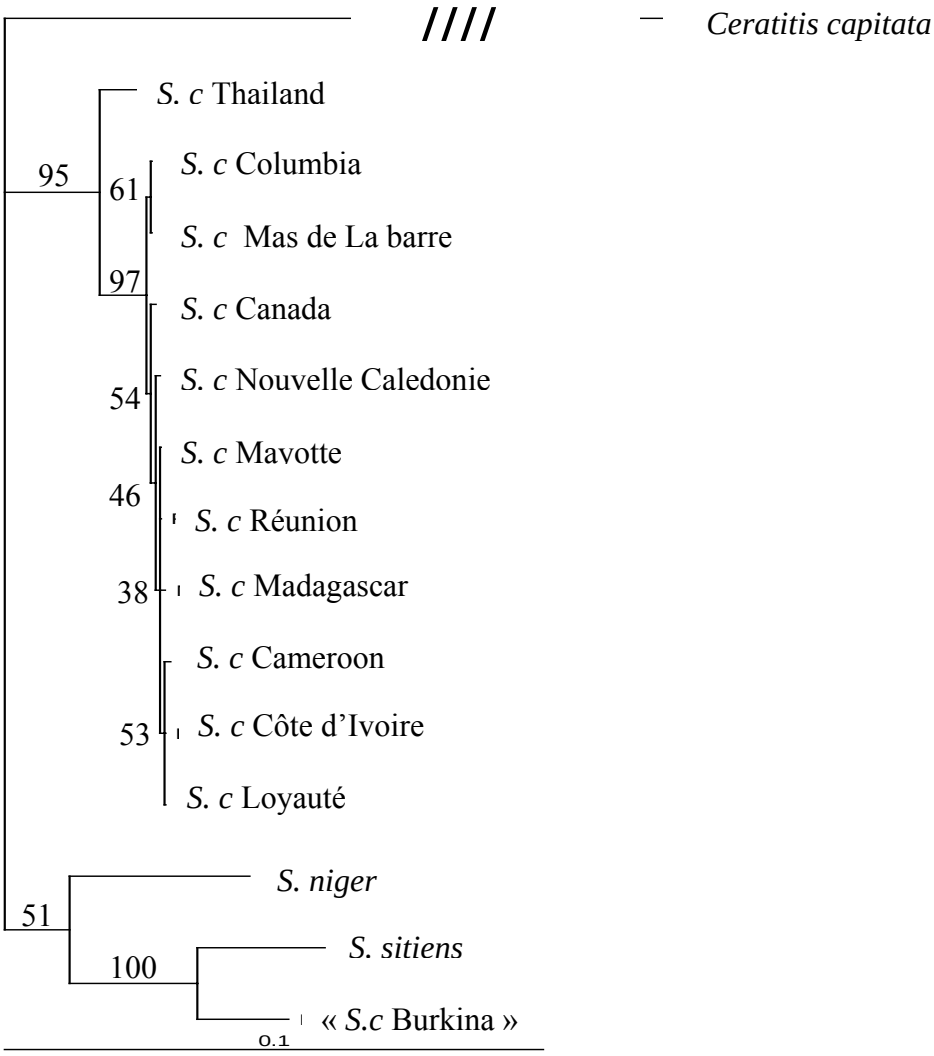
Sequencing

We got from a private company (Macrogen, South Korea) the sequences of CytB and CoxI mitochondrial genes for some of the studied populations of *S. calcitrans* (Table 2). Theses sequences were aligned and compared using "Bioedit Sequence Alignment Editor" and PAUP 4.0b10 (Swofford, 2000) softwares and the trees drawn using TreeView (V1.40). The sequence of the outgroup (*Ceratitis capitata*) has been got from GenBank.

Table 9 Sequences of CytB and CoxI genes for some of the studied populations

	Cyt B							COX I																							
Positions	26	20	25	22	22	23		15	14	19	17	18	01	16	01	17	18	13	16	18	23	36	33	11	17	18		
Colombie	A	A	C	T	T	C		T	A	G	C	A	C	G	C	C	T	T	T	A	A	A	C	T	T	T	T	Col
Mayotte	G	T	C	T	T	C		N	G	G	C	A	C	G	C	C	T	T	T	A	G	T	C	T	T	T	T	May
Canada	A	A	C	T	T	C		T	G	G	C	A	C	G	C	C	T	C	T	A	A	T	C	T	T	T	T	Can
M. de La barre	A	A	C	T	T	C		T	A	G	C	A	C	G	C	C	T	T	T	A	A	A	C	T	T	T	T	M.b.
Réunion	G	T	C	T	T	C		T	G	G	C	A	C	G	C	C	T	T	T	A	A	T	C	T	T	T	T	Réu
Cameroun	G	T	C	T	T	C		N	G	G	C	A	C	G	C	C	T	C	T	A	A	T	C	T	C	T	T	Cam
Madagascar	G	T	C	T	T	C		N	G	G	C	A	C	G	C	C	C	T	T	A	A	T	C	T	T	T	T	Mad
Côte d'Ivoire	G	T	C	T	T	C		T	G	G	C	A	C	G	C	C	T	T	T	A	A	T	C	T	C	T	T	C.I.
Thaïlande	A	T	T	C	T	T		C	A	A	T	G	T	A	T	T	T	T	C	T	A	T	T	A	T	C	C	Thaï
Loyauté	G	T	C	T	T	C		T	G	G	C	A	C	G	C	C	T	T	T	A	A	T	C	T	C	T	T	Loy
N. Calédonie	G	A	C	T	C	C		T	G	G	C	A	C	G	C	C	T	T	T	A	A	T	C	T	T	T	T	N. C.

Maximum likelyhood tree



S. c = Stomoxys calcitrans
S. = Stomoxys

Figure 19 Maximum likelyhood tree from concatenated sequences of mitochondrial genes COXI and CytB.

Source: Porco *et al.* (2007, in preparation)

Discussion

High quality and quantity of DNA are necessary for a good sequencing.

The mitochondrial cytochrome b gene has been found to be useful for phylogenetic relationships and identification of many organisms because of its relatively high mutation rate. The cytochrome b gene has both conservative and variable regions, useful for investigating relationships of both distantly and closely related taxa.

The PCR assay is rapid, cheap, sensitive and easy to use. Both male and female stable fly and any life stage can be identified, PCR product easily distinguishable by agarose gel electrophoresis. Preservation is simple since specimens can be stored in ethanol and only small quantities of material are needed for identification. This assay will help improving our knowledge in the vector control research and could be applied to other groups of insects or living organisms important for health or agriculture.

This study represents a rapid and efficient method that is applicable on a routine basis for the identification of members of the *S. calcitrans*. This one step PCR method will assist in the improvement of the current knowledge on species distribution. Moreover, this method does not rely on skillful interpretation; therefore, no subjective bias is introduced in the identification.

The main problem for DNA analysis is how to keep the samples for DNA extraction. Tissues can be preserved in alcohol for only short period (Kirby, 1990).

The problem that may be encountered when using ethidium bromide is that it promotes DNA damage under UV illumination. It is best to keep the viewing time to a minimum to prevent damage to the DNA molecule and subsequent smearing on re-electrophoresis.

From the sequencing results, it seems that the specimen from Thailand show the highest divergence. If this results could be confirmed in the future, using other samples, it would tell that the origin of the species *S. calcitrans* would be in the Oriental region and not in the Ethiopian region as usually thought.

CONCLUSION

The economic impacts of the Stomoxyine flies on livestock are more and more recognized in tropical regions with special emphasize on beef and dairy cattle. All the available information on the control methods is obtained mainly from North America. Eventhough several research projects have now been implemented in different countries to make an inventory of the different species and to get more knowledge of their biology as well.

This first report has shown that at least four different species of *Stomoxys* were present in the beef and dairy farms in central Thailand including *Stomoxys calcitrans* (L.), *Stomoxys sitiens* Rondani, *Stomoxys indica* Picard and *Stomoxys bengalensis* Picard.

Characterization of *Stomoxys* spp. were studied and recorded for the first time in Thailand. Specimens were collected at beef and dairy cattle farms in central Thailand. i.e. Suwanvajokkasikit Animal Research and Development Institute (SARDI), Kamphaengsaen campus of Kasetsart University, Ban Yang district, Nakhonpathom province; Thai-Denish dairy cattle farm, Moak lek district, Saraburi province and private dairy farm, Kanchanaburi province.

The external morphology of four *Stomoxys* species was described using the identification key of Zumpt (1973). It was found that the average frontal index of male and female of *S. calcitrans* was 0.33 ± 0.03 mm and 0.55 ± 0.04 mm, respectively. Two lateral dark round spots are clearly seen on the second and third abdominal segment. Body length was ranged from 4 to 7 mm.

In *S. sitiens*, the average frontal index in male was 0.19 ± 0.02 mm and 0.45 ± 0.02 mm in female. Head with a broad frons, frontal stripe black. Abdominal pattern with two lateral dark oval shaped spots on the second and third segment, body length 4-6 mm.

The average frontal index in male and female of *S. indica* was 0.13 ± 0.02 mm and 0.44 ± 0.03 mm respectively. Abdomen with broad transverse band on the second and third segment. Body length 4-6 mm.

Since only one female *S. bengalensis* was observed during the experiment the frontal index could not be calculated. The body length was 6.5 mm.

The male genitalia of *S. calcitrans* from Kanchanaburi province, *S. sitiens* from Nakhonpathom province, and *S. indica* from Saraburi province were dissected and mounted as described by Krantz (1975). It was found that the horned sclerites were clearly distinguished among the three species. The various sclerotized plates were also different in shape among *S. calcitrans*, *S. sitiens* and *S. indica*.
The average sizes of male genitalia of flies were observed. It seemed that the size of male genitalia taken from *S. calcitrans* was the biggest, followed by *S. sitiens* and *S. indica* respectively. The average width of horned sclerite of *S. calcitrans*, *S. sitiens* and *S. indica* was 171 ± 7.42 μm, 166 ± 11.39 μm and 143 ± 2.16 μm respectively. The average length of hypopygium of *S. calcitrans*, *S. sitiens* and *S. indica* was 360 ± 5.32 μm, 342 ± 34.81 μm and 282 ± 7.66 μm respectively.

Adult flies were captured with Vavoua traps during 24 hours every two weeks from July 2004 to June 2005. Flies specimens were collected every 2 hours at 6:00, 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hrs. respectively. The traps were left operational during the night until 06:00 am. The number, species and sex of Stomoxyine flies were recorded at each collection hour, before killing in a freezer and preserved in 95% ethyl alcohol for further studies. This study has shown that *S. calcitrans* had a more diurnal activity, while *S. sitiens* and *S. indica* presented a peak of activity at dawn and sunset.

Samples of Stomoxyine flies were identified to species level by using the key for identification developed by Zumpt (1973) in the laboratory both at Department of Entomology, Kasetsart University, Thailand and Département Ecologie des Arthropodes dans Les Agroécosystèmes méditerraneéns, Université Paul Valéry-Montpellier III, France. Four different species were observed including *Stomoxys calcitrans* (L.), *Stomoxys sitiens* Rondani, *Stomoxys indica* Picard and *Stomoxys bengalensis* Picard. Among the three provinces, it was observed that *S. calcitrans* was the most abundant and commonly found in every locality, followed by *S. indica* and *S. sitiens* respectively. This was the first report that *S. indica* could be found in Thailand. During this experiment only one *S. bengalensis* female was observed from Nakhonpathom province.

For the distribution of the four species of Stomoxyine flies in central Thailand during 2004 to 2005, *S. calcitrans* has been found in every province including Nakhonpathom, Kanchanaburi and Saraburi. *S. sitiens* can only be observed in Nakhonpathom province whereas *S. indica* in Nakhonpathom and Saraburi provinces.

Seasonal abundance and daily activity of *Stomoxys* spp. was conducted in a beef and dairy cattle farm during July 2004 to June 2005 at SARDI of Kasetsart University, Nakhonpathom province (14° 01’ N., 99° 58’ E.), about 90 km west from the city of Bangkok (Thailand). The beef and dairy cattle farms housed 380 and 123 cattle respectively at the time of the samplings. Temperature, relative humidity, rainfall and radiated sunshine data were obtained from Kamphaengsaen meteorological station. Few variations all along the year were observed which is typical of a tropical climate. During the period of this field survey, wet rainy season was observed from July to October 2004, while dry season was from November 2004 to April 2005 with a rain in March 2005, and a new rainy season beginning in May 2005.

During this investigation at the beef cattle farm *S. calcitrans*, *S. sitiens* and *S. indica* were more abundant in September 2004 while at the dairy cattle farm *S. calcitrans*, *S. sitiens* and *S. indica* were more abundant in June 2005. *S. calcitrans* was found in great number.

Fly abundance was compared between the beef and dairy cattle farms and no significantly difference observed. A total of 95 *Stomoxys* spp. have been trapped in the beef cattle farm while 118 flies in the dairy cattle farm, with the similar repartition between the three species, *S. calcitrans*, *S. sitiens* and *S. indica*.

The sequencing of two mitochondrial genes has been performed to initiate a phylogeographical study of *S. calcitrans*. The aim of this study was to discover the craddle of this species, which became cosmopolitan, all the other species of *Stomoxys* being only tropical. The first results we got have shown that the origin of *S. calcitrans* was probably in the Oriental region and not in the Ethiopian one, as previously thought. However, more samples from Thailand population should confirm or not. The proposal since the Thailand population seems to diverge from the most of the group.

RECOMMENDATION FOR FUTURE WORK

1. Genetic structure can be described as the distribution of genetic variation and the result of migration, selection, mutation, genetic drift and related factors.

2. Phylogenetic analysis of DNA sequence has become an important tool for studying the evolutionary history of organism. Since the rate of sequence evolution varies extensively with gene or DNA segment (Wilson *et al*., 1977; Dayhoff *et al*., 1978), one can study the evolutionary relationships of virtually all levels of classification of organisms by using different genes or DNA segments.

3. During this investigation *S. calcitrans* was the predominant species found during the day time while *S. sitiens* and *S. indica* tend to be the crepuscular activity and fewer specimens were obtained from Vavoua traps. It seems that the blue color are not attracted by these two species. Further study on trapping method should be conducted since the Vavoua trap was first developed by Laveissière and Grébaut (1990) for collecting the tsetse flies.

4. Reconstruction of phylogenetic trees by using statistical methods was initiated independently in numerical taxonomy for morphological characters (Sokal and Sneath, 1963) and in population genetics for gene frequency data (Cavalli-Sforza and Edwards, 1964).

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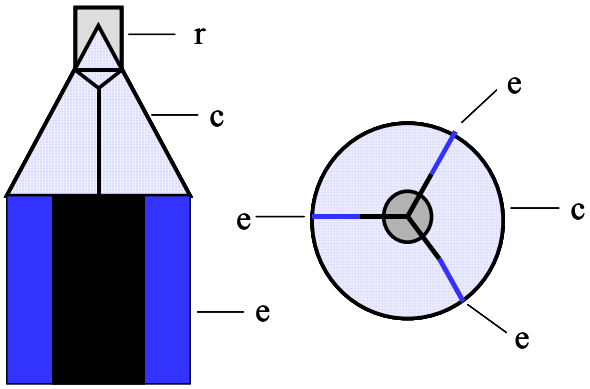
APPENDIX

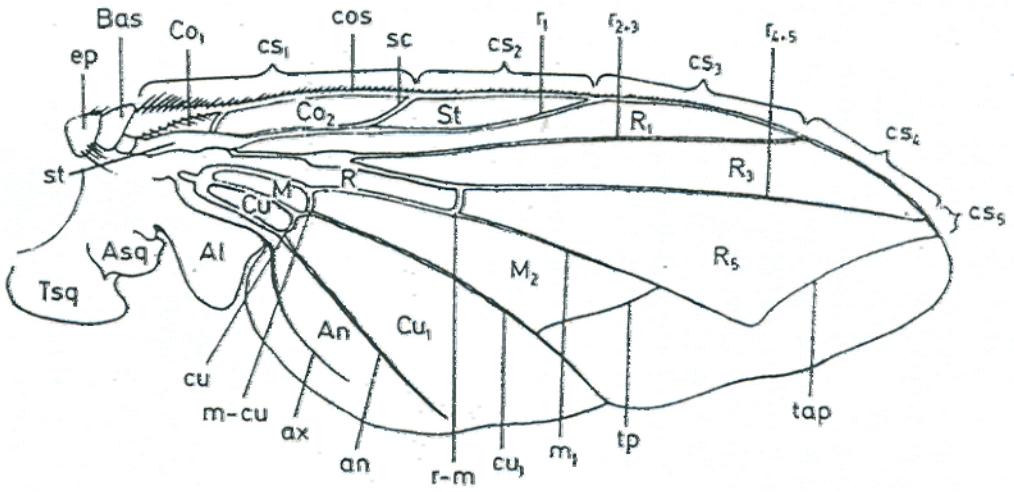
Appendix Figure1 Beef cattle shelter at Animal Research and Development Institute (SARDI), Kasetsart University, Kamphaengsaen campus, Ban Yang district, Nakhonpathom province

Appendix Figure 2 Dairy cattle farm at Animal Research and Development Institute (SARDI), Kasetsart University, Kamphaengsaen Campus, Ban Yang district, Nakhonpathom province

Appendix Figure 3 *Stomoxys* spp. feeding blood on the cattle's skin

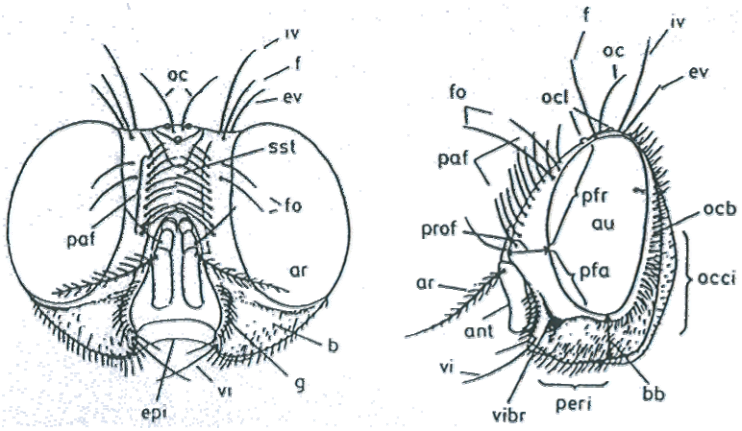


Appendix Figure 4 Diagram of Vavoua trap; c = cone of mosquitoes net, e = blabk cloth and blue cloth screen, r = capture box of *Stomoxys* spp. fly (Top); The Vavoua trap (Down)



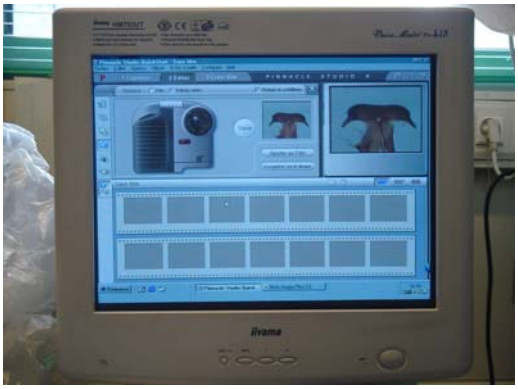
Appendix Figure 5 Wing of a musciform fly; ep = epaulet, Bas = basicosta, Co_1 = costal cell, St = subcostal cell, R = first basal cell, R_1 = marginal cell, R_3 = submarginal cell, R_5 = first posterior cell, M = second basal cell, M_2 = second posterior or discal cell, Cu = third basal cell, Cu_1 = third posterior cell, An = axillary cell, Al = aluta, Asq = alar squama, Tsq = thoracic squama, cos = costa, cs_{1-5} = segments of costa, st = stem vein, sc = subcosta, r_1 = first longitudinal vein, r_{2+3} = second longitudinal vein, r_{4+5} = third longitudinal vein, m_1 = fourth longitudinal vein (media), cu_1 = fifth longitudinal vein, an = sixth longitudinal vein, ax = axillary vein, r-m = discal cross vein, m-cu = upper cross vein, cu = lower basal cross vein, tap = upper marginal cross vein, tp = ower marginal cross vein

Source: Zumpt (1973)

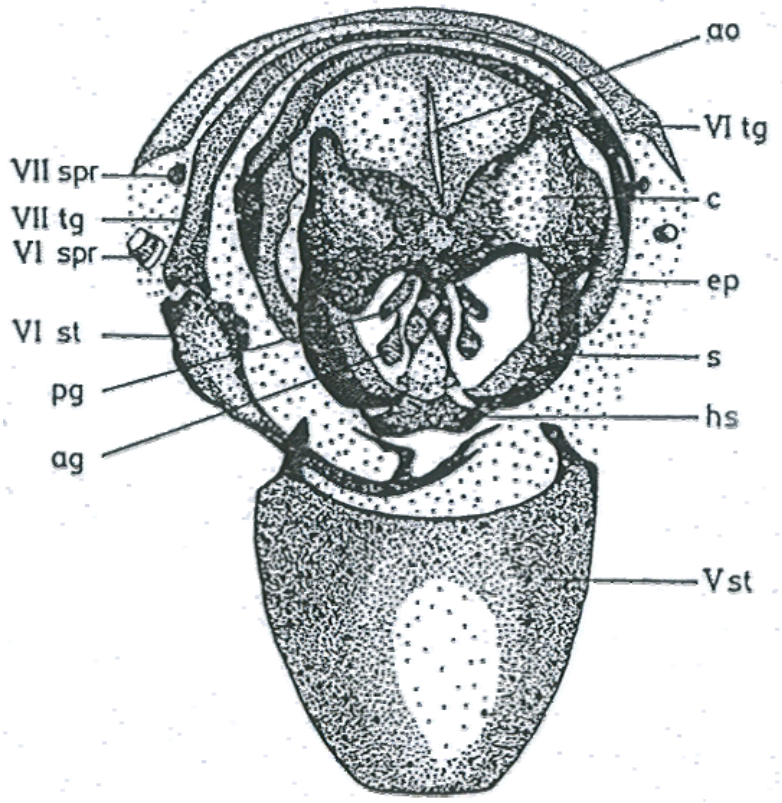


Appendix Figure 6 Frontal and lateral view of head of a musciform fly; ant = antenna, ar = arista, au = compound eye, b = bucca, bb = height of bucca, epi = epistome, ev = outer vertical bristle, f = frontal bristle, fo = fronto-orbital bristles, g = facial ridge, iv = inner vertical bristle, oc = ocellar bristle, ocb = postocular bristles, occi = occiput, ocl = ocelli, paf = parafrontal and parafacial bristles, peri = peristome, pfa = parafacialium, pfr = parafrontalium, prof = profrons, sst = frontal stripe, vi = vibrissa, vibr = vibrissarium

Source: Zumpt (1973)

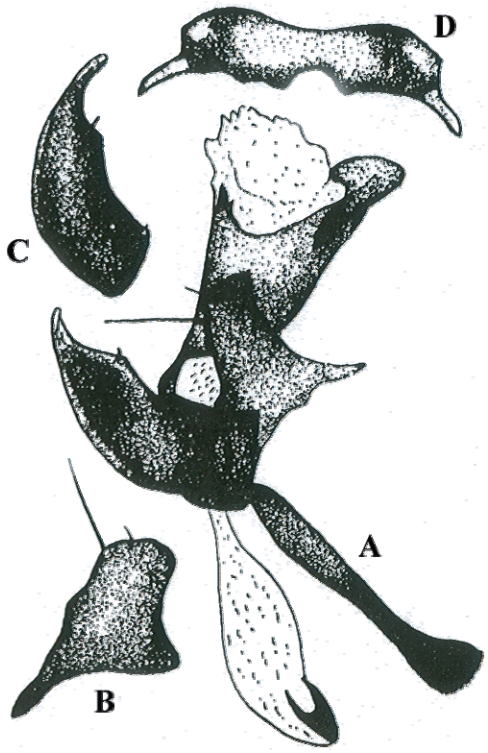


Appendix Figure 7 Left; The digital camera connecting with microscope, Right; The program Studio Quick Start Version 8 used for photographing the male genitalia of *Stomoxys* spp.



Appendix Figure 8 Male terminally of *Stomoxys calcitrans* (L.);
V st, VI st = fifth, sixth sternites;
VI spr, VII spr = sixth, seventh spiracles;
VI tg, VII tg = sixth, seventh tergites; ao = anal opening; c = cerci;
ep = epandrium; s = surstylus; hs = horned sclerite;
pg = postgonite; ag = pregonite

Source: Patton in Zumpt (1973)



Appendix Figure 9 Male genitalia of *Stomoxys calcitrans* (L.); A) aedeagus, B) right pregonite, C) right postgonite, D) horned sclerite

Source: Patton in Zumpt (1973)

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