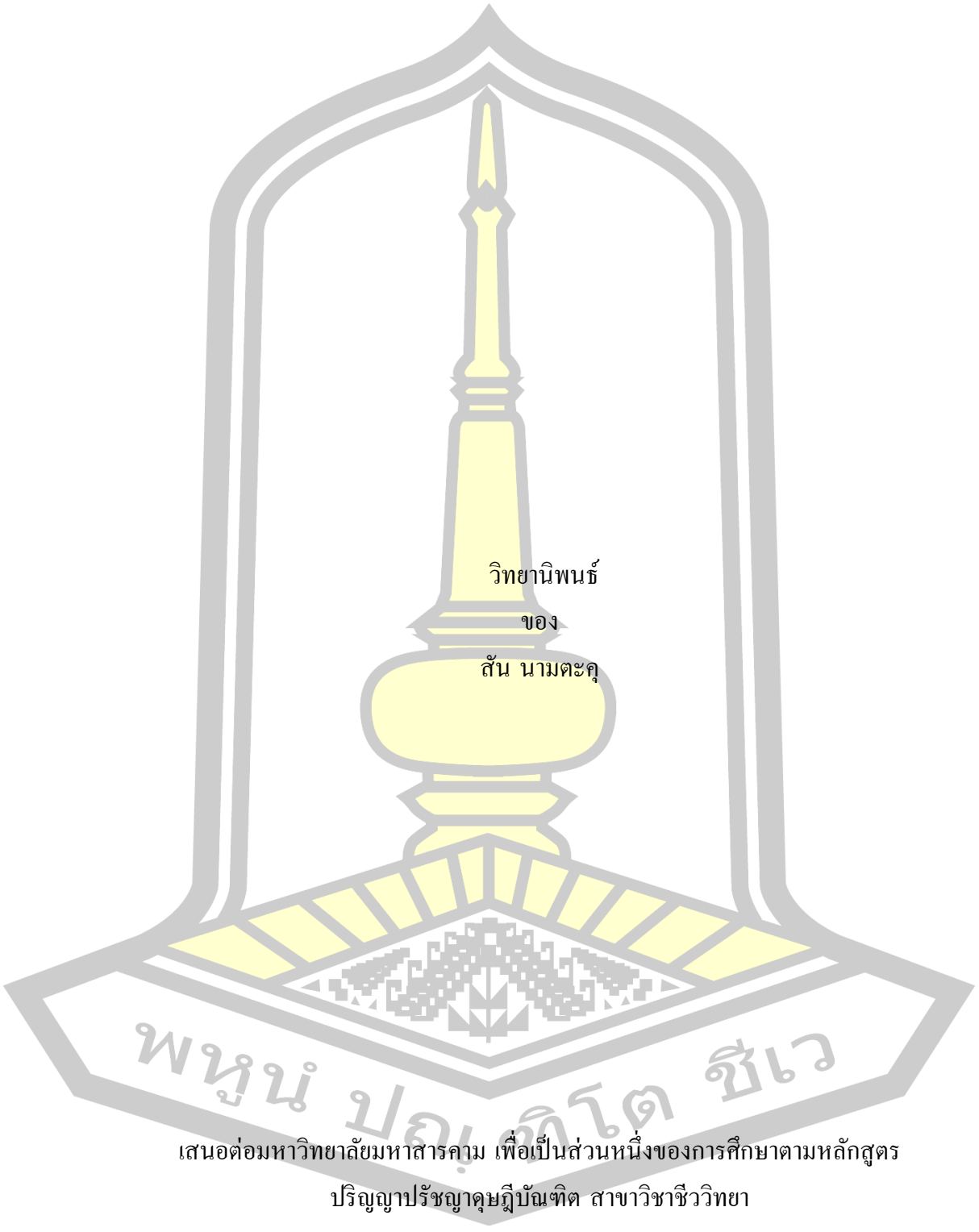
Genetic Diversity of *Hemerodromia* (Diptera: Empididae) in Thailand

San Namtaku

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Doctor of Philosophy in Biology
May 2019

ความหลากหลายทางพันธุกรรมของ *Hemerodromia* (Diptera: Empididae) ในประเทศไทย



วิทยานิพนธ์

ของ

สัน นามตะคุ

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The examining committee has unanimously approved this Thesis, submitted by Mr. San Namtaku , as a partial fulfillment of the requirements for the Doctor of Philosophy Biology at Mahasarakham University

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TITLE Genetic Diversity of *Hemerodromia* (Diptera: Empididae) in Thailand

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ABSTRACT

Species of *Hemerodromia* Meigen, 1822 (Diptera, Empididae) are important components of lotic habitats in freshwater ecosystems. The goal of this study was to examine genetic diversity and to test the efficiency of the mitochondrial cytochrome c oxidase subunit I (COI) barcoding region for species level identification of *Hemerodromia* in Thailand. Twelve *Hemerodromia* species were collected from 31 sites in North and North Eastern Thailand and 135 COI sequences obtained. Twenty eight sequences from the NCBI database were also included in the analysis. DNA barcoding identification analysis based on the best close match method performed well; with 100% of specimens agreeing with morphological identification. A phylogenetic tree based on the mitochondrial barcode sequences revealed a well-supported monophyly for all *Hemerodromia* species. Population genetic structure analyses of two geographically widespread species, *H. fusca* and *H. yunnanensis* revealed high level of genetic differentiation between populations. This result suggest limitation of gene flow that could be effected by patchy distribution of the forest habitat of these species.

Demographic history of *H. fusca* and *H. yunnanensis* revealed that both had undergone population demographic expansion. Lacking the information of the generation time preclude dating of expansion time but it is likely that the demographic expansion in both *Hemerodromia* species could be related to the Pleistocene climatic and environmental change like those of many other aquatic insects in Thailand.

Keyword : Hemerodromia, COI gene, DNA barcoding, Demographic history, Empididae

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CHAPTER 1
INTRODUCTION

1.1 Background

The superfamily Empidoidea of order Diptera represents about 7.8% of world diversity with 11,839 known species in about 400 genera (Yang *et al*., 2006; Yang *et al*., 2007) The superfamily is comprised of five families; Atelestidae, Dolichopodidae, Empididae, Hybotidae and Brachystomatidae (Sinclair BJ and Cumming JM, 2006) Brachystomatidae, Empididae and Hybotidae are conspicuous and often abundant part of this superfamily in Thailand (Plant, 2015).

Genus *Hemerodromia* Meigen (aquatic dance fly) belong to the family Empididae. *Hemerodromia* probably of recent origin in Eurasia (Plant *et al.*, 2012) but there are many undescribed species especially in the Neotropical Realm (Câmara *et al*.,2014) and insular Southeast Asia, which is considered a center of diversity of the genus (Adrain R. Plant, 2011). The immature stages are important components of freshwater ecosystems, especially in lotic habitats (Ivković *et al*.,2012). The tribe Hemerodromiini, in which the immature stages are strictly aquatic (Plant, 2011), living in usually well-oxygenated water. Some species of the *Hemerodromia* are predators of larvae and adults of several insects (Knutson LV and Steyskal GC, 1981; Ivković *et al*., 2012). Larvae of some *Hemerodromia* species prey on black fly larvae (Knutson LV and Steyskal GC, 1981), the vector of human onchocerciasis and some other livestock diseases. Adults of the *Hemerodromia* are poor fliers and are often found on bankside vegetation where they prey on insects(Wagner R, 2004; Ivković *et al*., 2012)

Hemerodromia Meigen is the largest aquatic genus of the family Empididae (Plant, 2015). The immature stages of *Hemerodromia* are strictly aquatic, usually found in well oxygenated lotic habitats such as streams and rivers but some species may occasionally be found in lentic waters (Adrain R. Plant, 2011). Some species of *Hemerodromia* are predators of larvae and adults of several insects such as black flies (Knutson LV and Steyskal GC, 1981), which are known as vectors of human onchocerciasis and some other livestock diseases. Adults of *Hemerodromia* have been

reported to be poor fliers and are often found on bankside vegetation where they prey on other insects (Wagner R, 2004). *Hemerodromia* are useful indicators for monitoring healthy aquatic environments.

Plant found 25 species of *Hemerodromia* from Thailand (Plant, 2015). Twenty new species were described. - *Hemerodromia alphalutea* Plant, 2015, *H. anisoserrata* Plant, 2015, *H. anomala* Plant, 2015, *H. attenuata* Plant, 2015, *H. betalutea* Plant, 2015, *H. conspecta* Plant, 2015, *H. deltalutea* Plant, 2015, *H. deminuta* Plant, 2015, *H. demissa* Plant, 2015, *H. epsilutea* Plant, 2015, *H. etalutea* Plant, 2015, *H. gammalutea* Plant, 2015, *H. isochita* Plant, 2015, *H. namtokhinpoon* Plant, 2015, *H. ocellate* Plant, 2015, *H. oriens* Plant, 2015, *H. phahompokensis* Plant, 2015, *H. songsee* Plant, 2015, *H. systoechon* Plant, 2015 and *H. zetalutea* Plant, 2015. Five species known previously from China were recognized. - *H. acutata* Grootaert, Yang & Saigusa, *H. flaviventris* Yang & Yang, *H. furcata* Grootaert, Yang & Saigusa, *H. fusca* Yang & Yang and *H. yunnanensis* Yang & Yang.

Population genetic study is important for understanding mechanisms underlying genetic structure and diversity of the species. Moreover, the study of population genetics can also determine the population history enabling us to understand the role of historical environmental change on genetic structure and diversity of living organisms. However, knowledge of population genetic structure and diversity in *Hemerodromia* is essentially lacking. Previous studies of many aquatic insect species in Thailand, such as Simuliidae indicated that Pleistocene climatic and environmental change play significant roles on the genetic structure, diversity and demographic history of simuliid faunas in Thailand. Because *Hemerodromia* is usually found in the same habitat as Simuliidae (which is usually water courses in pristine forest), its population genetic structure and population history analysis in *Hemerodromia* could be compare with previous study in Simuliidae as well as other insect such as mosquitoes. This comparative study would increase our understanding of the factors that influence genetic diversity of species in one of the most significant biodiversity hotspot regions.

In this study, genetic diversity and efficiency of mitochondrial cytochrome *c* oxidase I (COI) for morphological identification of *Hemerodromia* were tested. This is the first analysis of DNA barcodes of *Hemerodromia*. Previous studies in several families of the order Diptera e.g.Simulidae, Chironomidae, Hybotidae (Kondo *et al*., 2016; Nagy *et al*., 2013; Pramual *et al*., 2016) have shown that DNA barcoding sequences can effectively discriminate species and have also uncovered cryptic diversity that has not yet recognized based on traditional taxonomy (Changbunjong *et al*., 2018; Kondo *et al*., 2016; Kunprom & Pramual, 2016; Nagy *et al*., 2013; Pramual *et al*., 2016; Pramual P, 2014). Other advantage of the DNA barcode is that it possible to associate different life stages (Thaijarern *et al*., 2017) . Because the immature stages of many *Hemerodromia* species remain unknown, the DNA barcode library sequences provided in the present study will be very useful in expanding explorations of the diversity of these interesting insects to include their early stages.

1.2 Objectives of the research

The objective of this study is to investigate genetic structure, diversity and efficiency of mitochondrial cytochrome *c* oxidase I for species identification of *Hemerodromia* in Thailand.

1.3 Scope of the Research

Hemerodromia specimens will be collected from varied ecological conditions and habitats throughout Thailand. Species will be identified using morphology. DNA will be extracted from adult and polymerase chain reaction (PCR) will be used to amplify a fragment of cytochrome *c* oxidase subunit I (COI) gene. PCR products will be checked, purified and sequenced. Genetic diversity, genetic structure and population history and DNA barcoding will be examined using information from mitochondrial COI sequences.

CHAPTER 2
LITERATURE REVIEW

2.1 Classification of Hemerodromia

Genus *Hemerodromia* was classified into family Empididae (Subfamily Hemerodromiinae). The classification of this genus as follow:

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Oder Diptera

Superfamily Empidoidea

Family Empididae

Subfamily Hemerodromiinae (tribe Hemerodromiini)

Genus *Hemerodromia* Meigen

2.2 Biology and ecology of the *Hemerodromia* Meigen

Adult *Hemerodromia* prefer humid conditions and are almost never found more than a few meters away from the water. *Hemerodromia* are usually found restrictively close to well-oxygenated and clear water. Most species occur on clean, fast-flowing rivers and streams. They can be found on various stream sizes from those that are only 5-10 cm wide flowing under the bushes to 100 m wide rivers. *Hemerodromia* in temperate regions is univoltine with only one emergence of adults each year. However, in tropical region such as in Thailand, *Hemerodromia* is likely multivoltine as some species can be found throughout the year.

The life cycle of *Hemerodromia* includes immature stages (egg, larva, pupa) and a mature stage (adult) (Figure 1). The egg, larva and pupa live in clear water. Eggs of *Hemerodromia* are very small. The egg incubation period is 12-16 days. Larvae of *Hemerodromia* have a body length of ≤ 3 mm. Larvae have seven pairs of

abdominal prolegs. Larvae of some species of *Hemerodromia* live among mosses in flowing water where they have been reported to prey on simuliid larvae (Knutson LV and Steyskal GC, 1981), The pupa of the *Hemerodromia* has a body length of ≤ 3 mm with the apical pair integumentary horns short and lightly sclerotized. The pupal period is 12-14 days. Adults of *Hemerodromia* live in terrestrial ecosystems. The adult has a body length ≤ 3 mm. At least some adult *Hemerodromia* are known to be predators of simuliid larvae and the adults of several insects.

Apart from studies of mating behavior and swarming, little is known about the habits of Empididae (Knutson LV and Steyskal GC, 1981). Adults are most often found in vegetation in moist locations, on tree trunks or even on the surface of the water. Both sexes of most genera take a proteinaceous meal and some genera also feed on nectar. Living insects, particularly swarming or emerging Diptera (including many Empididae) can be important food sources for many predatory animals. Many adult empidids have restricted feeding zones, such as among swarming Diptera, on tree trunks, on the ground or on plant surfaces. The Empididae are probably important in natural control of some pest insects. Adults of several genera prey on emerging or swarming mosquitoes and black fly larvae and pupae.

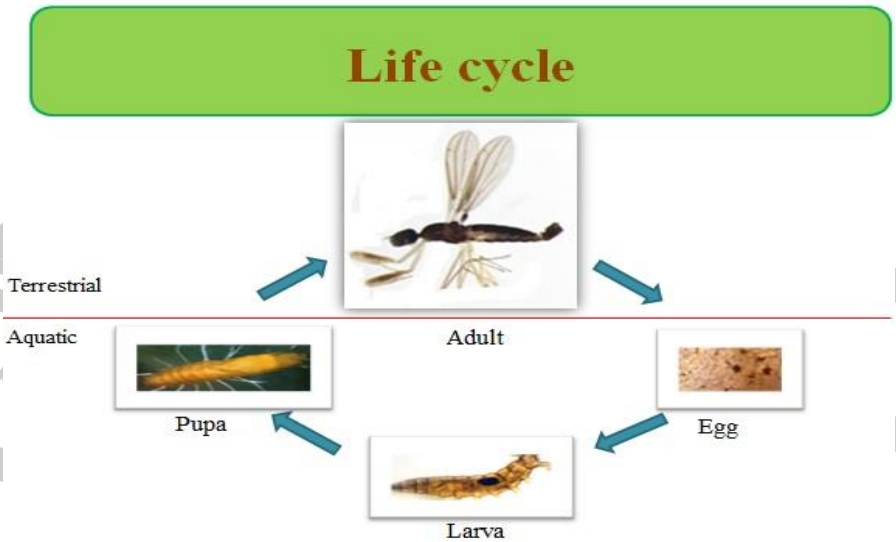


Figure 1 Life cycle of *Hemerodromia* spp. (diptera.info/forum/viewthread.php?forum_id=4&thread_id=69596Diptera.info - Discussion Forum: Diptera (eggs, larvae, pupae))

2.3 Population genetics

Population genetics is the study of genetic variation and genetic structure of a species. Two factors including contemporary factors (contemporary ecological conditions, geographical factors, distance between population size, etc.) and historical factors (climate and ecology conditions changing, population size changing in the past, etc.) effect the genetic structure and diversity of species (Pramual, 2014). Advances in both molecular biology and population genetic theory have revolutionized the field. Previous studies in Thailand revealed that both historical and contemporary factors are playing a role on patterns of genetic structure and diversity in Thai faunas. Several studies of black fly in Thailand were reported about population history analysis indicated that population expansions occurred during the Pleistocene. *Simulium tani* had population expansions during the mid-Pleistocene and the late Pleistocene suggesting that current population structure and diversity could be due in part to the species response to Pleistocene climatic fluctuations (Pramual *et al*., 2005). *Simulium siamense* Takaoka and Suzuki complex had population expansion in the late Pleistocene about 120,000 years ago (Pramual *et al*., 2011). *Simulium aureohirtum* had population expansion dating back to the last glaciations (Thaijarern *et al*., 2014). However, the population genetics and demographic history of guava fruit fly *Bactrocera correcta* (Kunprom *et al*., 2015) reported the demographic expansion occurred dating back to the end of the last glaciations of the Pleistocene.

2.4 DNA barcoding

DNA barcoding is a method that uses a short genetic marker in the DNA of an organism to identify unknown specimens to a particular species. The most commonly used barcode region, for animals is a fragment of approximately 600 base pairs of the mitochondrial cytochrome *c* oxidase I gene (COI). Applications include; identifying insect larvae (which may have fewer diagnostic characters than adults and are frequently less well-known), identifying the diet of an animal, based on its stomach contents or faeces and identifying products in commerce (herbal supplements, wood, or skins and other animal parts). Several studies of DNA barcoding in Diptera were reported around the world. In Colombia, synanthropic flesh flies (Diptera, Sarcophagidae) were studied for tested the efficiency of the barcode

region of the mitochondrial cytochrome oxidase subunit I (COI) gene for 100% several species identification. Kimura two-parameter genetic distances and reconstruct a Neighbor-Joining phylogenetic tree were used for genera data analysis. A tree shown monophyletic form clade indicated that COI barcodes had efficiency for species identification of flesh flies (Buenaventura *et al*., 2018). In India, DNA barcode was evaluated for species identification of Tabanidae (Diptera); the vectors of surra disease or trypanosomiasis. Horse flies and deer flies are common names applied to members of the family Tabanidae (Diptera). Tabanid flies are pestiferous and of veterinary and medical importance, with about 244 species in India. Major vectors of *Trypanosoma evansi* that causes trypanosomiasis. Neighbor-Joining and Bayesian tree were used for data analysis. Analysis revealed that all morphologically identifiable species can be discriminated using DNA barcoding data (Banerjee *et al*., 2015). Moreover, *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) from Southern Western Ghats of India were used for studied two new species by using DNA barcode. Two new species of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) were described on the basis of reared adult, pupal and larval specimens. Phylogeny of members in the genus *Simulium* was reconstructed based on DNA barcoding gene (cytochrome oxidase *c* subunit I). Tree analysis using maximum likelihood method is congruent with evidence of two new species in the subgenus *Gomphostilbia* and separated from other species (Anbalagan *et al*., 2015). In Japan, DNA barcode was evaluated the efficacy for identify and analyze five Japanese encephalitis mosquito vectors (*Culex fuscocephala, Culex gelidus, Culex tritaeniorhynchus, Culex pseudovishnui* and *Culex vishnui*). The intra- and inter nucleotide divergence and the maximum parsimony tree were observed for data analysis (Karthika *et al*., 2018). In Malaysia, pupa, larva and DNA barcoding of *Simulium* (*Simulium*) *hackeri* Edwards (Diptera: Simuliidae) were used for studied morphological characters (Ya'cob *et al*., 2018). In Peru, DNA barcoding was used for identification of sand fly species (Diptera: Psychodidae) from leishmaniasis-endemic areas. Phlebotomine sand flies were the only proven vectors of leishmaniases, a group of human and animal diseases. Accurate knowledge of sand fly species identification is essential in understanding the epidemiology of leishmaniasis and vector control in endemic areas. Classical identification of sand fly species based on morphological characteristics often remains

difficult and requires taxonomic expertise. Neighbor-joining (NJ) analysis based on Kimura 2-Parameter genetic distances and intraspecific genetic divergence were used for data analysis (Nzelu *et al*., 2015). Generally, DNA barcode was used for species identification and had high efficiency results in several Diptera including; phlebotomine sand fly (Diptera: Psychodidae) (Romero-Ricardo *et al*., 2016), *Oxysarcodexia* Townsend (Diptera: Sarcophagidae) (Madeira *et al*.,2016), sarcosaprophagous Diptera species (Rolo *et al*., 2013) and European Fanniidae (Diptera) (Grzywacz *et al*., 2017). Furthermore, DNA barcoding was used for exploring genetic variation in haplotypes studied in filariasis vector *Culex quinquefasciatus* (Diptera: Culicidae) (Vadivalagan *et al*., 2017). The intra- and inter-population polymorphism, Neighbor-joining (NJ) tree, the genetic diversity index Tajima' D and Fu's FS were used for data analysis.

For DNA barcodes in Thailand, black flies are among the most well studied insects. Black flies in the subgenus *Gomphostilbia* (Diptera: Simuliidae) were used for examined the efficacy of DNA barcoding. Both intraspecific and interspecific genetic divergence values, based on the Kimura-2 parameter were used for data analysis. Values of overlap in seven species owing to incomplete lineage sorting and imperfect taxonomy, implying that DNA barcoding to identify these species will be ambiguous. Despite a low level of success, they found that DNA barcoding is useful in revealing cryptic biodiversity, potentially facilitating traditional taxonomy (Pramual *et al*., 2011). Whereas, studied DNA barcoding of tropical black flies (Diptera: Simuliidae) found high intraspecific genetic divergence and barcodes also differentiated cytoforms of selected species complexes, albeit with varying levels of success. The differential efficiency of DNA barcodes to discriminate cytoforms was attributed to different levels of genetic structure and demographic histories of the taxa. DNA barcode trees were largely congruent with phylogenies based on previous molecular, chromosomal and morphological analyses, but revealed inconsistencies that will require further evaluation (Pramual & Adler, 2014). Moreover, DNA barcode was used for studied association of black fly (Diptera: Simuliidae) life stages by using mitochondrial cytochrome c oxidase subunit I (COI) barcoding sequences to associate unknown larvae with the known species of black flies of the *Simulium multistriatum*-

group. Black fly larvae remain unknown because they cannot be associated with the known species described from other life stages. Phylogenetic analyses and distance-based criteria were used to associate unknown specimens with the known species. The unknown larvae were separated into two monophyletic clades with strong support. Morphological descriptions of these larvae and characters for species diagnosis were provided. The results indicated that DNA barcoding sequences could used effectively to associate unknown life stage with the known species, and this ability will facilitate further study of all aspects of black fly biology (Pramual & Wongpakam, 2014). Additional, DNA barcode had efficiency for species identification including; hematophagous flies (Diptera: Muscidae: Stomoxyinae) and horse flies (Diptera: Tabanidae). DNA barcoding was able to discriminate between morphologically uncertain or misidentified and confirm the correct species, which were important steps for elucidating diversity (Changbunjong *et al*., 2016; Changbunjong *et al*., 2018). DNA barcoding also application for studied with wing morphometrics in distinguish three Aedes vectors; *Aedes aegypti* (Diptera: Culicidae) (L.), *Ae. albopictus* (Skuse), and *Ae. scutellaris* (Walker). These mosquito had morphologically similar and sympatric in some parts of their distribution; therefore, there was a risk of incorrect morphological identification. Any confusion could have a negative impact on epidemiological studied or control strategies. The result confirmed that these morphologically close species were valid, and that geometric morphometrics could considerably increase the reliability of morphological identification (Sumruayphol *et al*., 2016).

2.5 *Hemerodromia* spp. in Thailand

In Thailand, there are twenty five species of the *Hemerodromia* (Plant, 2015) including *H. alphalutea* Plant, 2015, *H. anisoserrata* Plant, 2015, *H. anomala* Plant, 2015, *H. attenuata* Plant, 2015, *H. betalutea* Plant, 2015, *H. conspecta* Plant, 2015, *H. deltalutea* Plant, 2015, *H. deminuta* Plant, 2015, *H. demissa* Plant, 2015, *H. epsilutea* Plant, 2015, *H. etalutea* Plant, 2015, *H. gammalutea* Plant, 2015, *H. isochita* Plant, 2015, *H. namtokhinpoon* Plant, 2015, *H. ocellata* Plant, 2015, *H. oriens* Plant, 2015, *H. phahompokensis* Plant, 2015, *H. songsee* Plant, 2015, *H. systoechon* Plant, 2015, *H. zetalutea* Plant, 2015, *H. acutata* Grootaert, Yang & Saigusa, 2000, *H. flaviventris*

Yang & Yang, 1991, *H. furcata* Grootaert, Yang & Saigusa,2000, *H. fusca* Yang & Yang, 1986 and *H. yunnanensis* Yang & Yang, 1988. Distribution patterns of *Hemerodromia* habitats in Thailand could be divided into four categories namely; the Northern mountains, the Northern lowlands, the South and the East. Species richness and the endemicity of *Hemerodromia* are shown in Figure 2 and Table 1, respectively (Plant, 2015).

Table 1 Species of the *Hemerodromia* in four habitat categories

Habitats	*Hemerodromia* spp.
Northern montane apparently endemic species.	*H. deltalutea*,
	H. deminuta,
	H. systoechon,
	H. isochita,
	H. phahompokensis,
	H. zetalutea
Northern lowland apparently endemic species	*H. demissa*,
	H. epsilutea
Northern montane widespread species	*H. acutata*
	H. flaviventris
	H. songsee
Southern apparently endemic species	*H. etalutea*
	H. ocellata
Eastern apparently endemic species	*H. anisoserrata*
	H. oriens
Widespread lowland species	*H. fusca*
	H. yunnanensis
	H. furcata
Limestone tufa specialist species	*H. anomala*
	H. namtokhinpoon
	H. conspecta

Source: Plant (2015)

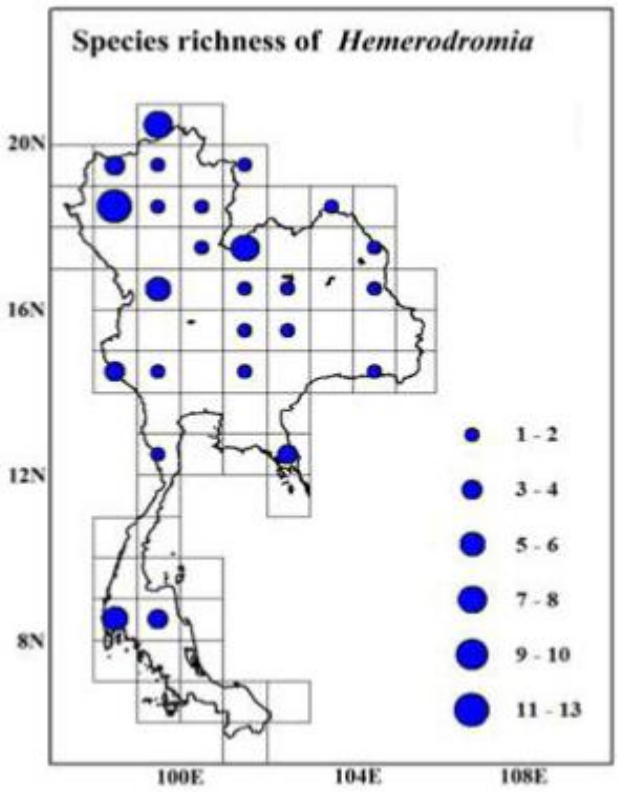


Figure 2 Species richness of *Hemerodromia* in Thailand

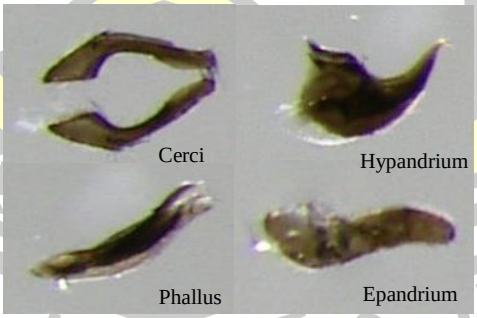
Source: Plant (2015)

For this study, twelve species of *Hemerodromia* were selected for DNA barcoding study including; *H. acutata* Grootaert, Yang & Saigusa, 2000, *H. anisoserrata* Plant, 2015, *H. anomala* Plant, 2015, *H. betalutea* Plant, 2015, *H. conspecta* Plant, 2015, *H. namtokhinpoon* Plant, 2015, *H. oriens* Plant, 2015, *H. songsee* Plant, 2015, *H. flaviventris* Yang & Yang, 1991, *H. furcata* Grootaert, Yang & Saigusa, 2000, *H. fusca* Yang & Yang, 1986 and *H. yunnanensis* Yang & Yang, 1988. These twelve species were selected because they are distributed widely throughout the country. These species occur near streams or rivers in diverse forest types and are also found in irrigation channels near rice fields at 95-1,306 meters and 51-1,152 meters, respectively (Plant, 2015)

2.5.1 *Hemerodromia acutata* Grootaert, Yang & Saigusa, 2000

Adult of *H. acutata* is shown in figure 3. Terminalia of *H. acutata* had black and elongate cerci, epandrium quite narrow, hypandrium look like quadrate shape. Hypandrium look like keel shape.

(A)



(B)

Figure 3 (A) Adult of male *H. acutata* Grootaert, Yang & Saigusa, 2000, (B) Terminalia of *H. acutata* Grootaert, Yang & Saigusa, 2000

Plant reported that found *H. acutata* in Kamphaeng Phet and Chiang Mai during September to December (Plant, 2015). In this study, found *H. acutata* from Siriphoom waterfall, Chom Thong, Chiang Mai, Muang Pond, Khum Yuam district, Mae Hong Son and Mae U-Kor, Khum Yuam, Mae Hong Son on October. Habitats of *H. acutata* are moist and variety of plants cover the stream. Distribution of *H. acutata* shown in figure 4.

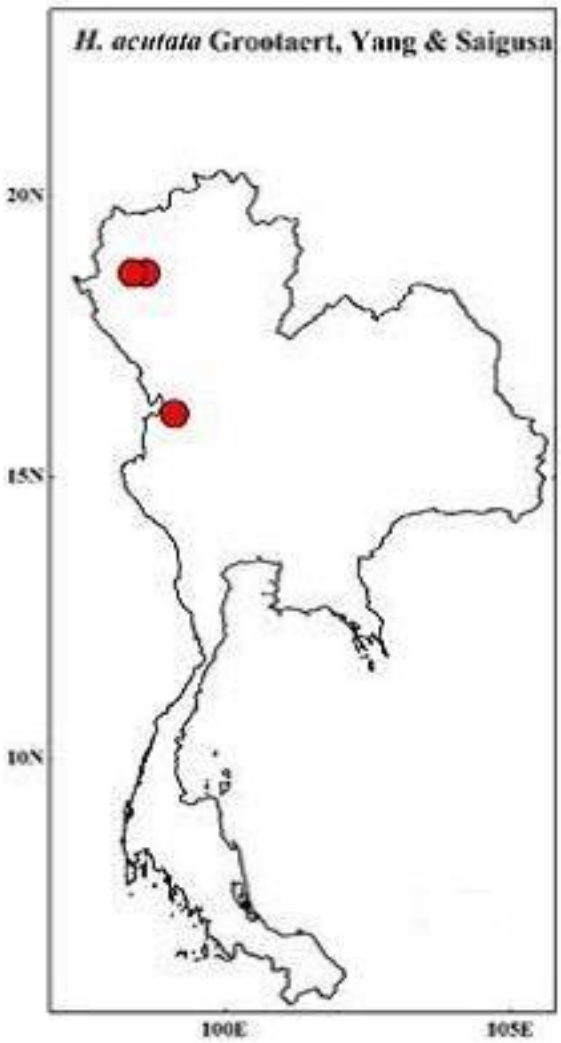
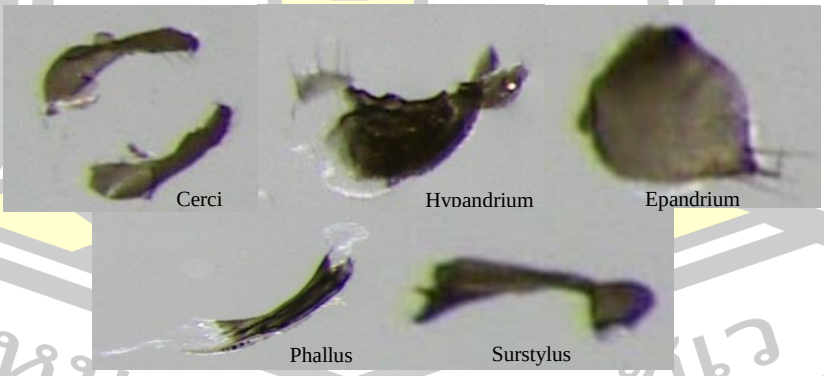


Figure 4 Distribution of *H. acutata* Grootaert, Yang & Saigusa, 2000 in Thailand
Source: Plant (2015)

2.5.2 *Hemerodromia anisoserrata* Plant, 2015

Adult of *H. anisoserrata* is shown in figure 5. Terminalia of *H. anisoserrata* had elongate cerci, epandrium look round and subquadrate shape, hypandrium look like keel shape.

(A)



(B)

Figure 5 (A) Adult of male *H. anisoserrata* Plant, 2015
(B) Terminalia of *H. anisoserrata* Plant, 2015

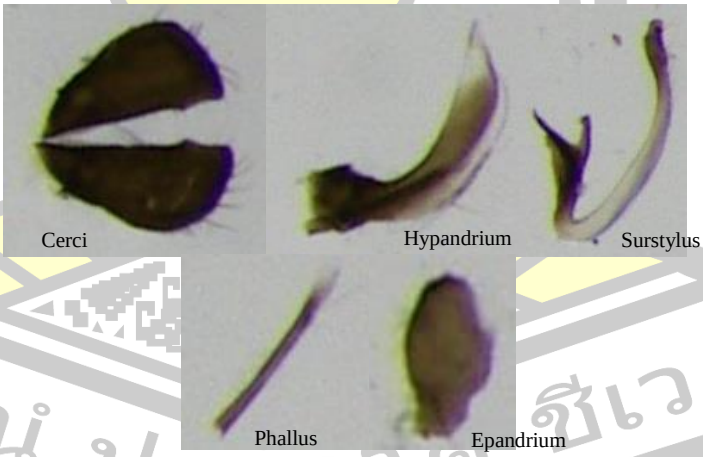
Plant reported that found *H. anisoserrata* from Loei on November (Plant, 2015). In this study, found *H. anisoserrata* from Song Kon waterfall, Phu Ruea, Loei on March. Habitats of *H. anisoserrata* are moist, big shade of stone and moderate plants cover the stream. Distribution of *H. anisoserrata* shown in figure 6.

Figure 6 Distribution of *H. anisoserrata* Plant, 2015 in Thailand
Source: Plant (2015)

2.5.3 *Hemerodromia anomala* Plant, 2015

Adult of *H. anomala* is shown in figure 7. Terminalia of *H. anisoserrata* had long and broad cerci, epandrium black with dark setae, hypandrium white and long narrow, surstylus very long up curve and pale. This species had a distinctive by wings had a strong black irregularly trapezoid stigma around R4.

(A)



(B)

Figure 7 (A) Adult of male *H. anomala* Plant, 2015

(B) Terminalia of *H. anomala* Plant, 2015

Plant reported that found *H. anomala* from tufa stream in Loei and Kanchanaburi during February, August, November and December (Plant, 2015). In this study, found *H. anomala* in tufa waterfall from Suan Hom, Nong Hin, Loei on September and March. Habitats of *H. anomala* are moist, open place, slope shade of stone and mild plants cover the stream. Distribution of *H. anomala* shown in figure 8.

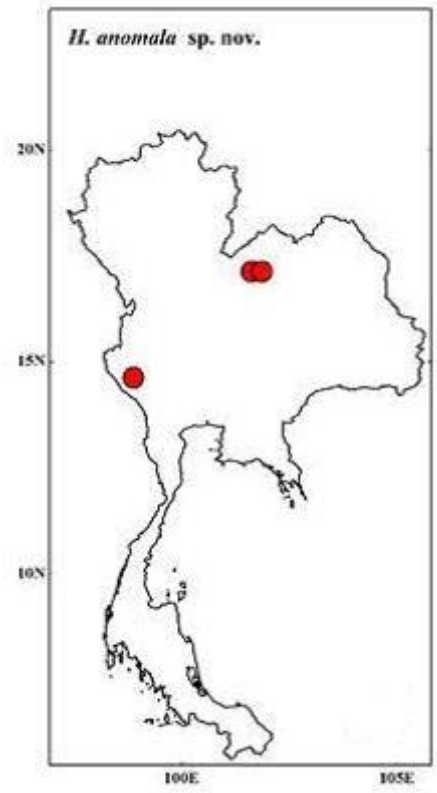


Figure 8 Distribution of *H. anomala* Plant, 2015 in Thailand

Source: Plant (2015)

2.5.4 *Hemerodromia betalutea* Plant, 2015

Adult of *H. betalutea* is shown in figure 9. Female of *H. betalutea* had whitish yellow legs, white halters and yellow abdomen

Figure 9 Adult of female *H. betalutea* Plant, 2015

Plant reported that found *H. betalutea* in Khampaeang Phet and Chiang Mai during December to July, the cool dry season (Plant, 2015). In this study, found only female from Chom Thong, Chiang Mai on June. Unfortunately could not found any male of *H. betalutea.* Distribution of *H. betalutea* shown in figure 10.

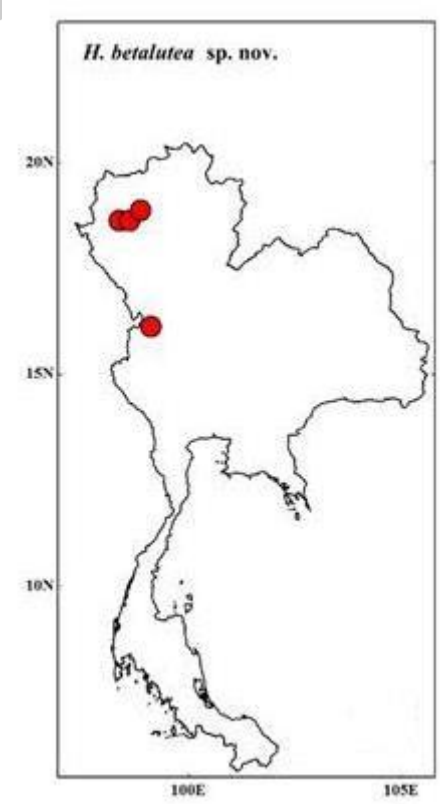
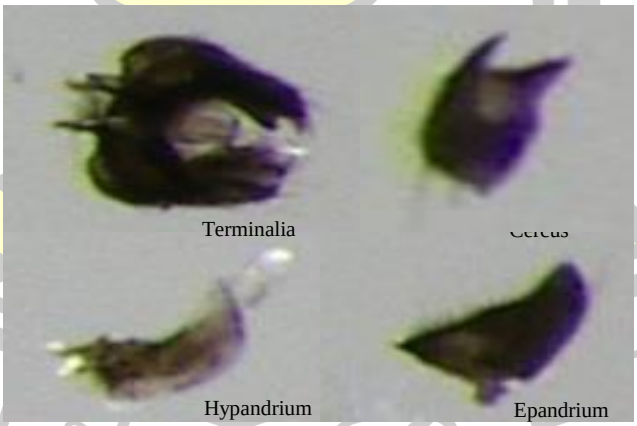


Figure 10 Distribution of *H. betalutea* Plant, 2015 in Thailand
Source: Plant (2015)

2.5.5 *Hemerodromia conspecta* Plant, 2015

Adult of *H. conspecta* is shown in figure 11. Terminalia of *H. conspecta* had compact and small size with short and broad cerci, hypandrium small and short, epandrium broad and look like subtriangular shape in dorsal view.

(A)



(B)

Figure 11 (A) Adult of male H. conspecta Plant, 2015

(B) Terminalia of *H. conspecta* Plant, 2015

Plant reported that found *H. conspecta* in Loei on November (Plant, 2015). In this study, found *H. conspecta* from Suan Hom, Nong Hin, Loei on March. Habitats of *H. conspecta* are moist, open place, slope shade of stone and mild plants cover the stream. Distribution of *H. conspecta* shown in figure 12.

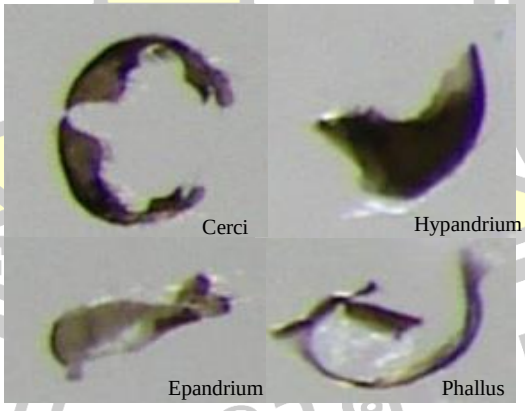
Figure 12 Distribution of *H. conspecta* Plant, 2015 in Thailand

Source: Plant (2015)

2.5.6 *Hemerodromia namtokhinpoon* Plant, 2015

Adult of *H. namtokhinpoon* is shown in figure 13. Terminalia of *H. namtokhinpoon* had black and small size, complex cerci, epandrium very elongate and narrow, hypandrium look like subovate shape.

(A)



(B)

Figure 13 (A) Adult of male *H. namtokhinpoon* Plant, 2015

(B) Terminalia of *H. namtokhinpoon* Plant, 2015

Plant reported that found *H. namtokhinpoon* in Loei in late November during the cool dry season (Plant, 2015). In this study, found *H. namtokhinpoon* from Suan Hom, Nong Hin, Loei on March. Habitats of *H. namtokhinpoon* are moist, open place, slope shade of stone and mild plants cover the stream. Distribution of *H. namtokhinpoon* shown in figure 14

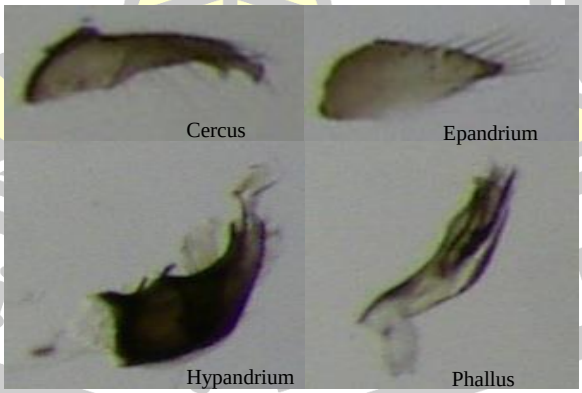
Figure 14 Distribution of *H. namtokhinpoon* Plant, 2015 in Thailand

Source: Plant (2015)

2.5.7 *Hemerodromia oriens* Plant, 2015

Adult of *H. oriens* is shown in figure 15. Terminalia of *H. oriens* had very elongate cerci, epandrium rather narrow and apically point, surstylus small and narrow but apically spatulate, hypandrium narrow and distal upcurve look like keel shape.

(A)



(B)

Figure 15 (A) Adult of male *H. oriens* Plant, 2015

(B) Terminalia of *H. oriens* Plant, 2015

Plant reported that found *H. oriens* from Mukdahan on November (Plant, 2015). In this study, found *H. oriens* from Mukdahan on November. Habitats of *H. oriens* are moist, small shade stream in deciduous lowland and dry evergreen forests. Distribution of *H. oriens* shown in figure 16

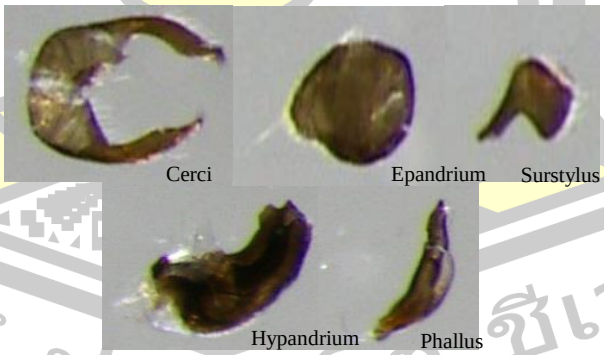
Figure 16 Distribution of *H. oriens* Plant, 2015 in Thailand

Source: Plant (2015)

2.5.8 *Hemerodromia songsee* Plant, 2015

Adult of *H. songsee* is shown in figure 17. Terminalia of *H. songsee* had elongate and black cerci, epandrium elongate look like subrectangular, slightly and apical point, hypandrium look like hemispherical shape, surstylus had strongly spatulate apically in lateral view.

(A)



(B)

Figure 17 (A) Adult of male *H. songsee* Plant, 2015

(B) Terminalia of *H. songsee* Plant, 2015

Plant reported that found *H. songsee* in Chiang Mai during February, April, May, September, November and December (Plant, 2015). In this study, found *H. songsee* from Chiang Mai (Doi Inthanon, Chomthong, and Doi Pha Hom Pok, Fang) on May and December. Habitats of *H. songsee* are stream in hill evergreen and moist hill evergreen forests. Distribution of *H. songsee* shown in figure 18.

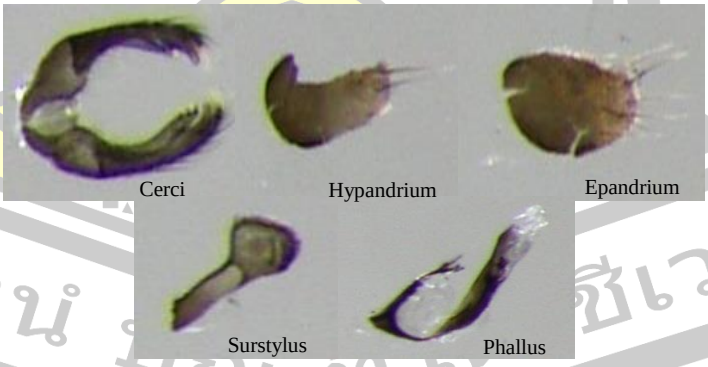
Figure 18 Distribution of *H. songsee* Plant, 2015 in Thailand

Source: Plant (2015)

2.5.9 *Hemerodromia flaviventris* Yang & Yang, 1991

Adult of *H. flaviventris* is shown in figure 19. Terminalia of *H. flaviventris* had black and elongate cerci, epandrium elongate look like ovoid shape with strong setae on outer face, hypandrium short and narrow, surstylus look like broad subovate shape.

(A)



(B)

Figure 19 (A) Adult of male *H. flaviventris* Yang & Yang, 1991

(B) Terminalia of *H. flaviventris* Yang & Yang, 1991

Plant reported that found *H. flaviventris* from Nan and Chiang Mai on November and December (Plant, 2015). In this study, found *H. flaviventris* from Siriphoom waterfall, Chom Thong, Chiang Mai on October. Habitats of *H. flaviventris* from are moist, variety of plants cover the stream and predominantly evergreen and pinus forest biotopes. Distribution of *H. flaviventris* shown in figure 20.

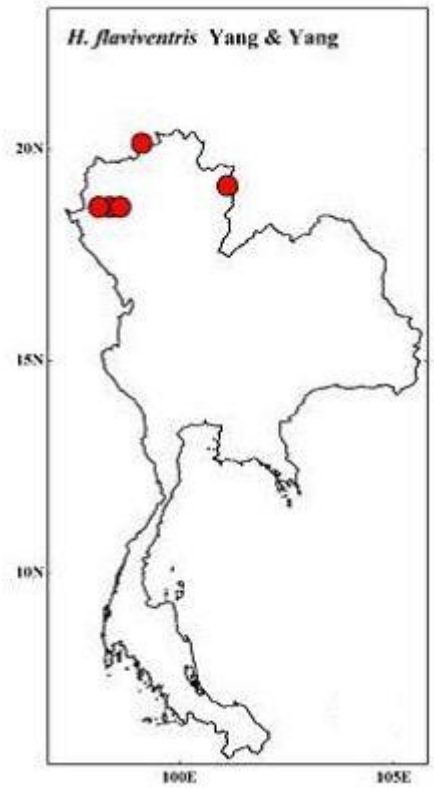


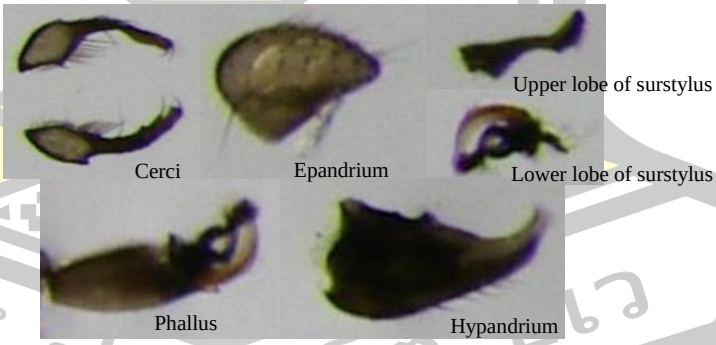
Figure 20 Distribution of *H. flaviventris* Yang & Yang, 1991 in Thailand

Source: Plant (2015)

2.5.10 *Hemerodromia furcata* Grootaert, Yang & Saigusa, 2000

Adult of *H. furcata* is shown in figure 21. Terminalia of *H. furcata* had black and elongate cerci with apical separate distinctive bifurcate, epandrium elongate look like subovate shape, surstylus compose of upper lobe and lower lobe, the upper lobe look like almost shoe-shape, hypandrium look like keel shape.

(A)



(B)

Figure 21 (A) Adult of male *H. furcata* Grootaert, Yang & Saigusa, 2000

(B) Terminalia of *H. furcata* Grootaert, Yang & Saigusa, 2000

Plant reported that found *H. furcata* in Nan, Chiang Rai, Chiang Mai, Mae Hong Son, Loei, Chantaburi and Surat Thani on March, July, October and November (Plant, 2015). In this study, found *H. furcata* from Doi Chiang Dao, Chiang Dao, Chiang Mai and Song Kon waterfall, Phu Ruea, Loei on October and March. Habitats of *H. furcata* are stream in hill evergreen forest with moist, variety of plants cover the stream and sometime found near mosses and plants where stand in the middle of the stream. Distribution of *H. furcata* shown in figure 22.

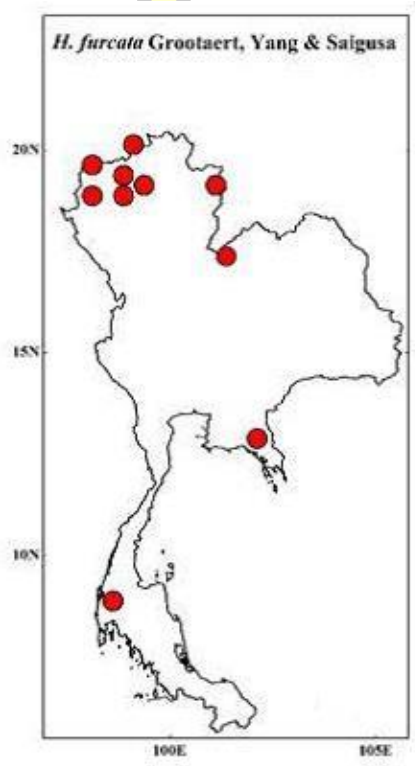
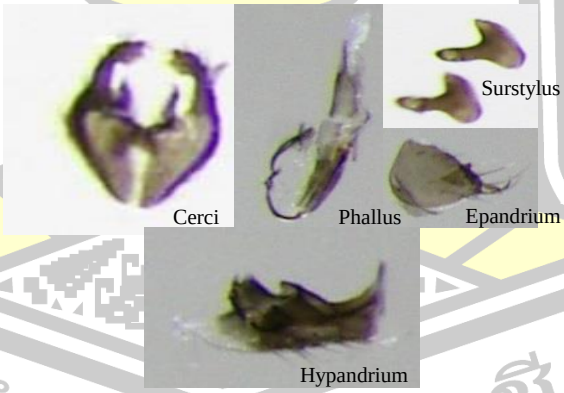


Figure 22 Distribution of *H. furcata* Grootaert, Yang & Saigusa, 2000 in Thailand Source: Plant (2015)

2.5.11 *Hemerodromia fusca* Yang & Yang, 1986

Adult of *H. fusca* is shown in figure 23. Terminalia of *H. fusca* had black and moderately elongate cerci with dark setae, epandrium bluntly pointed look like ovoid shape, surstylus very conspicuously inflate apical distinctive broadly T-shape, hypandrium look like keel shape.

(A)



(B)

Figure 23 (A) Adult of male *H. fusca* Yang & Yang, 1986
(B) Terminalia of *H. fusca* Yang & Yang, 1986

Plant reported that *H. fusca* is distributed throughout the country (Plant, 2015). Specimens were collected from Beung Kan, Chaiyaphum, Chantaburi, Chiang Mai, Kamphaeng Phet, Kanchanaburi, Khon Kaen, Lampang, Loei, Mukdahan, Nakhon Nayok, Nakhon Si Thammarat, Nan, Petchaburi and Surat Thani. Adults were found in every month all of the year but most abundant from September to March. In this study, found *H. fusca* from Huay Phrik Luang, Nan, Suan Sawan waterfall and Suan Hom waterfall, Nong Hin, Loei, Doi Inthanon, Chomthong, Chiang Mai, Nong Pok, Roi-Et, Huay Mae Sa, Mae Rim, Chiang Mai, Mae U-Kor, Khum Yuam, Mae Hong Son and Ban Song Kon, Phu Ruea, Loei. Specimens were collected on March and during September to December. Habitats of *H. fusca* are moist, variety of plants cover with small and middle stream, open places, big shade stone waterfall, slope stone waterfall and small stream near the rice field. Distribution of *H. fusca* shown in figure 24.

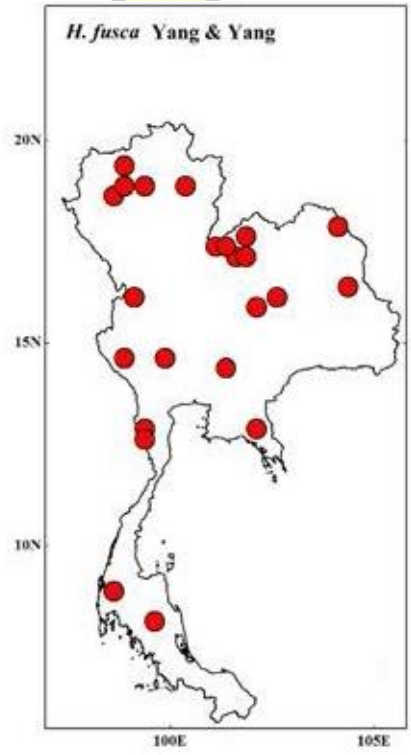
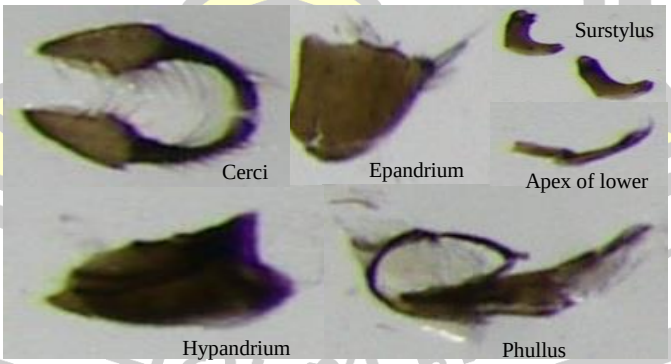


Figure 24 Distribution of *H. fusca* Yang & Yang, 1986 in Thailand
Source: Plant (2015)

2.5.12 *Hemerodromia yunnanensis* Yang & Yang, 1988

Adult of *H. yunnanensis* is shown in figure 25. Terminalia of *H. yunnanensis* had black and elongate cerci with dark setae, epandrium bluntly pointed look like ovoid shape, surstylus very conspicuously inflate apical distinctive broadly L-shape, hypandrium look like keel shape.

(A)



(B)

Figure 25 (A) Adult of male *H. yunnanensis* Yang & Yang, 1988
(B) Terminalia of *H. yunnanensis*, Yang & Yang, 1988

Plant reported that *H. yunnanensis* is distributed throughout the country (Plant, 2015). Specimens were collected from Loei, Chaiyaphum, Petchabun, Phayao, Nan, Sisaket, Bueng Kan, Chantaburi and Surat Thani. Adults were found most abundant during October and November but minority capturing in January, May, July and September. In this study, *H. yunnanensis* found from Pla Ba waterfall, Phu Ruea, Loei, Jet Sri waterfall, Sega, Beung Kan, Huai Tub Gor Sod, Na Haeo, Loei, Ban Song Kon, Phu Ruea, Loei, Huay Wang Yai waterfall, Kantharalak District, Sisaket, Phu Foi Lom National Park, Udon Thani and Doi Chiang Dao, Chiang Dao, Chiang Mai. Specimens were collected on March, May and during September to December. Habitats of *H. yunnanensis* are moist, variety of plants cover with small and middle stream, open places, big shade stone waterfall, slope stone waterfall and small stream near the rice field. Distribution of *H. yunnanensis* shown in figure 26.

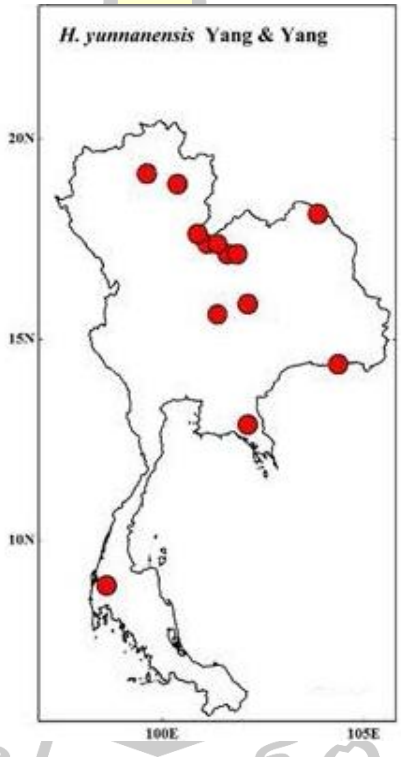


Figure 26 Distribution of *H. yunnanensis* Yang & Yang, 1988 in Thailand
Source: Plant (2015)

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Sample collections and identifications

Adult fly of *Hemerodromia* specimens were collected throughout Thailand using sweep net swung plane, up and down near bush areas around the waterfalls or stream banks from 31 sites in Thailand, which have a clean environment and without human disturbance (Figure 27; 28; 29, Table 2) Specimens were preserved in 80% ethanol and kept at -20° C for further study. *Hemerodromia* specimens was identified morphologically using the key of Plant (Plant, 2015).

3.2 DNA extraction, amplification and sequencing

Genomic DNA was extracted using the GF-1 Tissue DNA Extraction Kit (Vivantis, Selangor Dural Ehsan, Malaysia). A 650 bp mitochondrial DNA fragment of the cytochrome *c* oxidase I (COI) was amplified followed the method described in Rivera & Currie ((Rivera & Currie, 2009) using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAG GGTGACCAAAAAATCA-3′) (Folmer *et al*., 1994). Polymerase chain reaction (PCR) conditions as follows; ddH_2O 34 µl, buffer A 5 µl, $MgCl_2$ 3 µl, dNTP 1.6 µl, primers (forward 2 µl and reverse 2 µl) and Taq DNA polymerase 0.4 µl (Conflitti *et al*.,2010). PCR products were checked with 1% agarose gel and purify using the HiYield Gel/PCR DNA Extraction Kit (RBC Bioscience). Purified PCR products were send for sequencing at Macrogen sequencing service (Seoul, Korea) and first BASE laboratories Sdn Bhd (Selangor, Malaysia), using the same primers as in PCR.

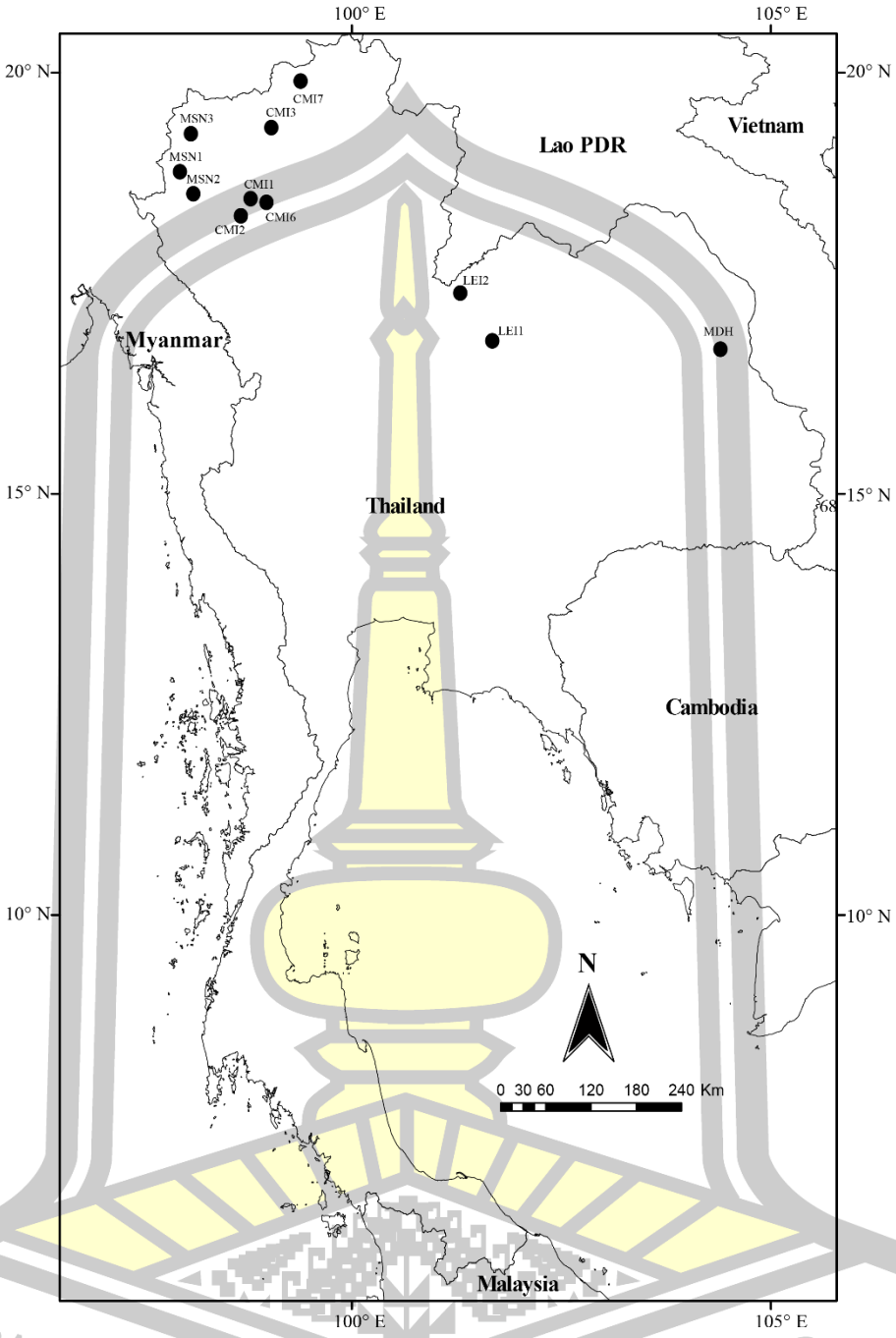


Figure 27 Collection sites of 9 species of *Hemerodromia* in Thailand. Details of sampling sites are given in Table 2 Abbreviations: MSN = Mae Hong Son; CMI = Chiang Mai; LEI = Loei; MDH = Mukdahan

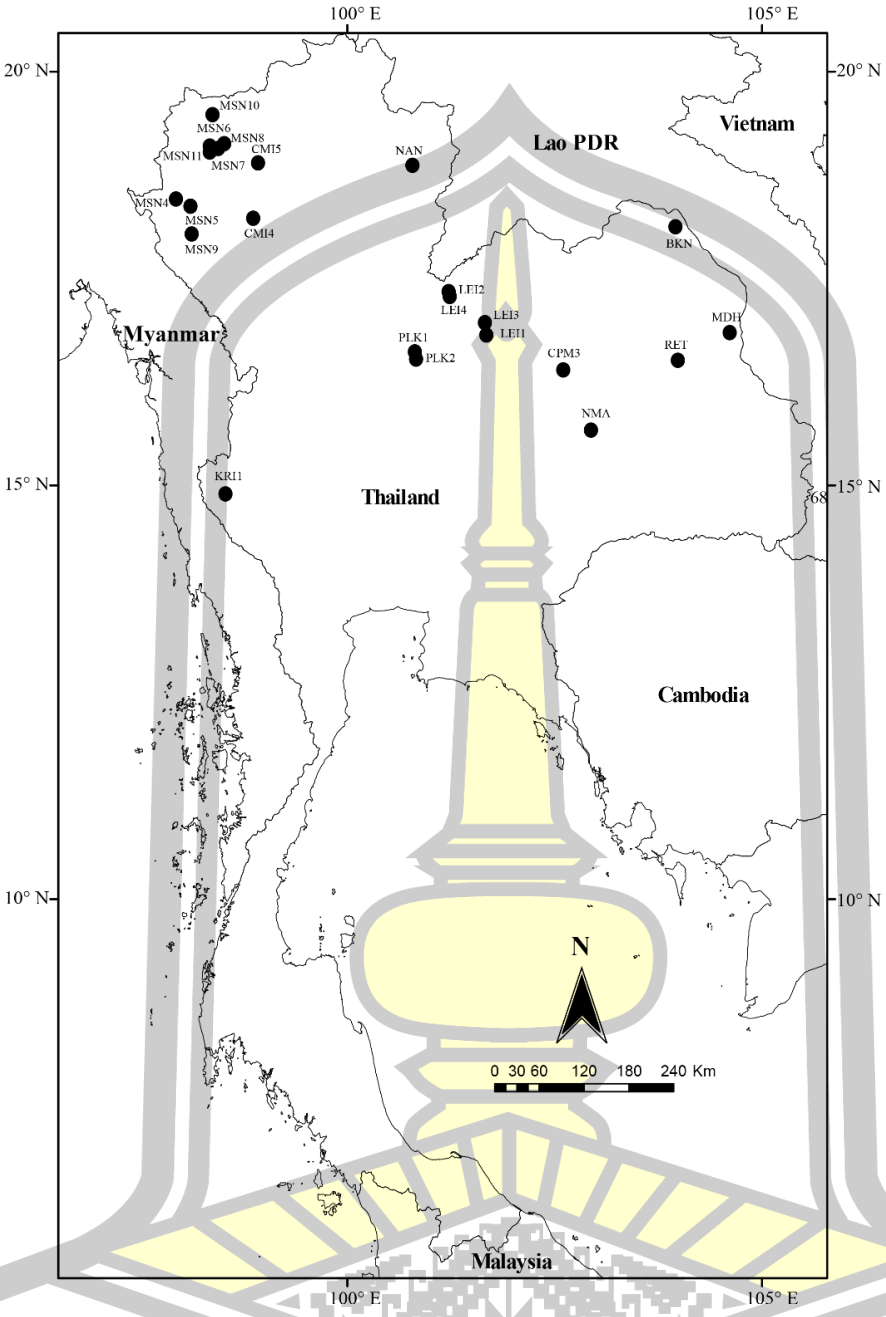


Figure 28 Collection sites of *Hemerodromia fusca* in Thailand. Details of sampling sites are given in Table 2 Abbreviations: MSN = Mae Hong Son; CMI = Chiang Mai; LEI = Loei; BKN = Bueng Kan; MDH = Mukdahan; RET = Roi Et; NMA = Nakhon Ratchasima; KRI = Kanchanaburi; PLK = Phitsanulok; NAN = Nan; CPM = Chaiyaphum

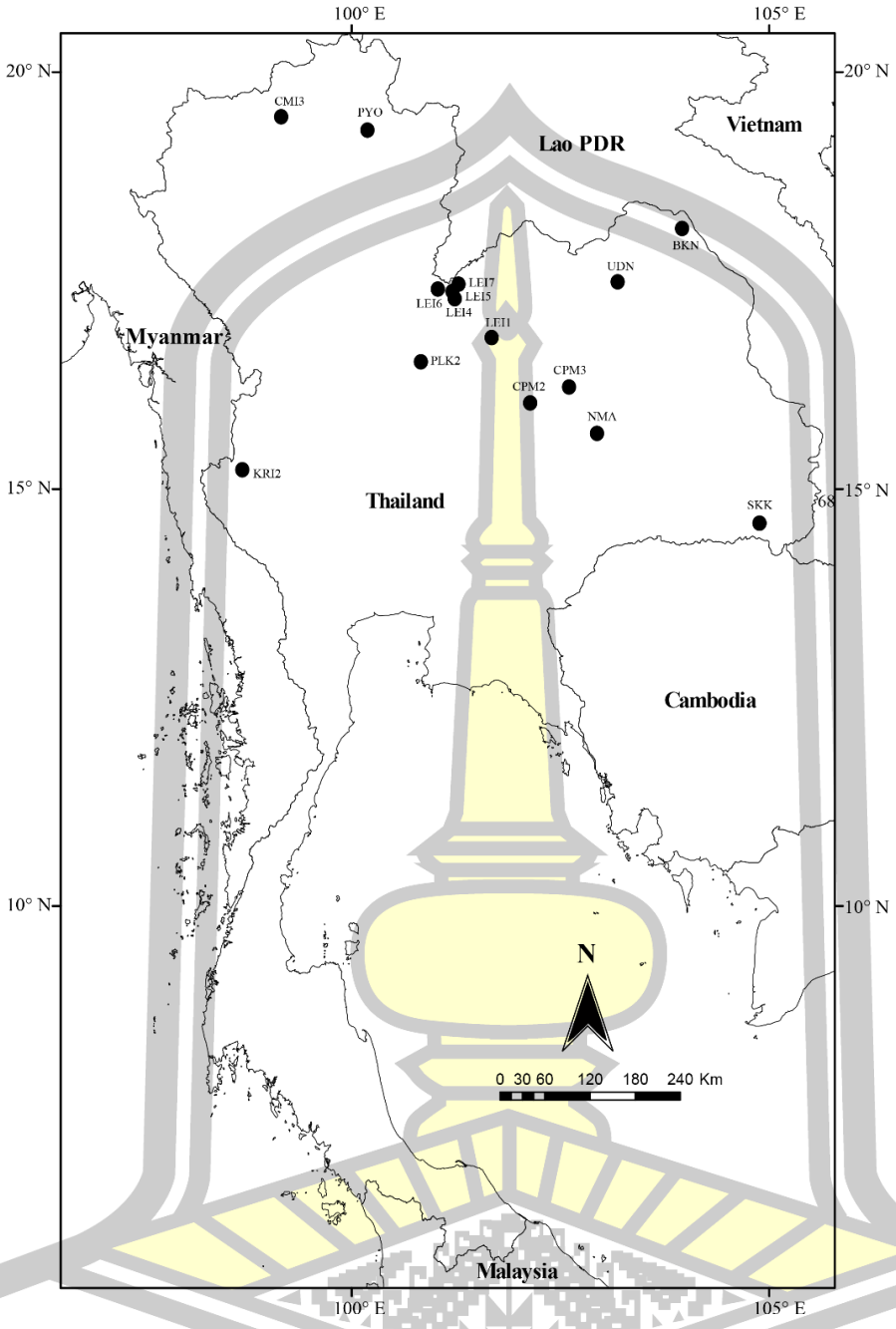


Figure 29 Collection sites of *Hemerodromia yunnanensis* in Thailand. Details of sampling sites are given in Table 2 Abbreviations: CMI = Chiang Mai; PYO = Phayao; LEI = Loei; BKN = Bueng Kan; NMA = Nakhon Ratchasima; UDN = Udon Thani; SKK = Sisaket; KRI = Kanchanaburi; PLK = Phitsanulok; CPM = Chaiyaphum

3.3 Data analysis

A total of 135 COI sequences from 12 species of *Hemerodromia* (Table 2) from Thailand were obtained. Twenty eight sequences of *Hemerodromia* from other geographic regions available in GenBank were included for data analyses. Sequences were aligned using CLUSTALW packaged in MEGA 7 (Tamura *et al*., 2016). Genealogical relationships between haplotypes were estimated using the median-joining (MJ) network algorithm (Bandelt *et al*., 1999) in the software NETWORK ver. 5.0.0.3 (www. fluxus-engineering.com). Intraspecific and interspecific genetic divergence values were calculated based on the Kimura 2-parameter (K2P) model, using MEGA 7. Phylogenetic relationships between species were calculated based on three methods including neighbor-joining (NJ), maximum likelihood (ML) and Bayesian analysis (BA). The NJ analysis was performed in MEGA 7. The ML analysis was implemented in PhyML 3.0 (Guindon *et al*., 2010) with approximate likelihood ratio tests (Anisimova & Gascuel, 2006) to calculate branch support. Bayesian analysis was performed using MRBAYES 3.04b (Huelsenbeck & Ronquist, 2001). The best-fit model for the Bayesian analysis was selected by hierarchical likelihood ratio tests implemented in MrModeltest (Nylander, 2004). For all phylogenetic analyses, *Chelifera frigelii* (GenBank Accession no. KR632271) was used as the outgroup as the morphological characters suggested that this genus most closely related to the *Hemerodromia*. To test the efficiency of the COI DNA barcoding sequence for species identification of the *Hemerodromia*, the best close match methods in the program TaxonDNA (Meier *et al*.,2006) was used to test the frequency of successful identification. Haplotype diversity (h) and nucleotide diversity (π) were calculated using Arlequin ver.3.5.1.2 (Excoffier & Lischer, 2010). The population genetic structure was estimated using population pairwise *F*st calculated in Arlequin ver.3.5.1.2 (Excoffier & Lischer, 2010). Mismatch distribution was used to test the demographic history of the populations. A population that in the recent past has undergone a demographic expansion shows a unimodal mismatch distribution (Rogers & Harpending, 1992). The sum-of-squares deviation and Harpending's raggedness index (Harpending, 1994) were used to test the deviation from the prediction of the sudden expansion model. Mismatch distribution was estimated using Arlequin. In addition, Fu's F_S test (Fu, 1997) and Tajima's *D*

(Tajima, 1989) statistical tests were also used to test for population equilibrium. The expectation is that these tests will yield large negative values during demographic population expansion.

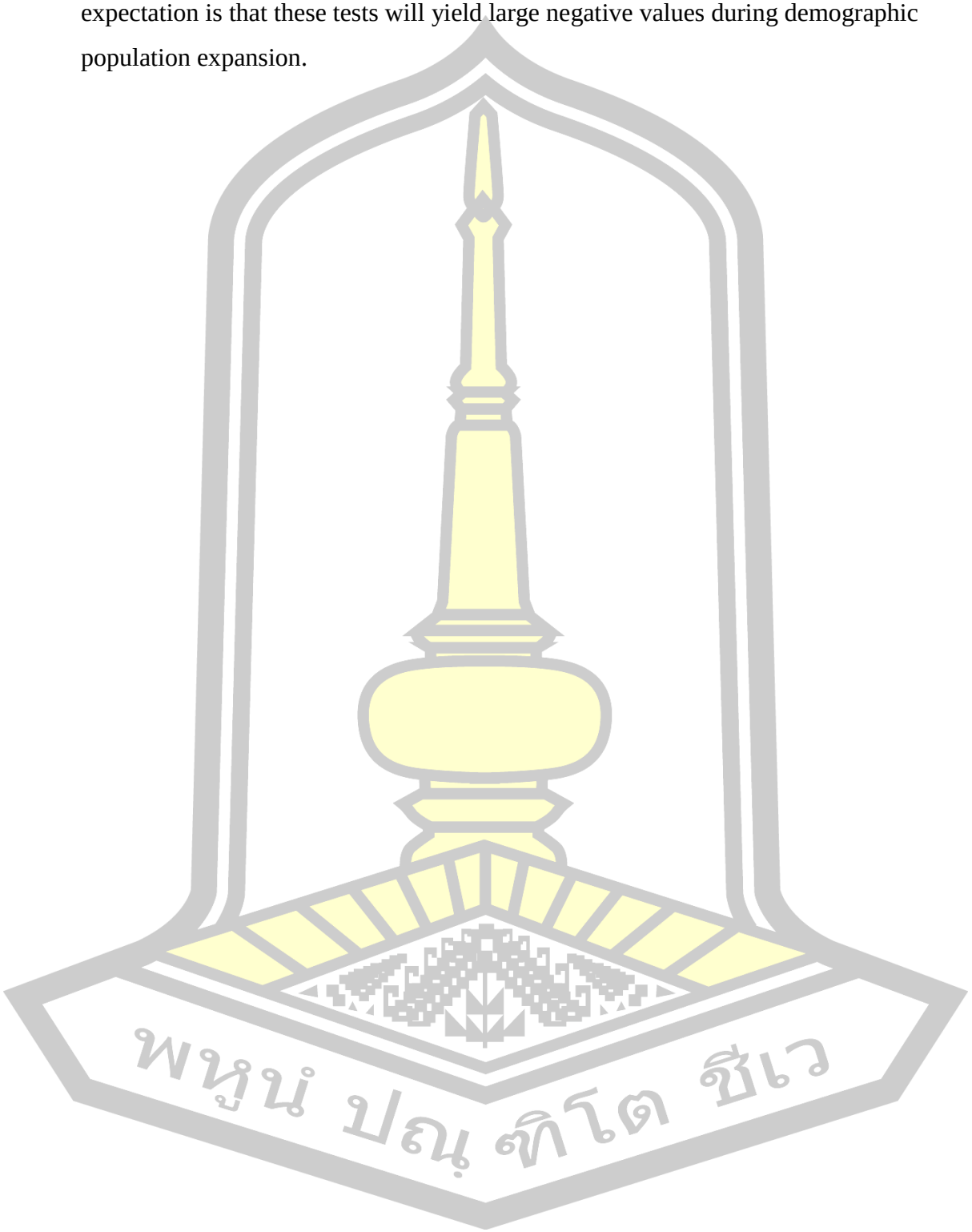


Table 2 Details of specimens collection sites of *Hemerodromia* in this study

	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
H. anomala Plant	Suan Hom waterfall, Nong Hin, Loei	LEI1	17°02'49"N 101°45'42"E	579	16/09/2016, 23/03/2017
H. acutata Grootaert, Yang & Saigusa	Siriphoom waterfall, Chom Thong, Chiang Mai	CMI1	18°32'48"N 98°30'56"E	1,305	13/10/2017
	Muang Pond Khum Yuam Mae Hong Son	MSN1	18°38'44"N 97°56'25"E	438	13/10/2017
	Mae Oor Kor Khum Yuam Mae Hong Son	MSN2	18°50'36"N 97°59'01"E	609	15/10/2017
H. conspecta Plant	Suan Hom waterfall, Nong Hin, Loei	LEI1	17°02'49"N 101°45'42"E	579	23/03/2017
H. betalutea Plant	Check point 2, Chomthong, Doi Inthanon, Chiang Mai	CMI2	18°31'39"N 98°29'59"E	1,639	15/06/2014
H. flaviventris Yang & Yang	Siriphoom waterfall, Jom Thong, Chiang Mai	CMI1	18°32'48"N 98°30'56"E	1,305	18/10/2016, 13/10/2017
H. furcata Grootaert, Yang & Saigusa	Doi Chiang Dao, Chiang Dao, Chiang Mai	CMI3	19°20'31"N 98°52'11"E	994	19/10/2016
	Song Kon waterfall, Phu Reua,Loei	LEI2	17°21'31"N 101°24'23"E	733	22/03/2017
	Ban Huai Sai Kaew, Tham Pla National park, Mae Hong Son	MSN3	19°35'28"N 98°00'06"E	590	24/10/2014

Table 2 (Continued)

Species	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
H. fusca Yang & Yang	Campground Pond, Chomthong, Doi Inthanon, Chiang Mai	CMI4	18°32'04"N 98°31'08"E	1200	15/12/2006
	Suan Sawan waterfall, Nong Hin, Loei	LEI3	17°03'58"N 101°44'53"E	627	26/11/2013
	Suan Hom waterfall, Nong Hin, Loei	LEI1	17°02'49""N 101°45'42"E	579	29/11/2013
	Agricultural land, Huai Phrik Luang, Nan	NAN	18°54'23"N 100°29'04"E	390	21/11/2012
	Route between Ban Song Kon and Ban Pla Ba, Phu Reua, Loei	LEI4	17°22'03"N 101°23'07"E	667	17/09/2016, 22/03/2017
	Huai Mae Sa, Queen Sirikit Botany garden, Mae Rim,Chiang Mai	CMI5	18°53'43"N 98°51'31"E	648	17/10/2016
	Muang Pond, Khum Yuam Mae Hong Son	MSN11	18°38'44"N 97°56'25"E	438	13/10/2017
	Beside corn farm, Mae Oor Kor Khum Yuam Mae Hong Son	MSN4	18°50'36"N 97°59'01"E	609	15/10/2017
	Tung Bua Tong NP, Mae Oor Kor Khum Yuam Mae Hong Son	MSN5	18°53'23"N 98°05'35"E	1,442	15/10/2017
	Mok Mi Whai cliff, Nong Pok, Roi-Et	RET	16°23'40"N 104°18'46"E	330	19/11/2017
	Ched Sri waterfall, Sega, Bueng Kan	BKN	18°09'38"N 103°57'01"E	197	13/11/2015

Table 2 (Continued)

Species	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
	Song Kon waterfall, Phu Reua, Loei	LEI2	17°21'31"N 101°24'23"E	733	22/03/2017
	Phu Pha Kham,Nong Sung, Mukdahan	MDH	16°26'09"N 104°25'11"E	360	25/11/2017
	Ban Nam Gud, Huai Pha, Muang, Mae Hong Son	MSN6	19°30'22"N 98°04'30"E	420	14/10/2017
	Pa Thum Wua Temple, Mae Hong Son	MSN7	19°31'18"N 98°05'30"E	405	14/10/2017
	San Sak Sit, Ban Nam Kud, Mae Hong Son	MSN8	19°30'38"N 98°04'44"E	430	14/10/2017
	Mae La Noi, Mae Hong Son	MSN9	18°27'08"N 97°56'18"E	328	13/10/2017
	Thong Pha Phum National Park, Kanchanaburi	KRI1	14°74'75"N 98°57'97"E	185	25/11/2018
	Nam Phut Na Lao, Chaiyaphum	CPM3	16°59'54"N 101°89'80"E	246	18/10/2018
	Noen Maprang tufa stream, Phitsanulok	PLK1	16°59'72"N 100°67'71"E	121	15/11/2018
	Noen Maprang, Khun Huai Tum stream, Phitsanulok	PLK2	16°58'41"N 100°68'58"E	77	15/11/2018

Table 2 (Continued)

Species	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
	Wang Tao waterfall, Tub Lan National Park, Nakhon Ratchasrima	NMA	14°19'57"N 102°14'08"E	248	23/11/2018
	Ban Na Poo Pom, Pang Ma Pha, Mae Hong Son	MSN10	19°32'06"N 98°06'24"E	357	14/10/2017
H. namtokhinpoon Plant	Suan Hom waterfall, Nong Hin, Loei	LEI1	17°02'49"N 101°45'42"E	579	23/03/2017, 27/05/2017
H. oriens Plant	Phu Pha Kham,Nong Sung, Mukdahan	MDH	16°26'09"N 104°25'11"E	360	25/11/2017
H. songsee Plant	Kiew maepan, Doi Inthanon, Chomthong, Chiang Mai	CMI6	18°33'29"N 98°28'51"E	2,210	19/12/2014
	Route to summit, Doi Pha Hom Pok National Park , Fang, Chiang Mai	CMI7	20°03'01"N 99°08'38"E	2,036	14/05/2014
H. yunnanensis Yang & Yang	Pla Ba waterfall, Phu Reua, Loei	LEI5	17°23'51"N 101°22'02"E	279	20/09/2015, 18/09/2016
	Ched Sri waterfall, Sega, Bueng Kan	BKN	18°09'38"N 103°57'01"E	197	13/11/2015 21/10/2016
	Huai Tub Gor Sord, Phu Suan Sai National Park, Na Haeo, Loei	LEI6	17°30'55"N 100°56'19"E	915	19/09/2015
	Route between Ban Song Kon and Ban Pla Ba, Phu Reua, Loei	LEI4	17°22'03"N 101°23'07"E	667	22/03/2017

Table 2 (Continued)

Species	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
	Huai Wang Yai waterfall, Kantararak, Sisaket	SKK	14°26'34"N 104°29'44"E	210	13/10/2016, 12/05/2017
	Phu Foi Lom National Park, Udon Thani	UDN	17°09'12"N 102°43'54"E	415	07/12/2017
	Doi Chiang Dao, Chiang Dao, Chiang Mai	CMI3	19°20'31"N 98°52'11"E	994	19/10/2016
	Lead Phob waterfall, Phu Reau, Loei	LEI7	17°29'53"N 101°20'07"E	1122	12/01/2018
	Suan Hom waterfall, Nong Hin, Loei	LEI1	17°02'49"N 101°45'42"E	579	23/03/2017
	Pha Hin Ngam National Park, Chaiyaphum	CPM2	15°58'18"N 101°42'76"E	444	22/11/2006
	Champa Thong waterfall, Doi Luang National Park, Pa Yao	PYO	19°20'11"N 99°73'44"E	382	21/11/2012
	Khao Laem National Park, Kanchanaburi	KRI2	14°99'83"N 98°62'03"E	197	26/11/2018
	Nam Phut, Na Lao, Chaiyaphum	CPM3	16°61'59"N 101°89'48"E	238	19/10/2018

Table 2 (Continued)

Species	Collection sites	Code	Latitude /Longitude	Elevation (m)	Collection date
	Wang Tao waterfall, Tub Lan National Park, Nakhon Ratchasrima	NMA	14°19'57"N 102°14'08"E	248	23/11/2018
H. anisoserrata Plant	Song Kon waterfall, Phu Reua, Loei	LEI2	17°21'31"N 101°24'23"E	733	22/03/2017

CHAPTER 4
RESULTS

4.1 COI sequence variation and DNA barcoding

Intraspecific genetic divergence based on the Kimura 2-parameter ranged from 0% to 4.0%, with an average of 1.1%. The maximum intraspecific genetic divergence value (4.0%) was found in *Hemerodromia yunnanensis* (Table 3). Minimum intraspecific genetic divergence value (0%) were found in *H. anomala*, *H. conspecta*, *H. flaviventris*, *H. furcata* and *H. namtokhinpoon*.

Interspecific genetic divergence ranged from 7.4% to 19%, with a mean of 12.7% (Table 4). A low level (7.4%) of minimum interspecific divergence occurred between *H. flaviventris* and *H. songsee* while a high (19%) level of maximum interspecific divergence occurred between *H. namtokhinpoon* and *H. furcata*. Intraspecific and interspecific genetic divergence values showed in Figure 30. All specimens (n = 135) were perfectly (100%) identified into species based on the COI sequences (Table 3).

4.2 Mitochondrial genealogy and demographic history

The Mitochondrial genealogy of 153 COI sequences of *Hemerodromia fusca* (Figure 31) and 80 COI sequences of *Hemerodromia yunnanensis* (Figure 32) revealed no major divergence lineage. Overall, both species show star-like shape with no geographic association of the haplotypes. Mismatch distribution analysis of *H. fusca* revealed a unimodal mismatch graph from mismatch distribution analysis (Figure 33) and the values of haplotype diversity and nucleotide diversity are show in Table 4. Both sum-of-squares deviation (SSD = 0.00389, *P* = 0.87000) and Harpending's raggedness index = 0.00554, *P* = 0.82000) were not significantly different from the simulated data under the sudden population expansion model. This is consistent with the star like shape of the median joining network. Population expansion was also supported by significant negative values for and Fu's *Fs* (-17.79831, *P* = 0.00000) test and negative value forTajima's *D* (-0.62915, *P* = 0.31700) test although the not statistically significant.

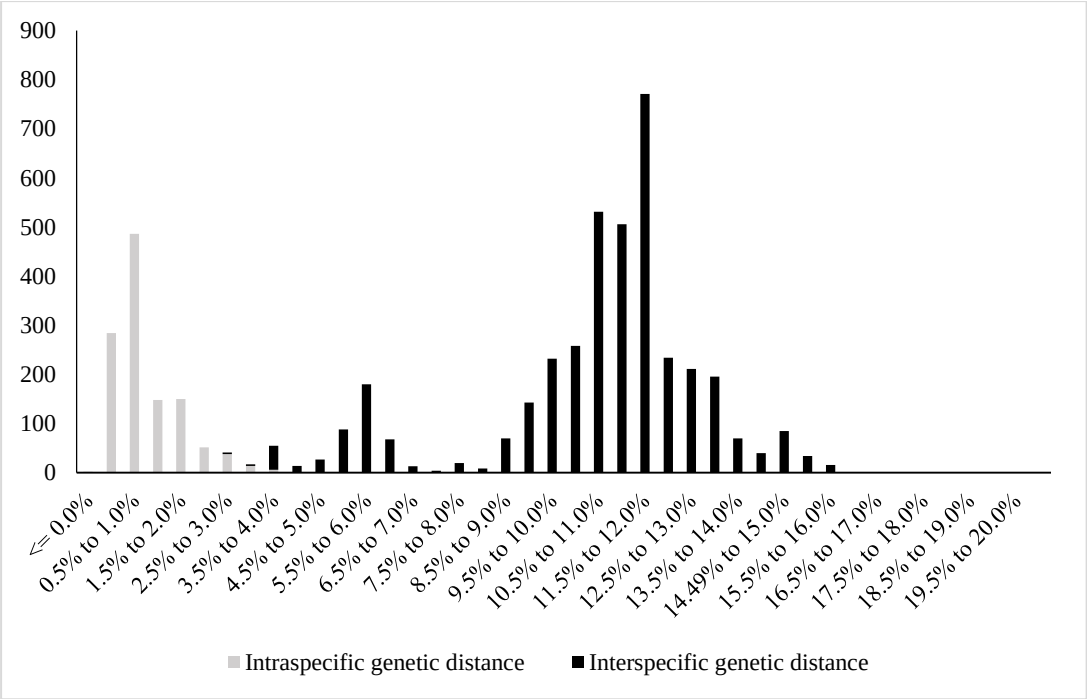


Figure 30 Distributions of intraspecific and interspecific genetic distances, based on 302 mitochondrial cytochrome c oxidase subunit 1 sequences from 12 species of *Hemerodromia* in Thailand.

Table 3 DNA barcode statistics for twelve species of *Hemerodromia* in Thailand. Percentage correct identification is based on best close match methods in TaxonDNA (Meier *et al*., 2006)

Species	Number of specimens	Number of haplotype	Range of intraspecific genetic divergence (mean) %	Range of interspecific genetic divergence (mean) %	Percent correct identification (n)	Percent misidentification (n)
H. anomala	7	3	0.0–0.8 (0.3)	8.4–16.9 (11.5)	100 (7)	0
H. acutata	4	4	0.1–2.4 (1.5)	10.1–17.3 (13.3)	100 (4)	0
H. conspecta	7	3	0.0–0.3 (0.2)	9.9–15.2 (11.7)	100 (7)	0
H. betalutea	4	4	0.3–3.2 (1.8)	9.7–16.2 (12.1)	100 (4)	0
H. flaviventris	7	5	0.0–1.7 (0.6)	7.4–16.4 (13.1)	100 (7)	0
H. furcata	8	5	0.0–3.4 (1.3)	10.4–17.9 (12.5)	100 (8)	0
H. fusca	30	22	0.1–2.1 (0.8)	9.2–14.6 (12.4)	100 (30)	0
H. namtokhinpoon	18	15	0.0–2.0 (0.8)	9.9–19.0 (14)	100 (18)	0
H. oriens	5	5	0.1–2.6 (1.1)	10.8–17.9 (14.2)	100 (5)	0
H. songsee	4	4	0.8–2.3 (1.6)	9.7–15.6 (11.8)	100 (4)	0
H. yunnanensis	36	20	0.1–4.0 (1.4)	10.8–13.8 (12.2)	100 (36)	0
H. anisoserrata	5	5	0.5–2.9 (1.7)	10.9–18.2 (13.6)	100 (5)	0

Table 4 Haplotype diversity (*h*) and nucleotide diversity (π) of the 16 populations of *Hemerodromia fusca* based on mitochondrial cytochrome c oxidase I (COI) gene sequence

Populations	Number of samples	Number of haplotype	Haplotype diversity	Nucleotide diversity
LEI1	11	8	0.8909 ± 0.0918	0.003968 ± 0.002582
LEI4	16	14	0.9833 ± 0.0278	0.007013 ± 0.004060
CMI5	5	4	0.9000 ± 0.1610	0.011659 ± 0.007652
RET	4	3	0.8333 ± 0.2224	0.002242 ± 0.002001
BKN	4	3	0.8333 ± 0.2224	0.003737 ± 0.003009
LEI2	10	6	0.8889 ± 0.0754	0.038831 ± 0.021080
MSN4	11	10	0.9818 ± 0.0463	0.015301 ± 0.008544
MSN5	3	3	1.0000 ± 0.2722	0.017937 ± 0.014022
MSN6	11	7	0.9091 ± 0.0656	0.002935 ± 0.002026
MSN7	15	15	1.0000 ± 0.0243	0.029767 ± 0.015646
MSN8	13	13	1.0000 ± 0.0302	0.038174 ± 0.020155
MSN9	5	4	0.9000 ± 0.1610	0.022571 ± 0.014271
MSN11	20	12	0.8105 ± 0.0918	0.014232 ± 0.007612
CPM3	3	3	1.0000 ± 0.2722	0.004983 ± 0.004331
PLK1	7	7	1.0000 ± 0.0764	0.010321 ± 0.006333
PLK2	7	7	1.0000 ± 0.0764	0.012528 ± 0.007571
Total	**145**	**119**	**0.9630 ± 0.115**	**0.020523 ± 0.010260**

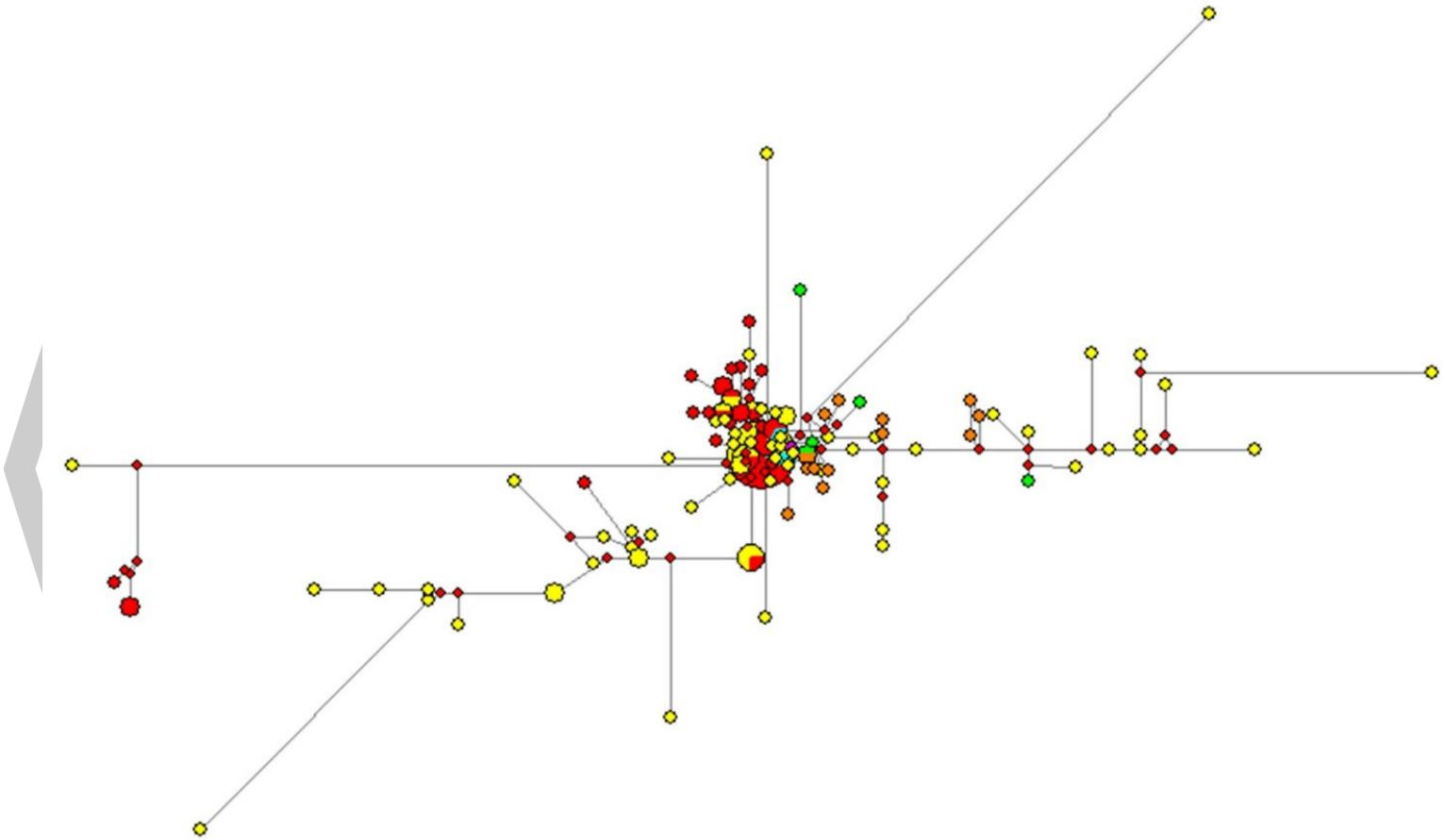


Figure 31 Median joining network for 153 COI sequences of *Hemerodromia fusca*. Size is relative to the number of individuals sharing a haplotype. The network has a star-like shape. Red =Upper North East, Yellow = North, Blue = Central North East, Green = Lower North East, Pink = West, Orange = Central

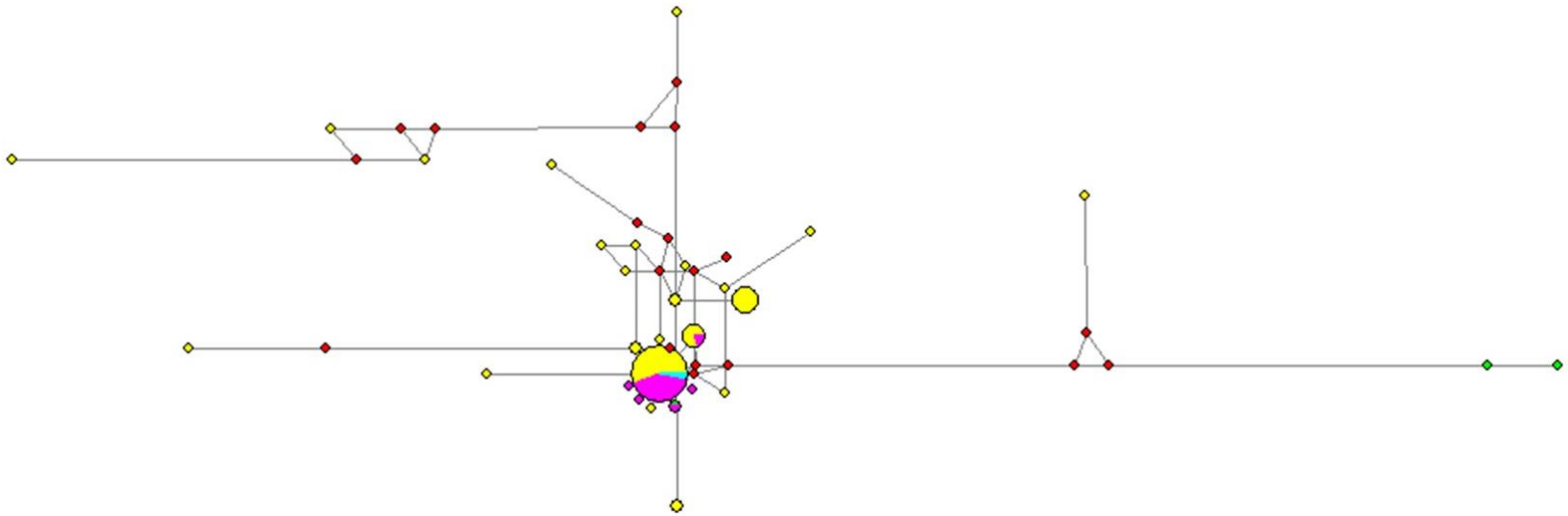


Figure 32 Median joining network for 80 COI sequences of *Hemerodromia yunnanensis*. Sizes of the circles are relative to the number of individuals sharing a specific haplotype. Yellow = Upper North East, Red = North, Pink = Lower North East, Green = West, Blue = Central

Hemerodromia yunnanensis population showed a unimodal mismatch graph (Figure 34) and the values of haplotype diversity and nucleotide diversity showed in Table 5. Both sum-of-squares deviation (SSD = 0.00757, *P* = 0.85000) and Harpending's raggedness index = 0.01443, *P* = 0.89000) were not significantly different from the simulated data under the sudden population expansion model. Thus, this species also has undergone recent demographic expansion that was also supported by significant negative values for Tajima's *D* (-2.19563, *P* < 0.0001) and for Fu's *Fs* (-24.82719, *P* < 0.0001) tests.

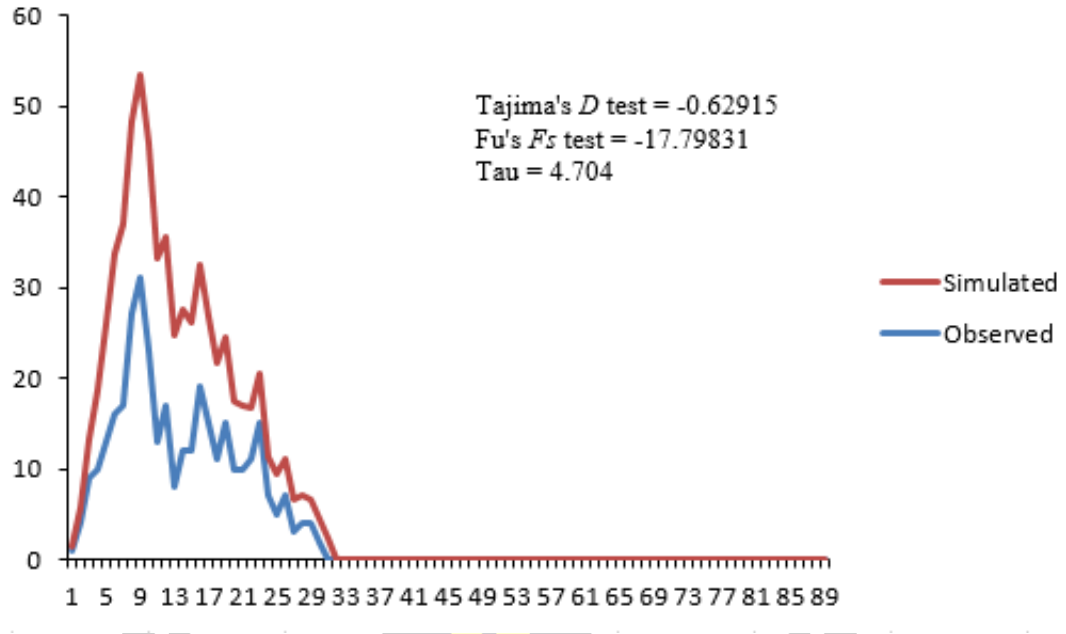


Figure 33 Mismatch distribution of the 153 COI sequences of *Hemerodromia fusca* demonstrating observed and expected pairwise differences based on the predictions of sudden population expansion model. Mismatch distribution of *H. fusca* is consistent with predictions of the sudden population expansion model (SSD = 0.00389, *P* = 0.87000; Harpending's raggedness index = 0.00554, *P* = 0.82000).

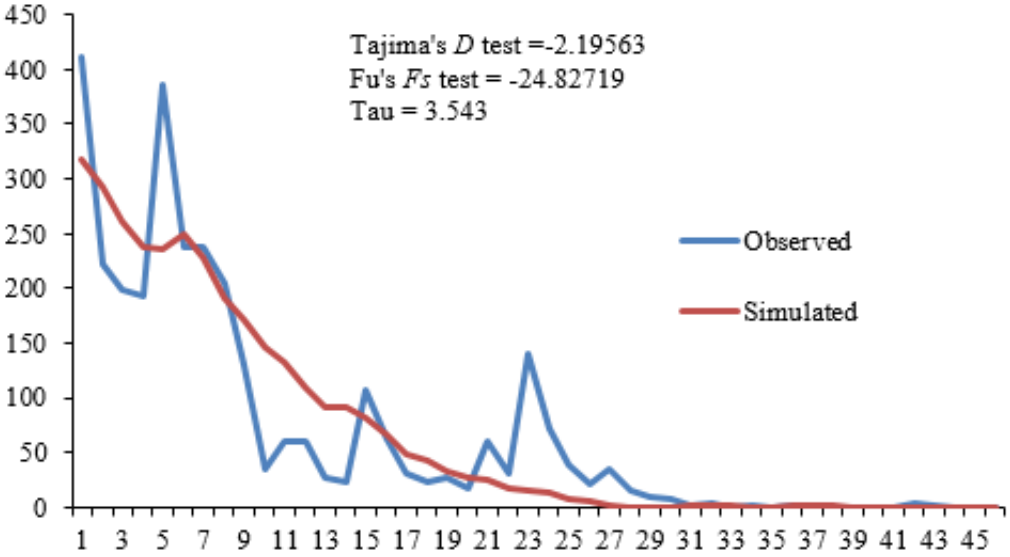


Figure 34 Mismatch distribution of the 80 COI sequences of *Hemerodromia yunnanensis*
demonstrating observed and expected pairwise differences based on the predictions of sudden population expansion model. Mismatch distribution of *H. yunnanensis* is consistent with predictions of the sudden population expansion model (SSD = 0.00757, $P = 0.85000$; Harpending's raggedness index = 0.01443, $P = 0.89000$).

4.3 Genetic diversity and genetic structure

Two geographically widespread species, *Hemerodromia fusca* and *H. yunnanensis* were subjected to population genetic analyses. A total of 145 sequences were obtained for *H. fusca* and 119 haplotypes were identified. Of these, 80 haplotypes were unique, and 16 haplotypes were shares by at least two individuals. The haplotype diversity in each population ranged from 0.8105 in Mae Hong Son (MSN11) to 1 in Mae Hong Son (MSN5, MSN7, MSN8), Chaiyaphum (CPM3) and Phitsanulok (PLK1, PLK2) with an average of 0.9630 (Table 4). Nucleotide diversity in each population range from 0.002242 in Roi Et (RET) to 0.038831 in Loei (LEI2) with an average of 0.020523 (Table 4). Population pairwise F_{st} analysis revealed that 54.17% of total comparisons were genetically statistically different, and the remaining (45.83%) were not (Table 6). Populations from Loei (LEI4) made major contributions

to genetic structure. Comparisons of these populations with others are all highly significantly different (Table 6).

A total of 70 sequences were obtained for *H. yunnanensis* and 38 haplotypes were identified among 70 sequences. Of these, 30 haplotypes were unique, and 8 haplotypes were shares by at least two individuals. The haplotype diversity in each population ranged from 0.2417 in Nakhon Ratchasima (NMA) to 1 in Loei (LEI6) with an average of 0.8696 (Table 5). Nucleotide diversity in each population range from 0.000374 2417 in Nakhon Ratchasima (NMA) to 0.030938 in Loei (LEI6) with an average of 0.013546 (Table 5). Population pairwise F_{st} analysis revealed that 57.14% of total comparisons were genetically statistically different, and the remaining (42.86%) were not (Table 7). Populations from Nakhon Ratchasima (NMA) made major contributions to genetic structure. With an exception, comparisons of these populations with others are all highly significantly different (Table 7).

4.4 DNA barcode trees

All tree analysis methods (NJ, ML and BA) revealed similar tree topologies therefore, only the NJ tree is presented (Figure 35). Minor differences between analysis methods included the placement between *Hemerodromia anomala* and *H. conspecta*, which were clustered into one clade in the ML tree but formed separate clusters in both NJ and BA trees. The NJ tree revealed that Thai *Hemerodromia* species were retrieved as a separate clade from the 28 sequences of *Hemerodromia* spp. obtained from GenBank. All 12 species formed monophyletic clades with strong support. *H. fusca* and *H. yunnanensis* formed a clade with strong support (>98%) suggested that they are closely related species.

Five species (*H. furcata*, *H. conspecta*, *H. betalutea*, *H. songsee*, *H. flaviventris*) formed another clade but weakly supported. *H. oriens* and *H. anomala* were isolated from other species and were placed in different clades. Three species, *H. anisoserrata*, *H. acutata* and *H. namtokhinpoon* clustered together to make another clade but with weak support.

Table 5 Haplotype diversity (*h*) and nucleotide diversity (π) of the 7 populations of *Hemerodromia yunnanensis* based on mitochondrial cytochrome *c* oxidase I (COI) gene sequence

Populations	Number of samples	Number of haplotype	Haplotype diversity	Nucleotide diversity
LEI5	7	4	0.7143 ± 0.1809	0.009695 ± 0.005908
BKN	16	10	0.8667 ± 0.0793	0.017203 ± 0.009174
LEI6	3	3	1.0000 ± 0.2722	0.030938 ± 0.022826
SKK	13	8	0.8846 ± 0.0699	0.002418 ± 0.001710
UDN	12	5	0.6667 ± 0.1409	0.003425 ± 0.002260
CPM	3	2	0.6667 ± 0.3143	0.002994 ± 0.002753
NMA	16	6	0.2417 ± 0.1353	0.000374 ± 0.000497
Total	**70**	**38**	**0.8696 ± 0.0349**	**0.013546 ± 0.006982**

Table 6 Population pairwise *F*st (below diagonal) between populations for 16 populations of *Hemerodromia fusca* from Thailand

Population	LEI1	LEI4	CMI5	RET	BKN	LEI2	MSN4	MSN5	MSN6	MSN7	MSN8	MSN9	MSN11	CPM3	PLK1	PLK2
LEI1	0															
LEI4	0.373**	0														
CMI5	0.517	0.166**	0													
RET	-0.114	0.369**	0.456*	0												
BKN	-0.027	0.375**	0.437**	-0.143	0											
LEI2	0.243**	0.358**	0.215*	0.097	0.097	0										
MSN4	0.107	0.311**	0.210**	-0.014	-0.004	0.140*	0									
MSN5	0.448**	0.494**	0.209	0.307	0.295	0.041	-0.055	0								
MSN6	0.006	0.439**	0.566**	-0.093	-0.036	0.232**	0.090	0.478*	0							
MSN7	0.178*	0.240**	0.160*	0.065	0.062	0.211**	0.106**	0.099	0.179**	0						
MSN8	0.296**	0.339**	0.171*	0.172*	0.173**	0.202**	0.147*	0.040	0.291**	0.053*	0					
MSN9	0.465**	0.448**	0.228*	0.320**	0.311**	0.198*	0.156*	0.087	0.492**	-0.011	-0.031	0				
MSN11	-0.003	0.236**	0.277**	-0.101	-0.088	0.179**	0.054	0.184	-0.012	0.116**	0.245**	0.261**	0			
CPM3	0.072	0.367**	0.377*	-0.035	-0.115	0.039	-0.052	0.207	0.034	-0.011	0.098	0.223	-0.096	0		
PLK1	0.071**	0.363**	0.381**	-0.065	-0.048	0.156	0.061	0.256	0.055	0.135*	0.223**	0.337**	0.023	-0.101	0	
PLK2	0.431**	0.437**	0.422**	0.338	0.331	0.278*	0.267**	0.406 *	0.466**	0.012	0.127*	0.133	0.207**	0.257	0.304**	0

*$P < 0.05$, ** $P < 0.01$

Table 7 Population pairwise *F*st (below diagonal) between populations for 7 populations of *Hemerodromia yunnanensis* from Thailand

Population	**LEI5**	**BKN**	**LEI6**	**SKK**	**UDN**	**CPM**	**NMA**
LEI5							
BKN	0.059						
LEI6	0.090	0.070					
SKK	0.351**	0.144**	0.410*				
UDN	0.054	0.136**	0.330**	0.279**			
CPM	-0.088	0.079	0.056	0.590**	0.152		
NMA	0.132*	0.277**	0.575**	0.733**	0.293*	0.355	

*$P < 0.05$, ** $P < 0.01$

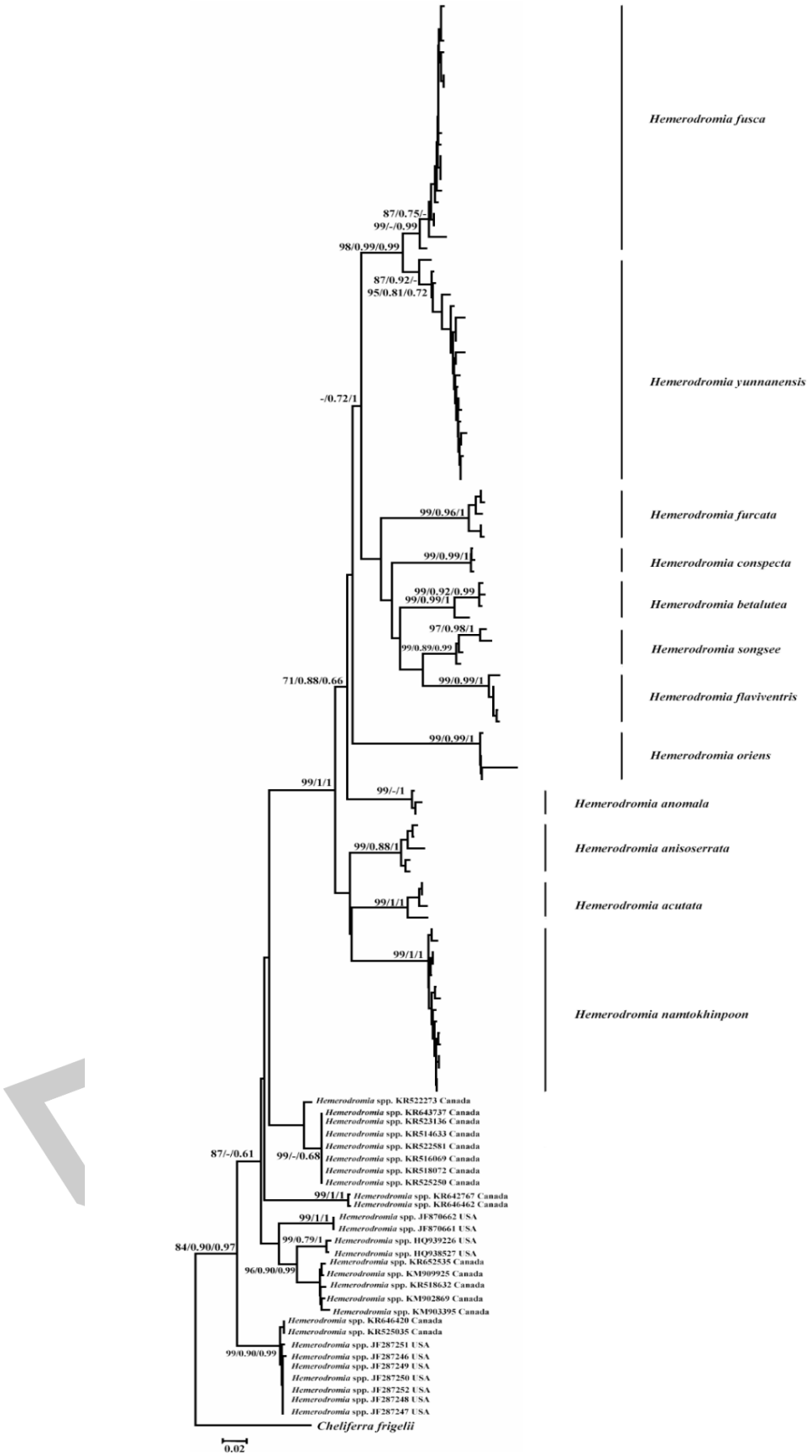

Hemerodromia fusca
Hemerodromia yunnanensis
Hemerodromia furcata
Hemerodromia conspecta
Hemerodromia betalutea
Hemerodromia songsee
Hemerodromia flaviventris
Hemerodromia oriens
Hemerodromia anomala
Hemerodromia anisoserrata
Hemerodromia acutata
Hemerodromia namtokhinpoon
87/0.75/-
99/-/0.99
98/0.99/0.99
87/0.92/-
95/0.81/0.72
-/0.72/1
99/0.96/1
99/0.99/1
99/0.92/0.99
99/0.99/1
97/0.98/1
99/0.89/0.99
71/0.88/0.66
99/0.99/1
99/0.99/1
99/1/1
99/-/1
99/0.88/1
99/1/1
99/1/1
87/-/0.61
99/-/0.68
99/1/1
99/1/1
99/0.79/1
84/0.90/0.97
96/0.90/0.99
99/0.90/0.99
Hemerodromia spp. KR522273 Canada
Hemerodromia spp. KR643737 Canada
Hemerodromia spp. KR523136 Canada
Hemerodromia spp. KR514633 Canada
Hemerodromia spp. KR522581 Canada
Hemerodromia spp. KR516069 Canada
Hemerodromia spp. KR518072 Canada
Hemerodromia spp. KR525250 Canada
Hemerodromia spp. KR642767 Canada
Hemerodromia spp. KR646462 Canada
Hemerodromia spp. JF870662 USA
Hemerodromia spp. JF870661 USA
Hemerodromia spp. HQ939226 USA
Hemerodromia spp. HQ938527 USA
Hemerodromia spp. KR652535 Canada
Hemerodromia spp. KM909925 Canada
Hemerodromia spp. KR518632 Canada
Hemerodromia spp. KM902869 Canada
Hemerodromia spp. KM903395 Canada
Hemerodromia spp. KR646420 Canada
Hemerodromia spp. KR525035 Canada
Hemerodromia spp. JF287251 USA
Hemerodromia spp. JF287246 USA
Hemerodromia spp. JF287249 USA
Hemerodromia spp. JF287250 USA
Hemerodromia spp. JF287252 USA
Hemerodromia spp. JF287248 USA
Hemerodromia spp. JF287247 USA
Cheliferra frigelii
0.02

Figure 35 Neighbor-Joining tree for 95 haplotypes of the mitochondrial cytochrome c oxidase subunit 1 sequences of 12 nominal species of *Hemerodromia* in Thailand and 28 unknown species of *Hemerodromia* from the GenBank. Bootstrap values for neighbor-joining, maximum likelihood and posterior probability of Baysian analysis are shown above or near the branches. Scale bar represents 0.02 substitutions per nucleotide position.

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Genetic diversity, genetic structure and demographic history of *Hemerodromia fusca* and *Hemerodromia yunnanensis*

Genetic diversity of the species could be affected by population history (Hewitt, 1996; Hewitt, 2000). Population genetic structure analysis of *H. fusca* and *H. yunnanensis* revealed that 54.17% and 57.14% of comparisons were genetically significantly different, respectively. Significant genetic differentiation between populations suggest a limitation of gene flow. Geographic barriers; large mountain ranges, habitat fragmentation and species dispersal have been found to be important factors that limit gene flow in insects (Aketarawong *et al*., 2007; Hu, Zhang *et al*., 2008; Shi *et al*., 2014). Although both species are widely distributed in Thailand and could occupy diverse habitats, they are mainly found in the forest areas. Because the patchy distribution of the habitat associated with forests thus facilitate population isolation.

Demographic history of *H. fusca* and *H. yunnanensis* revealed that both had undergone recent population demographic expansion. However, the population expansion time cannot be calculated because lack of the information on generation time for *Hemerodromia*. Although with this limitation, it is likely that the signals of population expansion in both species could be a response to the same historical event. Previous studies in aquatic insects in Thailand such as black flies suggest the importance of Pleistocene climatic and environmental fluctuation on species demographic history (Meeyen *et al*., 2014; Pramual *et al*., 2005) During the Pleistocene glaciations, although the ice sheet is not directly covered Southeast Asia but the climate conditions were cooler and drier (Penny, 2001; Voris, 2000). Several studies reported that Pleistocene affect genetic structure and diversity of species including; anopheles mosquito, *Anopheles dirus* (Morgan *et al*., 2011; O'Loughlin *et al*., 2008) fruit flies, *Bactocera latifrons* (Kunprom *et al*., 2015; Meeyen *et al*., 2014) and black flies, *Simulium tani* (Pramual et al., 2005). Because of both *H. fusca* and *H.*

yunnnanensis, which their life cycle are in the stream of the forest, thus the Pleistocene climatic change could also affect their population. Thus, signal of the population expansion in both *Hemerodromia* could be the respond of these *Hemerodromia* to more increasing after the recovery of the climate from cool and dry to warm and moist conditions.

5.1.2 DNA barcode of *Hemerodromia* in Thailand

The level of genetic variation within species of *Hemerodromia* in Thailand is low (0–4%) compared to those of family Hybotidae (Diptera, Empidoidea), which had a wider range (0–17.2%)(Nagy *et al*., 2013).

Hemerodromia yunnanensis possessed greatest intraspecific genetic diversity with maximum K2P genetic distance of 4.0%. This level of intraspecific genetic divergence has often found in the species complexes of the insect order Diptera (Pramual & Adler, 2014; Pramual & Pangjanda, 2015). However, no evidence found that specimens of *H. yunnanensis* are comprised of more than one species as the phylogenetic analysis based on COI sequences revealed that they are single lineage. High intraspecific genetic divergence is not uncommon in Diptera (Meier *et al*., 2006) This species is geographically widespread being recorded in China, Vietnam, Singapore and Malaysia (Plant, 2015) In Thailand, this species was found throughout the country and in diverse habitats, thus, a high level of genetic variation is not unexpected. However, more specimens from wider geographic regions are needed to test the hypothesis that this species is a species complex or single highly polymorphic species.

In contrast to the geographically widespread species, some *Hemerodromia* are geographically and ecologically restricted to particular areas or habitats. *H. anomala*, *H. conspecta* and *H. namtokhinpoon*, for example, are restricted to limestone streams. These species are only found in high calcareous streams, often with thick tufa formations (Plant, 2015). Levels of genetic diversity of these species are relatively low compare to other ecologically broader species. Previous studies of aquatic insects, (black flies, Simuliidae) in Thailand, found that species restricted to calcareous streams exhibited a low level of within population genetic variation but a high level of genetic differentiation between populations (Pramual & Pangjanda,

2015). However, the results for ecologically specializations of *Hemerodromia* species are based on single locations, and the genetic structure and diversity of these species requires further exploration.

H. fusca and *H. yunnnanensis* were retrieved as a clade with strong support and were thus clearly differentiated from others in this study. Morphological similarities also suggest a close relationship between these two species, which are distinguished primarily by differences in male terminalia (Plant, 2015). Additionally, in both *H. fusca* and *H. yunnnanensis* the ground color of the anepisternum and katepisternum is black or brown, while in other species (excepting *H. conspecta*) the ground color is yellow or brownish yellow. For other species, the phylogenetic inferred based on COI sequences seem not to be separated by morphological taxonomy. However, more species need to be include before firm conclusion can be made.

The results indicated that COI DNA barcoding sequences are highly effective for identification of the *Hemerodromia* in Thailand. All specimens were correctly identified into species. The results are consistent with the phylogenetic analysis that found that all species formed monophyletic clades with strong supports. Because high successful rate for species identification of *Hemerodromia* in Thailand, further studies could potentially benefit from the DNA barcode library provided in present study. DNA barcodes have been used successfully to associate different life stages of the insects (Thaijarern *et al*., 2017). This will be potentially by very useful for further study of the *Hemerodromia* because almost all of the immature stages of these species are unknown.

5.2 Conclusion

In conclusion, DNA barcode of *Hemerodromia* can be used effectively for species identification. The DNA barcoding sequences reported in this study will be very useful for associating adults with unknown immature life stages of *Hemerodromia* and possibly leading to the fuller understanding of the morphological characters and associated ecological conditions of these species.

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