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DNA Barcoding of *Hemerodromia* Meigen, 1822 (Diptera: Empididae) from Thailand

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ABSTRACT.– Species of *Hemerodromia* Meigen, 1822 (Diptera, Empididae, Hemerodromiinae) are important components of lotic habitats in freshwater ecosystems. The goal of this study was 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 Northeastern Thailand and 135 COI sequences obtained. 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 135 mitochondrial barcode sequences obtained here and 28 sequences from the NCBI database revealed a well-supported monophyly for all *Hemerodromia* species from Thailand.

KEY WORDS: cytochrome *c* oxidase I, DNA barcode, Empididae, *Hemerodromia*, Thailand

INTRODUCTION

Hemerodromia Meigen is the largest genus of the Empididae subfamily Hemerodromiinae (Plant, 2015), sometimes known as aquatic dance flies. 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 (Plant, 2011). Larvae of all species of *Hemerodromia* are probably predators of the larvae and pupae of several aquatic insect groups but predominantly of Chironomidae. They also feed on larvae of Simuliidae (Diptera) (Knutson and Steyskal, 1981), which are vectors of human onchocerciasis and certain livestock diseases. Adults of the *Hemerodromia* are often found on riparian vegetation where they prey on small adult insects (Wagner et al., 2004). *Hemerodromia* are sensitive to environmental perturbations and are useful indicators of the health of aquatic ecosystems.

In Thailand, 31 species of *Hemerodromia* are known (Plant, 2015; 2020). Among these, 25 were newly described from Thailand, namely, *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. ocellata* Plant, 2015, *H. oriens* Plant, 2015, *H. phahompokensis* Plant, 2015, *H. songsee* Plant, 2015, *H. systoechon* Plant, 2015 and *H. zetalutea* Plant, 2015. *H. aliaextriata* Plant, 2020, *H. deprimatura* Plant, 2020, *H. oretenebraea* Plant, 2020, *H. pairoti* Plant, 2020 and *H. samoha* Plant, 2020. Six species known previously from China. - *H. acutata*

Grootaert, Yang and Saigusa, *H. flaviventris* Yang and Yang, *H. furcata* Grootaert, Yang and Saigusa, *H. fusca* Yang and Yang and *H. yunnanensis* Yang and Yang and *H. menghaiensis* Yang and Yang.

Hemerodromia in temperate regions are probably univoltine with only one emergence of adults each year but some can have two generations per year (Ivković et al., 2007). However, in tropical regions such as in Thailand, *Hemerodromia* may be multivoltine as some species could be found throughout the year although others demonstrate seasonal adult emergence (Plant, 2015, 2021). Consideration of the climatic, ecological and biogeographic complexity of tropical Southeast Asia suggests that an extremely rich *Hemerodromia* fauna awaits discovery in the region (Plant, 2015).

In this study, we test efficiency of mitochondrial cytochrome *c* oxidase I (COI) for discrimination of specimens of *Hemerodromia* that had been determined on the basis of morphology. To the best of our knowledge, this is the first analysis of the use of DNA barcodes in support of species identification of *Hemerodromia*. Previous studies in several families of the order Diptera such as Simulidae, Chironomidae and Hybotidae (Pramual et al., 2016; Kondo et al., 2016; Nagy et al., 2013) have shown that DNA barcoding sequences can effectively discriminate species and have also uncovered cryptic diversity that had not yet been recognized based on traditional taxonomy (Hajibabaei et al., 2007; Nagy et al., 2013; Pramual and Adler, 2014; Kunprom and Pramual, 2016; Kondo et al, 2016; Pramual et al., 2016; Changbunjong et al., 2018). Another benefit of DNA barcodes is that they can be used to associate different life stages (e.g. 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 particularly useful in expanding explorations of the diversity of these interesting insects to include their early stages.

MATERIALS AND METHODS

Specimen collection and identification

Adult fly specimens were collected using a sweep net on or near vegetated areas around waterfalls and stream banks from 23 sites in Thailand (Table 1, Fig. 1). Specimens were preserved in 80% ethanol and kept at -20°C. Species were identified morphologically using the key and descriptions of Plant (2015).

DNA extraction, polymerase chain reaction 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 following the method described in Rivera and Currie (2009) using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGG TGACCAAAAAATCA-3′) (Folmer et al., 1994). PCR products were checked with 1% agarose gel and purified using the HiYield Gel/PCR DNA Extraction Kit (RBC Bioscience). Purified PCR products were sequenced at the Macrogen sequencing service (Seoul, Korea) and Apical Scientific (Selangor, Malaysia), using the same primers as in PCR.

Data analysis

A total of 135 COI sequences from 12 morphological species of *Hemerodromia* (Table 2) from Thailand were obtained. Sequences were deposited in GenBank under accession numbers MK070180 – MK070314.

TABLE 1. Details of specimen collection sites of *Hemerodromia* in this study

Species	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	Doi Inthanon, Chomthong, Chiang Mai (CMI2)	18°31'39"N/ 98°29'59"E	1,639	15/06/2014
H. flaviventris Yang & Yang	Siriphoom waterfall, Chomthong, 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, Mae Hong Son (MSN3)	11°19'59"N/16°98'00"E	590	24/10/2014
H. fusca Yang & Yang	Agricultural land, Huay Phrik Luang, Nan (NAN)	18⁰54'23"N/ 100˚29'04"E	390	21/11/2012
	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
	Campground Pond, Chomthong, Chiang Mai (CMI4)	18˚32'04"N/ 98˚31'08"E	1,200	15/12/2006
	Mok Mi Whai cliff, Nong Pok, Roi-Et (RET)	16˚23'40"N/ 104˚18'46"E	330	19/11/2017
	Huay Mae Sa, Queen Sirikit Botany garden, Mae Rim, Chiang Mai (CMI5)	18˚53'43"N/ 98˚51'31"E	648	17/10/2016
	Mae Oor Kor, Khum Yuam Mae Hong Son (MSN2)	18˚50'36"N/ 97˚59'01"E	609	15/10/2017
	Tung Bua Tong, Mae Oor Kor, Khum Yuam Mae Hong Son (MSN4)	18˚53'23"N/ 98˚05'35"E	1,442	15/10/2017
	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
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)	23°03'01"N/ 99°08'38"E	2,036	14/05/2014

TABLE 1. (Continue)

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, Beung Kan (BKN)	18°09'38"N/ 103°57'01"E	197	13/11/2015 21/10/2016
	Huay 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	17°22'03"N/ 101°23'07"E	667	22/03/2017
	Huay 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, Udonthani (UDN)	17°17'39"N/ 102°75'14"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
H. anisoserrata Plant	Song Kon waterfall Phu Reua, Loei (LEI2)	17°21'31"N/ 101°24'23"E	733	22/03/2017

Additionally, we also included 28 sequences of *Hemerodromia* from other geographic regions available in GenBank in the data analysis. Sequences were aligned using CLUSTAL W package in MEGA X (Kumar et al., 2018). Intraspecific and interspecific genetic divergence values were calculated based on the Kimura 2-parameter (K2P) model, using MEGA X. Phylogenetic relationships between species were calculated based on three methods, neighbor-joining (NJ), maximum likelihood (ML) and Bayesian analysis (BA). The NJ analysis was performed in MEGA X. The ML analysis was implemented in PhyML 3.0 (Guindon et al., 2010) with approximate likelihood ratio tests (Anisimova and Gascuel, 2006) to calculate branch support. Bayesian analysis was performed using MRBAYES 3.04b (Huelsenbeck and 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* (Zetterstedt) (GenBank accession no. KR632271) was used as the outgroup as the morphological characters suggested that this genus is closely related to *Hemerodromia* (Plant, 2011). 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) were used to test the frequency of successful identification.

RESULTS

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* Yang and Yang (Table 2). Minimum intraspecific genetic divergence values (0%) were found in *H. anomala*, *H. conspecta*, *H. flaviventris* Yang and Yang, *H. furcata* Grootaert, Yang and Saigusa and *H. namtokhinpoon*.

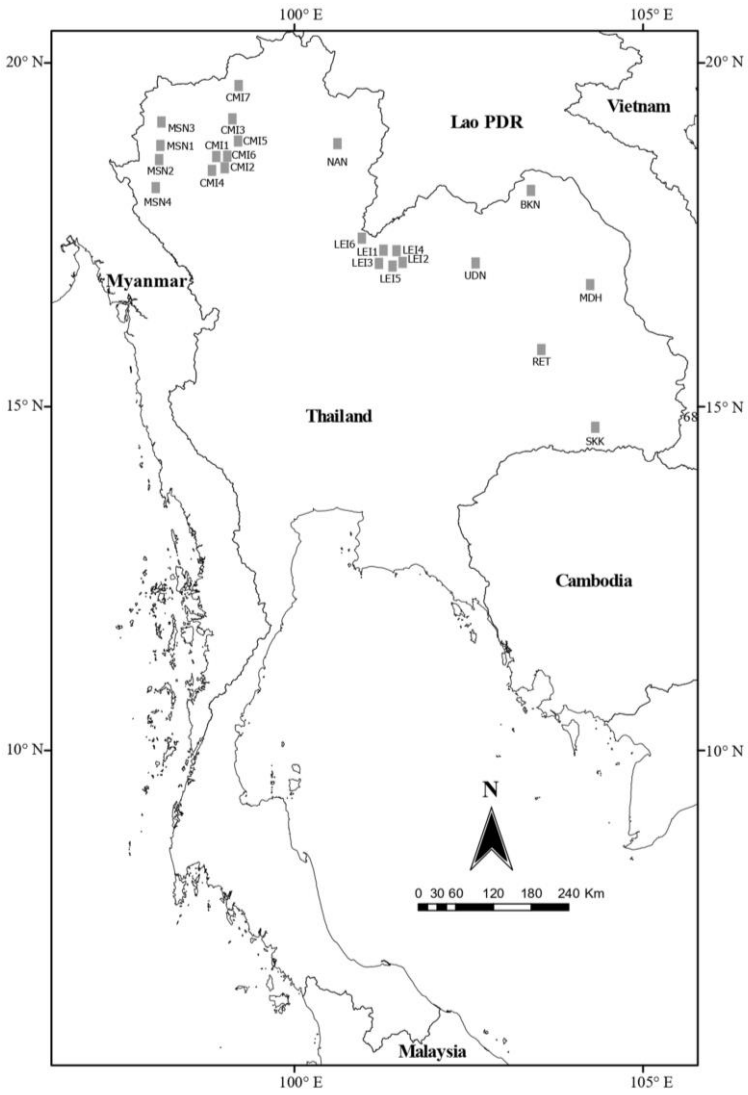

FIGURE 1. Collection sites of 12 species of *Hemerodromia* in Thailand. Details of sampling sites are given in Table 1.

Interspecific genetic divergence ranged from 7.4% to 19%, with a mean of 12.7% (Table 2). A low (7.4%) level of minimum interspecific divergence occurred between *H. flaviventris* Yang and Yang and *H. songsee* while, a high (19%) level of maximum interspecific divergence occurred between *H. namtokhinpoon* and *H. furcata* Grootaert, Yang and Saigusa. Intraspecific and interspecific genetic divergence values showed little overlap (Fig. 2). All specimens (n = 135) were perfectly (100%) identified into species based on the COI sequences (Table 2).

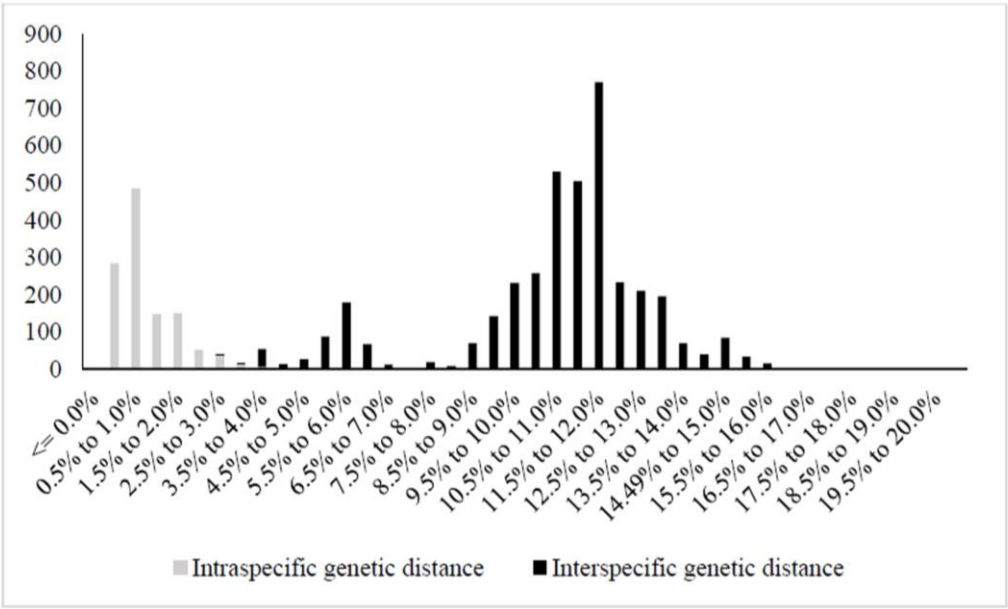

FIGURE 2. Distributions of intraspecific and interspecific genetic distances, based on 135 mitochondrial cytochrome *c* oxidase subunit 1 sequences from 12 species of *Hemerodromia* in Thailand.

TABLE 2. 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

DNA barcode trees

All three phylogenetic analysis methods (NJ, ML and BA) revealed similar tree topologies therefore, only the NJ tree is presented (Fig. 3). Minor differences included the placement between *Hemerodromia anomala* and *H. conspecta*. *H. anomala* and *H. conspecta* are 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 formed a separate clade from 28 sequences of extralimital *Hemerodromia* spp. obtained from the GenBank. All 12 Thai species formed monophyletic clades with strong support. *Hemerodromia fusca* and *H. yunnanensis* formed a clade with strong support (>98%) suggesting that they are closely related species.

Five species (*Hemerodromia furcata*, *H. conspecta*, *H. betalutea*, *H. songsee*, *H. flaviventris*) formed another clade but with weak support. *Hemerodromia oriens* and *H. anomala* were isolated from other species and were placed in different clades. Three species, *H. anisoserrata*, *H. acutata* and *H. namtokhinpoon* were retrieved in another clade but with only weak support.

DISCUSSION

Our results indicated that COI DNA barcoding sequences are highly effective for identification of *Hemerodromia* species 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 support. Because of the high success rate for species identification, further studies of *Hemerodromia* in Thailand could potentially benefit from the DNA barcode library provided in present study, for example as an aid to associate different life stages (Thaijarern et al., 2017) or to associate morphologically homogeneous females with males.

The level of genetic variation within species of *Hemerodromia* in Thailand is low compared to those of members of closely related family, Hybotidae (Diptera, Empidoidea), which has higher range (0-17.2%) (Nagy et al., 2013). The exception is high intraspecific genetic divergence in *H. yunnanensis*. This species possessed the greatest intraspecific genetic diversity with maximum K2P genetic distance of 4.0%. This level of within species genetic divergence is often found in species complexes of the insect order Diptera (Pramual and Adler, 2014; Meier et al., 2006). This species is geographically widespread being recorded in China, Vietnam, Singapore and Malaysia (Plant, 2015). In Thailand, *H. yunnanensis* has been found throughout the country and in diverse habitats. Thus, a high level of genetic variation is not unexpected. It should be noted that there are small differences in the morphology of the male terminalia in some populations, particularly in the south and west of Thailand, Vietnam, Singapore and Pulau Tioman (Malaysia) (Plant, 2015), indicating that different geographical populations might represent cryptic taxa withing a *H. yunnanensis*-complex. However, these populations were not studied here. Based on closely related morphology of the male cercus and surstylus, Plant (2015) raised an informal species-group to include at least, *H. yunnanensis*, *H. fusca*, *H. phahompokensis* Plant and *H. songsee* Plant from Thailand, *H. digitata* Grootaert, Yang and Saigusa from southern China and *H. serpa* Smith from Nepal. In contrast to the geographically widespread species, some *Hemerodromia* are geographically range-restricted. For example, local high-elevation endemics such as *H. phahompokensis*,

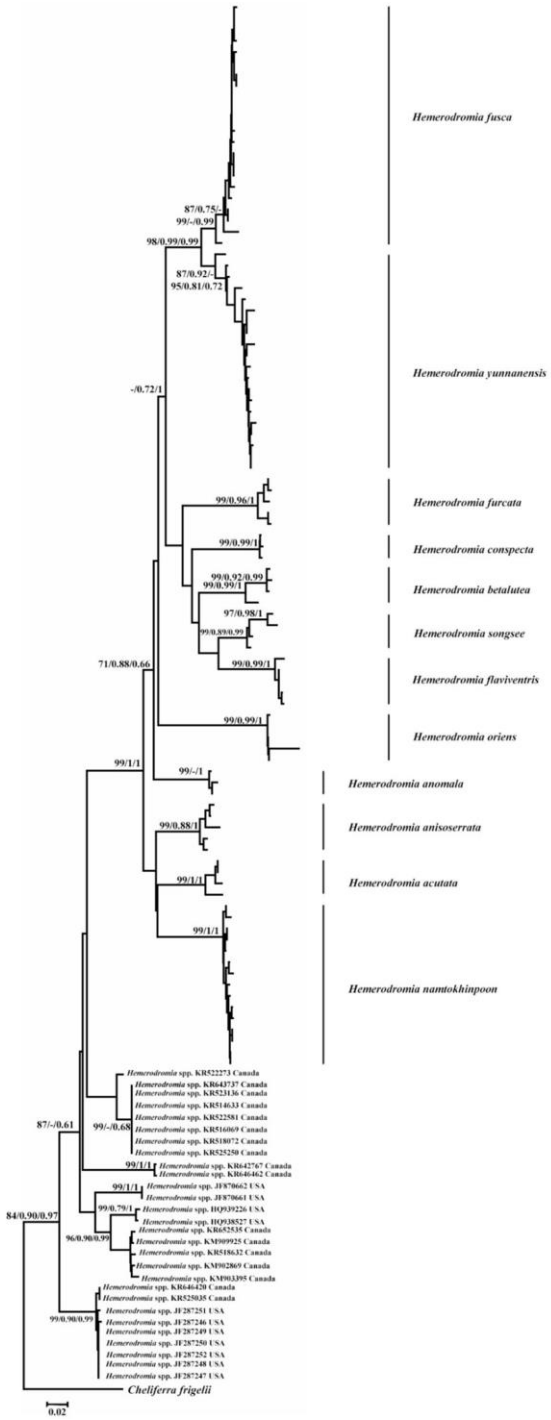

FIGURE 3. Neighbor-Joining tree for 95 haplotypes of the mitochondrial cytochrome *c* oxidase subunit 1 sequences of 12 nominal species of *Hemerodromia* in Thailand and 28 unknown species of *Hemerodromia* from the GenBank. Bootstrap values for neighbor-joining, maximum likelihood and posterior probability of Baysian analysis are shown above or near the branches. Scale bar represents 0.02 substitutions per nucleotide position.

which is entirely restricted to high mountain summits and which might have radiated historically from more widespread and essentially lowland forms, as typified by the modern species, *H. fusca* and *H. yunnanensis* (Plant, 2015). Other *Hemerodromia* spp. are highly stenotopic, showing strong niche-conservatism, being ecologically restricted to specialized habitats. *Hemerodromia anomala*, *H. conspecta* and *H. namtokhinpoon* are restricted to the limestone streams flowing over tufa formations (Plant, 2015, 2021). Levels of genetic diversity of these species are relatively low compared to other ecologically eurytopic species. Previous study of black flies from calcareous streams in Thailand, found low levels of within-population genetic variation but high levels of genetic differentiation between populations (Pramual and Pangjanda, 2015). Our sampling of highly stenotopic *Hemerodromia* species was restricted to single localities, yet they actually occupy fragmented patches of suitable habitat widely distributed in a matrix of unsuitable habitat. Wider sampling will enable a better appraisal of population genetic structure, as has already been reported for *H. conspecta* in which vicariant diversification is driving active processes of speciation and microendemism occurring within multiple microrefugia (Kunprom et al., 2021)

In conclusion, DNA barcodes of *Hemerodromia* can be used effectively for species identification. The DNA barcoding sequences reported in this study are potentially useful tools for association of the unknown life stages or uncovering cryptic species of *Hemerodromia*. Such tools could facilitate a better understanding of many aspects of the biology of these important aquatic insects. There are 31 species of *Hemerodromia* reported in Thailand (Plant, 2015; 2020) but only 12 were included in this study because of unavailability of specimens. It would be useful to conduct further DNA barcode examinations of the remaining species to expand testing of the efficacy of this method for species identification, and also to detect possible hidden diversity in these insects.

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