CHAPTER III
MATERIALS AND METHODS

3.1 Study Sites

The northeastern geographic formation of Thailand performs relative high areas comparing with the nearby regions. It contains several plateaus such as Khorat Plateau in the south and Sakon Nakhon Plateau in the north, covering 168,854 km^2 (Panichapong, 1985). The northeastern part is politically divided into 19 provinces, namely Amnat Charoen, Buri Ram, Chaiyaphum, Kalasin, Khon Kaen, Loei, Maha Sarakham, Mukdahan, Nakorn Phanom, Nakhon Ratchasima, Nong Bua Lamphu, Nong Khai, Roi Et, Sakon Nakhon, Sri Sa Ket, Surin, Ubon Ratchathani, Udon Thani, and Yasothon provinces. This region consists of three sub-basins of west-east draining the Chi and Mun Rivers into the Mekong River (Penning de Vries and Ruaysoongnern, 2010). The rice field covers almost 70% of the total areas, which is a targeted cultivation for commercial purposes in northeastern region (DAE, 2012). The paddy systems were mentioned of providing the good micro habitats for freshwater leeches (Moore, 1927; Nesemann and Sharma, 2001).

In this study, freshwater leeches were collected covering all river basins throughout northeastern Thailand, namely Chi, Mekong, Mun, Pa Sak, and Songkhram river basins. There were 27 localities; however leeches specimens were successfully collected from 17 localities as shown in Figure 3-1. The localities, GPS Data and specimen numbers of each species are shown in Table 3-1.

3.2 Leeches Sampling and Preservation

Four hundred and thirty five adult leeches were collected and examined from April 2012 to February 2014. Freshwater leeches were lured out from the burrow in the bottom of aquatic habitats by stirring or making vibration to the water. After appearing, leeches were collected by hand or a dip net. All collected leeches were photographed and kept alive in glass aquaria for observing body color and pattern and some behaviors of the leeches. Most specimens were then relaxed in 10% ethanol. After, they were straight, the Vernier caliper were used to measure the body length from oral sucker to caudal sucker, and the widest of the body for the body width (Figure 3-2). The rest of specimens were kept in 95% ethanol for further external and internal morphological studies, and for further molecular studies. Some specimens were brought back alive to Animal Systematics Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok for karyotypic analysis.

3.3 Leeches Identification

Identification of specimens were made on the basis of Lesson (1842), Wahlberg (1856), Whitman (1886), Moore (1927), Richardson (1969), Klemm (1972), and Lai and Chen (2010). In addition to the body pattern in live specimens, and other external characters and internal characters were also examined (Figures 2-1 and 2-3).

The observed external characters are as follows: body length and width, number of annuli, number of complete segment, body color, color pattern, location and the arrangement of eyes, distance between male and female pores, and number

of sensillae organ. For internal examination, number of testis, absent or present of vagina stalk, atrium shape, size of epididymis, size of common oviduct, and size and shape of vaginal caecum were observed and recorded. Both external and internal organs of each species were also illustrated.

3.4 Scanning Electron Micrographs (SEM)

Jaws of sacrificed specimens were subjected to scanning electron microscope (SEM). The specimens that preserved in 95% ethanol were dehydrated through a series of ethanol (30%, 50%, 70%, and 95%), 10-15 minutes each step. Hundred percent ethanol was used for washing the specimens 3 times, stayed in 10 minutes each time. After that, dehydrated specimens were subjected to critical-point-dry (Quorum model K850. UK), then put on stub and sputter coated with 35 nm of gold/palladium before examined with a LEO/Zeiss DSM982 Geminifield emission scanning electron microscope of The Scientific and Technological Research Equipment Centre, Chulalongkorn University.

3.5 Karyological Study

Chromosome preparations were made from the testisacs using hypotonic, fixation and air-drying techniques modified from Patterson and Burch (1978) and Kongim et al. (2013). Live leeches were injected with 0.1 mL of 0.1% (v/v) colchicine for 3-4 h and then dissected to remove the testisacs into 0.07% (w/v) KCl solution (hypotonic) for 30 min. The sample then fixed in fresh Carnoy's fixative (3:1 (v/v)

absolute ethanol: glacial acetic acid). The testisacs were cut into small pieces in fresh Carnoy's fixative and the separated cells were collected by centrifugation at 1,500 rpm for 10 min. The supernatant was removed and the cell pellet resuspended in 0.5 mL of fresh Carnoy's fixative. Cell suspensions were dropped onto clean pre-heated (60 °C) glass slides, air-dried, and stained in 4% (v/v) Giemsa sclution for 10 min. Photomicrographs of 10 to 15 well-spread metaphase cells were measured for their relative length and centromeric index. Mitotic karyotypes were arranged and numbered for chromosome pairs.

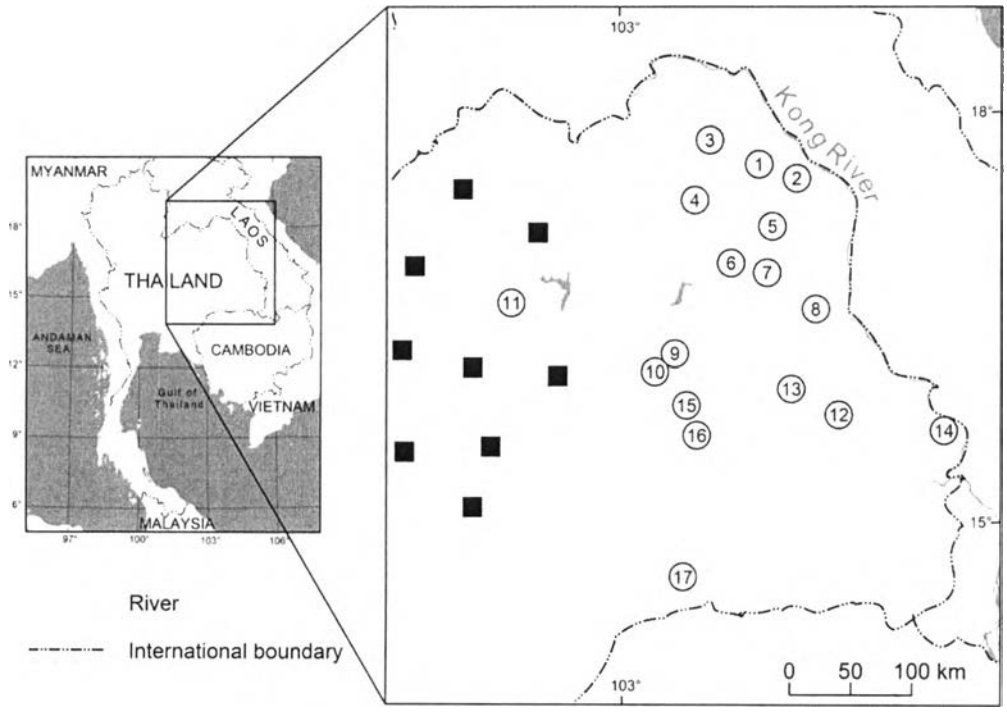

Figure 3-1. Map of northeastern boundary of Thailand showing sampling localities. Sampling localities are numbered referring to the list in Table 3-1 (Black squres indicate localities where failed to obtain specimens).

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Table 3-1. Locality, co-ordinations and sample size of each leech species used in the present study. Locality numbers refer to the localities shown in Figure 3-1.

No.	Locality	Coordinates	Number of specimens examined		
			H. javanica	*H. manillensis*	*Hirudinaria* sp.
1	Ban Donsala, Na Wa, Nakhon Phanom	17°34'27.22" N 104°7'18.64" E	44	82	5
2	Ban Majang, Na Wa, Nakhon Phanom	17°36'53.4"N 104° 8' 21.9"E	-	51	1
3	Ban Nongwang, Tao Ngoi, Sakon Nakhon	17°45'41.26"N 103°44'42.00"E	9	4	-
4	Phang Khon, Sakon Nakhon	17°22'29.02"N 103°40'26.81"E	-	2	-
5	Mueang, Sakon Nakhon	17°10'52.69"N 104°7'50.94"E	-	2	-
6	Phu Phan, Sakon Nakhon	16°54'14.64"N 103°54'7.50"E	-	6	-
7	Ban Janpen, Tao Ngoi, Sakon Nakhon	16°55'32.59"N 104°10'9.31"E	16	1	-
8	Ban Nonghai, Khamcha-i, Mukdahan	16°34'53.92"N 104°29'29.00"E	13	13	-

Table 3-1. Continued

No.	Locality	Coordinates	Number of specimens examined		
			H. javanica	*H. manillensis*	*Hirudinaria* sp.
9	Khong Chai, Kalasin	16°15'44.76"N 103°27'22.91"E	-	28	-
10	Ban Thatoom, Mueang, Mahasarakham	16°10'48.40"N 103°26'59.30"E	-	4	-
11	Huai E-pong, Phu Wiang, Khon Kaen	16°43'51.30"N 102°17'17.00"E	-	11	-
12	Tumbon Bung, Mueang Amnat Charoen	15°50'21.48"N 104°27'33.95"E	-	30	-
13	Pa Tio, Yasothon	15°57'2.81"N 104°25'12.78"E	-	3	-
14	Khemarat, Ubon Ratchathani	15°59'11.82"N 105°8'20.53"E	-	26	-
15	Chaturaphak Phiman, Roi Et	15°49'59.77"N 103°31'0.86"E	1	5	-
16	Kaset Wisai, Roi Et	15°39'13.70"N 103°35'58.39"E	-	67	-
17	Huai Saneng Reservoir, Surin	14°47'14.70"N 103°28'34.50"E	-	11	-