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Development and Characterization of 18 Microsatellite Markers for the Asian Buffalo Leech *Hirudinaria manillensis* (Lesson, 1842) and Cross-Species Amplification with Its Congeners

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ABSTRACT. – The Asian buffalo leech *Hirudinaria manillensis* is an obligatory ectoparasite freshwater leech, feeding on blood of vertebrates. Its natural populations have been threatened by habitat destruction and overexploitation for traditional medicine. In this study, we developed 18 species-specific microsatellite markers for *H. manillensis* using a next-generation sequencing approach. Microsatellite polymorphisms were screened in 30 individuals collected from four localities in Thailand. Number of alleles per locus was relatively high, ranging from 5 to 18, with an average of 9.94. The observed and expected heterozygosity values ranged from 0.200 to 0.900, and 0.567 to 0.932, respectively. The polymorphism information content (PIC) varied from 0.509 to 0.910. Ten out of the 18 loci showed significant deviation from Hardy-Weinberg Equilibrium. This might be caused by the Wahlund effect and small sample size. Null allele frequencies ranged between 0.000 and 0.396. There was no significant linkage disequilibrium between pairs of loci after a sequential Bonferroni correction. These markers were also successfully cross-species amplified in all of its congeners (*H. javanica*, *H. bpling* and *H. thailandica*), with the amplification success rate between 56–89% of the loci used. These highly polymorphic loci will be useful for further population genetics study of *H. manillensis* and its congeners.

KEYWORDS: Asian buffalo leech, cross-species amplification, *Hirudinaria manillensis*, microsatellite markers

INTRODUCTION

Buffalo leeches in the genus *Hirudinaria* are freshwater obligatory ectoparasites, feeding on blood of vertebrates. They mainly feed on mammals, and occasionally feed on fishes, amphibians, and reptiles (Moore, 1927). Their distribution range covers a wide area in south- and southeast Asia and the southern part of China. (Lai and Chen, 2010). Currently, there are four valid species in this genus (Jeratthitikul et al., 2020), including *H. manillensis* (Lesson, 1842), *H. javanica* (Wahlberg, 1856), *H. bpling* Phillips, 2012, and *H. thailandica* Jeratthitikul and Panha, 2020 in Jeratthitikul et al., 2020.

Asian buffalo leeches have been used as medical devices for blood circulation (Lent, 1986), as a source of biopharmaceuticals (Müller et al., 2017), as ingredients in traditional medicine (Enguang, 2008), as well as being a model organism in the laboratory (Elliott and Kutschera, 2011). Due to the high demand created by these uses, leech populations are overexploited in their natural habitat, although they can be cultured at the farm scale (Teh et al., 2011; Zhang et al., 2008). Additionally, natural populations are dramatically declining throughout their geographical ranges due to wetland habitat destruction and water pollution (Lai and Chen, 2010; Singhal and Davies, 1996). However, basic genetic information that could provide a guideline for conservation and captive management in *H. manillensis* is still limited.

Microsatellite DNA is one of the most informative markers for population genetic study. However, there are no microsatellite markers developed specifically for *H. manillensis*. Microsatellite markers have been developed and applied to analyze genetic diversity in some leech species, for example, a North American medicinal leech (*Macrobdella decora*) (Budinoff et al., 2004) and European medicinal leeches (*Hirudo medicinalis* and *Hirudo verbana*) (Siddall et al., 2007). Liu et al. (2015) applied the microsatellite markers for *Whitmania pigra* to *H. manillensis* collected from China and Vietnam, but their results showed low genetic variation among the examined populations.

We previously attempted a cross-species amplification using microsatellite primers developed by Liu et al. (2015) and Morishima et al. (2018), but the amplifications were not successful. In this study, we thus aimed to develop novel microsatellite markers for *H. manillensis* using a next-generation sequencing (NGS) approach. We also performed cross-species amplification in the three congeners of *H. manillensis*: *H. javanica*, *H. bpling*, and *H. thailandica.*

TABLE 1. Localities with geographic coordinates and number of samples used in this study (*N*).

Species	*N*	Locality	Coordinate
H. manillensis	15	Thailand, Udon Thani Province, Mueang District, Ban Chan, Nong Ban Chan	17°20.25'N 102°47.12'E
H. manillensis	5	Thailand, Bueng Kan Province, Phon Charoen District, Don Ya Nang, Nong Loeng	18°02.02'N 103°37.47'E
H. manillensis	5	Thailand, Nakhon Phanom, Ban Phaeng District, Phai Lom, Nong Wang	18°00.14'N 104°10.26'E
H. manillensis	5	Thailand, Sakon Nakhon Province, Mueang District, That Na Weng, Nong Han Kumphawapi Lake	17°13.29'N 104°08.36'E
H. javanica	5	Thailand, Nakhon Phanom Province, Tha Uthen District, Phanom, Nong Thum	17°41.55'N 104°21.88'E
H. bpling	5	Thailand, Phang Nga Province, Kapong District, Buffalo field near Phu Ta Jor	08°46.06'N 98°27.32'E
H. thailandica	5	Thailand, Chai Nat Province, Mueang District, Lotus pond near Ban Kluai	15°10.65'N 100°08.70'E

MATERIALS AND METHODS

Leech samples were collected from natural freshwater habitats in Thailand under the permission of the Faculty of Science, Mahidol University Animal Care and Use Committee SCMUACUC (MUSC65-003-596). Localities and number of individual samples used in this study are shown in Table 1. The 2-step method was used to euthanize the leeches. First, the animals were relaxed by gradually adding of 95% (v/v) ethanol (EtOH) into freshwater in the animal container, starting from approximately 5% (v/v) concentration until they became anesthetized. After the leeches were relaxed, they were moved to another container and fixed in 95% (v/v) ethanol. Approximately 0.5 cm^3 of caudal sucker or dorsal muscle was cut and stored in 95% ethanol at -20 °C until DNA extraction.

We extracted genomic DNA from the tissues using NucleoSpin Tissue extraction kit (Macherey-Nagel, Germany), following the manufacturer's protocol. One sample of *H. manillensis*, collected from Udon Thani Province, was used to perform Illumina Hiseq whole genome sequencing to produce 150 bp-length paired-end reads (Macrogen Inc., Korea). A total of 69,707,058 raw reads were obtained. Short or low-quality reads were removed by Trim Galore v0.6.2 (Krueger, 2019). The edited reads were assembled by ABySS v2.0.2 (Simpson et al., 2009). Microsatellite regions were mined using MISA (Thiel et al., 2003). The parameters were set to search for at least 10 repeats of di- and trinucleotide motif.

We designed 33 primer pairs for the unique microsatellite regions using Primer3web ver. 4.1.0 (Kõressaar et al., 2018). The target microsatellite fragments ranged from 90 to 300 bp in length. We initially tested for amplification success and polymorphism of these 33 primer pairs in 12 *H. manillensis* individuals using polymerase chain reaction (PCR). Eighteen out of the 33 loci were successfully amplified. They produced clear PCR product bands, with polymorphism among samples. The forward primers of these 18 loci were then labelled with 6-FAM, HEX or NED fluorescent dyes and used for screening the microsatellite polymorphisms in 30 *H. manillensis* samples from four localities (Table 1). Each locus was amplified separately in a 12.5-µL reaction, containing 1X PCR buffer, 1.25 µg BSA, 0.2 mM each dNTP, 2.4 mM $MgCl_2$, 0.32 µM of the fluorescent-labeled forward primer, 0.32 µM reverse primer, 0.4 U HotStarTaq DNA polymerase (Qiagen), and at least 25 ng DNA template. The PCR conditions were as follows: initial denaturation at 95 ºC for 15 min, followed by 40 cycles of denaturation at 95 ºC for 45 s, annealing at 53–56 ºC for 45 s and extension at 72 ºC for 45 s, followed by a final extension at 72 ºC for 10 min. The PCR products were visualized on 3.0% agarose gels. The successfully amplified PCR products were multiplexed according to the fragment sizes and fluorescent dyes, and fragment length analysis was performed using ABI 3730XL (Applied Biosystems) with a 400HD internal size standard. Microsatellite alleles were genotyped using Peak Scanner Software v1.0 (Applied Biosystems).

TABLE 2. Characterization of 18 microsatellite loci developed for *H. manillensis*.

Locus	Repeat motif	Primer sequence (5' to 3')	T_a (°C)	Size range (bp)	N_a	H_o	H_e	PIC	Freq. Null alleles	GenBank Accession No.
Hiru1	$(TC)_{23}$	F: AGCGTCTGTTGTCTTATTCTGTG R: CCACCCATAACCAGCCTGT	56	141–169	10	0.733	0.782	0.751	0.020	MT590616
Hiru2	$(AC)_{20}$	F: AAAGCCGGGAACATCAACAC R: CCTTCCAGGTCTGTGTTTGC	56	159–209	12	0.433*	0.820	0.783	0.206	MT590617
Hiru4	$(CAT)_{18}$	F: CCGTGATTCTTTGCCATCTT R: CAGCAGGACAAGGTTTGGTT	55	240–273	12	0.800	0.810	0.781	0.000	MT590618
Hiru8	$(ATC)_{14}$	F: AGAACAGATGGATGGACAGATGA R: TGACTTCAGGGAGGCTTACTATT	54	120–228	18	0.900	0.932	0.910	0.008	MT590619
Hiru9	$(TCA)_{15}$	F: CTCAGATGGGAGCCGAACT R: GTCAAAGAGAACTGATGATGACG	56	150–201	10	0.423*	0.814	0.774	0.396	MT590620
Hiru10	$(TCA)_{14}$	F: CAGCAGCAACGTGGATAAC R: TGACAAGTGGTTATGGCGAAG	55	205–301	7	0.207*	0.749	0.708	0.355	MT590621
Hiru12	$(CAT)_{14}$	F: CTGGAGGACACTTATTCGATGA R: GTGGTGATTGTGGTGGCAG	56	121–169	8	0.533	0.594	0.556	0.032	MT590622
Hiru15	$(TGA)_{10}$	F: CGGCTTTCTTTCGACACGA R: TCTGAAAGGCCACGTCATCA	55	90–117	8	0.633*	0.834	0.798	0.103	MT590623
Hiru16	$(TGA)_{12}$	F: GTCACGCTTCGCTGATCAC R: TCGGCTCAGACAGTTTTCCA	55	105–150	10	0.667	0.830	0.791	0.082	MT590624
Hiru19	$(ATG)_{12}$	F: ATGTCGAATGCTGGATGATG R: CGGTCTGTTTGCTCTGCTTA	55	136–157	8	0.633*	0.806	0.765	0.089	MT590625
Hiru20	$(CA)_{12}$	F: GTCACAGGAACAACGTCTGC R: TGAGGGACCACATATCATCG	55	150–180	7	0.567	0.567	0.509	0.000	MT590626
Hiru26	$(TA)_{10}$	F: CTTTTGGAAGGTAGGAGCTGA R: GATTAAGCGCCAAGTCACATTC	54	135–157	9	0.786*	0.825	0.786	0.150	MT590627
Hiru28	$(GAT)_{10}$	F: CAGCACCGCCTTAAGTCATC R: GGTGTCGGTCCATCAACATG	55	172–283	16	0.767*	0.895	0.869	0.060	MT590628
Hiru30	$(ATG)_{10}$	F: GATTGTGGTGATGGTCTGGC R: ACTGCTCATCTTTGTCATCTTTG	55	116–200	11	0.500*	0.833	0.798	0.368	MT590629
Hiru34	$(TGA)_{10}$	F: CGTCGGTCTTCATGTCCAAC R: ATCATCATCACGACCGACCA	53	92–119	5	0.667	0.726	0.665	0.028	MT590630
Hiru35	$(CT)_{13}$	F: GCGAAAGGATGCCATTGACT R: ACAGATGGACAAATGAACGAGT	54	140–158	8	0.567	0.660	0.610	0.050	MT590631
Hiru36	$(AT)_{14}$	F: TGATTGAGCCTGGGTTTAGC R: GGACCAATTTGAGACCTTGG	54	227–297	8	0.200*	0.729	0.692	0.301	MT590632
HiruB1	$(TA)_{19}$	F: TCCCATAATCTCCGCCATCT R: TTGTCACCGAGCGAAATGAA	53	179–241	12	0.333*	0.808	0.769	0.257	MT590633

T_a, annealing temperature; N_a, number of alleles per locus; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphic information content; * significant deviation from HWE at the significance level $P = 0.05$.

Microsatellite polymorphism was analyzed based on number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and polymorphism information content (PIC) using Microsatellite Toolkit (Park, 2001). The deviation from Hardy-Weinberg Equilibrium (HWE) at each locus and linkage disequilibrium (LD) between pairs of loci were examined using GenePop 4.7 (Raymond and Rousset, 1995; Rousset, 2008). A sequential Bonferroni correction (Holm, 1979) was applied at the significance level $\alpha = 0.05$ for the multiple comparisons of LD. Null allele frequencies were estimated using Micro-Checker version 2.2.3 (Van Oosterhout et al., 2004).

In addition, we performed cross-species amplification for each locus on five samples of the three congeners *H. javanica*, *H. bpling*, and *H. thailandica*, following the same protocol and analysis methods, except for the tests for deviation from HWE and LD, which were not possible due to small sample sizes.

RESULTS

Amplifications of the 18 microsatellite loci in 30 samples of *H. manillensis* produced clear and polymorphic bands on agarose gel. The number of alleles per locus ranged from 5 to 18, with an average of 9.94. The H_o and H_e ranged from 0.200 to 0.900 and 0.567 to 0.932, with an average of 0.575 and 0.779, respectively. The PIC varied from 0.509 to 0.910, with an average of 0.740. Ten out of the 18 loci showed significant deviation from HWE (P<0.05) (Table 2). There was no evidence for large allelic dropout. No

TABLE 3. Cross-species amplification in three congeners of *Hirudinaria*, number of alleles and allele size range (bp).

Locus	*H. javanica*		*H. bpling*		*H. thailandica*	
	N_a	Size range (bp)	N_a	Size range (bp)	N_a	Size range (bp)
Hiru1	2	139, 141	2	137, 139	5	157–165
Hiru2	6	155–201	3	155–169	2	157, 159
Hiru4	5	261–288	3	249–279	-	-
Hiru8	3	129–162	-	-	-	-
Hiru9	2	147, 180	3	168–186	2	135, 177
Hiru10	-	-	-	-	-	-
Hiru12	5	139–172	-	-	-	-
Hiru15	5	99–132	5	84–135	3	99–123
Hiru16	5	126–147	5	111–156	2	111, 114
Hiru19	5	121–133	2	94, 148	-	-
Hiru20	2	150, 154	2	176, 178	5	158–184
Hiru26	4	137–171	4	143–153	3	147–155
Hiru28	5	220–289	4	196–208	4	181–223
Hiru30	2	116–125	-	-	-	-
Hiru34	2	98, 104	3	89–304	2	110, 119
Hiru35	5	144–172	1	134	1	142
Hiru36	3	287–295	-	-	-	-
HiruB1	-	-	-	-	-	-

N_a, number of alleles per locus; -, unsuccessful amplification

significant linkage disequilibrium was found after a sequential Bonferroni correction. Null allele frequencies ranged between 0.000 and 0.396.

For cross-species amplification in the three congeners, 16 (89%) loci were successfully amplified for *H. javanica*, 12 (67%) loci for *H. bpling*, and 10 (56%) loci for *H. thailandica*. Some of the successfully amplified loci were monomorphic or dimorphic (Table 3). The unsuccessful amplification attempts included no amplification and poor amplification. Allele size ranges of the cross-species amplifications were similar to those of *H. manillensis* (Table 3). However, this data should be interpreted with caution due to the small sample sizes.

DISCUSSION

Variability of microsatellite markers developed for *H. manillensis* in this study was equal or even higher than those of the previous reports in other leeches. Allelic diversity of the microsatellite loci in this study was similar to values found for *Whitmania pigra* and *Hirudo nipponica* (N_a = 9.1 alleles/locus; Liu et al., 2013) and for *Haemadipsa japonica* (N_a = 10.0 alleles/locus; Morishima et al., 2018). The H_o and H_e of our 18 microsatellite loci were slightly higher than those reported in *Macrobdella decora* (H_o = 0.31, H_e = 0.43; Budinoff et al., 2004), *W. pigra* and *H. nipponica* (H_o = 0.348, H_e = 0.688; Liu et al., 2013), and *H. japonica* (H_o = 0.517, H_e = 0.786; Morishima et al., 2018). Similarly, the PIC values were comparable to those in previous studies; for example, *W. pigra* and *H. nipponica* (PIC = 0.640; Liu et al., 2013) and *H. japonica* (PIC = 0.766; Morishima et al., 2018). The PIC values of all loci in this study were higher than 0.5, indicating that these loci were highly informative and will be useful for population genetic studies (Botstein et al., 1980).

Our sample size was small and the samples were obtained from several localities; these factors could be responsible for deviation from HWE in 10 of the 18 loci. Approximately half of the loci showed relatively low frequency of null alleles, between 0.0 and 0.1. Relatively high frequency of null alleles (more than 0.3) was detected in Hiru9, Hiru10, Hiru30, and Hiru36, which may be caused by the excess homozygosity and therefore these loci should be used with caution. The presence of null alleles, in turn, may also lead to the significant deviations from HWE observed in these loci.

At least 56% of the microsatellite loci developed for *H. manillensis* can be amplified in the congeners. *Hirudinaria javanica* showed the highest success rate of cross-species amplification among the congeners. This is likely due to the close evolutionary relationship between *H. javanica* and *H. manillensis*, as they are a sister clade (Tubtimon et al., 2014; Jeratthitikul et al., 2020).

In conclusion, we were first to develop and characterize 18 microsatellite markers for *H. manillensis*. All of the 18 loci were polymorphic, with

moderate to high levels of microsatellite diversity. These markers were cross-species amplified in the congeners of this species (*H. javanica*, *H. bpling*, and *H. thailandica*) with successful amplifications between 56 and 89%. These microsatellite markers will be useful for population genetics study, conservation management, and culture of *H. manillensis* and its congeners.

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LITERATURE CITED

Botstein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics, 32: 314–331.

Budinoff, R., Siddall, A. and Siddall, M. 2004. Twelve variable microsatellite loci for the North American medicinal leech, *Macrobdella decora*. Molecular Ecology Notes, 4: 491–493.

Elliott, J.M. and Kutschera, U. 2011. Medicinal leeches: Historical use, ecology, genetics and conservation. Freshwater Reviews, 4: 21–41.

Enguang, T. 2008. Progress in the study of ecology, zoogeography, group, control repellent and medical usage of Hirudinea in China. Acta Ecologica Sinica, 28: 6272–6281.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics, 6: 65–70.

Jeratthitikul, E., Jiranuntskul, P., Nakano, T., Sutcharit, D. and Panha, S. 2020. A new species of buffalo leech in the genus *Hirudinaria* Whitman, 1886 (Arhynchobdellida, Hirudinidae) from Thailand. ZooKeys, 933: 1–14.

Kõressaar, T., Lepamets, M., Kaplinski, L., Raime, K., Andreson, R. and Remm, M. 2018. Primer3-masker: Integrating masking of template sequence with primer design software. Bioinformatics, 34: 1937–1938.

Krueger, F. 2019. Trim Galore, Babraham Bioinformatics. Available from: www.bioinformatics.babraham.ac.uk/projects/trim_galore/, (9 Dec 2019).

Lai, Y.T. and Chen, J.H. 2010. Leech fauna of Taiwan, National Taiwan University Press, 118 pp.

Lent, C. 1986. New medicinal and scientific uses of the leech. Nature, 323: 494.

Lesson, J.P. 1842. Description d'une nouvelle espèce de sangsue. Revue Zoologique par la Société Cuvierienne, 5: 1–8.

Liu, F., Guo, Q., Shi, H., Cheng, B., Lu, Y., Gou, L., Wang, J., Shen, W., Yan, S. and Wu, M. 2015. Genetic variation in *Whitmania pigra*, *Hirudo nipponica* and *Poecilobdella manillensis*, three endemic and endangered species in China using SSR and TRAP markers. Gene, 579: 172–182.

Liu, F., Shi, H.Z., Guo, Q.S. and Wang, T. 2013. Isolation and characterization of microsatellite loci for the analysis of genetic diversity in *Whitmania pigra*. Biochemical Systematics and Ecology, 51: 207–214.

Müller, C., Haase, M., Lemke, S., and Hildebrand, J. 2017. Hirudins and hirudin-like factors in Hirudinidae: implications for function and phylogenetic relationships. Parasitology Research, 116: 313–325.

Moore, J.P. 1927. The segmentation (metamerism and annulation) of The Hirudinea: Arhynchobdellae. In: Harding, W. A. and Moore, J. P. (Eds). The Fauna of British India, Taylor and Francis, London, 1–12, 97–302 pp.

Morishima, K., Suzuki, T. and Aizawa, M. 2018. Characterizationof 13 polymorphic microsatellite loci in the Japanese land leech. Parasitology International, 67: 13–15.

Park, S.D.E. 2001. Trypanotolerance in west African cattle and the population genetic effects of selection. University of Dublin, Ireland, 254 pp.

Phillips, A.J. 2012. Phylogenetic placement of a new species of Asian buffalo leech (Arhynchobdellida:Hirudinidae), and confirmation of human-mediated dispersal of a congener to the Caribbean. Invertebrate Systematics, 26: 293–302.

Raymond, M. and Rousset, F. 1995. GENEPOP (Version 1.2): Population genetics software for exact eests and ecumenicism. Journal of Heredity, 86: 248–249.

Rousset, F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources, 8: 103–106.

Siddall, M.E., Trontelj, P., Utevsky, S.Y., Nkamany, M. and Macdonald, III K.S. 2007. Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo medicinalis*. Proceedings of the Royal Society B, 274: 1481–1487.

Simpson, J., Wong, K., Jackman, S., Schein, J., Jones, S. and Birol, I. 2009. ABySS: A parallel assembler for short read sequence data. Genome Research, 19: 1117–1123.

Singhal, R.N. and Davies, R.W. 1996. Effects of an organophosphorus insecticide (Temephos) on gametogenesis in the leech *Hirudinaria manillensis* (Hirudinidae). Journal of Invertebrate Pathology, 67: 100–101.

Teh, J.C., Kamarudin, M.S., Rahim, A.A. and Saad, C.R. 2011. Performance of selected chemical compounds in eliciting feeding of Asian buffalo leech, *Hirudinaria manillensis*. Journal of Fisheries and Aquatic Science, 6: 846–851.

Thiel, T., Michalek, W., Varshney, R. and Graner, A. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics, 106: 411–422.

Tubtimon, J., Jeratthitikul, E., Sutcharit, C., Kongim, B. and Panha, S. 2014. Systematics of the freshwater leech genus *Hirudinaria* Whitman, 1886 (Arhynchobdellida, Hirudinidae) from northeastern Thailand. ZooKeys, 452: 15–33.

Van Oosterhout, C., Hutchinson, W., Wills, D. and Shipley, P. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4: 535–538.

Wahlberg, P. 1856. Neue Blutegel. Zeitschrift für die Gesammten Naturwissenschaften,8: 271–272.

Zhang, B., Lin, Q., Lin, J., Chu, X. and Lu, J. 2008. Effects of broodstock density and diet on reproduction and juvenile culture of the Leech, *Hirudinaria manillensis* Lesson, 1842. Aquaculture, 276: 198–204.