

Short Note

Preliminary Study of Genetic Diversity in the Giant Freshwater Stingray, *Himantura chaophraya* (Batoidea: Dasyatidae) from the Remnant Populations in Thailand

JENJIT KHUDAMRONGSAWAT^{1*}, TUCKSAORN BHUMMAKASIKARA²
AND NANTARIKA CHANSUE³

¹ Animal Systematics and Molecular Ecology Research Group, Department of Biology, Faculty of Science, Mahidol University, Rama 6 Road, Rajathewi District, Bangkok 10400, THAILAND

² Wildlife Conservation Office, Department of National Park, Wildlife and Plant Conservation, 61 Phaholyothin Road, Chatuchak District, Bangkok 10900, THAILAND

³ Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University, Henri Dunant Road, Pathumwan District, Bangkok 10330, THAILAND

* Corresponding Author: Jenjit Khudamrongsawat (khudamrong@gmail.com)

Received: 29 October 2016; Accepted: 5 February 2017

Himantura chaophraya, also known as the giant freshwater stingray, is one of the largest freshwater fishes in the world found in Thailand. The genetic distinctness of the *H. chaophraya* population in Thailand from the population of *H. chaophraya* in India was demonstrated¹, which is described and recognized the geographic uniqueness of each population. The presence of *H. chaophraya* in the central plain in Thailand has been known since 1983². The distribution of this specie from local people interview and literature search was compared with field surveys between 2009 and 2011. The result revealed the diminishing range as well as the decrease in abundance³. Nevertheless, the capture of this species is occasionally reported in the Mekong River near Nakhon Phanom Province and Mae Klong River near Amphawa Floating Market in Samut Songkhram Province. Causes of the reduction in population abundance are thought to include overfishing and by catch fishers, but the actual causes have not been identified^{4,5}. Water pollution evidently have caused several stingray deaths in the past

few years as seen in the sugar barge accident on 31st May 2011 in the Chaophraya River resulting in death of over 20 individuals of the giant freshwater stingrays⁶ and a most recent incident in the Mak Klong River in October 2016 causing death of at least 50 individuals of the stingrays⁷. All losses urge the Thai government to enforce legal protection of the species.

Currently, the Thai Cabinet has passed the new regulation that will include *H. chaophraya* into the protected lists in the Wild Animal Reservation and Protection Act. In addition, proposed criteria for IUCN Red List have considered using genetic diversity information of a species⁸. Therefore, information regarding this species will be useful for conservation and management. This study aimed to evaluate fine scale genetic variation of the remnant populations of *H. chaophraya* in Thailand. This information may provide supporting evidence for the appropriate national conservation status and legal protection designation of the species and serve as baseline data for future conservation.

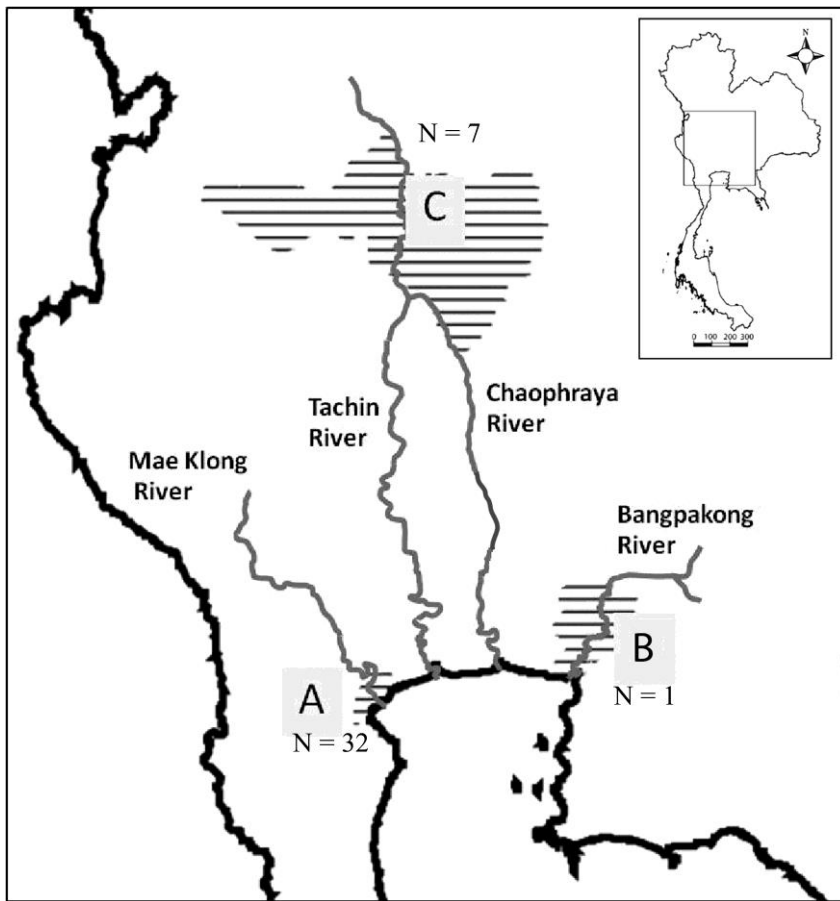


FIGURE 1. Thick lines = rivers where *H. chaophraya* reported in Central Thailand. Horizontal lines = areas where *H. chaophraya* found in this study. A = sampling area in Mae Klong River, B = sampling area in Bangpakong River, C = sampling area in Chaophraya River.

Samplings of *H. chaophraya* tissue were opportunistically collected from 2009 to 2011 (Fig. 1). Although these rivers drain into the Gulf of Thailand in nearby provinces within 100 km of each other, they constitute discrete river basins but occasionally connected through seasonal inundation and man-made canals. *Himantura chaophraya* was caught using hooks and line and tissue samples from the pectoral fin were obtained and preserved in 95% ethanol. After tissue sampling, the fish were labeled with microchip on the left side of the body for recapture identification, and

released. Thirty-two tissue samples were collected from the lower Mae Klong River in Samut Songkhram Province (only 28 were used for cytochrome b sequencing), seven from the upper Chaophraya River in Nakhon Sawan Province (only six were used for microsatellite DNA analysis), and one sample from the lower Bangpakong River in Chachoengsao Province (Fig.1).

The genomic DNA was extracted using the commercial kit (QIAGEN Valencia, CA, USA). Genetic diversity of microsatellite DNA and cytochrome b were investigated performed investigated using eleven

published microsatellite DNA primers⁹ as well as those for cytochrome b¹. All PCR reactions were repeated twice for result confirmation. PCR products were sent to MACROGEN (Korea) for fragment length analysis and sequencing. Genetic diversity as measured by the number of microsatellite alleles, heterozygosity, cytochrome b haplotype polymorphism, and nucleotide diversity was determined using ARLEQUIN version 3.5¹⁰.

Samples from all locations were pooled for the analyses. The average microsatellite allele per locus was 8.9 alleles per locus. Observed heterozygosity for each locus ranged from 0.231 to 0.769. Half of the studied loci (Hcha-02, Hcha-03, Hcha-04, Hcha-05, Hcha-10, and Hcha-11) showed significant deviation from Hardy-Weinberg equilibrium in contrast to the results from a previous screening using only samples collected from the lower Mae Klong River⁸.

Of these polymorphic loci, some, such as loci Hcha-09 and Hcha-10, were highly variable and exhibited more than 10 alleles (Fig. 2). On the other hand, some loci, such as Hcha-06, were less variable. Most alleles were present in the samples from the lower Mae Klong River where the majority of the samples were obtained with a few exceptions. For example, allele 274 at locus Hcha-05 was observed in the sample from the upper Chaophraya River regardless of a small sample size. Additional samples from these rivers and other rivers that *H. chaophraya* is reported may provide an opportunity for further investigation of genetic variation and population structure of wild populations in Thailand. Although genetic diversity inferred from microsatellite DNA cannot be compared among species due to questionable evolution of the loci used in studies, the diversity of *H. chaophraya* quantified in this study

appeared to similar to several threatened batoids^{11,12,13}.

In addition to the microsatellite DNA analysis, fragments of 1,002 base pairs (bp) of cytochrome b from 36 samples (28 samples from Mae Klong River, seven samples from Chaophraya River with one additional sample obtained from Nakhon Sawan Provincial Administrative Organization after microsatellite DNA analysis, and one sample from Bangpakong River) were used for the analyses after correction and alignment. Variations of cytochrome b sequences were obtained (Table 1). Overall nucleotide diversity was 0.00039 ± 0.00043 with eight polymorphic sites. Three haplotypes were identified. One dominant haplotype (pattern 1) was found in all sampling locations and accounted for 88.9% (32 samples) of total samples. Moreover, the dominant haplotype was the precisely same sequence that was previously reported in 1999 using samples from the Chaophraya River¹. The presence of a dominant haplotype, together with low haplotype diversity suggests recent or ongoing dispersal events. The very low level of nucleotide diversity in *H. chaophraya* indicated little genetic polymorphism at evolutionary scale, which likely influences the viability of natural populations¹⁴.

Because the great difference in numbers of samples from different sampling locations, the analysis of population structure could not be determined with statistic confidence. It is questionable about the natural population structure of *H. chaophraya* population since its divergence from the marine ancestor. The distribution of this species in large rivers that are not parts of the same basin, and its occurrence in a wide range of habitats from over a hundred kilometers inland freshwater to

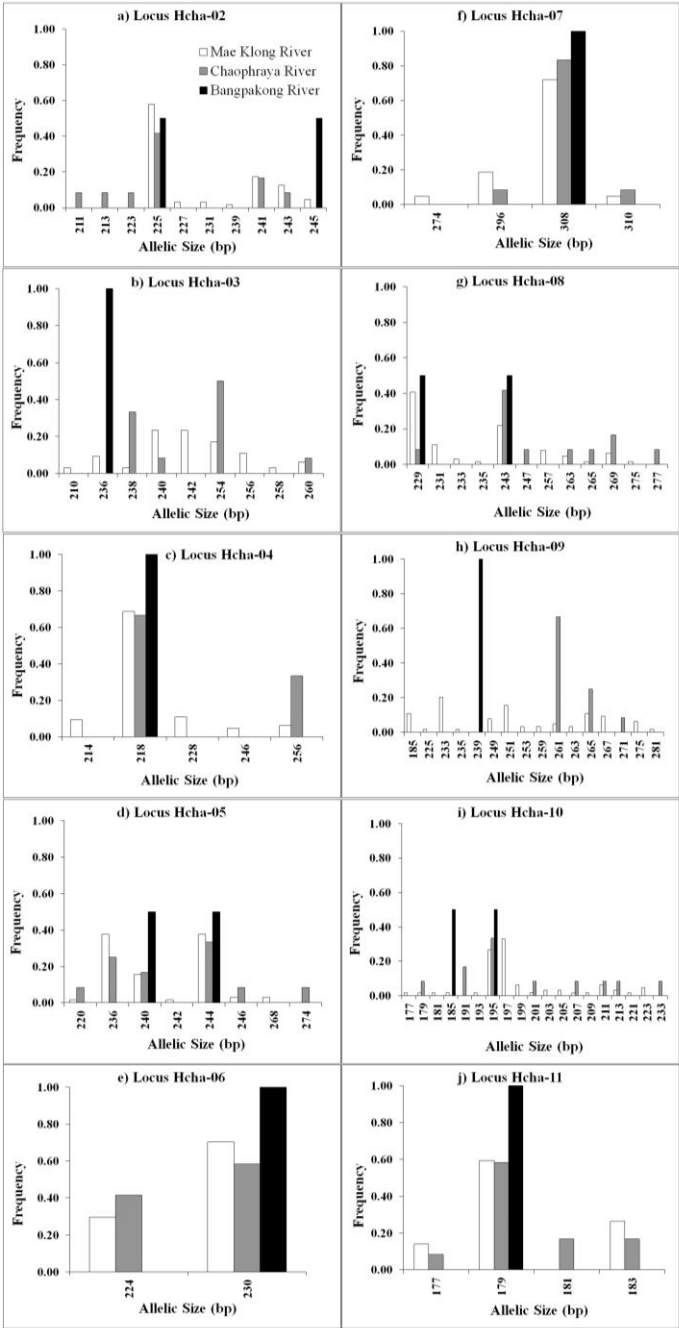


FIGURE 2. Microsatellite DNA allelic variation of ten polymorphic loci (a - j) from three sampling locations. White bars = samples from the lower Mae Klong River; grey bars = samples from the upper Chaophraya River; black bars = a sample from the lower Bangpakong River.

estuaries suggests dispersal and movement through seasonal river connections in the

Tertiary through Quaternary¹⁵ and use of the marine environment during sea level rise.

TABLE 1. Cytochrome b haplotype diversity of *H. chaophraya* in all sampling locations.

Haplotype	GenBank Accession	1	2	3	4	5	6	12	13	14	998	999	1000	1001	1002		
Pattern 1 (N = 32)	KX668133	.	.	A	A	T	A	...	A	.	T	...	C	T	.	.	.
Pattern 2 (N = 3)	KF111247	.	.	A	A	T	A	...	A	.	A	...	C	T	.	.	.
Pattern 3 (N = 1)	KF111248	.	.	G	T	C	T	...	C	.	A	...	T	C	.	.	.

Man-made canals connecting rivers and introduction by humans could occasionally contribute to movement between rivers.

Opportunistic capture of the giant freshwater stingrays in different rivers indirectly suggests that these rivers serve as habitats for this large fish, and the Mae Klong River likely harbors a large number of wild populations in Thailand. River pollutions have diminished wild population as well as exacerbated the viability of the species that has low level of genetic diversity as inferred from this study. Thus, genetic data that provide baseline evidence of pre-population reduction will facilitate future monitoring and restoration of wild populations.

ACKNOWLEDGEMENTS

Funding was provided by the Research Grant for New and Mid-Career Researchers of Mahidol University given to J. Khudamrongsawat. Special thank was given to W.Y. Brockelman, W. Akkarapatungwong, C. Kongrit for providing facility and comments, and P.D. Round for comments. Specimens of the stingrays and other rays were obtained with the help from VMARC, the Fish Siam Team (www.fishsiam.com), Nakhon Sawan Provincial Administrative Organization, Inland Fisheries Research and

Development—Supanburi Division, S. Sapwanna, and S. Manawattana (D.V.M.).

LITERATURE CITED

1. Sezaki, K., Begum, R.A., Wongrat, P., Srivastava, M.P., SriKantha, S., Kikuchi, K., Ishihara, H., Tanaka, S., Taniuchi, T. and Watabe, S. 1999. Molecular phylogeny of Asian freshwater and marine stingrays based on the DNA nucleotide and deduced amino acid sequences of the cytochrome b gene. *Fisheries Sci*, 65: 563-570.
2. Monkolprasit, S. and Robert, T. 1990. *Himantura chaophraya*, a new giant freshwater stingray from Thailand. *Jpn J Ichthyol*, 37: 203-208.
3. Khudamrongsawat, J. 2011. Population Genetics of the Giant Freshwater Stingray (*Himantura chaophraya*) in Mae Klong River and Bangpakong River, Final Report. Mahidol University, Bangkok. 33 pp.
4. Office of Natural Resources and Environmental Policy and Planning. 2007. Thailand Red Data: Vertebrates. Ministry of Natural Resources and Environment, Bangkok. 98 pp.
5. Stone, R. 2007. The last of the Leviathans. *Science*, 316: 1684-1688.
6. Daily News. 2011. Big Victim. Available from: <http://www.dailynews.co.th/newstartpage/index.cfm?page=content&categoryID=23&contentID=144169>, (accessed 15 June 2011).
7. Pollution Control Department. 2016. Environmental News: Proof of death of the giant freshwater stingrays in the Mae Klong River Number 59/2559 (in Thai). Ministry of Natural Resources and Environment. Available from: <http://www.pcd.go.th/Public/News/GetNewsThai.cf>

- m?task=lt2016&id=17523, (accessed 30 January 2017).
8. Wiloughby, J.R., Sundaram, M., Wijayawardena, B.K., Kimble, S.J.A., Ji, Y., Fernandez, N.B., Antonides, J.D., Lamb, M.C., Marra, N.J., and DeWoody, J.A. 2015. The reduction of genetic diversity in threatened vertebrates and new recommendations regarding IUCN conservation rankings. *Biol Conserv*, 191: 495-503.
 9. Bhummakasikara, T., Kongrit, C., Siripunkaw, C., Chansue, N. and Khudamrongsawat, J. 2013. Development of microsatellite DNA primers for the giant freshwater stingray, *Himantura chaophraya* (Batoidea: Dasyatidae) in Thailand, and cross-species amplification in other stingrays. *Conserv Genet Resour*, 5: 453-455.
 10. Excoffier, L.G. and Lischer, H.E.L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour*, 10: 564-567.
 11. Chapman, D.D., Simpfendorfer, C.A., Wiley, T.R., Poulakis, G.R., Curtis, C., Tringali, M., Carlson, J.K., and Feldheim, K.A. 2011. Genetic diversity despite population collapse in a critically endangered marine fish: the smalltooth sawfish (*Pristis pectinata*). *J Hered*, 102: 643-652.
 12. Kashiwagi, T., Broderick, D., Lance, S.L., Bennett, M.B., and Ovenden, J.R. 2012. Development and characterization of ten microsatellite loci for the reef manta ray *Manta alfredi*. *Conserv Genet Resour*, 4: 1055-1058.
 13. Yagashita, N. and Yamaguchi, A. 2009. Isolation and characterization of eight microsatellite loci from the longheaded eagle ray, *Aetobatus flagellum* (Elasmobranchii, Myliobatidae). *Mol Ecol Resour*, 9: 1034-1036.
 14. Grant, W.S. and Bowen, B.W. 1998. Evolutionary lineages of marine fishes: insight from sardines and anchovies and lessons for conservation. *J Hered*, 89: 415-426.
 15. Gupta, A. 2005. Landforms of Southeast Asia. In: A. Gupta (Ed.). *The Physical Geography of Southeast Asia*. Oxford University Press Inc., New York. 468 pp.
-