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Research Article

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Abstract

Previous genetic investigations of the Mekong giant catfish (*Pangasianodon gigas* Chevey, 1913) and striped catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) provided discordant results. Here, we sequenced mitochondrial (mt) DNA of the *cytochrome B* region, and a control region, to characterize the genetic variation of *P. gigas*, *P. hypophthalmus*, and hybrids of these two species. Among the three groups, *P. hypophthalmus* had the greatest diversity in both regions, yet all three studied groups showed lower genetic diversity compared to the results of previous studies. The Bayesian Skyline Plots showed a reduction in effective population sizes of the parental species. The hybrids were found to have a constant population size with a recent divergence time. As expected, the network and neighbor joining tree showed a close maternal genetic relationship between the hybrid and *P. hypophthalmus*, a reflection of the breeding between male *P. gigas* and female *P. hypophthalmus*. Our results provide genetic information on these endangered fish that will be useful for both conservation and commercial breeding programs.

Keywords

Mekong giant catfish, mitochondrial DNA, cytochrome B region, control region, Thailand

Introduction

The Mekong giant catfish (*Pangasianodon gigas* Chevey, 1913) and striped catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) are 2 of the 11 *Pangasianodon* species found in Thailand (Roberts & Vidthayanon, 1991). Endemic to the Mekong River, *P. gigas* is one of the largest freshwater fish, measuring up to 3 m in length and weighing up to 300 kg (Hogan, Moyle, May, Zanden, & Baird, 2004). With a dramatic decrease in population size of up to 90% over three decades (Hogan et al., 2004), *P. gigas* was classified as a Critically Endangered A4abcd ver 3.1 on the IUCN Red List of Threatened Species (Hogan, 2013) and listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The decreasing number of *P. gigas* also led the Thai Department of Fisheries to initiate a captive breeding program, which, since 1985 has released thousands of *P. gigas* to the wild (Hogan, 2013).

In contrast, *P. hypophthalmus* (commonly known as "Sawai" across Thailand) is much smaller than *P. gigas*, weighing 2 to 10 kg. The species is also a commercially important freshwater fish, serving as a food source for mainland Southeast Asian countries, for example, Thailand, Laos, Cambodia, and Vietnam. Striped catfish significantly declined in Thailand from 1980 to 1990, and

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in Cambodia in 2006; the declines led to it being classified as Endangered A2bd + 4bcd ver.3.1 on the IUCN species (The IUCN Red List of Threatened Species (Vidthayanon & Hogan, 2013). Intergenetic hybridization, or interspecies hybridization, has been used to create hybrids that receive a combination of beneficial parental characteristics, for example, hybrid vigor, fast development, and disease resistance (Burnside, 2004; Tave, 2003). Recently, interspecies hybridization between *P. gigas* and *P. hypophthalmus* has been initiated to counteract the economic disadvantages of both the giant and striped catfish; *P. gigas* grows slowly and is hard to rear, whereas *P. hypophthalmus* has an unpopular taste and yellow muscle color, making it unsuitable for export to western countries. The viable hybrid possesses commercially beneficial characteristics, for example, rapid development and the desired white muscle color (Panase, Amornlerdpison, & Mengumphan, 2013).

Previous genetic studies investigated *P. gigas* and its closely related species using mitochondrial (mt) DNA in the 16 rRNA region (Na-Nakorn et al., 2006) and in the mtDNA control region and autosomal microsatellites (Ngamsiri et al., 2007). The two studies provided discordant results in which the former stated that genetic variation in the Mekong giant catfish was commensurate with the other related species, including *P. hypophthalmus*, indicating that the Mekong giant catfish population might be more robust than currently thought and showed a genetic signature of the historically larger population due to wild origin. The latter study indicated low genetic variation in *P. gigas*, compared to other freshwater fish. However, to date, there have been no reports on the genetic variation and genetic sources of the hybrid species; in addition, no information has been published on the mtDNA of the *cytochrome B (cytB)* region of the parental species (*P. gigas* and *P. hypophthalmus*). Here, we report on sequencing data of the *cytB* and control regions from newly collected samples of *P. gigas* and *P. hypophthalmus* and their hybrids in Thailand, in order to provide a baseline for management strategies.

Materials and Methods

Sample Collection

We collected 117 tissue samples belonging to three fish groups, that is, (a) 48 samples of *P. gigas* from five farms: one from Sakon Nakhon Province (SK), in the north-east of Thailand, and four from Phayao Province (PY), Fisheries Stations at Chiang Rai Province (CR [FS]), Uttaradit Province (UT), and Chiang Rai Province (CR), in northern Thailand; (b) 44 wild samples of *P. hypophthalmus* from the Mun River, a tributary of Mekong River, in the Ubon Ratchathani Province (UB), north-eastern Thailand; and (c) 25 samples of hybrids from a farm in Chiang Mai Province (CM), northern Thailand (Figure 1; Table 1). All catfish were anesthetized with 0.1 mL/L Eugenol (in accordance with the EU Directive 2010/63/EU), and an approximately 1 cm^2 piece of anal fin or clip was collected and stored for around 1 year in absolute ethanol for further DNA extractions. Fish were then allowed to recover from the anesthetic and were released back into either the river (at the sampling site) or fish ponds.

In total, six farms were visited to collect captive samples (five for the *P. gigas* stock and one for the hybrid stock). The parent breeding stocks of *P. gigas* were from the Mekong River, whereas the parent breeding stocks for the hybrids were from different sources; the parental *P. gigas* group was from a farm in Chiang Rai Province, but the parental *P. hypophthalmus* was from an unknown origin in Ubon Ratchathani Province.

All samples were collected from 2016 to 2017 and were coded as PG for *P. gigas*, PH for *P. hypophthalmus* and HB for the hybrids. This research protocol was approved by the Laboratory Animal Research Centre, University of Phayao (No. UP-AE59-02–04-0005) and the Animal Ethics Committee of the Institute of Animals for Scientific Purpose Development of Thailand (No. U1-01205–2558).

DNA Extraction, Amplification, and Sequencing

We extracted genomic DNA using ZR Tissue & Insect DNA Mini Prep™ (Zymo Research Corp., USA). For the *cytB* region, 1,225 base pairs (bp) were amplified using modified primer pairs, that is, L14724 5′-GACTTGAAAAACCACCGTTG-3′ and H15915 5′-CGATCTCCGGATTACAAGAC-3′ (Xiao, Zhang, & Liu, 2001). For the control region, 855 to 859 bp were amplified using a modified primer pair, that is, L16007 5′-CCCAAAGCTAGGATTCTC-3′ (Kocher et al., 1989) and a modified R-D loop: 5′-GTTTAG GGGTTTGACAGG-3′ (Ngamsiri et al., 2007). A polymerase chain reaction (PCR) was performed with 38 to 40 cycles, each of which comprised a denaturation step at 94°C for 45 seconds; an annealing step at 58°C for the *cytochrome B*, and 56°C for the control region, for 45 seconds; and an extension at 72°C for 24 seconds. Each PCR reaction mixture had a total volume of 30 μL, including: 1 U *Taq* DNA polymerase, 0.1 mM dNTP, 1X buffer, 2.0 mM $MgCl_2$, 0.5 μM of each primer, and 50 ng DNA template. PCR amplicons were checked by electrophoresis using 1.5% agarose gel and then sent for sequencing at Macrogen Inc., South Korea. Both strands were sequenced with the same PCR primer pairs in both the *cytB* and control regions. An additional forward primer, namely L15519 5′-GGAGACCCAG AAAACTTTACCCC-3′ (Xiao et al., 2001), was also used for *cytB* sequencing.

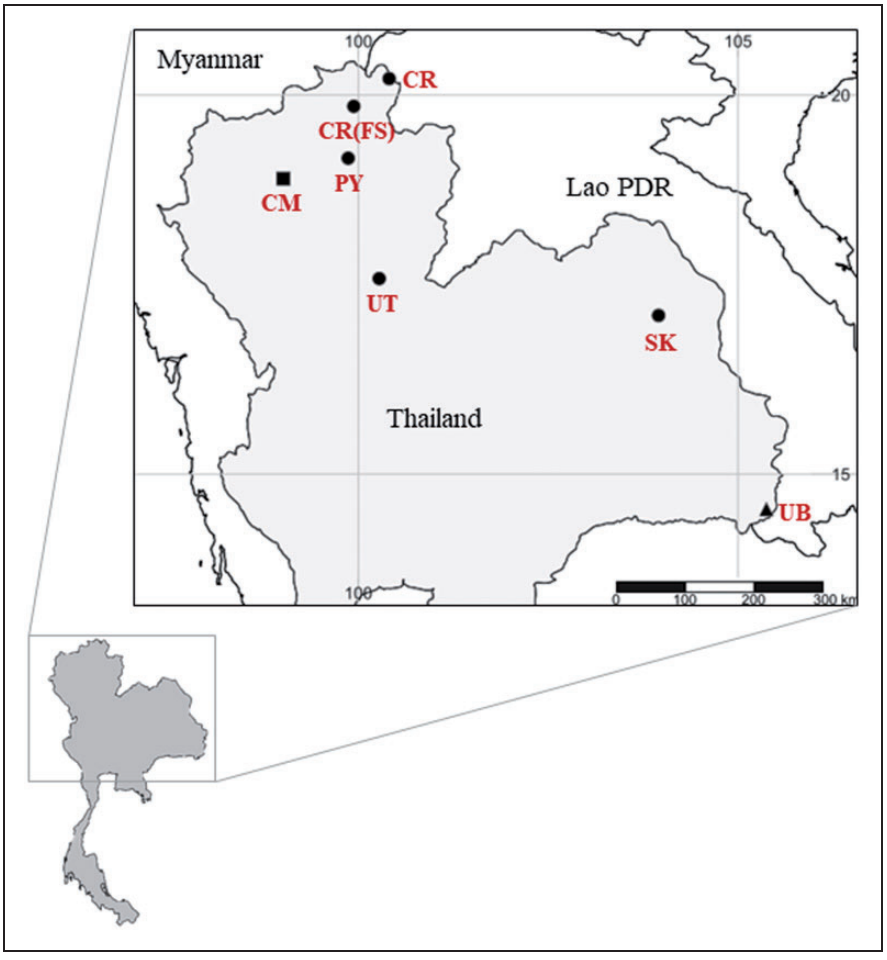

Figure 1. Locations where the samples were collected. Black circles (●) represent *P. gigas* samples. (PG_SK, PG_CR, PG_CR[FS)], PG_PY and PG_UT refer to *P. gigas* from Sakon Nakorn, Chiang Rai, Fisheries Station from Chiang Rai, Phayao and Uttaradit Provinces, respectively). Triangle (▲) represents *P. hypophthalmus* samples from Ubon Ratchathani Province (PH_UB). Square (■) represents hybrid samples from Chiang Mai Province (HB_CM). SK = Sakon Nakhon; PY = Phayao; CR (FS) = Fisheries station at Chiang Rai; UT = Uttaradit; CR = Chiang Rai; UB = Ubon Ratchathani; CM = Chiang Mai.

Table 1. Population Locations and Sizes of *P. gigas*, *P. hypophthalmus*, and Their Hybrids.

Species	Locations (population)	Code	Sample size	Latitude	Longitude
P. gigas (captive stock)	Sakon Nakhon Province	SK	11	17.092375	103.953983
	Phayao Province	PY	16	19.166961	99.866624
	Fisheries station at Chiang Rai Province	CR (FS)	2	19.851889	99.939976
	Uttaradit Province	UT	8	17.579447	100.275077
	Chiang Rai Province	CR	11	20.216885	100.406740
P. hypophthalmus (wild stock)	Ubon Ratchathani Province	UB	44	14.534353	105.374007
Hybrids (*P. gigas* × *P. hypophthalmus*) (captive stock)	Chiang Mai Province	CM	25	18.898045	99.012887

Statistical Analyses

The obtained sequences were edited and assembled using SeqScape software v2.7 (Applied Biosystem, USA). A multiple sequence alignment was executed with the reference sequences from Genbank—AY762971 for the Mekong giant catfish (Jondeung, Sangthong, & Zardoya, 2007) and KC846907.1 for the striped catfish (Zhao, Kong, & Zhou, 2014)—using MAFFT 7.271 (Katoh & Standley, 2013). Sequences were manually checked again with Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Because the number of sequences on the *cytB* and control region were not equal (Table 2), separate analyses were conducted; Arlequin 3.5.1.3 (Excoffier & Lischer, 2010) was used to obtain the following summary statistics: number of haplotypes, haplotype diversity (h), nucleotide diversity (π), mean number of pairwise difference, and the neutrality estimators, that is, Fu's *Fs* (Fu, 1997) and Tajima's *D* (Tajima, 1989). Pairwise genetic distance (Φ_{st}) was also computed by Arlequin 3.5.1.3. A neighbor joining (NJ) tree (Saitou & Nei, 1987), with 1,000 bootstraps, was constructed with the software MEGA 7 (Kumar, Stecher, & Tamura, 2016). Median-joining networks (Bandelt, Forster, & Röhl, 1999), without pre- and postprocessing steps, were constructed with Network (www.fluxus-engineering.com).

For the control region, the Bayesian Skyline Plots (BSP), based on Bayesian Markov Chain Monte Carlo analyses, were created using BEAST 1.8.0. We did not perform BSP for *cytB* due to very low genetic variation. The jModel test 2.1.7 (Darriba, Taboada, Doallo, & Posada, 2012) was run in order to choose the most suitable models to create BEAST input files by BEAUTi v1.8.0 (Drummond, Suchard, Xie, & Rambaut, 2012). The HKY substitution model was used for the analyses of *P. gigas* and the hybrids, whereas the HKY + G model was used for *P. hypophthalmus*. The BSP calculations were executed with strict clocks and a mutation rate of 3.6×10^{-8} (Donaldson & Wilson, 1999). The analysis was run for 3×10^7 steps, sampling every 10^3 steps, under the piecewise-constant skyline model with a random starting tree. Tracer 1.6 was used to generate the BSP plot from the BEAST results.

Results

We generated a total of 107 sequences, each with a length of 637 bp (15714–16350), for the control region, and 86 sequences of 917 bp from 14474 to 15390 for the *cytB* region (GenBank accession numbers for *cytB* is MN027096-MN027181 and for control region is MN017599-MN017705). Genetic diversity values from both mtDNA segments were highest in *P. hypophthalmus* (Table 2), that is, haplotype diversity ($h = 0.8070 \pm 0.0342$ and nucleotide diversity ($\pi = 0.0171 \pm 0.0088$ for the control region; $h = 0.5376 \pm 0.0822$ and $\pi = 0.0007 \pm 0.0006$ for *cytB*. Although the hybrids were the group with the lowest sample size, their extremely low genetic diversity ($h = 0.1000 \pm 0.0880$ and $\pi = 0.0005 \pm 0.0006$ for the control region; $h = 0.1053 \pm 0.0920$ and $\pi = 0.0001 \pm 0.0002$ for *cytB*) did not differ much from that of *P. gigas* ($h = 0.6729 \pm 0.0202$ and $\pi = 0.0088 \pm 0.0048$ for the control region; $h = 0.0000 \pm 0.0000$ and $\pi = 0.0000 \pm 0.0000$ for *cytB*).

The nonsignificant p values of Fu's *Fs* and Tajima's *D* ($p > .01$) infer that there has been no previous

Table 2. Genetic Diversity Values.

	cytB			D-Loop		
	P. gigas	*P. hypophthalmus*	Hybrid	*P. gigas*	*P. hypophthalmus*	Hybrid
Sample size	36	31	19	48	39	20
Number of haplotypes	1	3	2	3	6	2
Haplotype diversity	0	0.5376 (0.0822)	0.1053 (0.092)	0.6729 (0.0202)	0.807 (0.0342)	0.1 (0.088)
Nucleotide diversity	0	0.000657 (0.000598)	0.000115 (0.000223)	0.008822 (0.004782)	0.017068 (0.008822)	0.000478 (0.000581)
Mean number of pairwise differences	0	0.602151 (0.492565)	0.105263 (0.182826)	5.619681 (2.744395)	10.701754 (4.97868)	0.3 (0.326275)
Segregating site	0	2	1	12	31	3
Tajima's *D*	0 (1)	0.41134 (0.709)	−1.1648 (0.145)	3.21227 (1)	1.59234 (0.969)	−1.72331 (0.017)
Fu's *Fs* test	0 (NA)	0.46237 (0.55)	−0.83782 (0.085)	12.8821 (0.996)	12.0008 (0.997)	0.5439 (0.395)

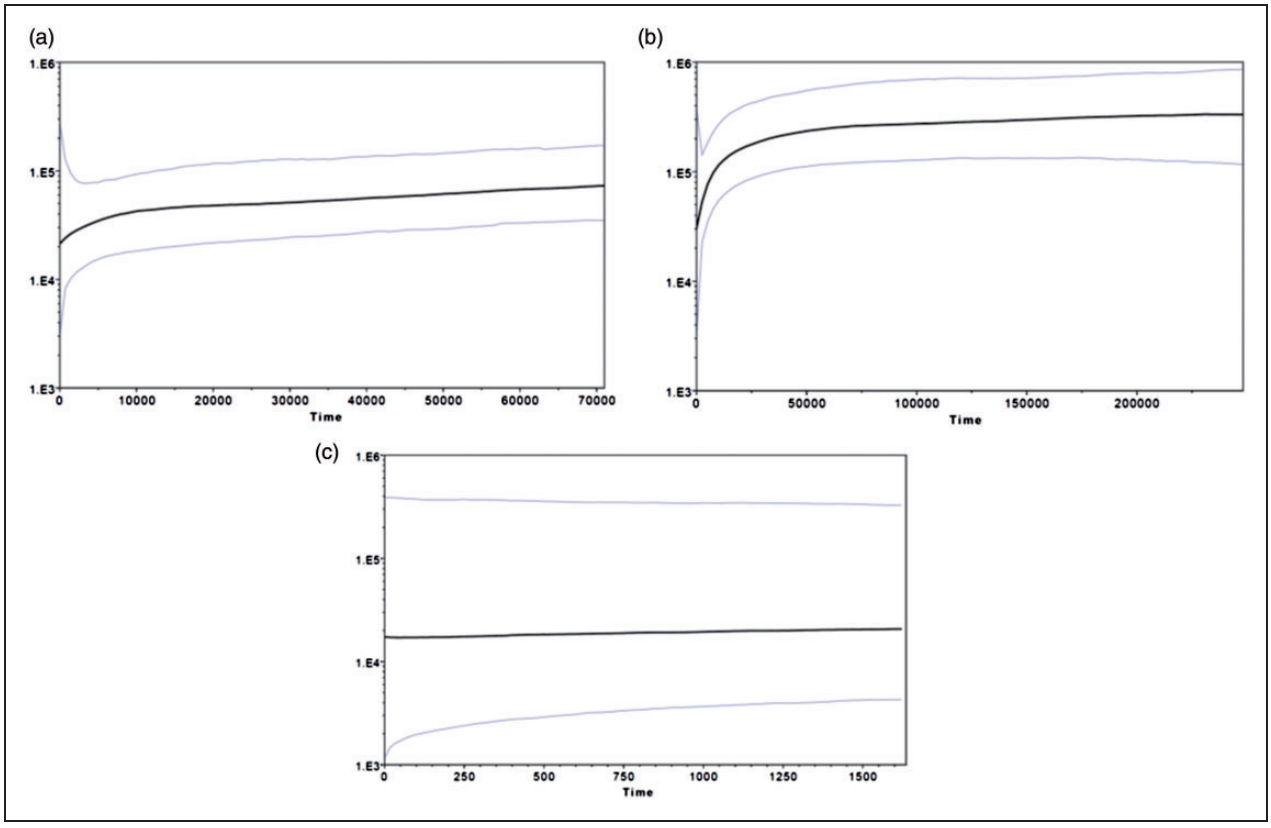

Figure 2. The Bayesian Skyline Plots based on mtDNA control regions showing the maternal effective population size changes of three populations, that is, *P. gigas* (a), *P. hypophthalmus* (b), and the hybrids (c). The maternal effective size and the time from present in years are shown in the *y*-axis and *x*-axis, respectively. The solid line indicates the median estimate and the thin lines are the 95% highest posterior density edge.

demographic expansion of these three groups. The BSP, based on the control region, showed population reduction in *P. gigas* and *P. hypophthalmus* (Figure 2(a) and (b)), whereas the hybrid group was found to have a relatively unchanged population size (Figure 2(c)). In addition, a short time of convergence indicated the recent origin of the hybrid group (Figure 2(c)).

As expected, analyses of the genetic relationships among the three fish groups indicated maternally genetic relatedness between the hybrid and *P. hypophthalmus*. The NJ tree and network showed the same clustering of the hybrid and *P. hypophthalmus* and a distinct clade of *P. gigas* (Figures 3 and 4). One shared haplotype between the hybrid and *P. hypophthalmus* was observed in both regions (Figures 3 and 4). Smaller values of pairwise genetic distances (Φ_{st}), with significant p value ($p < .01$), between the hybrid and *P. hypophthalmus* (0.3961 for the control region and 0.6941 for *cytB*) than the other two pairs ($\Phi_{st} > 0.9$) further supported a maternal genetic origin of the hybrid from *P. hypophthalmus*.

For the control region, three haplotypes of *P. gigas* are separated in different clusters in the NJ tree (Figure 3). Networks show that the haplotype of *P. gigas* from Payao Province (red) had diverged from that from Chiang Rai (green) (Figure 4). In addition, the haplotype of fish from the north-eastern province of Sakon Nakhon is shared with those of the Uttaradit and Chiang Rai Provinces located in northern Thailand.

Discussion

Here, we reported our sequencing data of the *cytB* and control regions, from newly collected samples of *P. gigas* and *P. hypophthalmus* and their hybrids from Thailand. The hybrids were genetically closer to *P. hypophthalmus* than *P. gigas*, possibly due to the hybridization between female *P. hypophthalmus* and male *P. gigas*. The reciprocal cross between male *P. hypophthalmus* and female *P. gigas* might produce either unsuccessful cross breeding or a low survival rate of the offspring, as exemplified by previous studies (Panase et al., 2013; Sutthi, Amornlerdpisan, Chitmanat, & Mengumphan, 2014). Hybridization results between the two *Clarias* catfishes (*C. gariepinus* × *C. batrachus*) showed that male *C. gariepinus* × female *C. batrachus* could produce viable

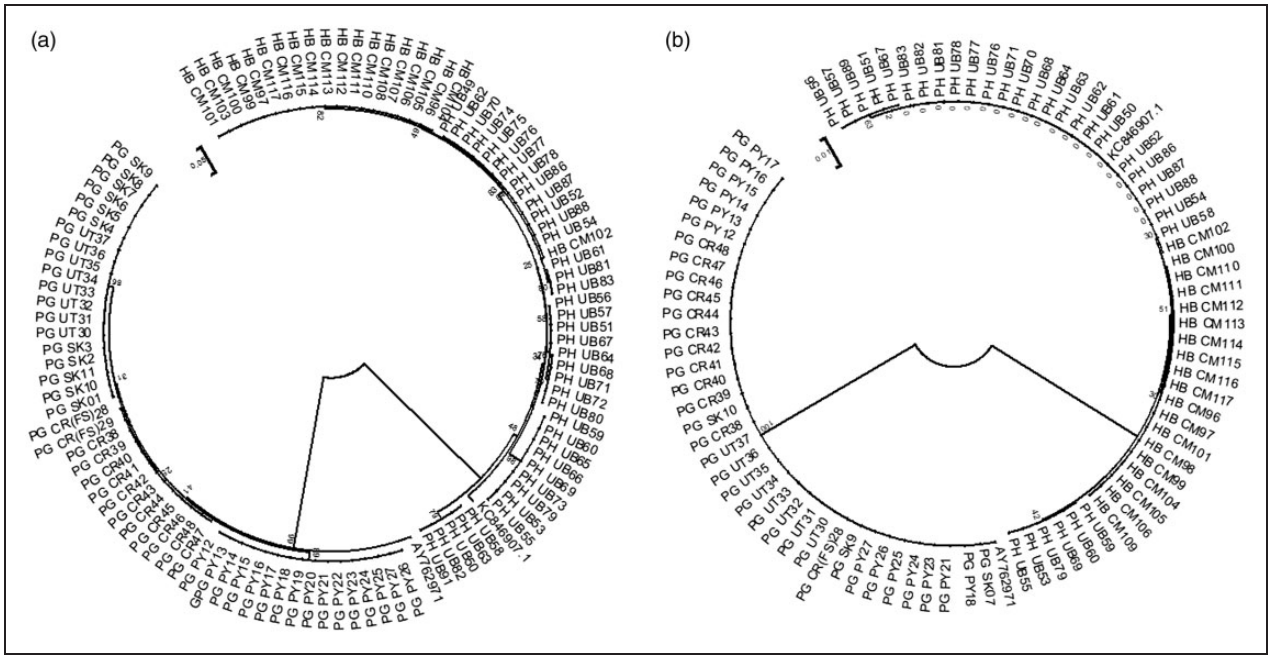

Figure 3. Neighbor joining tree of mtDNA sequences, based on the control region (a) and *cytB* (b). See population abbreviation in Figure 1.

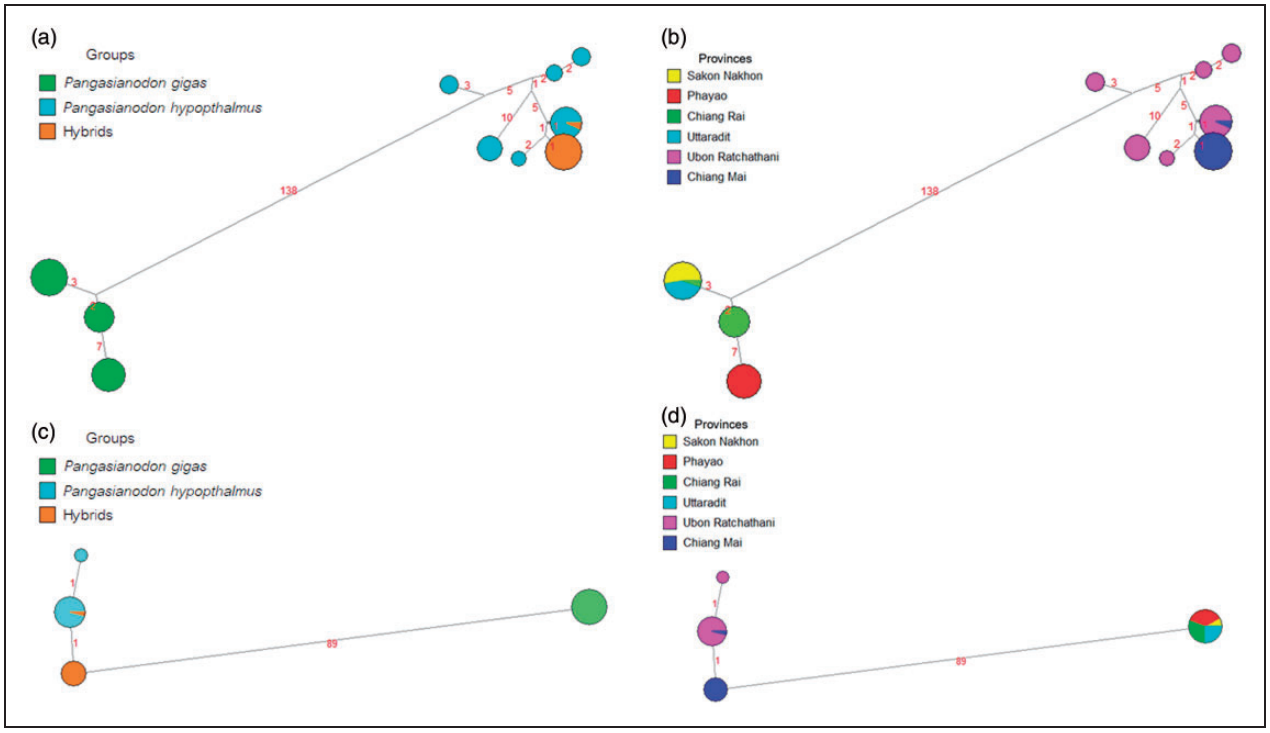

Figure 4. Networks of mtDNA sequences from the control region, based on groups (a) and geography (b) and from *cytB*, based on groups (c) and geography (d). Red numbers indicate number of base substitutions.

offspring, whereas all offspring from the reciprocal cross (female *C. gariepinus* × male *C. batrachus*) died within a few hours after hatching due to deformed morphology (Olufeagba & Okomoda, 2016). Another possible result of a reciprocal cross between female *P. gigas* × male *P. hypophthalmus* was hybrids lacking commercially positive heterosis, or less effective hybrids, as demonstrated in hybrids of male channel catfish (*Ictalurus punctatus*) ×

female blue catfish (*I. furcatus*) (Masser & Dunham, 1998) and hybrids of male *P. hypophhythalmus* × female *P. nasutus* (Hassan, Ambak, & Samad, 2011).

In general, the mtDNA diversities of all the fish groups were low; *P. hypophthalmus* showed highest genetic diversity in both mtDNA segments but, for *P. gigas*, genetic diversity in the control region (nucleotide diversity) was commensurate with that of a previous report (Ngamsiri et al., 2007); however, these values were higher than in the study by Na-Nakorn et al. (2006). The variation of the *cyt B* coding region showed extremely low genetic diversity in all groups. The very low internal diversity of *P. gigas* could be a result of captive stocks that were created from a limited number of founders, captured from the wild. However, we found a shared haplotype between *P. gigas* of the north and north-east of Thailand, indicating that geographic location had little influence on genetic diversity of wild *P. gigas* in the Mekong River, along the border of Thailand, before their use as a parent breeding stock.

The genetic diversity of *P. hypophthalmus* was greater than found in previous studies, in both regions (Na-Nakorn et al., 2006; So, Van Houdt, & Volkaert, 2006). This might be due to difference of mtDNA studied regions which higher mutation rate in this study than those previous reports (Nicolas et al., 2012). However, compared to other catfish (Nakagawa, Seki, Ishikawa, & Watanabe, 2016; Ochoa et al., 2015; Xu, Huang, Zeng, Li, & Peng, 2017; Zhong et al., 2013) and freshwater fish species (Hou et al., 2014; Kenthao, Wangsomnuk, & Jearranaipreprame, 2016; Song, Hou, Zhang, Yue, & Song, 2014; Swain et al., 2014), *P. gigas*, *P. hypophthalmus*, and their hybrids exhibited lower genetic diversity, which was also lower than those of other endangered and critically endangered fish species (Alves, Coelho, Collares-Pereira, & Coelho, 2001; Mandal et al., 2012; Wang et al., 2016).

Being discordant with earlier results (Na-Nakorn et al., 2006), our BSP plots supported the absence of demographic expansion. This might be explained by a wild origin and wide distribution of samples of *P. gigas* in previous study (Na-Nakorn et al., 2006). We detected a population reduction in *P. gigas* and *P. hypophthalmus* (Figure 2(a) and (b)) which agrees with So et al. (2006). However, many discordant results on genetic diversity values in both fish species could be clarified by further extensive sampling and studying more variable markers. The declining number of *P. gigas* caught from the Mekong River over many years (Hogan et al., 2004) supported our findings of population size reduction in this endangered species. Over-fishing, habitat destruction, habitat isolation, and migration route blockage might be causing the low genetic diversity of *P. gigas* and *P. hypophthalmus* (Hogan, 2013). Moreover, improper hatchery practices, for example, a low number of founders (founder effect), which promotes inbreeding; directional selection in breeding programs and the adaptation of reared populations to culture conditions could also lead to reduced genetic diversity (Cheng & Huang, 2013; Hsu, Takata, Onozato, & Gwo, 2015; Karlsson, Moen, & Hindar, 2010).

For captive fish, loss of genetic diversity within populations due to inbreeding might be minimized by introducing individuals from genetically distant wild stocks and carefully designing breeding programs based on cautious pedigree analysis (Vrijenhoek, 1998). Yet, due to the rarity of wild stocks of Mekong giant catfish, molecular relatedness estimators could be more feasible for conservation breeding schemes to avoid the mating of genetically close individuals (Fisch, Kozfkay, Ivy, Ryder, & Waples, 2015). If populations reared with high genetic homogeneity were released to the small natural populations, a decline of genetic diversity (Hsu et al., 2015; Karlsson et al., 2010; Yang et al., 2015) and reduction in population fitness (Vrijenhoek, 1998) might occur. Therefore, identifying population structure and genetic variation within and between populations were important for conservation program.

In summary, our results provided more genetic information on the hybrids of the endangered Mekong giant catfish (*P. gigas*) and striped catfish (*P. hypophthalmus*) in Thailand. We found low genetic diversity and a reduction in the effective population size in the parental species, as well as a constant population size of recent origin in the hybrid group.

Implications for Conservation

Our results support a decreased effective maternal population size with extremely low genetic diversity, which is putatively caused by several factors: inbreeding and improper hatchery practices for *P. gigas* and the hybrid species, and habitat destruction, habitat isolation, and migration route blockage for *P. hypophthalmus*. These results provide more mtDNA information not only for *P. gigas* but also for *P. hypophthalmus* and their hybrids, in Thailand. Our methods and results for the maternal genetic background of these catfish can be extended to further autosomal microsatellite studies and be applied to future farm and conservation management programs.

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Declaration of Conflicting Interests

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