Effects of Physical Barriers on Genetic Variation of Populations of Stone Lapping Minnow, *Garra cambodgiensis* (Tirant, 1884), in Wa River, Nan Province, Thailand

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ABSTRACT

Physical barriers within streams and rivers can lead to population fragmentation of fish species, and results in some undesirable genetic consequences. This study evaluated the impacts of three physical barriers (one large waterfall and two small concrete weirs) on genetic variation of *Garra cambodgiensis*, a small-bodied (4-10 cm) cyprinid native to Southeast Asia, and widely distributed in headwater streams of the Chao Phraya and Mekong river systems. In the upper Nan River drainage basin, especially in the Wa River, the populations are threatened by high fishing pressure and rapid habitat change. We analyzed five polymorphic microsatellite loci for 191 samples, which were collected during November-December 2016, from six locations: above and below Sapun Waterfall (SPA and SPB) in Pun Stream, Nakham Dam (NKA and NKB) in Mang Stream and Sawanua Dam (SWA and SWB) in Wa River. The sample above Sapan Waterfall (SPA) was most genetically distinct from other samples (F_{ST} = 0.097 - 0.307) and had the lowest genetic diversity. For the remaining samples, samples above and below the weirs were genetically similar (F_{ST} = 0.051 for SWA-SWB; 0.024 for NKA-NKB). STRUCTURE analysis suggested unequal admixture between the Nakham Dam and Sawanua Dam samples, suggesting some restrictions to downstream migration. The results suggest that a large barrier (>10 m high) has a significant impact on genetic differentiation, while smaller concrete weirs (< 5 m high) cause few genetic differences among populations. The barrier size should be an important consideration for the design of fish friendly structures.

Keywords: *Garra cambodgiensis*, physical barriers, gene flow, upper Nan River basin, genetic impact

INTRODUCTION

Dams, weirs and waterfalls are physical barriers that can restrict fish movements along a river and create fragmented fish habitats (Yamamoto *et al*., 2004; Neville *et al*., 2006; Neville *et al*., 2009). Depending on the design and operation, these physical barriers can cut off migration passage and alter the connectivity within a river. Habitat fragmentation can have severe consequences on population dynamics by reducing effective population size of separated populations and by restricting existing gene flow (Neville *et al*., 2006). Small, isolated populations are prone to loss of genetic variation due to genetic drift and may have lower population fitness (Roberts *et al*., 2013). Independent genetic changes among these small, isolated populations will eventually lead to population divergence.

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Received 10 October 2017 / Accepted 31 January 2018

The magnitude of the genetic effects of physical barriers depends on characteristics of the barriers, the length of population isolation (i.e., age of the barrier), size of isolated populations as well as species' life history characteristics (Yamamoto *et al*., 2004; Beneteau *et al*., 2009; Zhao *et al*., 2016). For example, in populations of white-spotted charr (*Salvelinus leucomaenis*) in southern Hokkaido, Japan, Yamamoto *et al*. (2004) detected that the degrees of genetic difference between populations above and below the dams in three rivers varied by the age of the dam (from 8 to 30 years). Beneteau *et al*. (2009) found strong evidence for an effect of river barriers (dams and weirs) on dispersal of greenside darter (*Etheostoma blennioides*) in Sydenham River, Southern Ontario. Zhao *et al*. (2016) showed that a high dam led to population fragmentation of a non-migratory fish species, *Sinibrama macrops*, in Min River, China whereas the 'low dams' did not. Hansen *et al*. (2014) illustrated that a 600-800 years old dam in the Gudena River system, Denmark, led to isolated lake populations of brown trout (*Salmo trutta*) compared with anadromous counterparts. Despite the wealth of knowledge on genetic effects of dams, very few studies have focused on tropical river systems where the rivers are under the influence of seasonal monsoons, and where most fish species migrate for only short distances.

To evaluate the effects of physical barriers on population genetics of a fish species, we focused on the stone lapping minnow (*Garra cambodgiensis*) in the mountainous streams within the Wa River, an important tributary of the upper Nan River in Nan Province, north Thailand. *G. cambodgiensis* is a small-bodied member (4-10 cm TL) of the family Cyprinidae, a highly diverse group of fishes in Asia. The species is widely distributed in the upper Nan River drainage basin, especially Bo Klua district, Nan Province as well as in other major rivers of Southeast Asia (Ligaray *et al*., 2015). This species inhabits rocky riffle bottoms of small and medium sized streams. During the rainy season, breeders congregate in flooded areas within the drainage basins to spawn semi-buoyant eggs. As the water recedes from these spawning areas, adults and fry are dispersed to streams. The breeding congregation makes this species vulnerable to heavy exploitation, especially from the fisheries that target gravid females. Because this species is a habitat specialist with a small size, their populations may be prone to population fragmentation due to physical barriers, especially those that are man-made. In addition, current fishing pressure imposed on breeder populations may drastically reduce effective population size, and subsequently accelerate genetic diversity loss.

This study aimed to evaluate the effects of physical barriers on genetic structure of populations of the lotic fish, *G. cambodgiensis*, a small-bodied, habitat-specialist species in the headwaters of the Wa River. We compared the effects of two extremes of barrier types, a large natural waterfall and smaller, more recently built concrete spillways. The insights obtained from this study can advance our knowledge about the extent to which the size of the barriers can lead to population fragmentation.

MATERIALS AND METHODS

Study area and sample collection

During November-December 2016, we obtained small pieces of caudal fin clips of 29 to 35 individuals of *G. cambodgiensis* from locations representing sections above and below three physical barriers in the Wa River system, a large tributary of the upper Nan River, in Bo Klua district, Nan Province (Figure 1, Table 1). The sampling locations were less than 200 m above and below the barriers. These barriers were Nakham Dam (a concrete weir, 5 m high, NK) in Mang Stream, Sapun Waterfall (natural waterfall, 10 m high, SP) in Pun Stream, and Sawanua Dam (a concrete weir, 3 m high, SW) in the Wa River, upstream of the Mang-Wa confluence. Two different barrier types included concrete weirs (NK, SW) and a natural waterfall (SP). Mang Stream is a large tributary of the Wa River (Figure 1). The fin clips were preserved in 95% ethanol for further analysis.

Table 1. Sampling locations of three barriers and barrier characteristics at Wa River, upper Nan River, Nan Province, Thailand

Stream	Name and type of barrier	Geographic co-ordinates (UTM)		Position relative to the barrier	Location code	Sample size (n)
		X	Y			
Mang Stream	Nakham Dam Concrete weir, 5 m high, ~ 40-50 years old	726355	2112478	Above	NKA	35
				Below	NKB	31
Pun Stream	Sapun Waterfall Natural waterfall, 10 m high > 100 years old	731079	2123372	Above	SPA	34
				Below	SPB	29
Main-stem Wa River	Sawanua Dam Concrete weir, 3 m high, ~ 40-50 years old	729096	2127669	Above	SWA	31
				Below	SWB	31

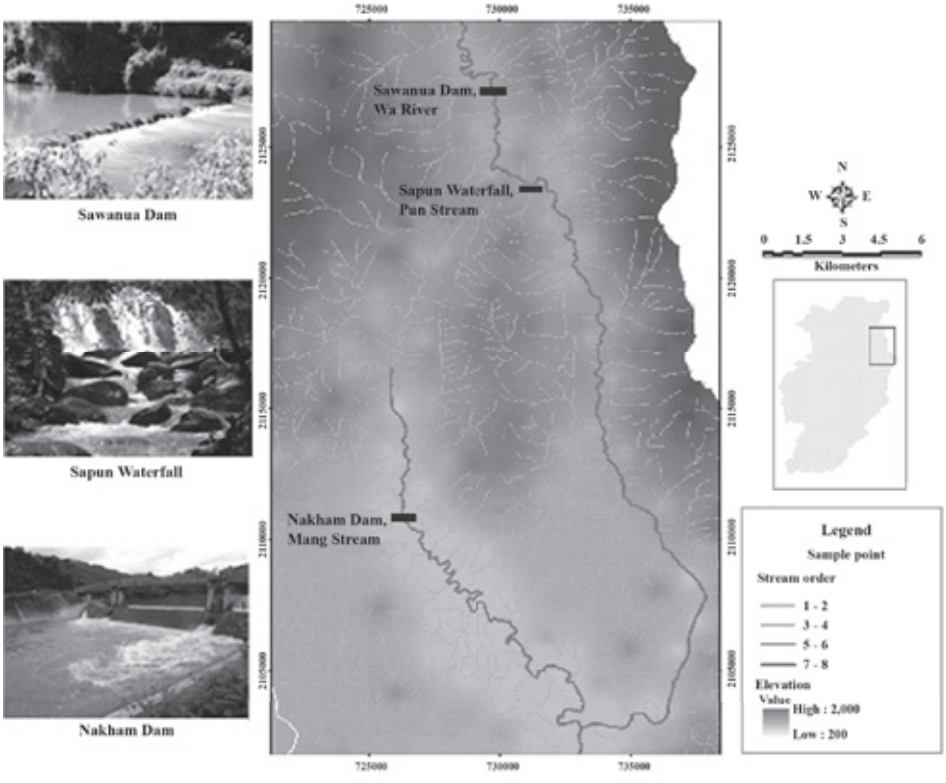

Figure 1. Locations of physical barriers examined in this study in the Wa River system, upper Nan River, Nan Province, Thailand. Sampling locations of *G. cambodgiensis* populations were less than 200 m above and below the barriers. The map also shows stream order, elevation (mean sea level, MSL), and physical appearance of barriers examined.

Microsatellite genotyping

Genomic DNA was extracted from fin clip tissue using salt extraction protocols (Aljanabi and Martinez, 1997). We analyzed five microsatellite loci, amplified using the polymerase chain reaction (PCR). These loci were a combination of markers developed for *G. cambodgiensis* and other *Garra* species. GC203 was developed for *G. cambodgiensis* (Jaisuk *et al*., 2014), HOLN was developed for *G. barreimiae* (Kirchner *et al*., 2014), and the three remaining loci, namely Gar3, Gar6 and Gar13, were developed for *G. orientalis* (Su *et al*., 2013). Each PCR reaction (10 µl) contained 10 ng of template DNA, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.6 units of Taq polymerase (5 $U \cdot \mu l^{-1}$, Vivantis, Malaysia). The temperature profile in a thermal cycler (MJ Mini Cycler, BioRad Laboratories, USA) consisted of three steps, i.e., 2 minutes of denaturation at 94˚C, 40 cycles of 30 seconds of denaturation at 94˚C, 30 seconds at annealing temperature, 30 seconds at 72˚C, and a final extension step for 5 minutes at 72˚C. PCR products were separated by 6% denaturing polyacrylamide gel electrophoresis (SCIE-PLAS EQ3341, United Kingdom). The DNA bands were visualized using a silver staining technique (Promega, USA). The DNA bands in the gels were estimated by comparison to a reference sequence of pGEM - 3Zf (+) Vector, with a 1 base-pair band increment (Promega, USA). An individual with a known genotype was used as a positive control for each gel.

Population genetic data analysis

Genetic diversity within populations and effective population size estimates

For genetic diversity indices, we estimated average number of alleles (A), effective number of alleles (A_e), heterozygosity (observed, H_o and expected heterozygosity, H_e), and inbreeding coefficient (F_{is}) using GenAlEx v.6.5 (Peakall and Smouse, 2006). To account for unequal sample sizes among population samples, we estimated allelic richness (A_r) based on the smallest sample size across all samples (n=30) based on a rarefaction method implemented in *F*stat v.2.9.3 (Goudet, 2001). To test the statistical significance of differences in genetic diversity measures among population samples, we used the Mann–Whitney U-test (Crooks and Shaw, 2016).

We tested for the deviations of observed genotypes from those expected under the Hardy-Weinberg equilibrium by the Markov chain method of exact probability test implemented in the software Genepop v.4.0 (Rousset, 2008). *P*-values were estimated from 10,000 dememorization numbers, in 100 batches with 5,000 iterations per batch. For the statistical inference, the *P*-value was adjusted using Bonferroni correction for multiple tests (Rice, 1989). We determined the presence of non-amplified alleles (null alleles), short allele dominance (large allele dropout) and stutter peaks using the Chakraborty (Chakraborty *et al*., 1992) and Dempster (Dempster *et al*., 1977) methods implemented in the programs MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al*., 2004) and FreeNA (Chapuis and Estoup, 2007), respectively. Moreover, to account for the effects of null alleles on the detection of population genetic structure, the FreeNA software estimated the pairwise F_{ST} values (Weir, 1996) based on allele frequencies corrected for null alleles (i.e. ENA, excluding null alleles, Chapuis and Estoup, 2007).

The contemporary effective population size (N_e) of each population sample was evaluated by the linkage disequilibrium (LD) method (Do *et al*., 2014) and sibship method (Wang, 2016) implemented in NeEstimator v.2 and COLONY v.2.05.1, respectively. For the LD method, the lowest allele frequency used was 0.01 and putative 95% confidence intervals were calculated by a parametric method (Do *et al*., 2014). The sibship approach uses maximum likelihood to estimate probabilities of full and half siblings of a sample of individuals taken from a population of interest.

Rapid increase in heterozygosities compared to those expected across loci under a mutation-drift equilibrium is an indication of a recent bottleneck event. To measure such reductions, we performed the analysis assuming a two-phase model of microsatellite evolution (TPM), which is the most appropriate for empirical microsatellite

data (Di Rienzo *et al*., 1994), with 90% single-step mutations and 10% multiple-step mutations and 1,000 replications in BOTTLENECK v.1.2.02 (Piry *et al*., 1999). The Wilcoxon's test was used to test for a significant heterozygosity excess (i.e., evidence of bottlenecks).

Genetic differentiation among populations

We assessed genetic divergence among population samples and the potential admixture level within a sample using two approaches, conventional and model-based. Analysis of molecular variance (AMOVA; Excoffier *et al*., 2006), an analogue to ANOVA, partitioned the overall variation into among regions (streams), among populations within regions, and within sample variation. We then estimated pairwise F_{ST} values using random permutation procedures (1,000 permutations) with an exact test *P*-value for a given F_{ST} using the software ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). The level of significance was adjusted for multiple simultaneous tests using the sequential Bonferroni procedure (Rice, 1989). A cluster analysis was performed based upon the Nei's genetic distance matrix (Nei, 1978) and a dendrogram was constructed with an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm using the Poppr R package (Kamvar *et al*., 2014). We presented a consensus dendrogram showing bootstrap supporting values for nodes (percentage based on 1000 bootstrap replicates).

Based on the model-based clustering approach, we analyzed multi-locus genotypes of individuals to determine a likely number of genetic clusters (K) assuming Hardy-Weinberg and linkage equilibrium within clusters and to determine genetic contribution of each cluster to individuals. The analysis was performed in the software STRUCTURE v.2.3.4 (Pritchard *et al*., 2000; Hubisz *et al*., 2009). To determine a likely K value for the dataset, we used a method proposed by Evanno *et al*. (2005) based on the difference in log probability of data between successive K values (i.e., ΔK statistics). A K value with the highest rate of change would be the probable K value for the data set. To obtain these probability values, we simulated a range of K values from 1 to 6, with 20 replicated runs for each value of K and 100,000 Markov Chain Monte Carlo (MCMC) iterations, following a burn-in period of 25,000. We used the admixture model with correlated allele frequencies, and default parameter settings. The ΔK statistics plot was generated by STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt, 2012). For each K, the clustering algorithm estimated a membership coefficient for each individual. These coefficients reflected the genetic admixture level within individuals (if existed). The display of membership coefficients of individuals for each K value was generated using a Pophelper R web app v.1.0.10 (Francis, 2016).

RESULTS

Genetic diversity within populations and effective population size estimates

All five microsatellite loci were polymorphic in all population samples. The allelic diversity was comparable among all samples. The average number of alleles per locus ranged from 6.00±1.41 (SWB) to 7.00±0.63 (NKA) whereas the effective number of alleles ranged from 2.70±0.86 (SPA) to 4.26±0.50 (NKA), and allelic richness ranged from 5.96±1.39 (SWB) to 6.91±0.68 (NKA). Average observed heterozygosity (H_o) across five loci was between 0.36±0.19 (SPA) and 0.63±0.16 (NKB) and expected heterozygosity (H_e) ranged from 0.59±0.13 (SPA) to 0.76±0.03 (NKA) (Table 2). SPA had significantly lower A_e and H_e values, than NKA, NKB and SPB ($P < 0.05$, Mann–Whitney U-test). We detected significant departure from the Hardy–Weinberg equilibrium (HWE) in 19 of the 30 tests (six samples x five loci), all of which had a deficiency of heterozygotes.

The MICRO-CHECKER analysis detected the presence of null alleles in most samples, but there was no evidence of stutter products or allelic dropout. The average of null allele frequencies ranged from 0.05±0.04 (GC203) to 0.19±0.07 (HOLN) (Table 2). However, the frequencies of null alleles at each locus were inconsistent across samples. The seeming presence of null alleles may

Table 2. Average allelic variability at five microsatellite loci of *Garra cambodgiensis* population samples. The indices include the number of alleles per locus (A), effective number of alleles (A_e), allelic richness (A_r), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{is}), and estimated null allele frequencies.

Samples	A	A_e	A_r	H_o	H_e	F_{is}	Null allele frequency
NKA	7.00±0.63	4.26±0.50	6.91±0.68	0.58±0.15	0.76±0.03	0.24±0.20	0.00 (GC203)-0.23 (HOLN)
NKB	6.80±0.75	4.02±0.34	6.76±0.72	0.63±0.16	0.75±0.02	0.24±0.13	0.00 (GC203)-0.22 (HOLN)
Mang Stream	6.90±0.70	4.14±0.44	6.84±0.70	0.60±0.16	0.76±0.03	0.24±0.17	
SPA	6.20±1.17	2.70±0.86	6.12±1.15	0.36±0.19	0.59±0.13	0.43±0.23	0.10 (Gar13)-0.26 (HOLN)
SPB	6.60±1.85	4.06±0.68	6.60±1.85	0.52±0.18	0.75±0.05	0.31±0.23	0.05 (GC203)-0.25 (Gar3)
Pun Stream	6.40±1.56	3.38±1.03	6.36±1.56	0.44±0.20	0.67±0.12	0.37±0.24	
SWA	6.40±1.20	3.71±0.75	6.35±1.16	0.55±0.11	0.72±0.05	0.22±0.18	0.00 (Gar13)-0.24 (HOLN)
SWB	6.00±1.41	3.37±1.24	5.96±1.39	0.58±0.16	0.64±0.18	0.08±0.11	0.00 (Gar6)-0.14 (HOLN)
Wa River	6.20±1.33	3.54±1.04	6.15±1.30	0.57±0.14	0.68±0.14	0.15±0.17	
All populations	6.50±1.28	3.69±0.94	6.45±1.27	0.54±0.18	0.70±0.12	0.26±0.21	
Each locus							
GC203	8.00±1.00	4.40±0.67	7.96±1.01	0.71±0.12	0.77±0.04	0.14±0.10	0.05±0.04
HOLN	6.00±1.15	3.70±1.09	5.99±1.14	0.37±0.15	0.69±0.14	0.50±0.19	0.19±0.07
Gar3	5.33±0.94	3.52±0.80	5.33±0.94	0.47±0.15	0.70±0.08	0.33±0.20	0.13±0.09
Gar6	6.50±0.76	3.09±0.99	6.40±0.70	0.55±0.15	0.63±0.16	0.10±0.10	0.06±0.03
Gar13	6.67±0.75	3..72±0.54	6.58±0.79	0.58±0.11	0.73±0.04	0.20±0.17	0.08±0.07

be a consequence of sampling errors or population properties. In addition, after adjusting the allele frequencies within each sample to account for the presence of null alleles, the FreeNA analysis showed only a slight change in pairwise F_{ST} values from the original data set (data not shown). We therefore used all loci for further analyses.

SPA and SPB samples had low effective population sizes of 3.4 (95% CI = 2.6 - 5.8) and 8.7 (95% CI = 5.1 - 14.2), respectively, based on the linkage equilibrium approach (Table 3). Other samples had comparable estimates of a very large population size. N_e estimates based on sibships suggested comparable values across all samples. Moreover, we detected significant heterozygote excess in one sample, NKA, under the two-phase mutation model (TPM) (P-value = 0.03). For the remaining samples, the mode-shift test showed a normal L-shaped distribution pattern of allele frequencies. The results implied the lack of bottleneck events in the recent history of most populations (Table 3).

Genetic differentiation among populations

AMOVA revealed significant genetic structure among streams and among groups within streams (upstream and downstream of the barriers). The genetic variation partitioned for among streams and for among groups within streams was 9% and 5% of total variation, respectively. Pairwise F_{ST} values were statistically significant for all sample pairs, with the values ranging from 0.02 (NKA-NKB in Mang Stream) to 0.31 (SPA-SWB in Pun Stream and the main-stem Wa River, respectively). SPA appeared to be the most genetically distinct in samples from other streams (F_{ST} = 0.16 - 0.31). Pairwise F_{ST} values among the three streams suggested the greatest difference to be between Pun Stream and the main-stem Wa River (0.18) (Table 4).

Table 3. Estimates and 95% confidence intervals of contemporary effective population size (N_e) based on linkage disequilibrium and sibship approaches and the detection of bottlenecks based on Wilcoxon's test for six population samples at five microsatellite loci.

Samples	Effective population size						Bottleneck test TPM (*P*- value)
	Based on linkage disequilibrium 95% Confidence intervals			Based on sibships 95% Confidence intervals			
	N_e	Lower	Upper	N_{eS}	Lower	Upper	
NKA	135.8	39.9	Infinite	25	14	24	0.031
NKB	Infinite	62.9	Infinite	24	14	47	0.062
SPA	3.4	2.6	5.8	17	9	34	0.156
SPB	8.7	5.1	14.2	21	12	41	0.063
SWA	433.3	42.2	Infinite	22	12	42	0.625
SWB	Infinite	106.8	Infinite	20	11	38	0.813

Table 4. Pairwise F_{ST} values among six *G. cambodgiensis* population samples and three streams*

	NKA	NKB	SPA	SPB	SWA	SWB
NKA	-					
NKB	0.024	-				
SPA	0.201	0.159	-			
SPB	0.046	0.055	0.097	-		
SWA	0.059	0.074	0.254	0.093	-	
SWB	0.106	0.133	0.307	0.139	0.051	-

	Mang Stream	Pun Stream	Main-stem Wa River
Mang Stream	0		
Pun Stream	0.094	0	
Main-stem Wa River	0.075	0.176	0

* Underlined values indicate statistical significance ($P < 0.0033$, *P*-values adjusted for multiple comparisons using Bonferroni correction = 0.05/15, 6 population samples; Adjusted $P < 0.0167$ for 3 stream pair comparisons).

Similarly, a UPGMA dendrogram based on Nei's genetic distance suggested SPA being the most genetically distant to the remaining samples. Two samples from the same stream were closely related. Samples from different streams were genetically different, with the exception of SPB, which was clustered together with NK samples (Figure 2).

The Bayesian clustering algorithm implemented in STRUCTURE suggested at least two possible genetic clusters, the sample above Sapun Waterfall (SPA) in Pun Stream and the remaining samples (Figure 3). At K = 2, we could not detect genetic difference between Pun and Mang streams. For K = 3, however, the STRUCTURE results agreed with the previously described analyses and suggested some differentiation among streams. The distribution of membership coefficients arranged by population samples suggested a shared ancestry between the samples above and below Sapun Waterfall (SPA and SPB). Also, there were some admixed individuals in the NK, SW and SPB samples. SPB contained genetic material from both all two or all three genetic clusters. SWA and SWB also contained genetic contribution from NKA and NKB, and vice versa.

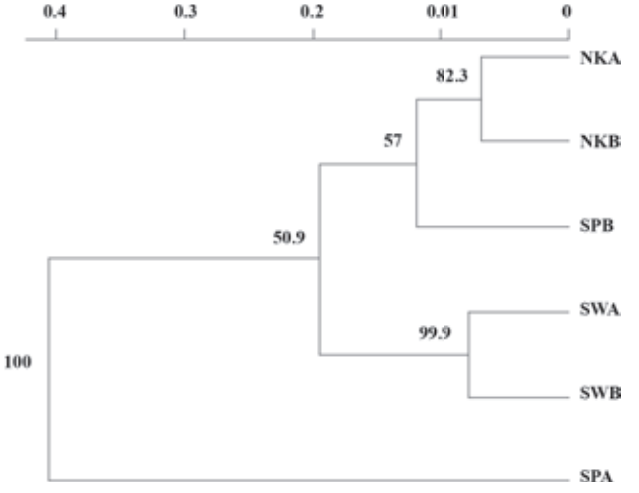

Figure 2. Dendrogram, generated from the Unweighted Pair Group with Arithmetic Mean (UPGMA) methods, based on Nei's genetic distances (Nei, 1978) among samples obtained from areas above and below physical barriers in the Wa River sub-basin of the upper Nan River drainage basin. The value supporting each node is a percentage of clustering generated from 1,000 bootstrap replicates.

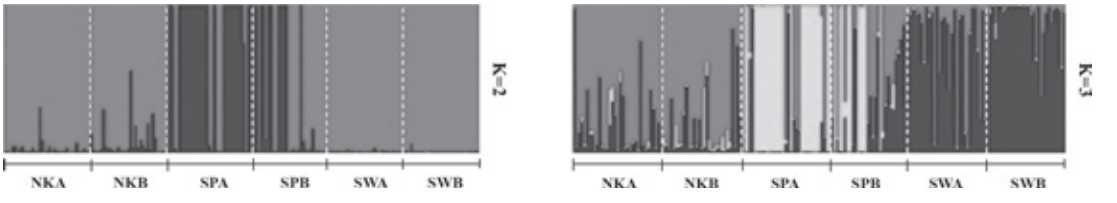

Figure 3. Membership coefficients of *G. cambodgiensis* individuals generated from a Bayesian clustering algorithm in the software STRUCTURE. The individual coefficients (vertical bars) were grouped by sampling locations. Membership to each cluster is represented by a different color. The bar plot illustrates two (K = 2) and three (K = 3) genetic clusters.

DISCUSSION

We detected genetic effects of the presence of physical barriers in the streams on genetic diversity of *G. cambodgiensis*, and barrier size determined the magnitude of the effects.

Effects of physical barriers on genetic diversity within populations

Small, isolated populations are prone to the loss of genetic diversity, due to genetic drift and the lack of incoming gene flow (Frankham *et al*., 2010). Our results suggested that Sapun Waterfall (>10 m high, age >100 years) had been an effective barrier to fish upstream migration and gene flow between *G. cambodgiensis* populations from above and below the waterfall. SPA, the sample from areas above Sapun Waterfall, had the lowest effective number of alleles and heterozygosity. Similar observations were reported for other stream-dwelling and riverine fish species, such as the white-spotted charr (*Salvelinus leucomaenis*, Yamamoto *et al*., 2006) and *Sinibrama macrops* (Zhao *et al*., 2016). Yamamoto *et al*. (2006) detected lower genetic diversity in above-dam populations compared to the below-dam populations in two rivers, namely the Kame and Hitozuminai Rivers, Japan (Kame River overall H_e = 0.35/0.71, H_o = 0.29/0.69 and the mean number of alleles 3.0/8.2 for the above- and below-dam samples respectively; Hitozuminai River overall H_e = 0.63/0.68, H_o = 0.65/0.67 and the mean number of alleles 6.0/7.2). Similarly, Zhao *et al*. (2016) reported the effects of dam structures on the freshwater fish species *Sinibrama macrops* in the Min River basin, China. The lowest amount of genetic diversity was found in a population above a high dam with a large drop, which created a reservoir.

Despite the presence of high quality habitats (pristine, forested streams) and high population abundance of *G. cambodgiensis* in Pun Stream (personal observations), where Sapun Waterfall is located, SP samples had stable, but low estimates of contemporary effective population size compared to other samples. We did not observe a recent bottleneck, although the *P*-value for the Wilcoxon's test was 0.063 in a population below the waterfall (SPB). Our observation implied that the small N_e estimates in these populations has been due to isolation imposed by the natural waterfall. This type of isolation leading to a small Ne can also be observed in other fish species. For example, in brown trout (*Salmo trutta*), two lake populations upstream of 400-500 years-old dams in the Gudena River system, Denmark, had lower N_e than for those downstream of the fall (Hansen *et al*., 2014).

We detected evidence of recent population bottlenecks in the Mang populations, especially the one above the weir (NKA, Wilcoxon's test *P*-value = 0.03). This recent reduction in population size may be due to anthropogenic activities. A dominant land use in Mang watershed is agriculture (Swidden cultivation) and the stream has been consistently dredged. These habitat disturbances can have a severe impact on a stream-dwelling fish, such as *G. cambodgiensis*.

Genetic differentiation

We detected fine-scale population genetic divergence among populations from tributary streams of the Wa River. All population genetic analyses suggested that the population above Sapun Waterfall (SPA) was the most genetically distinct. All samples above and below the barriers within the stream were genetically different. Also, STRUCTURE analysis revealed genetic distinctiveness among streams with some admixture among populations from Mang Stream, Wa River and below Sapun Waterfall in Pun Stream. The observed genetic divergence may be a consequence of the presence of a barrier isolating populations for an extended time period (Hansen *et al*., 2014) and/or a historical and contemporary hierarchical structure of a stream network (Pilger *et al*., 2017). Compared to the man-made weir structures in the Wa River system, the natural Sapun Waterfall is the largest and oldest barrier in the system. A combination of restricted gene flow for more than 100 years and low N_e of the populations above and below the waterfall may have facilitated high genetic distinctiveness between these two populations.

Size of a physical barrier affects the magnitude of the genetic isolation. While we detected high genetic distinctiveness between two populations isolated by a large natural waterfall, we observed subtler genetic effects of the smaller concrete weirs. On one hand, pairwise F_{ST} suggested genetic differences among all pairs of populations above and below the concrete weirs. On the other hand, STRUCTURE did not reveal differences between NKA and NKB and only slight differences between SWA and SWB. Our observations concurred with those observed in *Sinibrama macrops* populations isolated by dams in the Min River, China (Zhao *et al*., 2016).

Our results were confounded by the ages of the barriers. Being a natural barrier, Sapun Waterfall is much older than the concrete weirs, built in the 1960s and 1970s. Barrier age for a given size can also have a significant genetic impact. Yamamoto *et al*. (2004) found that the degree of genetic differentiation after habitat fragmentation by dams was correlated with the period of isolation. They found differences among populations isolated by large dams constructed 36 years ago (Ken-ichi River, $F_{ST} = 0.639$), 27 years ago (the Haraki Dam, $F_{ST} = 0.160$) and a more recent 8 year-old dam (Hitozuminai River dam, $F_{ST} = 0.023$).

The genetic divergence among streams observed in our study may be a consequence of the historical and contemporary stream connectivity within the Wa River system. The STRUCTURE analysis suggested admixture between populations in Mang Stream (NK) and the main-stem Wa River, especially the one above Sawanua Dam (SWA). The population below Sapun Waterfall (SPB) also consisted of admixed individuals from the Mang and Wa populations, indicating some possible gene flow. The upstream sections of Mang Stream and the Wa River (only about 6 km apart) before their confluence on the main-stem Wa River consist of numerous tributary headwater creeks (Figure 1). It is possible that these headwater creeks were connected in the past or occasionally are connected via seasonal floods although the contemporary terrain is quite mountainous. Examples of similar situations include Yazoo darters (*Etheostoma raneyi*) in North America (Sterling *et al*., 2012) wherepopulations above barriers were genetically similar, possibly due to headwater connections. To confirm these possibilities additional specimens from these headwater streams may be needed.

It is also interesting to note that samples from Pun Stream and those from the Wa River were more genetically distinct than either of them from Mang Stream despite a short geographic distance between Sapun Waterfall and the main-stem Wa River (0.3 km). The genetic data suggest the presence of an effective barrier for *G. cambodgiensis* restricting genetic input from the main-stem Wa River. Pun Stream is a low-order and high gradient stream; it is not likely to be affected by natural flooding in the main-stem Wa River. Also, a long stretch of white-water rapids (1.0 km) immediately upstream of the Pun Stream and Wa River confluence may act as another natural barrier.

CONCLUSION

A large natural waterfall has a large effect on genetic variation in a population of *G. cambodgiensis* above the fall and on genetic divergence between the isolated population and the remaining populations in the Wa River system, Nan Province. Smaller concrete weirs had a subtler genetic effect. We did not observe a difference in genetic diversity in the populations above versus below the weirs, but we detected significant genetic divergence between them. The weirs may also have restricted some fish movements (the genetic admixture was not identical between samples above and below Sawanua Dam). To minimize genetic impacts on a fish population, the size of a barrier should be an important consideration for the design of fish-friendly, man-made structures in a river.

ACKNOWLEDGEMENTS

This research was funded by the National Research Council of Thailand. We are grateful for the technical assistance from the staff of the Department of Animal Science and Fisheries, Faculty of Sciences and Agricultural Technology, Rajamangala University of Technology Lanna, Nan Campus and the Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand. Laboratory support was provided by the Biotechnology Center at Rajamangala University of Technology Lanna and the Department of Aquatic Science, Faculty of Science, Burapha University.

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