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Phylogeography of Endangered Bitterling *Acheilognathus melanogaster* Endemic to Eastern Japan

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The bitterling *Acheilognathus melanogaster* is a critically endangered primary freshwater fish endemic to the Pacific side of eastern Japan. To elucidate *A. melanogaster* genetic structure, we investigated phylogeography in nine populations, using gene sequences of mitochondrial Cytochrome b (*Cytb*), as well as nuclear Rhodopsin (*Rho*) and glycosyltransferase (*Glyt*). We found four *Cytb*-based geographical clusters unevenly divided between the northern and southern regions, with smaller groups in the south. Of the nuclear genes, *Glyt* did not show geographical differentiation, whereas *Rho* formed two clusters: one widely occurring and another restricted to central regions. Genetic diversity was generally higher in southern than in northern populations. Our results suggest that conservation of southern local populations is particularly important in maintaining the genetic diversity of this endangered fish.

Key words: Acheilognathinae, Cyprinidae, genetic structure, genetic diversity, conservation genetics

INTRODUCTION

Freshwater organisms in river systems differentiate due to isolation by oceans and landscape barriers. A central mountainous region divides the water catchment of Honshu Island of Japan into eastern and western parts, leading to different primary freshwater fish fauna in the two areas (Aoyagi, 1957; Watanabe et al., 2014; Watanabe et al., 2017). Several studies have reported genetic differentiation related to river systems and catchments in the numerous primary freshwater fishes of western Japan. (e.g., *Rhodeus atremius*, Miyake, 2011; *Gnathopogon* fishes, Kakioka et al., 2013; *Sarcocheilichthys* fishes, Komiya et al., 2014; various fishes inhabiting Lake Biwa, Tabata et al., 2016). In East Japan, studies on primary freshwater fish have focused both on those distributed throughout Honshu (e.g., *Lefua echigonia*, Miyazaki et al., 2011; *Liobagrus reinii*, Nakagawa et al., 2016; *Pseudogobio esocinus*, Tominaga et al., 2016) and those endemic to the region (e.g., *Pseudobagrus tokiensis*, Watanabe and Nishida, 2003; *Tanakia tanago*, Kubota et al., 2010; *Acheilognathus typus*, Saito et al., 2016). These fishes, excluding *T. tanago* and *L. echigonia*, show that there are low inter-regional genetic differentiations in eastern Japan, and it is expected that other species may also show low genetic differentiation among populations. Anthropogenic activity has resulted in large alteration and disturbance of freshwater environments in Japan, along with the introduction and establishment of several invasive species. These changes have led to declines in some potentially endangered endemic primary freshwater fish species (Kitamura, 2008). Eluciation of the genetic structure of these species is thus an urgent issue for conservation.

Bitterling (Cyprinidae, Acheilognathinae) are small, primary freshwater fishes that spawn in the gill chambers of living unionid freshwater mussels. Among most diversified primary freshwater fishes in Japanese Archipelago, bitterling distribution extends across Honshu, Shikoku, and Kyushu Islands. Many studies have been conducted on multiple aspects of this group, including breeding ecology, ethology, and morphological evolution (e.g., Smith and Reichard, 2005; Reichard et al., 2007; Kitamura et al., 2012; Smith, 2017). Conservation-related studies of bitterlings are also common, as most bitterling species are endangered (Kubota et al., 2010; Kitanishi et al., 2013; Kitazima et al., 2015). *Acheilognathus melanogaster* is a bitterling endemic

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to Japan, ranging in basins on the Pacific side of eastern Honshu. This species has an elongated body (6–10 cm), breeds in spring, and uses several unionid mussel species as hosts for spawning. They inhabit ponds, lakes and associated waterways leading to lakes, ponds, and the middle and lower basins of rivers. Most *A. melanogaster* populations are experiencing decline under a variety of pressures. These include predation by alien species, such as largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*); spawning-site competition with alien bitterling (e.g., *Rhodeus ocellatus ocellatus*: Hagiwara et al., 2017), as well as habitat destruction from human activity, specifically concrete revetment construction and large-scale land consolidation (Ministry of the Environment Japan, 2015). The species is now extinct in portions of its former southern distribution (Tokyo, Kanagawa, Chiba, and Gunma Prefectures). As a result, the bitterling has been listed in the Red List issued by Japan's Ministry of the Environment (MOE) since 1999 and categorized as endangered (EN) from 2007. However, no studies thus far have evaluated *A. melanogaster* geographical differentiation and genetic diversity.

Previous phylogenetic studies revealed that *A. melanogaster* forms a unique lineage within *Acheilognathus* (Chang et al., 2014; Kawamura et al., 2014), but we know little about its intraspecific phylogeny or genetic structure. Because the distribution area of *A. melanogaster* in eastern Japan extends from north to south, we expected the presence of geographic differentiation among basins. Clarifying the phylogeography of this species is important for conservation genetics and improved understanding freshwater biota characteristics in eastern Japan. Thus, in this study, we investigate phylogeography of *A. melanogaster* using mitochondrial and nuclear genes.

MATERIALS AND METHODS

We collected portions of the ventral fin from 107 *A. melanogaster* individuals representing nine populations (Fig. 1; Table 1), covering the species' entire distribution area. Then, the individuals were released immediately. The precise location of each population has not been disclosed here because describing detailed localities in

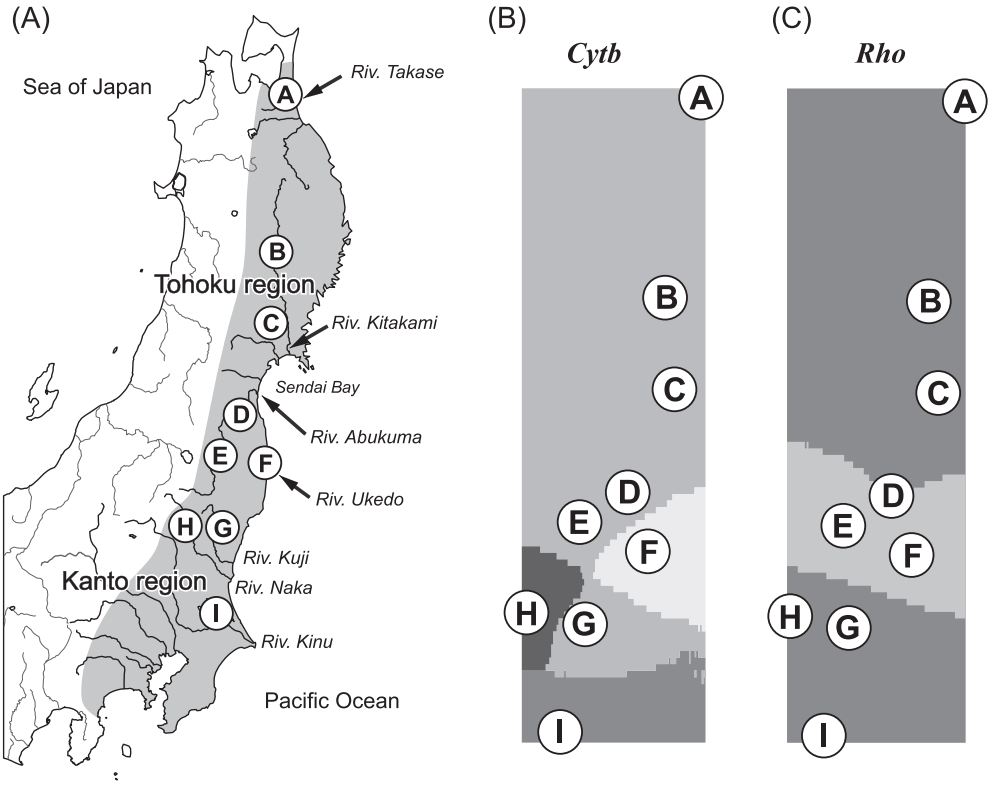

Fig. 1. Distribution area of *Acheilognathus melanogaster* (shaded regions), showing major river systems, sampling sites A–I **(A)**, as well as genetic clusters based on *Cytb* **(B)** and *Rho* **(C)**.

Table 1. Population ID, river system, specimen count (N), and haplotypes per gene (number of haploid in parentheses).

Population ID	River system	*Cytb*		*Glyt*		*Rho*	
		N	haplotype	N	haplotype	N	haplotype
A	Takase	17	C10(16), C11(1)	8	G01(16)	8	R01(13), R03(2), R09(1)
B	Kitakami	7	C03(5), C04(1), C05(1)	7	G01(14)	7	R01(14)
C	Kitakami	20	C03(5), C04(15)	8	G01(16)	8	R01(15), R07(1)
D	Abukuma	7	C04(6), C12(1)	7	G01(14)	1	R01(1), R08(1)
E	Abukuma	3	C04(3)	3	G01(6)	3	R01(1), R04(5)
F	Ukedo	13	C09(13)	8	G01(16)	8	R01(5), R09(5), R10(6)
G	Kuji	4	C04(4)	4	G01(8)	4	R01(8)
H	Naka	16	C01(11), C02(5)	8	G01(14), G02(2)	8	R01(16)
I	Kinu	20	C06(1), C07(1), C08(16), C13(1), C14(1)	8	G01(12), G03(1), G04(1), G05(1), G06(1)	8	R01(12), R02(2), R05(1), R06(1)

publications can hamper conservation (Lindenmayer and Scheele, 2017). Collected samples were stored in 99% ethanol at −20°C. Total DNA was extracted using a DNeasy tissue and blood kit (QIAGEN) or Wizard Genomic DNA Purification Kit (Promega). We analyzed one mitochondrial gene (Cytochrome b, *Cytb*) for all individuals and two nuclear genes (rhodopsin, *Rho*; glycosyltransferase, *Glyt*) for up to eight individuals per population. Fragments of each gene (*Cytb*: 1126 bp; *Glyt*: 802 bp; *Rho*: 821 bp) were amplified following methods from Kitamura et al. (2012), Li et al. (2007), and Chen et al. (2008), respectively. The PCR products were purified using either polyethylene glycol precipitation or ExoSAP-IT (USB), and then sequenced in an ABI 3130 Genetic Analyzer (Applied Biosystems). Haplotypes of *Cytb* were discriminated in PopART version 1.7 (Leigh and Bryant, 2015). Allele phases (haplotypes) for *Rho* and *Glyt* sequences were reconstructed in Phase 2.1.1 (Stephens et al., 2001; Stephens and Scheet, 2005) and seqPHASE (Flot, 2010). All sequences were deposited in DDBJ/GenBank/EMBOS (accession numbers: *Cytb*: LC369780–LC369886; *Glyt*: LC369887–LC369947, *Rho*: LC369948–LC370002).

To elucidate relationships between haplotypes, we reconstructed statistical parsimony networks (Templeton et al., 1992) in PopART. Haplotype diversity (h), nucleotide diversity (π), and molecular variance (F_{ST}) were calculated in Arlequin version 3.5.1 (Excoffier et al., 2005) to examine genetic diversity and between-population diversity. We assessed geographical associations among haplotypes using chi-squared tests in R version 3.3 (R Core Team, 2016). Genetic relationships among populations were examined using neighbor-joining (NJ) phylogenetic trees (Saitou and Nei, 1987), reconstructed with average between-population pairwise differences (Nei's dA: Nei, 1987) calculated from Arlequin. An NJ tree was calculated using the neighbor program in PHYLIP version 3.696 (Felsenstein, 2005). A Bayesian estimate of population spatial clustering across the distribution area was performed in GENELAND 4.0.8 (Guillot et al., 2005), with 5 MCMC runs (1×10^6 iterations, K = 1–9) under the following parameters: thinning of 100 and maximum nuclei number in the Poisson-Voronoi tessellation fixed to 300. The posterior probability of population membership for each pixel in the spatial domain (50 × 50 pixels) was computed after discarding the first 25% of saved iterations as burn-in.

RESULTS

Genetic diversity

We found 14 *Cytb* haplotypes (C01–C14) from 107 individuals (Table 1). Six of nine populations contained multiple haplotypes. Haplotype C04 was shared by 33 individuals from five populations, whereas seven haplotypes were unique. We obtained a single maximum parsimony network (Fig. 2A; 95% connection limit, 14 steps) with maximum connection length of 12 steps. Population I included five haplotypes, while populations E, F, and G had only one. Haplotype and nucleotide diversities were 0–0.52 and 0–0.0028, respectively (Table 2).

We found 10 *Rho* haplotypes (R01–R10) from 55 individuals (110 haploids) and 6 *Glyt* haplotypes (G01–G06) from 61 individuals (122 haploids) (Table 1). For *Rho*, seven of nine populations exhibited multiple haplotypes, and all nine shared R01. For *Glyt*, seven of nine populations were monotypic; G01 was shared across all populations, but the other five haplotypes were not. In the network analysis, *Glyt* and *Rho* haplotypes were connected within four and three steps, respectively (Fig. 2B and 2C). All haplotypes for both genes within the 95% connection limit of 12 steps. Haplotype/nucleotide diversities per population were 0–0.45/0–0.0014 for *Glyt* and 0–1/0–0.0012 for *Rho*, respectively (Table 2).

Geographical divergence

Haplotypes exhibited significant geographical association for *Cytb* (X^2 = 490.0, df = 104, P < 0.001) and *Rho* (X^2 = 266.08, df = 88, P < 0.001), but not for *Glyt* (X^2 = 45.84, df = 48, P = 0.56). Between-population genetic differentiation was significant for *Cytb* and *Rho* (*Cytb*: F_{ST} = 0.78, P < 0.001, *Rho*: F_{ST} = 0.35, P < 0.001), but not for *Glyt* (F_{ST} = −0.0019, P = 0.56). Thus, *Glyt* did not exhibit genetic

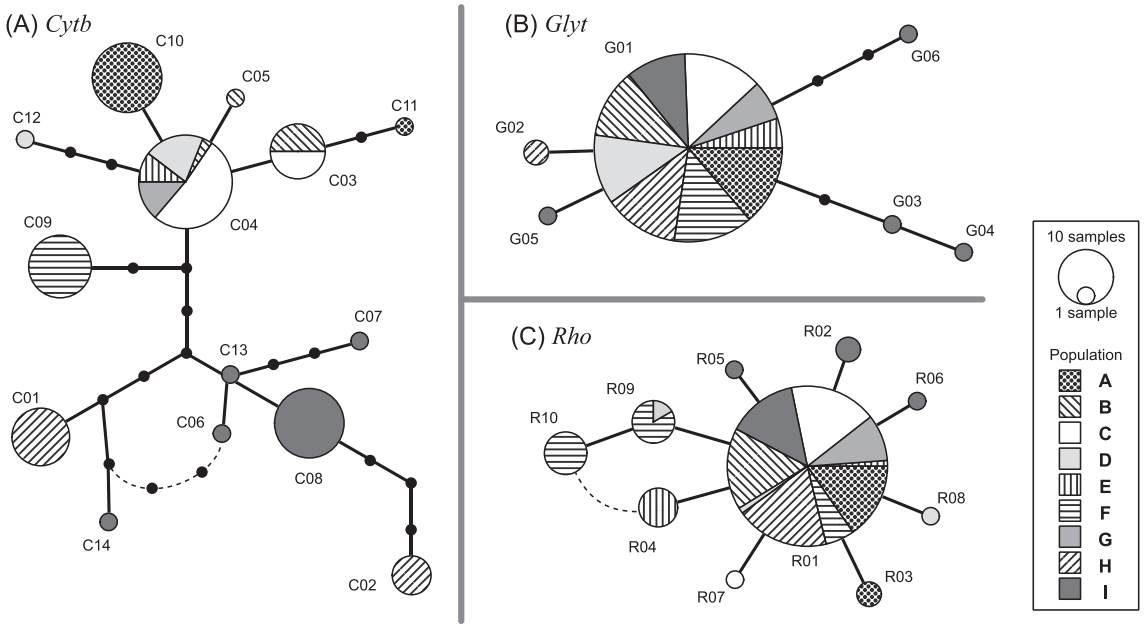

Fig. 2. Statistical parsimony networks based on *Cytb* **(A)**, *Glyt* **(B)**, and *Rho* **(C)**. Different patterns on the pie chart represent distinct populations, and circle size correlates with sample size. Small black circles indicate inferred missing haplotypes not observed in the data set.

Table 2. Genetic diversity indices of each population.

Population ID	*Cytb*		*Glyt*		*Rho*	
	h	π (%)	*h*	π (%)	*h*	π (%)
A	0.12 ± 0.10	0.042 ± 0.043	0	0	0.34 ± 0.14	0.045 ± 0.052
B	0.52 ± 0.21	0.068 ± 0.064	0	0	0	0
C	0.39 ± 0.10	0.035 ± 0.038	0	0	0.13 ± 0.11	0.016 ± 0.029
D	0.26 ± 0.20	0.076 ± 0.069	0	0	1.00 ± 0.50	0.12 ± 0.18
E	0	0	0	0	0.33 ± 0.22	0.042 ± 0.056
F	0	0	0	0	0.71 ± 0.05	0.12 ± 0.10
G	0	0	0	0	0	0
H	0.46 ± 0.10	0.28 ± 0.17	0.23 ± 0.13	0.029 ± 0.040	0	0
I	0.37 ± 0.14	0.11 ± 0.080	0.45 ± 0.15	0.14 ± 0.11	0.44 ± 0.15	0.061 ± 0.062

h haplotype diversity, π nucleotide diversity

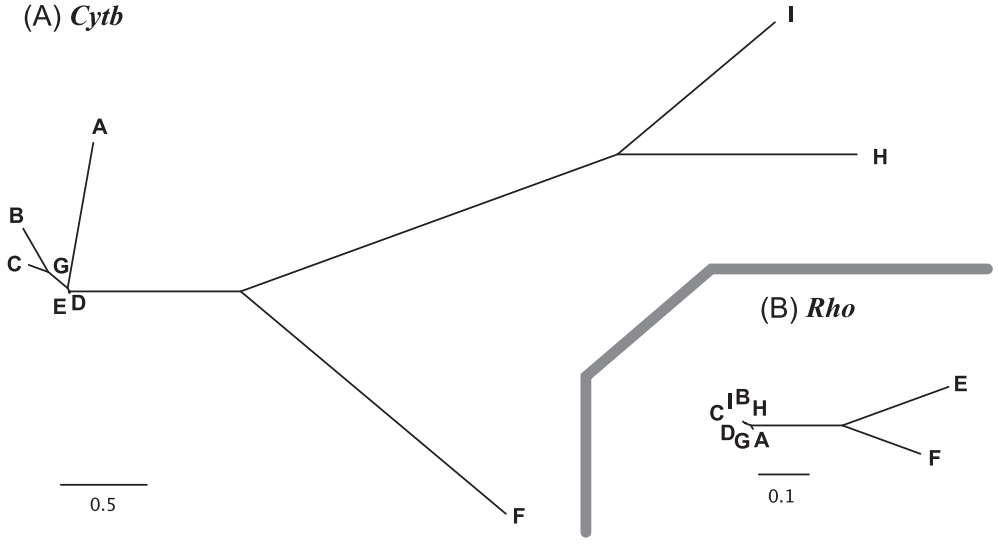

Fig. 3. Population-level phylogenetic trees depicting the genetic relationships among populations A–I based on *Cytb* **(A)** and *Rho* **(B)**.

structure and was excluded from subsequent analyses.

The *Cytb*-based phylogenetic tree revealed a large differentiation between Kanto and Tohoku populations; within-region differentiation was smaller in Tohoku than in Kanto (Fig. 3A). The *Rho*-based phylogenetic tree showed that populations F and E from southern Tohoku were highly differentiated from others, although overall differentiation was very low (Fig. 3B). Analysis using Geneland detected four genetic clusters for *Cytb* and two for *Rho*. Populations F, H, and I (all south of the distribution area) clustered differently based on *Cytb*, while the remaining populations belonged to one cluster (Fig. 1B). In contrast, populations E and F formed one cluster based on *Rho*; despite geographical division, the remaining populations all belonged to the second cluster (Fig. 1C).

DISCUSSION

In this study, we expected that *A. melanogaster* populations would reveal low genetic differentiation across the river systems along the Pacific side of eastern Honshu. Although we observed genetic differentiation and structure in *Cytb* sequences, the maximum distance between haplotypes was relatively small (1.07%). In addition, the two tested nuclear genes exhibited limited geographic divergence. These results are consistent with the low genetic differentiation of other fishes, which is also compatible with the lack of significant morphological differences among local *A. melanogaster* populations.

Nuclear and mitochondrial genes did not reveal consistent geographic divisions among tested populations; analysis with the nuclear gene found only two divisions, compared with four for the mitochondrial gene. This difference can probably be attributed to a slower evolutionary rate in the selected nuclear gene.

Acheilognathus melanogaster is unlikely to migrate between river systems that are not directly connected, so interpopulation gene flow is restricted by geographical distance. However, *Cytb*-based genetic differentiation was not always proportional to distance between river systems. For example, Naka River (containing population H), Kuji River (population G), and Kinu River (Lake Kasumigaura: population I) estuaries are close to each other, but exhibited large differences in genetic differentiation (Fig. 2A). Additionally, populations A–F are widely separated across several river systems in Tohoku, but showed little genetic differentiation (Figs. 1B and 2A). The Kitakami River (containing population B and C) and Abukuma River (populations D and E) systems flow down into Sendai Bay. Thus, when sea levels were lower during the last glacial period, gene flow between the populations of these river systems may have occurred downstream, through their confluence or flood plains. Similarly, *A. typus*, a species found throughout eastern Honshu, shows low genetic differentiation in areas where its distribution overlaps with *A. melanogaster* (Saito et al., 2016). However, gene flow during the glacial period is only one possible explanation for the observed patterns; other geographic factors, such as river capture, may have contributed to low genetic differentiation.

Despite being geographically close, population F belonged to a different *Cytb* genetic cluster from population E. However, population F was part of the same *Rho*-based genetic cluster as population E (Fig. 1B and C). These results suggest that the two populations may have once

been genetically close, but with the large genetic differentiation of *Cytb* resulting from a founder or bottleneck effect. The latter seems likely, as the Ukedo River system (containing F) flows directly to the Pacific Ocean and is isolated from other rivers. Unfortunately, the 2011 Great East Japan Earthquake and related Fukushima Daiichi nuclear disaster has drastically altered the environment of the Ukedo River. Therefore, *A. melanogaster* conservation in this system depends on further investigation of population F.

We observed *Cytb*-based genetic differentiation between Kanto populations H (Naka River) and I (Kinu River), consistent with genetic differentiation in *Tanakia tanago* populations across the same two rivers (Kubota et al., 2010; Saito et al., 2017). Overall, *Cytb* was differentiated in *A. melanogaster* across multiple regions; all populations exhibited low haplotype diversity, but populations H and I had relatively high nucleotide diversity. Overall genetic diversity of both nuclear genes was also low, but H and I possessed multiple haplotypes. These results suggest that Kanto populations had relatively high genetic diversity and endemicity. However, *A. melanogaster* in the southern four Kanto prefectures became locally extinct due to recent habitat degradation. For example, *A. melanogaster* abundance in Kanto region is highest in Lake Kasumigaura, where exotic predators (e.g., *Micropterus salmoides*, *Lepomis macrochirus*, and American channel catfish, *Ictalurus punctatus*) and alien bitterling competitors for hosts (e.g., *Rhodeus ocellatus ocellatus* and *A. macropterus*) are both increasing in number. These invasive species are major threats to *A. melanogaster* (Morosawa and Fujioka, 2007). In conclusion, to maintain this species' genetic diversity, we must pay particular attention to conserving local populations in the Kanto region.

Although the nuclear genes analyzed in this study were polymorphic, they may not be suitable for detailed exploration of *A. melanogaster* genetic differentiation because they exhibit low substitution number. Additionally, there is controversy regarding the effectiveness of mitochondrial DNA for such research (Bazin et al., 2006; McCusker and Bentzen, 2010; Kazancıoğlu and Arnqvist, 2014). Therefore, future research may need to examine *A. melanogaster* genetic structure using microsatellites or SNPs obtained from RAD-seq (Baird et al., 2008) and MIG-seq (Suyama and Matsuki, 2015).

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

NN and JK designed this study. JK, OI, MK and YF collected the materials. NN performed molecular experiments and analysis, and wrote manuscript, with contribute from TS. All authors contributed to the editing of the manuscript.

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