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Zoological Research

Isolation of Microsatellite DNA and Preliminary Genomic Analysis of Mud Carp（*Cirrhina molitorella*）

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Abstract：In order to determine the applicability of microsatellite primers developed from common carp（*Cyprinus-cario*）for genomic analysis in mud carp（*Cirrhina molitorella*），24 primer pairs from common carp were designed to amplify microsatellite loci in the mud carp containing CA，GA，AT and GGGA sequences．Thirteen primers（54%）successfully amplified specific products in the mud carp and 11 primers（48%）showed high polymorphism in the mud carp population．The results indicated that the average number of alleles per locus in the mud carp stocks was 5.2．Average heterozygosity（*Ho*），unbiased expected heterozygosity（*He*）and polymorphism information content（*PIC*）in the wild population were 0.61 ± 0.2，0.8 ± 0.09 and 0.72 ± 0.1 respectively．Several Hardy-Weinberg departure value were significantly departed from Hardy-Weinberg equilibrium．The study showed that microsatellite primers from a species of *Cyprinidae* can be used for mud carp genetic analysis without much cost or time input．

Key words：Mud carp（*Cirrhina molitorella*）；Microsatellite；Screening；Genomic polymorphism

鲮鱼的微卫星位点筛选和群体遗传多样性初步分析

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摘要：利用鲤科鱼类微卫星引物在鲮鱼中进行扩增，结果在 24 对引物中，13 对引物能成功扩增，且在鲮鱼中的扩增产物表现稳定，其中 11 对有较高多态性，等位基因数在 2—7 个之间，扩增的条带符合孟德尔遗传规律。随后利用筛选的微卫星座位对鲮鱼野生和养殖群体遗传多样性进行了初步分析。分析结果显示：鲮鱼野生群体的平均等位基因数 5.2 个；观测杂合度在 0.25 与 0.8 之间，平均观测杂合度（*Ho*）是 0.61 ± 0.2，平均期望杂合度（*He*）是 0.8 ± 0.09；群体座位平均多态信息含量（*PIC*）为 0.72 ± 0.1。相比之下，养殖群体的平均观测杂合度（*Ho*）和平均期望杂合度（*He*）都低于野生群体，分别是 0.59 ± 0.2、0.75 ± 0.1。两群体间的遗传相似度为 0.7774、遗传距离为 0.2518。研究表明：用其他鱼类分离出的微卫星引物可以快速筛选到适用于鲮鱼遗传分析的微卫星座位。

关键词：鲮鱼；微卫星；筛选；遗传多样性
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Cirrhina molitorella（Cypriniformes，Cyprinidae，Labeoninae，Labeo）is a fish species unique to southern China，which is mainly distributed in the Zhujiang River system，Hainan，and the Yuanjiang and Lancang River systems of Yunnan（Zheng et al，2005）．Few studies on *C．molitorella* have been reported，most focusing on aquaculture and basic biology，and very few on molecular biology of the species using techniques such as RAPD and DAF analysis．Recently，the population of *C．molitorella* has diminished due to over fishing and artificial releases have been increasing to supplement fish populations．This will lead to a decrease of

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genetic diversity of the original *C. molitorella* population and genetic erosion from other fish population. In order to provide more information and data for the protection and sustainable utilization of the *C. molitorella* genome, the microsatellite markers of *C. molitorella* were investigated.

Microsatellites are also called simple sequence repeats (SSR), being a simple repeat sequence consisting of one to six bases, distributed in the complete DNA of an organism. Microsatellites have many attributes making them excellent for scientific studies, such as abundant polymorphisms, co-dominant heredity and easy detection. Microsatellite markers are excellent for estimating the genetic diversify of a species. The isolation of microsatellite loci and design of relevant primers is the first step in the application of the marker. Much work needs to be done to construct a genomic DNA library in the traditional way of isolating microsatellite loci, restricting the application and development of the microsatellite marker technique for many populations. Since DNA sequences are highly conserved between closely related species, it can be effective to analyze the microsatellites of a fish species using microsatellite primers from other fish species (Lin et al, 2003). In this paper, the microsatellite primers of fishes of Cyprinidae were used to amplify the DNA of *C. molitorella*. Abundant polymorphic microsatellites were selected to analyze the population genetic structure of *C. molitorella* and provide reference for further genetic study of this species.

1 Materials and Methods

1.1 Materials and extraction of genomic DNA

Twenty fish samples were collected from a wild *Cirrhina molitorella* population from West River and were housed at the Guangdong *C. molitorella* breeding farm. Another 20 samples were collected from a fish farm in Panyu, Guangdong. Blood samples were extracted and stored in ACD (acid-citrate-dextrose) solution at −20℃. After extraction with TIANGEN, DNA was suspended to be used as the template DNA in polymerase chain reactions (PCR).

1.2 Origination and selection of primers

Twenty-two primer pairs (MFW-XX, HLJ-XX) originally developed for common carp and two (Koi-XX) developed for color carp were used to amplify the genomic DNA of *C. molitorella* (Lin et al, 2003; Liu et al, 2002; Lu et al, 2005). Microsatellite primers were selected according to their bands on electrophoretic patterns. Primers should amplify clear and polymorphic bands and follow Mendelian segregation patterns of gene inheritance for the microsatellite loci (Lin et al, 2003).

1.3 Reaction conditions of PCR

20 μL PCR amplification contained 2.0 μL 10 × PCR Buffer, 0.2 μL dNTP Mix (10 mmol/L each dATP, dCTP, dGTP, dTTP), 1.0 U Taq DNA polymerase, 50 ng of each template DNA and 0.5 mmol/L of each primer. PCR amplifications were performed with the following cycle parameters: initial denaturation (95℃ for 5 min); 35 cycles of denaturation (94℃ for 30 s), annealing (55℃ for 30 s); extension (72℃ for 45 s); final extension (72℃ for 7 min). Some parameters, such as annealing temperature and Mg^{2+} concentration, were adjusted according to the amplified products. Each sample was mixed with the loading buffers (5∶1), and 8 μL of the mixture were electrophoresed in 8% non-denaturing polyacrylamide gel with 1 × TBE buffers and 140 Volts for approximately 5 hours. Samples were then visualized on ultraviolet gel documentation systems after being tested using silver staining (Sanguinetti et al, 1994).

1.4 Statistical analysis

The bands of electrophoretic patterns were analyzed using ImageMasterID Elite (Version 3.01) software and artificial adjustment. The number of alleles (α), effective number of alleles (α_e), average heterozygosity (Ho), unbiased expected heterozygosity (He) (Nei, 1978), Hardy-Weinberg departure value and population heterozygosity (H) were used to study the genetic variations within the population. Inter-population genetic variations were estimated using the genetic similarity coefficient (I) and genetic distance. The polymorphism information content (PIC) of every locus was calculated based on the data from the wild population to analyze the applied value of the selected microsatellite markers (Wang et al, 2004; Quan et al, 2006).

2 Results and Analysis

2.1 Results of 13 microsatellite primers tested in *Cirrhina molitorella*

Twenty-four pairs of microsatellite primers of Cyprinidae fishes were used to amplify the DNA of *Cirrhina molitorella*. Thirteen primer pairs (54%) produced clear and stable bands from the fish after opti-

mizing the PCR conditions, 11 (46%) were polymorphic and two (MFW20 and Koi3) were monomorphic. Excluding the two monomorphic genes, MFW20 and Koi3, there were 2 – 7 genes in the 11 primer pairs, with an average of 4.4. The size of the excerpts was between 118 bp (MFW20) and 217 bp (MFW19), of which MFW7, MFW20, MFW26 and Koi3 were different from that of Cyprinidae. Koi3 differed from Cyprinidae by more than 300 bp (466 – 500 bp in Cyprinidae and 160 bp in *C. molitorella*). MFW7 in Cyprinidae was over 206 bp, but in *C. molitorella* ranged from 145 bp to 165 bp. MFW20 in Cyprinidae (205 bp) is longer than in *C. molitorella* (118 bp). Similarly, MFW26 was 209 bp in Cyprinidae and 125 – 170 bp alleles in *C. molitorella*. A description of the 13 microsatellite primers is given in Tab 1.

2.2 Results of microsatellite loci in *Cirrhina molitorella* population

Eleven of the 24 primer pairs were polymorphic. All others were abandoned as they were unable to produce specific, clear, stable or polymorphic bands. For instance, MFW20 and Koi3 only produced one allele (Tab. 3). The total number of alleles from the 11 primer pairs in *C. molitorella* was 48, ranging from 2 (MFW4, MFW5, Koi20) to 7 (MFW1). The average number of alleles each locus was 4.4.

2.3 Genetic diversity analysis of *Cirrhina molitorella* population

According to the protocol in range determination of farm animal intervarietal heredity globally, a microsatellite locus used to estimate genetic diversity should contain at least four alleles (Wang et al, 2005). Eight pairs of highly polymorphic primers were used to analyze the genetic characteristics of *C. molitorella* (Tab. 2 – Tab. 4).

The results show that the number of alleles per locus was between 3 (HLJ011) and 7 (MFW1), the average was 5.2 in each locus. Microsatellite polymorphism can provide information on the evolution of a species as alleles with high frequencies are the most conservative and primitive in a population, while other multiple alleles can form during the course of evolution. Therefore the allele with the highest frequency of the eight loci might be the most primitive allele in *C. molitorella*.

Tab. 1　The amplification conditions and results of 13 microsatellite primers in *Cirrhina molitorella*

Locus	Primers sequence	Repeats motif	Tm (℃)	Size range (bp)	No. of alleles	Mg^{2+} (mmol/L)
MFW1	F GTC CAG ACT GTC ATC AGG AG GAG GTG TAC ACT GAG TCA CGC	CA	62	180–210	7	1.0
MFW2	F CAC ACC GGC TAC TGC AGA G GTG CAG TGC AGG CAG TTT GC	CA	54	170–200	5	1.5
MFW4	F TCC AAG TCA GTT TAA TCA CCG GGG AAG CGT TGA CAA CAA GC	CA	56	144–146	2	1.2
MFW5	F GAG ATG CCT GGG GAA GTC AC AAA GAG AGC GGG GTA AAG GAG	CA	62	175–178	2	1.0
MFW7	F TCC AAG TCA GTT TAA TCA CCG GGG AAG CGT TGA CAA CAA GC	CA	60	145–165	6	1.2
MFW15	F CTC CTG TTT TGT TTT GTG AAA GTT CAC AAG GTC ATT TCC AGC	GA	48	162–190	6	1.2
MFW19	F GAA TCC TCC ATC ATG CAA AC GCA CAA ACT CCA CAT TGT GCC	CA	42	190–217	4	1.7
MFW20	F CAG TGA GAC GAT TAC CTT GG GTG AGC CAG CCC ACA TTG AAG	CA	42	118	1	1.5
MFW26	F CCC TGA GAT AGA AAC CAC TG CAC CAT GCT TGG ATG CAA AAG	CA	53	125–170	6	1.0
Koi3	F GTT TTC TGT TGT AGG CTC TG TAC TTC ATC TCT CGC ACT CA	GA	45	160	1	1.5
Koi20	F TGC CCT CTC TTT CCT TCA CAG GCT TCA ACA CAA ACA CA	GGGA	48	160–162	2	1.5
HLJ011	F TTA GCC AGC CAG AGAC AAG C CAC TGC CAC AAA CCC ATC TA	CA	50	175–217	3	1.5
HLJ017	F TGT CCG AGT GTT TTT GTC ATT C TGA CAA CAC ATT TGC CTC AA	AT	49	201–210	5	1.5

Primer sequence and repeat motifs refer to Lin et al (2003), Liao et al (2005, 2002) and Lu et al (2005).

Tab. 2 Allele frequencies of eight microsatellite loci in wild and cultured *Cirrhina molitorella* populations

Locus	Allele	Allele frequency of wild population	Allele frequency of cultured population	Locus	Allele	Allele frequency of wild population	Allele frequency of cultured population
MFW1	1	0.1111	0.0303	MFW15	1	0.0833	0.1034
	2	0.1111	0.1515		2	0.2499	0.2067
	3	0.0556	0.2727		3	0.2499	0.1724
	4	0.3333	0.1818		4	0.2499	0.1724
	5	0.0556	0.2121		5	0.0833	0.3103
	6	0.2778	0.1212		6	0.0833	0.0345
	7	0.0556	0.0303	MFW19	1	0.1429	0.0571
MFW2	1	0.1429	0.1200		2	0.2857	0.3714
	2	0.1429	0.1600		3	0.1142	0.2000
	3	0.2143	0.2800		4	0.2857	0.2286
	4	0.2858	0.3200		5	0.0571	0.1429
	5	0.2143	0.1200	MFW26	1	0.1667	0.1290
MFW7	1	0.0606	0.0606		2	0.1111	0.2258
	2	0.0606	0.0303		3	0.1667	0.1613
	3	0.303	0.2727		4	0.2778	0.3226
	4	0.2424	0.1212		5	0.1667	0.1290
	5	0.1818	0.0303		6	0.1111	0.0323
	6	0.1515	0.4848	HLJ017	1	0.1714	0.0513
HLJ011	1	0.2000	0.6897		2	0.2857	0.0769
	2	0.6333	0.2759		3	0.2000	0.5128
	3	0.1667	0.0345		4	0.1143	0.1538
					5	0.2286	0.2051

Tab. 3 Characteristics of eight microsatellite loci assessed for wild stocks of *Cirrhina molitorella*

Locus	Number of effective alleles of wild population (α_e)	Observed heterozygosity of wild population (Ho)	Unbiased expected heterozygosity of wild population (He)	D value of wild population (d)	The value of polymorphism information content of wild population (PIC)
MFW1	4.5	0.8	0.8187	−0.0228	0.7476
MFW2	4.7	0.35	0.8270	−0.5768	0.7585
MFW7	4.7	0.65	0.8275	−0.2145	0.7539
MFW15	4.8	0.25	0.8335	−0.7001	0.7603
MFW19	5.0	0.75	0.8421	−0.1094	0.7740
MFW26	5.4	0.8	0.8577	−0.0673	0.7333
HLJ011	2.1	0.5	0.5591	−0.1057	0.4745
HLJ017	4.6	0.75	0.8249	−0.0908	0.7489

Heterozygosity (H), or gene diversity, can highlight the genetic variations of many loci in a population. It is thus considered to be a suitable parameter to estimate the genetic variation of a population. The expected values of average heterozygosity were calculated using the formula of non-biased heterozygosity reported by Nei (1978) for small samples. The observed heterozygosity (Ho) of the wild *C. molitorella* population was between 0.25 and 0.8. The average Ho and average expected heterozygosity (He) were 0.61 ± 0.2 and 0.8 ± 0.09 respectively for the wild population, but were lower in the cultured population (0.59 ± 0.2 and 0.75 ± 0.1 respectively). This indicates medium genetic diversity in the wild *C. molitorella* population and a relatively lower diversity in the cultured population.

The polymorphic information content (PIC) is an index for analysis of the polymorphism of an amplified product. According to the protocol of Botstein (1980), $PIC > 0.5$ indicates highly polymorphic loci, $0.25 < PIC < 0.5$ indicates middling polymorphic loci and $PIC < 0.25$ indicates low polymorphism. The PIC of the eight loci in the wild population of *C. molitorella* ranged from 0.4745 to 0.774, and the average PIC was 0.72 ± 0.1. The PIC was over 0.7 in all cases, except with HLJ011 ($PIC < 0.5$), meaning that seven of the loci were highly polymorphic and could be used to calculate the genetic diversity of the *C. molitorella* population.

The Hardy-Weinberg departure value (D) is a fixed index, describing the departure of a locus in a population from Hardy Weinberg equilibrium. $D > 0$ means that there is heterozygote deficiency at a given locus, whereas $D < 0$ indicates heterozygote excess. All eight of the loci in the wild *C. molitorella* population

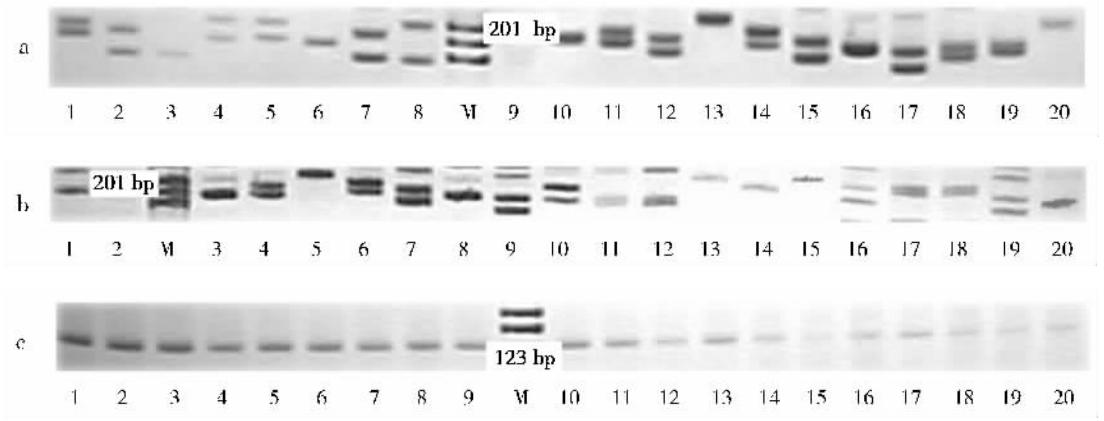

Fig. 1　Electrophoretic patterns of PCR products of *Cirrhina molitorella*

a：From wild population amplified by MFW1；b：From a cultured population amplified by MFW1；

c：From a cultured population amplified by MFW20.

M：pBR322 DNA/*Msp* I molecular weight marker，1−20：20 individuals of wild *Cirrhina molitorella* population.

Tab. 4　Characteristics of eight microsatellite loci assessed for cultured stocks of *Cirrhina molitorella*

Locus	Number of effective alleles of cultured population (α_e)	Observed heterozygosity of cultured population (H_O)	Unbiased expected heterozygosity of cultured population (H_e)	D value of cultured population (d)
MFW1	5.2	0.65	0.8507	−0.2359
MFW2	4.3	0.25	0.8051	−0.6895
MFW7	3.0	0.65	0.7057	−0.0789
MFW15	4.8	0.45	0.8312	−0.4586
MFW19	3.9	0.75	0.7854	−0.0451
MFW26	4.6	0.55	0.8258	−0.3398
HLJ011	1.8	0.45	0.4705	−0.0436
HLJ017	3.0	0.95	0.6977	0.3616

had excess heterozygosity，with some of them departing from Hardy Weinberg equilibrium.

The genetic similarity coefficient and genetic distance based on allele frequencies between the two populations were 0.7774 and 0.2518 respectively，indicating a slight heredity differentiation.

3　Discussion

3.1　Selection of microsatellite loci in *Cirhinna molitorella*

Much work needs to be done to construct a genomic DNA library in the traditional way of isolating microsatellite loci，restricting the application and development of the microsatellite marker technique for wild populations. Since single-copy DNA sequences which pair with microsatellites are highly conservative between closely related species，analyzing the microsatellite of a fish using microsatellite primers from other related fishes is effective.

Sun & Liang (2001) analyzed the genetic heterogeneity of two local carp populations (*Cyprinus carpio haematopterus* Temminck et Schlege and *Cyprinus pellegrini* Tchang) with SSLP makers from the zebra fish. Lin and Luo (2003) found that seven of 28 pairs of microsatellite primers which originated from common carp produced relevant specific bands in the DNA of Xiangjiang grass carp. Liao et al (2005) also found six highly polymorphic microsatellite loci in grass carp with 45 primer pairs from the common carp. Zhu et al (1999) tested the applicability of four pairs of microsatellite primers from *Acipenser fulvescens* on *Acipenser sinensis* Gray，two of which generated stable and polymorphic bands. Shao et al (2002) reported that 14 of 21 microsatellite primers from *Scaphirhynchus platorynchus* could be used in *Acipenser sinensis* Gray and 10 primer pairs were highly polymorphic. All of these studies have shown that using related species to provide microsatellites is an effective way to analyze the microsatellite of rarer fish species. Eleven of 24 primer pairs from Cyprinidae were identified to analyze the microsatellite in *C. molitorella* in this paper；the ratio of the successful primers was similar to that of Zhu et al

(1999) and Shao et al (2002) but higher than that of Lin & Luo (2003) and Liao et al (2005).

Although the primers of Cyprinidae successfully amplified in *C. molitorella*, the size and number of alleles were significantly different. Koi3 identified four alleles (466 – 500 bp) in the common carp but only identified a single allele with 160 bp in *C. molitorella*. MFW7, MFW20 and MFW26 also produced different bands in *C. molitorella*. This means that it is necessary to screen the products and design primers for further study of the species, although it saves time and energy to use an applicable microsatellite primer from another species for the unique conservative microsatellite flanking sequences and the variable repeated sequences. MFW1 produces polymorphic bands in many fishes such as carp, silver carp, *Paramisgurnus dabryanus*, *Labeo rohita* and *C. molitorella* in this research (seven genes in total), indicating that MFW1 is a relatively primitive locus with conservative microsatellite flanking sequences and less selective pressure on its repeated sequences. Liao et al (2005) proposed that duplication events have happened to MFW1 during the course of evolution of the fishes.

3.2 Genetic analysis of *Cirrhina molitorella* population

The values of observed heterozygosity (Ho) of the wild *C. molitorella* population were between 0.25 and 0.8, the average Ho and average expected heterozygosity (He) were 0.61 ± 0.2 and 0.8 ± 0.09 respectively, but were lower in the cultured population (0.59 ± 0.2 and 0.75 ± 0.1 respectively). Similar results have been obtained in other studies. Hedgecock & Sly (1990) reported that a wild pacific oyster population had more alleles on a certain locus than a cultured population, using electrophoresis technology of isoenzymes. Li et al (2004) compared the genetic diversity of three cultured abalone populations and two wild abalone populations with microsatellite markers and found that the number of alleles in the cultured populations was 76% less than that in the wild populations. The average expected heterozygosity was also remarkably low in the cultured populations. Li (2004) proposed that a low number of breeding adults in the cultured populations caused the decrease of genetic diversity and intensified genetic drift in the populations. However Skaala et al (2004) found that 42% of alleles were lost in cultured Atlantic salmon populations and there was a lower difference in heterozygosity, and therefore reported that the founder effect (Quan et al, 2005) was the main reason for the loss of alleles in the cultured populations. The reasons for lower average heterozygosity and no allele loss in the cultured *C. molitorella* population in this research needs to be study further. Both the average observed heterozygosity (Ho) and average expected heterozygosity (He) were over 0.5 and departed from the standard of panmictic population heterozygosity of Hardy Weinberg equilibrium law. Quan et al (2005) proposed that genotype depletion from environmental pressures, human behavior or sampling errors caused this departure. Xie et al (1999) believed that small sampling variance for assessing population changes at DNA level when the sample size $n \geqslant 10$ caused similar results, according to the DNA sequence sampling theory reported by Tajima (1983). It was less possible in this research for sampling error lead to the reported departure from equilibrium, as the sample size was 20.

The statistical data showed medium genetic diversity in the wild *C. molitorella* population and a bottleneck effect and inbreeding depression in the cultured population, enhancing the germplasm homogenization and decreasing the genetic diversity of the two populations (genetic distance between populations : 0.2518). Zheng et al (2005) reported low genetic diversity in a wild *C. molitorella* population with 98 information points and 35.7% polymorphism fragment ratio of 23 paired microsatellite primers using RAPD and DAF techniques. Zhu et al (2005) studied mud carp in the Xijiang branch of the Pearl River using RAPD and reported a high genetic diversity with a higher average heterozygosity at 0.1281.

The results indicate a medium genetic diversity level in the wild *C. molitorella* population and a relatively lower one in the cultured population, which differs from formerly reported studies. This may firstly be due to different sampling locations. Zheng et al (2005) collected their samples from different sites of the Zhujiang River with none being cultured in farm ponds. The wild *C. molitorella* population analyzed in this research had been cultured in farm ponds for approximately three years. Secondly, microsatellite DNA and RAPD are different genetic markers. Microsatellite markers are a stable and reliable research technique, which is why it was used in the present study. However RAPD, which Zheng et al (2005) and Zhu et al (2005) used, does not produce stable bands when the

lab conditions are changed. In addition, the standards for the numbers, length, sequence of the primers used in RAPD are not certain (Zheng et al, 2005). Therefore it is difficult to compare the results of RAPD method.

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