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Genetic characterization of far eastern species of the genus *Crepidostomum* (Trematoda: Allocreadiidae) by means of 28S ribosomal DNA sequences

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ABSTRACT

Genetic divergence and phylogenetic relationships of four species of the genus *Crepidostomum* Braun, 1900 sensu Caira, Bogĕa (2005) were revealed using partial sequences of 28S ribosomal RNA gene. Genetic divergence between *C. cf. farionis* (Muller, 1784) and *C. nemachilus* Krotov, 1959 was 3.1%, which corresponds to the mean value of interspecific divergence between *Crepidostomum* species. These two species, therefore, can be recognized as bonafide species. However, we found no genetic differences between 28S rRNA gene sequences of *C. nemachilus* and *C. cf. metoecus* Braun, 1900 in spite of considerable morphological and ecological differences. Maximal values of genetic *p*-distances were revealed between *C. cf. auriculatum* Wedl, 1857 and *C. cf. farionis*. Phylogenetic relationships of *Crepidostomum* spp. for which sequence data are available, along with species in other related genera (*Bunodera* Railliet, 1896 and *Allocreadium* Loss, 1900) showed a paraphyly of the genus *Crepidostomum*. Considerable differentiation of *C. cf. auriculatum* from other *Crepidostomum* species was revealed, which may reflect the original description of this species in a separate genus *Acrolichanus* Ward, 1917. Our results are consistent with the conventional systematics that places the four genera (*Crepidostomum*, *Bunodera*, *Megalogonia* and *Allocreadium*) within the same family.

KEYWORDS

DNA; Sequencing; *Crepidostomum*; *Acrolichanus*; Trematoda; Digenea; Phylognetic Relationships

1. INTRODUCTION

Species of the Allocreadiidae are important components of the parasite fauna of the freshwater fishes and their systematics have not been clarified yet. The membership of these trematodes to either family Allocreadiidae Stossich, 1903 or family Bunoderidae Nicoll, 1914 has been discussed in the literature [1-5]. However, molecular phylogenetic analyses that have included these trematodes have recovered them in a monophyletic Allocreadiidae [6-8]. The genus *Crepidostomum* sensu Caira, Bogĕa, 2005 is one of the most taxonomically confused groups of trematodes of the family Allocreadiidae, which contains 40 nominal and 24 valid species. The life cycle of *Crepidostomum* spp. involves freshwater bivalves as first intermediate hosts, freshwater arthropods as second intermediate hosts, and freshwater fishes from a variety of families as definitive hosts. The most widely distributed species of this genus are *C. auriculatum* Wedl, 1857, *C. farionis* (Muller, 1784) and *C. metoecus* Braun, 1900. It is not surprising that these three species have been known by numerous synonyms. For example, a trematode of salmons was reported as *C. ussuriensis* by Layman (1930) [9] for south territory of Russian Far East from *Salvelinus* sp. Later this species was recognized as synonym of *C. farionis* by Skrjabin and Koval (1966). Another established species for Russian Far East—*C. nemachilus*, described by Krotov (1959) [10] from *Barbatula toni*, was recognized as synonymous taxon of *C. ussuriensis* [1]. After that the taxonomical status of this species was not discussed in the Russian literature. Shi-

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mazu (1990) touched upon issue of taxonomic status of *C. nemachilus* and considered this species as synonymous with *C. farionis* [11]. The problem of validity of *C. nemachilus* still needs to be resolved.

The taxonomy of *Crepidostomum auriculatum*, an intestinal flatworm of sturgeons, has a confusing history, and was reviewed by Choudhury (1997) [12]. It was first described as *Distoma auriculatum* by Wedl from the sterlet *Acipenser ruthenus* from Austria (River Danube). This species was first reported in North America by Linton in 1897 under the same name [13]. Pratt was the first who used the name "*Crepidostomum auriculatum*" for description of this species [12]. The first detailed description of *C. cf. auriculatum*, based on whole mounts and histological sections, was given by Skwortzoff (1927, 1928) from sterlet *Acipenser ruthenus* from Volga River [14,15]. Taxonomical study of Hopkins (1933) resulted in the recognition of all North American forms from sturgeons as *C. lintoni* (Pratt in Linton, 1901) and recognition of *C. auriculatum* from sterlet as separate species [16]. Later, these two species were recognized as synonymous by many researchers [1,3,17-19]. *Crepisodostomum auriculatum* was also considered by Hopkins (1934) as synonymous with *Acrolichanus similis*, described by Wisniewski in 1933 from *Salmo trutta* and *Oncorhynchus mykiss* [20]. But further taxonomical studies showed that *A. similis* and *C. auriculatum* are separate species [3,12]. According to Shul'man (1954) and Choudhury (1997), there are trends in a few differences between European and North American *C. auriculatum* specimens in spite of agreement in all essential details [12,19]. Thus, placing *C. auriculatum* in either *Crepidostomum* or *Acrolichanus* is still a topical problem, as the identity of specimens of this species from different locations North America, Europe and Northern Asia, including Russian Far East. The aim of our study is to clarify the taxonomy of far eastern *Crepidostomum* species by genetic characterization using patrial sequences of the 28S rRNA gene.

2. MATERIALS AND METHODS

Trematodes *C. cf. auriculatum*, *C. cf. farionis*, *C. cf. metoecus* and *C. nemachilus* were obtained during parasitological field work in 2008-2010 from south of Russian Far East (water bodies of Primorsky Region and Amur River near Nikolaevsk-na-Amure city). Morphology of 1000 specimens (alive and mounted in Canada balsam) of trematodes was examined and four species were revealed. For molecular analysis adults of each species, obtained from the intestine of definitive host species, were preliminary checked under slight pressure between two glass slides and then fixed in 96% ethanol. Species identification was performed according to different authors [1-3,10]. Additionally it was examined nine syntypes of *C. nemachilus* from muzeum of K. I. Skrjabin All-Russia Institute of Helminthology, Moscow, Russia (№ 7506 and 7507). List of species, incorporated into analysis, presented in **Table 1**. We used symbol "*cf.*" for the names of three species (*C. cf. auriculatum*, *C. cf. farionis*, *C. cf. metoecus*) because of these specimens weren't compared with the trematodes from type location in spite of preliminary identification of these species by morphology.

Table 1. List of the taxa incorporated in sequence analysis.

Species	n	Host	Author	EMBL reg. number
		Family. Allocreadiidae		
Crepidostomum cf. auriculatum	15	*Acipenser schrenkii*	This study	FR821371 - FR821385
Crepidostomum cf. auriculatum	3	*Acipenser schrenkii*	This study	FR821386 - FR821388
Crepidostomum cf. auriculatum	11	*Huso dauricus*	This study	FR821389 - FR821398
Crepidostomum cf. farionis	6	*Oncorhynchus masou*	This study	FR821399 - FR821404
Crepidostomum cf. metoecus	3	*Salvelinus leucomaensis*	This study	FR821405 - FR821407
Crepidostomum nemachilus	2	*Barbatula toni*	This study	FR821408 - FR821409
Crepidostomum cornutum	1	*Lepomis gulosus*	Curran *et al*., 2006	EF032695
Megalogonia ictaluri	1	*Ictalurus punctatus*	Curran *et al*., 2006	EF032694
Bunodera acerinae	1	*Gymnocephalus cernuus*	Petkeviciute *et al*., 2010	GU462122
Bunodera luciopercae	1	*Perca fluviatilis*	Petkeviciute *et al*., 2010	GU462124
Allocreadium lobatum	1	*Semotilus corporalis*	Curran *et al*., 2006	EF032693
Allocreadium isoporum	1	*Alburnus alburnus*	Petkeviciute *et al*., 2010	GU462126
		Family Apocreadiidae		
Callohelmis pichelinae	1	*Hemigymnus melapterus*	Bray *et al*., 2009	FJ788495

DNA extraction was performed from whole worms with silica technique as follows: 1) Trematode specimens were dried and homogenized in 3 M Guanidine thyocyanate. 2) Homogenate was incubated for 10 minutes at 57˚C. 3) Water suspension of silica (silicium dioxide) was added to homogenate (5 µl of 50% suspension per 2 µg of expected amount of DNA), mixed and incubated about 5 minutes at 57˚C. 4) Silica/Guanidine solution was centrifuged for 15 sec. at 10,000 rpm, supernatant was elucidated. 5) Sediment was washed using cold washing buffer (20 mM TRIS-HCl, pH = 7.4, 1 mM EDTA, 50 mM NaCl, 50% ethanol) for four times by adding of 500 µl of the washing buffer, mixing and centrifuging as in previous step. 6) Sediment was dried in air for about one hour and then was resuspended in 100 - 150 µl of nuclease free water, mixed and incubated for 10 minutes at 57˚C. 7) Water suspension was centrifuged for 2 minutes at 12,000 rpm, supernatant was transferred to the clean tubes and used for PCR.

The 28S rRNA gene was amplified using the polymerase chain reaction (PCR) with the following primers: DIGl2 (5'-AAG CAT ATC ACT AAG CGG-3') and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') [21]. Initial PCR reaction was carried out in a total volume of 20 µl. Each reaction contained 0.25 mM of each primer pair, combined with 1 µl of aqueous solution of DNA, 10x *Taq* buffer, 1.25 mM dNTP, 1.5 mM magnesium, and 1 unit of *Taq* polymerase. Amplification of a 1200-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) with 3 min denaturation hold at 94˚C; 40 cycles of 30 s at 94˚C, 30 s at 52˚C, 2 min at 72˚C and 7 min extension hold at 72˚C. PCR contamination control was performed by including negative controls alongside positive controls, using both primers. PCR products were initially directly sequenced using ABI Big Dye Terminator v.3.1 Cycle Sequencing kit (as instructed by manufacturer) and internal sequencing primers: 300F, ECD2, 900F, 1200R [21]. Reading of the cycle PCR products was performed with ABI 3130 genetic analyzer of Institute of Biology and Soil Sciences FEB RAS. Sequences of *Crepidostomum* spp. in this study were aligned with 28S sequence data of other species downloaded from NCBI GenBank database, representing following genera of the family Allocreadiidae: *Crepidostomum*, *Megalogonia*, *Allocreadium*, *Bunodera*. Also we used *Callohelmis pichelinae* as an outgroup [22] (**Table 1**).

DNA sequences were assembled with SeqScape v.2.6 software and aligned using MEGA 5.0 [23] alignment explorer with default options. Regions that could not be aligned unambiguously were excluded from the analyses. The number of variable, parsimony-informative sites, nucleotide composition and substitutions were obtained in MEGA 5.0. Frequencies of different substitution types and substitution rate were calculated using test of sub stitution pattern 4 by 4. Genetic divergence was estimated using genetic p-distance values, which were calculated by including all substitution types. Phylogenetic analysis of nucleotide sequences was undertaken, using all accessible methods, including maximum parsimony, maximum likelihood and Bayesian methods. All distance and parsimony trees were reconstructed with MEGA 5.0 and Bayesian inference was obtained using MrBayes v. 3.1.2 software [24]. The resulting networks were rooted with the outgroup taxa. Phylogenetic algorithms ML and BI performed using the GTR + G model (general-time reversible model with gamma distribution). This model showed the best fit to the data using Modeltest v. 3.07 software [25] using Akaike's information criterion [26]. Bayesian inference was employed using the following nucleotide substitution parameters: lset nucmodel = 4 by 4, nst = 6, rates = gamma. Mcmc algorithm was performed using 4,000,000 generations and 2 independent runs. Burn-in (sump and sumt) values were 80000. A nonparametric bootstrap with 1000 replicates was used to evaluate the robustness of the clusters through nodal support [27] for maximum parsimony and maximum likelihood algorithms. Nodal support for Bayesian algorithm was estimated by posterior probabilities [28]. Kishino-Hasegawa [29] and Shimodaira-Hasegawa [30] tests for phylogenetic trees were performed using PAUP 4b10 [31].

3. RESULTS

In the present study data on the genetic divergence and systematic of the far eastern species of the genus *Creidostomum* were obtained, using 28S rDNA partial sequences. Amplicons were about 1200 bp in length and the sequences, using for analysis after alignment were 1151 bp, including 70 variable and 66 parsimony-informative sites. There were no significant differences in nucleotide composition of 28S rDNA of the *Crepidostomum* species in comparison with the specimens from another genera and families of trematodes: A = 20.7%, T = 25.6%, C =21.7%, G = 32%.

The substitution pattern analysis revealed that significant differences of transition/transwersion rate ratio bias values (R) obtained by pairwise comparision of 28S rDNA sequences of different *Crepidostomum* species (**Table 2**). Minimal R values were obtained by comparision of *C. cf. auriculatum* and other *Crepidostomum* species (R = 2.26 - 2.74). Values of this parameter were 22.38 and 31.33 between *C. cf. farionis*/*C. cf. metoecus* and *C. cf. farionis*/*C. nemachilus*, respectively, indicating bias towards transitions between sequences.

Genetic divergence between far eastern *Crepidostomum* species ranged from 0 (*C. cf. metoecus*/*C. nema-*

chilus) to 3.8% (*C. cf. auriculatum*/*C. cf. farionis*) with a mean value 3.2% (**Table 3**). Divergence between 28S rDNA sequences between *C. cf. farionis* and *C. nemachilus* was 3.1% that close to the mean interspecific divergence between *Creidostomum* species. However, there are no divergence between 28S rDNA sequences of *C. nemachilus* and *C. cf. metoecus* in spite of considerable morphological differences. Genetic divergence between *C. cf. auriculatum* and *B. luciopercae* was 3.1% that corresponds with interspecific divergence level of *Crepidostomum* species. Maximal values of genetic divergence between far eastern *Crepidostomum* species covered with minimal values of intergeneric divergence (**Table 3**).

Phylogenetic analysis revealed six clusters with a high statistical support (**Figure 1**). First one was a basal and formed by the genus *Allocreadium*, and the second one included *C. cf. auriculatum*, a parasite of the sturgeons. Third cluster included the genus *Bunodera*, which was represented by two species: *B. luciopercae* (Muller, 1776) and *B. acerinae* Roitman and Sokolov, 1999, which were recognized early [32-34]. The fourth cluster included *C. cornutum* and *M. ictaluri*. Last two clusters corresponded with different *Crepidostomum* species: *C. nemachilus* and *C. cf. metoecus* gathered in a single cluster and *C. cf. farionis* formed a distinct cluster with a high statistical support, which was closely related to the (*C. nemachilus* + *C. cf. metoecus*) cluster. Genetic divergence between obtained clusters ranged from 3.3% (clusters IV/V and V/VI) to 6.0 (clusters II/VI). To test of significance of the tree topology, which showed paraphyly of the genus *Crepidostomum*, we compared this topology with tree constraining this genus as monophyletic using Kishino-Hasegawa and Shimodaira-Hasegawa tests (**Table 4**). Obtained results showed significant differences between unconstrained phylogenetic tree, showed a paraphyly of *Crepidostomum* and phylogenetic tree with constraint monophyly of *Crepidostomum* for three phylogenetic algorithms used.

Table 2. Transition/transversion ratio bias (R^*), obtained by pairwise comparision of 28S rDNA sequences of different *Crepidostomum* species.

		1	2	3	4
1	*C. cf. auriculatum*				
2	*C. cf. farionis*	2.31			
3	*C. cf. metoecus*	2.26	22.38		
4	*C. nemachilus*	2.74	31.33	228.51	

$^*R = [A \cdot G \cdot k_1 + T \cdot C \cdot k_2] / [(A+G) \cdot (T+C)]$, where k_1 и k_2 – frequences of transitions between purines and pyrimidines, respectively. *R* becomes 0.5 when there is no bias towards either transitional or transversional substitution because, when the two kinds of substitution are equally probable, there are twice as many possible transversions as transitions.

Table 3. Genetic divergence (%) between different species of the family Allocreadiidae using 28S rDNA partial sequences.

	1	2	3	4	5	6	7
1. *C. cf. auriculatum*							
2. *C. cf. farionis*	3.8						
3. *C. cf. metoecus*	3.3	3.2					
4. *C. nemachilus*	3.2	3.1	0				
5. *C. cornutum*	4.3	4.8	2.9	2.9			
6. *B. lucioperca*	3.1	5.2	4.0	4.0	4.3		
7. *A. isoporum*	4.1	5.8	5.1	5.1	5.5	5.6	

4. DISCUSSION

This is the first time that 28S sequence data have been used to elucidate the systematics of the far eastern *Crepidostomum* spp. and our data were useful for delineating some species boundaries and questioning at least one nominal taxon (*C. nemachilus*). All of the phylogenetic trees demonstrated the same topology and showed a paraphyly of the genus *Crepidostomum* (**Figure 1**). Firstly, *C. cf. auriculatum*, a parasite of the sturgeons, appears as more divergent species, that was reproduced on phylogenetic trees as a distinct cluster, which was not related to other *Crepidostomum* species. This cluster has a high bootstrap support as so as high mean value of genetic divergence (3.8%) in comparison with other *Crepidostomum* species from the Russian Far East (3.08%). Moreover, *C. cf. auriculatum* is characterized by lower value of transition/transversion ratio bias (R = 2.26 - 2.74) in comparison with the other *Crepidostomum* species (**Table 2**), indicating slight bias from equal contents of transitions and transversions, *i.e.* prevalence of transversion substitutions type, which is typical for high level taxa. Results of KH and SH tests confirm significance of high differentiation of *C. cf. auriculatum* with other *Crepidostomum* species. In our opinion, presented results showed a currency of the question about systematic position of *C. cf. auriculatum* that was thought to be considered by Skwortzoff (1927) within the genus *Acrolichanus* [14].

Analysis of 28S rDNA from 29 specimens of *C. cf. auriculatum*, collected from three different fishes (two specimens of *Acipenser schrenckii* and one of *Huso dauricus*) showed an absence of variation between obtained sequences. It can be interpreted by limited genetic affinity between *C. cf. auriculatum* and the host species and suggests that sturgeons were used as definitive host for *C. cf. auriculatum* not so far. Such genetic stability irrespective of the host species was shown for other trematode species which infect different species of animals [35-37]. Moreover, *C. cf. auriculatum* differentiated from *Bunodera luciopercae* at interspecific divergence level (3.1%). Species *B. luciopercae* is a basal species on

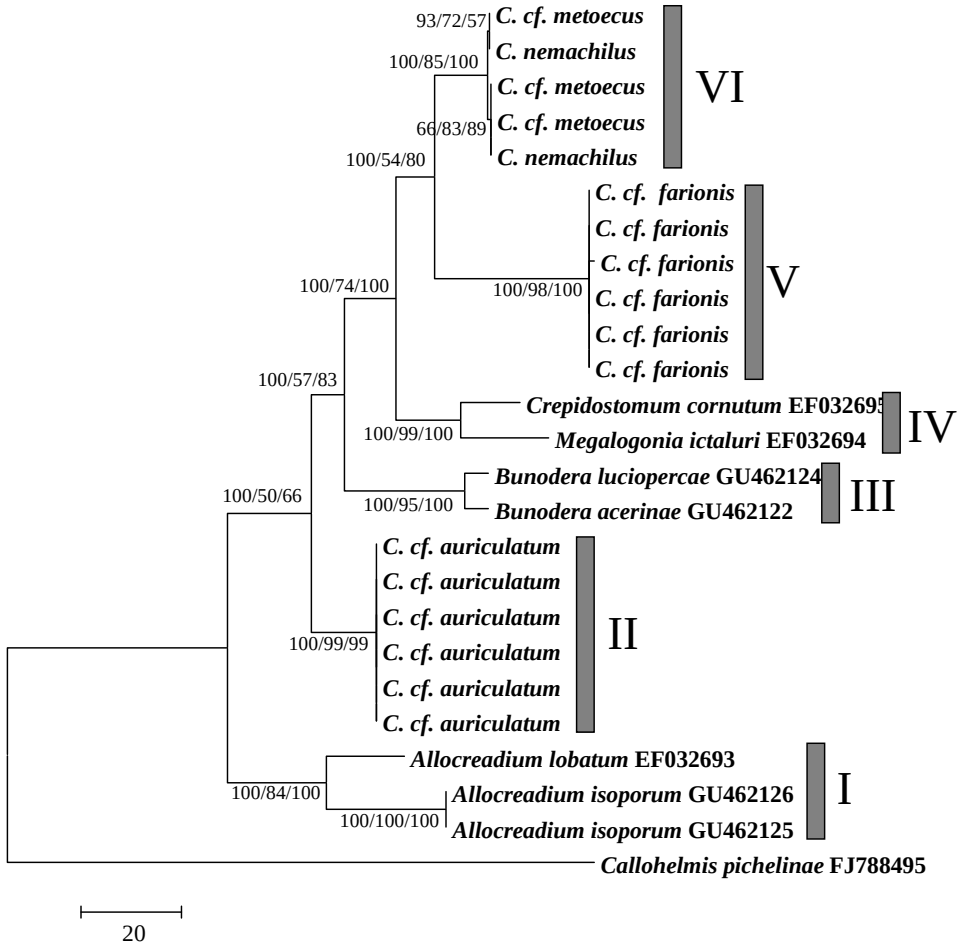

Figure 1. Phylogenetic tree of the family Allocreadiidae, obtained using maximum parsimony algorithm. Nodal numerals are the bootstrap statistics values (%) for MP/ML/BI algorithms, respectively. Roman numerals correspond to number of cluster, discussed in the text.

Table 4. Comparison by KH and SH test of log-likelihood scores among unconstrained and constrained monophyletic trees of the genus *Crepidostomum*. *P < 0.05. Both tests showed significant differences between unconstrained tree, showed a paraphyly of *Crepidostomum* and phylogenetic tree with constraint monophyly of *Crepidostomum* for three phylogenetic algorithms used.

Algorithm	Tree	–ln L	Diff –ln L	P (KH-test)	P (SH-test)
Maximum likelihood	Unconstrained	4162.43	(best)	0.000*	0.033*
	Constraint monophyly	4203.16	40.73		
Maximum parsimony	Unconstrained	4140.55	(best)	0.000*	0.002*
	Constraint monophyly	4205.68	65.13		
Bayesian inference	Unconstrained	4150.63	(best)	0.000*	0.002*

the phylogenetic relationships of the genus *Bunodera*, which considered as the first parasite of the percid fishes ancestor [38]. Such close relationships possibly suggested that percid fishes could be common to *Bunodera* species and *C. cf. auriculatum* in the past. Sturgeons, therefore, possibly become definitive host for *C. cf. auriculatum* during recent host-switching processes.

Secondly, our results showed close relationships of *Megalogonia ictaluri* and *Crepidostomum cornutum*, obtained from USA (GenBank data). This result confirms the point of Caira (1989) [3] about the membership of *Megalogonia* species to the genus *Crepidostomum* in spite of the point of Curran *et al*. (2006), which accept the genus *Megalogonia* [39].

Species *C. cf. farionis* and *C. cf. metoecus* have considerable external morphological differences between themselves, as so distribution and size of dorsal and dorsolateral oral lobes and tegumental papillae on anterior

end of body [3,4,40]. Also these two species differ by form of the genital bursa [1]. *C. nemachilus*, included in our investigations, has the same form of the oral lobes and tegumental papillae as so as *C. cf. farionis*, and the form of genital bursa resembles with *C. cf. metoecus*. However, *C. nemachilus* is characterized by irregularity of testicles surface unlike both *C. cf. farionis* and *C. cf. metoecus*. *C. nemachilus*, therefore, can be recognized as a possible distinct species by general morphology, which will be presented in a separate article. In the present study, genetic divergence between *C. cf. farionis* and *C. cf. metoecus* was 3.1% as so as between *C. cf. farionis* and *C. nemachilus* that corresponds to mean value of interspecific values range of *Crepidostomum* species (3.2%).

Species *C. cf. farionis* and *C. nemachilus*, therefore, cannot be recognized as synonymous taxa using both morphological and genetic criteria. However, this conclusion needs to be examined using *C. cf. farionis* from type location, because of ambiguous taxonomy of *C. cf. farionis* from Russian Far East, which was firstly described as *C. ussuriensis* Layman, 1930 and reduced to synonym with *C. cf. farionis* without sufficient evidence. On the other hand, our results show high genetic identity of 28S rDNA sequences of *C. nemachilus* and *C. cf. metoecus* (one substitution per 1151 bp) in spite of their considerable morphological distinctions. This taxonomical problem need to be resolved by complex approach, including detailed description of life cycle and using more variable molecular markers for estimation of genetic divergence between these two species.

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