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ORIGINAL ARTICLE

Construction and Characterization of the BAC Library for Common Carp *Cyprinus Carpio* L. And Establishment of Microsynteny with Zebrafish *Danio Rerio*

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Abstract A bacterial artificial chromosome (BAC) library of common carp *Cyprinus carpio* L. was constructed as a part of ongoing common carp genome project, which is aiming assembly of common carp genome. The library, containing a total of 92,160 BAC clones with an average insert size of 141 kb, was constructed into the restriction site of *Hin*d III on BAC vector CopyControl pCC1BAC, covering 7.7 X haploid genome equivalents. Three dimension pools and superpools of the BAC library were established and 23 positive clones of 14 targets were identified from one-fifth of the BAC library. Pilot project of BAC end sequencing was conducted on 2,688 BAC ends from 1,344 clones and harvested 2,522 high-quality Q20 sequences with average length of 677 bp. The sequencing success rate was 93.8% and pair-end success rate was 92.3%. A total of 212 micro-syntenies had been established between common carp and zebrafish genomes as a trial for genome-wide comparative genomics in these two closely related species.

Keywords BAC · Common carp · Genome · Comparative mapping · Synteny

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Introduction

Cyprininae carps are the most cultivated species in aquaculture. Common carp is the third most cultivated species worldwide (David et al. 2003), and plays an important role in the world freshwater aquaculture. In the past decade, molecular genetics and genomics study of common carp had been conducted productively for environmental biology, evolutional biology and genetic improvement in breeding. To date, a large number of polymorphic genetic markers, including RAPD, AFLP, microsatellite, and SNP, had been developed for genetic linkage mapping and marker-assisted selection in breeding (Zhang et al. 2008a; Zhou et al. 2004; Li et al. 2007). Several linkage maps had been constructed based on polymorphic RAPD and/or microsatellite markers (Sun and Liang 2004; Cheng et al. 2010). Quantitative trait loci analysis had been conducted on various economic important traits including growth, cold-tolerance, muscle quality, amino acid content, etc. (Mao et al. 2009; Zhang et al. 2007). Expressed sequence tags (EST) with gene functional annotation were collected with traditional Sanger method and the next generation sequencing technology of Roche 454. Some of the ESTs were applied in the microarray studies for various transcritomic analysis (Williams et al. 2008; Moens et al. 2007a, b). However, there is still no large insert genomic DNA library available for common carp research community in China. A large-scale whole genome sequencing project for common carp had been funded and initiated by multiple institutes in China with a 454/Solexa mixed strategy. However, considering the short read of Solexa, even Roche 454, it would be difficult to assemble short pieces into large contig and scaffold without pair-end sequences of large insert genomic DNA libraries. Although the mate-pair

library can jump 20 kb on Roche 454 and Solexa platforms which may replace pair-end sequencing of fosmid library soon, the BAC library spanning over 100 kb genome region is still very useful for whole genome assembly. Besides, BAC libraries are also particularly useful not only for the construction of BAC fingerprint-based physical maps, but also for generation of chromosomal markers for fine mapping of regions of interest, for integration of physical and linkage maps, and as the material basis for position-based candidate gene cloning.

A number of BAC libraries have been constructed and characterized in aquaculture species including crucian carp, salmon, rainbow trout, channel catfish, grass carp, and shrimp, etc. (Geng et al. 2005; Wang et al. 2007; Jang et al. 2010; Thorsen et al. 2005; Shao et al. 2009; Zhang et al. 2010; 2008b), some of them had been actively used on marker development, physical mapping, and candidate gene cloning (Huan et al. 2010; Liu et al. 2009; Xu et al. 2006; Palti et al. 2009; Xu et al. 2007). Here, the BAC library of mirror carp, a cultivated variant of common carp, was constructed with *Hin*d III partial digestion and characterized. Insert size and genome coverage of the BAC library were evaluated. The positive clones of target genes or markers were identified using three-dimension pools and superpools. End sequences of 2,688 BAC clones were collected and used to construct the microsyntenies between common carp and zebrafish (*Danio rerio*).

Materials and Methods

Sample Collection

A female common carp (*Cyprinus carpio* L.) of weight of 4 kg, the founder of a common carp gynogenetic population, was collected from Songpu hatchery station at Harbin, Heilongjiang province, China. Blood was collected using sterile syringes and stored in 5 ml vacuum blood tube with EDTA anticoagulant at 4°C for high molecular weight (HMW) genomic DNA preparation.

BAC Library Construction

The BAC library of common carp was constructed from HMW genomic DNA processed at Amplicon Express, Pullman, Washington. The HMW DNA was partially digested with *Hin*d III and size selected. Ligations in pCC1BAC vector (Epicentre, Madison, WI, USA) were transformed into DH10B *E. coli* cells (Invitrogen, Carlsbad, CA, USA) and plated on LB agar with appropriate chloramphenicol, X-gal, and IPTG concentrations. In order to obtain longer inserts, library construction described above was conducted twice. Clones of both constructions were robotically picked with a Genetix QPIX (San Jose, CA, USA) and arrayed into 384-well plates containing LB freezing media. Plates were incubated for 16 h, replicated and then frozen at −80°C.

Insert Size Estimation

To estimate insert sizes, 10 μl aliqots of BAC miniprep DNA were digested with 5 U of *Not* I enzyme during 3 h at 37°C. The digestion products were separated by pulsed-field gel electrophoresis on CHEF-DRIII system (BioRad, Hercules, CA, USA) in a 1% agarose gel with TBE buffer. Insert sizes were compared to those of the MidRange II PFG marker (New England Biolabs, Ipswich, MA, USA). Electrophoresis was carried out for 18 h at 14°C with an initial switch time of 5 s, a final switch time of 15 s, in a voltage gradient of 6 V/cm.

Carp BAC Library Pooling

For quick and effective PCR screening of BAC clones with specific DNA sequences and evaluating the BAC library, a BAC three-dimension pool containing 18,432 BAC clones was constructed which cover around 1.5 haploid genome equivalents.

Two identical copies of the original BAC library were inoculated to 2×YT liquid medium, then cultured at 37°C for 12 h. Twenty-four clones from the same row of a 384-well plate were combined into one well of a 96-deep-well plate, namely primary row pool. Using the other BAC copy, primary column pool was constructed in a similar way: 16 clones from the same column of a 384-well plate were combined into one well of a 96-deep-well plate. To avoid potential pollution across target wells, pipette tips were replaced after each target well was finished.

Secondary pools for each group were constructed based on the primary pools. Eighty microliter bacterial suspensions from each well of vortexed primary row pool were collected and combined by plate. The plate pool consisted of 48 wells, each of which contained all the 384 BAC clones from one original plate. Thirty microliter bacterial suspensions from each well of vortexed primary row pool were collected and combined into secondary row pool with 16 wells, each of which contained all the 1,152 BAC clones from the same row in one group. Thirty microliter bacterial suspensions from each well of vortexed primary column pool were collected and combined into secondary column pool with 24 wells, each of which contained all the 768 BAC clones from the same column in one group. To avoid

potential pollution across different wells of the primary pools, pipette tips were replaced per sample during the construction of secondary pools. All the 88 wells of secondary pools for a BAC group were placed on one 96-deep-well plate. The whole BAC library pooling procedure was executed on a JANUS automated workstation (PerkinElmer, Waltham, MA, USA).

PCR Screening

Eighty-eight PCR reactions were necessary for screening positive clones from one BAC group. One microliter bacterial suspensions were used as DNA templates in a 20 μl PCR system. While there was one positive clone among the 18,432 clones of one BAC group, there would be three positive PCR results among the 88 wells of secondary pools, respectively in plate pool, secondary row pool, and secondary column pool. And the positive wells would serve as three-dimensional coordinates to locate the original positive clone. Sometimes there was more than one positive well in each secondary pools, suggesting that there was more than one positive clone in this group, thus additional PCR reactions for candidate clones in the original BAC library would be necessary for further validation.

BAC End Sequencing and BES Processing

BAC clones were inoculated into 2.0-ml 96-well culturing blocks containing 1 ml 2× YT medium and 12.5 μg/ml chloramphenicol from 384-well stocking plates using 96-pin replicator (V&P SCIENTIFIC, San Diego, CA, USA). Blocks were covered by air permeable seal (Excel Scientific, Wrightwood, CA, USA) and incubated at 37°C for 20 h with shaking at 300 rpm. The blocks were centrifuged at 3,000×*g* for 10 min in Beckman Avanti J-26 XP centrifuge (Beckman Coulter, Brea, CA, USA) to precipitate bacteria. The culture supernatant was decanted and the blocks were inverted and tapped gently on paper towel to remove remaining liquid. BAC DNA was then isolated according to the alkaline lysis protocol (Sambrook 2001). BAC DNA was collected in 96 plates and stored in −20°C before use.

Sanger's sequencing reactions were conducted in 96-well semi-skirt plates using the following ingredients: 2 μl 5× sequencing buffer, 1 μl sequencing primer (3 pmol/μl), 1 μl BigDye v3.1 Dye Terminator, 3 μl BAC DNA, and 3 μl water. The cycling reactions were conducted with GeneAmp® PCR System 9700 Thermal Cyclers (Life Technology, Foster City, CA, USA) under the following conditions: initial 95°C for 5 min, then 100 cycles of 95°C for 30 s, 55°C for 10 s, and 60°C for 4 min followed by incubation at 4°C before clean-up. The T7 and pIBRP primers were used for PCR reactions (T7 primer: TAATACGACT CACTATAGGG; pIBRP primer: CTCGTATGTTGTGTG GAATTGTGAGC). After PCR reactions were completed, 1 μl of 125 mM EDTA and 25 μl prechilled 100% ethanol were added to each well. After mixing and incubating at room temperature for 10 min, the plate was spun on 3,500×*g* at 4°C for 30 min followed by washing in 50 μl of 70% ethanol at 2,500×*g* for 10 min. Hi-Di formamide (10 μl) was added to each well to re-suspend DNA. The DNA was denatured at 95°C, and then the samples were analyzed with an ABI 3730XL automated capillary sequencer (Life Technology).

The BAC end sequences base calling and quality assessment was performed using Phred software (Ewing and Green 1998; Ewing et al. 1998) using $Q \geq 20$ as a cutoff. Vector sequences and short sequences were trimmed by LUCY2 (Li and Chou 2004). The refined BESs were then stored in a local MySQL database. Mate-paired BESs were identified by using database query. Repeats were masked using Repeatmasker software (http://www.repeatmasker.org) and BLASTX search was conducted against non-redundant (nr) protein database with a cut off value of 10^{-5}. Mate-paired BES were searched against zebrafish whole genome assembly version 8 (zv8) with program BLASTN and e-value cutoff 10^{-5}. A perl script was used to parse BLAST results into a tab-delimited file.

Results and Discussion

BAC Library Construction and Characterization

The BAC library of common carp consists of 92,160 BAC clones and is represented by a total of 240 384-well microtiter plates, including 192 plates from first construction and 48 plates from second construction (Table 1). In order to

Table 1 A brief statistics of common carp BAC library

Total clones	92,160
1st construction	73,728
2nd construction	18,432
Average insert size	141 kb
1st construction	131 kb
2nd construction	180 kb
Haploid genome equivalent	7.7 X
1st construction	5.7 X
2nd construction	2 X
Insert-empty BACs	2,350 (2.55%)

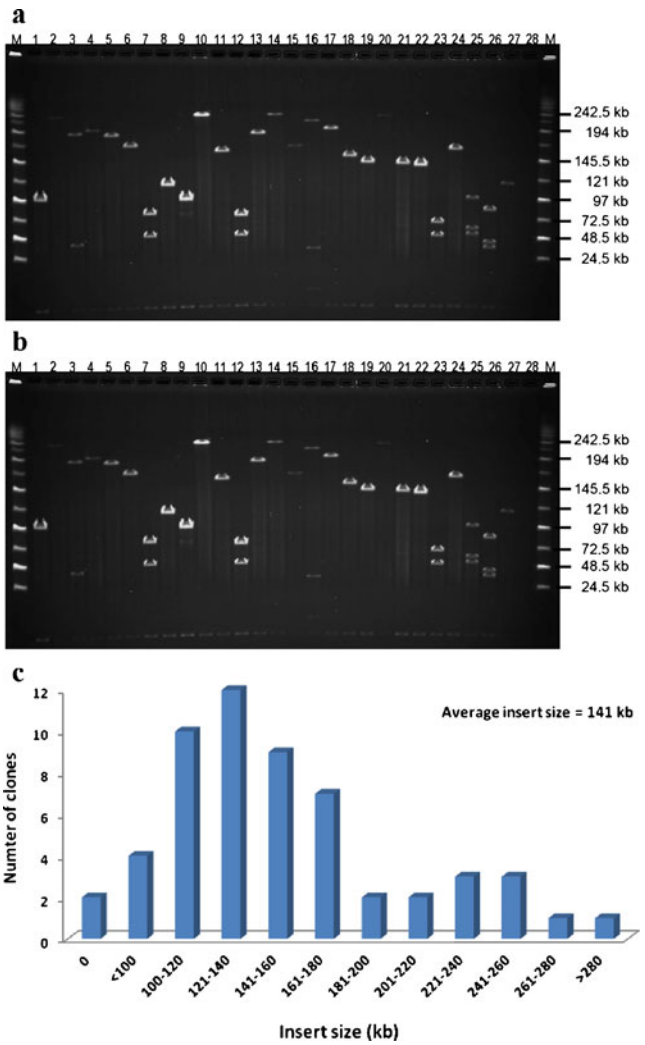

Fig. 1 Analysis of the insert sizes of common carp BAC library. **a** Randomly selected 28 BAC clones from first construction were digested with *Not* I, the bands were separated by pulsed-field gel electrophoresis. "*M*" represents MidRange II PFG marker from NEB. **b** Randomly selected 28 BAC clones from second construction were digested with *Not* I, the bands were separated by pulsed-field gel electrophoresis. "*M*" represents MidRange II PFG marker from NEB. In **a** and **b**, the lowest band of each lane with identical length was pCC1BAC vector band of 8,128 bp. **c** Insert size distribution of common carp BAC library. A total of 56 clones were analyzed from common carp BAC library

evaluate the average BAC insert sizes, 56 clones, 28 from each construction, were randomly chosen and the corresponding DNA was extracted, digested with the rare cutter *NotI* enzyme and analyzed by PFGE (Fig. 1). All fragments generated by *NotI* digestion contained an 8.1-kb vector band and various insert fragments, except two non-insert clones. The estimated insert sizes ranged from 87 to 237 kb in first construction with an average of 131 kb, and ranged from 99 to 296 kb in second construction with an average of 180 kb (Fig. 1). The average insert size of the whole library was 141 kb according to the calculation on both constructions. The haploid genome size of common carp is estimated to be 1,700 Mbp (Tiersch et al. 1989), hence the coverage of the first and second constructions were predicted to be 5.7 and 2 haploid genome equivalents. Thereby, the probability of finding any specific sequence of common carp genome in this library is estimated to be greater than 99.9% according to the formula $P=1-e^{((N\times\ln(1-i/GS)))}$ where P=probability, N=number of clones, i=average insert size of clones, GS=haploid genome size. Insert-empty clones were assessed and a total of 2,350 (2.55%) BAC clones were recognized as insert-empty clones in the library. Besides, there are 135 wells identified as missing well in 240 384-well plates.

BAC Library Pooling and PCR Screening

To further validate and characterize the library, a set of primer pairs for seven genes and ten microsatellite markers, which are currently studied in our other projects, were selected to be used in the library screening in a three-dimension pool containing 18,432 BAC clones. After PCR screening and further verification, 14 primer pairs, except primer pairs of two genes (melanocortin two receptor accessory protein 2 and Follistatin 3) and one microsatellite marker, had identified 23 positive BACs from 18,432 BAC clones, with an average of 1.64 per target. In these 23 positive clones, seven positive clones were for five genes and 16 were for nine microsatellite markers, respectively (Table 2). The success rate for the screening is 82%, which is higher than the estimated 78% possibility calculated from the formula listed above. This suggested that the BAC library had a good representation of the common carp genome. It may also imply that some of the targets have more than one copy in common carp genome, which had been proved in other teleosts (Wang et al. 2010).

Those 16 positive BAC clones of the randomly selected nine microsatellites illustrated the feasibility of map integration strategy via mapping genetic markers onto BAC clones. Once microsatellite markers in a linkage map are anchored to their positive BAC clones, the physical map contigs holding those positive BAC clones would be integrated into the linkage map.

The BAC pools containing more BAC clones will be further constructed once budget and man power are allowed, which would be much easier and efficient to identify the positive BAC clones of each individual target for both gene cloning and map integration.

BAC End Sequences

To better evaluate the common carp BAC library, both ends of 1,344 random selected BAC clones were sequenced.

Table 2 Positive BAC clones of specific targets identified from 18,432 clones of common carp BAC library

Targets[a]	Forward primers	Reverse primers	Positive BAC clones
FST1	AGAGCCACATGCTTGCTTGG	GGTTTTGGGAAGGTGGTTGC	019_G21, 041_H05
MSTN	TCGTGAACCAGTGCCCAGAC	AGATTGGACTCCTGTGGCAG	036_O07, 044_J07
AgRP	GAAGACCTTGAATCCTATGATG	GAGTGTAGAAATAGGCTCTATGC	027_E22
Leptin	AGCAGGTATCGCTGCTCTGTTG	CGATAGACCCGAGACCTTGGAT	002_N04
MC4R	GCGCTACCACAACATCATGACC	GTCCATACCAGCAGCAGCAGAT	011_I18
HLJE334	GGCTGTCAAGAGGCTACGAC	TGAGTTCATTTCTGCGTTGG	001_N07, 016_K10, 039_J20
HLJE182	GAGGAGCTGGAGGACTGGAT	CAATGCAATCTCCGGAAAGA	038_P23, 047_J15
HLJE323	GGTTGGACGAGTGGATGTTA	TTGTCATGCGGCTTCTTATG	023_F18
HLJE27	GAAACTCCATACGGACTGA	AGTGTCCCTGCCTCTTCT	012_F16, 037_H18
HLJE24	CTTCTTCGGTGATGACTT	GACTTGCCTCCCATGTTT	006_B04, 006_F12, 010_F12, 032_D10
HLJE146	TGAGCAGACCAACTCACCTG	TTGGTCTGTTTCCTGGCTCT	040_C13
HLJ473	CCCCTCTTCATCCTTCTC	ATCTGTCAGACCCAACCC	033_C03
HLJE203	TCTATGTAGTGCCCGATGAGC	CAAATGCCTGGTGTATTGTCC	007_O03
HLJE324	AACCTGTTCACGCAGGTCTC	GGACTGGAACTCGCTAGTGG	040_B22

[a] FST1, MSTN, AgRP, and MC4R are follistatin 1, myostatin, agouti-related protein, and melanocortin 4 receptor, respectively. HLJ series are microsatellite markers selected from published common carp linkage map

After quality filtering and vector trimming, a total of 2,522 high-quality (quality score>Q20) BESs with minimum length of 100 bp and average length of 677 bp were collected (GenBank accession no. HN150714-HN153235). The sequencing success rate is 93.8%. All BESs were loaded into a MySQL database. Database query further identified that a total of 1,241 BAC clones were successfully sequences on both ends and the mate-pair successful rate was 92.3%.

Repeatmasker with the latest Repbase was used to analyze 1,706,216 bases of the 2,522 high-quality BESs and evaluate the genome structure and organization of common carp first time. Apparently, common carp genome is an A/T-rich genome with the 63.2% AT and 36.8% GC. The GC content is very similar to the 36.5% of the closely related zebrafish (Han and Zhao 2008), and lower than those of channel catfish (39.3%) (Xu et al. 2006) and tetraodon (45.9%) (Han and Zhao 2008). A total of 14.61% bases of surveyed BES were masked, including 4.93% retroelements (0.09% SINEs, 2.55% LINEs, and 2.29% LTR elements), 5.22% DNA transposons, 1.55% simple repeats, and 1.78% low complexity sequences (Table 3).

Repeat masked BES were searched against non-redundant protein database with BLASTX program an e-value cutoff of 10^{-5}. A total of 543 BES had at least one significant hit on the non-redundant protein database, which occupied around 21.6% of those 2,522 BES. The BLASTX results were parsed into tab-delimited format and the top hits of each BAC ends sequence were collected in a spreadsheet. Although repetitive elements had been masked using closely related zebrafish database, there had been still 184 BES identified that contain putative retroelements and transposons in the BLASTX results, including transposable element Tc1 transposase (ACO51862), reverse transcriptase (BAE46430), and polyprotein (AAC33526, AAN12398), etc. It had been reported that transposable elements may play the important roles in driving the evolution of eukaryotic genome complexity, which perhaps evolved as a means to achieve adaptive diversification (Bowen and Jordan 2002). The high proportion of transposable elements may imply the genome complexity of common carp genome, which may be also supported by the diversification of common carp on various traits. After removing redundant hits and putative repetitive element hits, a total of 356 unique genes were identified from the BES (supplemental Table 1).

Establishing Microsyntenies of Common Carp and Zebrafish

Repeat masked mate-pair BESs were then analyzed by using BLASTN program against zebrafish assembly zv8 with e-value cutoff 10^{-5}. BLASTN results were collected and parsed into a spreadsheet containing only the top hit of each BES. From 1,241 pairs of BES, 745 BESs of T7 orientation, and 690 BESs of PIBRP orientation had been mapped on zebrafish genome with significant similarity, respectively. After database manipulation, a list of 265 BACs had been identified which had both ends mapped on the same zebrafish chromosomes. Then the BACs harboring novel repetitive elements on both ends were filtered. A total of 212 microsyntenies were then constructed between common carp and zebrafish genomes with a threshold that the distances of both hits of two pair-end BESs

Table 3 Repetitive elements of the common carp genome BAC end sequences as assessed by REPEATMASKER

Repetitive elements	Number of elements	Length occupied (bp)	Percentage of sequence (%)
Retroelements	263	84,060	4.93
SINEs	10	1,466	0.09
Penelope	0	0	0
LINEs	138	43,464	2.55
CRE/SLACS	0	0	0
L2/CR1/Rex	118	37,068	2.17
R1/LOA/Jockey	8	1,778	0.1
R2/R4/NeSL	0	0	0
RTE/Bov-B	3	376	0.02
L1/CIN4	6	2,905	0.17
LTR elements	115	39,130	2.29
BEL/Pao	13	6,661	0.39
Ty1/Copia	2	1,097	0.06
Gypsy/DIRS1	74	23,982	1.35
Retroviral	10	3,804	0.22
DNA transposons	622	88,981	5.22
hobo-Activator	241	25,709	1.51
Tc1-IS630-Pogo	84	19,144	1.12
En-Spm	50	4,214	0.25
MuDR-IS905	0	0	0
PiggyBac	24	4,825	0.28
Tourist/Harbinger	24	2,598	0.15
Other	1	71	0
Unclassified	29	2,438	0.14
Total Interspersed repeats		176,546	9.67
Small RNA	21	1,755	0.1
Satellites	111	15,931	0.93
Simple repeats	534	26,386	1.55
Low complexity	674	30,419	1.78

on a same zebrafish chromosome are equal or less than 500 kb (Fig. 2 and supplemental Table 2). These microsyntenies would serve as an important role on comparative genomics once the physical map and high-density linkage map are available in common carp, which had been demonstrated in the comparative genomics study in animals including several teleosts (Liu et al. 2009; Larkin and Lewin 2008; Leeb et al. 2006). A large-scale BAC end sequencing and physical mapping is in progress as part of "common carp genome project". The primary target is to generate over 40,000 BESs and the first generation physical map for the whole genome sequencing assembly as well as synteny construction in comparative genomics. It will be allowed a large-scale comparative analysis between common carp and zebrafish and better understanding of common carp genome.

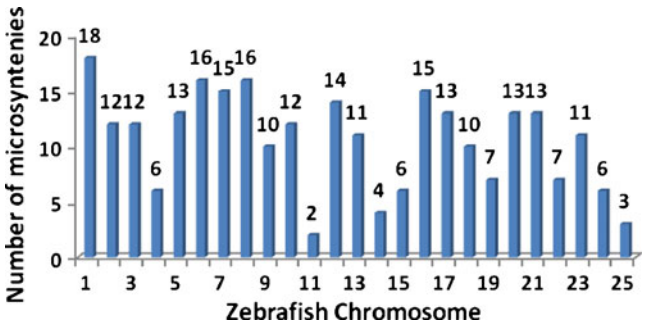

Fig. 2 Microsyntenies between common carp and each of zebrafish chromosomes

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