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# Characterization, polymorphism assessment, and database construction for microsatellites from BAC end sequences of channel catfish (*Ictalurus punctatus*): A resource for integration of linkage and physical maps

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**Abstract**

To apply genome-based technologies for genetic improvements using marker-assisted selection, genome research involving genetic linkage mapping and physical mapping is required, and integration of genetic and linkage maps would significantly enhance the capacities for genome research. In catfish, the major aquaculture species in the United States, linkage and physical maps have been constructed. However, integration of genetic linkage and physical maps demands large-scale, genome-wide hybridizations, or genetic mapping of polymorphic markers derived from bacterial artificial chromosome (BAC) clones whose locations are known from the physical map. In this work, we identified a large number of microsatellites from BAC end sequences of channel catfish, characterized the microsatellites, tested their utility for linkage mapping in a resource family used for genetic mapping, and constructed a web-searchable database for BAC end sequences, their linked microsatellites, microsatellite primers, PCR conditions, and polymorphic information. A total of 3652 microsatellites in 2744 distinct BACs were identified. Of these, 1100 had sufficient and complex flanking sequences for PCR primer design. We have tested 500 primer pairs and found 211 (42.2%) were polymorphic and segregating in the resource family used for genetic mapping. These microsatellites represent a major fraction of co-dominant polymorphic markers identified to date in catfish, and should be a valuable resource for genetic mapping to increase linkage map resolution, and for integration of genetic linkage and physical maps.


*Keywords:* Microsatellite; Marker; Linkage map; Map integration; BAC; Physical map; BAC end; Channel catfish; *Ictalurus punctatus*

## 1. Introduction

Integration of genetic linkage and physical maps is essential for the understanding of genes underlining performance traits, and it can be achieved using two different approaches. First, DNA markers that have already been mapped to genetic linkage maps can be used as probes to hybridize to high-density BAC filters. This approach can be made more effective by the adoption of two dimensional hybridizations (Han et al., 2000; Gardiner et al., 2004), but can be complicated by the presence of repetitive sequences, gene families, and pseudo-genes associated with the probes. While efforts have been devoted to hybridization studies in catfish (Bao et al., 2005; Peatman et al., 2006), several major technical problems limit large-scale, genome-wide hybridization of microsatellite markers to BAC contigs. Second, polymorphic DNA markers can be developed from the known locations on the physical maps. In this approach, polymorphic markers such as microsatellites can be

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identified from BAC clones that are already fingerprinted for the construction of physical maps. The polymorphic markers can then be genetically mapped.

In channel catfish (*Ictalurus punctatus*), linkage maps have been constructed using microsatellite markers (Waldbieser et al., 2001; Liu et al., submitted for publication), and a BAC contig-based physical map has been constructed (Xu et al., 2007) using the CHORI 212 BAC library (Wang et al., 2007). Over 20,000 BAC end sequences have been generated (Xu et al., 2006). In this project, our objectives were to characterize microsatellites identified from the BAC end sequences, to test their polymorphism in our resource family used for the construction of the genetic linkage map, and to develop a database for the BAC-anchored microsatellites, making them a useful resource for the integration of the genetic linkage and physical maps.

## 2. Materials and methods

### 2.1. Mining microsatellites from BAC end sequences

The FASTA file of the BAC end sequences was downloaded from NCBI and stored on the local computer for microsatellite mining. A Perl-based script *Msatfinder* (freeware, downloaded from http://www.genomics.ceh.ac.uk/msat-finder/) was used for microsatellite mining from the FASTA file (Thurston and Field, 2005). As mononucleotide repeats are not useful for mapping, they were manually excluded from the search output file.

### 2.2. Characterization of microsatellites

First, the microsatellite-containing BAC end sequences were searched to determine whether they had sufficient flanking sequences for primer design by harboring at least 50 bp flanking sequences on either side of microsatellites. The resulting unique set of BAC end sequences containing microsatellites was used to design primers using *Msatfinder*. Only a fraction of these so-called microsatellite-containing BAC end sequences with sufficient flanking sequences supported successful primer design as many flanking sequences contain sequences of low complexity that prohibit generation of PCR primers using *Msatfinder*.

### 2.3. Assessing the utility of BAC-anchored microsatellites for linkage mapping

The usefulness of the identified microsatellites depends on their polymorphism. For genetic linkage mapping, their usefulness can be tested in the resource families. We have tested a fraction of the identified microsatellites in one of resource families, $F_1$-2 x channel catfish-6. PCR primers was designed to have a product of G/C content of 40–60%, and the PCR product length of 100–300 bp, and purchased from Sigma Genosys (The Woodlands, TX).

PCR amplification was conducted using a thermocycler (Eppendorf AG, Brinkmann Instruments, Inc., Westbury, New York) using the following amplification profiles: 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTPs, 4 ng upper PCR primer, 6 ng lower PCR primer, 1 pmol labeled primer, 0.25 units of JumpStart *Taq* polymerase (Sigma, St. Louis, MO), 20 ng genomic DNA, in a total reaction volume of 5 μl. After an initial incubation at 94°C for 90 s, PCR was carried out at 94°C for 30 s, 30 s at the appropriate annealing temperature specific with primer sets (50–60°C, see database for details), 72°C for 45 s, for 35 cycles. Upon the completion of PCR, the reaction was incubated at 72°C for an additional 10 min. The PCR products were analyzed on 7% sequencing gels using a LI-COR automated DNA sequencer.

After gel electrophoresis, the positions of both alleles from the sire and the dam were determined, and their segregation was confirmed by genotyping eight fish of the mapping population. Upon genotype calling and determination of allele segregation, polymorphism in the resource family was determined.

### 2.4. Development of a database for BAC end sequences and their associated microsatellites

A database for BAC end sequences and microsatellites was developed based on the Apache/MySQL/PHP/CGI platform. The microsatellite information was categorized to four Excel sheets for the database. Sheet 1 contains the ID information including the GenBank BAC end sequence accession ID, Microsatellite ID, and contig information. Sheet 2 contains the microsatellite information including Microsatellite ID, type of repeat motifs, numbers of repeats, total length, position, and primer ID (if available for design primers). Sheet 3 contains the PCR primer information including primer ID, upper primer sequences and lower primer sequences. Sheet 4 contains the PCR condition information including primer ID, annealing temperature, cycles, and other reaction information. The primary key microsatellite ID, the foreign keys accession ID and primer ID were used to establish the relationship among the sheets in the database. This database will be available for query of the microsatellite information, including the BAC end sequences, motif type and numbers, primers, PCR conditions, and polymorphism information related to the linkage group.

## 3. Results

### 3.1. Identification of microsatellites in BAC end sequences

A total of 20,366 BAC end sequences were used as the source for the identification of BAC-anchored microsatellites. A total of 5553 microsatellites (including multiple microsatellites per BAC end sequence) was identified from the 20,366 BAC end sequences. Of these, some BAC end sequences harbor more than one microsatellite. A total of 3652 distinct BAC clones were found to harbor at least one microsatellite. In order to be useful for mapping, the microsatellite-containing BAC end sequences must have sufficient flanking sequences for the design of PCR primers. Analysis using *Msatfinder* revealed that 605 BAC end sequences harbored microsatellites at the very beginning of the BAC end sequences, and 1296 BAC end sequences harbored microsatellites at the end of the BAC end sequences (Table 1). These will not be useful for testing as markers unless additional sequencing is conducted. After eliminating these, a total of 2744 distinct BAC end anchored microsatellites had sufficient flanking sequences for primer design (Table 1).

### 3.2. Characterization of the BAC-anchored microsatellites

The majority of the microsatellites identified from the BAC end sequences were dinucleotide repeats (63.5%), while the tri- and tetra-nucleotide repeats accounted for 22.0% and 14.5%, respectively. Of the dinucleotide repeats, the most abundant types were AC (27.1%), AT

Table 1
A summary of the microsatellites identified from BAC end sequences

| | |
|---|---|
| BAC end sequences | 20,366 |
| Microsatellites found | 5553 |
| Microsatellites at the beginning of BES | 605 |
| Microsatellites at the end of BES | 1296 |
| BES with microsatellites and enough flanking sequences for primer design | 3652 |
| Distinct BAC clones harboring microsatellites with enough flanking sequences for primer design[a] | 2744 |
| Number of distinct BAC harboring microsatellites with quality flanking sequences allowing for primer design[b] | 1100 (40.1%) |

[a]Number of distinct BAC end sequences with at least 50 bp flanking sequences both upstream and downstream of microsatellites.
[b]Quality flanking sequences were defined as sequences that support primer design using *Msatfinder*.

(27.0%), and GT (23.6%), while AG (14.0%) and CT (8.1%) were much lower; and the CG type was very rare (0.06%). As the BAC end sequences were obtained from both strands of the catfish DNA and the true orientation of the BAC end sequences was unknown, the four distinct dinucleotide repeat types are AC/GT: 50.7%, AG/CT: 22.1%, AT/TA: 27.0%, and CG/GC: 0.06%. Clearly, the AC/GT type of dinucleotide repeats was the most abundant type in the catfish genome, accounting for over 50% of all dinucleotide repeats.

The tri-nucleotide repeats are uneven in distribution, with ATT (35.8%) and AAT (27.8%) being most abundant. These two types of tri-nucleotide repeats accounted for over 63.6% of all tri-nucleotide repeats. It is apparent that all A/T-rich repeat types were more abundant than G/C-rich repeat types. For instance, after the most abundant ATT and AAT (both are 100% A/T repeats), all tri-nucleotide repeats with two of their three bases of the repeats being A or T had a representation of at least 2.6%, whereas all G/C-rich tri-nucleotide repeats with two of their three bases being G or C were all below 0.6% of the tri-nucleotide repeats with the exception of AGG (1.5%).

Very similar to the situation of the tri-nucleotide repeats, the distribution of tetra-nucleotide repeats was not even. They were most abundant with AAAT (18.9%) and TTTA (16.4%). In general, it was also true that tetra-nucleotide repeats with greater A/T had a greater representation. For instance, tetra-nucleotide repeats with at least three bases being A or T accounted for almost 80% of all tetra-nucleotide repeats with AAAG (13%), AAGT (10%), AAAC (6.7%), GTTT (5.7%), CTTT (2.8%), AACT (2.6%), and AGTT (1.7%) among the most abundant types. The only exception appeared to be AATT which accounted for only 1.3% of all tetra-nucleotide repeats. G/C-rich tetra-nucleotide repeats were rare with many types not detected at all.

### *3.3. Assessment of the utility of the BAC-anchored microsatellites for linkage mapping*

In order to be mapped on the genetic linkage map, microsatellites must be polymorphic in the resource families used for genetic linkage mapping. To assess the proportion of the BAC-anchored microsatellites useful for linkage mapping, PCR analysis was conducted using the parents of the mapping population. A total of 500 pairs of primers were ordered for testing. As shown in Table 2, of the 500 microsatellites tested, 211 (42.2%) were polymorphic within the ($F_1$2 x Channel 6) resource family.

It seems that tri-nucleotide microsatellites produced the highest percentage of polymorphism within the resource family. Of the 349 tested dinucleotide repeats, 133 (38.1%) were polymorphic; of the 80 tested tri-nucleotide microsatellites, 46 (57.5%) were polymorphic; and of the 71 tested tetra-nucleotide microsatellites, 33 (46.5%) were polymorphic in the resource family. While polymorphic levels were similar (and also in some cases the numbers were too small to make a meaningful assessment, Table 2) among various types of tri-nucleotide and tetra-nucleotide microsatellites, it appeared that CT (48.5%) and AG (43.9%) types of dinucleotide repeats were most polymorphic, whereas the AT type (20.9%) of dinucleotide repeats was least polymorphic in the resource family.

### *3.4. Database construction for the BAC-anchored microsatellites*

A web-based searchable database was constructed for the BAC end sequences, and their associated microsatellites. Information in the database included BAC clone name, BAC end sequences, contig information, GenBank accession numbers, microsatellite motifs and locations, microsatellite primer names, primer sequences, and PCR conditions. This database is accessible to the public through the Internet link http://www.animalgenome.org/aquaculture/catfish/projects/auburn/suppl2007089.html.

Table 2
The number and polymorphism tested from various types of microsatellites

| Microsatellite types | Number of microsatellite primer pairs tested | Number of polymorphic microsatellites | % polymorphic |
|---|---|---|---|
| AC | 129 | 51 | 39.5 |
| AG | 41 | 18 | 43.9 |
| AT | 43 | 9 | 20.9 |
| TC | 33 | 16 | 48.5 |
| TG | 103 | 39 | 37.9 |
| Sub-total | 349 | 133 | 38.1 |
| AGG | 7 | 3 | 42.9 |
| AAT | 27 | 14 | 51.9 |
| AAC | 3 | 1 | 33.3 |
| ATC | 2 | 2 | 100 |
| CTG | 1 | 0 | 0 |
| GGA | 1 | 0 | 0 |
| ATG | 7 | 4 | 57.1 |
| ATT | 27 | 18 | 66.7 |
| GTT | 1 | 0 | 0 |
| TCC | 1 | 1 | 100 |
| TTC | 1 | 1 | 100 |
| TGG | 1 | 1 | 100 |
| GTT | 1 | 1 | 100 |
| Sub-total | 80 | 46 | 57.5 |
| AACA | 8 | 3 | 37.5 |
| AATA | 13 | 7 | 53.8 |
| TAAC | 2 | 1 | 50 |
| AATC | 2 | 1 | 50 |
| TAAT | 1 | 0 | 0 |
| GACA | 1 | 0 | 0 |
| ATCT | 4 | 2 | 50 |
| ATGG | 5 | 0 | 0 |
| TCCA | 4 | 3 | 75 |
| TATT | 21 | 9 | 42.9 |
| TGTT | 6 | 4 | 66.7 |
| TTTC | 2 | 1 | 50 |
| TGAA | 2 | 2 | 100 |
| Sub-total | 71 | 33 | 46.5 |
| Total | 500 | 211 | 42.2 |

## 4. Discussion

In this work, a large number of microsatellites were identified from BAC end sequences of channel catfish. These microsatellites represent a major fraction of microsatellites in catfish identified to date. These microsatellites will be significant not only as potential polymorphic markers for genetic mapping, but also as the marker resource for integration of genetic linkage and physical maps as they were developed from BAC clones that are already fingerprinted for the construction of a physical map (Xu et al., 2007). Mapping of these BAC end-derived microsatellites will not only add additional markers on the linkage map thereby increasing map resolution, but may also improve the coverage of the linkage map because BAC end sequences are more randomly distributed along the genome than are gene-containing regions.

Earlier efforts in microsatellite marker development in aquaculture species were accomplished by the construction of

microsatellite-enriched libraries (e.g., Liu et al., 1999; Carleton et al., 2002; Coulibaly et al., 2005). However, recently, it has been shown that the identification of microsatellites through data mining is a very effective way for marker development (Serapion et al., 2004; Ju et al., 2005; Schwenkenbecher and Kaplan, 2007; Garnica et al., 2006; Blenda et al., 2006; Perez et al., 2005). In many instances, microsatellite markers were identified using EST resources. Here we demonstrate that data mining is also very effective using BAC end sequences for the purpose of identifying microsatellite markers. The limiting factor is the availability of BAC end sequences. In catfish, we previously generated 20,366 BAC end sequences. In order to integrate the linkage and physical maps to the fullest extent, microsatellite markers need to be developed from as many BAC clones as possible among those BACs that have been fingerprinted for the construction of the physical map (Xu et al., 2007). Efforts for the sequencing of additional BAC end sequences are ongoing in our laboratory. To fully integrate the physical map with the genetic linkage map, multiple polymorphic markers are needed from a single contig to both integrate and orient the linkage map with physical map.

Analysis of the utility of the BAC-anchored microsatellites for linkage mapping was determined by testing the polymorphic status in the resource family used in catfish linkage mapping. In spite of the large numbers of microsatellites identified from BAC end sequences, their utility for linkage mapping depends on the nature of flanking sequences to support PCR primer design, the amplifiability of the designed microsatellite primers for the generation of PCR products with high fidelity, and the polymorphism of the microsatellites within the resource family. Clearly, the largest loss of the number of microsatellites useful for linkage mapping resulted from the flanking sequences with low sequence complexities. Of the 2744 distinct BAC harboring sufficient flanking sequences as defined by the presence of at least 50 bp flanking sequences on either side of the microsatellites, only 1100 (40%) supported primer design using *Msatfinder*. The next major reduction of useful microsatellite for linkage mapping resulted from the lack of PCR products or PCR products without fidelity, or the lack of polymorphism within the resource family. Of the 500 pairs of PCR primers tested, 211 microsatellites (42.2%) were polymorphic in the resource family. It appeared that the tri-nucleotide (57.5%) and tetra-nucleotide repeats (46.5%) had a higher level of polymorphism in the resource family than the dinucleotide repeats (38.1%). Among dinucleotide repeats, it appeared that the AT repeats had the lowest polymorphic rate in the resource family tested. Such information concerning repeat types and polymorphic rates will allow us in the future to pick the microsatellites most likely to be polymorphic as our BAC end sequence resource expands. Obviously, the tri-, and tetra-nucleotide repeats are favored because of their greater polymorphic rates and much reduced problems in stutter bands, a common problem for dinucleotide repeats. The tested polymorphic microsatellites are ready for mapping. Due to financial limitations, genotyping of the resource family was not accomplished in this work, but is of the highest priority once funding is available.

Based on this polymorphic rate, an estimated 460 polymorphic microsatellites will be available for linkage mapping from the present set of BAC end sequences. In spite of their significance for linkage mapping and for the integration of the linkage and physical maps, many more BAC-anchored markers are required for full integration of linkage and physical maps. Catfish has 29 pairs of chromosomes; the estimated 460 markers will provide approximately 16 markers per chromosome, or approximately one marker per 8 cm. It is obvious that many more markers are needed to bring a greater level of map resolution for detailed analysis of aquaculture traits. From the perspective of physical mapping, the current assembly of the BAC contig-based physical map has 3307 contigs. Therefore, just one marker per contig requires 3307 polymorphic markers, and multiple markers per contig are needed to orient the contigs on linkage maps. Clearly, more BAC ends should be sequenced. Additional efforts are ongoing in our laboratory in BAC end sequencing, and in refinement of the physical map to bring the number of contigs to a smaller scale.

The distribution of microsatellites in the catfish genome is highly biased toward A/T-richness. This is particularly true for tri- and tetra-nucleotide repeats as almost all microsatellites with a higher A/T have a larger representation than the G/C-rich microsatellites. This is probably due to the fact that the catfish genome is AT-rich, estimated to be 60.7% (Xu et al., 2006).

Long term genome research requires establishment of various databases such that linkage information, BAC clones, their associated sequences and markers can be easily accessed and tracked. In this work, we have constructed a database presenting BAC end sequences, microsatellite location, microsatellite types, microsatellite primer location and sequences, PCR conditions, and polymorphic information in the resource families. This database can be amended upon generation of additional information related to linkage and physical maps. The microsatellites developed from BAC end sequences, along with this database, will provide a valuable resource for the integration of genetic linkage and physical maps in catfish. This resource will be open for the catfish genome community to map to these microsatellites to the linkage map.

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