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Development of Microsatellite Markers by Data Mining from DNA Sequences

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1. Introduction

1.1 What are microsatellites

Microsatellites are tandem repeats of 1-6 nucleotides found at high frequency in the nuclear genomes of most taxa (Beckmann and Weber, 1992). As such, they are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). For example, (A)₁₁, (GT)₁₂, (ATT)₉, (ATCG)₈, (TAATC)₆ and (TGTGCA)₅ represent mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats, respectively. A microsatellite locus typically varies in length between 5 and 40 repeats, but longer strings of repeats are possible. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotides are the dominant type of microsatellite repeats in most vertebrates characterized so far, although trinucleotide repeats are most abundant in plants (Beckmann & Weber, 1992; Chen et al., 2006; Kantety et al., 2002).

Despite the fact that the mechanism of microsatellite evolution and function remains unclear, SSRs were being widely employed in many fields soon after their first description (Litt & Luty 1989; Tautz 1989; Weber & May 1989) because of the high variability which makes them very powerful genetic markers. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms (Schuler et al., 1996; Knapik et al., 1998), but their applications span over different areas ranging from kinship analysis, to population genetics and conservation/management of biological resources (Jarne & Lagoda 1996).

Microsatellites can be amplified for identification by the polymerase chain reaction (PCR), using two unique sequences which are complementary to the flanking regions as primers. This process results in production of enough DNA to be visible on agarose or polyacrylamide gels; only small amounts of DNA are needed for amplification as thermocycling in this manner creates an exponential increase in the replicated segment. With the abundance of PCR technology, primers that flank microsatellite loci are simple and quick to use, but the development of correctly functioning primers is often a tedious and costly process. However, once they are developed and characterized in an organism, microsatellites are powerful for a variety of applications because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage (Liu & Cordes, 2004).

Unlike conserved flanking regions, microsatellite repeat sequences mutate frequently by slippage and proofreading errors during DNA replication that primarily change the number

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of repeats and thus the length of the repeat string (Eisen 1999). Because alleles differ in length, they can be distinguished by high-resolution gel electrophoresis, which allows rapid genotyping of many individuals at many loci for a fraction of the price of sequencing DNA. Many microsatellites have high-mutation rates (between 10^{-2} and 10^{-6} mutations per locus per generation, and on average 5×10^{-4}) that generate the high levels of allelic diversity necessary for genetic studies of processes acting on ecological time scales.

1.2 Progress in the development of microsatellites

As aforementioned, the major drawback of microsatellites is that they need to be isolated and characterization before to be used for the first time. Generally, microsatellites can be developed by the following approaches:

1. Cross-species amplification

Because the sequences of flanking region are generally conserved across individuals of the same species and sometimes of different species, a particular microsatellite locus can often be identified by its flanking sequences. The presence of highly conserved flanking regions has been reported for some microsatellite loci in cetaceans (Schlötterer et al., 1991), turtles (FitzSimmons et al., 1995) and fish (Rico et al., 1996), allowing cross-amplification from species that diverged as long as 470 million years ago (Ma).

In this way, the first step is to search published literature and public databases for any existing microsatellite primers for the target species or closely-related species. The availability of microsatellite markers for a given species will be a combination of past interest in that species (and related species) and the inherent success rate of microsatellite development for that taxon. There are clear differences in the frequency of microsatellite regions in the genomes of plants, animals, fungi and prokaryotes (Toth et al. 2000), and the success rate of isolating microsatellite markers often scales with their frequency in the genome (Zane et al. 2002).

Currently, many microsatellite markers are reported as primer notes in a specialized journal "Molecular Ecology Notes" (now changed as "Molecular Ecology Resources"). There is a searchable database online for any microsatellite primers published in this journal (<http://tomato.bio.trinity.edu/>). The sequences themselves are archived in GenBank, and are often submitted long before their use appears in published studies. GenBank can be searched with a web-based engine run by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) by typing in the species, genus or family name, the term microsatellite and selecting the Nucleotide database (Benson et al. 2008; Wheeler 2008).

2. Genomic library- based method

Traditionally, microsatellite loci have been isolated from partial genomic libraries (selected for small insert size) of the species of interest, screening several thousands of clones through colony hybridization with repeat-containing probes (Rassmann et al. 1991). Although relatively simple, especially for microsatellite-rich genomes, this approach can turn out to be extremely tedious and inefficient for species with low microsatellite frequencies. Therefore, several alternative strategies have been devised in order to reduce the time invested in microsatellite isolation and to significantly increase yield.

Conventional library-screening methods, established before 2000, had low efficiency and they could be time-consuming. A repeat-enriched method by using an AFLP procedure, named as FIASCO, was reported and increased the efficiency of microsatellite isolation significantly (Zane et al. 2002).

1.3 Mining microsatellites from nucleotide sequences

Methods of SSR-mining have gone through a rapid evolution during the past few years. The first approaches relied on visual inspection of sequence. Although manual comparison of a small number of sequences is feasible, standard accuracy criteria are hard to establish, and this method does not scale well for multiple sequences and many microsatellite location. The efficiency of visual inspection is increased when it is performed aided by computer programs that are capable of displaying sequence traces. Computer-aided manual examination was used in the analysis of overlapping regions of genomic clone sequences to detect microsatellites. Although visual inspection remains an integral part of software testing and tuning, demands for fast and reliable detection in large data sets have necessitated the development of automated, computational methods of microsatellites discovery.

Once batches of nucleotide sequences with the length higher than approximate 200 base pairs have been accumulated in a species, mining microsatellites from them would be a cheapest way. Recently, with the great progress in genomics and bioinformatics, many *in silico* approaches are increasingly being used for the development of microsatellite markers in many species. Structured, classified and easy to use microsatellite data have been compiled in various microsatellite databases that have been developed and made available online by various institutions in recent years (Table 1). Many of these resources are dedicated to mine microsatellites, although they are sometimes by-products of completed or ongoing genome-sequencing projects.

A number of algorithms already existed which either directly or indirectly detect tandem repeats, all suffer from significant limitations. One group of algorithms was based on computing alignment matrices, and their primary limitation was excessive running time. Another group of algorithms found tandem repeats indirectly using methods from the field of data compression, which may require that the approximate pattern size and a range for the number of copies be specified. Benson (1999) overviewed microsatellite-finding softwares and presented a new algorithm for finding tandem repeats which works without the need to specify either the pattern or pattern size. The algorithm presented in this paper is designed to overcome many of the aforementioned limitations: (i) it uses the method of *k-tuple matching* to avoid the need for full scale alignment matrix computations; (ii) it requires no *a priori* knowledge of the pattern, pattern size or number of copies; (iii) there are no restrictions on the size of the repeats that can be detected; (iv) it uses percentage differences between adjacent copies and treats substitutions and indels separately; (v) it determines a consensus pattern for the smallest repetitive unit in the tandem repeat. The program has already been used as a preprocessor in a new alignment algorithm where tandem duplication augments the standard mutation set of insertion, deletion and substitution.

This chapter aims to give readers basic concept and know-how about the development of microsatellite markers by data mining from DNA sequences.

Database	Species	Host	Description	Weblink
Mouse Microsatellite Database of Japan (MMDBJ)	Mouse	National Institute of Genetics, Japan	Collection of 6119 microsatellites. Also includes PCR conditions for all entries of primer sets and keyword searches for the information	www.shigen.nig.ac.jp/mouse/mmdbj/

Simple-Sequence Repeat Database (SSRD)	Human	Center for Cellular and Molecular Biology, India	Provides summary and detailed view of SSRs, the flanking genomic regions and their associations with genes and sequence tagged sites (STS) markers	www.ccmb.res.in/ssr
Satellolog	Human	Michael Smith Genome Science Center, Canada	Catalogs 1-16 repeat-unit perfect repeats in the human genome	http://satellolog.bcgsc.ca
Microsat2006	Human	King's College, UK	Catalogs human microsatellite repeats	www.microsatellites.org/db_search.php
Molecular Mycology SSR Database	Nine fungal genomes	Westmead Hospital, UK	Mono- to hexa-nucleotide repeats of fungal genomes with complete or draft sequences available	www.mmrl.med.usyd.edu.au/ssr.html
TRBase	Human	University of Exeter, UK	Perfect and imperfect repeats of 1-2000 bp unit lengths from human-sequence data and annotation files for 11 chromosomes	http://trbase.ex.ac.uk
InSatdb	Five fully sequenced insect genomes	Center for DNA Fingerprinting and Diagnostics, India	Microsatellite information according to size, genomic location, nature and sequence composition (repeat motif and GC%) as well as microsatellite cluster	http://210.212.212.8/PHP/INSATDB/home.php
TRDB	Data imported from genome.ucsc.edu	Center for Advanced Genome Technology (CAGT), Boston University, USA	Microsatellite collection along with information on their primers, marker potential, etc., in addition to the facility to screen user's sequence resources, while enabling a user to store and organize their data in allocated 100 Mb of storage space	http://cagt.bu.edu/page/TRDB_about

Table 1. Some online microsatellite resources (from Prakash 2007)

2. Mining microsatellites from nucleotide sequences

2.1 Sources of the data

Sequences from both genomic DNA and cDNA can be used for microsatellite mining. Though some researchers produced DNA sequences and kept them in their own laboratories, publicly accessible nucleotide databases are the major source in many studies today including microsatellite mining. The GenBank sequence database is an annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced at National Center for Biotechnology Information (NCBI) as part of an international collaboration with the European Molecular Biology Laboratory (EMBL) Data Library from the European Bioinformatics Institute (EBI) and the DNA Data Bank of Japan (DDBJ). GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. GenBank continues to grow at an exponential rate, doubling every 10 months. Release 134, produced in February 2003, contained over 29.3 billion nucleotide bases in more than 23.0 million sequences. GenBank is built by direct submissions from individual laboratories, as well as from bulk submissions from large-scale sequencing centers. GenBank nucleotide records are located in separate databases that must be searched independently. These include dbEST and dbGSS, plus multiple databases for the CoreNucleotide division, including nr, htgs, wgs and env_nt. ESTs [<http://www.ncbi.nlm.nih.gov/dbEST/>] are generally short (<1 kb), single-pass cDNA sequences from a particular tissue and/or developmental stage. However, they can also be longer sequences that are obtained by differential display or Rapid Amplification of cDNA Ends (RACE) experiments. ESTs are particularly attractive for marker development since they represent coding regions of the genome and putative function can often be deduced by homology searches although little is known about many of the ESTs. While ESTs provide means for the identification of genes, microsatellites provide high level of polymorphism. Microsatellites identified in ESTs are typically referred to as EST-SSRs or genic SSRs, contrasting to type II SSRs which come from random sequences of the genome. The identification of ESTs has preceded rapidly, with approximately 39 million ESTs sequences now available in public databases (e.g. GenBank 4/2008, all species). As a by-product of EST or BAC sequencing projects in many organisms, microsatellite-mining from SSR-containing ESTs is inexpensive and time-saving, and has proved to be an effective approach to develop microsatellites for genetic map and population genetics studies in animals and plants (e.g. Yue et al., 2004; Wang et al., 2005; Caire et al., 2005).

STS [<http://www.ncbi.nlm.nih.gov/dbSTS/>]s are short genomic landmark sequences (1). They are operationally unique in that they are specifically amplified from the genome by PCR amplification. In addition, they define a specific location on the genome and are, therefore, useful for mapping.

GSS [<http://www.ncbi.nlm.nih.gov/dbGSS/>]s are also short sequences but are derived from genomic DNA, about which little is known. They include, but are not limited to, single-pass GSSs, BAC ends, exon-trapped genomic sequences, and AluPCR sequences.

EST, STS, and GSS sequences reside in their respective divisions within GenBank, rather than in the taxonomic division of the organism. The sequences are maintained within GenBank in the dbEST, dbSTS, and dbGSS databases.

ESTs are particularly attractive for marker development represent coding regions of the genome and putative function can often be deduced by homology searches. While ESTs provide means for the identification of genes, microsatellites provide high levels of polymorphism.

2.2 Finding and characterizing repeat motifs

Traditionally, SSR isolation has relied on the screening of genomic libraries using repetitive probes and sequencing of positive clones in order to develop locus-specific primers. These processes are necessary for many organisms but normally time-consuming and labor-intensive. Mining SSR from public databases has been streamlined with technological advance and protocol optimization to make the process cheaper, more efficient and more successful, and has proved to be an effective approach to develop microsatellites for genetic map and population genetics studies in animals (Serapion et al., 2004; Yue et al., 2004; Wang et al., 2005; Chen et al., 2005; Pérez et al., 2005; Maneeruttanarungroj et al., 2006) and plants (Cordeiro et al., 2001; Kantety et al., 2002; Chen et al., 2006).

Here, we demonstrate how to mine SSRs from common carp EST data step by step.

1. Download EST sequences from public databases

The target ESTs from the NCBI dbEST database were downloaded into VectorNTI software (InforMax Inc.). First, "common carp EST" was used as a keyword to search nucleotide sequences at the NCBI databases (<http://www.ncbi.nlm.nih.gov>). EST sequences of common carp were downloaded from GenBank, DDBJ and EMBL databases between January 1, 2002 and October 18, 2005. All matched sequences were downloaded by changing the "display" window to FASTA, and the "send to" window to FILE. A file containing 10,088 sequences was saved as a text file.

2. Tools for microsatellite mining

In general, microsatellite-finding tools can be classified broadly into three subcategories based on their architecture: first, such as MISA and TROLL etc; second, Tandem-Repeats Finder (TRF) etc; third, ATR and ETR, etc (Table 2). (Prakash et al., 2007)

Name, acronym and weblink of the tool	Salient features	Limitations
Repeatmasker www.repeatmasker.org	Available online and stand-alone; mines perfect, imperfect and compound repeats; accepts data in multiple formats; presents statistical analysis; returns flanking sequences; MaskerAid, a performance enhancement is available	Runs only on Unix/Linux systems; not specific for microsatellites
Sputnik (http://espressoftware.com/pages/sputnik.jsp and http://cbl.labri.fr/outils/Pise/sputnik.html)	C-language program available online and stand-alone; mines perfect, imperfect and compound repeats; accepts data in multiple formats; improved versions include Modified Sputnik-I and Modified Sputnik-II	Automated statistical analysis files not generated; runs only on Unix/Linux systems; hexanucleotide repeats are not screened
Tandem Repeats Finder (TRF) (http://tandem.bu.edu/trf/trf.html)	Both online and stand-alone versions are GUI; mines perfect, imperfect and compound repeats; platform	Accepts input as fasta files only; automated statistical analysis file not generated (TRAP;

	independent	www.coccidia.icb.usp.br/trap/ [54] can be used); process limited-size files only; output files are numerous and difficult to manage
Repeatfinder (www.cbcb.umd.edu/software/RepeatFinder/)	Available online and stand-alone; mines perfect, imperfect and compound repeats; accepts multiple formats as input	Runs on Unix/Linux systems; not specific for microsatellites
eTandem and eQuicktandem (http://bioweb.pasteur.fr/seqanal/interfaces/etandem.html)	Perl script available online and stand-alone; parts of EMBOSS suite; mines perfect, imperfect and compound repeats; accepts input in multiple formats; generates statistics	Runs only on SGI Irix, Linux, Sun solaris and Tru64 Unix
REPuter (http://bibiserv.techfak.uni-bielefeld.de/reputer/)	Available online and stand-alone; stand-alone version can handle large genomic sequences; output cataloged in a format similar to BLAST; statistical and graphical analysis provided; excellent connectivity to BLAST, FASTA.	Limited capacity of online version; accepts data in fasta/plain format only; runs only on Unix; not specific for microsatellites
Simple-Sequence Repeat Identification Tool (SSRIT) and Clemson University Genomics Institute Simple-Sequence Repeat Tool (CUGI SSR) (www.gramene.org/db/searches/ssrtool)	Perl scripts available online and stand-alone; platform independent (CUGI SSR is a modified version of SSRIT)	Finds only perfect repeats; accepts only fasta-formatted files; automated statistical analysis not generated
Tandem Repeats Occurrence Locator (TROLL) (http://wsmartins.net/cgi-local/webtroll/troll.cgi) and WebTROLL (http://wsmartins.net/webtroll/troll.html)	C++ program available online and stand-alone (TROLL downloadable, WebTROLL web interface); identifies perfect, imperfect and compound repeats; also designs primers	Accepts fasta-formatted files only as input; executes only on Linux systems; statistical analysis not provided
Microsatellite Analysis Server (MICAS)	An exclusively web-based utility	Scans only one file at a time;

(http://210.212.212.7/MIC/index.html)		compound and imperfect repeats are not identified; statistical analysis is not performed
MISA (http://pgrc.ipkgatersleben.de/misa/)	Perl script executing only offline; large sequences are handled easily; statistical analysis is generated; platform independent; can design primers using Primer3 by running supplementary scripts	Inappropriate clustering of microsatellite motifs in statistical analysis file; only fasta-formatted files are taken as input; identifies only perfect repeats and compound repeats
mreps (http://bioinfo.lifl.fr/mreps/mreps.php and http://bioweb.pasteur.fr/seqanal/interfaces/mreps.html)	Available online and stand-alone; identifies compound and imperfect repeats; accepts data in multiple formats; platform independent; can design primers	Statistical analysis is not performed
Search for Tandem Repeats in Genomes (STRING) (http://www.caspur.it/_castri/STRING/)	C-language program available online and stand-alone; finds perfect, imperfect and compound repeats; runs well with large genomic sequences; platform independent	Only fasta files taken as input; no automated statistical analysis
Search for Tandem Approximate Repeats (STAR) (http://atgc.lirmm.fr/star)	Available online and stand-alone; searches for 'approximate' tandem repeats of a given motif; platform independent	Does not generate statistical analysis
MicrosatDesign (http://daphnia.cgb.indiana.edu/wfleabase/software)	Perl scripts executing as a stand-alone tool; builds database and designs primers from the nascent DNA-sequencer outputs; DNA-sequence trace files are taken as an input; combination of phredPhrap, Primer 3 and GCG software/eTandem software; identifies compound repeats and imperfect repeats as well	Specific in its use; does not generate statistical analysis
Poly (http://bioinformatics.org/poly/)	Downloadable Python script; statistical analysis is provided; platform independent	Slow

<p>Exact Tandem Repeats Analyzer (E-TRA) and Tandem Repeats Analyzer (TRA) (ftp.akdeniz.edu.tr/Araclar/)</p>	<p>C++ program available online and stand-alone; search microsatellites in ESTs combining with key-word match searches; multiple sequences and multiple files can be handled simultaneously; provide flanking sequences and capable of designing primers; fast; GUI; find perfect, imperfect and compound repeats; accept input in multiple formats; provides statistical analysis</p>	<p>Redundancy in output</p>
<p>msatminer (www.genomics.ceh.ac.uk/msatminer/)</p>	<p>Perl scripts executing online and stand-alone; finds compound repeats and imperfect repeats also; accepts input in multiple formats; statistical analysis can be obtained on executing additional scripts; separate scripts for designing primers</p>	<p>Runs on Unix and Mac OS environment; stand-alone version complicated owing to requirements to execute as many as four scripts for complete analysis</p>
<p>msatcommander (http://code.google.com/p/msatcommander/)</p>	<p>Python script available for download; GUI; capable of searching perfect, imperfect and compound repeats with flexibility; output in CSV format; platform independent; primer designing utility available</p>	<p>No online interface; only fasta formatted files accepted as input; statistical analysis is not generated automatically</p>
<p>SciRoko (www.kofler.or.at/bioinformatics/SciRoKo/index.html)</p>	<p>C-language program available for stand-alone execution; identifies perfect, imperfect and compound repeats; highly flexible; extremely fast; GUI; provides statistical analysis; platform independent</p>	<p>Depends on .NET framework</p>
<p>Imperfect Microsatellite Extraction (IMEx) (http://203.197.254.154/IMEX/)</p>	<p>C-language program executing stand-alone; finds perfect and imperfect repeats; efficient, fast and user-</p>	<p>Executes on Linux</p>

	friendly; returns the coding/ noncoding information of microsatellites; highly flexible; can design primers as well; statistics are generated	
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Table 2. Characteristics of some important microsatellite search tools

In our study in common carp (*Cyprinus carpio*), we use software “Tandem Repeat Finder” (Benson, 1999). All the ESTs were screened for potential microsatellites by using the TRF with the following parameters: match: 2; mismatch 7; indel: 7; PM: mini-score; 30; and max period size 500. Strings of oligo sequences were used to search for microsatellites: 6 repeats for dinucleotides; 4 repeats for trinucleotides, and 3 repeats for tetranucleotides and pentanucleotides as described by Stalling et al (1991).

3. Frequency and distribution of microsatellites

A total of 10,088 ESTs of common carp with an average length of 531 bp were downloaded from public databases and subject to bioinformatic analyses. The results showed that 555 (about 5.5%) of these ESTs contained SSRs inside, which is lower than values reported in some aquaculture animals e.g. black tiger shrimp (*Penaeus monodon*) (13.7%, Maneeruttanarungroj et al., 2006), Japanese pufferfish (*Fugu rubripes*) (11.5%, Edwards et al., 1998) and channel catfish (11.2%, Serapion et al., 2004), but higher than those in some other species e.g. Chinese shrimp (*Fenneropenaeus chinensis*) (2.2%, Wang et al., 2005), bay scallop (*Argopecten irradians*) (3.9%, Zhan et al., 2005), and red sea bream (*Chrysophrys major*) (4%, Chen et al., 2005). The abundance of EST-derived microsatellites seems to be highly species-specific in aquacultured animals studied.

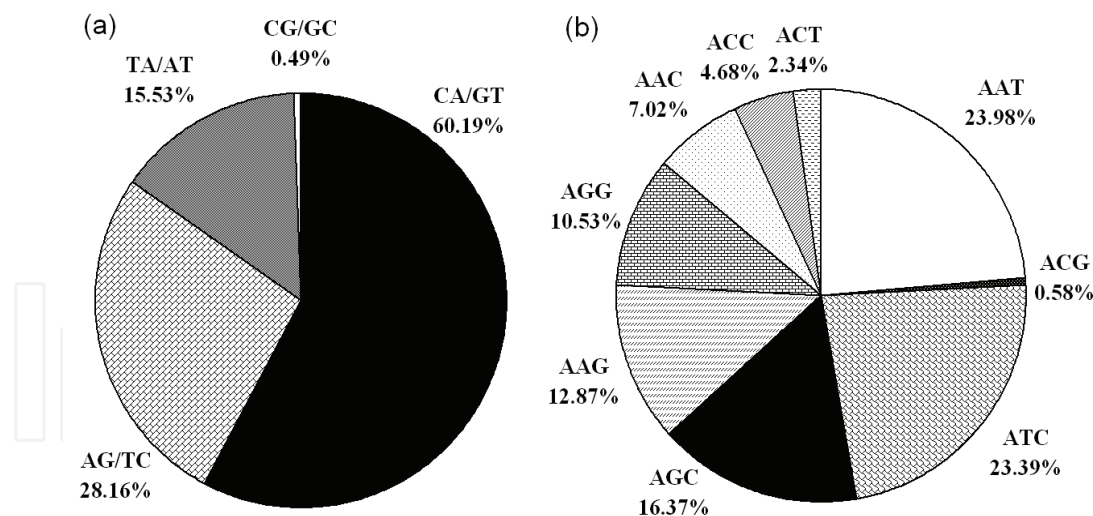


Fig. 1. Distribution of the repeat types of dinucleotides (a) and trinucleotides (b) in common carp EST-SSRs identified by mining public expressed sequence tags databases.

Most of these common carp EST-SSRs were composed of dinucleotide and trinucleotide repeats. Specifically, the abundance of di-, tri-, tetra-, and penta-nucleotide motifs among these ESTs is 37.2%, 30.8%, 20.4%, and 11.7%, respectively. For dinucleotides, AC/TG is the most abundant (Figure 1a), which is consistent with previous findings for both Type I and Type II microsatellites in fish (Edwards et al., 1998; David et al., 2001; Serapion et al., 2004),

various plant species (Gupta & Varshney, 2000), and vertebrates as a whole (Neff & Gross, 2001). The proportion of the trinucleotide repeats was also not evenly distributed, with the two most frequent types (AAT and ATC) accounting for 24.0% and 23.4% of the total motifs, respectively (Figure 1b).

Dinucleotides are the dominant type of microsatellite repeats in most aquaculture species characterized so far, although trinucleotide repeats are most abundant in plants (Cho et al. 2000; Chen et al., 2006; Kantety et al., 2002) (Fig.2).

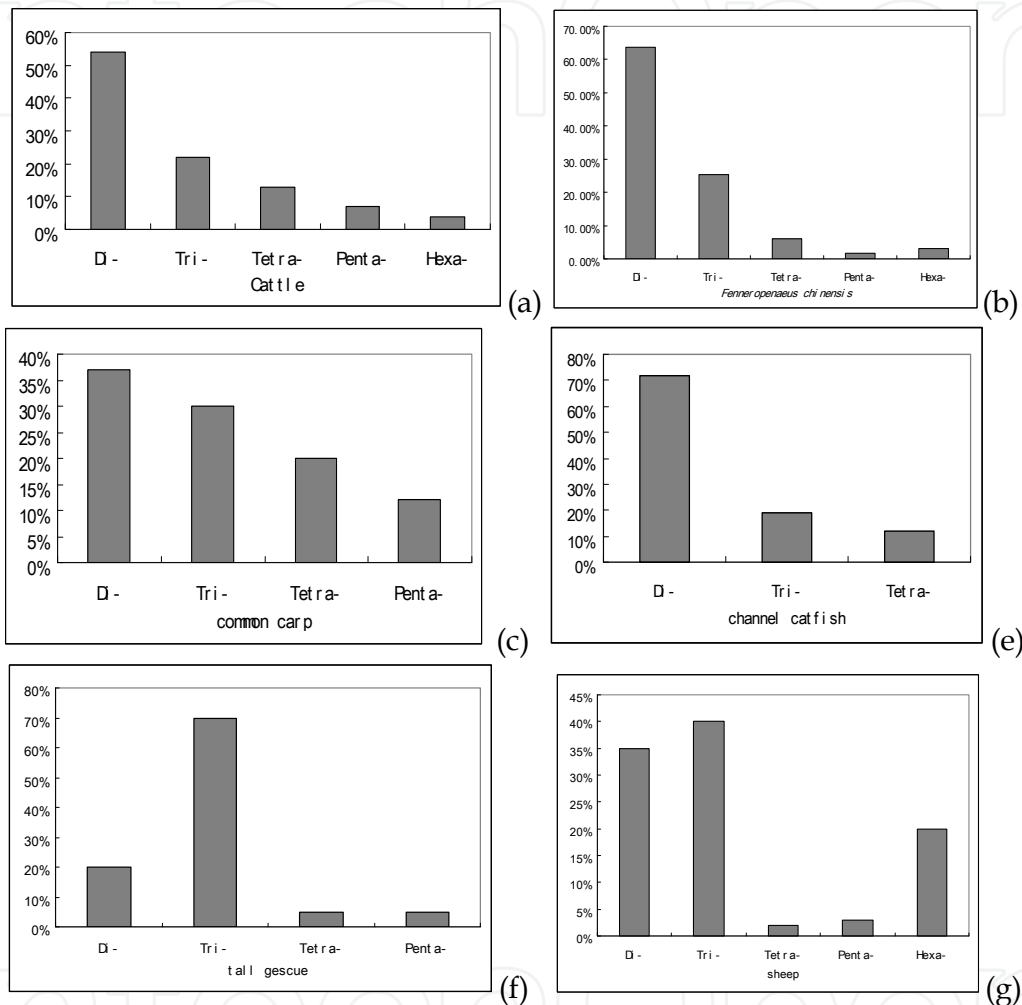


Fig. 2. Distribution of microsatellites in EST sequences from various species.

2.3 Other related bioinformatic work

1. Clustering analysis

EST sequences were analyzed by cluster analysis using the ContigExpress module in VectorNTI package (available at <http://download.invitrogen.com>) and linear assembly algorithm was applied. The criteria for clustering were set at a minimum overlap of 30 bases (default is 20 bases). Each cluster was visually inspected to ensure the fidelity of alignment to avoid pseudo-clusters caused by repetitive elements or long strings of microsatellite repeats. In our study, after clustering and assembly, 465 unique microsatellite-containing ESTs were identified, including 400 singletons and 65 contigs (Wang et al., 2007).

2. Identification of the known genes

The unique ESTs were then subjected to BLASTx search against the GenBank (protein database) for putative identification of gene function. When accumulated probability of sequence similarity was less than 1×10^4 , the tentative identities were established. The BLASTx results revealed that about 165 of these ESTs showed similarity to genes or proteins of known function (Wang et al., 2007).

3. Primer design for microsatellites

In our study, 60 of the 465 unique ESTs or genes were randomly chosen for pilot tests for primer design, locus amplification and polymorphism. Software 'Primer 3' (<http://www.genome.wi.mit.edu/cgi-bin/primer/>) was used to design primers for the amplification of repeat regions of interest across the flanking regions. During the primer design, the range of annealing temperature was set up to be between 45 and 55°C, and that of expected size of PCR products 150-250 bp. A single pair of "best" primers was designed and synthesized for each unique EST or gene that contains SSR, and no repeated designs and syntheses of primers were carried out. Here we introduce several tools for primer design (Table 3).

Name of the tools	Features	Limitations
Primer 3	Work on line.	web; C-language
Primer 5	Designing primers for long PCR of sequences up to 50 kb is possible.	Windows
Oligo 6	The graphic features allow screens to be displayed in either a bar or a dot graph.	Windows; Macintosh
DNASTAR	Sequence assembly and SNP discovery; gene finding; utility for importing unusual file types. Primer design function included.	Windows
FASTPCR	Automatically SSR loci detection; direct PCR primers design	Windows

Table 3. Characterize important software for microsatellites design

3. Laboratory verification of predicted microsatellites

3.1 PCR amplification and polymorphism test for microsatellites

In our study, PCR amplifications of microsatellites were carried out on a thermocycler (PTC-100, MJ Research) by using the following program: 94°C for 5 min, followed by 34 cycles of 94°C for 35s, appropriate annealing temperature for 35s, and 72°C for 50s, and a final extension of 72°C for 10 min. The PCR reactions were performed in a 25 µl-reaction mixture, which contained 2.5 µl 10×reaction buffer, 2 µl Mg^{2+} ($1.5 \text{ mmol} \cdot \text{L}^{-1}$), 1 µl dNTP ($10 \text{ mmol} \cdot \text{L}^{-1}$), 0.5U *Taq* polymerase ($2 \text{ U} / \mu\text{l}$), 2 µl template DNA, 0.25 µl each of the primer ($5 \mu\text{mol} \cdot \text{L}^{-1}$), and 17 µl sterile water. PCR products were separated in 6% denaturing polyacrylamide gel and visualized by silver staining. Allele sizes were determined by comparison with pBR322

DNA/*Msp* I markers (Sino-American, Luoyang, China) combined with image analysis as described previously (Tong et al., 2005).

Out of the 60 common carp EST-SSRs for which primers were designed, 54 primers worked (25 polymorphic, 11 monomorphic, 18 with multiple bands) and 6 failed in the common carp. Some of polymorphic EST-SSRs are shown in Fig 3.

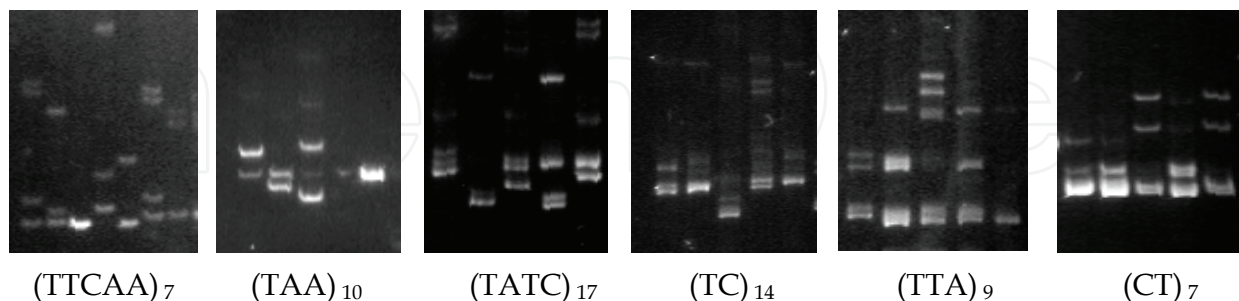


Fig 3. Polymorphism test for parts of EST-SSRs in common carp (Wang et al. 2007).

Twenty-five of the 60 EST-SSRs were found to be polymorphic in a common carp population. The observed heterozygosity of these polymorphic loci ranged from 0.13 to 1.00, and expected heterozygosity ranged from 0.12 to 0.91. The number of alleles of the polymorphic EST-SSRs in common carp ranged from 3 to 17 (mean 7).

Of the 60 common carp EST-SSRs, 10 (17%) of them showed polymorphism in a pilot panel in crucian carp (*Carassius auratus*). In silver carp (*Hypophthalmichthys molitrix*), only 3 (5%) of these loci were found to be polymorphic. In general, these loci are less polymorphic in crucian carp and silver carp than in their source species (common carp).

3.2 Hardy-Weinberg Equilibrium (HWE)

In our study, when the frequencies and distributions of the alleles and genotypes were compared under the HWE expectation for an ideal population (random mating, no mutation, no drift, and no migration), 6 of the 25 loci showed significant departure after Bonferroni correction ($P < 0.002$), and the remaining 19 EST-SSRs were in HWE.

A heterozygote excess (also known as homozygote deficit) occurs when the data set contains fewer homozygotes than expected under HWE, and a heterozygote deficit (also known as homozygote excess) occurs when there are more homozygotes than expected under HWE. Currently, tests used to determine statistically significant deviation from HWE have low power when allelic diversity is high and sample sizes are moderate (Guo & Thompson 1992). However, failure to meet HWE is not typically grounds for discarding a locus. Heterozygote deficit, the more common direction of HWE deviation, can be due to biological realities of violating the criteria of an ideal population, such as strong inbreeding or selection for or against a certain allele. Alternatively, when two genetically distinct groups are inadvertently lumped into a single sampling unit, either because they co-occur but rarely interbreed (unbeknownst to the sampler), or because the spatial scale chosen for sampling a site is larger than the true scale of a population, there will be more homozygotes than expected under HWE. This phenomenon is called a Wahlund effect and may be a common cause of heterozygote deficit in population genetic studies. Both of these causes of heterozygote deficit should affect all loci, instead of just one or a few.

3.3 Null alleles

Null alleles are those that fail to amplify in a PCR, either because the PCR conditions are not ideal or the primer-binding region contains mutations that inhibit binding. In our study, primers of the six loci failed to amplify in the common carp, and primers of some other loci could not amplify specific products. This could be due to one or both primers being designed across the junction of the spliced ends of exons in the EST sequence, which in genomic DNA is interrupted by an intron (Cordeiro et al., 2001), or due to the inaccuracy of some EST sequences.

As a result of null alleles, some heterozygotes are genotyped as homozygotes and a few individuals may fail to amplify any alleles. Often the mutations that cause null alleles will only occur in one or a few populations, so a heterozygote deficit might not be apparent across all populations. A simple way to identify a null allele problem is to determine if any individuals repeatedly fail to amplify any alleles at just one locus while all other loci amplify normally (suggesting the problem is not simply poor quality DNA). If re-extraction and amplification still fail to produce any alleles at that locus, it is likely that the individual is homozygous for a null allele. In addition, a statistical approach to identifying null alleles can match the pattern of homozygote excess to the expected signatures of several different causes of homozygote excess and estimate the frequency of null alleles for each locus. The software MICROCHECKER (Van Oosterhout et al. 2004) is designed for this aim. A more technical way to detect null alleles is to examine patterns of inheritance in a pedigree (e.g. Paetkau & Strobeck 1995). Redesigning primers to bind to a different region of the flanking sequence, or adjusting PCR conditions can often ameliorate null allele problems. Many researchers are quick to use highly stringent PCR conditions without considering the downside that it inflates the chances for null alleles. A low incidence of null alleles is usually only a minor source of error for most types of analyses, but for certain analyses e.g. parentage analysis, even rare null alleles can confound results and any loci with strong evidence of null alleles should be excluded.

3.4 Mendelian inheritance

Mendelian inheritance of alleles is a requirement for almost all population genetic analyses for diploid vertebrate species (Jarne & Lagoda 1996). Because relatively few studies report tests for Mendelian inheritance, it is still unclear how common non-Mendelian inheritance is across taxa. Potential causes of true non-Mendelian behaviour are sex linkage, physical association with genes under strong selection, centres of recombination, transposable elements, or processes during meiosis such as non-disjunction or meiotic drive (segregation distortion). These processes can have severe effects, such as only one parental allele being passed on to all offspring. Performing defined crosses and genotyping a large number of offspring can be quite challenging or impractical in some species, and straightforward in others, such as those that brood their young. Microsatellite loci in any polyploidy species have a high likelihood of occurring multiple times throughout the genome and this will confound analysis, so in particular inheritance should always be examined for polyploidy. Even in diploid or haploid species, duplication of loci can be common and potentially problematic. Any case of a locus displaying more than two alleles per individual (that is not traceable to cross

contamination of samples) should be discarded from most analyses. It is important to note that automated sequencers are set by default to call only two alleles per locus, and will return apparently valid allele calls regardless of the actual number of amplification products produced; for this reason, automated sequencer allele calling should always be double checked by an experienced operator.

3.5 Gametic disequilibrium

When two loci are very close together on a chromosome, they may not assort independently and will be transmitted to offspring as a pair. Even if loci are not linked physically on a chromosome, they can be functionally related or under selection to be transmitted as a pair (hence the more accurate term gametic disequilibrium is starting to replace the term linkage disequilibrium). While functional linkage would be unusual for microsatellite loci, microsatellites can be clustered in the genome and gametic disequilibrium should always be tested. Gametic disequilibrium creates pseudo-replication for analyses in which loci are assumed to be independent samples of the genome. Like tests of HWE, gametic disequilibrium testing has low power for highly polymorphic loci, so examining confidence intervals on estimates is recommended. Several user-friendly software programs (most of them are accessible online), such as ARLEQUIN, FSTAT, GENEPOP, GENETIX, and MICROSATELLITE ANALYZER, include tests for gametic disequilibrium by searching for correlations between alleles at different loci. One type of linkage that this test will not catch is sex linkage; however, sex linkage will produce an apparent heterozygote deficit that resembles a null allele problem. Lastly, there are many ecological questions that can benefit from the study of linked loci (Gupta et al. 2005). For instance, inter population variation in linkage can correlate with the history of bottlenecks (Tishkoff et al. 1996).

4. Prospects

The option of mining microsatellites from DNA-sequence databases has clearly advanced our understanding of evolutionary processes, leading to the formation of repeats in the genome and their selective advantage for the organism. Information on microsatellite distribution in the genomes is a prerequisite for an in-depth understanding of processes determining the formation of microsatellite regions in genomes. This can be obtained either by de novo mining of repeats in genomic sequences or by accessing a database cataloging microsatellite repeats along with their genomic positions.

Despite many advantages, microsatellite markers also have several challenges and pitfalls that at best complicate the data analysis, and at worst greatly limit their utility and confound their analysis. For example, there are some taxa for which new marker isolation is still fraught with considerable failure rate, such as some marine invertebrates (Cruz et al., 2005), lepidopterans (Meglecz et al., 2004) and birds (Primmer et al., 1997). If mutations occur in the primer region, some individuals will have only one allele amplified, or will fail to amplify at all (Paetkau & Strobeck 1995). Several taxa seem more often beset by amplification problems than others, notably, bivalves, corals and some other invertebrate taxa (Hedgecock et al., 2004). On the other hand, because the cDNAs from which ESTs are derived lack introns, one possible concern with EST-SSRs is that unrecognized intron splice

sites could disrupt priming sites, resulting in failed amplification. Alternatively, large introns could fall between the primers, resulting in a product that is either too large or, in extreme cases, failed amplification. In some cases, it may be possible to redesign the primers to exclude troublesome introns.

A large amount of organisms on the earth are directly or indirectly important to human life. However, only a small fraction of them are under comprehensive studies using modern science and technology. Due to the limitation of investment and funding, only a very low percentage of organisms have enough DNA or protein sequences, although they may be economically or ecologically important. Sequence data are expected to accumulate in more diverse species.

An optimistic trend in recent years is that with the advance in sequencing technique (e.g. 454 sequencing by Roche) and the increase of invest by government and private companies, full genomic sequences, EST or BAC sequences, have been increasing rapidly, especially in some domestic animals and plants as well as some model organisms.

The recent trend is to cross-amplify molecular markers across a set of closely related genomes. Microsatellites associated with quantitative trait loci (QTLs) and agronomically important genes remain a good candidate for the development of specific markers. The low cost of their generation and ease in documentation are two of the important relative advantages of these sequences over equally promising single nucleotide polymorphisms (SNPs). Microsatellites can thus firmly be expected to have an important role in genomics research in the future and mining microsatellites from DNA databases is likely to take center stage to come.

5. Conclusion

With the increasing accumulation of the nucleotide sequence data in both private and public databases, and the invention of more efficient computer-based tools, mining some valuable biological resources, such as microsatellites and SNPs, from the raw DNA data, has become one of the most popular areas of biological studies today, bioinformatics. Development of SSRs by data mining from sequence data is a relatively easy and cost-saving strategy for any organisms with enough DNA data. This is a very good example from data to knowledge, and from knowledge to basic and applied studies for biology, production, conservation and management of many organisms.

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This book presents four different ways of theoretical and practical advances and applications of data mining in different promising areas like Industrialist, Biological, and Social. Twenty six chapters cover different special topics with proposed novel ideas. Each chapter gives an overview of the subjects and some of the chapters have cases with offered data mining solutions. We hope that this book will be a useful aid in showing a right way for the students, researchers and practitioners in their studies.

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