A novel method for large-scale identification of polymorphic microsatellites through comparative transcriptome analysis

Running title: A method for SSR identification

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ABSTRACT Microsatellite (SSR) is one of the most popular markers for applied genetic research, but generally the current methods to develop SSRs are relatively time-consuming and expensive. Although high-throughput sequencing (HTS) approach has become a practical and relatively inexpensive option so far, only a small percentage of SSR markers turn out to be polymorphic. Here, we designed a new method to enrich polymorphic SSRs through the comparative transcriptome analysis. This program contains five main steps: 1) transcriptome data downloading or RNA-seq; 2) sequence assembly; 3) SSR mining and enrichment of sequences containing SSRs; 4) sequence alignment; 5) enrichment of sequences containing polymorphic SSRs. A validation experiment was performed and the results showed almost all markers (> 90%) that were indicated as putatively polymorphic by this method were indeed polymorphic. The frequency of polymorphic SSRs was significantly higher ($P < 0.05$) but the cost and running time were much lower than those of traditional and HTS approaches. The method has a practical value for polymorphic SSRs development and might be widely used for genetic analyses in any species.

INTRODUCTION

Microsatellites (SSRs) have been emerged as one of the most popular markers for a wide range of applications in population genetics, conservation biology and marker-assisted selection (Abdelkrim et al., 2009; Luo et al., 2012). Classically, microsatellite marker development requires: the construction of a genomic library enriched for repeated motifs; isolation and sequencing of microsatellite containing clones; primer design; optimization of PCR amplification for each primer pair; and a test of polymorphism on a few unrelated individuals. Most of these steps are either expensive, time-consuming, or both. With the wide application of high-throughput sequencing (HTS) technology, especially the whole transcriptome sequencing, development SSRs by HTS has become a practicable alternative for many species in recent years (Wu et al., 2014). It has greatly reduced the running time and cost requirement for SSR development. However, the frequency of polymorphic SSR markers developed by this method is much low in some species, which means that most of the loci cannot be effectively applied in genetic analysis (Iorizzo et al., 2011; Luo et al., 2012). According to our best knowledge, there is no records addressed the low frequency of polymorphic SSRs. Here we provided a new method for development of polymorphic SSRs through comparative transcriptome analysis.

RESULTS AND DISCUSSION

Three, two and four transcriptomes of rice, grass carp and lined seahorse respectively were used and a total of 299, 206 and 956 putatively polymorphic SSRs were obtained by this method, respectively (Table 1; Table S1). Twenty, thirty and sixty loci were randomly selected for primer design, and 19 (95.00%), 26 (92.86%) and 50 (90.91%) loci showed polymorphic in rice, grass carp and lined seahorse, respectively (Table 1; Table S2). One-way ANOVA showed the frequency of polymorphic SSRs identified by this method was significantly ($P < 0.05$) higher than that of traditional approach and HTS (Fig. 2). In addition, we recently developed polymorphic SSR markers for lined seahorse by HTS approach, and the ratio of polymorphic SSRs was 17.93% (Arias et al., 2016). While using the same transcriptomes to develop SSRs by this method, the ratio was raised to 90.91%.

This method, which is based on the idea of enriching homologous sequences containing the same SSR with a different number of repeats, could identify polymorphic SSRs directly through comparative transcriptome analysis. Compared with traditional methods and HTS, this method have eliminated the most intensive wet lab steps, and the time and cost for primer synthesis and experimental validation by

identifying and separating the many "monomorphic" SSRs from the minority polymorphic ones, cutting the running time and cost by half or more (Tang et al., 2008; Iorizzo et al., 2011). The fact that almost all tested SSRs predicted to be polymorphic were indeed validated as polymorphic demonstrates that it is an efficient and reliable method to develop polymorphic SSR markers. The method will play an important role in developing polymorphic SSR markers, providing a better service for the selective breeding and genetic studies.

MATERIALS AND METHODS

Materials

Each ten specimens of rice (*Oryza sativa*), grass carp (*Ctenopharyngodon idella*) and lined seahorse (*Hippocampus erectus*) were used to investigate the ratio of polymorphic SSRs developed by our method. The leaves of rice and the dorsal fin of grass crap and seahorse were used for DNA extraction.

Architectural structure

The pipeline of this method consists of five steps (Fig. 1): 1) transcriptome data downloading or RNA-seq; 2) sequence assembly; 3) SSR mining and enrichment of SSR containing sequences; 4) sequence alignment; 5) enrichment containing polymorphic microsatellite sequences.

Transcriptome data gaining

The precondition of this method is to detect polymorphic SSRs in two or more transcriptomes from different samples. Three and two transcriptomes of rice and grass carp were downloaded from NCBI (Table 1). Four transcriptomes of seahorse were sequenced by us.

De novo assembly

The raw reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep). Then clean data was used to perform RNA *de novo* assembly with Trinity using default parameters.

SSR mining and enrichment of SSR containing sequences

We took rice as an example to enrich polymorphic SSRs. The three transcriptomes assembled were renamed "T1", "T2" and "T3", respectively.

MIcroSAtellite identification tool (MISA; http://pgrc.ipk-gatersleben.de/misa) was employed for SSR mining from different transcriptomes with the following settings (SSR motifs and number of repeats): dimer-6, trimer-5, tetramer-5, pentamer-5 and hexamer-5. In order to reduce the rate of false

positives, a Python code was written to rule out the sequences which only contain mononucleotide repeats, compound SSRs, or end with SSRs. The command line is written as follows:

```
from Bio import SeqIO
import os
samples=['T1','T3','T4']
for sample in samples:
ids=[]
faD=SeqIO.to_dict(SeqIO.parse(open(sample+'.fa'),'fasta'))
for la in open(sample+'.ssr'):
    if 'ID' not in la:
        aL=la.split('\t')
        ln=len(faD[aL[0]].seq)
        if aL[2]!='p1' and aL[5]!=1 and aL[6]!=ln:
            ids.append(aL[0])
comp_ids=[]
for lb in open(sample+'.ssr'):
    bL=lb.strip().split('\t')
    if 'c' in bL[2]:
        comp_ids.append(bL[0])
fas=SeqIO.parse(open(sample+'.fa'),'fasta')
for fa in fas:
    if fa.id in ids and fa.id not in comp_ids:
        open(sample+'.ssr.fa','a').write('>%s\n%s\n' % (fa.id,str(fa.seq)))
```

And then we renamed all the transcriptomes and all the sequences containing SSRs detected with MISA software by adding different prefixes. Finally, we combined all the sequences containing SSRs from different transcriptomes into a file. The command line is written as follows:

```
from Bio import SeqIO
samples=['T1','T3','T4']
for sample in samples:
```

```
fas=SeqIO.parse(open(sample+'.Trinity.fasta'),'fasta')
for fa in fas:
    open(sample+'.fa','a').write('>%s.%s\n%s\n' % (sample,fa.id,str(fa.seq)))
for la in open(sample+'.Trinity.fasta.misa'):
    if 'ID' not in la:
        la=sample+'.'+la
open(sample+'.ssr','a').write(str(la))
```

Alignment of containing SSR sequences

Sequences containing SSRs were clustered using the default parameters of the CD-HIT tool at 90% sequence identity level.

Rename the SSR file

We then edited a Python code that generated the reverse complement of the minus strand transcripts, according to the strand information in the output of CD-HIT:

```
import re
from Bio import SeqIO
faD=SeqIO.to_dict(SeqIO.parse(open('all.ssr.fa'),'fasta'))
baseD={'A':'T','T':'A','G':'C','C':'G'}
samples=['T1','T3','T4']
for sample in samples:
for line in open(sample+'.ssr'):
    lst=line.strip().split('\t')
    if lst[0] in faD:
        if 'c' not in lst[2] and lst[2]!='p1' and 'ID' not in line and lst[-2]!="1" and int(lst[-1])!=len(faD[lst[0]].seq) and int(lst[-1])!=len(faD[lst[0]].seq)-1 and int(lst[-1])!=len(faD[lst[0]].seq)-2 and int(lst[-1])!=len(faD[lst[0]].seq)-3:
            ma=re.findall('\((.+)\)',lst[3])[0]
            maL=list(ma)
            rc=''
```

```
                for base in maL:
                    if base in 'ACGT':
                        rc+=baseD[base]
                rc=rc[::-1]
                ss=[ma,rc]
                ss.sort()
                ss='('+'/'.join(ss)+')'
                newline=re.sub('\(([ACGT]+)\)',ss,line)
                open(sample+'.ssr.reformed','a').write(str(newline))
```

And then, we edited a Python code that generated reverse complementary of minus strand transcripts, according to the strand information in the ouput of CD-HIT:

```
from Bio import SeqIO
import re
sD={}
for la in open('cd-hit.clstr'):
 if 'at' in la:
    id=re.findall('>(.+)\.\.\.',la)[0]
    strand=re.findall('([+-])+\/',la)[0]
    sD[id]=strand
 if '*' in la:
    id=re.findall('>(.+)\.\.\.',la)[0]
    sD[id]='+'
fas=SeqIO.parse(open('all.ssr.fa'),'fasta')
for fa in fas:
 if fa.id in sD:
    if sD[fa.id]=='+':
        open('plus.ssr.fa','a').write('>'+str(fa.id)+'\n'+str(fa.seq)+'\n')
    if sD[fa.id]=='-':
        seq=fa.seq.reverse_complement()
```

```
        open('plus.ssr.fa','a').write('>'+str(fa.id)+'\n'+str(seq)+'\n')
```

Enrichment of sequences containing polymorphic SSRs

A script was executed to enrich sequences with different repeats from all the sequences containing SSRs:

```
import re
from collections import defaultdict
from Bio import SeqIO
import os
def getD(ssr):
s=[]
for la in open(ssr):
    if 'ID' not in la:
        aL=la.strip().split('\t')
        ma=re.findall('\(.+\)\d+',aL[3])
        s.append((aL[0],ma[0]))
d=defaultdict(set)
for k, v in s:
    d[k].add(v)
return d
t1D=getD('T1.ssr.reformed')
t2D=getD('T3.ssr.reformed')
t3D=getD('T4.ssr.reformed')
allD={}
allD.update(t1D)
allD.update(t2D)
allD.update(t3D)
page=open('cd-hit.clstr').read()
clusters=re.findall('(.+?)>Cluster',page,re.S)
for cluster in clusters:
```

```
224         trans=re.findall('T\d\.TR\d+\|c\d+_g\d+_i\d+',cluster,re.S)
225         if len(trans)>1:
226             tt=[]
227             ss=[]
228             for tran in trans:
229                 if tran in allD:
230                     tt.append(str(tran)+':'+str(list(allD[tran])))
231                     ss+=list(allD[tran])
232             if len(tt)>1:
233                 ma=re.findall('\)\d+\'',str(ss))
234                 ma=set(ma)
235                 mas=re.findall('\((.+?)\)',str(set(ss)))
236                 ssr=''
237                 for mm in list(set(mas)):
238                     if mas.count(mm)>1:
239                         ssr=mm
240                 if len(ma)>1 and len(mas)>len(set(mas)):
241                     ttt=[]
242                     for t in tt:
243                         if ssr in t:
244                             ttt.append(t)
245                     na=str(ttt).lstrip('[').rstrip(']').strip('"').replace('", "','\t')
246                     open('enrichment.SSRs','a').write(str(na)+'\n')
247     for ll in open('enrichment.SSRs'):
248         ma=re.findall('([a-zA-Z0-9]+)\.TR\d+',ll)
249         ma=set(ma)
250         if len(ma)>1:
251             open('enrichment.SSRs.txt','a').write(str(ll))
252     faD=SeqIO.to_dict(SeqIO.parse(open('plus.ssr.fa'),'fasta'))
253     n=0
```

```
    for la in open('enrichment.SSRs.txt'):
    aL=la.strip().split('\t')
    for it in aL:
        id=it.split(':')[0]
        open('cluster'+str(n),'a').write('>'+str(id)+'\n'+str(faD[id].seq)+'\n')
    os.system('muscle -msf -in cluster'+str(n)+' -out cluster'+str(n)+'.muscle')
    n+=1
os.system('mkdir muscle; mv cluster* muscle; rm enrichment.SSRs')
```

Validation experiments

Primers were designed using Primer premier 5 software. The PCR products were separated by capillary gel electrophoresis using the ABI 3100 Genetic Analyser. The peak heights and fragment sizes were analyzed using GeneMarker software.

Data analysis

Previous studies on SSR development in animals and plants, along with their frequency of polymorphic SSR markers, were randomly downloaded from the internet (Table S3). Differences in mean value of the frequency of polymorphic SSRs developed by three methods were analyzed using one-way analysis of variance (ANOVA).

Competing interests

The authors declare no conflict of interest.

Author contributions

W.L. and Q.L. conceived and designed the experiments; H.Q., X.W. and Q.Z. performed the experiments and analyzed data; W.L., and Q.L. prepared the manuscript. All authors read and approved the final manuscript.

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Supplementary information

Table S1. The putative polymorphic SSRs enriched by this method in rice (*O. sativa*), grass carp (*C. idella*) and lined seahorse (*H. erectus*).

Table S2. The SSR loci validated by experiments and characteristics of the primers.

Table S3. The species and the frequency of polymorphic SSRs cited in this study.

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313 **Table 1** Number of SSRs, polymorphic SSRs frequency of three verified species

	Species for verification		
	Rice	**Grass carp**	**Lined seahorse**
Source of the transcriptome	SRR1799209 SRR1974265 SRR2048540	SRR1618540 SRR1618542	SCSIO-CAS
Total number of SSRs in transcriptome	29,517	21,959	19,006
Number of putatively polymorphic SSRs enriched by this program	299	206	600
Number of primers designed for validation experiments	20	30	60
Number of primers amplified with clear product bands (%)	20 (100%)	28 (93.33%)	55 (91.7%)
Number of polymorphic SSRs (%)	19 (95.00%)	26 (92.86%)	50 (90.91%)

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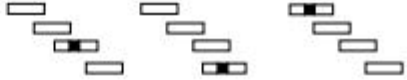
317 **Figure Legends**：

318 **Figure 1. Flowchart of polymorphic markers enrichment and development.**

319 **Figure 2. Comparison of polymorphic marker frequency developed by different methods.** A, B
320 and C represents SSRs developed by traditional methods, HTS approach and the method designed in
321 this study, respectively; * and ** represents significance at P = 0.05 and P = 0.01, respectively; #
322 represents SSR developed by HTS; † represents SSR developed by this method. The data of the
323 frequency of polymorphic SSRs used in the figure was cited from the published references (Table S1).

Step 1. Transcriptome data downloading or RNA-Seq

Transcriptome data are downloaded from the database;
or total RNA is sequenced by next-generation sequencing technology.



Step 2. Sequence assembly

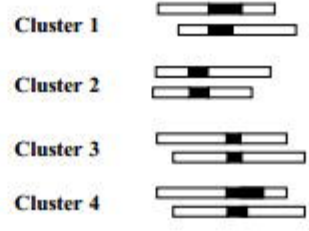
Raw reads are assembled using Trinity.

Step 3. SSR mining and enrichment of containing SSR sequences

MISA is used to detect the reads containing microsatellite repeated motifs;
The containing SSRs reads are enriched by this peogram.

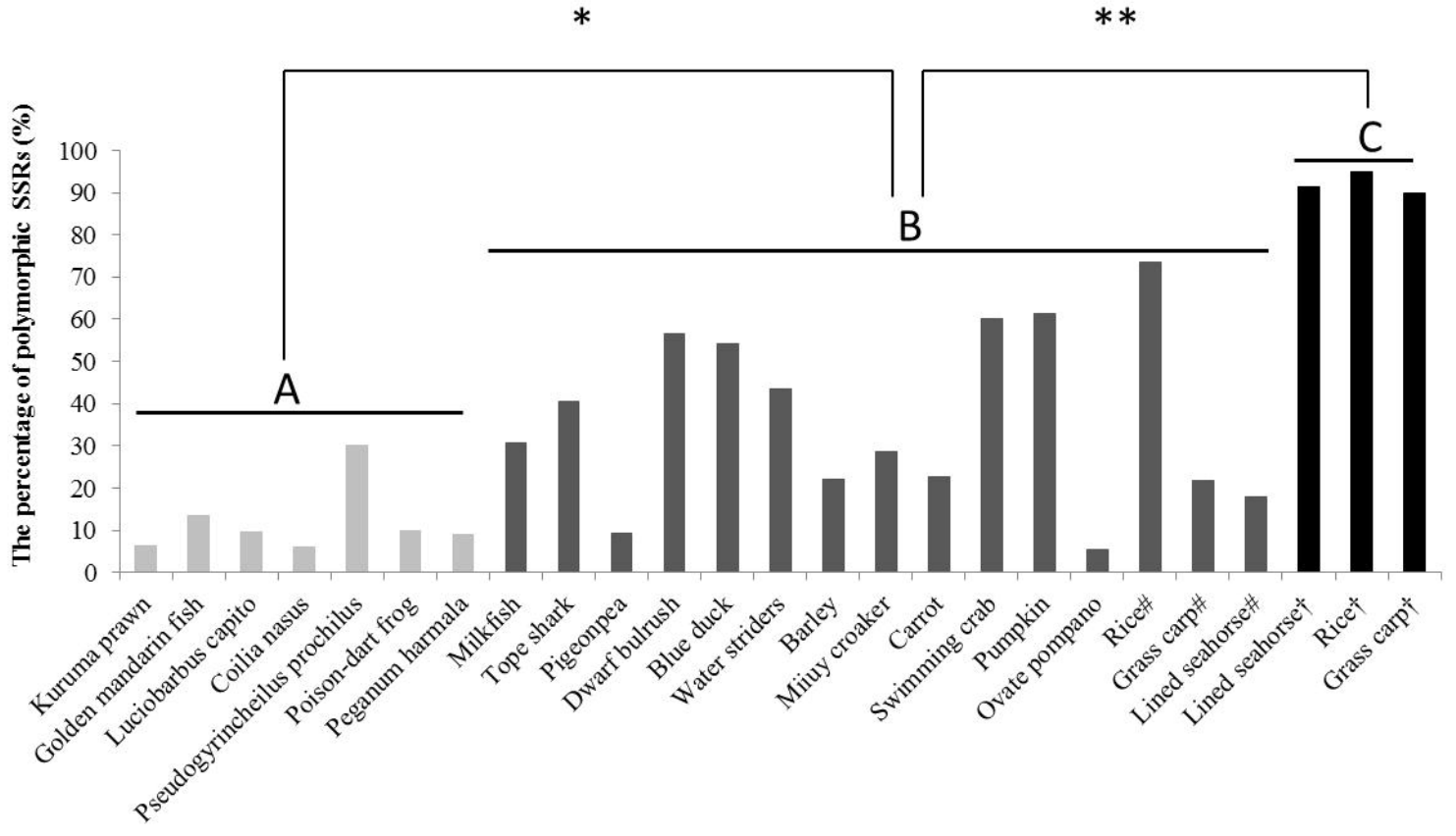
Step 4. Sequence alignment

Sequences which contain SSRs are done sequence alignment by this program.



Step 5. Enrichment containing polymorphic microsatellite sequences

Sequences which contain SSRs with different repeat numbers are enriched.


*
**
A
B
C
The percentage of polymorphic SSRs (%)
100
90
80
70
60
50
40
30
20
10
0
Kuruma prawn
Golden mandarin fish
Luciobarbus capito
Coilia nasus
Pseudogyrincheilus prochilus
Poison-dart frog
Peganum harmala
Milkfish
Tope shark
Pigeonpea
Dwarf bulrush
Blue duck
Water striders
Barley
Miiuy croaker
Carrot
Swimming crab
Pumpkin
Ovate pompano
Rice#
Grass carp#
Lined seahorse#
Lined seahorse†
Rice†
Grass carp†