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Research Article

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Effect of Different Probe Hybridization Methods on the Yield of Simple Sequence Repeat Loci in *Clarias batrachus*

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ABSTRACT

Probe hybridization is one of the most critical steps in the identification of simple sequence repeat loci from a repeat enriched genomic DNA library further used for Sangers or next generation sequencing. Here we report the comparative analysis of three different approaches used for probe hybridization in a repeat enriched genomic DNA library of Clarias batrachus. The percentage of SSR containing sequence for Bloor et al., 2002, Glen et al., 2007and modified method was found to be 26%, 29.42% and 42% respectively. The maximum number of di (101) and trinucleotide (18) repeat motif was found with Glen et al., 2007 protocol. Tetranucleotide repeat motif was found to be maximum (7) with the modified protocol. We conclude from our study that the modified protocol described in this paper increases the yield of specifically non-targeted loci.

Key words: *Simple sequence repeat motif, Probe hybridization, Comparison, Efficiency, Non targeted.*

INTRODUCTION

Last two decades have witnessed the major transformation in fisheries and aquaculture but without looking into several important issues such as sustainability and impact on the environment. Large attention is required for both conservation and aquaculture and it is high time to incorporate the concept of genetic management.

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Genetic management not only help in the diagnosis of genetic health through genetic surveillance of natural stocks but also play a role in the identification of genetic processes responsible for bringing about genetic changes in stocks such as inbreeding, inappropriate selection of brooder, artificial selection, monitoring of sea ranching etc[1]. Microsatellite markers are a marker of choice since a long time for genetic management due to abundance, high polymorphism and codominance. Several protocols were developed time to time for efficient development of microsatellite markers. Some of the widely used protocols are screening small insert DNA library, microsatellite enrichment library, public database mining etc. Comparative study of different methodologies was performed by several researchers. Enrichment library method is widely used for microsatellite marker development. The success of library enrichment is directly dependent upon the step of probe hybridization and hence it is one of the most critical steps with a direct effect on the yield. Different probe hybridization protocols are available such as single or multiple incubation temperatures and one or mixture of probes at a time etc. Present study is aimed to compare a modified protocol with two widely used probe hybridization protocols Bloor *et al*., and Glen *et al*., using tri and tetranucleotide repeat probes[2,3].

MATERIAL AND METHODS

Generation of genomic DNA fragments through partial digestion

Clarias magur muscle or fin tissue (~100 mg) was collected in a fresh 2 ml microfuge tube using sterile scissors and forceps and cut into small pieces. Genomic DNA (gDNA) was isolated using phenol-chloroform method following Sambrook *et al.*[4] The integrity of the isolated gDNA was checked by 1% agarose gel electrophoresis and 260/280 value (approximately 1.8) in a spectrophotometer. Partial restriction endonuclease (RE) digestion was performed to yield maximum fragments in the range of 400-1000 bp. Restriction enzyme digestion conditions were optimized by varying the incubation time. For this 2µg of gDNA was digested with *Sau*3a1 (5U/µl) for 5, 10, 15, 20, 25 and 30 min at 37°C. The restriction enzyme digest was heat inactivated at 85°C for 20 minutes. The reaction product was checked on 1% agarose gel along with 100 bp ladder. The RE conditions were further fine-tuned by testing incubation times between 5 – 10 min, viz. 6, 7, 8, 9 and 10 min. Bulk gDNA partial RE digestion was set up based on the results to yield DNA fragments in 400-1000 bp size range.

Adapter Ligation and Amplification of Fragments

Sticky end adapters with *Sau*3a1 compatible ends was prepared by annealing Oligo A (GGCCAGAGACCCCAAGCTTCG; 21mer) and Oligo B (GATCCGAAGCTTGGGG TCTCTGGCC; 25mer) as reported by Bloor *et al*. (2001). For adapter preparation 1000 pmol of each of oligonucleotide A and B were mixed from a 10pmol/µl stock in a microfuge tube by gentle pipetting and denatured at 80°C for 2-5 min in a thermocycler (BioRad Pvt Ltd., USA). The denatured mixture was allowed to re-anneal by slowly cooling to room temperature for 1 h. The adapter was diluted to a final concentration of 25 pmol/µl with nuclease-free water and stored at -20°C until further use.

Adapter quantity was optimized by setting pilot ligation reactions using varying quantities of the adapter (25, 50, 75, 100 pmol) per µg of RE digested DNA in 25 µl ligation reaction volumes. The ligation was performed by overnight incubation at 16^{o}C. Adapter ligation was confirmed by a test PCR reaction using 3 µl of the ligation mixture, 30pmol of OligoA and 12.5 µl of 2x PCR master mix (ThermoScientific, USA) in a final volume of 25 µl. The PCR cycling conditions included an initial denaturation at 95^{o}C for 5 min followed by 30 cycles of denaturation at 95^{o}C for the 50s, annealing at 56^{o}C for 1 min, extension at 72^{o}C for 2 min and final extension for 10 min at 72^{o}C. The adapter concentration that yielded PCR products concentrated within 400-1000bp range with minimal amplification

beyond this size range was considered optimal. The optimal adapter concentration identified from the pilot studies was used to set up the final adapter ligation reaction. The ligation mixture included 10 µg of RE digested genomic DNA, optimized quantity of adapter, 50U of T4 DNA ligase, 1x ligase buffer, and nuclease free water to makeup volume to 150ul. This ligated product was amplified by PCR in a PCR reaction mix containing 8 µl of the ligation mix, 20 picomoles of OligoA, 25 µl of 2x PCR mastermix and nuclease free water in a final volume of 50 µl. PCR conditions were the same as that for pilot reactions. Bulk PCR product (400µl) was run on 2% NuSieve GTG (LONZA, USA) agarose gel (7 x 10 cm) using a preparative comb along with 100 bp plus DNA ladder at 60V for 1h and the agarose gel containing 400-1000 bp fragments was excised using a sterile microscope cover-slip. DNA was extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen, NL) following the supplier's guidelines. Eluted DNA was quantified using Nanodrop (Thermo Scientific, USA) and stored at -20^{o}C till further use.

Microsatellite Probe Selection

The probes were selected based on the type of microsatellites to be developed, in this case tri and tetra-nucleotide repeats. Based on the available information regarding the abundance of the type of microsatellites , 5 tri nucleotide [$(ACT)_{8,}$ $(TGA)_8$, $(GTT)_8$, $(TAT)_8$ & $(TAA)_{8,}$] and 4 tetra-nucleotide [$(AGAT)_{6,}$ $(GTTT)_6$ $(CTGT)_6$ & $(ACAG)_6$] probes were designed with biotinylation at 3' end. Lyophilized oligo was dissolved in sterile 1X TE buffer, pH 7.0 to obtain a stock solution of 100pmol/µl (100µM) and stored at -20°C.

Enrichment for Microsatellite Containing DNA Fragments

The success of microsatellite marker development is largely governed by this step. We have initially followed method of Bloor et al., and Glen et al., and further modifications were done to assess its effect on final yield[2,3]. In addition to established protocols from Bloor et al., and Glenn et al., we used an additional modified protocol that incorporated elements from both of these protocols for probe hybridization[2,3]. While the protocol of Bloor et al., relies on the use of a single probe and a single hybridization temperature, Glen et al., employs an equimolar mixture of 3' biotinylated oligos with a range of hybridization temperatures at a time[2,3]. Our protocol employed a mixture of probes, but all with the same hybridization temperature for probe hybridization at a time. The same stock of adapter ligated DNA was used for validation of the three protocols. In the protocol by Bloor et al., the individual probes were first captured on the streptavadin-coated magnetic beads (M-280 Dynabeads, Dyna l) which were then used to hybridize to the adapter ligated DNA[2]. Conversely in the other two protocols, the probe mixture was hybridized to the adapter ligated DNA first and the hybridized mixture was captured by Dynabeads.

Probe Hybridization (Based on Bloor et al.[2])

Briefly, 100 µl of M-280 Dynabeads (10mg/ml) was washed stringently in an equal volume of 1 X Washing/Binding (W/B) buffer (0.5M NaCl, 5mM Tris HCl and 0.5mM EDTA, pH 7.5) and 200pmol of 3'-biotinylated oligonucleotide was captured onto the washed beads as per manufacturer's instruction. These beads were washed once in 400 µl of 1 X W/B and twice in 400 µl of 6 X SSC (prepared from 20X SSC- 3 M Nacl and 0.3 M Sodium Citrate, pH 7.0), re-suspended in 50 µl of 6X SSC and incubated at the probe-specific hybridization temperature. A mixture of 30 µl of adapter ligated DNA ,0.25 µl of Oligo A (20 pmol), 15 µl of 20X SSC and 4.75 µl of nuclease free water was prepared and incubated at 95°C for 10 min in a thermocycler. As the temperature ramped down to 60°C the prepared probe suspension was added and mixed by gentle pipetting. The mixture was incubated at probe specific hybridization temperature for 30 min with gentle agitation after every 5 min. To enhance the specificity of hybridization the temperature was increased to 10^{o}C above the hybridization temperature and incubated for 2 h. Subsequently the hybridized beads were

washed repeatedly using SSC buffer, aliquoted and resuspended in 100 µl 1x SSC buffer, and incubated at the probe-specific temperatures for 10 min. The beads were subsequently rinsed with 400ul each of 1x TE and 50 mM NaCl at room temperature. Finally, each aliquot was re-suspended in 50µl PCR-grade water (final bead concentration was 5 µg/µl). This suspension was used for PCR.

Probe Hybridization (Based on Glen *et* al.[3])

The reaction mixture constituted of 10 µl of adapter ligated DNA, 25 µl of 2x Hyb solution, nuclease free water and 10 pmol of the oligo probe mix. The incubation conditions that were set in a thermocycler included 95°C for 5 min followed by a quick ramping to 70°C and extremely gradual cooling to 50°C by reducing 0.2°C every 5 s for 99 cycles. The sample was kept at 50°C for 10 min before gradual cooling to 40°C, this time by reducing 0.5°C every 5 s for 20 cycles. This was followed by a quick ramping down to 15°C. The logic was to denature everything, quickly go to a temperature slightly above the annealing temperature of the oligos in the mixes used, and then slowly decrease, giving the oligos opportunity to hybridize with DNA fragments that they most closely match when the solution is at or near the oligo's annealing temp.

Probe Hybridization with Modified Protocol

The reaction mixture was similar to the protocol of Glenn et al. except that different pairs of probes [$(ACT)_8$ & $(TGA)_8$; $(ACT)_8$ & $(GTT)_8$; $(TGA)_8$ & $(GTT)_8$; $(TAT)_8$ & $(TAA)_8$; $(AGAT)_6$ & $(GTTT)_6$ and [$(CTGT)_6$ & $(ACAG)_6$] was used based on their similarity of Tm[3]. 3 µl of each probe in a paired combination was used from a 10pmol/ µl stock. Incubation conditions for probe hybridization were selected based on the Tm of the probe pair. For the first three probe combinations, the conditions were - 95°C for 15 min; 70°C for 30 min; 65°C for 30 min; 60°Cfor 30 min; after which temperature is reduced by 2°C after every 30 min until it reaches to 44°C where the mixture was placed for 30 min and then removed for further step. For the fourth probe combination a similar strategy was followed only that the minimum temperature used was 40°C.

Collection of Probe Hybridized DNA Fragment

50 µl of streptavadin-coated magnetic beads (10mg/ml) (M-280 Dynabeads, Dynal) was taken in a 1.5 ml sterile microcentrifuge tube and washed in 250 µl of 1XTE by gentle pipetting. The tube containing mixture was placed on magnetic particle concentrator (MPC) stand for 3 min until the magnetic beads got accumulated on one side then the liquid was remove. Beads were further washed thrice with 1X TE buffer and then once with 1X Hyb solution and finally suspended in 150 µl 1X Hyb. The DNA and probe mixture (50µl) from above were added to the 150 µl of dynabead suspension. The mixture was incubated on orbital shaker at slow speed (130 rpm) for 1 hr at room temperature. Subsequently, the beads were captured using MPC and supernatant was removed by pipetting. Dynabeads were washed twice with 400 µl 2X SSC, containing 0.1% SDS. During each washing step the beads were re-suspended properly by pipetting before harvesting. Again beads were washed twice with 400 µl of 1XSSC/ 0.1% SDS. Two final washes were done with the same wash buffer warmed to 50°C (within 5-10°C of the Tm for the oligo). Finally, the beads were suspended in 200 µl 1X TE, vortexed and incubated at 95°C for 5 min. A fresh autoclaved 1.5 ml tube was placed in MPC and beads were transferred from above to this tube without delay. From here supernatant was pipetted out immediately and transferred to a new labeled tube (~150µl) (This supernatant contains enriched fragments of DNA). To this supernatant 22µl of NaOAc/EDTA solution was added and mixed by pipetting. Enriched DNA was precipitated by adding 500 µl of 95% ethanol. The contents were mixed by inverting the tube and then placed on ice for 30 min. The DNA was pelletized by centrifugation at 13000 rpm for 12 min. The supernatant was discarded and 500 µl of chilled 70% ethanol was added and

centrifuged at 13000rpm for 10 min. Supernatant was discarded carefully using pipette and sample was placed in laminar flow for air drying (around 20 min). The pellet was resuspended in 25µl of 1X TE and stored at 4°C overnight for rehydration.

Amplification of Enriched DNA Fragments and T/A cloning

Eight PCR reactions (PCR mastermix, Fermentas, USA-25 µl, Oligo A (10 pmol/ µl)-3 µl, DNA- 5 µl and Nuclease-free water-17 µl to make up the final volume to 50 µl) were prepared with 50 µl reaction volume. PCR cycling conditions were as following - initial denaturation at 95°C for 3 min followed by 5 cycles of denaturation at 95°C for 30 s, 60^{o}C for 30s and extension at 72°C for 45s, followed by 30 cycles of initial denaturation at 92°C for 30s, 60 °C for 30s and extension at 72°C for 55s and final extension for 30 min at 72^{o}C. PCR product was separated on agarose gel and fragments of 400-1000 bp were excised and eluted using QIAquick Gel extraction kit (Qiagen, NL). The concentration of eluted DNA was estimated using Nanodrop spectrometer. Eluted DNA was cloned following the principle of T/A cloning and transformed in to *E. coli* DH5α using InsTAclone™ PCR Cloning Kit (Thermo Scientific, USA).

Sequence analysis

White colonies were tested for the presence of insert using colony PCR (2X PCR master mix (Fermentas, USA)- 12.5 µl, M13 forward and reverse primers (10 pmoles/ µl)-1 µl each, Colony suspension- 1 µl, and Nuclease-free water- 9.5 µl). The PCR cycling conditions were as following - initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for the 50s, annealing at 55°C for 30s and extension at 72°C for 2 min and final extension for 10 min at 72°C. The insert size was determined by running the PCR product on 1% agarose gel against a molecular size standard. Clones with insert size above 600 bp were grown on freshly prepared LB-Amp stab (in 2 ml microfuge tube) and sent for sequencing. Sequences were analyzed using three software namely vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), clustalw (http://www.ebi.ac.uk/ Tools/msa/clustalw2/) and Gene Runner v. 5 to remove vector sequences as well as adapter sequences. After this step, the sequences were analyzed for the presence of microsatellite repeats by using three online software http://mail.nbfgr.res.in/fishmicrosat/repmap.php, http://wsmartins.net/websat/ and http://www.biophp.org/minitools/microsatellitrepeatsfinder/demo.php.

RESULTS AND DISCUSSION

Microsatellite markers have proven to be extremely important tools in genetic management because of their high polymorphism, abundance and co-dominance Liu and Cordes[5]. Although di-nucleotide repeats are dominant in vertebrate genomes and have been widely used, alleles that differ by a single repeat unit are confused with stutter bands and hence pose difficulties while scoring. Hence, researchers are now focusing towards developing tri and tetra nucleotide repeats Naish and Skibinski[6]. These repeats are more useful as they can be transformed into Type-1 markers after analysis Toth et al.[7].

Microsatellite loci have largely been identified by screening genomic DNA libraries enriched for repeat sequences by hybridization with synthetic repeat containing probes[8,9,2,3]. Some researchers also mined ESTs available in public databases for repeats associated with genes[10,11]. High throughput sequencing is becoming a boon for SSR mining in fish and several loci have been identified from NGS data[12,11]. However, identification of novel microsatellite loci from repeat enriched genomic DNA library is still the most popular methodology due to simplicity and cost-effectiveness. As a first step in the generation of repeat enriched genomic DNA library, partial digestion of genomic DNA by *Sau*3A1 was standardized to generate fragments largely in the range of 400 to 1000 bp. The reaction mixture was incubated for different durations including 30, 20, 10 and 5 min and the results required further fine tuning of incubation time between 5 and 10 min. Hence, the second set

of digests was set up for 6, 7, 8 and 9 min. Best result was obtained when the digest was incubated for 9 min (Fig. 1). These conditions were used for bulk digestions. The optimum level of the adapter was further estimated and best results were obtained when adapter concentration was kept at 25 pmol/ µg of RE digested DNA (Fig. 2).

The probes for hybridization were selected based on the available information from literature survey on the type of microsatellites to be developed. In this study, our aim was to develop tri and tetra nucleotide repeats from enriched genomic DNA library of *C. magur*. It has been reported that among the tri-nucleotide repeats, AAT, TTG, TAA and TGA are the most abundant in *C. magur* accounting for 75.24 % each followed by CTT, GAA, CAT and AAC (4% each). Remaining six types of tri-nucleotide repeat types (CAC, GCA, CCT, GTG, GCT &TAG) are found at low level 1.9% Mohindra *et al*.[13]. Among the tetranucleotide repeats reported from *C. magur*, GTTT, TAAA and TTCT are reported to be the most abundant. Whereas other types of tetra-nucleotide repeats (CTTC, TCTA, TATT, TTAA, TGGA, TCTG, TGTA, GTGC and AGAA) were found only once in ESTs derived microsatellites[13]. This information was used for the designing of biotynilated probes in this study. The probe hybridized DNA was PCR amplified (Fig. 3) and further used for T/A cloning and then positive clones with fragment size more than 400 bp were sent for sequencing.The protocol for repeat enrichment by hybridization depends heavily on the probe hybridization efficiency. We report the comparative analysis of efficiency of three different protocols for probe hybridization in this study. Initially, protocol of Bloor et al., (2001) was followed (only one probe at a time and single probe hybridization temperature) which yielded 26 repeat-containing sequences out of 100 clones. Following the protocol of Glen et al.[3] where a mixture of the probes with a range of probe hybridization temperatures was used yielded 33 repeat containing sequences out of 112 random clones sequenced (29.46%). A modified protocol where probes with same hybridization temperature were used together yielded 42% repeat containing sequences (34 out of 81). Out of a total of 424 white colonies screened, 298 contained inserts $\geq$400 bp and were commercially sequenced. Sequences were analysed for quality and the presence of tandem repeats using the online software as mentioned earlier. In several sequences more than one microsatellite locus was present. Repeats separated by more than 70 bp were counted as a separate locus and totally 174 loci were identified out of 93 repeats containing sequences of which 60.34 % were perfect microsatellite loci. Apart from these, 9 imperfect, 49 complex, and 11 compound loci were also identified. The number of mono-, di-, tri- and tetra-nucleotide repeats were 78, 203, 27 and 11, respectively (Table 1). Several workers have observed that the microsatellite repeats in the cloned sequences do not necessarily match the repeat sequences used as probes. In the modified approach used in this study, hybridization was carried out at higher temperatures for a longer duration in order to minimize non-specific binding and allow sufficient time for binding with the specific probe. This not only resulted in the better recovery of repeat sequences, also allowed us to trap repeats that matched the probe sequences. Nunome *et al*.[14]reported that high hybridization temperature allows enrichment of specific and fewer repeat sequences and that the level of enrichment was essentially constant for temperatures from 55 to 65°C. Therefore, lower hybridization temperature may be used to avoid the loss of less specific repeats like compound microsatellite repeats as can be seen with the protocol of Glen *et al.*[3] and the modified protocol. In the current study, many sequences had stretches of two or more different type of repeats, which made them complex microsatellite loci. Hence, the hybridization temperature may further be optimized to increase the specificity of the repeat sequences. We identified a total of 319 repeat motifs and the dominating type are the dinucleotides repeats (63.6 %) as expected, TG/GT repeats being the most abundant

(33.66% of total dinucleotide repeats). Similar results were recently reported by Mohindra *et al*., for *C. magur*, who found di-nucleotide repeats to be the dominant type (53.2%) with GT being the most abundant repeat[13]. It is also reported that the majority of microsatellites (30-67%) found in the genome of vertebrates are di-nucleotides with AC/GT repeats being predominant[7]. Dinucleotide repeat motif yield was maximum with the protocol of Glen *et al.*[3] 49.75% than other two protocols 21.67% in Bloor et al.[2] and 28.57 % with the modified method. Total nine tetranucleotide repeats were identified here predominantly by modified method seven and two by the method of Glen *et al*[3]. The possible reason is could be lower hybridization temperature that causes nonspecific binding and capture of repeat motifs of smaller size against larger probe size[15].

Table 1: Sequence analysis

	Total	**Bloor *et al*., 2002**	**Glen *et al*., 2007**	**Modified method**
Clones sequenced	293	100	112	81
Sequences with repeats	93	26 (26%)	33 (29.4%)	34 (42%)
Total no of loci	174	31 (31%)	79 (70.5%)	64 (79%)
Perfect loci	105	18 (58%)	47 (59%)	40 (62%)
Imperfect loci	9	4	1	4
Compound loci	11	1	2	8
Complex loci	49	8	29	12
Mononucleotide repeat	78	5	62	11
Dinucleotide repeat	203	44	101	58
Trinucleotide repeat	27	0	18	9
Tetranucleotide repeat	11	2	2	7

Captions to illustrations

Fig. 1: Partially digested genomic DNA of *C. batrachus.* Lane M: 100 bp plus ladder (Fermentas); L1: *Sau*3A1 digest after 9 min incubation

Fig. 2: Test PCR of adapter ligated DNA fragments. Lane M: 100 bp plus (Fermentas);
1-4: PCR product of adapter ligated DNA at 25, 50,75 and 100 pmol adapter / µg DNA,respectively

Fig. 3: PCR product of probe captured DNA. Lane M: 100 bp plus ladder(Fermentas); L1 2:PCR products of probe captured DNA

Fig. 1

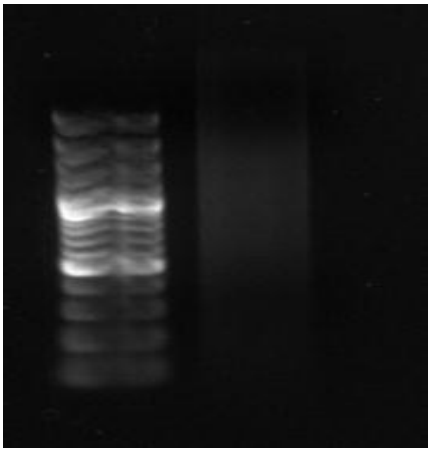


Fig. 2

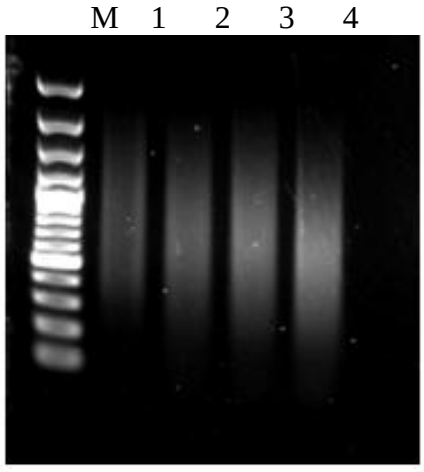
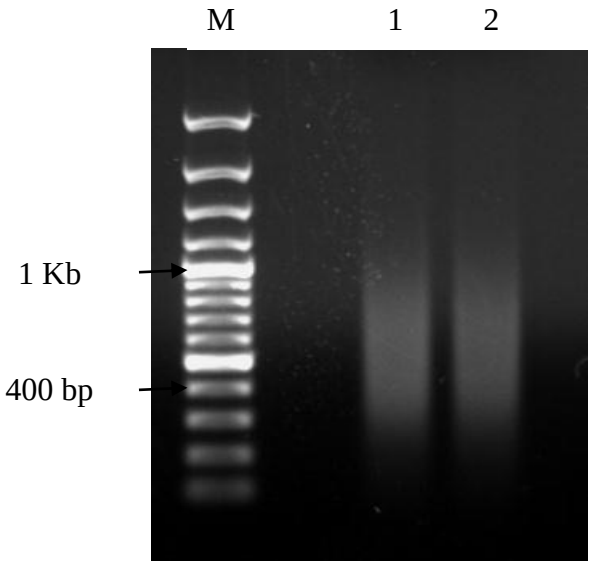


Fig. 3



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REFERENCES

1. Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P., Mormède, P., Prunet, P. and Vandeputte, M., Genetics of adaptation and domestication in livestock. *Livestock Production Science*, **93(1):** 3-14 (2005).
2. Bloor, P.A., Barker, F.S., Watts, P.C., Noyes, H.A. and Kemp, S.J., Microsatellite library by enrichment. *School of Biological Sciences, University of Liverpool, Liverpool, UK*. (2001).
3. Glenn, T.C. and Schable, N.A., Isolating microsatellite DNA loci. *Methods in enzymology*, **395:** 202-222 (2005).
4. Sambrook J, Fritsch E F, Maniatis T, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press (1989).

5. Liu, Z.J. and Cordes, J.F., DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, **238(1):** 1-37 (2004).
6. Naish, K.A. and Skibinski, D.O.F.Tetranucleotide microsatellite loci for Indian major carp. *Journal of Fish Biology*, **53(4):** 886-889 (1998).
7. Toth, G., Gaspari, Z. and Jurka, J. Microsatellites in different eukaryotic genomes: survey and analysis. *Genome research*, **10(7):** 967-981 (2000).
8. Yue, G.H., Kovacs, B. and Orban, L. Microsatellites from Clarias batrachus and their polymorphism in seven additional catfish species. *Molecular Ecology Resources*, **3(3):** 465-468 (2003).
9. Swain, S., Das, S.P., Bej, D., Patel, A., Jayasankar, P., Das, P.C. and Das, P. Isolation and characterization of sixteen microsatellite loci for fringe-lipped carp, Labeo fimbriatus. *Conservation Genetics Resources*, **4(4):** 913-915 (2012).
10. Liu, X., Luo, W., Zeng, C., Wang, W. and Gao, Z., Isolation of new 40 microsatellite markers in mandarin fish (Siniperca chuatsi). *International journal of molecular sciences*, **12(7):** 4180-4189 (2011).
11. Xu, D., Zhang, Y. and Peng, Z. High-throughput microsatellite marker development in Amur catfish (Silurus asotus) using next-generation sequencing. *Conservation Genetics Resources*, **5(2):** 487-490 (2013).
12. Sahu, B.P., Patel, A., Sahoo, L., Das, P., Meher, P.K. and Jayasankar, P. Rapid and cost effective development of SSR markers using next generation sequencing in Indian major carp, Labeo rohita (Hamilton, 1822). *Indian J. Fish*, **59(2):** (2012).
13. Mohindra, V., Singh, A., Patangia, R., Tripathi, R.K., Singh, R.K., Sah, R.S. and Lal, K.K., Characterization of 27 novel gene-associated SSR markers in Indian catfish, Clarias batrachus (Linnaeus, 1758) and their application in genetic diversity analysis. *Mol. Ecol. Resour*, **12:** 1196-1197 (2012).
14. Nunome, T., Suwabe, K., Iketani, H., Hirai, M. and Wricke, G. Identification and characterization of microsatellites in eggplant. *Plant Breeding*, **122(3):** 256-262 (2003).
15. Zhan, A., Bao, Z., Hu, X., Lu, W. and Hu, J. Methods comparison for microsatellite marker development: different isolation methods, different yield efficiency. *Journal of Ocean University of China (English Edition)*, **8(2):** 161-165 (2009).