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Research Article

DNA Fingerprinting and Molecular Marker Development for *Baliospermum montanum* (Wïlld.) Muell. Arg.

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ABSTRACT

Baliospermum montanum is one of the medicinal plants, commonly known as 'Denti', belongs to the family *Euphorbiaceae* found in India, Nepal, Burma and Malaya. It has many medicinal values as the pharmaceutical compounds isolated from its secondary metabolites are used for the treatment of various diseases. In the present study, the result shows that the *B. montanum* genomic DNA extracted, which were randomly amplified using 20 set of primers by arbitrary PCR-based DNA fingerprinting (RAPD) for screening, whereby only 16 primers were able to produce bands at a different score while the remaining three primers remain unamplified. However, out of reproducible primers (MAP-1-16), three primers (MAP-1, 12 and 14) were found to produce more specific bands, which were selected for specific amplification analysis. Prior to the sequence analysis of specific amplicons (BM MAP-1, BM MAP-13, and BM MAP-14), the following sequences was retrieved at different size per band, i.e BM MAP-1 (604bp), BM MAP-13 (493bp) and BM MAP-14 (506bp) respectively. However the origin of replication fragments (ORFs) shows BM MAP 01- 604bp has 7 ORFs, BM MAP 13-494bp with 3 ORFs, and BM MAP-506bp has 10 ORFs. In addition, these sequences were submitted to the NCBI website after been analyzed (BLAST) (GenBank accession numbers KX139410.1, KX139411.1 and KX139412.1) that they have no any similarities by the available literature. Thus, could be specifically applicable as a molecular marker of *B. montanum* DNA fingerprint for identification of medicinal plant adulteration or other molecular analysis.

Keywords: *Baliospermum montanum*; RAPD; DNA sequencing; Molecular marker development

INTRODUCTION

Baliospermum montanum is belong to the family *Euphorbiaceae* commonly known as 'Denti' found in Nepal, Burma, Malaya, and India[1], which has so many medicinal value as the pharmaceutical compound isolated from its secondary metabolite such as codeine, L-dopa, reserpine, digitalis, and the anticancer drugs vincristine, taxol, are used in ovarian and breast cancers treatment[2], therefore the plant based formulations are utilized by most of the national and international pharmaceutical companies for many disorders and disease treatment globally[2]. Their root contained phorbol ester and can also be used as an anti-inflammatory while the root past are applied to painful swelling and piles. Seeds are used as powerful hydragogue which promotes watery evacuation of bowels, the seed can also use extensively in rheumatism. However, the seeds are used externally as in snake bite. Leaves used in asthma, abdominal tumor and bronchitis. The root leaves and the seeds were used Ayurveda in liver disorder, snake bite poisoning and abdominal disorder[3]. Due to the invention of molecular markers currently, gives a feasible straight evidence in relation to genetic divergence as well as the inter-relationships amongst organisms at their DNA level, therefore the improvement of a range of molecular marker system was absolutely helpful for genetic assessment of plant species, ecological research, for taxonomy and evolutionary. Although some molecular marker system may principally differ from one another, but their main function is to express the genome-wide variability[4]. Therefore molecular markers were used for identification of plant species genetic diversity, as different types of plant DNA fingerprinting techniques are available[5], such as RAPD which is a PCR-based method of DNA profiling that basically involves the amplification of DNA sequences using random Primers[6]. Although the RAPD method uses arbitrary primer sequences, many of these primers must be screened in order to select primers that provide useful amplification products[7].

MATERIALS AND METHODS

Plant material

The plant was collected from Western Ghats of Tamil Nadu, in the year June 2013 and preserved in the green house of parental institution.

Isolation of genomic DNA

The fresh leaves are collected from green house of grown *B. mantanum*, 3g of plant's leaves were measured and cuts into small pieces, then crushed gently using a glass

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Table 1: DNA finger printing of *B. montanum* using arbitrary PCR method

Primers	Primer Sequence (5'- 3')	No. of bases	No. of bands obtained	Relative Percentage of bands	Range of Amplicons size	Approximate size of amplicons
MAP-1	GTG CAA TGA G	10	07	9.59	380-1800	380,550,850,950, 1100,1300,1800
MAP-2	AGG ATA CGT G	10	02	2.74	400-900	400,900
MAP-3	AAA TCG GAG C	10	04	5.48	375-1200	375,400, 700,1200
MAP-4	AAG ATA GCG G	10	-	-	-	-
MAP-5	GGA TCT GAA C	10	04	5.48	700-1700	700,800, 200,1700
MAP-6	TTG TCT CAG G	10	-	-	-	-
MAP-7	GTC CTA CTC G	10	01	1.37	750	750
MAP-8	GTC CTT AGC G	10	-	-	-	-
MAP-9	TGC GCG ATC G	10	07	9.59	350-1400	350,700,850,900, 1100,1300,1400
MAP-10	AAC GTA CGC G	10	-	-	-	-
MAP-11	GCA CGC CGG A	10	06	8.22	500-3400	500,800,1300,1500,1800,3400
MAP-12	CAC CCT GCG C	10	05	6.85	750-2200	750,800,1200,1700,2200
MAP-13	CAT CCC GAA C	10	04	5.48	500-2300	500,900, 1700,2300
MAP-14	GGA CTC CAC G	10	04	5.48	600-1800	600,700, 1200,1800
MAP-15	AGC CTG ACG C	10	06	8.22	300-1300	300,400,600,750, 900,1300
MAP-16	CTA TCG CCG C	10	02	2.74	500-750	500,750
MAP-17	CGG GAT CCG C	10	05	6.85	350-1800	350,500,750,1500, 1800
MAP-18	GCG AAT TCC G	10	06	8.22	300-1500	300,540,750,800, 1400,1500
MAP-19	CCC TGC AGG C	10	04	5.48	400-1600	400,750, 1200,1600
MAP-20	CCA AGC TTG C	10	06	8.22	300-1100	300,400,500,750, 900,1100

bead, 3ml of extraction buffer was also added, the entire material was transfer into 10 ml polypropylene tube and mixed gently. The mixture was incubated for 1 hour at 60 ^{0}C in a shaking water bath with intermittent mixing for each 10 min. A 3ml of chloroform: isoamylalcohol (24:1) was added and mixed gently by inversion for 15 min, after spins at 8000 rpm for about 10 min at 30 ^{o}C, then the upper clear aqueous layer was carefully transferred to a fresh polypropylene tube. 1.5 ml of 5 M NaCl and an equal volume of isopropanol (0.6 volume) was added and mixed gently without any vortex, then was allowed to stand for 1 hour. The DNA pellet was transferred to 1.5ml microfuge tube, after mixing with isopropanol, the sample was centrifuged at 10,000 rpm for 10 min and discarded the supernatant, then the pallet was washed with 70% ethanol twice, by adding 0.5ml and centrifuged for 5 min at 10,000 rpm, then the supernatant was discarded and dried the pallet using an air blower till the entire alcohol smelling was completely disappear. 0.5ml of high salt TE buffer was added to the pallet and mixed by tapping to dissolve[8].

DNA purification

5µl of DNase was added to the DNA sample and kept in water bath at 37^{o}C, and allowed to stand for 1 hour of the incubation period. After incubation, 1ml volume of chloroform: isoamyl alcohol (24:1) was added and carefully mixed for 10 min then centrifuged at 10,000 rpm for 10 min, the upper layer was transferred to another fresh tube and mixed with a 1/10th volume of 3 M sodium acetate (pH 4.8). 2.5ml of chilled absolute ethanol was also added for precipitation. The pellet was dried using air blower then dissolved by addition of 0.5ml of TE buffer. After purification, the DNA isolate was subjected to electrophoresis for 1 hour at 50V in order to check their quality, by mixing 2µl of DNA isolate with 2µl of loading dye and loaded onto an agarose gel (0.8% w/v) containing 0.5% of ethidium bromide, and DNA maker (1kb DNA ladder) was also loaded to a separate well. Therefore after completing the entire process the gel was visualized by gel documentation system under UV light, which shows a qualitative thick, single band of DNA double-stranded with high molecular weight[5].

DNA fingerprint

RAPD Analysis

RAPD analysis was conducted using 20 random primers which were arbitrarily selected (MAP-1, MAP-2……MAP-20) (MAP=Medicinal and Aromatic plant/primer) for random amplification of DNA sequences. The polymerase chain reaction(PCR) were performed based on the following mixture: 8 µl of 2X master mix; primer (map-1 to 20) 1.5 µl; 1µl of DNA template and 5.5 µl of double distilled water, therefore the entire mixture (16 µl) was centrifuged by microcentrifuge before the PCR amplification process.

Table 2: DNA sequences of specific amplified product of modified arbitrary PCR.

Sequence Name	Size (bp)	Sequence
BM MAP 01	604	1 GGGTACACCC TCCACTCTCC TCTTCCCCTC CGAATGCCTT CCTGCTAATC CCCTGTGCTC 61 CAATTTCTTT TACTACTGGA ACTTATCCAA CAGAGTTCAC TCAAGGATCG TTCCTAATGG 121 CTCCGGGGCG GGTCAATTCG CCTCCGTCTT CGACGTTCTC CGCTCCTTCC CCTGGCCTTT 181 GCTAGGCCGT GCTGTTCCTC CCCTTCGAGA GCATGTTTGA ATATCTTAAC GTTGTGTTGA 241 CTTATTTGCT GCTTTCGATT AGAGGAAATT TGTGTACTTA TTGTTTGGAT AGCTTTACTT 301 TAGAAAGTAG GGAAAAAACT CAACCGCTAG CAAACAATGG GATTGATTAT ATTACGATCT 361 AATTTAATCC TTTATCGGAC AGGGAAGTTC TCACAATGAA TGTGGAGTTA CCCAGTTATG 421 AGTTACAGTG GGAAAAGAAT ATTAATCTGA ATGCATACTC ATCTCAATCT CTATAAGAGA 481 ATGCTAAACC AATTGTCCTG TGGCCCAGCG CCTATTGCAC AGCACAACAA CACCACACAC 541 CCCACCTACT TAAACATATT CTTCTTAACA GGGCTCAGGT TATAAAAACG GATCCTACCA 601 GGGT
BM MAP 13	493	1 CTTGGGTGCC GCAACGGTTT TCCACTAGAT CTTGAATAAT TTTTTGCCTA CTTTTTGCCA 61 CTCTGTTATT TCTCAAATAC GACTTTATTT TCCTTTTTAA TCGGTAAATA TTTCTTTTTG 121 CATTATTTTT TTGATGAGAT GATTTATTTT TATGTGGCTA CTTGTATTTG TTGTTTTGGT 181 TGGTTGGTTG TTTTTTTTTT GTTATTTTTT CACAAAAAAA TGAACAAATT AACTTCATTG 241 CATTTGCATT TTCAGGTGTT CCCACCAAAC CTCTATACCC AACCTAAATA TCTTTTTAGA 301 ATAAAAAAGG AGGTGATTAA GGGTGTCGGG GAAACGGCGG CCCTCCTTAC CAAAAAGGAA 361 ACCCCCTACC CGACGGTTAT CCCTGTCATA TACGGAAATG GCCCGTGGCT AATCCTCATT 421 ACCGTTGGAA TAATTTTTGG AGGGTTCGCA AAATGGGTTC TTTCCATTAA TTGCGAGGGC 481 AGAGGCGGGA GGA
BM MAP 14	506	1 TGCAACCCCC CCAACCTCCG GTTGCGCGGC CCTGACGTTA CCCGCCTATC CTACGGACTC 61 AATCGATTGA CTCTGGAGCA TGACCACCCC TAGCATACCC GATGTTTAAT CAGCGGCTCC 121 CATTCAATCT TACAGCTAAG TCAGTTGGTT ACCACTAGAA ATTTGTCCAT TGATGCTTGC 181 TTACTGAAGG TAGATGGTTG AAGGATAAAA CGTAATGCAA GAAACAGGTA TACTACAACA 241 ATTCAATATA AAGTATTCCT GATGACCCTC CACCATGGGC ATAGTTCACC CATCTTCAAT 301 GGACAAATTT TTACCAGAGA CCAACTGCCA TTTCTGAATG ATTGAATTTG AGAACCTGAT 361 GTTCACATCC GGAATGTCAT TGATGGATAT GCTCCAGAAA TTAATCCATT AAGAATGTAT 421 GATATTCAAA TACTGCCTGC TTCAGCAAGC AGAGAATAAA GATTTATTAG AGGTCTATCC 481 GCCTTCATTC ATGGTGGAGT CCTCCA

However the DNA amplification was programmed to perform 40 cycles under the following setup: first cycle (initial denaturing) was set at 90ºC for 5 min, second denaturation 95ºC for 1 min, annealing for 1 min at 40ºC and extension at 72ºC for 2 min. While the second cycle has a separate set up as follow: denaturation at 95ºC for 30 sec, annealing at 40ºC for 30 and extension at 72ºC for 1 min. Then go to 5 Rep 38 cycles. Final extension 72ºC for 10 min. Hold at 10ºC and finally ended the program were by the amplified product was stored at 20ºC.

Agarose gel electrophoresis

The amplified products were subjected to electrophoresis to separate the band using 1.2% w/v agarose gel. 72 mg of agarose was dissolved in 60 ml of TAE (1X) buffer and boiled for 10 min in a microwave oven then was shaken gently and repeated for 5 min again, after it was completely dissolved was allowed to stand up to 40ºC, then 5 µl of ethidium bromide was added and mixed gently on a surface to avoid vortex formation and finally was poured into gel casting tray, after solidification was transferred to the gel tank containing 1X TAE buffer with wells at negative charge direction. The comb was removed and the amplified product containing separate primer was loaded to respective wells with first well loaded with DNA maker (1kb DNA ladder). Then electrophoresis was run for 1 hour and 30 min at 110V. After electrophoresis, the gel was transferred and visualized by gel documentation system under UV light.

Data scoring

RAPD gel images were analyzed for the number of bands given the range of the amplification and variable amplified product. Primers have been selected for the secondary method of amplification based on single, double and multiple band production.

Specific amplification of selected bands

Primers which are yielding single and double band in uniform size alone were selected for the specific amplification by the following PCR conditions. The mixture was preferred as follow: 8µl of the 2X master mix (PCR), 1.5µl of primer (map-1, map-13, map-14 and map-19), 1 µl of DNA template and 5.5 µl of double distil water, which makes the total of 16 µl. The DNA amplification was programmed to perform 44 cycles under the following setup: first cycle initial denaturing was set at 95ºC for 15 sec, second denaturation 95ºC for 30 sec, annealing for 30 min at 40ºC and extension at 72ºC for 20 sec. While the second cycle was found to have a different setup as follow: denaturation at 95ºC for 25 sec, annealing at 40ºC for 20 and extension at 72ºC for 20 sec. Then go to step 5 Repeat 44 cycles while the Final extension was set to be 72ºC for 8 min. Hold at 10ºC and finally ended the program were by the amplified product was stored at 20ºC. Also, the time required was found to be two and half hour ($1^1/_2$ hour) to complete the entire cycles. The products were resolved on agarose (1.2% w/v) gel electrophoresis as mentioned earlier.

DNA sequencing analysis

The three amplicons that produced specific bands (amplified from three set of primers i.e map-1, map-13 and map-14) were sent for sequencing analysis by sangar method, and the retrieved sequences were submitted to NCBI Database (GenBank accession numbers KX139410.1, KX139411.1 and KX139412.1).

2.6 *Protein sequencing analysis*

The retrieved sequences were analyzed on NCBI database to check whether they are having any important protein by converting the nucleotide sequence into amino acid sequences.

RESULT

The extracted genomic DNA from *B. montanum* were initially randomly amplified using 20 set of primers by arbitrary PCR-based DNA fingerprinting (RAPD) for screening, in which only 16 primers were able to produce bands at the different score while the remaining three primers remain unamplified (Figure 1). However, out of reproducible primers (MAP-1-16), three primers (MAP-1, 12 and 14) were found to produce more specific bands, which were selected for specific amplification analysis (Figure 2).

Although the entire primers have equal number of base pair sequences (10bp) but the obtained data score were totally different, even though some shared equal number of bands and relative percentage of bands per primer as follow: MAP-1 & 9 (9.59); MAP-3,5,13,14 & 19 (5.49); MAP-2 & 16 (2.74); MAP-7 (1.37); MAP-12 & 17 (6.85); MAP-11,15,18 & 20 (8.22) and MAP-4,6,8 & 10 (no band amplified at all). But other parameters (range of amplicon size and their approximate size) were entirely different (Table 1).

DNA fingerprinting

With regards to the 16 reproducible primers, three primers were selected and re-amplified by a modified arbitrary PCR (RAPD) for DNA fingerprinting construction. Using these selectable primers (MAP-1, MAP-13, and MAP-14), then specific amplicons of selected bands were constructed (Figure 2).

Sequencing analysis

Prior to the sequence analysis of specific amplicons (BM MAP-1, BM MAP-13 and BM MAP-14) generated by specific re-amplification, the following sequence were retrieved at different size per band, i.e BM MAP-1 (604bp), BM MAP-13 (493bp) and BM MAP-14 (506bp) respectively (Table 2), in which can also be used as a molecular marker of that particular plant (*B. montanum*). In addition to that, three separate set of sequences were initially subjected to FASTA BLAST, then letter submitted to the NCBI (GenBank accession numbers KX139410.1, KX139411.1 and KX139412.1).

However the origin of replication fragments(ORFs) was pointed out and calculated, which shows a varies numbers of fragments per sequence, as BM MAP 01- 604 bp has 7 ORFs and BM MAP 13-494bp with 3 ORFs, while BM MAP-506bp has 10 ORFs (Table 3) (Figure 3).

DISCUSSION

In the present study, the DNA fingerprinting marker of *B. montanum* was developed by RAPD marker analysis which is a common tool used for medicinal plant DNA fingerprinting. The RAPD-DNA profiling has a wide rate

Table 3: Calculated ORF details of *B. montanum* DNA sequences.

Sequence	ORF No.	Location		Length	Strand
		Start	Stop		
MAP 01 product	1	117	185	69	Positive
	2	182	214	33	Negative
	3	213	362	150	Positive
	4	337	399	63	Positive
	5	396	476	81	Positive
	6	400	450	51	Positive
	7	451	585	135	Positive
MAP 13 product	1	152	223	72	Positive
	2	158	238	81	Negative
	3	366	467	102	Negative
MAP 14 product	1	46	96	51	Negative
	2	102	158	57	Positive
	3	181	282	102	Negative
	4	215	262	48	Positive
	5	243	293	51	Negative
	6	262	345	84	Positive
	7	275	337	63	Positive
	8	338	412	75	Positive
	9	415	459	45	Positive
	10	426	488	63	Negative

of application as normally used for specific differentiation between a closely related medicinal plant species[9] and clonal differences of plant species[5]. RAPD analysis is one of the suitable technique used for medicinal plant authentication (quality control) of herbal medicine[10], it was also an applicable tool for plant adulteration analysis which was applied in the previous studies for DNA Profiling analysis of various plant species[11], *Gylycyrrhiza globra*[12], *Tridax procumbens; Curuma longa; Sesbania grandiflora and Prenna integrifolia*[13], *S. asoca*[14] and *Bacupa* monnieri[15,16]. However, a DNA fingerprinting of *Helicunthus elastic* was also developed by RAPD[5]. Therefore in addition to RAPD, another study revealed that other molecular technique can be included together for medicinal plant authentication, such as AFLP, SNP, SSR, ISSR, ESTs[4]. RAPD was found to be a less cost and more rapid DNA profiling analysis tool[13] as well as even less time consuming[17].

However, with regards to the specific bands and their number of base pairs, in present study reveals that, out of the 20 set of primers(MAP-1 to MAP-20) used, three of them (MAP-1, MAP-13 and MAP-14) were found to be able to produce a specific single band, which were further re-amplified (using modified arbitrary PCR), and subjected to a sequencing analysis whereby highly qualitative amplicons were retrieved with the following different length of sequences basepairs: 604bp (BM MAP-1) ; 494bp (BM MAP-13) and 506bp (BM MAP-14). This confirmed with the work done by Rajagopal *et al*[13] used 20 set of primers also got three selectable primers that produce a single band with the following different length of sequence base pairs: 250bp (OPA8); 300bp (OPA18) and 1000bp (OPA18) from three different plant species *Tridax procumbens; Sesbania grandiflora;* and *Curuma longa;* respectively, out of four species used in their study. While Yadav *et al*[15] got only one single band with 589 bp from *Bacupa monnieri.* In addition, three set of a specific band were separately observed from a separate number of species i.e five, two and two set of bands from *W. tintoria, H. pubscence* and *W. rothi* respectively[14].

In the present study, the BLAST result shows that no homology and nor similarity either with already known plant sequence on NCBI Database, from both the three set of the nucleotides sequence of different length [(604bp (BM MAP-1); 494bp (BM MAP-13) and 506bp (BM MAP-14)]. In contrast to work done by Malay Das *et al*[18] in which their nucleotide sequence (Bb_{836}836) BLAST result shows a homology as well as similarity with another nucleotide sequence of known plant on the NCBI database. However, prior to the ORF in the present study, it shows BM MAP 01- 604bp with 7 ORFs and BM MAP 13-494bp with 3 ORFs, while BM MAP-506bp with 10 ORFs. Therefore, this is confirmed by another study where they found the ORF of a derived amino acid sequence was signified between the range of amino acid 85 to 201, although the second sequence with 609bp (Bb_{609}) has an ORF from amino acid 93 to 184, contains hypothetical protein similar with *Caemorhabditis briggsae* (CAE65839)[18].

CONCLUSION

The DNA amplified products (using 20 RAPD primers) from *Baliosperm montanum* were modified and successfully developed three highly molecular weight DNA fingerprint markers which were confirmed specifically for *B. montanum*. However, they were also confirmed that no similar sequence been identified from previous literature as well as nor any hypothetical protein

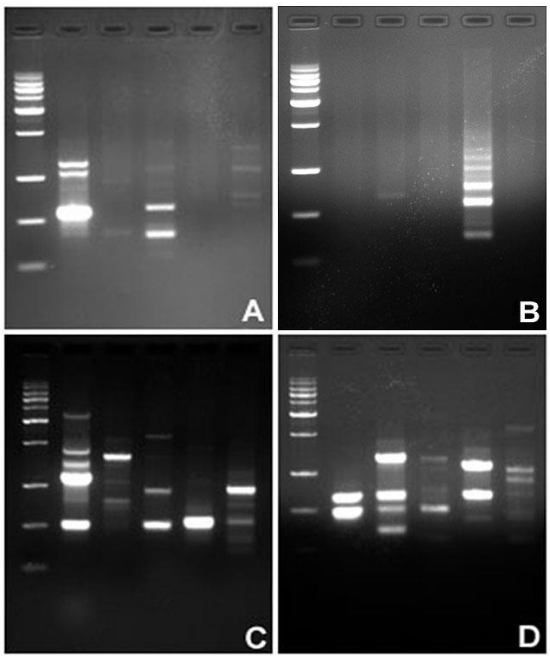

Figure 1: Arbitrary PCR based DNA finger printing of *Baliospermum montanum*. Lane 1 in A, B, C & D: DNA Marker (1 kb ladder); (A). Lane 2-6. Amplified product by MAP primers 1-5; (B) Lane 2-6. Amplified product by MAP primers 6-10; (C) Lane 2-6. Amplified product by MAP primers 11-15; (D) Lane 2-6. Amplified product by MAP primers 16-20

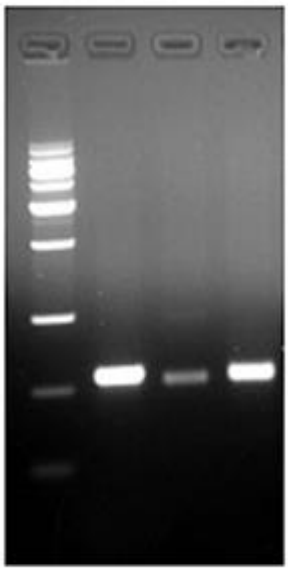

Figure 2: Specific amplication of selected bands using modified arbitrary PCR. Lane 1: DNA Marker; Lane 2. *B. monatnum* DNA with MAP 1; Lane 3. *B. monatnum* DNA with MAP 13; Lane 4. *B. monatnum* DNA with MAP 14.

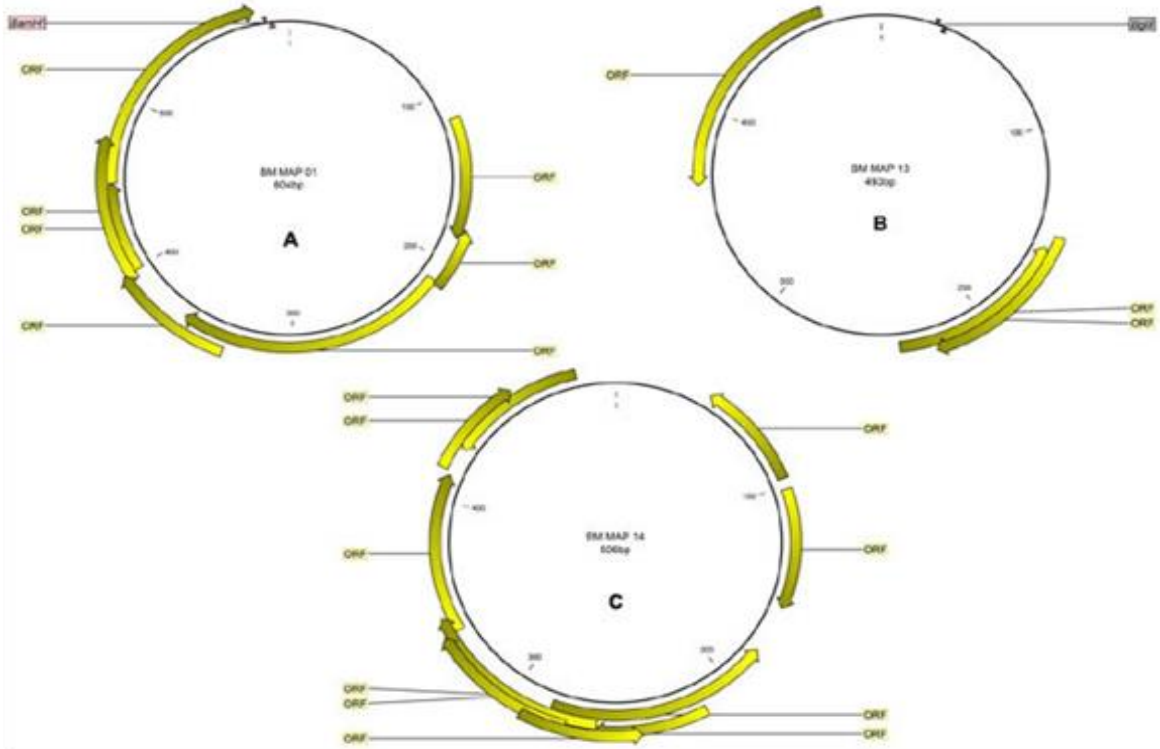

Figure 3: Circular view of calculated ORF of three DNA sequences of *B. montanum*. (A) BM MAP 01- 604bp with 7 ORFs; (B) BM MAP 13 – 494bp with 3 ORFs; (C) BM MAP 14 – 506bp with 10 ORFs

after subjected to BLAST analysis on NCBI Database. Thus, come to conclude that, they could be specifically applicable as a molecular marker of *B. montanum* DNA fingerprint for identification of medicinal plant adulteration or other molecular analysis.

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