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Transferability of Genic-SSR markers developed from the transcriptome data of *Selaginella bryopteris* (L.) Baker

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Abstract

In the present study, the experimental validation of Genic-SSR markers developed from transcriptome data of *Selaginella bryopteris* was done, and their cross-species transferability was examined across genus and species of pteridophytes. Twenty primer pairs were designed to amplify the various repeats of SSR markers, and first validated with complementary DNA (cDNA) of *S. bryopteris*. Out of twenty primer pair, fourteen primer pairs (70%) could amplify the DNA of *S. bryopteris*. Among *Azolla* spp., four SSR markers (SbSSR4, SbSSR12, SbSSR14 and SbSSR18) were found to be 100% transferable, while others SbSSR13 (80%), SbSSR3 and SbSSR20 (50%) and four markers SbSSR6, SbSSR10, SbSSR15 and SbSSR19 (10%). Out of twenty validated primers, 11 primers were found to be polymorphic. The value of polymorphism information content (PIC) ranged from 0.47 to 0.86 with an average of 0.49. The Jaccard's similarity coefficient ranged from 0.10 to 0.93 which revealed a wide range of genetic identity. The similarity between *A. fuliculoids* and *A. maxicana* (0.93), and *A. pinnata* and *A. rubra* (0.93) was high and while low similarity coefficient of *S. bryopteris* with *P. vittata* (0.18), local fern (0.20) and with *Azolla* spp. (0.10- 0.25). Two major Clusters formed based on UPGMA i.e. *S. bryopteris*, *P. vittata*, local fern in Cluster I and *Azolla* spp. in Cluster II, a clear divergence between *S. bryopteris* and other pteridophytes. BAUS1 was found to be more genetically divergent followed by BAUS2, BAUS1 and BAUS3 in comparison to known species of *Azolla*. The transferability of Genic-SSR markers of *S. bryopteris* demonstrated the utility for elucidating genetic relationship among selected pteridophytes.

Keywords: *Selaginella bryopteris*, *azolla*, genic-ssr, transcriptome, transferability

1. Introduction

Selaginella bryopteris (L.) Baker [family Selaginellaceae; 2n=20] is a heterosporous pteridophyte and considered to be the first vascular plant on earth. This is one of the 'Sanjeevani-like' plants commonly found on hilly terrains in the state of Rajasthan, Uttar Pradesh, Uttarakhand, Bihar and Jharkhand. The whole plant is enriched with phytochemicals of medicinal importance, and being used for curing heat strokes, irregular menstrual cycle, and jaundice since time immemorial (Sah *et al.*, 2005; Singh *et al.*, 2018) ^[1]. This plant is also being used in traditional Indian systems of medicine for the prevention and cure of several disorders and for the treatment of patients suffering from spermatorrhoea, venereal disease, constipation, colitis, urinary tract infections, fever, epilepsy, leucorrhoea, beriberi and cancer. It is also used as a strength tonic and has anti-inflammatory effects (Paswan *et al.*, 2017) ^[10]. Plants have microphylls, opposite, alternate or whorled, simple, one veined, sometimes dimorphic, with scale like ligule and stems dichotomously branched. This medicinally important plant is also xerophytic in nature and can withstand low moisture level and, therefore, it is excellent reservoir of gene resources related to secondary metabolism and abiotic stresses particularly drought and heat (Singh *et al.*, 2018; Kumar *et al.*, 2018) ^[11]. *Azolla* is an aquatic fern belonging to family Salviniaceae. In comparison to other ferns, it is highly reduced in size, and harbours within its leaves cyanobacteria, *Anabaena azollae* and *Nostoc azollae* in symbiotic association. Both bacteria are capable of fixing atmospheric nitrogen for the fern and carbohydrate is provided by fern to bacterium and this association process makes *Azolla* an agriculturally important.

It is used for maintaining soil fertility as Green manure or Bio-fertilizer in rice fields in South-East-Asia (Lumpkin and Plucknett, 1982) [12]. Rice farmers have been using it as bio-fertilizer as it provides nitrogen to rice. It can double its biomass in 3-10 days and yield about 8-10 tonnes fresh matter/ha in Asian rice fields reported in *Azolla pinnata* in India (Hasan *et al.*, 2009) [13]. For the first time, last year (2018) genomes of two tiny ferns, *Azolla filiculoides* and *Salvinia cucullata* got sequenced, both have extremely small genomes [*A. filiculoides* is 0.75 Gb while *S. cucullata* is 0.26 Gb] (Li *et al.*, 2018). Ferns can have as many as 720 pairs of chromosomes and genomes as big as 148 billion base pairs of DNA sequences (Gb), in contrast to *Arabidopsis thaliana*, the first plant to have its genome sequenced, has only five pairs of chromosomes and a genome size of 0.135 Gb. *Pteris vittata* has ornamental appearance and used in gardens and decoration purposes. There are several reports of phytoextraction of arsenic by its hyperaccumulator *P. vittata* (Caille *et al.*, 2004; Gonzaga *et al.*, 2008) [7, 8].

SSRs are clusters of short tandem repeats of nucleotides bases. SSRs are multi-allelic and show co-dominant segregation pattern so; most appropriate in accessing genetic diversity and give way to evolutionary study. Genomic SSRs are identified from random genomic sequences and are not suitable for assessment of functional diversity whereas, Expressed Sequence Tags (ESTs) or Genic are developed from cDNA (Complementary DNA) libraries to obtain gene expression information in contrasting environmental conditions or across developmental stages. Genic SSRs actually regulate gene expression and function as they are derived from transcriptome sequences. As it exists in transcribed part of genome, it can be used for gene-based maps which may help to identify candidate functional genes and also enhance efficiency of Marker Assisted Selection (MAS). Genomic SSRs are more abundant in non-coding region so, show high level of polymorphism in comparison to genic SSRs due to their concentration in gene rich region. EST-SSRs show high level of transferability among distantly related species which is not possible with genomic SSRs. Transcriptome sequencing (RNA sequencing, RNA-seq) is an alternative to whole genome sequencing as it can target genomic regions with corresponding EST sequences which can be exploited to develop EST-SSR markers.

Earlier, there were no reports on molecular markers in *S. bryopteris*. Simple Sequence Repeat (SSR) markers were identified and reported in *S. bryopteris* for the first time by Singh *et al.* (2018) [1] but were not experimentally validated. A total of 27,490 and 12,063 SSRs were identified in fronds and root transcriptome respectively. Further, after filtration, 9074 SSRs for fronds and 3811 SSRs for root sample were predicted containing flanking region of 15bp that was used for PCR-based validation. Among the total SSRs identified, dinucleotide repeat motifs represented the largest fraction i.e. in fronds 25,869 (94.1%) and in roots 10,387 (86.1%), trinucleotides in fronds, 1185 (4.3%) and in roots, 1438 (11.9%), tetranucleotides, 379 (fronds) and 204 (roots), pentanucleotide, 47 (fronds) and 21 (roots) followed by hexanucleotides, 13 in fronds and 10 in roots. It is expected that the experimental validation may yield some potential markers that would serve as the repertoire of DNA markers. These markers could be used for genetic studies, molecular taxonomy, Intellectual Property Right (IPR) disputes etc. in *S. bryopteris*, related species and in other ferns. The genic SSR markers are transferable among

closely related species in comparison to genomic SSRs (Cordeiro, 2001) [14].

In the present study, the experimental validation of Genic-SSR markers developed from transcriptome data of *S. bryopteris* was done, and their cross-species transferability was examined across genus and species of pteridophytes.

2. Materials and Methods

2.1 Plant material

The plants of *Selaginella bryopteris* were collected from hills of Seikhpura, Bihar and maintained in pots, *P. vittata* and local fern collected from nearby areas of Sabour, Bhagalpur. Ten species of *Azolla* were collected from Biofertilizer unit, BAU Sabour, Bhagalpur, namely, *A. microphylla*, *A. filiculoids*, *A. maxicana*, *A. caroliniana*, *A. pinnata*, *A. rubra* and four selections namely, BAUS1, BAUS2, BAUS3 and BAUS4.

2.2 DNA Isolation

The pteridophytes contain secondary metabolites, colored inhibitors and mucilage. *Azolla* has high content of mucilage and proteins that hinders with DNA to get extracted. So, different protocols were used for DNA isolation in different plants. The genomic DNA was extracted from the *S. bryopteris*, *P. vittata* and local fern following the method described by Doyle and Doyle (1987) [15] with a few modifications. In brief, the protocol was as follows: (1) Grinding of 150-200mg of leaves in 2% CTAB with 2% PVP (polyvinylpyrrolidone) and transferred to eppendorf tube, (2) Adding of equal amount of chloroform-isoamylalcohol. Mixed properly by inversion, (3) Centrifuged at 10000rpm for 10 minutes and aqueous phase transferred with slant cut tip to new tube, (4) Addition of 1/5th volume of 5% CTAB, mixed and chloroform isoamylalcohol equal to the previous volume, (5) Centrifuged for 5 min at 10000 rpm, aqueous phase transferred and added equal amount of CTAB precipitation buffer, (6) Centrifuged for 1 min at 10000 rpm for pelleting down of DNA, pellet dissolved in 200 µl of high salt T.E. buffer, (7) Adding of chilled 95% ethanol 2.5 times volume and mixed gently, (8) Centrifuged for 10 min at 10000 rpm supernatant discarded and pellet washed in 200µl of cold 70% ethanol by centrifuging for 5 min at 10000 rpm, (9) Supernatant discarded, pellet is dried for 30 min and dissolved 0.1X T.E buffer.

The mucilage and protein content are usually very high in *Azolla*, and during crushing it forms a gum like substance that latter hinders in DNA isolation. Therefore, in case of *Azolla*, the protocol of Ramos *et al.* (2014) was followed with some modification. In brief, the protocol was as follows: (1) Surface sterilization of fronds in 0.12% sodium hypochlorite for 15 min and washing it for 5-6 times with water, (2) Grinding of 150-200mg tissue in 2% CTAB extraction buffer with 2% PVP and 10% β-mercaptoethanol, (3) Sonicated for 15 seconds then paused for 30 sec and again sonicated for 15sec, (4) added 300µl of saturated NaCl and centrifuged at 10000 rpm for 10 min, (5) supernatant collected with slant cut tip and 160µl of 5M NaCl mixed gently, (6) 600 µl of chloroform Isoamyl alcohol (CIA) added and centrifuged at 10000 rpm for 10 min, (7) 50µl of 10% CTAB added to supernatant collected, shaken well and 600µl of CIA added and centrifuged at 10000 rpm for 10 min, (8) Supernatant taken out and equal amount of cold isopropanol added and precipitated at -20 °C for 1 hr, (9) washing of pellet with 70% ethanol and pellet dried at 70 °C for 3-4 min and dissolved in 0.1X T.E.

Absorbance of DNA was measured using a spectrophotometer Spectrophotometer (Genova Plus, JENWAY, U.K.) and the purity of DNA was determined by calculating the ratio of absorbance at 260 and 280 nm. A value for the ratio less than 1.8 was considered ideal for finding the purity of DNA. The integrity of DNA was checked on 0.8% agarose gel. For this, 5µl of DNA was mixed with 2 µl of loading dye and electrophoresed on 100 volt for 20 min in 1X TAE buffer.

2.3 Selection of Genic-SSR marker and BLAST analysis

The unigene transcripts for fronds and roots were screened for microsatellite by search tool MISA (Micro Satellite, <http://www.pgrc.ipk-gatersleben.de/misa>) for SSR markers. Genic-SSRs were selected on the basis of abundance, type of repeats, length of repeats and their presence in protein encoding unigene sequences (Table 1). Genic-SSR containing the unigene sequences were analysed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for their similarity to any known gene in the GenBank database.

2.4 Primer designing

Primers for SSR repeats were designed using online programme Primer 3 Input (<http://bioinfo.ut.ee/primer3-0.4.0/>) and oligonucleotide property calculator (<http://biotools.nubic.northwestern.edu/OligoCalc.html>) keeping ~100 bp minimum amplicon size that can be easily resolved on 2% agarose gel.

1. Validation of designed SSR primers

Primer pairs were validated first for their specificity by Polymerase chain reaction (PCR) using cDNA of *S. bryopteris*. The PCR was carried out on a thermocycler (Veriti R#9902, ABI, Singapore) as followed for 25µl of PCR reaction. 12.5µl of 2X Primix Taq (Xcelris Genomics, India), 1µl of forward and reverse primers each, 1µl of DNA (50 ng) and 9.5 µl of distilled autoclaved water with 35 cycles of denaturation at 94 °C for 30 sec, annealing temperature at 52-55 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The PCR product was resolved by gel electrophoresis on 1.5% Agarose-EtBr gel. Gel was viewed on a UV trans-illuminator and captured on gel documentation system (UVITEC, Cambridge, U.K.).

2.5 PCR amplification of SSR markers

The PCR was carried out with DNA of *S. bryopteris* P. vittata and local fern, *A. microphylla*, *A. filiculoids*, *A. maxicana*, *A. caroliniana*, *A. pinnata*, *A. rubra* and four selections namely, BAUS1, BAUS2, BAUS3 and BAUS4 on a thermal cycler (Veriti R#9902, ABI, Singapore). The volume of reaction mixture was 15µl. 7.5µl of 2X Primix Taq (Xcelris Genomics, India), 0.5µl of forward and reverse primers each, 1µl of DNA (50ng) and 5.5µl of distilled autoclaved water constituted the whole reaction mixture. Initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing temperature of 52-55 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The PCR product was resolved by gel electrophoresis on 1.5-2.5% Agarose-EtBr gel. Gel was viewed on a UV trans-illuminator and captured on gel documentation system (UVITEC, Cambridge, U.K.).

3. Statistical analysis

3.1 Polymorphism information content (PIC)

The term PIC was originally introduced in the human genetics by Botstein *et al.* 1980. It refers to the value of a marker for

detecting polymorphism within a population or set of genotypes by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus. The PIC was calculated using PIC-CALC software (<https://www.liverpool.ac.uk/~kempsj/pic.html>).

3.2 Phylogenetic analysis

Based on genetic similarity matrices, a dendrogram was constructed using NTSYS pc version 2.10 software by the unweighted pair group mean (UPGMA) analysis method or by online phylogenetic analysis tool available at <http://genomes.urv.cat/UPGMA>.

4. Result and Discussion

In this section, the outcomes of the research work on “Transferability of genic-SSR markers developed from the transcriptome of *Selaginella bryopteris* (L.) Baker.” has been discussed keeping in view the experiments conducted on experimental validation of Genic-SSR markers developed from the transcriptome data, analysis of transferability of these markers in other pteridophytes, analysis of polymorphism percentage of these markers, phylogenetic analysis of selected pteridophytes using these markers. Most of pteridophyte including agriculturally important *Azolla* spp., are lesser studied at genomic level, and therefore reliable molecular markers are lacking. The cross-species transferable SSR markers are useful in such cases. In the present study, the validation of Genic-SSR markers developed from transcriptome data of *S. bryopteris* was done, and their transferability was examined across genus and species of pteridophytes. Each Genic-SSR containing unigene sequences were putatively identified by BLAST similarity search of genes/transcription factors related to flavonoid and other biosynthesis pathway.

4.1 Validation of SbSSR primers in *S. bryopteris*

SSRs identified and reported in *S. bryopteris* for the first time after transcriptome analysis of fronds and root (Singh *et al.* 2018)^[1] were not experimentally validated. A total of 27,490 and 12,063 SSRs were identified in fronds and root transcriptome, respectively. Further, after filtration, 9074 SSRs for fronds and 3811SSRs for root sample were predicted containing flanking region of 15 bp that was used for PCR-based validation. Among the total SSRs identified, dinucleotide repeat motifs represented the largest fraction i.e. in fronds 25,869 (94.1%) and in roots 10,387 (86.1%), tri-nucleotides in fronds, 1185 (4.3%) and in roots, 1438 (11.9%), tetranucleotides, 379 (fronds) and 204 (roots), pentanucleotide, 47 (fronds) and 21 (roots) followed by hexanucleotides, 13 in fronds and 10 in roots (Singh *et al.*, 2018)^[1]. It was expected that the experimental validation may yield some potential markers that would serve as the repertoire of DNA markers. These markers could be used for genetic studies, molecular taxonomy, Intellectual Property Right (IPR) disputes etc. in *S. bryopteris*, related species and in other ferns. As the Genic-SSR markers are transferable among closely related species in comparison to genomic-SSRs (Cordeiro, 2001)^[14]. The high reproducibility, scorable bands per marker, relative abundance, good genome coverage, transferability, discriminating genotypes or cultivars and polymorphism demonstrate the potential utility of Genic or EST-SSR markers. In the present study, sixty Genic-SSR containing unigenes sequences from the transcriptome of *S. bryopteris* were putatively identified for BLASTN and BLASTX. On the basis of abundance of SSR, repeat motif

length and type and BLAST analysis result, twenty primer pairs were selected, designed and synthesized. As the primers were designed from transcriptomic sequences of *S. bryopteris*, they were first validated with cDNA of *S. bryopteris* for the confirmation of the product size. PCR amplification of the primers revealed that fourteen out of twenty primers i.e. 70% of the total primers amplified genomic DNA of *S. bryopteris*.

4.2 Validation of SbSSR primers in *S. bryopteris* and their transferability in other pteridophytes

The primers designed for *S. bryopteris* were found to amplify the genomic DNA of selected pteridophytes and thus, transferability of markers was achieved. The transferability of the SSR loci ranged from 13 to 100% in all accessions. SbSSR4 and SbSSR18 exhibited transferability of 100%, SbSSR12 with 92%, SbSSR12 and SbSSR13 with 84%, SbSSR3 with 53% and rest markers had transferability less than 50% (Fig. 1). Among *Azolla* spp., four SSR markers SbSSR4, SbSSR12, SbSSR14 and SbSSR18 were found to be 100% transferable, while others SbSSR13 with 80%, SbSSR3 & SbSSR20 with 50% transferability. Range of transferability observed in *Azolla* spp. was in range of 10 to 100% (Fig. 2). It has been reported that EST-SSR markers are more transferable in closely related species due to their homology in the conserved sequences. SSR markers were reported in *Osmanthus* having high transferability rate (84%) in other uncharacterised species of *Osmanthus* (Alexander *et al.* 2017). SSR markers developed in *Capsicum annum* were 80% transferable in forty-eight germplasms of capsicum (Buso *et al.* 2016) [18]. While, SSR markers reported in zinger, large cardamom and turmeric were found to be transferable in small cardamom (*Elettaria cardamomum*) (Cyriac *et al.* 2016) [19]. (Li *et al.* (2013) [20] reported that the EST-SSRs developed in mosco bamboo showed transferability twelve bamboo species and sixty four per cent of was observed ninety per cent of Genic-SSRs of Nules clementine exhibited transferability in other citrus species (Luro *et al.* 2008) [16]. Transferability was accessed in crops of different genus belonging to same family. SSR markers developed in rice had transferability of 45% in sorghum (Krupa *et al.* 2017);

transferability of EST-SSRs of *Camellia sinensis* was also observed with seventy per cent in other species of theaceae family (Xiao-mao *et al.* 2011); SSRs of adzuki bean showed the 100% of transferability twenty cultivars of pigeon pea (Agbagwa *et al.* 2015) [17]; twenty five per cent of soybean markers were transferable to peanut genome (He *et al.* 2006). EST-SSR markers have also been found to exhibit transferability across families, markers of safflower and pulses were found to be transferable in cotton genotypes (Harijan *et al.* 2017). It was also reported that EST-SSRs markers developed in *Jatropha* were reported to be 57.0 to 95.6% transferable among five species of *Jatropha* and 47% transferability across genera in *Ricinus communis* (Yadav *et al.* 2011).

4.3 Phylogenetic analysis of selected pteridophytes using these markers

Jaccard's similarity coefficient ranged from 0.10 to 0.93 which revealed a wide range of genetic identity. *A. filiculoids* and *A. maxicana* (0.93) and *A. pinnata* and *A. rubra* (0.93) were found to be more genetically similar (Table 2 and Fig. 3). *A. rubra* and *A. filiculoids* were found to have similarity of 0.80 and both being 0.73 and 0.67 of similarity. *A. rubra* and *A. filiculoids* were found to be in same cluster and genetic similarity of 0.70 was found and more diverge from *A. microphylla* using arbitrary markers (Coppennolle *et al.* 1993); *A. microphylla*, *A. mexicana*, *A. caroliniana*, *A. filiculoides* and *A. rubra* were grouped in one cluster and *A. pinnata* in other cluster when diversity study was done on RAPD amplification (Pereira *et al.* 2011); SCAR marker was developed from RAPD markers for identifying different species of *Azolla* using molecular markers. 709, 490, 390 and 182 bp of SCAR amplicon were generated for *A. microphylla*, *A. filiculoids*, *A. rubra* and *A. pinnata*, respectively (Abraham *et al.* 2013).

In conclusion, the transferability demonstrated the utility of Genic-SSR markers of *S. bryopteris* for elucidating genetic relationship among selected pteridophytes used in the present study. It revealed transferability of these markers across families and species within one genus or family.

Table 1: List of transferable SSR markers from the fronds and roots transcriptome

Transcript ID	Repeat motif	Name of SSR	Sequence	Tm (°C)	Expected size (bp)	% Transferability	PIC value
FRONDS							
Unigene_19_Transcript_26	(TC)6	SbSSR1F	CTGTAAAACAGTAC TCTATGTCCTC	52	193	23	0.56
		SbSSR1R	TTTTGAAGACCTCTC TCTCC				
Unigene_3581_Transcript_4232	(CGG)5	SbSSR3F	TTGAGGCAAGTATT GCTGTTG	51	200	53	0.65
		SbSSR3R	CCAGGAAATCCGTA GTGTTG				
Unigene_10_Transcript_10	(TGCG)7	SbSSR4F	CTGGAATGAATTGC GAAAGA	46	161	100	0.47
		SbSSR4R	AACGATGGATGACA CGAAAC				
Unigene_3443_Transcript_4062	(CACG)5	SbSSR6F	AAGAATTATGGTGT GCCTGCT	51.5	163	15	0.75
		SbSSR6R	CTTGCGAGTGTGTAT AATCGTCT				
Unigene_18404_Transcript_21068	(AGAGAC)6	SbSSR10F	ACGAGTTTGTGTCTA TGTGT	48	172	13	0
		SbSSR10R	CCGTTATAAGATTTCG GAAAG				
ROOTS							
Unigene_1063_Transcript_1345	(GA)11	SbSSR12F	TTTACCTGCATAAAC CTCGT	53	198	92	0.66

		SbSSR12R	TGAAGCTATAGAAG ACGTAGCC				
Unigene_1065_Tra nscript_1347	(TG)11	SbSSR13F	TAAAGTCGGAGAGA CCAAAA	52	159	84	0.59
		SbSSR13R	TAGAGAACTCCGA CCAAAA				
Unigene_35063_Tr anscript_41449	(GCAG)6	SbSSR14F	GAAACATGTCTCA CCAGCA	54	198	84	0.15
		SbSSR14R	GGCGGAAGAAGAGA GAGAAG				
	(TGCG)6			50		15	0.15
Unigene_5203_Tra nscript_6406		SbSSR15F	GACTGTCTCAGCAAGA GTGAGATG			217	
		SbSSR15R	TTCTAGCTTCCTTTG CTCTTGT				
		SbSSR17R	AGTGGCAGGTCTGG AATGTT				
Unigene_52458_Tr ansript_59904	(GTCCT C)5	SbSSR18F	GAACGACGACGAGG CAAAC	54.5	242	100	0.86
		SbSSR18R	GAAGGAGGCAGGAA GACGAC				
Unigene_22601_Tr ansript_27370	(AT)10tt(GA)8	SbSSR19F	CAGGTCACAGTATG TGTGTCAA	52	192	23	0.86
		SbSSR19R	ATACAGCTGGACTT CTCTGCT				
Unigene_16124_Tr ansript_19616	(GT)10(G A)13	SbSSR20F	GTCATAACTAATGC ACGTCCTG	51	251	46	0.53
		SbSSR20 R	TAACACCTGGC ATCTAACTTC				

Table 2: Jaccard’s Similarity coefficients of different plants used in the present study

	<i>S. bryopteris</i>	<i>P. vittata</i>	<i>local fern</i>	<i>A. microphylla</i>	<i>A. filiculoids</i>	<i>A. maxicana</i>	<i>A. caroliniana</i>	<i>A. pinata</i>	<i>A. rubra</i>	BA US -1	BA US -2	BA US 3	BA US- 4
<i>S. bryopteris</i>	1												
<i>P. vittata</i>	0.18	1.00											
<i>local fern</i>	0.20	0.23	1.00										
<i>A. microphylla</i>	0.16	0.24	0.19	1.00									
<i>A. filiculoids</i>	0.15	0.22	0.18	0.67	1.00								
<i>A. maxicana</i>	0.15	0.21	0.17	0.63	0.93	1.00							
<i>A. caroliniana</i>	0.10	0.14	0.10	0.59	0.75	0.81	1.00						
<i>A. pinata</i>	0.15	0.22	0.18	0.79	0.73	0.80	0.75	1.00					
<i>A. rubra</i>	0.15	0.21	0.17	0.73	0.80	0.87	0.81	0.93	1.00				
BAUS -1	0.25	0.13	0.14	0.43	0.48	0.52	0.50	0.55	0.60	1.0 0			
BAUS -2	0.16	0.17	0.20	0.42	0.29	0.27	0.25	0.38	0.36	0.2	1.0		
										8	0		
BAUS -3	0.16	0.24	0.19	0.71	0.92	0.86	0.69	0.79	0.86	0.5	0.3	1.0	
										0	1	0	
BAUS -4	0.14	0.21	0.15	0.43	0.50	0.47	0.44	0.50	0.57	0.3	0.6	0.5	1.0
										7	3	4	0

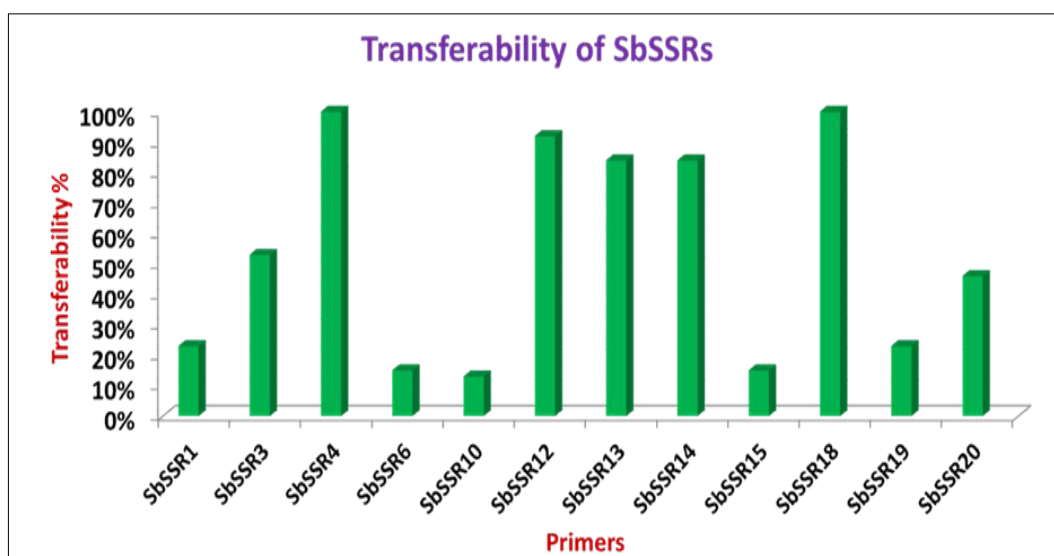


Fig 1: Cross transferability of SbSSR markers to *P. vittata*, local fern and *Azolla spp.*

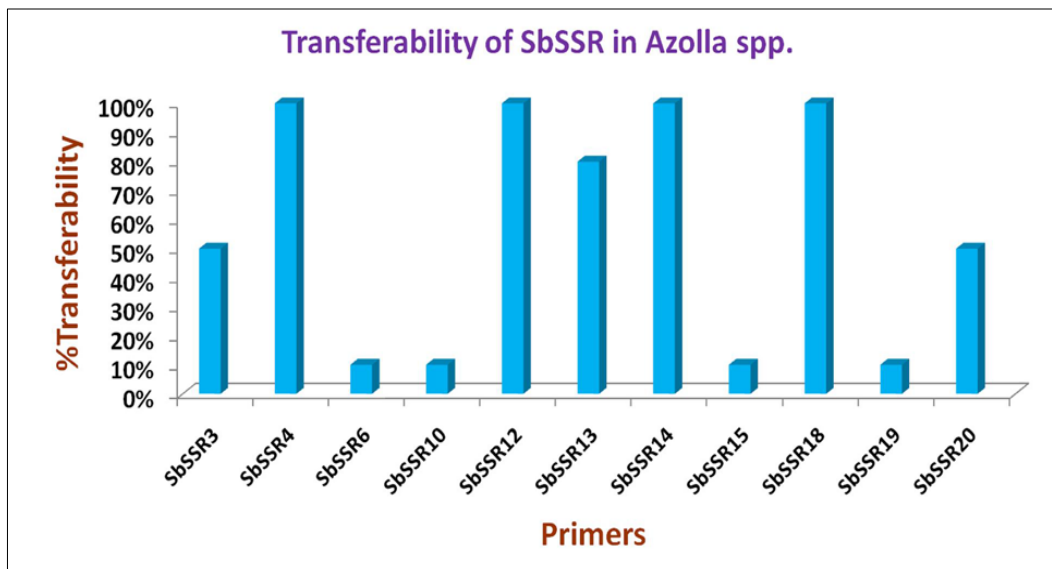


Fig 2: Cross-transferability of SbSSR markers among *Azolla* spp.

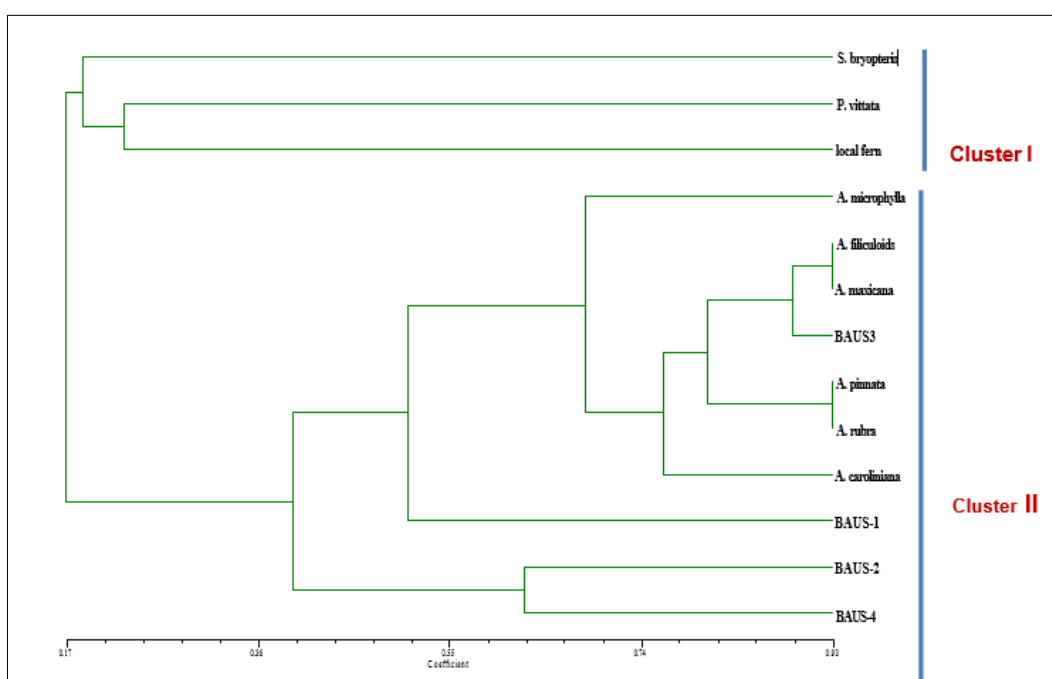


Fig 3: Dendrogram produced by Jaccard's coefficient and UPGMA clustering method based on Genic-SSR markers of *S. bryopteris*.

5. Acknowledgement

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