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# DEVELOPMENT OF MALE SPECIFIC DNA BASED MARKER IN *SIMAROUBA GLAUCA* DC (PARADISE TREE)

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**ABSTARCT:** *Simarouba glauca* DC, is a poly-gamo-dioecious tree belongs to family Simaroubaceae and a potential source of biodiesel. There is an urgent requirement to develop a tool needed for sex identification at pre-flowering stage to support selection and breeding of this plant because of its sex specific economic values. As there are no sex specific morphological markers and no allosomes to identify the sex through cytological methods, an easy, rapid and reliable molecular tool for sex identification at pre-flowering stage in *S. glauca* is reported in the present study. A total of 50 random decamer primers were used for screening of specific Random Amplified Polymorphic DNA (RAPD) markers in male and female populations. Only one primer OPA-18 amplified genomic DNA in different patterns in male and female genotypes. Pair of Sequence Characterized Amplified Region (SCAR) primers designed based on RAPD sequence, amplified a single 1110 base pairs DNA band only in male populations. These SCAR primers may be efficiently used as effective, convenient and reliable molecular markers for sex identification in *S. glauca* at pre-flowering stages. This would pave the way to screen male and female seedlings for the mass cultivation which in turn save time and economic resources.
**Key words:** Molecular marker, Sex determination, RAPD, SCAR.

**Abbreviations:** RAPD- Random Amplified Polymorphic DNA
RFLP - Restriction Fragment Length Polymorphism
SCAR - Sequence Characterized Amplified Region

## INTRODUCTION

Sex determination is the developmental decision that occurs during the plant life cycle that leads to the differentiation of two organs or cells that produce two gametes. In plants, there is great variation in where, when and how this decision occurs [1]. Most angiosperm species produces bisexual flowers and some are dioecious. Sex determination and the evolution of sexual specialization and dioecy in plants has also been the topic of several recent reviews [12, 18]. Sexual dimorphism is the rule in most animals. However, in plant kingdom, dioecy is found only in 10% of the angiosperms [48]. Dioecism has originated independently in different families and genera and several distinct genetic mechanisms regulating dioecy have been found in different plant species [1]. Sex is the queen problem in evolutionary biology and tracing of molecular markers for sex expression has potential importance in basic and applied research. Molecular mechanism of sex determination in dioecious plants is not well understood. Neither the genetic nor the physiological basis of gender differentiation has not been completely resolved in any plant species, in spite of the striking progress made over the floral development [45]. The presence of sex chromosomes has been documented in some plants [46]. More often, the sex ratio in dioecious plant species is controlled by the expression of alleles at one to several loci [23]. Genetic marker system based on direct analysis of the genomic DNA have been used widely for genetic mapping, disease diagnostics and evolutionary studies and they could prove very useful in the study of sexual determination in dioecious plants such as pistachio [22], hemp [28], basket willow [2] etc. Plants exhibit three major sex strategies in angiosperms like, bisexual, monoecious and dioecious forms. Apart from this, they also exhibits intermediate stages like andromonocious, gynomonocious, androdioecius and gynodioecious. Another interesting group known as poly-gamo dioecious plants consisting of exclusively 5% staminate flowers, few (40-50%) male flowers, few bisexual flowers the remaining 40-50% plants produces only the pistillate flowers [39]. *Simarouba glauca* is a poly-gamo-dioecious tree species belongs to family Simaroubaceae [5].

*S. glauca* is also commonly known as paradise tree, aceituno and is a native of Central and South America and found under a wide range of conditions and at low to medium elevations from Southern Florida to Costa Rica, Caribbean Islands, Bahamas, Jamaica, Cuba, Hispaniola, Puerto Rico, Nicaragua, Mexico, El Salvador etc.
*S. glauca* is an important tree species growing in the forests of Central and South America. National Bureau of Plant Genetic Resources first introduced it in to India during 1960s in the Research Station at Amaravathi, Maharashtra. This was brought to the University of Agricultural Sciences, Bangalore in 1986 and systematic Research and Developmental activities began from 1992 onwards. Both male and female plants have sex specific economic values. Seeds of *S. glauca* are the important source of biodiesel and exhibits considerable and significant medicinal properties. The main active groups of chemicals in Simarouba are called quassinoids, which belong to the triterpene chemical family. The antiprotozoal and antimalarial properties of these chemicals have been documented for many years. Several of the quassinoids found in Simarouba, such as ailanthinone, glaucarubinone and holacanthone are considered the plant's main therapeutic constituents and are the ones documented to be antimicrobial [35], antiviral [30], antiprotozal, anti-amoebic [15], antimalarial [16] and even toxic to cancer and leukemia cells [44]. Therefore, looking to the economic and medicinal values of *S. glauca,* its perennial habit and lack of sex specific morphological characters, identification of the sex at pre-flowering stage plays a key role in breeding of this taxa. Karyomorphological studies in both male and female populations, revealed that both the taxa exhibit homomorphic chromosomes with 2n= 30. Therefore sex cannot be identified based on karyomorphological analysis [8] and thus sex determination mechanism in *S. glauca* is may be at gene level. Identification of sex through RAPD markers have been reported in *S. glauca* [38], *Momordica dioica* [7,9], *Carica papaya & Cycas circinalis* [17], *Borassus flabellifer* [25]. Similarly SCAR marker for gender identification have been reported in *Momordica dioica* [34], *Carica papaya* [19], *Salix viminalis* [20], *Ginkgo biloba* [27]. The objective of the present study is to investigate the molecular basis of genotypic differentiation between male and female plants during pre-flowering stages through RAPD based SCAR markers in poly-gamo-dioecious species *S. glauca* which would facilitate breeding and selection with the saving of time and economic resources.

## MATERIALS AND METHODS

### Plant material collection and isolation of genomic DNA

Both male and female populations, with well defined sex were personally collected from 20 different geographical locations. Plants were maintained in the experimental garden of the department. Identified and vouchered specimens have been deposited in the herbarium, Botany Department, Karnatak Science College, Dharwad.
Genomic DNA was isolated from fresh, young leaves of sexually differentiated plants by modified CTAB method following the standard protocol [14]. Quantification and purity checking of isolated DNA is done through UV-spectrometer and confirmed by running genomic DNA through 0.8% agarose gel. In all cases, the extracted DNA was diluted to a final concentration of 10 ng/µl.

### PCR amplification

RAPD reactions were performed and the results were analyzed as described by Williams et al. (1990) [47]. RAPD analysis was performed with random decamer primers (Operon Tech., Alameda, USA). Each 20µl of the PCR reaction mixture consists of 50ng genomic DNA, 1.5mM $MgCl_2$, 200µM each dNTP, 15pM primer, 0.5 units of *Taq* DNA polymerase (Banglore GeNei™, Bangalore, India). Amplifications were carried out in a Gradient Palm Cycler (Corbett Research, Australia) with initial denaturation temperature of $94^0$C for 4 minutes and each cycle with 15 seconds denaturation at $94^0$C, 15 seconds annealing at $35^0$C, 75 seconds for extension at $72^0$C. The reaction continued for 40 cycles followed by 7 minutes at $72^0$C to ensure the completeness of the primer extension. Amplified products were separated by electrophoresis on 1.2% (w/v) agarose gel, λ DNA double digest with EcoRI and HindIII (GeNei™, Bangalore, India) served as a molecular weight marker and visualized by staining with ethidium bromide and documented.

### Cloning and sequencing of male-specific DNA fragment

The putative male-specific DNA band (1100 bp) amplified by RAPD primer OPA-18 was eluted and purified DNA fragment was cloned using TOPO TA cloning kit (INVITROGEN). The chimeric plasmid was transferred to *E. coli* strain DH5α by chemical transformation [37]. Cloned fragments were sequenced and SCAR primers were subsequently developed (Table-1) using the Primo 3 ver 4.0.

### Validation of SCAR

PCR amplification of both male and female genomic DNA with SCAR primer was carried out with the following recipe. 25ng genomic DNA, 1.5mM $MgCl_2$, 200µM each dNTP, 10pM each of forward primer and reverse primer, 0.5 units of *Taq* DNA polymerase. Amplifications were carried out with initial denaturation temperature of $94^0$C for 4 minutes and each cycle with 15 seconds denaturation at $94^0$C, 30 seconds annealing at $62^0$C, 75 seconds for extension at $72^0$C.

The reaction continued for 40 cycles followed by 7 minutes at $72^0$C for final extension. Amplified products were separated by electrophoresis on 1.2% (w/v) agarose gel, λ DNA double digest with EcoRI and HindIII served as a molecular weight marker and visualized by staining with ethidium bromide and documented.

## RESULTS

Among the 50 random decamer primers used in the present investigation, OPA-18 with the sequence 5′-AGGTGACCGT -3′, amplified genomic DNA in different patterns in both staminate and pistillate DNA samples. A specific amplicon of approximately1100 bp appeared only in staminate samples (Fig 1: lanes 1, 2, 3, 4, 5, 6), but not in pistillate samples (Fig 1: lanes 7, 8, 9, 10, 11, 12). This amplicon was named as OPA-$18_{1100.}$ This male specific DNA band was extremely reproducible under a broad range of amplification conditions.

## SCAR primer development

As the fidelity and the reproducibility of RAPD markers is often questionable, the current trend is to convert the RAPD sequence in to SCAR marker which is more reliable and reproducible. Several SCAR markers have been developed from RAPD sequences [32]. Forward primer MSSMS-01F contained 18-mer and reverse primer MSSMS-01R contained 20-mer (Table-1) (MSSMS- Male Specific Scar Marker of *Simarouba glauca*, F-Forward, R- Reverse). SCAR primers yielded a single distinct and brightly resolved band of 1100 bp in the genomic DNA of all the male plants collected from 50 different geographical locations of Karnataka state (Result of six locations shown in Fig 3) . This SCAR marker band was named as MSSMS (Male Specific Scar Marker of *Simarouba glauca*). Variation in the annealing temperature did not generate any fragment other than the SCAR confirming the specificity of the SCAR primer for male sex.

**Table 1. *Simarouba glauca* sex specific SCAR primer pair sequence derived from cloned RAPD fragment. Optimal annealing temperature for each set of SCAR primers is noted.**

| RAPD Primer used for amplification | SCAR Primer | Number of base pairs | Sequence (5′------3′) | G+C content (%) | Annealing temperature |
|---|---|---|---|---|---|
| OPA-18 | MSSMS-01F | 18 | GTTAGCAGGTGACCGTAG | 55.6 | $60^0$C |
| | MSSMS-01R | 20 | TAGGTGACCGTCCATGAATG | 50.0 | |

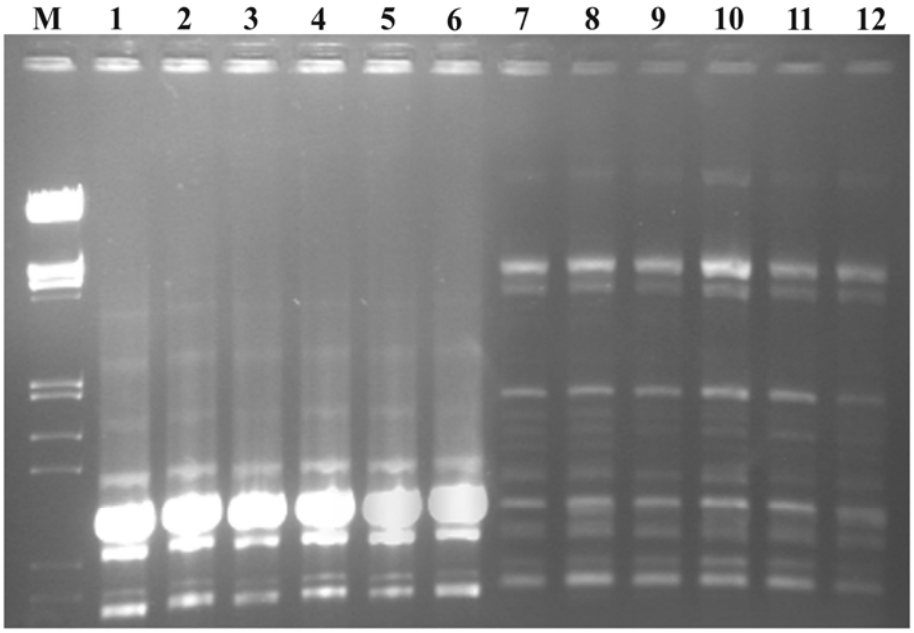


Fig. 1

**Fig. 1 RAPD profiles of *S. gluaca* generated by OPA - 18 primer. Lane M- λ DNA double digest with EcoRI and HindIII. Lanes 1, 2, 3, 4, 5,6 male accessions; Lanes 7, 8, 9, 10, 11, 12 female accessions.**

**Sequence analysis of male-specific RAPD marker OPA-$18_{1100}$**

The cloned OPA-$18_{1100}$ was sequenced which has 1113bp with 44.1% G+C content and 55.9% A+T content (A=357; G=263; T=265; C=228) (Fig 2). DNA sequence of OPA-$18_{1100}$ was deposited in GenBank (Accession number: HM240852). BLASTn [3] results revealed that the sequence has no perfect homology with known plant nucleotide sequences at different sequence similarity levels. However considerable similarity was found with *Medicago truncatula* clone mth2-5h18, complete sequence with accession number AC144406.17(185 bits and E-value=3e-118) and *Intsia palembanica* clone J11 microsatellite sequence with accession number FJ448431.2 (351bits and E-value=3e-93). blastx analysis revealed that OPA-$18_{1100}$ sequence has homology with *Oryza alta* isolate 1 retrotransposon Atlantys, complete sequence with accession number EU257654.1 (52.8 bits and E-value=8e-04) and *Oryza officinalis* retrotransposon Atlantys, partial sequence with accession number EU257656.1 (51.9 bits and E-value= 0.001). tblastx analysis of OPA-$18_{1100}$ sequence has homology with retrotransposon protein, putative, unclassified [*Oryza sativa* (japonica cultivar-group)] with accession number ABA97725.2 (45.1 bits and E-value=0.016).

**Analysis of SCAR-MSSMS**

Analysis of SCAR-MSSMS genomic sequence was analyzed through Gene tool lite *ver.*1 revealed that one Open Reading Frame is present in the region 790-990. Pairwise percentage identity analysis was performed for comparison between SCAR-MSSMS sequence with sequence of MADS box gene *SlAP3* of *Saline latifolia* (GenBank Accession number-AB519803) revealed 59.2 % similarity, comparison between SCAR MSSMS sequence with sequence *APETALA3* (GenBank Accession number- M86357.1) exhibited 57.4% similarity. Further SCAR MSSMS sequence compared with some repetitive sequences like *RAYS*I (GenBank Accession number- FJ534486) and *RAE180* (GenBank Accession number- AM398639) of *Rumex acetosa* that revealed 57.6 and 61.5% similarity respectively.

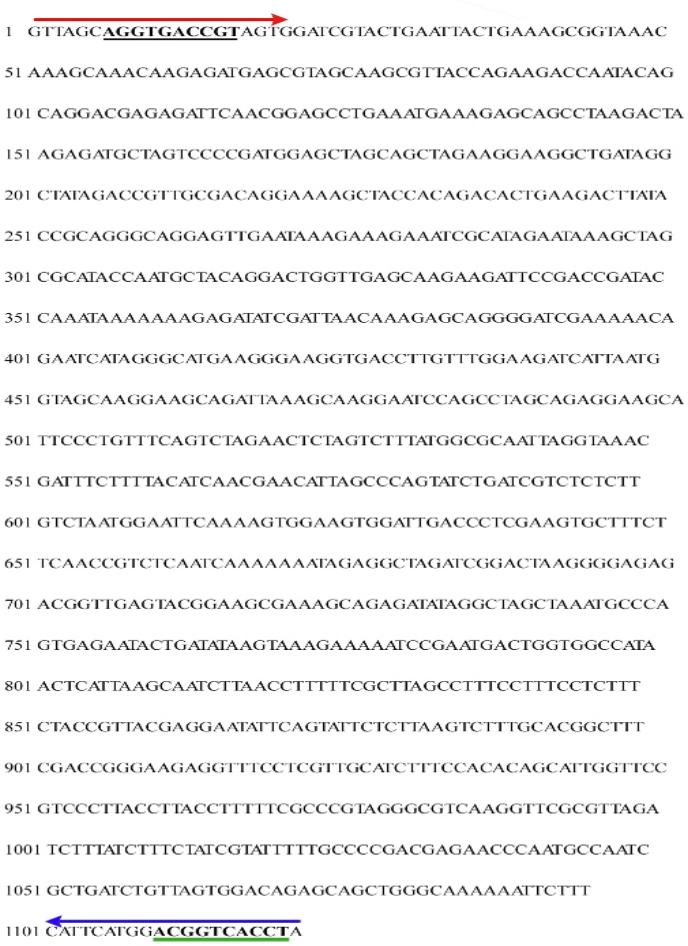


**Fig.2**

**Fig. 2 Complete DNA sequence of the specific RAPD marker OPA-$18_{1100.}$ The sequence of OPA-18 existed at the 5′ and 3′ ends. Red arrow indicates Forward primer MSSMS-01F and Blue arrow indicates reverse primer MSSMS-01R were SCAR primers. Black bar indicate sequence of OPA-18 primer. Green bar indicate complementary sequence of OPA-18 primer**

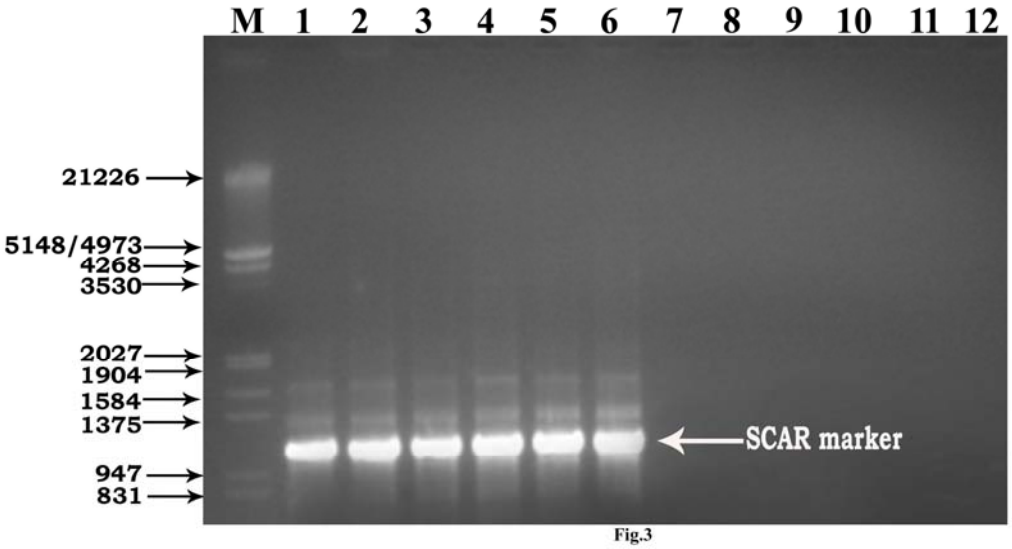


**Fig. 3 SCAR marker analysis of *S. glauca* Lane M- λ DNA double digest with EcoRI and HindIII Lanes 1-6 male accessions; Lanes 7-12 female accessions.**

## DISCUSSION

Identification of sex in dioecious plants during early stages of development is important for selecting female or male plants for mass cultivation, to conserve time and to reduce cost of production. It is more significant in dioecious perennial tree species with sex specific economic and medicinal values. To date, several molecular markers for sex type discrimination in dioecious plants, including papaya, have been reported in Japan [43], Hawaii [13] and India [33].

In the absence of morphological and genetic information on sex determination in dioecious plants, the use of molecular markers for discriminating staminate and pistillate genotypes is worthwhile. SCAR markers are sometimes advantageous over RAPD markers because they can detect a single locus and their amplification is less sensitive to reaction conditions. The literature contains many reports highlighting the use of molecular markers such as RAPD, RFLP, AFLP, SSR, SCAR for gender identification in higher plants. Hormaza et al. (1994) [22] reported a 945 bp band unique to females of *Pistachio vera*, on amplification with OPA-08. In *Piper longum*, two RAPD bands of 905 and 757 bp generated by OPA-10 and OPC-12, respectively were male-specific [6,41] reported six female specific and two male specific RAPD markers in *Actinida deliciosa*. Generation of sex-specific bands has not been restricted only to RAPD primers, but AFLP primers in *Ficus fulva* also reported [42]. Savitha et al. (2008) [38], reported that primer OPS-6 can be used to detect male sex in ***S. glauca*** . In the present investigation RAPD amplicon OPA-$18_{1100}$ was found to be linked with male sex. It is highly reproducible and uniformly appeared in all male populations. This SCAR marker MSSMS (**M**ale **S**pecific **S**car **M**arker of ***S****imarouba glauca)*; can be used to identify the male seedlings at pre-flowering stage. blastx and tblastx analysis reveals that SCAR-MSSMS sequence exhibit homology with some retrotransposon Atlantys of *Oryza species,* which represents repetitive DNA**.** Atlantys is the LTR (long terminal repeat) retro- transposable element family in *Oryza* [4]**.** Homology with such retro-elements in SCAR-MSSMS genomic sequence seems to be linked with sex chromosomes in *S. glauca.* Because it is clear that the evolution of plant sex chromosomes is associated with large increases in the DNA amount as junk DNA [29]. The reasons behind these increases and the types of sequences are not well understood. In *Rumex acetosa,* sex chromosome specific repeated sequences have been described [40] and the sex chromosomes appear to contain large amounts of retroviral and viral related sequences. There is emerging evidence [21] that, plant sex chromosomes show some sequence degeneracy, as is the case in animal Y chromosomes, where the non-pairing segment of the Y chromosome largely lacks functional loci. The application of the techniques of molecular biology will shed further light on the sex chromosomes of plants, the genes carried on them and the ways in which they have evolved.

Since *S. glauca* exhibit homomorphic chromosomes, presence of such retroelements in SCAR-MSSMS sequence paves the way for investigation towards linkage of such sequence with sex determining chromosome. There by such chromosomes can be distinguished by FISH technology [26]. The on-going genome project of papaya further revealed that the male-specific Y chromosome locus represents genetic degeneration; that is low gene density, a high density of retro-elements and repetitive sequences and inhibition of recombination [11]. This concept reveals the association of this junk DNA sequence with sex determination mechanism in *S. glauca*.

Analysis of SCAR-MSSMS through Gene Tool lite *ver.* 1.0. revealed that they exhibit considerable homology with MADS box gene *SlAP3* of *Saline latifolia* (GenBank Accession number-AB519803). The MADS box is a common DNA-binding domain of transcription factors which are crucially regulated in plant development, and perhaps comparable in importance to HOX homeobox transcription factor genes in animals [31]. Further SlAP3 has similarity with an *Arabidopsis* floral identity gene, *APETALA3,* which functions in organ identity and morphogenesis of petals and stamens [24].

Analysis of SCAR-MSSMS through Gene Tool lite *ver.* 1.0. Revealed that this sequence exhibit considerable homology with *APETALA3* (GenBank Accession number- M86357.1). The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes for MADS box and is expressed in petals and stamens. Thus more detailed investigation can reveal SCAR-MSSMS sequence linked to floral fate, as the flower-meristem-identity gene *LEAFY* of *Arabidopsis* is constitutively expressed. *LEAFY* is sufficient to determine floral fate in lateral shoot meristems of *Arabidopsis,* with the consequence that flower development is induced precociously[45]. SCAR-MSSMS genomic sequences were compared with two repetitive sequences like *RAYS*I (GenBank Accession number- FJ534486) and *RAE180* (GenBank Accession number- AM398639) of *R. acetosa*. Pairwise percent identity analysis through Gene Tool lite *ver.* 1.0, revealed that SCAR-MSSMS genomic sequences exhibit homology with these two repetitive sequences. Existence of repetitive sequences is key information as it helps for designing probes for identification of sex chromosomes in *S. glauca* as this taxa exhibit homomorphic chromosomes. Because it is reported that Y chromosome painting of *R. acetosa* and *S. latifolia* has been successfully conducted using FISH with micro-dissected Y chromosomes as probes [36]. These findings strongly suggest that repetitive sequences accumulate in plant Y chromosomes, indicating that the Y chromosome in some dioecious plants is degraded like those of mammals and fruit flies [10]. The localization of two repetitive sequences on the Y chromosome, *RAYS*I, *RAE180*, suggests that dynamic structural changes including reciprocal traslocation and inversion frequently occur on the Y chromosome of *R. acetosa* [40]. Practically this marker can be used to screen large population of plants at very early developmental stages to support breeding programs. In case of dioecious perennial tree species with the sex specific economic value, development of a molecular tool to differentiate the sexes at pre-flowering stage will be a value addition.

## CONCLUSION

SCAR-MSSMS exhibits considerable homology with genes like MADS box gene *SlAP3* of *Saline latifolia, APETALA3* of *Arabidopsis thaliana, LEAFY* of *Arabidopsis* and similarities with retro elements like *RAYS*I and *RAE180* of *R. acetosa* strongly suggests that this DNA sequence plays significant role in morphogenesis of flowering phenomenon. As the sex identification is not possible at chromosomal level, it is better to use molecular markers. Therefore SCAR markers developed in this research work can be used fruitfully to identify sex of the *S. glauca* seedlings in the pre-flowering stage. Present study further suggests the development of SCAR primers from SSR analysis of genomic DNA, analysis of repetitive DNA sequences linked to sex determination and Y chromosome painting.

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