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An Alternative Method for Cytotype Determination in *Acorus calamus* L., Accessions by PCR- RFLP Technique

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***Acorus calamus* is a well-known medicinal and aromatic plant used for many industrial and pharmaceutical purposes. In our current study, twenty different accessions of *Acorus calamus* collected from North-East India and South India have been studied and an attempt has been made to classify them according to their ploidy level by cytogenetic analysis and to correlate them via PCR-RFLP technique for the Non- Transcribed Spacer (NTS) region of the 5S-rRNA gene. β-asarone which is the major phyto-chemical found in *Acorus* sp. when present in higher concentrations was found to be carcinogenic. The scope of this study is to identify diploid *Acorus calamus* accessions as they are reported to have less concentration of β-asarone from those of triploids. Cytoyping was performed by staining of root metaphase chromosomes. For PCR-RFLP analysis, the NTS region of 5S rRNA gene was amplified by using primers, designed from the coding regions of 5S rRNA gene. The PCR products were digested with *Eco*RI restriction enzyme. Triploid accessions showed restriction digestion at 586 bp resulting in an additional fragment of 127 bp, which is absent in diploids. Ten diploid accessions and 10 triploid accessions were identified by cytogenetic technique and PCR-RFLP method. The sequences of triploid and diploid were analyzed using bioinformatics tools.**

Key words: *Acorus calamus, Eco*RI, PCR-RFLP, 5S-rRNA gene, NTS region, cytogenetics, β-asarone.

Generally environmental factors such as morphological, chemical, anatomical and developmental factors are used for the identification, detection and quantification of herbal and aromatic plants[1]. Quantification of compounds by techniques such gas chromatography, liquid chromatography is necessary for plant identification. These methods take up time and is very cumbersome, whereas DNA based molecular methods is relatively fast and quantification is easier and these molecular methods are effective in genotypic discrimination[2-3]. Since genetic method requires genotype instead of phenotype, DNA-based experiments have become widely employed techniques for rapid identification of herbal medicines. Using PCR approaches, nanogram quantities of DNA are sufficient to amplify and yield sufficient amounts of template DNA for molecular genetic analysis[4].

Recently, 5S rRNA gene spacer region has been used for determining the phylogenetic relationships of some higher plants. The 5S-rRNA gene is present in all ribosomes, except in the mitochondria of few species[5]. 5S-rRNA undergoes transcription from hundreds to thousands of genes in all eukaryotes. These sequences are tandem repeats of alternative arrays of sequences coding 5S-rRNA and Non-Transcribed Spacers (NTSs) located separately from the 18S-26S rRNA gene

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clusters [6]. The NTS region shows a high variation in base composition and length which has been speculated as it is not under the same selection procedure as the 120bp 5S-rRNA gene. The specific function of 5S-rRNA as a component of the large ribosomal subunit in eukaryotes may be attributed due to its high level of conservation. This 5S-rRNA gene and NTS region can be considered as a good model for organization and evolution[7]. On the basis of these analysis and assumptions, variation in the NTS region has been used in a number of plant species for studying intraspecific variation, mapping 5S-rDNA arrays, genome evolution and phylogenetic reconstruction[8-9].

In our present study we have used the principle of 5S rRNA PCR-RFLP technique for determining the ploidy level of the regionally endangered and threatened species, traditionally and economically important aromatic and medicinal plant, *Acorus calamus* L.,

Sweet flag, *Acorus calamus* L., is a semi-aquatic, tall perennial herbaceous plant. It grows well in temperate to sub temperate regions. It is one of the highly valued medicinal and aromatic plants in India commonly known as Bach in Hindi. The plant generally grows in high altitude regions. The rich ethno botanical aspects of *A. calamus* have been reviewed [10]. On the basis of its ploidy status and geographical distribution, *A. calamus* has been classified as (i) diploid variety (2n = 2x = 24), (ii) triploid variety (2n = 3x = 36), (iii) the tetraploid variety (2n = 4x = 48) and (iv) hexaploid variety (2n = 6x = 72).

Studies have reported that the species appears to follow a geographical pattern of distribution with respect to ploidy level and the accessions growing in North America are diploid, whereas those growing in Europe and temperate Asia are primarily triploids and the ones growing in eastern and tropical Asia are tetraploids [11][12]. Reports have stated that the constituents of the essential oil and also particularly the â-asarone content depend on the ploidy level of the taxons. It is stated that tetraploids contain highest content of β-asarone with around 70-96%, triploids around 5-19% and diploids 0-2% [13-15]. In the Indian *Acorus calamus* oil, it was reported to have higher percentage of β- asarone and assumed to be of teraploid origin[16]. The use of *Acorus calamus* with toxic β-asarone content has been reported to be unsafe on account of its tumour inducing activity[17].

MATERIALS AND METHODS

Collection of Plant Materials

In our present study 20 different accessions of *Acorus calamus* collected from different geographical areas of South and North Eastern parts of India were used. The geographical location data (Table 1) for 20 *A. calamus* samples were identified and mapped using DIVA GIS [18] to create geographical distribution map for *A. calamus* in various regions of South and North East India (Fig. 1). These plants are classified morphologically and maintained at the Division of Plant Genetic Resources, Indian Institute of Horticultural Research, Bangalore, India.

Cytogenetic Analysis

For the study of mitotic divisions in chromosomes, actively growing young root tips of the *Acorus calamus* plant were excised and pretreated with 2 mM solution of 8-hydroxy quinoline for 4 hours at 10°C to arrest cell growth at metaphase and enhance chromosome observation, subsequently fixed in acetic acid:alcohol (1:3) mixture (Carnoy's fixative) to preserve the material. These were hydrolyzed in 1 N HCl for 10–15 seconds on a flame prior to staining wit aceto-orcein stain to soften cell walls and make the root tip malleable. The staining was performed by using 2% acetoorcein. Maceration was done with squash preparations by dissecting a well stained portion of the root tip and a drop of 1% aceto-orcein was added. It was covered with a cover slip and gently tapped so that it is adequately pressed. Then temporary slides were prepared by sealing the edges of the coverslip with sealing wax. These slides were observed and recorded by an Olympus IXV0I oil immersion microscope (DSS Imagetech Pvt. Ltd, India).

PCR-analysis

Young leaves of the plant were grinded using a pestle and mortar by freezing them in liquid nitrogen. Good quality genomic DNA from all the samples was isolated by CTAB buffer method with few modifications [19]. Intact and pure genomic DNA was assessed with 1 % agarose gel electrophoresis. Then, it was quantified with fluorimeter (DyNA QuantTM200, Hoefer, CA, USA). Based on the

quantification data, DNA dilutions were made in 1 X TE buffer for a volume of 250 µl (working solution) to a final concentration of 30 ng per ìl and stored in 4°C. The NTS region of 5S rRNA gene was amplified by PCR using primers for the coding region of 5S rRNA gene for all the accessions[4]. The primers that were used are: forward primer (5'GTGCTTGGGCGAGAGTAGTA'3) reverse primer (5'TTAGTGCTGGTATGATCGCA'3). The amplification reaction was carried out with 60 nanograms of DNA, 5ìl of 10X assay buffer, 0.2mM dNTPs, 20 picomol forward and reverse primers, 1 unit of *Taq* DNA polymerase. All the PCR reagents were purchased from Merck Genei, India. The PCR conditions consisted of: Initial denaturation for 5min at 4°C; Denaturation for 1min at 94°C; Annealing for 2min at 57°C; Final elongation for 2min at 72°C. This was repeated for 35 cycles and a final Extension for 10min at 72°C, in Applied Biosystems Veriti™ Thermal Cycler, CA, USA. PCR was performed with 50 µl reaction volume. After PCR amplification, 10 µl of the amplified products were resolved using 1.5 % agarose gel for individual accessions, stained with ethidium bromide and documented using (Alpha Imager ™1200, Alpha Innotech Corp., California, USA). Reproducibility of the results was confirmed by repeating the PCR amplification once and resolving the same by agarose gel electrophoresis.

Isolation, purification and sequencing of the amplified PCR fragments.

Around 20 µl of the amplified PCR products were used. The amplified fragments were excised from the agarose gel and purified using a gel clean up kit. For elution of DNA fragments, 1.2 per cent low melting agarose was used, since gels from low melting agarose exhibit low background fluorescence when stained with ethidium bromide. The band corresponding to the desired DNA fragment was cut using a sterile scalpel. Care was taken to avoid much exposure to ultra violet radiation on a UV trans-illuminator. The sliced gel pieces were purified using 'GenElute™ Gel Extraction Kit' (SIGMA-Aldrich Inc., USA). These purified gel products were taken in a clean eppendorf tube sealed well and then forwarded for sequencing to Merck Genei Pvt. Ltd, Bangalore, India. The sequencing was done using BigDye® Terminator v3.1 Sequencing Kit (Applied Biosystems, USA) and analyzed on ABI PRISM® 377 Genetic Analyzer (Applied Biosystems, USA). Sequencing the purified products was done using both forward and reverse primer separately.

Restriction digestion of the amplified PCR product

After confirming the amplification of single product (around 700bp) with gel electrophoresis, the choice of the restriction endo-nucleases for digesting the amplified products was made after comparing the restriction sites of the corresponding 5S-rRNA gene of both diploid and triploid using NEB CUTTER online tool [20]. The samples were subjected to restriction digestion to confirm whether they are diploid or triploid. Digestion of the amplified DNA product was performed with the restriction enzyme *Eco*RI (Genei Merck Pvt.Ltd, India) with10 ìl of the PCR amplified product ~0.1-0.5 µg of DNA), 16 µl of nuclease free water, 2 µl of 10X reaction buffer and 2 µl of (20 units) of the enzyme was used for restriction digestion. Incubation time was 2 hrs at 37°C.

Sequence analysis using online tools

The triploid and diploid DNA sequences analysis was performed separately using NCBI online statistical program BLASTn[21]. These sequences showed high percentage of similarity with the already deposited sequences for the *Acorus calamus* cytotypes from NCBI GENBANK. Evolutionary relationships among the sequences were charted out by cluster analysis using T-COFFEE[22] and CLUSTAL W[23] tools for multiple sequence alignment from the EBI and the phylogenetic analysis and the dendograms for 1000 boot strap[24] values were constructed using the MEGA version 5.0 software tool[25] and by maximum parsimony method[26].

RESULTS

Cytogenetic Analysis

In all the slide preparations the root metaphase chromosomes were stained and observed, their mitotic divisions were found to be normal. The different stages of mitosis [prophase, anaphase, metaphase and telophase] were regular. No aneuploidy was observed. The size of the chromosomes was considerably very small. Clumping tendency was commonly observed. All the 20 preparations that we observed

Table 1. Place of collection, morphological data, geographical co-ordinates (latitude, longitude) of all 20 *Acorus* accessions

S. No	Accession no	Place of collection	Plant height (cm)	leaf length (cm)	leaf width (cm)	No. of leaves	Latitude	Longitude
1	TNRET-3	Doddabetta, Tamil Nadu	40.0±0.8	36.5±0.5	0.9± 0.0	5.6±0.6	11° 26’ 42.1"N	76° 41’14.9"
2	KARRET-147	Agumbe, Karnataka	37.5±0.4	34.0±1.0	1.0±0.1	5.3±0.6	13°302 31.323 N	75° 52 45.243 E
3	KARRET-28	Belgaum, Karnataka	50.2±0.1	45.0±0.5	1.3±0.0	6.6±0.6	15°51'.0"N	74° 302 03 E
4	TNRET-5	Doddabetta, Tamil Nadu	47.6±0.3	43.3±1.5	0.7±0.1	4.6±0.6	11° 242 8.73 N	76° 442 12.23 E
5	APRET-9	Rajahmundry,Andhra Pradesh	50.3±0.2	46.6±0.6	0.9±0.1	7.0±1.0	16° 582 483 N	81° 462 483 E
6	TNRET-4	Parsons Valley, Tamil Nadu	45.3±0.1	42.5±0.6	1.2±0.1	6.0±0.0	11° 242 03 N	76° 422 03 E
7	TNRET-6	Ooty, Tamil Nadu	49.5±0.5	46.3±0.3	1.2±0.1	7.3±0.6	11°24’42.2"N	76° 41' 44"E
8	TNRET-7	Thambettu, Tamil Nadu	48.5±0.2	45.0±1.0	1.0±0.1	6.3±0.6	11° 26' 42.1"N	76° 41' 14.9"E
9	KARRET-179	Devanahalli ,Karnataka	49.8±0.2	45.7±0.2	1.0±0.1	7.0±0.0	13° 132 483 N	77° 422 03 E
10	KARRET-181	BR,Hills, Karnataka	48.0±0.9	42.9±2.6	1.0±0.2	5.3±0.6	11° 592 383 N	77° 82 263 E
11	KARRET-229	Koratagere, Karnataka	48.5±0.1	44.3±0.6	1.2±0.1	6.3±0.6	13° 312 123 N	77° 132 483 E
12	KERRET-83	Kalpetta, Kerala	48.6±1.5	44.6±0.1	0.9±0.1	7.0±0.0	11°372 21.18"N	76° 42 52.53 E
13	KERRET-84	Thrissur, Kerala	50.2±0.1	45.0±0.1	1.4±0.1	6.6±0.6	10° 312 123 N,	76° 122 363 E
14	KARRET-203	Bakala, Karnataka	47.9±0.7	43.6±0.2	1.3±0.0	7.3±0.6	13° 55 ’44.7"N	75 °34' 5.2"E
15	KARRET-228	Attigundi, Karnataka	51.4±0.1	46.2±1.3	1.1±0.1	5.6±0.6	13°25’ 44.8’N	75° 44’32.1"E
16	AS RET-5	Nunmathi, Assam	37.5±0.5	33.0±1.0	0.7±0.0	3.0±0.0	26°5’26"N	91°32’14"E
17	AS RET-6	Dharbaum, Assam	49.2±0.2	45.4±0.1	1.2±0.0	5.6±0.6	26°4’49"N	91°33’34.6"E
18	AS RET-8	Sorupthar, Assam	47.0±0	43.6±0.1	1.4±0.1	6.3±1.2	26°192 54.53 N	93°862 24.23 E
19	AS RET-14	Dhansiri, Assam	40.0±0.5	36.0±0.3	0.9±0.0	6.0±1.0	25°48’ 6.2"N	93°36’23.7"E
20	AS RET-21	Karbi, Assam	37.5±0.2	36.0±3.3	0.9±0.0	4.6±0.6	25°45’55.3"N	93°9’30.6"E

were either diploid or triploid. The details of the observed slides are given (Table 2).

PCR-RFLP Analysis

PCR amplification resulted in an amplified product of around 700bp for all the accessions. The amplified products were subjected to sequencing (one diploid accession and one triploid accession). The sequenced results were subjected to BLAST search analysis from NCBI and the sequences matched with already reported sequences for *Acorus calamus* chemotypes.

The amplified products were also digested with *Eco*RI restriction enzyme and the restriction profile varied for triploids and diploids with the triploid showing restriction digestion with *EcoR*I and the diploid did not show any digestion profile. (Fig 2&3)

Sequence Analysis

The sequenced results of both diploid and triploid accessions showed high similarity to the already submitted DNA sequences of the 5S-rRNA sequences of *Acorus calamus* chemotypes. The sequences obtained from sequencing a diploid accession ASRET-21, showed 96% identity with the partial 5S- rRNA sequence of similar diploid chemotype B (Table 3). Similarly, by sequencing TNRET – 3, a triploid accession showed maximum identity with similar triploid chemotype A (Table. 4). The coding regions located just before the NTS region of both triploid and diploid sequences were found to be identical with few other species such as *Vitis vinifera, Corylus americana, Callistenom salignus, Clivia nobilis, Clivia miniata, Clivia clauscens, Triticum aestivum, Populus alba,*

Table 2. Ploidy status of all 20 accessions of *Acorus calamus* based on cytogenetic analysis

S.no	Accession no	Ploidy level
1	ASRET-14	Diploid
2	ASRET-21	Diploid
3	TNRET-5	Diploid
4	TNRET-6	Diploid
5	ASRET-6	Diploid
6	TNRET-4	Diploid
7	KARRET-28	Diploid
8	ASRET-5	Diploid
9	APRET-9	Diploid
10	KARRET-181	Diploid
11	KARRET-179	Triploid
12	KERRET-84	Triploid
13	KARRET-229	Triploid
14	TNRET-7	Triploid
15	TNRET-3	Triploid
16	KERRET-83	Triploid
17	KARRET-228	Triploid
18	KARRET-147	Triploid
19	KARRET-203	Triploid
20	ASRET-8	Triploid

Table 3. Alignments details obtained by nblast analysis of sequenced 5s rRNA gene (partial sequence) of a diploid *Acorus calamus* accession

S. No	Description	Max score	Query cover	E - value	Identity	Accession
1	*Acorus calamus* 5S ribosomal RNA genes, chemotype B, partial sequence,	1123	100%	0.0	96%	AY214463.1
2	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	835	100%	0.0	88%	AB017424.1
3	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	835	100%	0.0	88%	AB017421.1
4	*Acorus calamus* 5S ribosomal RNA genes, partial sequence	824	100%	0.0	88%	AY812747.1
5	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	824	100%	0.0	88%	AB017422.1
6	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	813	100%	0.0	88%	AB017423.1
7	*Acorus gramineus* gene for 5S rRNA, partial sequence, chemotype B,	486	100%	9e-134	80%	AB017425.1

Chemotype A - Triploid; Chemotype B - Diploid

Table 4. Alignments details obtained by nblast analysis of sequenced 5s rRNA gene (partial sequence) of a triploid *Acorus calamus* accession

S. No	Description	Max score	Query cover	E - value	Identity	Accession
1	*Acorus calamus* 5S ribosomal RNA genes, chemotype A, partial sequence	1179	100%	0.0	97%	AY812747.1
2	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	1179	100%	0.0	97%	AB017424.1
3	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	1179	100%	0.0	97%	AB017422.1
4	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	1179	100%	0.0	97%	AB017421.1
5	*Acorus calamus* gene for 5S rRNA, chemotype A, partial sequence	1157	100%	0.0	96%	AB017423.1
6	*Acorus calamus* 5S ribosomal RNA genes, chemotype B,partial sequence	625	100%	2e-175	83%	AY214463.1

Chemotype A - Triploid; Chemotype B - Diploid

Hordeum lelcheri, Gossypium harkenessi, Victoria regia etc. Since the coding regions of these species are similar, it is possible to use the same primers as we have used to amplify their respective NTS regions. These sequences of diploid and triploid cytotypes were aligned using T-COFFEE server (Fig.4) and a boot strap analysis for 1000 pseudo samples was performed using MEGA version 5.0 software. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 3 most parsimonious trees (length = 366) is shown. The consistency index is (0.743), the retention index is (0.630), and the composite index is 0.612 (0.468) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches[24] in Fig. 5. The MP tree was

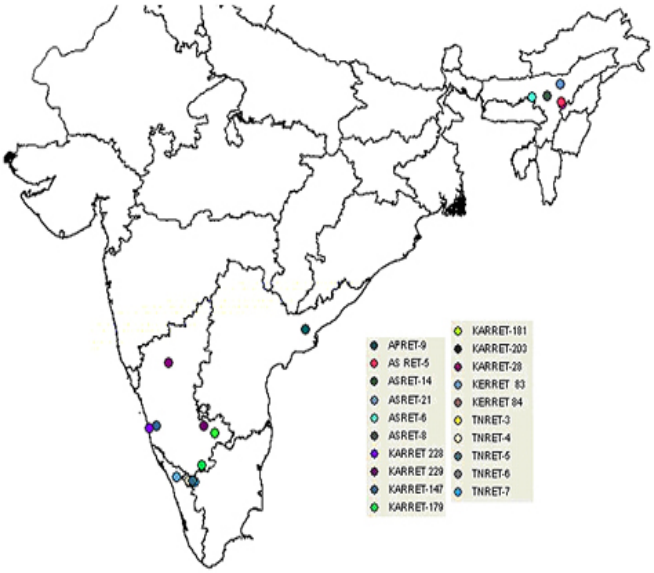

Fig. 1. Distribution map showing the *Acorus calamus* accessions collected from 20 different sites in the present study

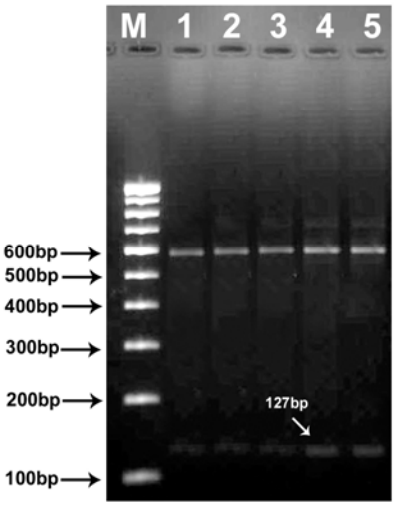

Fig. 2. Restriction digestion profile of Triploid Acorus calamus accessions with *Eco*RI enzyme showing a restricted fragment of 127bp. The lanes represented with legends are M- DNA reference Marker, 1 - KARRET-179; 2 - KERRET-84; 3 - KARRET-229; 4 - TNRET-7; 5. TNRET-3

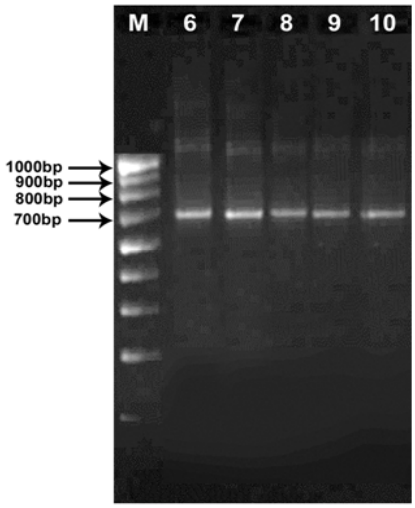

Fig. 3. Restriction digestion profile of Diploid *Acorus calamus* accessions with *Eco*RI enzyme with no restricted product. The lanes represented with legends are M- DNA reference Marker, 6 - KARRET-181; 7 - APRET-9; 8.ASRET-5; 9 - TNRET-5 AND 10 - ASRET-21

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Triploid    GTGCTTGGGCGAGAGTAGTACTGGGATGGGTGGGGGTCATCATGGGA-GTCATCGTGTTG 59
diploid     GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGA----CCTCCTGGGAAGTCCTCGTGTTG 56
            ********************** *********     * ** ***** *** ********

Triploid    CACCCCCCGAAATCTCCTTTTTCTATTTTTTCCCTCCTCCGTCCCATTTTTTCACGTC 119
Diploid     CACCCCCCGAAATCTCCTTTTTCCGAT-------CCCATCCGTCCC-TTTTTTCACGTC 108
            ************************   *       * * ******** *************

Triploid    CCATTCTTCATCCACGTGTCGGAACCCCACGTGGAAGGGGCGCAAACGGAGACCGACTGG 179
Diploid     CCATTCTTCTTCCACGTGTCGGATCCTCCCGTGGACGGGCCCCCGACGGAGACCGGCCGC 168
            ******** ************ ** * ****** *** * *  ********** * *

Triploid    TCAAGCTAATTTTCCACGTGACCGAATCTCATAGGAATGCCCGTCTGCGCCA---CGTGG 236
Diploid     TCACGCTGCTTTTCCACGTGACCGTTTTTTAAAGGAGTGCC-GTATGCGGTAGAACGTGG 227
            *** ***  ***************  * * * **** **** ** ****  *   *****

Triploid    TTCTTAGCGGCCGCAGGAGTTGGCGGTGCCGGCAGATCCGAGGAAAAACGTCTGCGGGGA 296
Diploid     GTCATAGCGGCCGCATGAGTTGCCGGTGCCGGCAAATCCGAGTAAAATTGTCTCATGGGG 287
             ** *********** ****** *********** ******* ****  ****   ***

Triploid    CCCGCATTGTG-CCGAAACTTCATAGGGCCGTAACTTTCGCTACGGTCGTCAGAACGGCT 355
Diploid     CCCGCAGGGGGGCCGAAACTTCATTGGGCCGTAACTTTCGCTACGGGCGTCGGAACGGCT 347
            ******  * * ************ ********************* **** ********

Triploid    CGTATTACATATGTTTTTGGGGCAGCTCGACGAGCCGGTCGCGATGGGCAATGTGCCGGA 415
Diploid     CATATTACATATGTTTTTGGGGCAGCTCGACGAGCCGGTCGCGATGGGCAATGTGCCGAG 407
            * ************************************************************

Triploid    GGCACGTTGCCGCCGTCTCGGATCCCAAAATTTCCATCGTTTCGGCCCCCGAAACGCTGT 475
Diploid     GGCACGTTGCAGCCGTCTCGGATTCCAAAATTTCCACCGTTTCGGCCCCCGAAACTCGGT 467
            ********** ************ ************ ****************** * **

Triploid    TTTTTCCCTGAAATTTCCTTTGCCTGTCGTTCTTTTGTAAAAGATCTCACCCTCCGGTGC 535
Diploid     TTTCTCCTGAAAATTTCCTTTCCCTGTCGCTCTTTTGGATAAGATCCCATCC-------C 520
            *** ***   *********** ******* ******* * ****** ** **       *

Triploid    TGGGTTTGCTATTTCGATTCTCGAGCATGTCCCGAGGTTCCGTATAGTTCGAATTCTTCT 595
Diploid     TGGGTTTGCTATTTCGGTTCTCGAGCATGTCCCGAGGTTCCGTATAGTTCTAATTCCTCT 580
            *************** ********************************** ***** ***

Triploid    GCTGCGCCGTTGCCTGTCTGAATTTAGTTTTTTTATCCGTGACAGACGCGGGCCCGCTA 655
Diploid     GCTGCGCCGTTGCCT-TCTGAATTTAGTTTTTTTATCCGTGACAGACGCGGGCCCGCTA 639
            *************** ********************************************

Triploid    CGATGCCATTTAAGGGATAAGTCCGCGTTCTTCACGGGTGCGATCATACCAGCACTAA 713
Diploid     CGATGCCATT--AAGGATATGTTCGCATTCTGTACGGGTGCGATCATACCAGCACTAA 695
            **********  * ***** ** *** ****  ************************
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Fig. 4. Alignments of the nucleotide sequences of 5S-rRNA gene spacer region of diploid and triploid *A. calamus* cytotypes. The coding regions are underlined. Primer sequences are indicated in bold. Identical sequences are indicated by (*). Gaps (-) are introduced for the best alignment. EcoRI site is evidenced in the squared

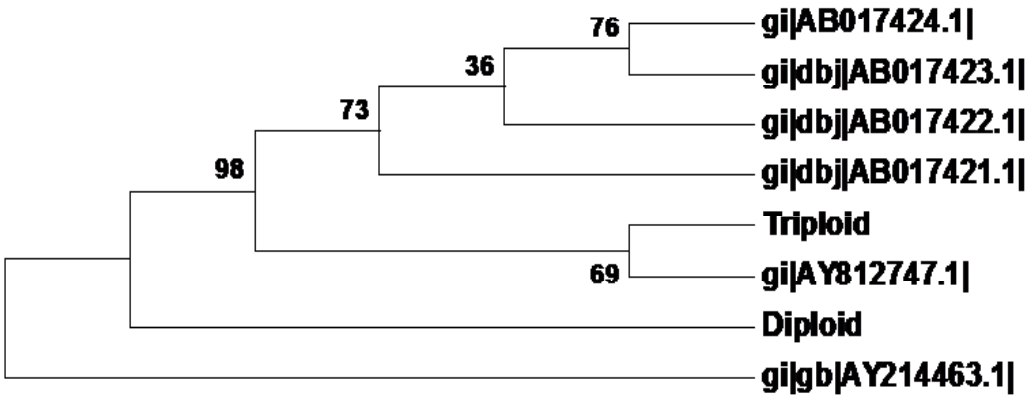

Fig. 5. Maximum Parsimony bootstrap analysis of triploid and diploid *A. calamus* sequences obtained after blast search. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. For ploidy status of the accession, refer the accession numbers listed in Table 3 and 4

obtained using the Subtree-Pruning-Regrafting (SPR) algorithm[27] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 634 positions in the final dataset.

DISCUSSION

Acorus calamus or sweet flag is a well-known traditional medicinal and aromatic plant whose essential oil is also used for various purposes. The US FDA and also the EC interdicted the utilization of sweet flag in therapeutic formulations and food and beverages industries due to the potential carcinogenic effects of its essential oil, with particular reference to β-asarone which was found to be toxic in rats[28-29]. Since studies have stated that tetraploids contain highest content of â-asarone with around 70-96%, triploids around 5-19% and diploids 0-2%, the *Acorus calamus* varieties with very low percentage of â-asarone were permitted to be used.

In our present study, we have compared all the 20 accessions of the *A. calamus* by cytogenetic analysis. The ploidy level for each accession was determined. In all 20 accessions, all the stages of cell division *i.e* prophase, metaphase, anaphase and telophase were normal. Out of all accessions that were observed 10 were found to be diploids and the rest triploids, ruling out any tetraploids. The accessions from North-East i.e from Assam (4 accessions) were found out to diploids and whereas the South Indian varieties were a mixture of both diploids and triploids. These results were similar to a study that Indian *Acorus calamus* is not a tetraploid[30].

In higher eukaryotes, the 5S-rRNA gene occurs in tandemly repeated units consisting of 120 bp coding region separated by a non-transcribed spacer of various sizes. Although the coding region is highly conservative, the non-transcribed spacer varies from species to species in both sequence and length, since it is apparently not under the same rigorous selection pressure as in the coding region[4]. Thus, the diversity of the spacer region can be used as an identification basis. In our present study, we have used two primers (coding sequences) flanking the spacer region of 5S-rRNA gene, which was already being utilized for identifying the differing chemotypes of *A. calamus* by PCR analysis[31]. These coding sequences were also found identically present in other plants, which were listed already. This indicates the coding sequences of 5S rRNA genes are conserved across many plant species. After careful observation of both diploid and triploid sequences, one *Eco*RI restriction site (GAATTC) found in triploids was absent in diploids. A single base was found to be replaced (TAATTC) in diploid. These results were further confirmed by analyzing them *in silico* using

the NEB CUTTER tool. Similar were results were also reported [4]. Further the amplified products were subjected to restriction digestion with *Eco*RI enzyme and a restriction profile which varied for the triploid and diploid varieties was evident with an additional 127bp fragment. The restriction digestion results for all accessions were found to correlate with the results that we had obtained from cyto-genetic analysis.

CONCLUSION

The importance of *Acorus calamus* as a medicinal plant and also its benefits in the food and beverage industry is well known. Considering the health effects caused due to the presence of high concentration of carcinogenic β-asarone in the triploid and tetraploid cytotypes, the PCR-RFLP technique with the detection of an *Eco*RI restriction site in the Non-Transcribed Spacer region (NTS) can be considered a valuable tool for identification of the triploid varieties and also account to beneficial and safer selection of diploid cytotypes.

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