Genetic diversity analysis and chemical profiling of Indian *Acorus calamus* accessions from South and North-East India

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Acorus calamus L. (Family: Acoraceae) is a well-known traditional, endangered, medicinal and aromatic plant mainly found in India and China. The plant is also widely used in industrial, pharmaceutical and food industries. In the present study, 20 different accessions of Indian *A. calamus* were subjected to the study of genetic diversity (RAPD), and cytogenetic and phytochemical (β-asarone) analysis. For RAPD analysis, 9 primers were chosen, which generated 107 DNA fragments. The average percentage of polymorphism was recorded to be 67.23%. The primer OPA 12 showed the highest (100%) polymorphism, whereas the lowest (38.2%) polymorphism was observed for the primer OPBB 6. The polymorphism information content (PIC) values ranged 0.44 (OPA 7) to 0.18 (OPA11), while marker index (MI) values ranged 4.74 (OPA 7) to 0.36 (OPA 11). A dendogram was constructed by UPGMA method and the robustness of the tree was confirmed by bootstrap analysis with 1000 pseudo samples. For cytogenetic analysis, the 20 *A. calamus* accessions were screened for their ploidy status. The accessions were found to be either diploid or triploids. The phytochemical analysis of β-asarone content was determined through by HPLC method. The β-asarone concentration varied in the range of 2.2 to 7.2 mg/100 mg. The results of present study indicated the presence of low level of polymorphism among the *A. calamus* accessions of South India and North-East India. The phytochemical and cytogenetic analysis revealed that both diploid and triploid have low concentration of β-asarone irrespective of their geographical location.

Keywords: β-Asarone, HPLC, medicinal plant, ploidy, RAPD

Introduction

Medicinal plants are being threatened worldwide due to habitat fragmentation, deforestation, over harvesting and exploitation to meet demands of raw drug markets and industries[1]. This over exploitation of medicinal plants has resulted in reduced population density in many species and some of these plants are critically endangered[2]. Adaptability to environmental changes decreases when there is reduction or loss of genetic diversity within populations. The level of genetic diversity also affects individual fitness and potential population persistence. This has resulted in reduced population size, increase in genetic drift, decreased population adaptability and also increase in the risk of extinction of the valuable medicinal plants[3-4]. Thus the assessment of genetic diversity is the first step in the evaluation of long-term conservation need of any plant species[5].

Acorus calamus L. (Family: Acoraceae), commonly known as 'Sweet Flag', is one such important medicinal plant, which has been traditionally used in Ayurvedic and Unani medicines. The phyto-chemical ingredients of the plant are utilized worldwide for various purposes. The plant is being threatened due to deforestation and overexploitation and is red-listed and categorized as species of conservation concern in South India by the Foundation for Revitalization of Local Health Traditions, (FRLHT)[6], Bengaluru. The plant is a semi-aquatic herb, growing in temperate to sub-temperate regions. Rhizomes, leaves and roots of the plant are used as traditional medicines in India and China. The plant possesses insecticidal[7-8], antifungal[9], antibacterial[10] and allelopathic properties. It is widely employed in modern medicine due to its sedative, laxative, diuretic, and carminative properties[11]. The plant also possesses a characteristic sweet smell due to the presence of a component known as β-asarone [(Z)-1,2,4-trimethoxy-5-prop-1-enyl-benzene]. Pharmacological studies have brought into evidence the toxicity concern of the plant too for the presence of β-asarone, which at higher

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concentrations was reported to be carcinogenic in rats[7,11-13]. It is also believed that the β-asarone content and its concentration vary with the ploidy level of the plant as well as its geographical location[14]. With this background, the present study focuses on the following objectives: (a) Analysis of genetic diversity by RAPD markers in the population of different accessions of *A. calamus* collected from North-East India and South India; (b) determination of ploidy level of the *A. calamus* accessions using cytogenetic techniques; and (c) estimation of β-asarone content through HPLC analysis. The results of the present study would help us to understand the genetic and cytogenetic relatedness of the different accessions of Indian *A. calamus* and the relationship with β-asarone content and to develop strategies for the conservation of the species.

Materials and Methods
Plant Materials

In the present study, 20 different accessions of *A. calamus* collected from different geographical areas of South and North-Eastern parts of India were used. Using the passport data (Table 1), the accessions mapped at various regions of South and North-East India using DIVA GIS software[15] and a geographical distribution map was drawn (Fig. 1). These plants were further maintained at the Division of Plant Genetic Resources, Indian Institute of Horticultural Research, Bangalore, India.

For the morphological analysis, three plants from the population of each accession were considered and their average is shown in Table 1.

DNA Isolation and RAPD Analysis

Young, disease-free leaves of each accession were collected, frozen by liquid nitrogen and stored at −70°C until used. Leaves were powdered with liquid nitrogen in pre-cooled sterile mortars and pestle. DNA was isolated from approx 2 g of leaf powder
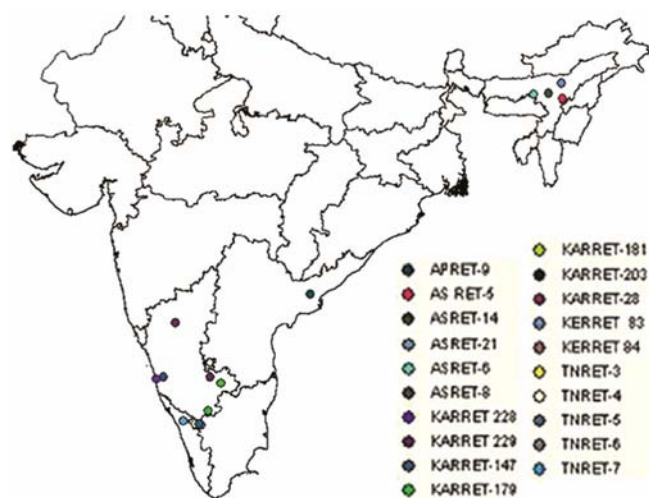

Fig. 1—A distribution map showing locations (South & North-East India) of collection of 20 *A. calamus* accessions used in the present study.

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

No.	Acc. 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°30′31.32″N	75° 5′ 45.24″ 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° 30′ 0″ E
4	TNRET-5	Doddabetta, Tamil Nadu	47.6±0.3	43.3±1.5	0.7±0.1	4.6±0.6	11° 24′ 8.7″ N	76° 44′ 12.2″ E
5	APRET-9	Rajahmundry,Andhra Pradesh	50.3±0.2	46.6±0.6	0.9±0.1	7.0±1.0	16° 58′ 48″ N	81° 46′ 48″ 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° 24′ 0″ N	76° 42′ 0″ 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° 13′ 48″ N	77° 42′ 0″ E
10	KARRET-181	BR,Hills, Karnataka	48.0±0.9	42.9±2.6	1.0±0.2	5.3±0.6	11° 59′ 38″ N	77° 8′ 26″ E
11	KARRET-229	Koratagere, Karnataka	48.5±0.1	44.3±0.6	1.2±0.1	6.3±0.6	13° 31′ 12″ N	77° 13′ 48″ E
12	KERRET-83	Kalpetta, Kerala	48.6±1.5	44.6±0.1	0.9±0.1	7.0±0.0	11°37′21.18"N	76° 4′ 52.5″ E
13	KERRET-84	Thrissur, Kerala	50.2±0.1	45.0±0.1	1.4±0.1	6.6±0.6	10° 31′ 12″ N,	76° 12′ 36″ 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±0.0	43.6±0.1	1.4±0.1	6.3±1.2	26°19′54.5″N	93°86′24.2″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

using CTAB method[16]. The purified DNA samples were quantified with fluorimeter (DyNA Quant™200, Hoefer, CA, USA). Based on the quantification data, DNA dilutions were made (25 ng/μL) in 1× TE buffer and subjected to RAPD analysis. Approx 40 ng of DNA was used as template for PCR reaction. Reaction volume of 25 μL was consisted of: 1× PCR assay buffer, 0.2 mM dNTPs, 0.5 units of *Taq* DNA polymerase, 20 pmol of primers. The details of the primers used are mentioned in Table 2.

Of 40 primers screened for optimization of PCR amplification, 9 primers showed clear unambiguous, polymorphic and reproducible banding pattern. We used the following amplification conditions: Initial denaturation for 3 min at 94°C, 40 cycles for 30 sec at 94°C, 1 min at 43°C and 1 min at 72°C, and final extension for 10 min at 72°C, in a Thermal Cycler (Applied Biosystems Veriti™, CA, USA). Amplified products were resolved using 2% agarose gel 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.

Data Analysis

Clearly resolved, unambiguously amplified amplicons from the documented gel images were scored for their presence or absence of bands for each primer. The scores were obtained in the form of a matrix with '1' and '0', which indicated the presence and absence of bands, respectively. The binary data scored was used to construct a dendrogram. The genetic associations between genotypes were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers[16]. Similarity matrix was generated using the NTSYSpc software, version 2.0[17]. The similarity coefficients were used for cluster analysis and dendrogram was constructed by the Unweighted Pair Group method (UPGMA)[18] and SHAN clustering in the NTSYSpc program. Bootstrap analysis was performed for 1000 pseudo samples using FREETREE software[19] and the trees were viewed by TREEVIEW program. On the basis of the scored data, the parameters that were calculated for each primer are as follows, percentage polymorphism; effective multiplex ratio (EMR) = $np(np/n)$, where "np" is number of polymorphic loci and "n"= total number of loci; polymorphism information content (PIC) = $1−f^2+(1−f)^2$, where 'f' is the frequency of the marker in the data set; marker index (MI) is calculated by multiplying PIC value with EMR value for each primer.

Sample Preparation for Cytogenetic Analysis

For the study of mitotic divisions in chromosomes, actively growing young root tips of the *A. calamus* plant were excised and pretreated with 2 mM solution of 8-hydroxy quinolinone for 4 h at 10°C to arrest cell growth at metaphase and enhance chromosome observation. Subsequently, these were fixed in acetic acid:alcohol (1:3) mixture (Carnoy's fixative) to preserve the material. These were then hydrolyzed in 1 N HCl for 10-15 sec on a flame prior to staining with aceto-orcein to soften cell walls and make the root tip malleable. The staining was performed by using 2% aceto-orcein. 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 get adequately pressed. Then

Table 2—Details of primers used and estimation of percentage polymorphism, effective multiplex ratio (EMR), polymorphism information content (PIC) and marker index (MI) in *Acorus* accessions

Primer used	Total polymorphic bands	Total monomorphic bands	Total no. of bands	Primer sequence	GC content (%)	Percentage polymorphism	EMR	PIC	MI
OPA3	12	1	13	5′-AGTCAGCCAC-3′	60	92.31	11.04	0.43	4.74
OPA5	7	7	14	5′-AGGGGTCTTG-3′	60	50.00	3.50	0.19	0.66
OPA7	11	1	12	5′-GAAACGGGTG-3′	60	91.67	10.01	0.45	4.50
OPA16	7	5	12	5′-CCAGCTTACG-3′	60	58.33	4.06	0.30	1.22
OPA9	8	4	12	5′-GGGTAACGCC-3′	70	66.67	5.28	0.32	1.69
OPA12	9	0	9	5′-TCGGCGATAG-3′	60	100.00	9.00	0.40	3.60
OPBB5	5	8	13	5′-TGCGCCCTTC-3′	70	38.46	1.95	0.23	1.17
OPBB6	5	8	13	5′-TGCTCTGCCC-3′	70	38.46	1.95	0.19	0.44
OPA11	4	5	9	5′-CAATCGCCGT-3′	60	44.44	1.76	0.20	0.360
			108#	Average	63	64.48	5.39	0.30	2.04

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).

HPLC Analysis

For this study, we have used the external standard method. β-Asarone standard was obtained from M/s Natural Remedies, Bangalore, India. Standard solutions of β-asarone known concentrations were prepared by dissolving in methanol. They were filtered through 0.2 µm nylon membrane filter and injected in to HPLC. A standard curve was obtained by plotting the peak area against concentration. A linear fit was chosen with regression coefficient of r^2=0.993. The concentration of the sample was determined by comparison with the standard curve[20].

All the rhizome samples were dried at 35°C for 60 h in hot air oven, Serwell Instruments Inc., India. Dried samples were ground in a Mixer Mill MM 400 (RETSCH, Germany) to get fine powder. About 20 mg of the sample powder was taken in a test tube and 15 mL of methanol was added. The test tubes containing sample solutions were sonicated (Vibra Cell VC 505, Sonics & Materials, USA) for 5 min. The sample solution was filtered using 0.2 µm nylon membrane (Advanced Micro Devices India Pvt. Ltd., India) and injected in to HPLC.

The HPLC analyses were carried out on a Shimadzu Series LC-10A system (Shimadzu, Kyoto, Japan) consisting of a liquid chromatograph connected to a UV-VIS detector (10 A), binary pump and controlled by Shimadzu Class VP Workstation software. The column used was C18 Gemini, 250×4.6 mm^2, 5 µm (Phenomenex, USA) with security guard column. The flow rate was 1 mL/min and mobile phase consisted of water with 0.1% TFA (solvent A) and methanol (solvent B). The instrument was run in an isocratic mode (A: 35; B: 65). The detection was monitored at 210 nm.

Results and Discussion

Initially 40 RAPD primers were screened for genetic diversity analysis, of which 9 primers showed clear amplified profiles. These 9 primers generated a total of 107 DNA fragments (Table 2). The number of polymorphic bands ranged from 14 to 9 with an average of 12 bands per primer, with a product range 830 to 1200 bp. The average polymorphism percentage was found to be 63.0%. The primer OPA 12 showed the highest polymorphism percentage (100%), whereas the lowest was for the primer OPB 6 (38.2%). The average PIC value was found to be 0.30 and the average MI value 1.92. The highest MI value was observed to be 4.3 for OPA3 and the lowest was 0.36 for OPA11. Higher the MI value more is the probability of using the particular primer for studying specific traits. A similarity matrix was constructed on basis of Jaccard's coefficient, which revealed the genetic distances. The highest Jaccard's coefficient value among the South Indian accessions observed was 0.86, while it was 0.76 between the North-East Indian accessions. The lowest Jaccard's coefficient value was observed between APRET-9 and KERRET-84. Higher Jaccard's coefficient values indicated higher genetic similarity. The dendogram indicated low to medium level of diversity among the *A. calamus* accessions (Fig. 2). The analysis of the clusters revealed the formation of one major cluster (1) and two sub clusters (1B & 1A), which are mixture of both North-East Indian and South Indian accessions. The highest bootstrap value of 100 was observed between TNRET-4 and TNRET-5, followed by KARRET-228 and KARRET-229 with a value of 82, ASRET-8 and ASRET-5 with a value of 80, and KARRET-203 and KARRET-28 with a value of 74. These higher bootstrap values indicate that the accessions are closely related. The Principal Coordinate Analysis (PCA) showing the clustering pattern of all accessions is represented with their positions in four different quadrants (Fig. 3).

The dendogram also revealed that the accessions displayed low intra population diversity within the clusters and differences between them were less significant. Individuals from the same population mostly formed tight clusters. Genetic variation within
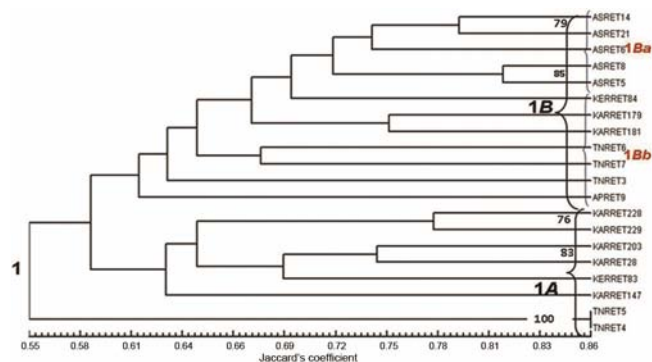

Fig. 2—Phylogenetic tree representing 20 accessions of *A. calamus*. [Bootstrap values are indicated in numerical as percentage]
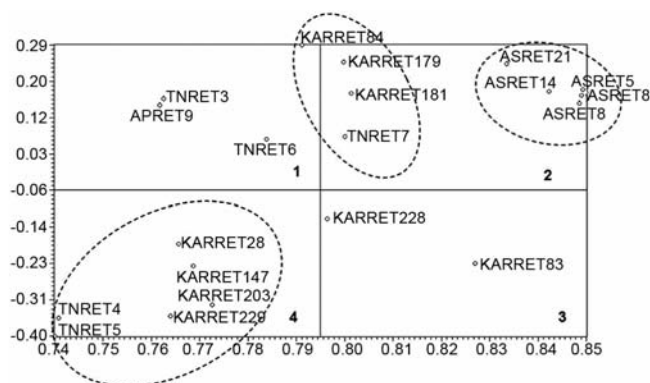
Fig. 3—Principal Coordinate Analysis (PCA) showing the clustering pattern indicated by dotted ellipses and their positions in four different quadrants.

Table 3—Ploidy status and β-asarone content of *Acorus* accessions

No.	Acc. no.	Ploidy level	β-Asarone content from HPLC analysis (µg/mL)	β-Asarone content (% w/w)
1	ASRET-14	Diploid	24.45	2.18
2	ASRET-21	Diploid	47.5	4.19
3	TNRET-5	Diploid	68.49	5.92
4	TNRET-6	Diploid	69.47	5.97
5	ASRET-6	Diploid	51.99	4.62
6	TNRET-4	Diploid	50.56	4.29
7	KARRET-28	Diploid	83.05	7.22
8	ASRET-5	Diploid	82.43	7.24
9	APRET-9	Diploid	58.31	4.92
10	KARRET-181	Diploid	61.81	5.22
11	KARRET-179	Triploid	56.98	5.07
12	KERRET-84	Triploid	53.81	4.93
13	KARRET-229	Triploid	59.17	5.49
14	TNRET-7	Triploid	52.39	4.69
15	TNRET-3	Triploid	58.84	4.99
16	KERRET-83	Triploid	51.11	4.28
17	KARRET-228	Triploid	62.00	5.27
18	KARRET-147	Triploid	62.62	5.43
19	KARRET-203	Triploid	54.64	4.63
20	ASRET-8	Diploid	52.77	4.52

a population was homogenous. Earlier studies on genetic diversity on *A. calamus* have also revealed that the individuals within a population were homogenous in terms of their genetic composition[21]. Using ISSR markers, it has been observed that a high amount of genetic variation and divergence among populations, and moderate variation within populations of *A. calamus*, existed in South Eastern Ohio, USA[21]. Even though loss of heterozygosity is a disadvantage in a changing environment, the emergence of homozygosity due to the dominance of clonal propagation may preserve the adaptive gene complexes in stable aquatic environment[22], especially with respect to the evolutionary primitive monocots[23]. Further, genetic diversity study on *Bacopa monnieri* species, collected from India, also indicated low level of genetic variation as a result of 'inbreeding' within the population[24]. *A. gramineus* species of the genus *Acorus* also revealed a comparable pattern of genetic variations with RAPD markers[25]. Thus, low level of genetic diversity and high genetic differentiation between geographical regions could be attributed to the clonal nature of *A. calamus*[21]. Recently, *A. calamus* accessions from South and North-East India were analyzed using ISSR markers and reported that, even though a high genetic divergence was present, a low level of genetic variation was observed. Though loss of heterozygosity is a disadvantage in a changing environment, the emergence of this homozygosity due to the dominance of clonal propagation may preserve the adaptive gene complexes in stable aquatic environment, especially with respect to the evolutionary primitive monocot *A. calamus*[26-27]. Similarly, RAPD markers have been used on Indian *A. calamus* accessions and reported that the clustering of the

populations was not particular to their geographical regions[27]. This reflected a high genetic differentiation and low level of genetic variation among genotypes from the same locations. This low level of RAPD marker variations could be due to high heterozygous nature of plants, which might not be uniform across the different populations throughout the country.

In the present study, even though the accessions used represent the natural populations from two different agroclimatic zones of India, *i.e.*, one from South India and the other from North East India, the accessions showed genetic relatedness among each other. Similarly, RAPD analysis of *Iris setosa* showed genotypes from one geographical location sharing higher level of genetic relatedness with other geographic location[28]. This corroborates the fact that geographically and genetically distinct populations can exchange genetic information and are not completely isolated. The cytogenetic studies on *A. calamus* accessions revealed that the different stages of mitosis prophase, anaphase, metaphase and telophase observed were regular. The size of the chromosomes was very small and no aneuploidy was observed. The cytological analysis also indicated that all 20 accessions of the *A. calamus* were either diploid or triploid (2n or 3n, where x=12) irrespective of their geographical locations. The ploidy status of the observed 20 accessions is described in Table 3.

The HPLC chromatogram of the β-asarone standard showed a retention time of 7.13 (Fig. 4). For the evaluation of β-asarone content, the *A. calamus* samples were subjected to HPLC analysis (Figs 5 & 6). The concentration of each accession was determined using the area under the curve method. The results obtained are depicted in Table 3. The range of concentration varied around 2.80 to 7.3 mg/100 mg of dry wt of the powdered rhizomes. The lowest value was observed for the sample ASRET-14 with concentration of 2.80 mg/100 mg, while the highest was observed for the sample ASRET-6 with a concentration of 7.3 mg/100 mg. In a recent report, *A. calamus* plants were observed to possess β-asarone content ranging from 65.96 to 92.12% in their essential oils[29].

The present analyses revealed that both the triploid and diploid accessions showed rather lower concentration of β-asarone and no significant correlation existed between the ploidy level and the β-asarone content in the rhizome samples. The present results also indicated a very low chemical variation with reference to β-asarone content even though the accessions studied belong to different geographical locations. Earlier, similar results were observed, wherein both diploid and triploid Indian *A. calamus* accessions possessed low β-asarone content[23]. However, the present results are contradictory to
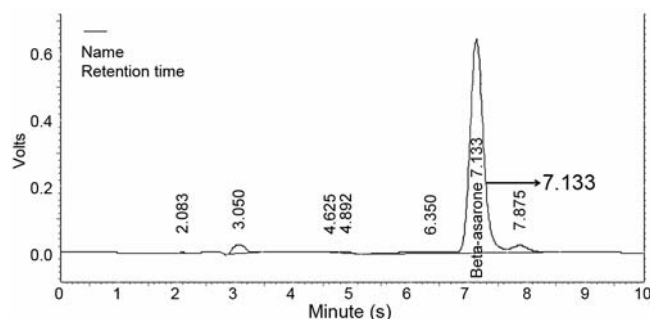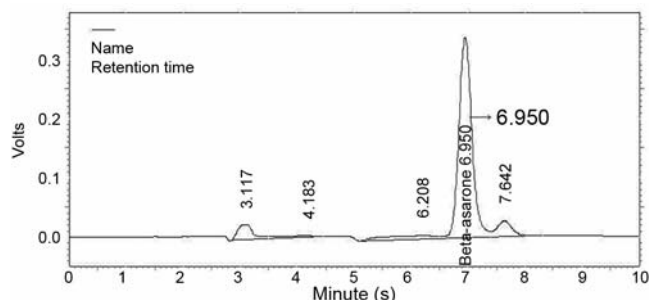
several previous reports, which have stated that Indian *Acorus* is a tetraploid. They have also reported that the triploid accessions of *A. calamus* possess high concentration of β-asarone, whereas the diploid accessions possess negligible or trace amount of it[10-13]. However, in another study, triploid plants are reported with low β-asarone content and tetraploid cytotypes of high β-asarone content in the analysed samples[26].

The pattern of low diversity within populations of *A. calamus* can be attributed to widespread monomorphism and weak population differentiation, which is catalyzed by inefficient pollen transfer, loss of sexual reproduction, insufficient seed production and widespread clonal growth[30]. The species proliferate through rhizomes, grow gregariously and seldom solitary plant is found in nature. The diaspore initiating the population might have been the rhizome. The further growth of the plant populations occurs due to clonal reproduction through the rhizome[22]. Even though the *Acorus* species reproduces both sexually (by seed) and asexually (by rhizome), the Indian population is derived mostly by clonal propagation as the species very rarely sets flowers and seeds. This may be attributed to be the major reason for low diversity among the accessions. Clonal propagation is common among monocots and emergent macrophytes. It is characteristic of plants growing in stressful environments[31-32]. Propagation through rhizomes coupled with inbreeding has attributed to the high levels of homozygosity observed in North American *Typha* populations[33]. With these factors, we can assume that even though the accessions taken for the current study are from different geographical locations, they would have originated from the same common ancestor. Hence, low level of genetic diversity and high genetic differentiation were observed among the accessions.

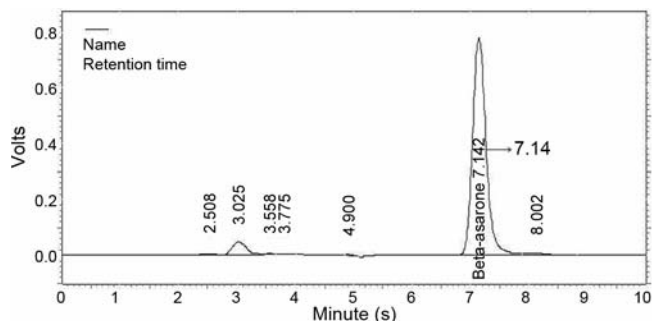The present study reveals low genetic variation and inbreeding among populations, which could be a

Fig. 4—HPLC Chromatogram of β-asarone standard.

Fig. 5—HPLC chromatogram of KERRET-13 (triploid) showing β-asarone peak.

Fig. 6—HPLC chromatogram of KARRET-181 (diploid) showing β-asarone peak.

serious threat to the species. The results of this study provide a valuable base line data to population genetics to address the conservation concerns of this threatened and medicinally important plant species. Sweet Flag is used today for perfumes, pot-pourris, and in flavoring alcoholic beverages and vinegar. But the use is limited due to the carcinogenic β-asarone present in the extract. Its oils are being employed as antifungal and anti-bacterial agents, used as a flavoring ingredient and as a natural pesticide. Since our findings revealed low concentration of β-asarone among the accessions these accessions can be utilized and conserved for its medicinal purposes. For the conservation of this medicinal plant few immediate steps need to be undertaken such as: (a) collection and conservation of the available accessions, (b) promoting cultivation of the species to protect the species from over harvesting, (c) reintroducing the species into its natural habitats, (d) use of proper harvesting methods to extract the phyto compound without damaging the entire plant, and (e) selecting the high yielding varieties of this plant for phytochemical uses.

Acknowledgment

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