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RAPD AND ISSR MOLECULAR MARKER VARIATIONS IN *ACORUS CALAMUS* LINN.

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ABSTRACT
Of the four cytotypes found worldwide, *Acorus calamus* Linn. in Manipur were found to be diploid and triploid with 24 and 36 chromosome numbers, respectively. In the present study, *A. calamus* accessions across 19 different populations were studied and correlated on the basis of their ploidy level. Randomly Amplified Polymorphic DNA (RAPD) and Inter Specific Sequence Repeat (ISSR) molecular markers were employed to reveal the genetic variability of the species in Manipur. Amplification of genomic DNA using 25 primers (18 RAPD and 7 ISSR) yielded 238 bands, of which 84 bands were polymorphic revealing 35.30% polymorphism. The average polymorphic information content obtained from RAPD and ISSR markers were 0.19 and 0.22, respectively. Marker index (RAPD = 0.078; ISSR = 0.106) and resolving powers (RAPD = 0.22 and ISSR = 0.26) indicated that the ISSR markers were more efficient than the RAPDs. The similarity matrix was used to construct a dendrogram based on UPGMA analysis and grouped accessions into two clusters. The dendrogram clustered the accessions as per their ploidy level.

KEYWORDS: *Acorus calamus* Linn, RAPD, ISSR, genetic diversity, β-asarone, ploidy level.

INTRODUCTION

Acorus calamus Linn. (sweet flag) is a wetland perennial monocot plant in which the aromatic rhizomes and leaves have been traditionally used against different ailments. It belongs to the family Acoraceae and is an important medicinal and aromatic plant with a global distribution.[1] The Indian subcontinent has a rich repository of medicinal and aromatic plants that are used by various indigenous health care systems. As per the estimates, over 7000 species of medicinal plants are used for medicinal purposes. However, due to an indiscriminate use of these resources over time and fragmentation of habitats, many of these species are increasingly threatened and face the risk of being genetically improverised.[2]

A. calamus has been recorded throughout India in marshy areas, either in wild or cultivation, ascending to an altitude of 2200 m in Himalayas.[3] Dried rhizomes of this plant have been used for flavouring bitter liquors and appetizers.[4] Various bioactive compounds present in *A. calamus* are acorone, acorenone, α- and β- asarone, asaryldehyde, caryophylene, isoasarone, methyl isoeugenol, and safrol.[5,6,7,8] In India, it is used in several drugs of the Unani and Ayurvedic health care systems.[9] In the Ayurvedic system of medicine, the rhizomes are considered to possess antispasmodic, antidiarrhoic, carminative and antihelminthic, antidepressant, CNS anxiolytic properties.[3,10,11] In an early report, it has been shown that the ethanolic extract of rhizomes of this plant possesses sedative, analgesic, moderately hypotensive and respiratory depressant properties.[12] *A. calamus* extract has also been used in traditional Chinese prescriptions and its effects on memory disorder, learning performance and anti-aging effect have been reported.[13,14] Four cytotypes - diploid (2n=2x=24), triploid (2n=3x=36), tetraploid (2n=4x=48) and hexaploid (2n=6x=72) of this species are found world-wide.[11]

The proportion of the essential oil particularly β-asarone obtained from the rhizome varies between the varieties of *A. calamus* and corresponds to the ploidy level.[10] The compound β-asarone is known to be toxic,[15,16] which is responsible for carcinogenic effects involving duodenal tumour induction,[17] unscheduled DNA synthesis in hepatocytes,[18] as well as antiproliferative and immunosuppressive,[19] CNS inhibitory,[20] sedative and hypothesmic[21] effects. Diploid varieties are characterized by the absence of β-asarone.[11] In the triploid cytotype, present in central Europe and Kashmir, the β-asarone content of the rhizome varies from 9-13%, while in the tetraploid cytotype, found in India, East Asia and Japan, the essential oil of the rhizome is 70-96% β-asarone.[22] The quality of the diploid essential oil, as evaluated by experts was defined as high quality and

suitable for applications in the food and beverage industry.[11] In previous reports, the varieties of *A. calamus* screened from Manipur was triploid[23,24] and it has relatively low level (7-7.8%) of β-asarone.[23] In this present study, *A. calamus* cytotype having the lowest β-asarone content was reported. Because of the varying level of β-asarone content, a precise identification of the *A. calamus* cytotypes in Manipur is essential for proper documentation and commercial application. In this report DNA-based dominant molecular marker techniques, RAPD and ISSR were chosen for assessing genetic variation of *A. calamus* in Manipur as well as to identify the cytotypes with varying ploidy level. This would be useful for plant breeding, quality control, intellectual property rights and eventually for pharmacological studies.

MATERIALS AND METHODS

Plant materials

Specimens of *A. calamus* (Fig. 2A) were collected from nineteen different locations in Manipur (Fig. 1) and maintained in the Experimental Garden of the Department of Life Sciences, Manipur University (Table 1).

Ploidy level analysis

For cytogenetic studies, the fresh root tips of the collected plants were pre-treated with saturated solutions of p-dichlorobenzene for 4 h at 20^{o}C. After thoroughly washing three times in distilled water, the root tips were re-suspended in freshly prepared Carnoy's fluid and stored at 4^{o}C for 4 h. The root tips were then placed in aceto-orecein for tissue staining. Squash preparations were made in acetocarmine and chromosome analysis was done at metaphase stage with a Dialux 22 microscope (Leitz, Germany) and photographed with D-LUX digital camera (Leitz, Germany).

DNA Extraction

Genomic DNA was extracted from leaves of the respective *A. calamus* specimens which were listed in Table 1. Total DNA was isolated using 4% (w/v) sodium dodecyl sulphate (SDS) instead of CTAB with slight modification from Doyle and Doyle DNA exatraction protocol,[25] and purified after RNase treatment. The extraction buffer contained 4% SDS, 1.42 mM NaCl (pH 8.0), 100 mM Tris-Hcl (pH 8.0), 20 mM EDTA, 4% (w/v) PVP and 2% (v/v) β–mercaptoethanol. Quantification was done using Biophotometer (Eppendorf, Germany) and quality was checked on 1.0% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) at 80 V for 1 h.

RAPD analysis

Twenty five decamer RAPD primers (Table 2) synthesized at Xcleris Genomics Prime X Company (Ahmedabad, India) by providing sequences of Operon Technologies (USA) and University of British Colombia (Canada) were initially screened to generate RAPD profiles. PCR amplification was carried out at 94^{o}C for 1 min, primer annealing for 1 min at 53^{o}C, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 7 min. Reaction mixture (25µl) contained 10 ng genomic DNA, 1X reaction buffer, 200 µM of each dNTPs (Promega, Medison, USA), 0.4 µM of each primer and 1 Unit of Taq DNA Polymerase (Bangalore Genie, Bangalore, India). The reactions were carried out in a DNA thermocycler (Gene Amp PCR System 9700, Applied Biosystems, USA). The amplification products were analysed on 1.8% agarose gel with a 100-bp DNA ladder (Bangalore Genei, Bangalore, India) and photographed using a Gel Documentation System (Vilber Lourmat, France). All PCR results were tested for reproducibility for at least three times.

ISSR analysis

For ISSR analysis, seven primers (Table 3) purchased from Sigma-Genosys, USA were initially screened. PCR amplification was carried out at 94^{o}C for 4 min for initial denaturation, followed by 35 cycles of denaturation at 94^{o}C for 1 min, primer annealing for 1 min at 20^{o}C for UBC 802, 42^{o}C for UBC 808, 40^{o}C for UBC 815, 47^{o}C for UBC 818, 46^{o}C for UBC 820 and UBC 824 and 50^{o}C for UBC 827, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 10 min.

Data scoring and analysis

Only clear, reproducible and unambiguous bands were considered for data analysis. Data were scored as '1' for presence and '0' for absence. The percentage polymorphism, polymorphism information content (PIC), effective multiplex ratio (EMR), resolving power (Rp) and marker index (MI) were calculated. Percentage polymorphism was calculated as percentage of polymorphic loci from total loci obtained per primer. Polymorphism information content (PIC) value of individual primers were calculated based on the formula PIC= 2 x F (1-F).[26] Marker index, a product of information content and EMR was calculated following.[27] Rp of each primer combination was calculated according to (Prevost A and Wilkinson M J 1999).[28] The Jaccard's similarity index was calculated using NTSYS-PC 2.02e (Applied Biostatistics Inc., Setauket, NY, USA) to compute pairwise Jaccard's similarity coefficient[29] and this similarity matrix was used in cluster analysis using an unweighted pair group method with arithmetic averages (UPGMA) and sequential, algomerative, heirarchiral and nested (SAHN) clustering algorithm to obtain a dendrogram.[30]

RESULTS AND DISCUSSION

Ploidy level analysis

Investigation on chromosome numbers and ploidy levels of *A. calamus* was made and it was found that *A. calamus* accessions collected from Keirenphabi and Wangkhem were diploid (2n=2x=24) with 24 chromosome numbers (Fig. 2B-C) whereas samples from remaining collection sites viz., Hengbung, Keibul Lamjao-1, Keibul Lamjao-2, Lamlai, Langol, Lilong, Mayang Imphal, Ningthoukhong, Nongren, Oinam,

Pangei, Patsoi, Sekmai, Singjamei, Tentha, Thoubal and Yumnam Huidrom were all found to be triploid (2n=3x=36) with thirty six chromosome numbers (Fig. 2D-2E). No tetraploid and hexaploid cytotypes were observed.

Table 1: Details of *Acorus calamus* accessions collected from different locations in Manipur with their ploidy level.

Sl. No.	Accession code	Collection site	Latitude (N)	Longitude (E)	Altitude (m)	Chromosome no./Ploidy level
1	MU/AC-01	Pangei	24°51'37"	93°58'33.43"	785	3x=36/Triploid
2	MU/AC-02	Keibul Lamjao-1	24°32'49.67"	93°50'5.90"	780	3x=36/Triploid
3	MU/AC-03	Thoubal	24°38'20.26"	94°0'0.84"	795	3x=36/Triploid
4	MU/AC-04	Keirenphabi	24°27'33.97"	93°47'17.34"	777	**2x=24/Diploid**
5	MU/AC-05	Ningthoukhong	24°34'36.39"	93°45'47.05"	772	3x=36/Triploid
6	MU/AC-06	Keibul Lamjao-2	24°31'55.44"	93°31'55.44"	787	3x=36/Triploid
7	MU/AC-07	Oinam	24°41'44.77"	93°48'22.64"	778	3x=36/Triploid
8	MU/AC-08	MayangImphal	24°35'53.29"	93°52'17.21"	772	3x=36/Triploid
9	MU/AC-09	Nongren	24°52'11.05"	94°3'51.37"	789	3x=36/Triploid
10	MU/AC-10	Tentha	24°34'30.03"	93°57'59.11"	774	3x=36/Triploid
11	MU/AC-11	Yumnam Huidrom	24°39'42.16"	93°54'14.18"	779	3x=36/Triploid
12	MU/AC-12	Patsoi	24°47'26.65"	93°53'13.09"	786	3x=36/Triploid
13	MU/AC-13	Lilong	24°43'11.36"	93°56'3.84"	780	3x=36/Triploid
14	MU/AC-14	Lamlai	24°52'1.18"	94°3'3.79"	792	3x=36/Triploid
15	MU/AC-15	Wangkhem	24°38'42.70"	94°0'23.30"	783	**2x=24/Diploid**
16	MU/AC-16	Sekmai	24°57'56.05"	93°53'12.17"	885	3x=36/Triploid
17	MU/AC-17	Langol	24°49'40.98"	93°54'8.93"	789	3x=36/Triploid
18	MU/AC-18	Singjamei	24°46'47.97"	93°56'18.41"	784	3x=36/Triploid
19	MU/AC-19	Hengbung	25°13'34.45"	94°0'0.16"	1169	3x=36/Triploid

Table 2: Marker parameters calculated for each RAPD primer used with *Acorus calamus*.

Sl. no.	Primer	Sequence(5' to 3')	TB	PB	PPB (%)	Product size (bp)	PIC	RP	EMR	MI
1	OPA-4	AATCGGGCTG	8	3	30.00	630-270	0.223	0.264	0.500	0.111
2	OPA-9	GGGTAACGCC	10	5	50.00	560-230	0.187	0.211	0.500	0.093
3	OPA-11	CAATCGCCGT	10	3	30.00	1000-210	0.235	0.281	0.300	0.070
4	OPA-15	TTCCGAACCC	5	2	40.00	520-390	0.187	0.211	0.400	0.074
5	OPC-5	GATGACCGCC	4	2	50.00	500-130	0.187	0.211	0.500	0.093
6	OPC-7	GTCCCGACGA	10	4	40.00	1500-180	0.187	0.211	0.400	0.074
7	OPC-11	AAAGCTGCGG	12	4	33.34	870-150	0.187	0.211	0.333	0.062
8	OPD-3	GTCGCCGTCA	11	5	45.45	1000-280	0.187	0.210	0.454	0.084
9	OPD-5	TGAGCGGACA	12	4	33.34	850-210	0.187	0.210	0.333	0.062
10	OPD-11	AGCGCCATTG	14	4	28.57	910-200	0.187	0.210	0.285	0.053
11	OPQ-5	CCGCGTCTTG	11	4	36.36	800-240	0.187	0.211	0.363	0.067
12	OPU-5	TTGGCGGCCT	7	1	14.28	720-270	0.187	0.212	0.142	0.026
13	OPU-16	CTGCGCTGGA	2	1	50.00	380-280	0.187	0.212	0.500	0.093
14	UBC-1	CCTGGGCTTC	13	3	23.08	1000-190	0.187	0.212	0.230	0.043
15	UBC-2	CCTGGGCTTG	14	5	36.42	790-190	0.245	0.295	0.357	0.087
16	UBC-4	CCTGGGCTGG	13	5	38.46	710-210	0.187	0.211	0.384	0.071
17	UBC-5	CCGGCCTTAA	7	5	71.42	810-220	0.216	0.211	0.714	0.154
18	UBC-6	CCGGCTGGAA	8	4	50.00	1500-210	0.187	0.211	0.500	0.093
Total			188	64	34.04		0.196	0.22		0.078

TB: total band, PB: polymorphic band, PPB (%): percentage polymorphic band (%), PIC: polymorphism information content, RP: resolving power, EMR: effective multiplex ratio, MI: marker index

Table 3: Marker parameters calculated for each ISSR primer used with *Acorus calamus*.

Sl. no.	Primer no.	Sequence (5' to 3)	TB	PB	PPB (%)	Product size (bp)	PIC	RP	EMR	MI
1	UBC-802	ATATATATATATATATG	8	2	25	210-810	0.187	0.21	0.25	0.046
2	UBC-808	AGAGAGAGAGAGAGAGC	11	3	27.27	250-660	0.187	0.21	0.27	0.050
3	UBC-815	CTCTCTCTCTCTCTCTG	3	2	66.66	290-800	0.187	0.21	0.66	0.123
4	UBC-818	CACACACACACACACAG	10	4	40	390-940	0.259	0.31	0.40	0.103
5	UBC-820	GTGTGTGTGTGTGTGT	8	4	50	400-900	0.223	0.26	0.50	0.110
6	UBC-824	TCTCTCTCTCTCTCTCTG	4	3	75	200-790	0.332	0.42	0.75	0.249
7	UBC-827	ACACACACACACACACG	6	2	33.33	450-1900	0.187	0.21	0.33	0.061
Total			50	20	40		0.223	0.26		0.106

TB: total band, PB: polymorphic band, PPB (%): percentage polymorphic band (%), PIC: polymorphism information content, RP: resolving power, EMR: effective multiplex ratio, MI: marker index

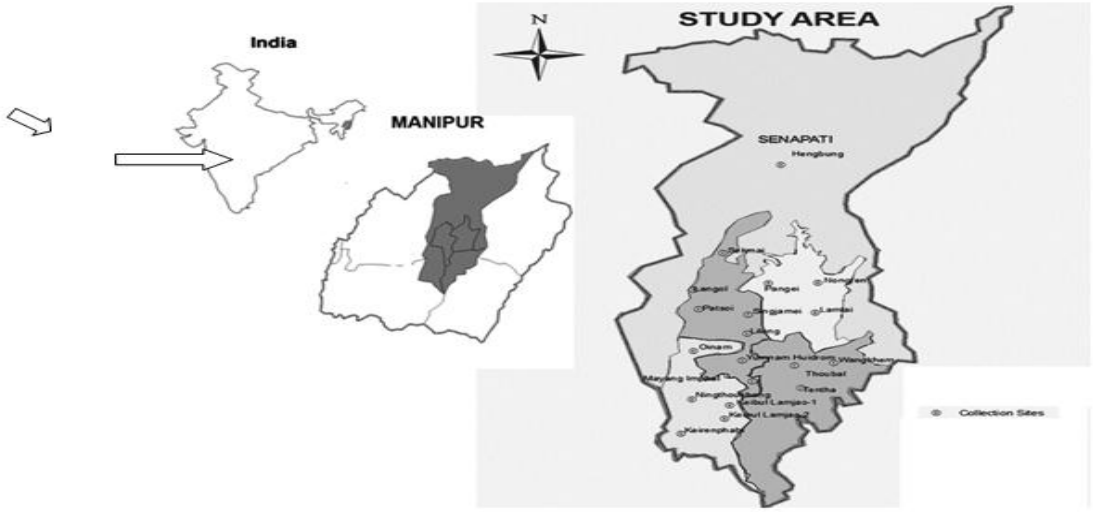

Figure 1: Geographical map showing the collection sites of 19 accessions of *Acorus calamus* in Manipur.

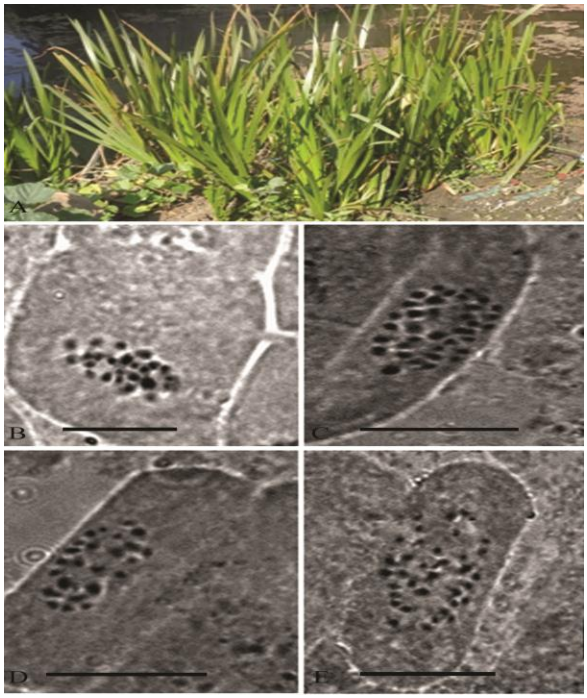

Figure 2: A. *Acorus calamus* in natural habitat. B- E. Photomicrograph of *Acorus calamus* root tipchromosomes, B) Keirenphabi (2n=2x=24); C) Wangkhem (2n=2x=24); D) Thoubal (2n=3x=36); E) Yumnam Huidrom (2n=3x=36), Bar (B-E) = 10 µm.

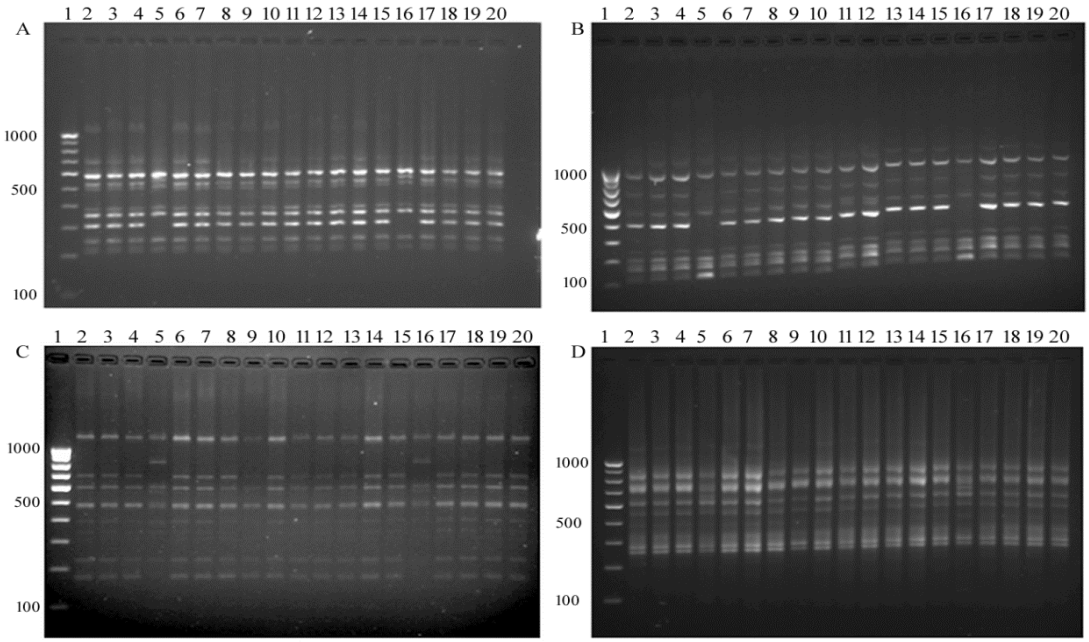

Figure 3: PCR products of genomic DNA from *Acorus calamus* accessions with RAPD primers, OPA-9(A), OPA-11 (B), OPQ-5 (C) and ISSR primer UBC-802 (D) Lane1=100 bp DNA marker, Lane 2-20(MU/AC 01-19).

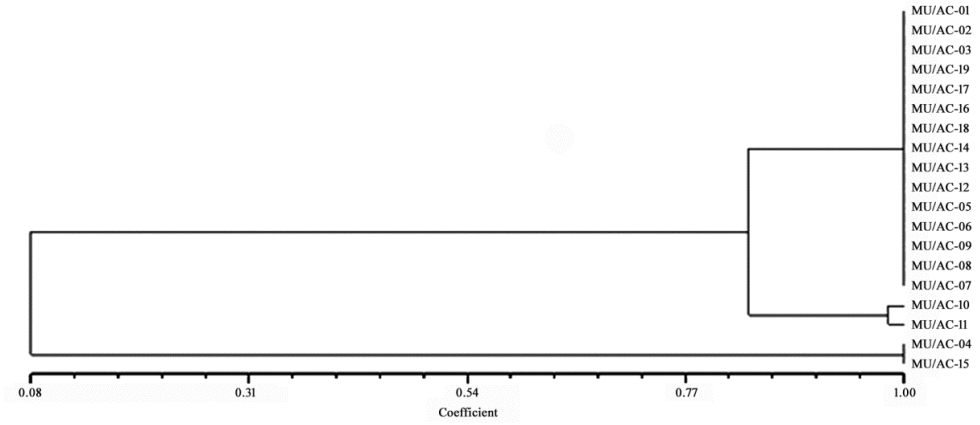

Figure 4: Dendrogram demonstrating the relationship among 19 different accessions of *Acorus calamus* collected from different parts of Manipur based on RAPD.

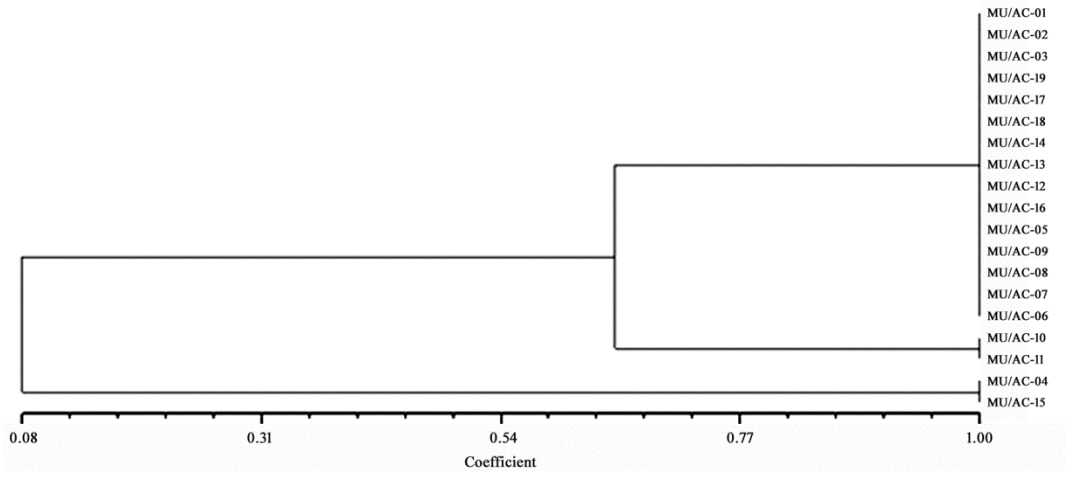

Figure 5: Dendrogram demonstrating the relationship among 19 different accessions of *Acorus calamus* collected from different parts of Manipur based on ISSR.

RAPD analysis

The intraspecific genetic diversity was studied among 19 accessions of *A. calamus* collected from different locations in Manipur. Of the 25 RAPD primers screened, 21 gave reproducible and consistant amplification and of these, 18 primers viz., (OPA-4, OPA-9, OPA-11, OPA-15, OPC-5, OPC-7, OPC-11, OPD-3, OPD-5, OPD-11, OPQ-5, OPU-5, OPU-16, UBC-1, UBC-2, UBC-4, UBC-5 and UBC-6) showed polymorphism while the rest of the seven primers showed monomorphism. The amplified PCR fragment size ranged from 130 to 1500 bp. A total of 188 RAPD markers were detected, of which 64 bands showed polymorphism. The average proportion of polymorphic markers across the primers was 34.04 % ranging between 14.28% (OPU-5) to 71.42% (UBC-5). The respective values for overall genetic variability for PIC, Rp, EMR and MI across all the 19 genotypes are given in Table 2. Highest PIC value (0.245) was observed for the primer UBC-2 and lowest PIC value (0.210) was recorded for majority of the primers. Average PIC value was 0.198. The MI values ranged from 0.026 to 0.154 with an average of 0.078. The highest value (0.154) was given by UBC-5 and the lowest value (0.026) was scored by OPU-5. Rp ranged from 0.210 to 0.295 with an average of 0.222 per primer. Highest value (0.295) was scored by UBC-2 and the lowest value (0.210) was scored by OPD-3, OPD-5 and OPD-11. EMR value ranged from 0.142 (OPU-5) to 0.714 (UBC-5). The patterns of RAPD fragments produced by the primer OPA-9 (Fig. 3A) showed two minor bands in lane 4 and lane 15 at 510 bp and 530 bp respectively whereas major band was missing in both the lanes at 310 bp; primer OPA-11 (Fig. 3B) showed an extra minor band at 610 bp in lanes 4, 10, 11 and 15 and a major band was found missing in lane 4 and 15 at 500 bp, primer OPQ-5 (Fig. 3C) showed an extra band in lanes 4 and 15 at 250 bp and a minor band missing in the same lane at 400 bp. Primer OPC-7 (Fig. 3D) showed two extra minor bands (590 bp and 850 bp) in lane 4 and 15 and two bands (180 bp and 700 bp) missing in the same lane.

The UPGMA dendrogram based on RAPD showed two main clusters (Fig. 4). The first cluster included all the accessions which were triploid and the second cluster has two accessions with diploid number of chromosomes and showed JSI of 0.08 with the first cluster. Again the first cluster contained two sub-clusters. The first sub cluster contained accessions comprising of 15 accessions which are triploid and collected from Hengbung, Keibul Lamjao-1, Keibul Lamjao-2, Lamlai, Langol, Lilong, Mayang Imphal, Ningthoukhong, Nongren, Oinam, Pangei, Patsoi, Sekmai, Singjamei and Thoubal whereas the second sub cluster contained two accessions from Tentha and Yumnam Huidrom. The first sub cluster showed maximum similarity between them at JSI of 1.00. In the second sub-cluster, the accession had a JSI=0.977 with the accessions. Based on cluster analysis, all accessions which were triploid (3x=36) were clustered in the first cluster whereas those accessions which were diploid (2x=24) were grouped in the second cluster. Cluster analysis revealed a strong distinctiveness of the accessions with same chromosome number. In this study, there was a strong correlation for karyotype with RAPD data among 19 intraspecific accessions of *A. calamus* from Manipur. Similarly, on the basis of 700 bp sequence of 5S-rRNA gene spacer region, three chemotypes of *A. calamus* (chemotype A- predominant Z-asarone, chemotype B- predominant sequiterpenoids, chemotype M-various ratio of Z-asarone and sequiterpenoids) were clustered separately.[31]

ISSR analysis

All the seven ISSR primers resulted in a number of amplified fragments which varied in size from 210-1900 bp. Of the 50 bands amplified 20 showed polymorphism. The average percentage polymorphism resulted by the 7 ISSR primers was 40% ranging between 25% (UBC-802) to 75% (UBC- 824) (Table 4). The respective values for the overall genetic variability for PIC, RP, EMR and MI across all the 19 genotypes were given in Table 3. Highest PIC value (0.332) was scored by the primer UBC-824 and lowest PIC value (0.187) was recorded for the primers UBC-802, UBC-808, UBC-815 and UBC-827. Average PIC value was 0.223. Highest RP (0.422) was scored by the primer UBC-824 and the lowest value (0.21) for the primer UBC-802. Highest EMR value (0.75) was recorded with UBC-824 and the lowest value (0.25) was scored with UBC-802. Highest MI value (0.249) was scored with the primer UBC-824 and the lowest value (0.046) for the primer UBC-802 (Fig. 3D).

The UPGMA dendrogram (Fig. 5) as revealed by ISSR primers also showed similar result. The first cluster included all the accessions which were triploid and the second cluster has two accessions with diploid number of chromosomes and showed JSI of 0.08 with the first cluster. Again the first cluster contained two sub-clusters. The first sub cluster contained accessions comprising of 15 accessions which are triploid and collected from Hengbung, Keibul Lamjao-1, Keibul Lamjao-2, Lamlai, Langol, Lilong, Mayang Imphal, Ningthoukhong, Nongren, Oinam, Pangei, Patsoi, Sekmai, Singjamei and Thoubal whereas the second sub-cluster contained two accessions from Tentha and Yumnam Huidrom. The first sub cluster showed maximum similarity between them at JSI of 1.00. In the second sub-cluster, the accession had a JSI=1.00 with the accessions.

CONCLUSION

The multifarious medicinal properties of *A. calamus* make it a potential plant for various industrial applications. Previous reports indicated Manipur having only triploid accessions as well as they did not compared the ploidy level with the molecular profiling of the plant. The present study enhances the knowledge of ploidy level in *A. calamus* found in Manipur. Two cytotypes viz., diploid (2n=2x=24) and triploid (2n=3x=36) are found. RAPD and ISSR profiling clustered separately the

diploid *A. calamus* accessions and triploid *A. calamus* accessions. Hence, the identification and evaluation of ploidy level of the germplasm are pre-requisites for its commercial utilization. In spite of these substantial outcomes, there are several areas where a considerable amount of research efforts are still demanded towards Acorus genetic improvement and conservation.

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