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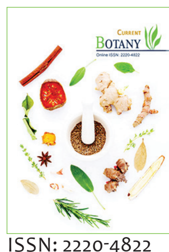

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Genetic diversity in *Cymbopogon khasianus* (Hack.) Stapf ex Bor collected from Meghalaya, Northeast India, using PCR-based dominant markers

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ABSTRACT

Cymbopogon is a globally recognized genus of medicinal, aromatic, and economically important grasses within the monocots, cultivated primarily for its high-value essential oil. The essential oil extracted from this crop has significant commercial and pharmaceutical applications. In the present study, a total of 48 germplasm lines of *Cymbopogon khasianus* (Hack.) Stapf ex Bor were collected from the Meghalaya region of Northeast India. Although these germplasms belong to the same species and chemotype, considerable morphological variation was observed among them. Therefore, 41 DNA-based dominant molecular markers, including 21 RAPD and 20 ISSR primers, were employed to assess and confirm the genetic diversity of the germplasm. Dendrograms generated through UPGMA and cluster analysis based on ISSR, RAPD, and combined datasets were largely similar and effectively separated the genotypes into distinct clusters. ISSR markers demonstrated higher efficiency, showing greater average PIC, MI, and Rp values (0.129, 2.28, and 2.13, respectively) compared to RAPD markers (0.097, 0.95, and 1.15). Additionally, ISSR primers exhibited a higher percentage of polymorphic bands (91.50%) than RAPD primers (81.59%). AMOVA results further supported the superiority of ISSR markers over RAPD markers. Overall, the findings of this study will contribute to the conservation and improvement of *C. khasianus* germplasm.

KEYWORDS: *Cymbopogon khasianus*, Northeast India, ISSR, RAPD, Genetic diversity

INTRODUCTION

The genus *Cymbopogon*, a member of the family Poaceae, is a universally accepted oil-bearing grass. *Cymbopogon*, commonly known as lemon grass, is an important raw material for various industrial sectors, especially the medicinal sector. The essential oil of lemon grass has massive industrial applications, *viz*., as in flavours, fragrances, soaps, cosmetics, perfumery, detergents and pharmaceuticals (Dutta *et al*., 2016, 2018). The different species of lemon grass exhibited great variation in morphology and chemotypes (Bor, 1960; Soenarko, 1977; Gupta & Jaffer, 1982). Approximately 140 species of *Cymbopogon* has have been reported globally, of which 45 are found in tropical Asian countries, including India (Dutta *et al*., 2016; Lal *et al*., 2016a, b). The North East part of the Indian subcontinent is home to three interesting morphotypes which were significantly different from typical lemon grass, i.e., *C. flexuosus* (RRLJ 1279) (Nath *et al*., 2002). Similarly, another species of *Cymbopogon* collected from the Meghalaya region of Northeast India was identified as *C. khasianus* (Hack.) Stapf ex Bor having high content of elemicin (~70%) (Dutta *et al*., 2018; Lal *et al*., 2018). *C. khasianus* is such a species which has the same chemotype, but variation has been found in its morphology. This species is propagated vegetatively and methyl eugenol was found as a major constituent in the leaf essential oil of *C. khasianus* collected from certain localities bordering the states of Arunachal Pradesh, Bhutan, Meghalaya and Assam (Rabha *et al*., 1986). Germplasm of *C. khasianus* from the Garo hills are is rich in geraniol content (Rabha *et al*., 1988). The other components present in *C. khasianus* essential oil are cis-asarone, germacrene-D, neryl acetate, methyl eugenol, camphene, β-ocimene, limonene, caryophyllene, geranyl acetate, *trans*-β-farnesene, etc. (Dutta *et al*., 2018). This species shows a wide range of morphological variation as well as in terms of essential oil

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content. As this plant is of utmost importance for the aromatic industry, pharmaceutical and household industries, therefore, the conservation and improvement of this species is necessary.

Though the morphological traits and essential oil analysis can be used to assess the diversity of the germplasm but these traits are usually affected by the environmental factors (Kumar *et al.*, 2009). Cross-pollination in *Cymbopogon* makes the genus vulnerable to adulteration of the cultivars at the genomic level, eventually loss of genomic purity (Bishoyi *et al.*, 2016). Molecular markers are one of the reliable tools which can be exploited to detect the purity of genotypes and hybrids. Similarly, assessment of intraspecific genetic variation is very helpful in the conservation genetics, genecology and breeding programmes (Nybom & Bartish, 2000).

DNA-based molecular markers like RAPD, ISSR are still being used to study and confirm the genetic diversity of the germplasms, especially in orphan crops like medicinal crops, as both these markers are cost-effective and simple (Baruah *et al.*, 2017). Compared to RAPD, ISSR is highly reproducible and polymorphic in nature due to long semi-arbitrary SSR primers (Adhikari *et al.*, 2015). Earlier RAPD analysis of the different *Cymbopogon* species was performed for the study of the genetic diversity (Shasany *et al.*, 2000; Adhikari *et al.*, 2013), but the molecular diversity assessment of *C. khasianus* germplasm has not been reported. Therefore, there is a need to study the genetic diversity of *C. khasianus* using the molecular markers for the maintenance of the germplasm and the marker-assisted improvement of the crops. With respect to this need, the present investigation deals with the intraspecific genetic diversity of *C. khasianus* collected from different regions of Meghalaya, Northeast India, using the molecular markers ISSR and RAPD.

MATERIALS AND METHODS

Collection Sites and Planting Material

A total forty eight genotypes were collected from different localities of Meghalaya, North East India, followed by planting in CRBD with three replications at CSIR-NEIST experimental farm. The collection sites are shown in Figure 1.

Identification of the Plant Specimens

During the flowering phase, we studied the floral anatomy and morphology of all genotypes and prepared herbaria for further study. For the identification of this species, herbaria were prepared and confirmed as *Cymbopogon khasianus* (Hack.) Stapf ex Bor by BSI, Shillong. Holotype- Herbarium, Botanical Survey of India, Shillong (Meghalaya) and Sibpur (Calcutta); Isotype- Herbarium, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India.

Extraction of DNA

Tender leaf sample from each of the forty-eight genotypes of *C. khasianus* was collected for the extraction of DNA. 50 mg lyophilized plant samples were weighed and tissuelyzed with liquid nitrogen. After that DNA extraction procedure was followed with the help of a DNA extraction kit (Himedia). The concentration and purity of the extracted DNA were determined by a Nanospectrophotometer (Eppendorf, Germany) using an aliquot of 5 µL of the sample from the stock DNA. The purity of the extracted DNA was also confirmed by using 1% agarose gel and the DNA was observed by using a gel documented system (Syngene, UK).

PCR Amplification

A total of twenty-one RAPD and twenty ISSR primers were selected for genetic diversity analysis after preliminary primer testing of 82 ISSR and 90 RAPD. ISSR and RAPD analysis was carried out with the final volume of 20 µL containing 15 ng and 10 ng DNA, respectively. 3 µL plant DNA samples, 10 µL 1x PCR master mix, 5.4 µL autoclaved molecular grade water and 1.6 µL primer were taken for PCR amplification.

The amplification reaction was carried out in a thermal cycler (Himedia Prima-96) using initial denaturation of 94 °C for 4 min followed by 35 cycles of annealing temperature 95 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min and a final extension of 72 °C for 5 min. All PCR products were separated by 1% (w/v) agarose gel containing ethidium bromide. The amplified bands were documented under the gel doc system.

Statistical Analysis

The observed bands were marked as 1 and 0 on the basis of their presence and absence, respectively (Figure 2). The total number of bands obtained from ISSR and RAPD markers, the number of polymorphic bands and the percentage of polymorphic bands were calculated.

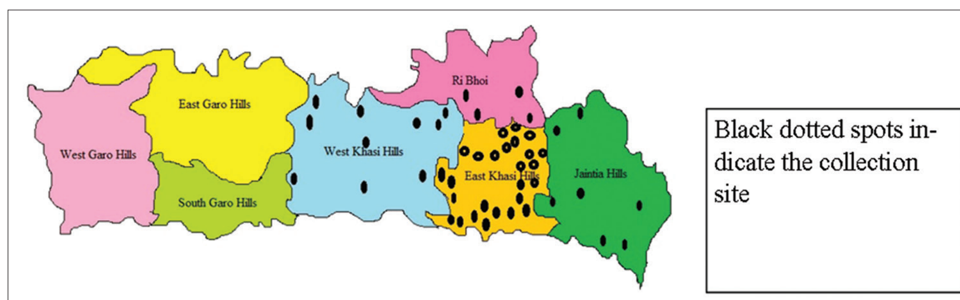

Figure 1: Collection site of *C. khasianus* from Meghalaya, Northeast India

Further, the effectiveness of different primers (both ISSR and RAPD) was calculated by using different indices, such as Polymorphism Information Content (PIC), Marker Index (MI) and Resolving Power (Rp). PIC value was calculated following the formula of Botstein *et al.* 1980 as—

PIC = $1 - \Sigma fi^2$ where f is the frequency of ith allele. Marker Index (MI) was calculated using the formula PIC×EMR (Milbourne *et al.*, 1997; Prevost & Wilkinson, 1999) where EMR is the product of fraction of polymorphic loci and number of polymorphic loci and Resolving Power according to the formula $Rp = \Sigma I_b$, where I_b denotes band informativeness of the primers with $I_b = 1 - [2 \times |.5 - p|]$, where '$p$' denotes the proportion of clones containing the bands (Prevost & Wilkinson, 1999).

Different genetic parameters such as- number of alleles per locus (Na), effective number of alleles (Ne), Nei's gene diversity (He), Shannon's diversity index (I) were determined with the help of POPGENE software (version 1.32) by Yeh *et al.* (2000). To determine genetic similarity among all possible pairs a dendrogram was constructed using NTSYS software programme version 2.1 (Rohlf, 2000). To further differentiate the groups, principal coordinate analysis (PCA) was done using Rohlf NTSYS Software. Analysis of Molecular Variance (AMOVA) was done using the software GenAIEx6.5b2 to determine the percentage of variance obtained within and among populations.

RESULTS

ISSR and RAPD Banding Pattern, PIC, MI and Rp

ISSR and RAPD techniques were used to find out the extent of genetic diversity in *C. khasianus* and primers used in the experiment are shown in Tables 1 and 2. A total of 20 ISSR primers yielded a total of 88 bands, of which 82 were polymorphic. Polymorphic bands produced per primer ranged from 1 (ISSR 82) to 6 (ISSR 13 and 40) and a total of 21 primers (ISSR 6, 13, 17, 20, 40, 46, 47, 54, 55, 68, 74, 75, 77, 80 and 81) showed 100% polymorphism (Table 1). Similarly, 80 bands were produced by 21 RAPD primers, of which 64 were found to be polymorphic for the entire data set. Number of polymorphic bands produced by per primer ranged from 2 (RAPD 2, 4, 5, 16 and 19) to a maximum of 4 (RAPD 1, 7, 33, 36 and 81). Primer 3, 6, 7, 22, 32, 33, 34, 36, 73 and 81 resulted in 100% polymorphism (Table 2). Overall percentage polymorphism revealed by both primers (ISSR and RAPD) was 91.5% and 81.59% respectively

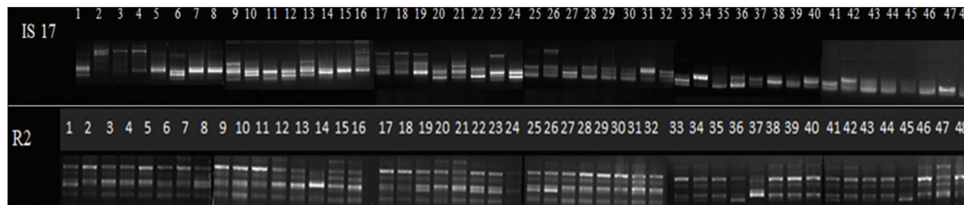

Figure 2: Gel electrophoresis of amplification products obtained with ISSR primer-17 and RAPD primer-2 of forty eight germplasm of *C. khasianus*

Table 1: Characteristics of primer sequences, number of polymorphic bands, total number of amplified bands, percentage of polymorphic bands, marker index, resolving power and polymorphic information content for ISSR marker in *C. khasianus*

ISSR Primers	Primer sequence	No. of polymorphic bands	Total no. of bands	PPB	MI	RP	PIC Value
ISSR 6	$(AG)_8CA$	5	5	100	3.51	3.04	0.140
ISSR 7	$(GA)_8T$	3	4	75	0.98	2.00	0.109
ISSR 13	$(CA)_8AT$	6	6	100	4.48	2.62	0.124
ISSR 17	$(GA)_7RC$	5	5	100	3.3	2.66	0.166
ISSR 20	$(CA)_6GG$	4	4	100	2.83	1.38	0.078
ISSR 21	$(TC)_8A$	2	3	66.67	0.64	0.67	0.159
ISSR 40	$(GA)_8YC$	6	6	100	3.33	3.83	0.111
ISSR 46	$(TG)_8A$	4	4	100	3.23	3.5	0.202
ISSR 47	$(AG)_8YC$	5	5	100	1.87	2.96	0.100
ISSR 54	$(GA)_9T$	5	5	100	4.22	3.8	0.168
ISSR 55	$(GAA)_6$	4	4	100	1.49	1.65	0.093
ISSR 63	$(CAC)_6T$	4	5	80	1.04	0.71	0.065
ISSR 68	$G(ACAG)_3ACA$	4	4	100	1.19	0.69	0.074
ISSR 74	$CCCGGATCC(CA)_9$	4	4	100	2.38	2.17	0.149
ISSR 75	$AGTACGAGT(TC)_7$	5	5	100	1.90	0.42	0.074
ISSR 76	$ACTTCGTAGT(AC)_7$	3	4	75	0.93	1.61	0.103
ISSR 77	$CCCGGATCC(CT)_9$	4	4	100	2.19	2.47	0.137
ISSR 80	$GATCAGT(GAGT)_5$	5	5	100	3.02	3.63	0.120
ISSR 81	$GATCGTA(GGTA)_5$	3	3	100	2.64	1.93	0.293
ISSR82	$GATC(TCTG)_8$	1	3	33.33	0.10	0.96	0.105
Total		82	88	-	-	-	-
Minimum		1	3	33.33	0.10	0.42	0.065
Maximum		6	6	100	4.48	3.83	0.293
Average		4.1	4.4	91.5	2.28	2.13	0.129

PPB=percentage of polymorphic bands, MI=marker index, RP=resolving power, PIC=polymorphic information content

Table 2: Characteristics of primer sequences, number of polymorphic bands, total number of amplified bands, percentage of polymorphic bands, marker index, resolving power and polymorphic information content for RAPD marker in *C. khasianus*

RAPD Primers	Primer sequence	No. of polymorphic bands	Total no. of bands	PPB	MI	RP	PIC Value
RAPD1	AATCGGGCTG	4	5	80	0.58	0.96	0.036
RAPD2	GGTCCCTGAC	2	4	50	0.35	0.39	0.089
RAPD3	GGGTAACGCC	4	4	100	0.68	0.72	0.042
RAPD4	GTGATCGCAG	2	3	66.67	0.28	0.67	0.069
RAPD5	GTGATCGCAG	2	4	50	0.21	0.87	0.051
RAPD6	CAATCGCCGT	3	3	100	1.34	1.54	0.149
RAPD7	TTCCGAACCC	4	4	100	0.56	0.59	0.035
RAPD8	AGCCAGCGAA	3	6	50	0.54	2.25	0.060
RAPD12	GTTGCGATCC	3	4	75	2.20	0.54	0.117
RAPD13	GGACTGGAGT	3	4	75	0.71	1.38	0.078
RAPD15	GTCCACACGG	3	4	75	1.27	1.46	0.140
RAPD16	CTGCTGGGAC	2	4	50	0.06	0.25	0.015
RAPD19	GGAGGGTGTT	2	3	66.67	0.51	1.13	0.263
RAPD22	GGACCCTTAC	3	3	100	1.61	1.41	0.178
RAPD27	TGGACCGGTG	3	4	75	0.71	1.3	0.078
RAPD32	TGCGTGCTTG	3	3	100	1.40	1.63	0.156
RAPD33	GACGGATCAG	4	4	100	2.30	2.63	0.144
RAPD34	AGGGCGTAAG	3	3	100	0.99	0.35	0.105
RAPD36	ACTTCGCCAC	4	4	100	0.94	1	0.058
RAPD73	GTGGTCCGCA	3	3	100	0.48	0.5	0.053
RAPD81	GGAGAGACTC	4	4	100	2.14	2.55	0.133
Total		64	80	-	-	-	-
Minimum		2	3	50	0.06	0.25	0.015
Maximum		4	5	100	2.30	2.63	0.263
Average		3	3.81	81.59	0.95	1.15	0.097

PPB=percentage of polymorphic bands, MI=marker index, RP=resolving power, PIC=polymorphic information content

(Tables 1 & 2). PIC values calculated for ISSR primers ranged from 0.065(ISSR 63) to 0.293(ISSR 81) and RAPD primers ranged from 0.015 (RAPD 16) to 0.263 (RAPD 19). Average PIC, MI and RP values for ISSR primers (0.129, 2.28, 2.13) were found to be higher than RAPD primers (0.097, 0.95, 1.15), indicating greater efficiency of ISSR primers.

Genetic Variability in *C. khasianus*

Genetic parameters, *viz*, observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (He), Shannon's diversity index (I) were shown in Table 3. All the parameters revealed by ISSR primers (1.93, 1.58, 0.33, 0.49) were found to be higher than RAPD primers (1.79, 1.36, 0.22, 0.34), which leads to an efficacy of ISSR markers over RAPD.

Genetic Similarity, Cluster Analysis and Principal Coordinate Analysis

Jaccard's similarity coefficient calculated for both ISSR and RAPD data showed that the similarity value for ISSR data ranged from 0.4886364 to 0.8636364 whereas the genetic similarity for RAPD data varied from 0.56 to 0.84 and their combined data varied from 0.57 to 0.89.

AMOVA Analysis

AMOVA analysis showed that for the ISSR marker percentage of variance obtained within and among populations is 92 and 8% respectively. Similarly, for RAPD data percentage of total variance obtained is 90 and 10% respectively (Table 4). ISSR and RAPD analysis showed that the variance within the population was 18.04 and 8.47 respectively, which was much higher than the variance among the population (1.63 and 0.94). Graphical representation of both the markers showing among and within the population of forty-eight germplasm of C. *khasianus* was shown in Figure 3a and 3b.

Table 3: Analysis of genetic parameters based on ISSR and RAPD markers

Marker	Mean Na	Mean Ne	Mean He	Mean I
ISSR	1.93	1.58	0.33	0.49
RAPD	1.79	1.36	0.22	0.34

Na=observed number of alleles, Ne=effective number of alleles, He=Nei's gene diversity or expected heterozygosity, I=Shannon's diversity index

Table 4: Analysis of molecular variance (AMOVA) table based on IISR and RAPD marker in *C. khasianus*

Marker	Source of variation	Degree of freedom (df)	Sum of square (SS)	Variance component	% of total variance	P value
ISSR	Among population	3	101.84	1.63	8	0.08
	Within population	44	793.56	18.04	92	0.005
	Total	47	895.40	19.67	100	
RAPD	Among population	3	52.75	0.94	10	0.1
	Within population	44	372.67	8.47	90	0.001
	Total	47	425.42	9.41	100	

Significance level based on 999 permutations

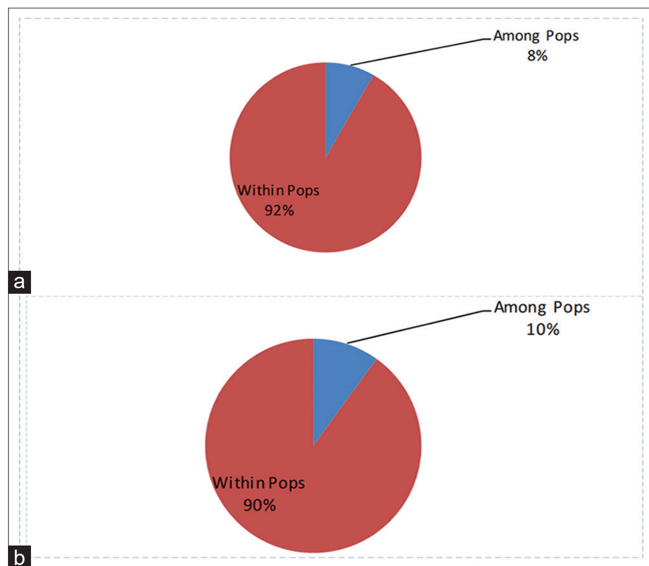

Figure 3: Percentage of molecular variance within and among population of *C. khasianus* using a) ISSR and b) RAPD markers

Clustering Analysis

The Unweighted Pair Group Method of Arithmetic Mean (UPGMA) derived dendrogram obtained from ISSR data divided the cluster into six main groups based on a 0.67 cut-off value. Group A consists of two genotypes, group B consists of fifteen genotypes, group C consists of ten genotypes, group D consists of four genotypes and group E and F includes fifteen and two genotypes, respectively (Figure 4a.). However, unlike ISSR data, RAPD data showed different grouping. At a 0.79 cut-off value dendrogram is divided into 3 groups. Group A consists of 34 genotypes, group B consists of seven genotypes and group C includes two genotypes. The rest five genotypes (RRLJM-534, RRLJM-521, RRLJM-543, WN-3, RRLJM- 542) came as separate individuals in the dendrogram (Figure 5a.). Similar to the ISSR data, the combined dendrogram consists of five groups on the basis of a cut-off value of 0.75. Group A includes eight genotypes, group B includes fifteen genotypes, group C includes five genotypes, group D includes two genotypes and group E and F include fifteen and two

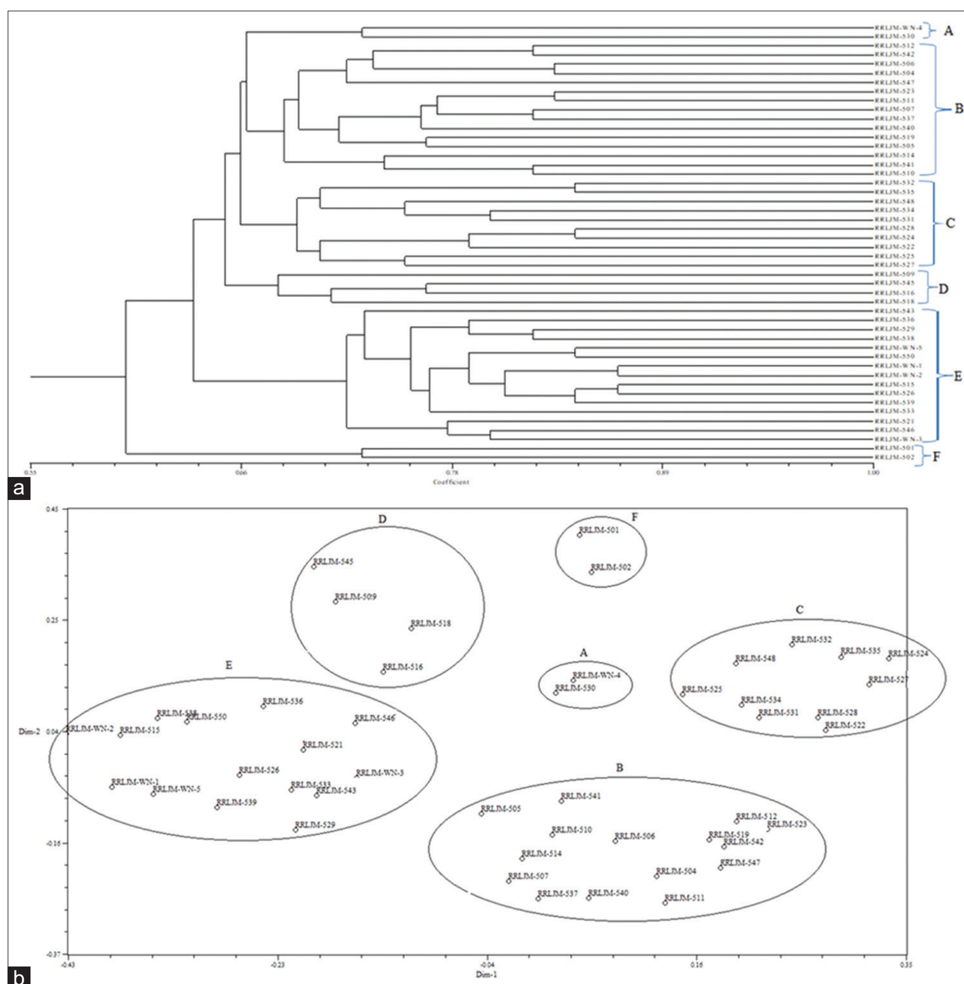

Figure 4: a) Dendogram and b) 3D PCA of forty eight germplasm of *C. khasianus* based on ISSR marker

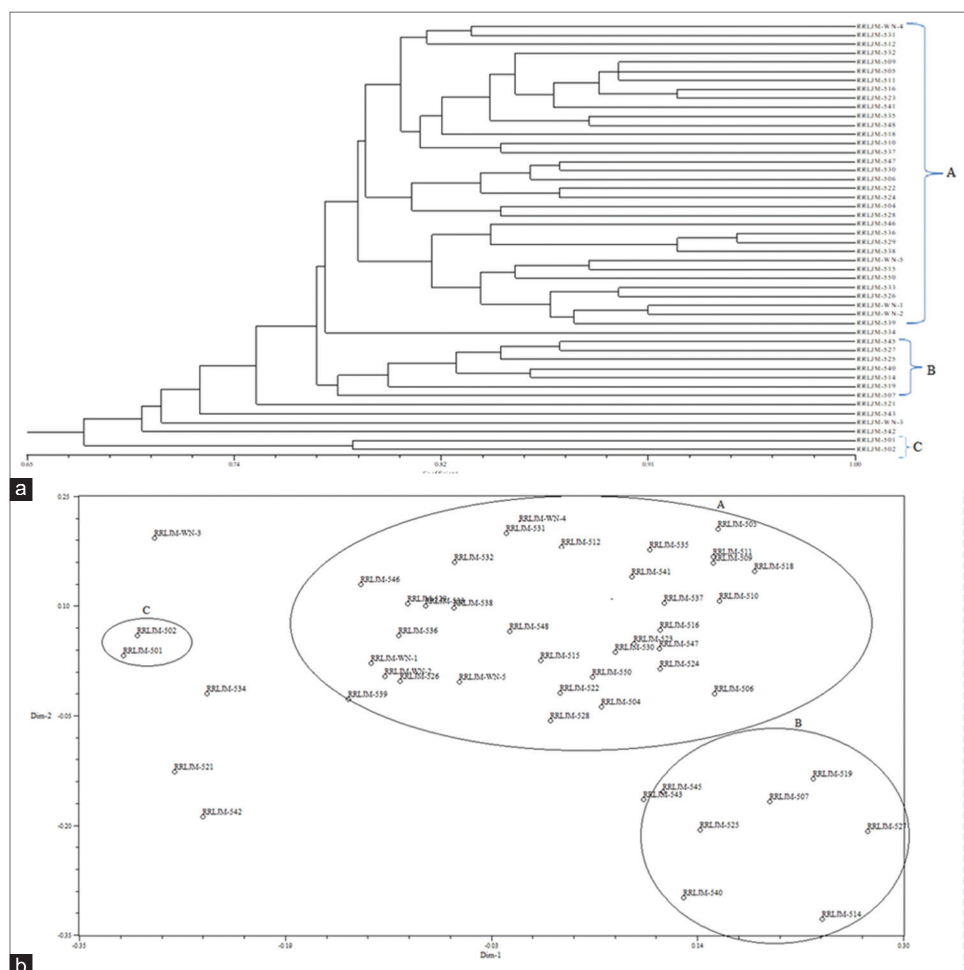

Figure 5: a) Dendogram and b) 3D PCA of forty eight germplasm of *C. khasianus* based on RAPD marker

genotypes, respectively. The genotype RRLJM-514 emerged as a single individual in the dendrogram (Figure 6a).

Principal coordinate analysis (PCA) of these germplasm for both the markers showed clear and distinct diversity among the population (Intraspecific). It was observed that a few genotypes in the dendrogram lying in the same sub-group showed slight variation in the PCA plot. Distinct groups found in three plots of PCA clearly resemble with UPGMA-generated three dendrograms. The only exception is that four genotypes, RRLJM-534, RRLJM-521, RRLJM-WN-3 and RRLJM-542 were scattered, and RRLJM-543 was lying along with group B in the RAPD-PCA plot, which were found as separate individuals in the dendrogram obtained from RAPD. Therefore, considering this minor exception same conclusion can be drawn for principal coordinate analysis from the view of cluster analysis. ISSR, RAPD and combined PCA-plot are shown in Figures 4b, 5b and 6b.

DISCUSSION

ISSR and RAPD markers were routinely employed for studying genetic diversity among populations because of their low cost and high reproducibility (Baruah *et al.*, 2017). Previously, several studies on the molecular diversity of *Cymbopogon* species have been reported, but to date, no such reports on *C. khasianus* have been made. To the best of our knowledge, this is the first report on a genetic diversity study of *C. khasianus* from North East India. ISSR markers, popularly known as Random Amplified Microsatellites (RAM), are highly variable and ubiquitously distributed throughout the genome, at the same time it achieves higher reproducibility compared to using RAPDs (Ng & Tan, 2015), which can be used as mapping tool and can be used in genomic finger printing in a variety of organism and its genetic as well as phylogenetic relationships and biodiversity (Hasibe *et al.*, 2009; Govarthanan *et al.*, 2011). In the present study, both ISSR and RAPD markers were successful in discriminating the genotypes under study. However, ISSR primers were more effective than in RAPD primers with average PIC, MI and Rp values of 0.129, 2.28, 2.13 than the latter (0.097, 0.95, 1.15). The numbers of bands produced per primer vary from 3 to 6 for both markers, with an average of 4.4 bands for ISSR primers and 3.81 for RAPD primers. The percentage of polymorphic bands in ISSR primers (91.50) was also higher than RAPD primers (81.59), showing the high effectiveness of both markers. The average MI value of ISSR primers (2.28) was found to be higher than RAPD primers (0.95) indicated that ISSR primers are selective in their amplification. Similar results were previously reported by

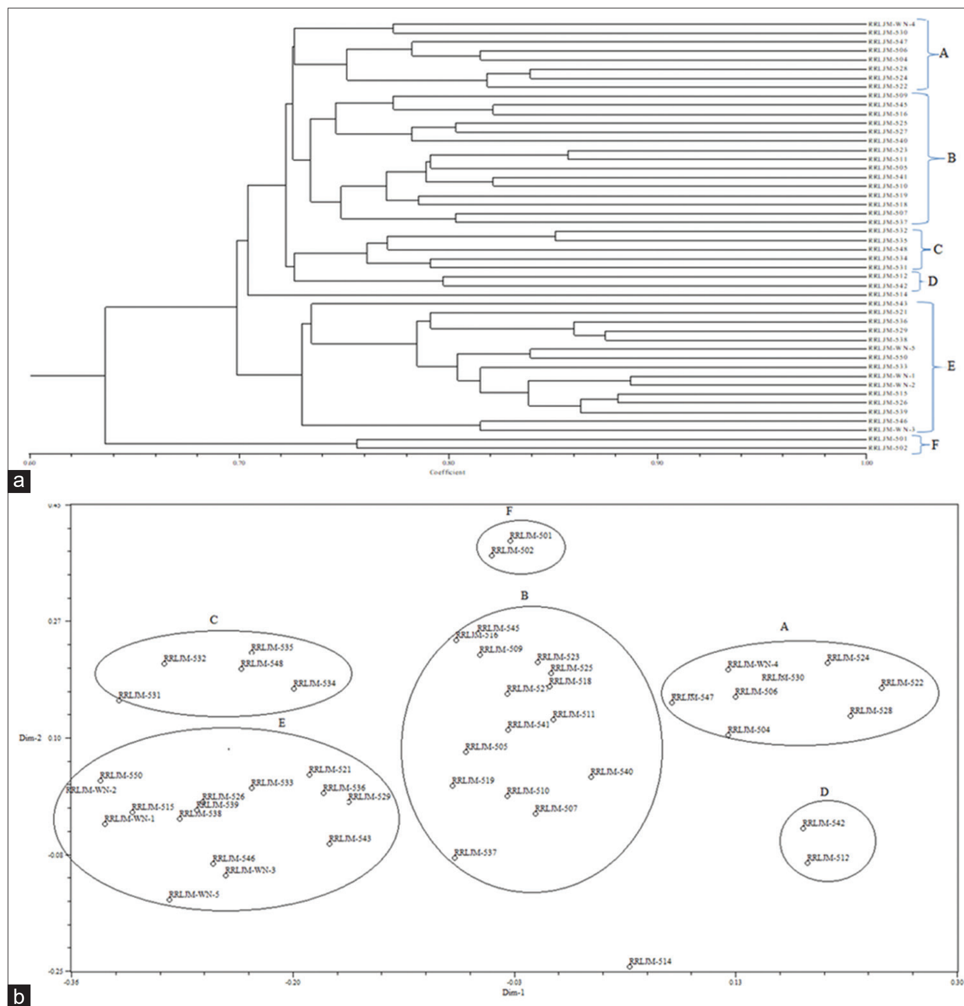

Figure 6: a) Dendogram and b) 3D PCA of forty eight germplasm of *C. khasianus* based on combined data

Baruah *et al.* (2017) for C. *flexuosus* and C. *winterianus* species. More potentiality of ISSR marker over RAPD was also formerly supported by the findings of Fang and Roose (1997). Our present results were comparatively higher than the previous results of 81.33% and 75.11% polymorphism using RAPD (Debajit *et al.*, 2015; Lal & Awasthi, 2015), 74.07%, 47.50% (Bhattacharya *et al.*, 2010) and 69%, 63% polymorphism using ISSR and RAPD, respectively (Bishoyi *et al.*, 2016). Similarly, Adhikari *et al.* (2015) showed 67% polymorphism by ISSR marker in *Cymbopogo*n spp.

The genetic variability parameters observed for *C. khasianus* showed that the average observed number of alleles, effective number of alleles, Nei's gene diversity or expected heterozygosity and Shannon's diversity index value were higher for ISSR marker than RAPD. Similar results were also obtained for *Cymbopogon* sp. and high-yielding *Zingiber officinale* lines by Baruah *et al.* (2017, 2019) where the values obtained were higher for ISSR than RAPD markers. AMOVA result for the present data also showed that percentage of total variance obtained within population is higher for ISSR maker than RAPD marker.

Cluster analysis and PCA plot obtained for both the markers showed different grouping. ISSR and combined data showed somewhat similar grouping. However, the groupings were not in accordance with the place of collection. Such condition may be due to adjacent geographical conditions or xenogamy nature of *C. khasianus* (Dutta *et al.*, 2018).

CONCLUSION

C. khasianus is a medicinal, aromatic and economic grass as its essential oil is rich in elemicin content, which is mainly used in the synthesis of an important alkaloid, i.e., mescaline. The species having the same chemotype shows variation in its morphology, which may be due to both the origin and geographical distribution of these genotypes or its cross-pollination behaviour. This is the first report of detecting molecular diversity and relationship of 48 germplasm of *C. khasianus* using ISSR and RAPD markers demonstrating the potentiality of ISSR marker towards genetic variation among the germplasm. Therefore, genetic diversity study at the intraspecific level using different markers is helpful to discriminate the groups on the basis of similarity index for the conservation, maintenance and the marker-assisted improvement of the crops.

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