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Efficiency of RAPD and ISSR markers in assessment of molecular diversity in elite germplasms of *Cymbopogon winterianus* across West Bengal, India

S. Bhattacharya[1], T. K. Bandopadhyay[1] and P. D. Ghosh[2*]

[1]Department of Molecular Biology & Biotechnology, [2]Department of Botany
University of Kalyani, Kalyani -741235, West Bengal, India

Abstract: Molecular characterization of eleven clones of *C. winterianus* collected from eleven districts of West Bengal was carried out with 10 RAPD and 9 ISSR primers. The RAPD primers developed 81 robust loci, which revealed 74.07% polymorphism and 61 ISSR markers generated 47.50% polymorphism. Out of the different anchored primer combinations, ISSR primers with AG and GA motifs produced clear and maximum scorable markers, thus revealing a better coverage of the genome. Genetic diversity parameters [average and effective number of alleles, percent polymorphism, average heterozygosity, intralocus gene diversity, polymorphic information content (PIC)] for RAPD, ISSR, and RAPD+ISSR along with UPGMA clustering based on Jaccard's coefficient were estimated with a view to assess efficiency of the marker system in *Cymbopogon*. RAPD markers were more efficient than ISSR markers with regards to detection of polymorphism, number of loci scored and PIC values. However resolving power (Rp), Shannon's index, mean coefficient of gene differentiation (Gst), and gene flow estimates were better ISSR markers. Genetic variations detected among the geographically different populations of *C. winterianus* could be of much use for the introgression of new characters from wild counterparts to the cultivars, isolation of stable segregating markers, and selection of improved varieties and conservation of germplasm resources.

Keywords: Genetic diversity, *Cymbopogon winterianus*, RAPD and ISSR, plant breeding.

كفاءة استخدام معلمات التضاعف العشوائي المتعدد الأشكال لسلسلة الدنا RAPD ومؤشرات المقاطع البسيطة المتكررة ISSR في تقييم التباين الجزيئي لمصادر وراثية متميزة في نبات حشيشه الليمون *Cymbopogon winterianus* L. في غرب البنغال (الهند)

س. باتاشاريا[1]، ت. ك. باندوباديايا[1]، ب. د. جوش[2*]

[1]قسم البيولوجيا الجزيئية والتقانات الحيوية؛ [2]قسم علم النبات، جامعة كالياني، كالياني – 741235، غرب البنغال، الهند

الملخص: تم عمل توصيف جزيئي بواسطة 10 باديء RAPD و9 باديء ISSR لأحد عشر صنف من حشيشه الليمون حيث جمعت من عشر مقاطعات في ولاية البنغال الغربية في الهند . باديء RAPD طور 81 موقع نشط والتي كشفت عن تعدد الأشكال في الدنا 74.07٪ و61 معلمة من ISSR كشفت عن تعدد الإشكال في الدنا 47.50٪. بادئات ISSR مع (AG) و (GA) المتكررة تنتج معلمات واضحة وبحدود قصوى، وبذلك تكشف تغطية أفضل للجينوم. تم تقدير قياسات التنوع الجيني [متوسط وفاعلية عدد الأليلات، نسبة تعدد الأشكال في الدنا، متوسط تخالف اللواقح، تنوع مواقع الجينات، تعدد أشكال محتوى المعلومات] لـ RAPD، ISSR، ISSR + RAPD مع UPGMA بناءا على معامل جاكارد بهدف تقييم كفاءة نظام المعلمات في نبات حشيشة الليمون. معلمات RAPD كانت أكثر كفاءة من معلمات ISSR فيما يتعلق بالكشف عن تعدد الأشكال في الدنا، وعدد المواقع المسجلة وتعدد أشكال محتوى المعلومات. بينما كانت معلمات ISSR أفضل من حيث قوة التبين، ومؤشر شانون، ومتوسط معامل الاختلاف الجيني، وتقديرات التدفق الجيني. الاختلافات الوراثية التي تم تحديدها بين أصناف حشيشه اليمون من مختلف المواقع يمكن أن تكون ذات فائدة كبيرة لإدخال مورثات من أصناف برية إلى أصناف مزروعة، وعزل معلمات انعزالية ثابتة، واختيار الأصناف المحسنة وصيانة الموارد الوراثية.

* Corresponding Author, *Email*: pdgbot@gmail.com

Introduction

The genus *Cymbopogon* possesses a large number of odoriferous species of the grass family (Poaceae), and is characterized by plants bearing aromatic essential oils in all parts. These oils are widely used in cosmetics, soap, perfumery and beverage industries. Many of them have considerable pharmaceuticals values and some also bears insect repellant properties as well (Mathur et al., 1988). Cymbopogons are highly heterozygous plants due to its cross pollination. Thus profound genetic variations are prevalent in the species (Sreenath and Jagadishchandra, 1991) that always demands better germplasm management and conservation practices.

Genetic diversity assessment is one of the key step in any plant breeding programmes. Knowledge of the genetic relationships among different accessions is essential for developing appropriate strategies for breeding, germplasm-management, and utilization of genetic resources (Paterson et al., 1991). Many previous workers reported the genetic diversity among different taxa of *Cymbopogon* species based on oil constituents as well as molecular analysis (Sangwan et al., 2001). However genetic characterization of common *Cymbopogon winterianus* cultivars found throughout different districts of West Bengal, India using molecular markers such as RAPD & ISSR has not been documented. With thorough characterization of cultivars, it is possible to study the level of diversity existing within a species and to establish an index of genetic similarities among different populations and varieties. Molecular markers have a number of perceived advantages over the morphological characters assessment of genetic diversity. Most of the morphological characters are sensitive to environmental conditions and growth stages whereas molecular markers are insensitive to such factors and are abundantly present. Marker assisted selection (MAS) has been the mainstay of any modern breeding programmes as can be used at early stages of plant development and is independent of the growth conditions (Soller and Beckmann, 1983). However, molecular markers do have certain problems like reproducibility across laboratories. Another limitation of RAPD markers is their dominant nature. These peculiarities of RAPDs impede direct estimations of allele frequency and can bias calculations of population differentiation (Neale and Harry, 1994). Another molecular system, Inter-simple sequence repeat (ISSR) markers, developed by Zietkiewicz et al. (1994) based on the amplification of a single primer containing a microsatellite 'core' sequence anchored at the 5′or 3′ end by a set of 2–4 purine or pyrimidine residues and offers a high degree of reproducibility with the detection of rich level of polymorphism in a relatively simple procedure. Hence, it has been widely used in assessments of genetic diversity (Bornet and Branchard, 2001) and cultivar identification (Prevost and Wilkinson, 1999).

Considering the potentials of the DNA marker based genetic diversity analysis, the present study aimed to evaluate the congruency of molecular markers system viz. RAPD and ISSR, in assessing and analyzing the nature and the extent of genetic diversity among the different elite germplasm of *Cymbopogon winterianus* collected from a narrow geographical region primarily from eleven different districts of West Bengal, India.

Materials and Methods

Eleven mature tillering elite germplasms of *Cymbopogon winterianus* (cv. Jorlab 2) were collected from eleven districts (Figure 1) of West Bengal, India (Table 1). The collected germplasms (slips) were grown initially in sterile sand and soil mixture in glass house at the experimental garden, Department of Botany, University of Kalyani, West Bengal, India and leaves for molecular studies were harvested from three weeks old seedlings.

Table 1. Clones of *Cymbopogon winterianus* collected from eleven districts of West Bengal used in the study.

Place of Collection (Districts)	Geographical location	Taxonomic series	Chromosome no. (2n) & ploidy	Clone designation
Mungpoo (Darjeeling)	27° 03' N, 88° 18' E	Citrati	2n, diploid	WB/CW1
Toralpara (Jalpaiguri)	26° 32' N, 88° 46' E	Citrati	2n, diploid	WB/CW2
Pundibari (Coochbehar)	26° 20' N, 89° 29' E	Citrati	2n, diploid	WB/CW3
Raiganj, (Uttar Dinajpur)	26°35' N, 87°48' E	Citrati	2n, diploid	WB/CW4
Kanchanpur (Bankura)	23° 14' N, 87° 07' E	Citrati	2n, diploid	WB/CW5
Orgram (Bardhaman)	23° 16' N, 87° 54' E	Citrati	2n, diploid	WB/CW6
Khannan, (Hooghly)	23 ° 01' N, 88° 30' E	Citrati	2n, diploid	WB/CW7
Kalyani (Nadia)	22° 59' N, 88° 29'E	Citrati	2n, diploid	WB/CW8
Sankrail (Howrah)	22° 35' N, 88° 23' E	Citrati	2n, diploid	WB/CW9
DumDum (Kolkata)	22° 38' N, 88° 38' E	Citrati	2n, diploid	WB/CW10
Nimpith (South 24 Parganas)	22° 31' N, 88° 38' E	Citrati	2n, diploid	WB/CW11

Genomic DNA was extracted from fresh leaves (200 mg) and rhizome (100 mg) using Cetyl trimethyl ammonium bromide (CTAB) based procedure with some modifications in the extraction buffer (Khanuja et al., 1999). DNA was isolated at least three times from same line of germplasms and quantity and quality of the extracted DNA samples were estimated by comparing band intensities against standard DNA ladder on 0.8% agarose gel. DNA samples were diluted to a final concentration of 25 ng/µl before PCR amplification using 10 RAPD (out of 40 primers tested) and 9 ISSR primers (out of 22 tested) which were procured from Genie, Bangalore, India, with GC content of 60%. Reproducibility of the RAPD primers was tested by repeating PCR reactions for at least three times under same PCR conditions with same set of chemicals according to Nayak et al. (2003). All the reactions of RAPD and ISSR were carried out in *Perkin elmer,* 2400 Gene Amp PCR system. The amplified PCR products were resolved on 1.5% agarose gel using 1X TBE buffer and stained with Ethidium bromide (0.5 µg/ml) before viewing in Gel Doc 1000 (*Biorad*). For ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. PCR mixture (25 µl) contained 25 ng of genomic DNA as template, 1X PCR buffer (Genei, Bangalore, India), 200 µM dNTPs (Genei, Bangalore, India), 1 unit (U) of Taq DNA polymerase (Bangalore Genei, India), 1µM of each primer (Genei, Bangalore, India) with various concentrations of $MgCl_2$ (1.5-2 mM) depending on the primer. PCR was performed at an initial denaturation temperature of 94ºC for 4 min followed by 40 cycles of 1 min denaturation at 94ºC, 1 min annealing at 2ºC lower than melting point for each primer and 2 min extension at 72ºC with a final extension of 72ºC for 10 min using thermal cycler.

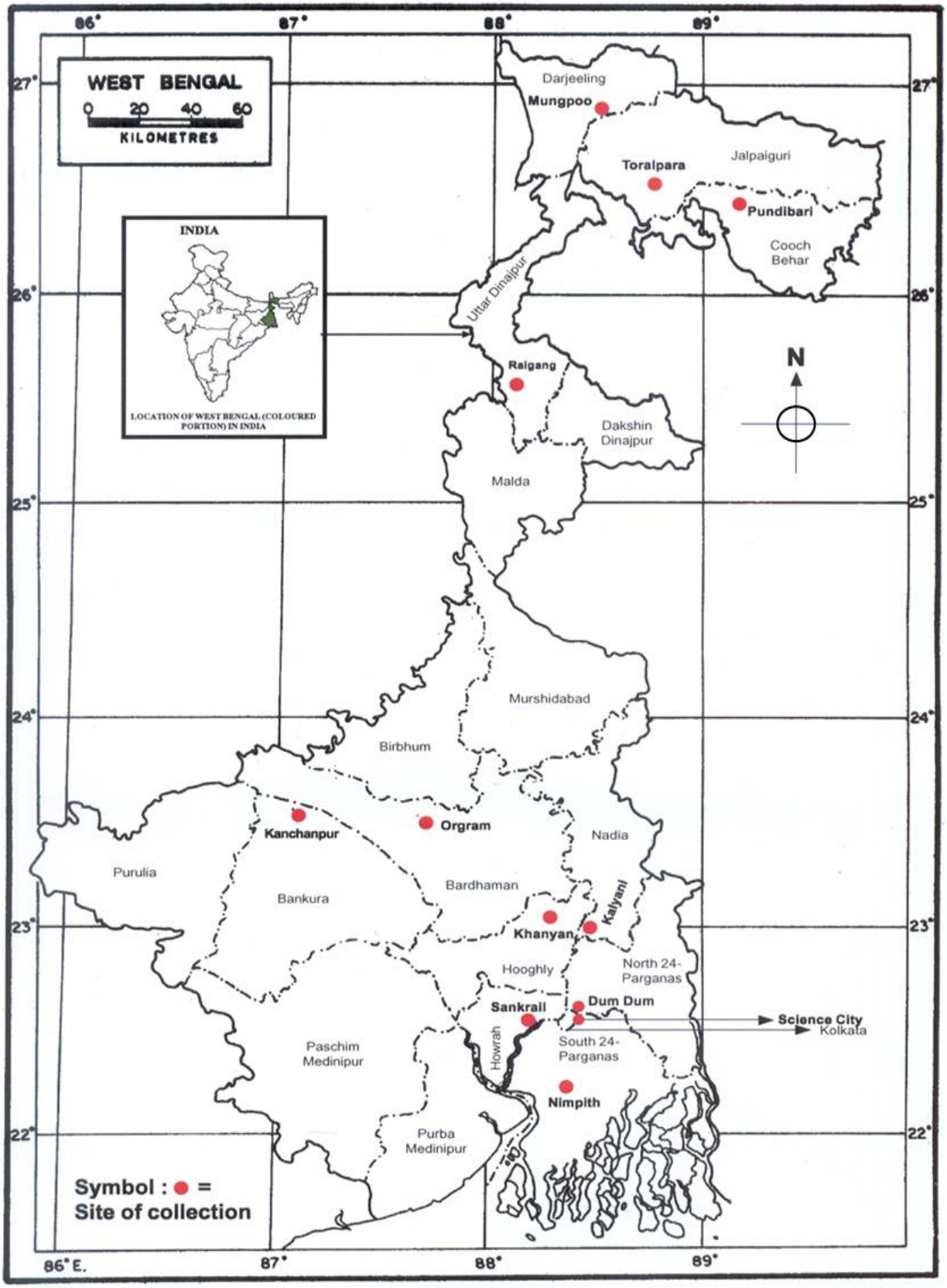

Figure 1. Map of West Bengal, India showing location of site of collection (eleven districts) of *Cymbopogon winterianus* germplasms. Red symbols indicate place of collection.
Note: map not to scale.

Data analysis

Reproducible, well resolved, unambiguous fragments in the range of 200–2900 b.p were scored manually. Each scorable band was scored as presence (1) or absence (0). The profiles generated in different *Cymbopogon* ecotypes were analyzed to compute polymorphic information content (PIC), average expected heterozygosity (H_i), Intralocus gene diversity (H_j), Shannon's index and gene flow estimates (Zhao et al., 2006) using appropriate mathematical derivations of population studies (Excoffer et al., 1992). Jaccard's similarity coefficient (J) (Jaccard, 1908) was used to calculate similarity between pairs of accessions, where, [$J = n_{x,y} / (n_t - n_z)$], $n_{x,y}$ is the number of bands common to sample A and sample B; n_t the total number of bands present in all samples and n_z the number of bands not present in A and B but found in other samples. Based on the proximity matrix obtained from Jaccard's coefficient, sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) method. Data analysis was done using Popgene (Yeh et al., 1997) software version 1.31 and XLSTAT software version 2008.4.02 (*Addinsoft, USA)*. The product-moment correlation (r) based on Mantel (Mantel, 1967). Z-value was computed to measure the degree of relationship between similarity index matrices produced by any two-marker systems. The Resolving power (Rp) (Prevost and Wilkinson, 1999) of RAPD and ISSR is determined by the formula Rp = Σ IB where IB (band informativeness) takes the value of: 1 – [2 x (0.5 – p)], p being the proportion of the 11 population of *Cymbopogon winterianus* containing the band.

Results

Eleven germplasms of *Cymbopogon winterianus* collected from eleven districts of West Bengal, India were amplified with 10 RAPD (from 40 decamers) and 9 ISSR (22 anchored & unanchored) markers to ascertain the level of genetic diversity within different germplasms different geographical regions (Table 2). A total 81 RAPD loci and 61 ISSR loci were scored in different plant populations collected from eleven different regions of West Bengal (Figure 2).

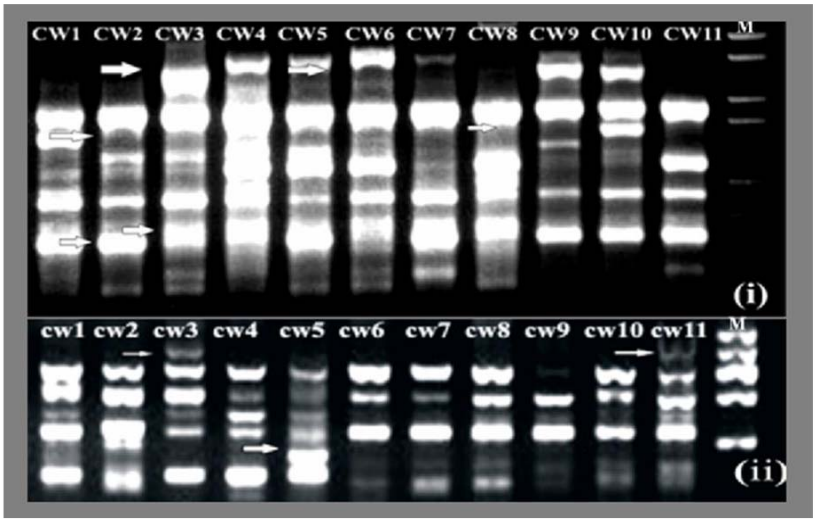

Figure 2. (i) RAPD profile of eleven germplasms of *C. winterianus* with primer MS10G9 (GGTATTACTT) resolved on 1.5% agarose gel. CW1-CW11 indicates *C. winterianus* clones obtained from eleven districts of West Bengal. The lane M corresponds to weight marker of known molecular weight (200b.p – 3000 b.p) obtained from *Eco*RI and *Hind*III digested genomic DNA of λ-phage. (ii) ISSR profile generated with primerBG-ISSR-1 [$(AG)_8A$] separated on 1.5% agarose gel stained with ethidium bromide (0.5μg/ml). Arrow indicates polymorphic bands.

Most of the PCR products were in the size range of 250-2900 b.p with 8.1 bands per RAPD and 6.7 bands per ISSR primers. In RAPD analysis 74.07% of scored loci were polymorphic; however in ISSR analysis only 47.5% loci were found to be polymorphic with an average number of polymorphic bands for RAPD and ISSR primer were 6.0 and 3.2 respectively (Table 3). Both 3′ anchored and unanchored primers were used for ISSR analysis to screen germplasms representing eleven locations. In case of unanchored primers, the SSR motif tends to slip within the repeat units during amplification leading to smearing instead of clear band. However extending the primer with 1-4 degenerate nucleotide at 3′ or 5′ end prevents internal priming and smear formation (Reddy et al., 2002). The anchored nucleotides also allow only a subset of microsatellite to serve as priming site. Our results also suggested that primers anchored at 3′ end give better amplification and clearer banding profiles than primers anchored at 5′ end and which is in accordance with previous workers (Blair et al., 1999). In the present study dinucleotide and trinucleotide SSR motifs AG, AC, CT, GA, GGA and CCA were used. Out of these AG and GA motifs produced clear and maximum scorable fragments thus revealing more coverage of genome (Figure 2). The above observations were in conformity with previous workers working on *Swertia chirayita* (Joshi and Dhawan 2007) and in *Gerbera* (Bhatia et al., 2008).

Table 2. PCR Amplification performance of eleven clones of *Cymbopogon winterianus*, using selected RAPD and ISSR primers.

Primer	Mono-morphic Bands	Poly-morphic Bands	Poly-morphism (%)	Total no. of bands amplified	Resolving power
RAPD SETS					
MS10G-1 (GTCCTACTCG)	2	4	66.6	43	6.272
MS10-G2 (GTCCTTAGCG)	1	4	80.0	38	4.277
MS10-G3 (CGGGATCCGC)	3	8	72.7	44	8.682
MS10-G4 (CCCTGCAGGC)	2	8	80.0	78	11.691
MS10-G5 (GGATCTGAAC)	2	6	75.0	65	7.110
MS10-G6 (CATCCCGAAC)	2	7	77.7	45	8.261
MS10-G7 (ATGGCATTGC)	2	6	75.1	69	7.451
MS10-G8 (TTCTGGCATA)	2	5	71.4	58	7.396
MS10-G9 (GATTCGCGAT)	3	7	70.0	61	10.302
MS10-G10 (GGAATCCGTG)	2	5	71.4	72	7.313
ISSR SETS					
BG-ISSR-1 $(GT)_8A$	4	3	42.8	42	8.852
BG-ISSR-2 $(GC)_8TG$	4	2	33.3	37	9.017
BG-ISSR-3 $(GA)_8T$	3	4	57.1	22	6.471
BG-ISSR-4 $(CA)_8GT$	5	4	44.4	35	11.139
BG-ISSR-5 $(GCA)_8AG$	4	2	33.3	42	8.827
BG-ISSR-6 $(CTG)_8G$	4	2	33.3	35	8.951
BG-ISSR-7 $(AG)_8CA$	3	4	57.1	49	7.132
BG-ISSR-8 $(CT)_8GAC$	5	3	37.5	39	11.348
BG-ISSR-9 $(TG)_8GA$	6	5	45.4	35	13.843

Table 3. Data showing comparative profiles of RAPD, ISSR and pooled RAPD+ISSR amplification in *Cymbopogon winterianus.*

Parameters	RAPD	ISSR	RAPD + ISSR
Total Number of bands amplified	**573**	336	909
Total number of loci scored	81.0	61	142
Total number of polymorphic loci	60.0	29	89
Percentage of polymorphism (%)	74.07	47.5	62.6
Average number of bands per primer	8.10	6.77	7.47
Average number of polymorphic bands per primer used	6.0	3.2	4.68
Resolving power	7.87	9.5	8.64

Wide genetic variation between each individual of *Cymbopogon winterianus* from different regions of West Bengal, India was evident from high number of polymorphic marker and appearance of unique bands, even though the study was limited to a small number of individuals. The values obtained from mean coefficient of gene differentiation (Gst) from RAPD (0.371) suggested that 62.9% of genetic diversity resided within the population, while 57.9% of genetic diversity within population was estimated from ISSR data (Table 4). The same degree of genetic heterogeneity was discerned through Shannon's information index. The product moment correlation (r) and the Mantel test statistic (Z) were calculated to measure the degree of relationship between the similarity matrices obtained and combined (RAPD and ISSR) data and the correlation was significant (0.873).

Table 4. Genetic diversity parameters characterizing eleven clones of *C. winterianus* derived from RAPD, ISSR and pooled data.

Parameters	RAPD	ISSR	RAPD + ISSR
Sum of Effective no. of alleles (SENA)	11.723	9.601	9.841
Shannon's Information index	0.205	0.221	0.201
Polymorphic information content (PIC)	0.248	0.217	0.232
Intralocus gene diversity (Hj)	1.11	0.94	0.84
Average gene diversity (Hi)	0.14	0.11	0.10
Gst	0.371	0.421	0.483
Gene flow estimate	0.438	0.519	0.520

Where Hj, Intralocus gene diversity = 1-p2-q2; where, p and q are the corresponding allele of gene. Hi, Average gene diversity = Hj/total no. of loci. Gst, Mean coefficient of gene flow differentiation.

The product moment correlation (r) and the Mantel test statistic (Z) were calculated to measure the degree of relationship between the similarity matrices obtained by RAPD and integrated RAPD and ISSR data and the correlation value (r) was significant (0.965). The product moment correlation (r) and the Mantel test statistic (Z) obtained by ISSR and integrated RAPD and ISSR data also showed significant correlation value (0.821). The matrix obtained from band sharing data of RAPD and ISSR and combination of RAPD and ISSR obtained from Jaccard's coefficient was used to construct cluster based on UPGMA

method (Figure 3). Both RAPD and ISSR marker data divided the eleven individuals into two separate large cluster group. The plants collected from the districts of North Bengal (CW1, CW2, and CW6) were grouped together into a single cluster indicating their common place of occurrence and sharing the common gene pool.

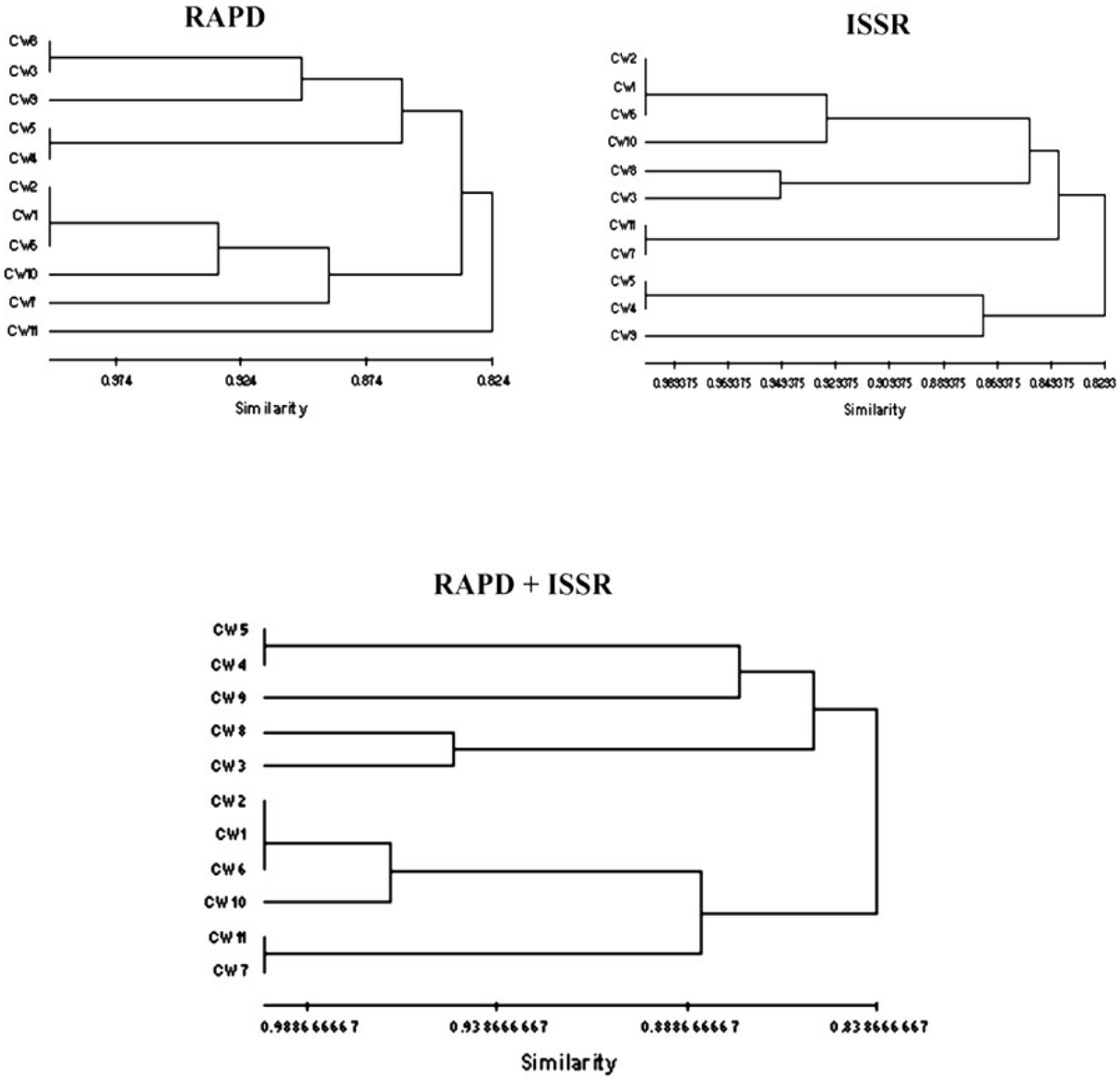

Figure 3. Cluster analysis using UPGMA method depicting genetic similarity (Jaccard's coefficient) between eleven clones of *Cymbopogon winterianus* derived from band sharing data of RAPD, ISSR and pooled RAPD+ISSR data.

Hence from our observations the clones of Mungpoo and Jalpaiguri and Coochbehar were more phylogenetically related with that of clones obtained from Nimpith (CW11) than the clones from Raiganj (CW4) and Bankura (CW5). Similarly plants collected from Kolkata and adjoining areas were similar in genetic make up and were clustered together. Clusters obtained from ISSR marker were more overlapping and indicating comparatively lesser extent of genetic diversity assessment by ISSR marker than RAPD in the present case. Combined cluster analysis of RAPD and ISSR data revealed similar results as of RAPD with

the segregation of individuals into two major cluster, one includes the plants CW5, CW4, CW9 (one small subcluster), CW8 and CW3 (second small sub cluster); while the second large cluster group accommodates CW2, CW1, CW6, CW10 (grouped into a single small sub cluster) and CW11 and CW7 (into another subcluster). It is infact interesting to note that clustering method with three types of marker system efficiently grouped CW1, CW2, CW6 and CW10 together indicating that these plants are genetically similar and are most related. However the plants CW11 (South 24 Parganas), CW5 (Bankura), CW3 (Coochbehar) were genetically distant the plants of North Bengal. The CW4 and WB/CW10 were distantly placed and were the least similar (71%) showing major genetic distance between them. Plants obtained from Hooghly (CW7) and Howrah (CW9) were placed apart though they are related geographically and showed less similarity coefficient indicating high heterozygosity within gene pool of *Cymbopogon winterianus* owing to its cross pollinated nature.

Discussion

RAPD marker was found to be more efficient in estimation of molecular diversity of different ecotypes of *Cymbopogon winterianus* than ISSR marker as evident from large values of polymorphic loci, PIC and average number of polymorphic bands per primer. Comparison of PIC values for two marker systems (a parameter associated with the discriminating power of markers) indicated that the PIC values for RAPD primers was 0.248, while of ISSR it was 0.247 indicating better resolving power of RAPD marker over ISSR. This may be due to the polyallelic nature of RAPD markers. Polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population. Heterozygosity corresponds to a probability that two alleles taken at random from a population can be distinguished using the marker in question. Thus a convenient quantitative estimate of marker utility and the polymorphism detected can be given in terms of Shannon's information index, average heterozygosity, coefficient of population differentiation (Gst) and estimate of gene flow. A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome.

The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. With this study we can conclude that the molecular analysis of different geographically scattered population of *Cymbopogon winterianus* across West Bengal through ISSR and RAPD fingerprinting provides a powerful tool for the generation of potential diagnostic markers for cultivar analysis. Also the phylogenetic analysis on the basis of RAPD and ISSR derived dendrogram supports the fact that region specific variations are there, which is because of the multiple generations of selection carried out after their introduction. The differences found among the dendrogram generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (573 for RAPDs and 336 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among *Cymbopogon* cultivars. Similar results have been observed by Loarce et al. (1996) in barley. Another explanation could be the low reproducibility of RAPDs. The observed high proportion of polymorphic loci suggests that there is profound genetic diversity heterogeneity in the populations. Such genetic polymorphism may be

accounted due to high degree of heterozygosity in *Cymbopogons* due to out crossing behavior that may lead to gene pool imbalance. Hence the present paper reports the occurrence of genetic diversity within different populations of *Cymbopogon winterianus* grown at eleven districts of West Bengal. From studies made from RAPD and ISSR marker, RAPD marker was found to give better polymorphism results than ISSR.

The results suggested that genetic base utilized in their breeding programmes has been restricted and that introgression of genes from unexploited sources deserves attention. Conventional breeding efforts have been made for improvement of the cultivars belonging to several species of *Cymbopogon* but these efforts did not involve assessment and consideration of genetic diversity for the selection of parents. Knowledge of molecular marker aided genetic diversity profiles, parallel to morphological and biochemical relatedness and differences among the *Cymbopogon* species (Sharma et al., 2000), could offer added advantages of strategic combination of traits and exploitation of the germplasm diversity. Such regional diversity may be exploited for the generation for potential hybrid lines with controlled breeding and hybridization strategies for expression of agronomically useful traits. Therefore studies on other chemotypic traits of wild counterparts and other cultivated species and varieties of *Cymbopogon* along with the use of combination of sensitive marker systems such as AFLP and SCAR and SSLP should be considered to screen and develop more suitable and tightly linked markers for improved traits and its further utilization in plant improvement and breeding programmes for exploitation of genetic resources for the sake of commercial and academic needs.

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