Indian Journal of Biotechnology
Vol 12, January 2013, pp 109-114

Assessment of genetic diversity of certain Indian elite clones of *Cymbopogon* species through RAPD analysis

Sinchan Adhikari[1], T K Bandopadhyay[2] and P D Ghosh[1]*

[1]Department of Botany and [2]Department of Molecular Biology and Biotechnology
University of Kalyani, Kalyani 741 235, India

Ten elite Indian cultivars of *Cymbopogon*, aromatic grasses of essential oil trade types—citronella, palmarosa and lemongrass, were characterized isolated from fresh leaf tissues were amplified with twelve 10-mer arbitrary primers. The amplification produced overall 64 scorable bands in the cultivars studied. Of which 52 were polymorphic and 12 were monomorphic. RAPD markers proved to be the efficient marker system with regard to detection of polymorphism, number of loci scored and PIC values. Polymorphism differed substantially within the discrete groups of cultivars and was approximately 71.88% in palmarosa, 6.25% in citronella and 46.88% in lemongrasses. Genetic variations detected among the elite cultivars could be of much use for the introgression of new characters from wild counterparts to the cultivars, isolation of stable segregating markers, selection of improved varieties and conservation of germplasm resources.

Keywords: *Cymbopogon*, diversity analysis, DNA markers, essential oils, RAPD

Introduction

The genus *Cymbopogon* (Family: Poaceae) is known to have about 140 species. Among these more than 52 have been reported to occur in Africa, 45 in India, 6 each in Australia and South America, 4 in Europe, 2 in North America and the remaining are distributed in South Asia[1]. Most of these species produced characteristics aromatic essential oils that have commercial importance in perfumery, cosmetics and pharmaceutical applications. The *Cymbopogon* essential oils are characterized by monoterpenes like citral, citronellol, citronellal, linalool, elemole, limonene, geraniol,1,8-cineole, β-carophyllene, methyl heptenone, geranyl acetate and geranyl formate. Essential oils of *Cymbopogon* species (aromatic grasses) have become profitable export products for many developing agrarian nations. Their cultivation in terms of area cultivated has rapidly expanded during past few decades. Apart from elite cultivars of the aromatic grasses, genetic resource support to the development of cultivars has remained limited compared to the situation in traditional crops, where enormous genetic knowledge and even saturated linkage maps have become available. Although more than 50 species of genus occur in nature, only a few of them like *C. flexuosus, C. pendulus, C winterianus,* and *C. martinii* are commercially cultivated as modern cash crops for essential oil production.

Cymbopogon is highly cross pollinated, heterozygous, graminaceous aromatic plant, which possesses problem through conventional breeding. In past, various efforts have been made to group various accessions into clusters, but all theses are based on the correlated phenotypic expression, herbage yield and identification of oil quality. Introgression of various traits, intermittent mutations and selection through human intervention may lead to variation in chemotypic characters across geographical distributions[2]. While natural hybridization may lead to the formation of morphological or chemotypic intermediates, defining taxa purely on this basis may not be appropriate. With the advent of molecular biological techniques with particular reference to DNA fingerprinting, a positive direction can be outlined, which cannot be possible through conventional breeding with special reference to qualitative and quantitative characters. DNA markers are independent of environment, age and tissue, and expected to reveal the genetic variation more conclusively. The techniques have been employed for cultivar identification, phylogenetic analysis, genetic mapping, estimation of out-crossing rates and population differentiation in a number of food, forage and fibre crops[3-5]. A number of DNA based markers are now available for the effective quantification of genetic variation in plant species. Restriction

*Author for correspondence:
E-mail: pdggene@rediffmail.com

fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been applied successfully and have provided considerable genetic information in a number of plant species[6,7]. These techniques are slow and expensive and are not amenable for assessment of genetic variation in large scale population genetic studies. More recently, PCR-based RAPD and simple sequence repeat (SSR) markers requiring small amounts of DNA have also been developed[8]. SSR markers have proved to be polymorphic but require nucleotide information for primer design[9]. RAPD has been used widely because it requires no DNA probe and no sequence information for the design of specific primers. RAPD analysis can be used to identify genetic variation[10,11], genetic relatedness[12,13], genetic diversity analysis[14,15] and phylogenetic relationship[16,17]. So far, few reports on the application of DNA fingerprinting for molecular characterization of *Cymbopogon* genome are available in literature. However, reports are lacking on genome sequencing of *Cymbopogon* with special reference to aromatic compound gene linked marker, which is a prerequisite for marker assisted selection breeding technique for the improvement of the crop. Moreover, there is urgent need to standardize a *Cymbopogon* genome database for strengthening molecular biological research for gene conservation and exploitation. Considering the potential of the DNA marker based genetic diversity analysis, the present study was aimed to estimate genetic diversity of certain Indian elite cultivars of *Cymbopogon* with special reference to identification of biotype specific markers.

Materials and Methods

Plant Material

The plant material used in this study consisted of 10 elite cultivars belonging to 3 species of *Cymbopogon* (Table 1). The cultivars were collected from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow; National Bureau of Plant Genetic Resources (NBPGR), Cuttack; and B N Mahavidyalaya, Hooghly, West Bengal. Each cultivar was vegetatively propagated from the tussocks (slips) and maintained in the experimental garden, Department of Botany, University of Kalyani.

DNA Extraction

Genomic DNA was extracted from fresh leaves (100 mg) using Cetyl trimethyl ammonium bromide (CTAB) based procedure with some modifications in the extraction buffer[18]. DNA was isolated at least three times from same line of germplasm 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.

PCR Amplification and Fragment Analysis

The DNA samples were subjected to RAPD assay, using 12 arbitrary decamers procured from Genei, Bangalore, India with GC content of 60% (Table 2). Different concentrations and combinations of PCR components like dNTP, $MgCl_2$, primer concentration

Table1—Details of *Cymbopogon* species and their cultivars subjected to diversity analyses

Cultivar	*Cymbopogon* species	Major oil constituents	Collection area
Tripta	*C. martinii*	Geraniol	CSIR-CIMAP, Lucknow
PRC-1	"	"	CSIR-CIMAP, Lucknow
Trishna	"	"	CSIR-CIMAP, Lucknow
ACC-01	"	"	NBPGR, Cuttack
ACC-02	"	"	NBPGR, Cuttack
Manjusha	*C. winterianus*	Citronellal, citranellol	CSIR-CIMAP, Lucknow
CIM Jeera	"	"	CSIR-CIMAP, Lucknow
Nima	*C. flexuosus*	Citral	CSIR-CIMAP, Lucknow
Pragati	"	"	CSIR-CIMAP, Lucknow
OD-19	"	"	B N Mahavidyalaya, Hooghly (WB)

Table2—List of RAPD primers, their sequences and PIC values

No.	Code	Sequence (5′-3′)	PIC value
1	OPS-01	GGTGACGCAG	0.317
2	OPS-02	GGTCCCTGAC	0.431
3	OPS-03	GTGACGTAGG	0.425
4	OPS-04	CCGACAAACG	0.000
5	OPS-05	GTCCTTAGCG	0.196
6	OPS-06	CCAAGCTTGC	0.330
7	OPS-07	AACGTACGCG	0.336
8	OPS-09	AGGATACGTG	0.435
9	OPS-10	CACCCTGCGC	0.148
10	OPS-12	GAAACGGGTG	0.000
11	OPS-13	TGCTCTGCCC	0.264
12	OPS-14	TGGGGGACTC	0.222

were used in different PCR cycles to optimize the best PCR conditions and to obtain the best amplification results. The amplification reaction mixture contained 400 µ*M* each of dNTP, 10 pmoles primers (1.5 µL), 1.0 m*M* $MgCl_2$, 0.2 U Taq polymerase (0.2 µL) (Bangalore Genei, India), 2.5 µL of Taq buffer and 25 ng of genomic DNA (2.0 µL). PCR amplification was carried out at 94°C (4 min), followed by 40 cycles at 94°C (15 sec), 40°C (15 sec) and 72°C (1.15 min), with a final extension for 7 min at 72°C. 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. All the reactions were carried out in Perkin Elmer, 2400 Gene Amp PCR system. The amplified PCR products were resolved on 1.5% agarose gel using 1× TAE buffer and stained with ethidium bromide (0.5 µg/µL) before viewing in Gel Documentation System (Uvitec, UK).

Data Analysis

Amplified fragments were scored as binary data, *i.e.*, presence as 1 and absence as 0, for the homologous bands. The polymorphic information content (PIC) values for each primer were calculated using the following formula: $PIC = 1-\sum SPij^2$, where Pi is the relative frequency of j^{th} allele for i^{th} marker, and summed over n number alleles. The matrix of RAPD phenotypes was analyzed on the basis of several indices of population genetics, such as, number of polymorphic loci, percentage of polymorphic loci, observed number of alleles (n_o), effective number of alleles (n_e), Nei's gene diversity (h), Shannon's information index (I) using POPGENE program version 1.32[19]. A dendrogram using unweighted pair group method (UPGMA) was constructed for estimating the genetic similarity based on Nei's coefficients among populations using NTSYS version 2.02[20].

Results and Discussion

Ten germplasms of *Cymbopogon* were amplified with 12 RAPD markers to ascertain the level of genetic diversity. Of the 12 primers screened, 10 primers produced reproducible results (Table 3). A total of 64 RAPD loci were amplified from different plant populations. Most of the PCR products were in the size range of 200-2000 bp with 6.4 bands per RAPD primers. Of the 64 bands scored, 52 (81.25 %) were found to be polymorphic (either occurring in or absent in less than 95% of all accessions) and 12 bands (18.75%) were found to be monomorphic in nature (Table 4). A total of 52 polymorphic loci were identified with an average

Table 4—Distribution of amplified fragments in *Cymbopogon* spp.*

Parameters	Values
Total no. of primers screened	12
No. of primers producing polymorphism	10
Total no. of loci scored	64
Total no. of polymorphic loci	52
Size of amplified bands	200 bp-2 kb
Av. no. of polymorphic bands per primer	5.2
% bands which are polymorphic	81.25

*Data pooled from studies on 10 cultivars of *Cymbopogon* spp.

Table 3—Primer-wise score of PCR amplification products scored in the elite Indian cultivars of *Cymbopogon*

Primer	No. of PCR amplification fragments generated in:							
	Palmarosa		Citronella		Lemon grass		All genotypes	
	Monomorphic	Polymorphic	Monomorphic	Polymorphic	Monomorphic	Polymorphic	Monomorphic	Polymorphic
OPS-01	0	7	7	0	4	3	0	7
OPS-02	1	7	8	0	4	4	1	7
OPS-03	0	8	8	0	3	5	0	8
OPS-04	0	0	0	0	0	0	0	0
OPS-05	3	2	5	0	3	2	2	3
OPS-06	1	5	3	3	2	4	0	6
OPS-07	1	5	6	0	3	3	5	1
OPS-09	1	6	6	1	3	4	0	7
OPS-10	0	0	0	0	0	0	0	0
OPS-12	4	1	5	0	3	2	3	2
OSP-13	3	2	5	0	4	1	2	3
OPS-14	4	3	7	0	5	2	3	4
Total Score	18	46	60	4	34	30	16	48

of 5.2 bands per primer. The frequencies of polymorphic bands obtained varied from primer to primer. Wide genetic variation between cultivars of the species (except citronella) was evident from the high number of polymorphic markers and unique bands, even though the survey was limited by the small number of cultivars available. The randomly primed PCR approach not only facilitated molecular distinction of the genotypes (cultivars) of *Cymbopogon* species but also provided some biotype specific markers. The biotype-specific markers (by presence) included OPS-13 (~500 bp) and OPS-03 (~200bp) for the palmarosa cultivars (Tripta and Trishna) (Fig. 1). Another biotype specific marker was scored for the lemongrass cultivars reflected from the RAPD profile of OPS-13 (~700bp) (Fig. 1). OPS-05 (~350bp) and OPS-06 (~250bp) also produced a specific marker for palmarosa variety ACC-01 and ACC-02, respectively. However, no such positive marker could be scored for citronella cultivars.

All the PCR products primed by OPS-01, OPS-06 and OPS-09 were polymorphic. However, polymorphism differed substantially within the discrete groups of cultivars and was approx. 71.88% in palmarosa, 6.25% in citronella and 46.88% in lemongrasses. Analysis of RAPD revealed that palmarosa had more allelic variability per locus (1.72) than lemongrass (1.46) and citronella (1.06). Estimates of gene diversity[21] values were much lower in citronella (0.026) than lemongrass (0.172) and palmarosa (0.251). The same order of genetic heterogeneity was observed through Shannon's information index (Table 5). RAPD marker data divided ten elite cultivars into three large cluster groups (Fig. 2). Manjusha and CIM Jeera of *C. winterianus* were grouped in the same cluster sharing the common gene pool. Tripta, Trishna, ACC-01 and ACC-02 of *C. martnii* shared a single cluster, whereas PRC-1 of *C. martinii* shared the cluster with the cultivars of *C. winterianus*, indicating the

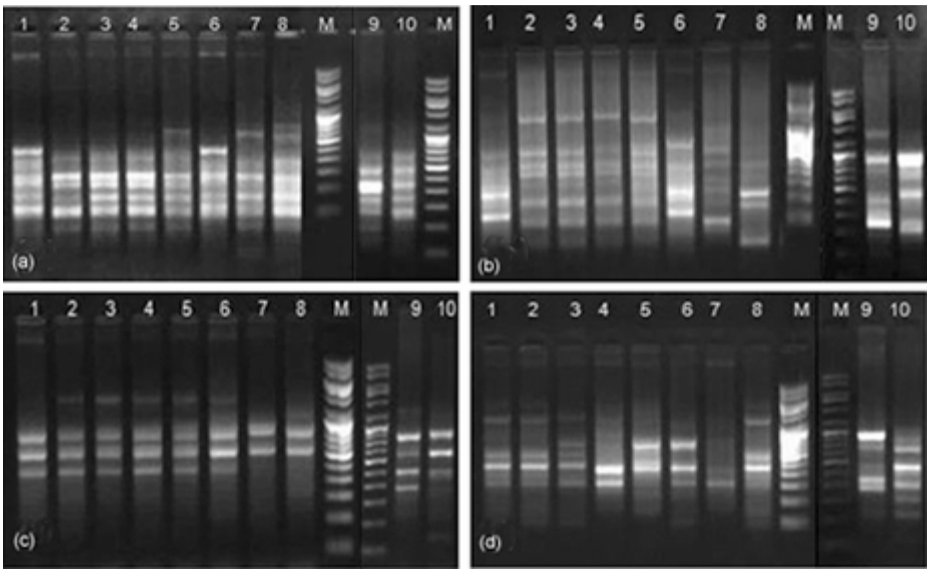

Fig. 1 (a-d)—RAPD profile of ten cultivars of *Cymbopogon* spp.: RAPD amplification generated with primers OPS-13 (a), OPS-03 (b), OPS-05 (c) and OPS-06 (d). [Lane 1, Tripta; lane 2, PRC-1; lane 3, Manjusha; lane 4, CIM Jeera; lane 5, Nima; lane 6, Trishna; lane 7, Pragati; lane 8, OD-19; lane 9, ACC-01; lane 10, ACC-02; & M, Marker (100 bp-3 Kb).

Table 5—Genetic variability across the Indian elite *Cymbopogon* genotypes as well as within the discrete biotypic groups of cultivars as discerned through randomly primed PCR

Parameter	Diversity value for cultivars of the *Cymbopogon* biotypes			
	Palmarosa	Citronella	Lemongrass	All genotypes
Observed no. of alleles (n_o)	1.7188	1.0625	1.4688	1.8125
Effective no. of alleles (n_e)	1.4267	1.0442	1.2909	1.5108
Nei's gene diversity (h)	0.2514	0.0259	0.1728	0.2971
Shannon's information index (I)	0.3781	0.0378	0.2586	0.4413
% polymorphic loci	71.88	6.25	46.88	81.25

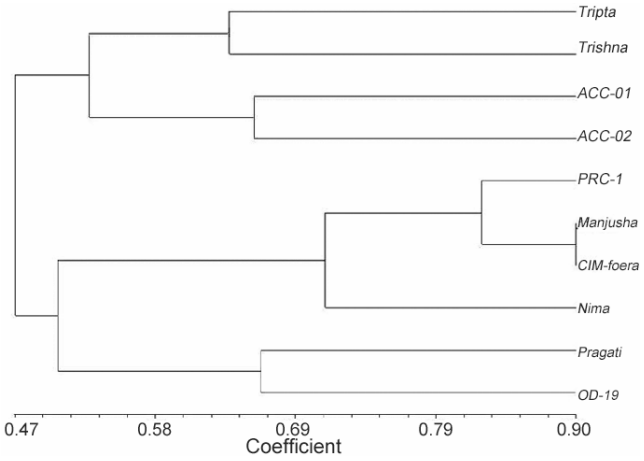

Fig. 2—UPGMA dendrogram based on Nei's genetic distance for the ten elite cultivars of *Cymbopogon* spp.

homology in the genome of cultivars. Pragati and OD-19 of *C. flexuosus* are grouped in another major cluster. The sharing of the gene pool was also observed between the *C. flexuosus* and *C. winterianus* as one of the cultivar of *C. flexuosus* (Nima) was grouped with the cultivars of *C. winterianus*.

DNA-based molecular markers can demonstrate similarities and differences between cultivars and accessions even when a morphological description is severely limited. Among these, RAPD, despite having certain disadvantages (dominant nature and stringent optimization of assay), can produce multilocus profiles, widely spanning the genome even in the absence of any prior genetic/sequence information. Also, they can be helpful in defining parental combinations (for distant gene introgression) to obtain better agronomic and oil trait cultivars. Therefore, RAPD was employed in the investigation presented here to estimate genetic relatedness and diversity among the cultivars of different essential oil trade types of *Cymbopogon* grasses. The observed high proportion of polymorphic loci suggests that there is a profound genetic heterogeneity in the species. The lower level of polymorphism in citronella is understandable, as rare and scanty flowering might have limited outcrossing-mediated heterozygosity enhancement. Chemotypically also, citronella displays far less variability in oil composition than the other *Cymbopogon* species[22]. The cultivars can be distinguished visibly at the interspecies level. The identity of plants as biotypes of lemongrass, citronella and palmarosa is also quite clear. The distinctions remain intact even when extremes of oil chemotypic variants are encountered for the species. However, at intra-species (inter-cultivar) level morphological distinctions are very minute and may require a suitable score of unique RAPD markers (positive) as 'stand along' (if not 'stand alone') fingerprints to aid in cultivar recognition.

Molecular diagnosis strongly suggests that the cultivars of citronella differ very little among themselves. It has also been observed that genetic base utilized in their breeding programmes is restricted and introgression of genes from unexploited sources deserves attention. Therefore, their wild counterparts of the Indian subcontinent (centre of genetic diversity) should be considered for utilization in the plant improvement programme, especially for minimizing the imbalance of recessive alleles in the heterozygous state. 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. RAPD, in combination with agronomic, morphological and oil chemotypic characteristics, can provide a catalogue of *Cymbopogon* cultivars for the identification of duplicate accessions, thereby defining core collections and strengthening exploitation of their genetic resources for horticultural and curatorial needs.

Acknowledgement

The authors thankfully acknowledge CSIR-CIMAP, Lucknow, NBPGR, Cuttack and Professor M L Ghosh, B N Mahavidyalaya, Hooghly (WB) for providing the elite cultivars. The authors would also like to thank the University Grants Commission (UGC), New Delhi for financial support and University of Kalyani (under SAP of DST-FIST) for central instrumentation facilities for this research project.

References

1 Jagadish Chandra K S, Recent studies on *Cymbopogon* Spreng. with special reference to Indian Taxa, *J Plant Crops*, 3 (1975) 1-5.

2 Kuriakose K P, Genetic variability in East Indian Lemongrass (*Cymbopogon flexuosus* Stapf.), *Indian Perfum*, 39 (1995) 76-83.

3 Chalmers K J, Waugh R, Sprent J I, Simons A J & Powell W, Detection of genetic variations between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers, *Heredity (Edinb)*, 69 (1992) 465-472.

4 Fritsch P & Rieseberg L H, High outcrossing rates maintain male and hermaphrodite individuals in populations of the

flowering plant *Datisca glomerata*, *Nature (Lond)*, 359 (1992) 633-636.

5 Kresovich S, Lamboy W F, Li R, Ren J, Szewc-Mcfadden A K *et al*, Application of molecular methods and statistical analysis for discrimination of accessions and clones of vetiver grass, *Crop Sci*, 34 (1994) 805-809.

6 Vos P, Hogers R, Bleeker M, Reijans M, Lee T V D *et al*, AFLP: A new technique for DNA fingerprinting, *Nucleic Acids Res*, 23 (1995) 4407-4414.

7 Xu X, Peng M, Fang Z & Xu X P, The direction of microsatellite mutations is dependent upon allele length, *Nat Genet*, 24 (2000) 396-399.

8 Williams J G K, Kubelik A R, Livak K J, Rafalski J A & Tingey S V, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, *Nucleic Acids Res*, 18 (1990) 6531-6535.

9 Sun G L, Salomon B & Bothmer R V, Characterization and analysis of microsatellite loci in *Elymus caninus (Triticeae: Poaceae)*, *Theor Appl Genet*, 96 (1998) 676-682.

10 Bered F, Barbosa-Neto J F, da Rocha B M & de Carvalho F I F, Genetic variability in common wheat based on morphological traits, coefficients of parentage and RAPDs, *J New Seeds*, 3 (2001) 73-87.

11 Taghian A S & Abo-Elwafa A, Multivariate and RAPD analyses of draught tolerance in spring wheat (*Triticum aestivum* L.), *Assiut J Agril Sci*, 34 (2003) 1-25.

12 Monte J-V, Casanova C & Soler C, Genetic variation in Spanish populations of the genus *Aegilops* revealed by RAPDs, *Agronomie*, 19 (1999) 419-427.

13 Wu X-H, Li Y-X, Song S-J & Guo P-Z, The RAPD marker analysis of wheat outbreeding population due to selection differentiation, *Hereditas (Beijing)*, 23 (2001) 33-37.

14 Zheng Y L, Chen Y O, Wei Y M, Zhou Y H & Zhang Z O, Esterase, gliadins and RAPD variations among Sichuan wheat (*Triticum aestivum*) cultivars, *Wheat Inf Serv*, 92 (2001) 5-8.

15 Maric S, Boloric S, Mortincic J, Pejic I & Kozumplik V, Genetic diversity of hexaploid wheat cultivars estimated by RAPD markers, morphological traits and coefficient of parentage, *Plant Breed*, 123 (2004) 366-369.

16 Tsuji K, Ohnishi O & Xiu P, Origin of cultivated Tatary buckwheat (*Fagopyrum tataricum* Gaertn.) revealed by RAPD analyses, *Genet Resour Crop Evol*, 47 (2000) 431-438.

17 Goriunova S V, Kochieva E Z, Chikida N N & Pukhal′skii V A, Phylogenetic relationships and intraspecific variation of D-genome *Aegilops* L. as revealed by RAPD analysis, *Genetika*, 40 (2004) 642-651.

18 Murray M G & Thompson W F, Rapid isolation of high molecular weight plant DNA, *Nucleic Acids Res*, 8 (1980) 4321-4325.

19 Yeh F C, Yang R C, Boyle T B J, Ye Z H & Mao J X, *POPGENE. The user friendly shareware for population genetic analysis* (Molecular Biology and Biotechnology Center, University of Alberta, Canada) 1997.

20 Rohlf F J, *NTSYSpc—Numerical taxonomy and multivariate analysis system*, version 2.02 (Exeter Publications, New York, USA) 1998.

21 Nei M, Analysis of gene diversity in subdivided populations, *Proc Natl Acad Sci USA*, 70 (1973) 3321-3323.

22 Sangwan R S & Sangwan N S, Metabolic analysis of oil-chemotypic diversity in *Cymbopogons*, in *Cymbopogons: The aromatic grass monograph*, edited by S Kumar, S Dwivedi, A K Kukreja, J R Sharma & G D Bagchi (CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India) 2000, 223-247.