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A COMPARATIVE ASSESSMENT OF MOLECULAR MARKER ASSAYS (AFLP AND RAPD) FOR *CYMBOPOGON* GERMPLASM CHARACTERIZATION

Nirupama Lal[1*] and S.K. Awasthi[1]

[1]Department of Lifesciences, Chhatrapati Shahu Ji Maharaj University Kanpur- 208024 UP, India.

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***Correspondence for Author**
Dr. Nirupama Lal
Chhatrapati Shahu Ji Maharaj University, Kanpur, Department of Lifesciences, Kanpur, U.P., India.

ABSTRACT

Several DNA-based marker systems are available for genetic fingerprinting of plants but information on their relative usefulness for characterization of *Cymbopogon* germplasm is lacking. The efficiency of RAPD and AFLP markers for the assessment of genetic relationships (intra- and interspecific variation), for cultivar identification and discrimination among eighteen *Cymbopogon* accessions belonging to eleven species, five varieties, one hybrid and one unidentified species were investigated. Dendograms were produced based on band patterns scores using UPGMA method. Results showed that each of the two techniques could unequivocally identify each accession but the techniques differed in the amount of polymorphism generated per primer (or primer pair) per accession. *Cymbopogon* genotypes classified in the same species status based on morphology or different species status based on chemical markers were often genetically different, emphasizing the need for molecular fingerprinting in *Cymbopogon* germplasm characterization. AFLPs showed the highest efficiency in detecting polymorphism and revealed genetic relationships that most closely reflected morphological and chemical classification.

Keywords: Cymbopogon; Genotyping; Genetic diversity; RAPD; AFLP; Molecular markers.

INTRODUCTION

The genus *Cymbopogon* (*Poaceae*) is known to include about 140 species. Among these, more than 52 have been reported to occur in Africa, 45 in India, six each in Australia and

South America, four in Europe, two in North America and the remaining are distributed in South Asia (Jagadish Chandra, 1975b). Most of these species produce characteristic aromatic essential oils that have commercial importance in perfumery, cosmetics and pharmaceutical applications. The *Cymbopogon* essential oils are characterised by monoterpene constituents like citral, citronellol, citronellal, linalool, elemol, 1,8-cineole, limonene, geraniol, β-carophyllene, methyl heptenone, geranyl acetate and geranyl formate. Citral is one of the important components of the oil present in several species of *Cymbopogon* with wide industrial uses such as raw material for perfumery, confectionery and vitamin A. After Sprengel named this genus in 1815, a number of taxonomists have attempted to classify the species of *Cymbopogon*. Hackel (1887) and Hooker (1897) have treated this genus as a subgenus of *Andropogon*. However, Stapf (1906) raised *Cymbopogon* to its original rank of a genus and this has been accepted by all the later taxonomists. Taxonomically, the species of *Cymbopogon* have been divided into three series viz. 'Shoenanthi', 'Rusae' and 'Citrati' (Stapf, 1906). The leaves of the species in 'Schoenathi' series are thin, in 'Rusae' subcordate and in 'citrati' lanceolate. The identification and classification of *Cymbopogon* species have become difficult because of the occurrence of numerous transitional forms which are supposed to have arisen by hybridization (Bor, 1960) and the existence of many varieties and races (Jagadish Chandra, 1975a). Some species, such as *Cymbopogon citratus*, are not known to flower, others like *Cymbopogon nardus* and *Cymbopogon winterianus* flower only rarely and the polyploid forms of *Cymbopogon flexuosus* and *Cymbopogon coloratus* flower, but do not set seed. The morphological variation and oil characteristics of various species and varieties of *Cymbopogon* have been reported (Husain, 1994), but such information is not sufficient to define precisely the relatedness among the morphotypes and chemotypes. For instance, *C. martinii* var. *sofia* and *C. martinii* var. *motia*, are morphologically almost indistinguishable, but show distinct chemotypic characteristics in terms of oil constituents (Guenther, 1950). Conversely, phenotypically and taxonomically well distinguishable species produce oils of almost identical chemical compositions. For example, *C. citratus* and *C. flexuosus* (Anonymous, 1988) produce lemongrass oil as major constituents. Such phenotypic traits, whether morphological or chemotypic are basically the phenotypic expression of the genotype, whereas, DNA markers being independent of environment, age and tissue are expected to reveal the genetic variation more conclusively in assessing such variations. Introgression of various traits, intermittent mutations and selection through human intervention may lead to variation in chemotypic characters across geographical distributions (Kuriakose, 1995). While natural hybridization may lead to the formation of morphological

or chemotypic intermediates, defining taxa purely on this basis may not be appropriate. The earlier works on phytochemical (Patra et al., 1990; Dhar et al., 1993), biochemical and physiological parameters (Nandi and Chatterjee, 1987), development of agrotechnology (Nair et al., 1979; Rao et al., 1985), genetic improvement (Ganguly et al., 1979; Maheshwari and Sethi, 1987) and breeding approaches (Kulkarni and Rajgopal, 1986; Rao and Sobti, 1987a; Shyalaraj and Thomas, 1993) of different *Cymbopogon* species and varieties appear scattered, and do not address the question of relationships among them. Molecular markers provide extensive polymorphism at DNA level and can be used for differentiating closely related genotypes (Pecchioni et al., 1996) and also to find out the extent of genetic diversity (Jain et al., 2003). DNA based markers such as Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) have been widely used in a number of plant groups for variety of purposes such as cultivar identification, diversity studies, parentage determination, developing breeding programmes and conservation strategies.

For several years, RAPD and AFLP have been found to be very useful in studying the phylogenetic relationship between plants, e.g., variation study among thirty four accessions of *Capsicum annum* (Paran et al., 1998) and nine cultivars of *Brassica campestris* (Das et al., 1999), diversity analysis in the different accessions of *Vetiveria zizanioides* (Lal et al., 2003), a comparative assessment of molecular marker assays (AFLP, RAPD and SSR) for white yam (*Dioscorea rotundata*) germplasm characterization (Mignouna et al., 2003), a comparative assessment of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines (Garcia et al., 2004), analysis of intra- and interspecific variation in some *Vigna* subgenus *Ceratotropis* (*Leguminosae*) species (Saravanakumar et al., 2004) and identification of inter- and intraspecific hybrids of *Mentha* (Shasany et al., 2005).

Earlier, Shasany et al., (2000) did RAPD analysis to trace the ancestors of cultivar *Java II* within *C. winterianus* and Khanuja et al (2005) investigated the extent of genetic diversity using RAPD analysis in relation to chemo-variation in oil constituents among nineteen different commercially and agronomically species of *Cymbopogon* in India. Considering the morphological, essential oil quality and diversity of the available germplasm in India, it is essential to asses the extent of diversity at genetic level for understanding the genetic base and reproductive behavior of the plant across the geographical regions. Also, such analysis can provide scope and methods that can be useful not only for conservation but also systematically breeding superior varieties in future. Approaches of RAPD followed by

selective AFLP analysis have been employed in this study to assess the gene bank accessions of *Cymbopogon* species available at Central Institute of Medicinal and Aromatic Plants (CIMAP) for diversity at genetic level.

In this study, the discriminatory power of AFLP fingerprinting has been compared with RAPD performed with protocols previously applied by Khanuja et al. (2005) for *Cymbopogon* species. Our preliminary study is aimed at investigating the possibility of *Cymbopogon* inter- and intra-species genotyping and evaluating the taxonomic potential of AFLP and RAPD in the delineation of species-specific identification.

MATERIALS AND METHODS

Plant material

Eighteen *Cymbopogon* accessions belonging to eleven species, five varieties, one hybrid and one unidentified species collected from the wild were included in this study. The representative accessions were collected either from the wild or received through the National Genebank in the genotype exchange programmes from other laboratories. These with individual identification are maintained in the National Genebank (field genebank) for medicinal and aromatic plants at Central Institute of Medicinal and Aromatic Plants (Sponsored by Department of Biotechnology, Government of India). The plants analysed in this study were also maintained in the glass house and are listed in Table-1. All the accessions concerned in the study were generally propagated vegetatively through slips except *C. martinii*.

DNA isolation and Polymerase Chain Reaction (PCR)

DNA was isolated from leaf tissue essentially according to the protocol described earlier (Khanuja et al., 1999). Polymerase chain reactions (PCRs) for RAPD analysis were carried out in 25 µl volume. Each reaction tube contained 25 ng of DNA, 0.2 unit of Taq DNA polymerase, 100 µM of each dNTPs, 1.5 mM $MgCl_2$ and 5 pmol of decanucleotide primers. The amplifications were carried out using the DNA Engine thermal cycler (MJ Research, USA) using 94°C, 35°C and 72°C temperatures for 40 cycles (Khanuja et al., 2000). The amplified products were separated on 1.2% agarose gel containing 0.5 μgml^{-1} of ethidium bromide and photographed with Image master VDS (Pharmacia). Custom-made decanucleotide primers were synthesised in the laboratory on Applied Biosystems 392 DNA–RNA Synthesizer and were designated as MAP01 to MAP20 (Table-2). Other two kits for commercial sets of primers were used were OPJ and OPT, each consisting of twenty random

decamer primers, (Operon Technologies, Alameda, CA, USA). The amplifications with primers generating the fragments were repeated thrice for all the accessions.

AFLP Analysis

Genomic DNA was digested with two restriction endonucleases, EcoRI and Tru9I (an isoschizomer of MseI), and double-stranded adaptors were ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplifications (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction tube (Vos et al., 1995). To carry out the reaction, an enzyme master mix for ten reactions was prepared containing 1 µl 10X T_4 DNA ligase buffer, 1 µl 0.5 M NaCl, 0.5 µl 1 mg/ml BSA, 1 µl Tru9I (10 U/µl), 4.25 µl EcoRI (12 U/µl), 0.5 µl T4 DNA ligase (20 U/µl high concentration), and 1.75 µl water. The restriction-ligation reaction consisted of 300 ng of DNA (5.5 µl), 1 µl 10X T_4 DNA ligase buffer, 1 µl 0.5 M NaCl, 0.5 µl 1 mg/ml BSA, 1 µl MseI adaptors (Applied Biosystems), 1 µl EcoRI adaptors (Applied Biosystems), and 1 µl enzyme master mix, as described above. The reaction mix was incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ (10 mM Tris and 0.1 mM EDTA) buffer. The ligated adaptors served as primer binding sites for low-level selection in preselective amplification of the restriction fragments. The MseI complementary primer had a 3'-C and the EcoRI complementary primer a 3'-A. Only the genomic fragments having an adaptor on each end amplified exponentially during PCR. The preselective amplification mixture was prepared by adding 4 µl of 20-fold diluted DNA from the restriction-ligation reaction, 0.5 µl AFLP preselective primer (EcoRI, Applied Biosystems), 0.5 µl AFLP preselective primer (MseI, Applied Biosystems), and 15 µl AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed at 72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, 60°C for 30 min, and finally incubated at 4°C. The preamplified DNA was diluted 20-fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out by using different MseI and EcoRI primer combinations (Applied Biosystems). Primers chosen for the amplifications were from 16 available AFLP selective primers (8 fluorescently labeled EcoRI primers and 8 unlabeled MseI primers). The EcoRI primers contained three selective nucleotides with the sequence 5'-[Dye-Primer-Axx]-3', while the MseI primers had the selective nucleotides starting with C, that is, 5'-[Primer-Cxx]-3'. The ''explorer'' gel for all 64 reactions was run with accession *C. nardus* (L.) Rendle var. *confertiflorous* (CIMAP/CN01) to determine the most responsive primer pairs (generating more number of fragments) for the *Cymbopogon* genome.

Multiplexing of PCRs was designed to set up all 64 (8 x 8) reactions in 24 tubes. For selective amplification, the reactions were set up as follows: 3 µl of 20-fold diluted preselective amplification reaction product, 15 µl AFLP core mix, 1 µl MseI primer 5'-[Primer-Cxx]-3', 1.5 µl EcoRI primers 5'-[Dye-Primer- Axx]-3' (0.5 µl of three EcoRI primers each were pooled here). Selective amplification was carried out in a thermal cycler programmed at 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 66°C (-1°C/cycle) for 30 s and 72°C for 2 min, and 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min with a subsequent hold for 30 min at 60°C and final incubation at 4°C. The samples were loaded on a 5% polyacrylamide gel on an ABI Prism 377 DNA sequencer (Applied Biosystems). The selective amplification reaction product (3 µl) was mixed with 4 µl of loading buffer (ROX500 size standard [10%], blue dextran [10%], deionized formamide [80%]) from which 1.5 µl was finally loaded on the gel. Five primer combinations—*Mse* I – CTA / *Eco* RI – ACT , *Eco* RI – AGG, *Eco* RI – AGC, *Mse* I – CTG / *Eco* RI – ACT , *Eco* RI – AGG, *Eco* RI – AGC, *Mse* I – CAC / *Eco* RI – ACA , *Eco* RI – AGG, *Eco* RI – AAG, *Mse* I – CAT / *Eco* RI – ACT , *Eco* RI – AGG, *Eco* RI – AGC, *Mse* I – CTT / *Eco* RI – ACT , *Eco* RI – AGG, *Eco* RI – ACC—generating more fragments than others, were chosen after analyzing the explorer gel. All the accessions were then subjected to selective amplification with these primer combinations. Selective amplification products obtained with primers labeled with three different fluorescent dyes were pooled together along with a loading buffer containing a size standard for loading, as stated above. For AFLP reactions, the AFLP amplification modules and the guidelines supplied by Applied Biosystems were used.

Data analysis

RAPD profiles were analysed by scoring the presence and absence of bands of different taxa. The AFLP profiles were analyzed using Gene-Scan analysis software (version 3.1; Applied Biosystems). For quantification of similarity, pairwise comparisons of banding patterns were made by calculating indices of similarity using the matching coefficient method of Nei and Li (1979). The average similarity matrix was used to generate a tree for cluster analysis by UPGMA (unweighted Pair Group Method with Arithmetic average) using NTSYS 2.1.

RESULTS AND DISCUSSSION

The *Cymbopogon* species have been divided into three series according to dominant essential oil components (Stapf. 1906). In the present analysis, most of the species are from series 'Citrati' except *C. martinii* and *C. caesius* which belong to 'Rusae' and *C. jwarancusa* to

series 'Schoenanthi'. Characteristic oil composition for long has acted as marker for each series. However, Khanuja et al. (2005) used RAPD analysis to study the extent of genetic diversity in relation to chemo-variation observed in different accessions. In the present study, comparative RAPD and AFLP analysis has further provided elaborate insights into variations among these accessions leading to refinements in the taxonomic understanding of *Cymbopogon*.

Random Amplified Polymorphic DNA (RAPD) analysis

All the eighteen accessions of the *Cymbopogon* species were analyzed at DNA level to assess the extent of molecular diversity and to compare the intra- and inter-specific similarity through RAPD. Using a set of sixty random decamer primers (20 each from MAP, OPJ, and OPT kits) a total of 150 amplification products were obtained of which 81.33% polymorphic, 8.00% monomorphic and 11.33% were unique bands (Table-3).

The graphic phenogram (Fig.1) obtained from average similarity matrix (Table-4) out grouped North-Indian lemongrass, *C. pendulus* (CP01) (2n=60) from rest of the accession of different species. East-Indian lemongrass *C. flexuosus* var. *flexuosus* (CF01) (2n=20, 40) and West-Indian lemongrass, *C. citratus* (CCi01) (2n=40, 60) (J. Christopher *et al.,* 1986) showing close similarity (57.1%) clustered together as the next out group. Thus, these two outgroups represented citral rich lemongrass accessions of series 'Citrati' (Jagadishchandra, 1975 and Stapf, 1906). Rest of the accessions was distributed into two subclusters. The accessions, *C. winterianus* Jowitt (CW01), *C. winterianus* var. *manjari* (CW02), *C. nardus* (L.) Rendle var. *nardus* (CN02) and *C. nardus* (L.) Rendle var. *Java II* (CN03) grouped together in one subcluster representing different varieties of *C. winterianus* and *C. nardus*. It represented 'Nardus complex' of series Citrati. In this subcluster, the accessions of *C. winterianus* Jowitt (CW01) was more closer to CN03 representing *C. nardus* (L.) Rendle var. *Java II* (76.1% similarity). The accession CN02 representing *C. nardus* (L.) Rendle var. *nardus* out grouped from the rest in this subcluster whereas, the accession *C. winterianus* var. *manjari* (CW02) was more similar to CW01, *C. winterianus* Jowitt. (71.7%) followed by *C. nardus* (L.) Rendle var. *Java II* (CN03) 66.3% and *C. nardus* (L.) Rendle var. *nardus* (CN02) (51%). Also, the similarity of *C. winterianus* Jowitt. (CW01) was more towards *C. nardus* (L.).

In the second subcluster, both the varieties of *C. martinii* showing close similarity with each other (68.8%) out grouped from the rest indicating a completely different genetic entity of

series 'Rusae'. Both the *C. caesius* accessions clustered together (similarity 70.8%) and were in the group containing *C. travancorensis*, CT01 and *C. flexuosus* var. *microstachy*, CF02 (similarity 77.1%). The accession *C. confertiflorus* (CCo01) clustered together with an unidentified species (CS01) showing the highest similarity (80.9%) which together grouped with *C. nardus* (L.) Rendle var. *confertiflorus* (CN01). The other accessions *C. jwarancusa* subsp. *jwarancusa* (CJ01) was found to be closer to the cymbopogon hybrid, *Jamrosa* (CH01) and were present in the same subcluster (similarity 73.8%).

The accessions *C. flexuosus* var. *flexuosus* (CF01) and *C. flexuosus* var. *microstachys* (CF02) grouped separately that is, *C. flexuosus* var. *flexuosus* (CF01) with *C. citratus* (CCi01) with a similarity of 57.1% and *C. flexuosus* var. *microstachys* (CF02) with *C. travancorensis* (CT01) with a similarity 77.1%. However, the similarity between *C. flexuosus* var. *flexuosus* (CF01) and *C. flexuosus* var. *microstachy* (CF02) was 3.3% indicating two completely different genotype with similar species name.

Amplified Fragment Length Polymorphism (AFLP) analysis

All the eighteen accessions analyzed for diversity by RAPD analysis were also subjected to detailed AFLP analysis with a view to further refine the taxonomic position of different accesssions. In this analysis a total of 113 unique (Table-5) and 291 monomorphic (Table-6) fragments were obtained. The similarity indices (Table-7) generated based on analysis of electro-phenogram showed significant diversity (47.1 to 97.7%) among the accessions. In the first cluster (Fig. 2), accessions of *C. winterianus* (CW01 and CW02) grouped together with *C. nardus* (L.) Rendle (CN02 and CN03). In the first cluster, accessions *C. winterianus* Jowitt. (CW01) and *C. winterianus* var. *manjari* (CW02) formed one subcluster with a similarity of 36.8% and *C. nardus* (L.) Rendle var. *Java II* (CN03) and *C. nardus* (L.) Rendle var. *nardus* (CN02) sharing 41.7% similarity grouped into another subcluster. *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) paired with *C. confertiflorus* (CCo01) showing higher similarity of 52.9%. *C. jwarancusa* subsp. *jwarancusa* (CJ01) and *Jamrosa* (CH01) grouped together with a similarity of 50%. In this subcluster, the unidentified species (CS01) outgrouped from the rest but, was associated with *C. nardus* (L.) Rendle var. *confertiflorus* (CN01), *C. confertiflorus* (CCo01), *C. jwarancusa* subsp. *jwarancusa* (CJ01) and *Jamrosa* (CH01). *C. travancorensis* (CT01) and *C. flexuosus* var. *microstachys* (CF02) grouped together with a similarity of 50% and both the varieties of *C. caesius* (CCa01 and CCa02) grouped together with a similarity of 45%. *C. martinii* var. *motia* (CM01) and *C. martinii*

var. *sofia* (CM02) sharing a similarity of 40% outgrouped in a different cluster. In the similar way, *C. flexuosus* var. *flexuosus* (CF01) paired with *C. citratus* (CCi01) with a similarity of 32% grouped together with an outgrouping of *C. pendulus* (CP01).

The results obtained from both RAPD and AFLP in the present genetic analysis suggests a separate larger cluster of 'Citrati' genotypes interrupted by 'Rusae' (*C. caesius*) and 'Schoenanthi' (*C. jwarancusa* subsp. *jwarancusa*) indicating similar or related genotypes producing different essential oil components. Though *vis-à-vis* correlation is not apparent between the genetic dendrogram and the cluster based on oil constituents (Khanuja, 2005), the combined data sets provide more insights to the relationship of *Cymbopogon* species studied. The species *C. flexuosus* var. *flexuosus* (CF01) and *C. citratus* (CCi01) together form a distinct cluster in both the types of analyses (RAPD and AFLP). Both of these species exhibit similar morphology, 'citral' rich oil content (more than 45%) and similarity in their geographical distribution substantiating the above distinctiveness. The other 'citral' rich species are *C. pendulus* (CP01) and *Cymbopogon* unidentified species (CS01) showed genetic similarity of about 13.3% (of CP01 with CF01) and 26% (of CP01 with CCi01) and 57.1% (of CS01 both with CF01 and CCi01) respectively in RAPD and 26% (of CP01 with CF01) and 30% (of CP01 with CCi01) and 2.9% (of CS01 with CF01) and 18.8% (of CS01 with CCi01) respectively in AFLP. So, in this analysis, *C. pendulus* (CP01) was found to be the most diverse species.

Also, in this RAPD and AFLP analyses, *C. martinii* varieties combined to form a single sub-cluster and distinct from the rest. A genetic similarity of 68.8% in RAPD and 40% in AFLP was observed between these two species in spite of a higher degree of diversity in oil constituents (64%, Khanuja *et al*., 2005). The elite variety *motia* which has high economic value possess higher geraniol (75%) content than var. *sofia* (12%) in their essential oil. These changes in chemotypic characters may be attributed to habitat and ploidy levels since *C. martinii* var. *sofia* is a tetraploid as compared to the diploid *C. martinii* var. *motia* (Rao and Sobti, 1987a). Variety *sofia* is reported to be developed as tetraploid form of *C. caesius* (Narayana *et al.,* 1966) having karyotypic and chemotypic similarity (Rao and Sobti, 1976b).
In spite of being in the same series, present analysis shows that *C. caesius* var. narrow leaf (CCa01) has similarity of only 45.7% (with CM01) and 37.4% (with CM02) whereas; *C. caesius* var. broad leaf (CCa02) shows similarity of 62.4% (with CM01) and 58% (with CM02) in RAPD. But, in AFLP, CCa01 shows 29.8% (with CM01) and 30.1% (with CM02)

similarity and CCa02 shows 15.9% (with CM01) and 11.4% (with CM02) similarity. However, only 39-44% similarity in oil constituents of *C. caesius* (CCa01 and CCa02) with *C. martinii* (var. *motia* and *sofia*) has been observed (Khanuja *et al*., 2005). Though the origin of species and phenotypic expression do not support the species status, the present analyses comprising of RAPD and AFLP variability along with essential oil constituents (Khanuja *et al*., 2005) do support the separate species status of these varieties as proposed by Rao and Sobti (1987b). Moreover, the diversity between these two species is much higher (46%) compared to the diversity among other species, where they share higher genetic similarity.
The distribution of *C. witerianus* and *C. nardus* in the first cluster was much more informative in AFLP analysis compared to the RAPD analysis as the accessions *C. winterianus* Jowitt. (CW01) and *C. winterianus* var. *manjari* (CW02) forming one subcluster with a similarity of 36.8% was clearly different from the subcluster of *C. nardus* (L.) Rendle var. *Java II* (CN03) and *C. nardus* (L.) Rendle var. *nardus* (CN02) shared 41.7% similarity between them and grouped into another subcluster. *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) paired with *C. confertiflorus* (CCo01) showing higher similarity of 52.9% and formed a subcluster unlike RAPD analysis (Fig. 2), where *C. confertiflorus* (CCo01) was much more similar to CS01, the unidentified species than to *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) (Fig.1). But, both the cluster analysis (RAPD and AFLP) indicated closer association between *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) and *C. confertiflorus* (CCo01). Shasany *et al*., 1999 had described the evolution of *C. winterianus* Jowitt. (CW01) from *C. confertiflorus* (CCo01) through *C. nardus* (L.) Rendle. Rendle. var. *Java II*, CN03. In this study also, the similarity of C. winterianus Jowitt. (CW01) was more towards C. nardus (L.) Rendle. var. Java II, (CN03) (with a similarity of 76.1% in RAPD and 31.6% in AFLP) compared to *C. nardus* (L.) Rendle var. *nardus*, CN02 (where a similarity of 52.1% in RAPD and 29.9% in AFLP) indicating the development of *C. winterianus* Jowitt. (CW01) through *C. nardus* (L.) Rendle. var. *JavaII* (CN03). As described earlier (Khanuja *et al*., 2005), *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) was also found to be similar to *C. confertiflorus* (CCo01) with a similarity of 68% in RAPD and 52.9% in AFLP and merger of these two varieties had been suggested.

Sobti *et al*., (1981), had reported the geraniol rich '*Jamrosa*' (CH01) is a hybrid between *C. jwarancusa* subsp *jwarancusa* (CJ01) of series 'Schoenanthi' and *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) of series 'Citrati'. They share 73.8% and 50% genetic similarity in RAPD and AFLP analysis respectively with the hybrid.

The RAPD and AFLP analysis also clarify some taxonomic disputes in *Cymbopogon* species. The elevation of a variety *C. flexuosus* var. *microstachys* (CF02) to a species status (Soenarko, 1977) has been substantiated as a distinct subcluster in the phylogeny. *C. travancorensis* (CT01), a recently described species was considered as a variant of *C. flexuosus* var *flexuosus* (CF01) (Karthikeyan *et al*., 1989) formed distinct cluster quite away from *C. flexuosus* var *flexuosus* (CF01) with 94% and 77.3% diversity in RAPD and AFLP respectively. Though these two species show maximum similarity in the morphology except the color of the stem (Bor, 1960), the genetic and essential oil constituent diversity (Khanuja *et al*., 2005) do support the separate species status of the endemic species (*C. travancorensis*, CT01). Also, the species *C. travancorensis* (CT01) has a very low (4%) citral content in the essential oil (Khanuja *et al*., 2005) compared to *C. flexuosus* var *flexuosus* (CF01) and distantly placed from the main cluster. The oil profile (Khanuja *et al*., 2005) of this species seems very similar to *C. nardus* (L.) Rendle var. *Java II* (CN03).

Of particular interest is the number of species reported by different authors in the genus *Cymbopogon* which varies from 55 to 140 (Bor, 1960). These differences may be due to the presence of morphologically similar species, with distinct chemical profiles considered as single species or formation of a number of natural hybrids or occurrence of a number of varieties in a single species identified as different species (Rao and Sobti, 1987a). Present study resolves some of the issues in this context. For example, morphologically similar varieties of *C. martinii* (var. *motia* and var. *sofia*) are diverse genetically and chemotypically, and these two varieties may be recognized as two different species as proposed by Rao and Sobti (1987b). In another instance, *C. travancorensis* (CT01), a species described recently and later considered conspecific to *C. flexuosus* var *flexuosus* (CF01) (Soenarko, 1977), formed a distinct group in the present analysis, indicating the necessity of a separate identity for this endemic species, so that it can be conserved in its natural habitat in order to maintain the original genotypes *in situ*. The elevation of *C. flexuosus* var. *microstachys* (CF02) to a new species *C. microstachys*, was also substantiated in this study. In addition, *C. confertiflorus* (CCo01) and *C. nardus* (L.) Rendle var. *confertiflorus* (CN01) were found to be similar and we propose merger of these two species into one, as *C. confertiflorus* (CCo01).

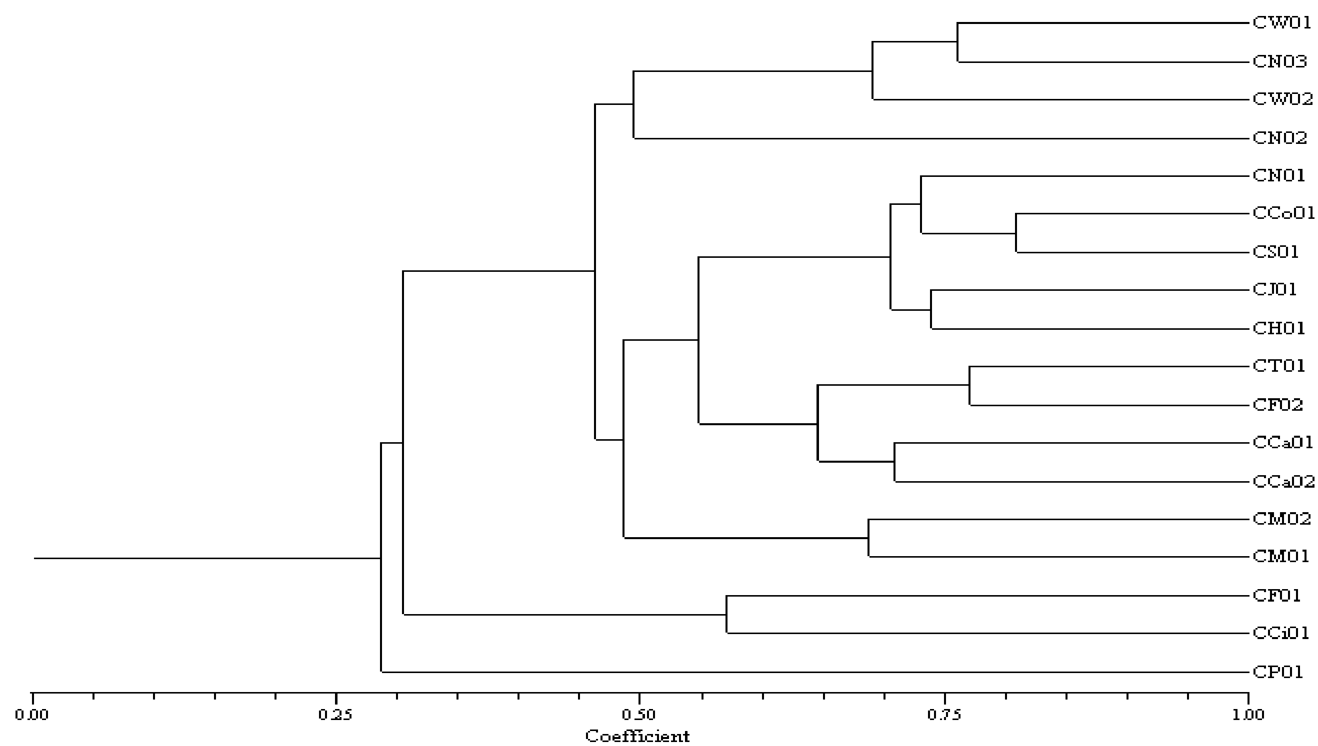

Fig. 1: Cluster diagram showing RAPD analysis of eighteen accessions of *Cymbopogon* species.

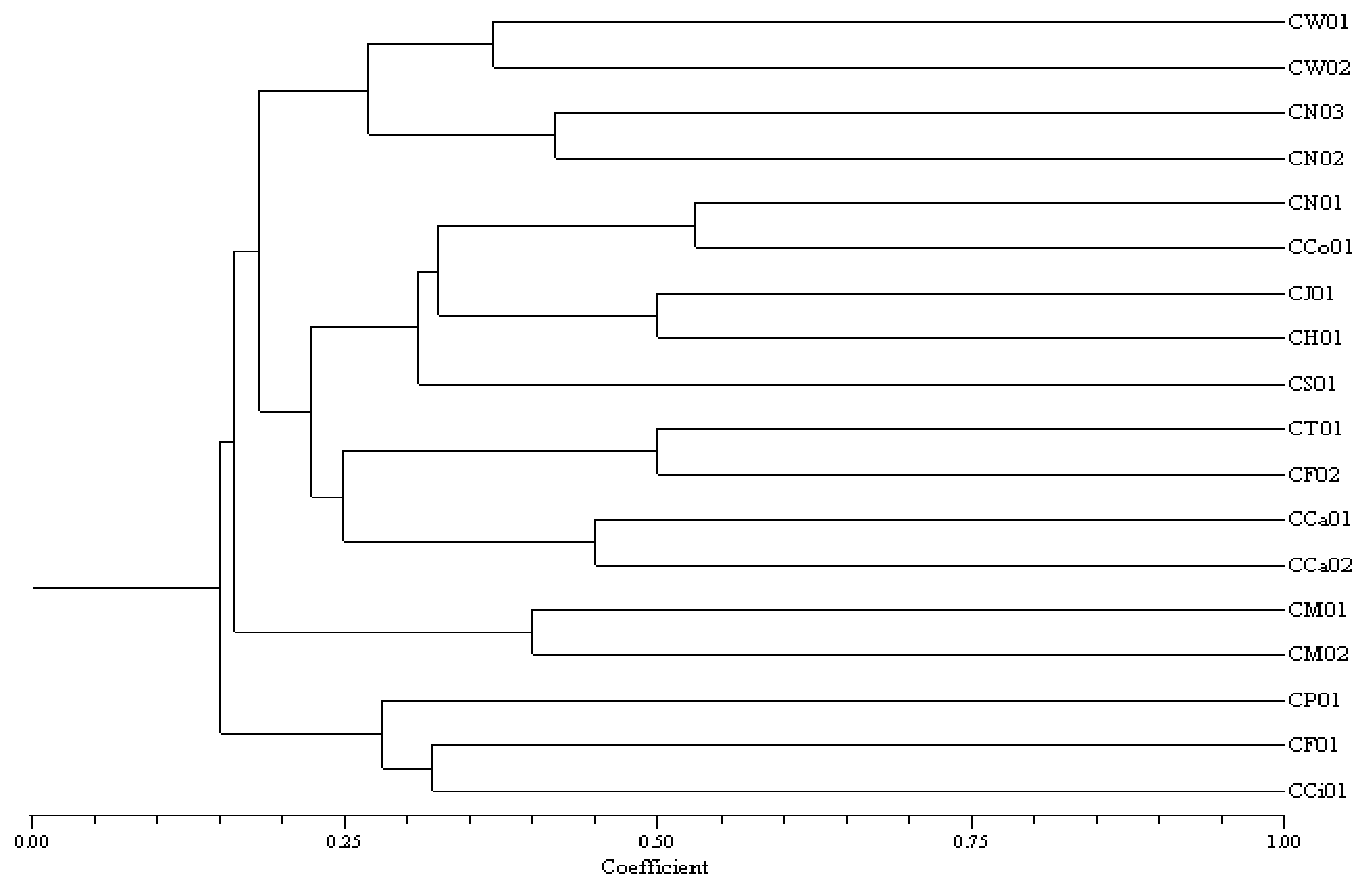

Fig. 2: Cluster diagram showing AFLP analysis of eighteen accessions of *Cymbopogon* species.

Table-1: Sourcing and characteristics of *Cymbopogon* accessions investigated.

S. No.	Accession(s)	Name of species	Taxonomic series (Stapf, 1906)	2n Chrom. no.	Refs.
1.	CIMAP/CCa01	*Cymbopogon caesius* (Nees) var. narrow leaf.	Rusae	20	3
2.	CIMAP/CCa02	*Cymbopogon caesius* (Nees) var. broad leaf.	Rusae	20	3
3.	CIMAP/CCi01	*Cymbopogon citratus* (D.C.) Stapf.	Citrati	60	3
4.	CIMAP/CCo01	*Cymbopogon confertiflorous* (Steud.) Stapf.	Citrati	20	3
5.	CIMAP/CF01	*Cymbopogon flexuosus* (Steud.) Wats. var. *flexuosus.*	Citrati	20, 40	3
6.	CIMAP/CF02	*Cymbopogon flexuosus* (Steudel) Wats. var. *microstachys* (Hook f.) Bor.	Citrati	20	11
7.	CIMAP/CH01	*Jamrosa*	-	-	-
8.	CIMAP/CJ01	*Cymbopogon jawarancusa* (Jones) Schult.subsp. *jawarancusa*	Schoenanthi	20	3
9.	CIMAP/CM01	*Cymbopogon martinii* (Roxb.) Wats. var. *motia* (B.K. Gupta).	Rusae	20	47
10.	CIMAP/CM02	*Cymbopogon martinii* (Roxb.) Wats. var. *sofia* (B.K.Gupta) .	Rusae	40	47
11.	CIMAP/CN01	*Cymbopogon nardus* (L.) Rendle var. *confertiflorous* (Steud.) Stapf..	Citrati	20	11
12.	CIMAP/CN02	*Cymbopogon nardus* (L.) Rendle var. nardus.	Citrati	20	11
13.	CIMAP/CN03	*Cymbopogon nardus* (L.) Rendle var. *Java II.*	Citrati	-	-
14.	CIMAP/CP01	*Cymbopogon pendulus* (Nees ex Steud.) Wats.	Citrati	60	11

15.	CIMAP/CS01	Unidentified *Cymbopogon species*	-	-	-
16.	CIMAP/CT01	*Cymbopogon travancorensis* Bor.	Citrati	20	11
17.	CIMAP/CW01	*Cymbopogon winterianus* Jowitt.	Citrati	40, 20	3, 11
18.	CIMAP/CW02	*Cymbopogon winterianus* var. *manjari.*	Citrati	-	-

Table-2: Primers synthesized in the laboratory.

Primers	Sequences
MAP01	5' AAATCGGAGC 3'
MAP02	5' TGCGCGATCG 3'
MAP03	5' GTCCTACTCG 3'
MAP04	5' GTCCTTAGCG 3'
MAP05	5' AACGTACGCG 3'
MAP06	5' GCACGCCGGA 3'
MAP07	5' CACCCTGCGC 3'
MAP08	5' CTATCGCCGC 3'
MAP09	5' CGGGATCCGC 3'
MAP10	5' GCGAATTCCG 3'
MAP11	5' CCCTGCAGGC 3'
MAP12	5' CCAAGCTTGC 3'
MAP13	5' GTGCAATGAG 3'
MAP14	5' AGGATACGAG 3'
MAP15	5' AAGATAGCGG 3'
MAP16	5' GGATCTGAAC 3'
MAP17	5' TTGTCTCAGG 3'
MAP18	5' CATCCCGAAC 3'
MAP19	5' GGACTCCACG 3'
MAP20	5' AGCCTGACGC 3'

Table-3: Fragments generated through RAPD analysis of eighteen different accessions of *Cymbopogon* species.

Primers	No. of Polymorphic fragments	No. of Monomorphic fragments	No. of Unique fragments
MAP	45	5	1
OPJ	37	3	6
OPT	40	4	10
Total	122	12	17

Table- 4: Average similarity matrix generated in RAPD analysis of eighteen different accessions of *Cymbopogon* species.

	CCa01	CCa02	CCi01	CCo01	CF01	CF02	CH01	CJ01	CM01	CM02	CN01	CN02	CN03	CP01	CS01	CT01	CW01	CW02
CCa01	1.000																	
CCa02	0.708	1.000																
CCi01	0.037	0.468	1.000															
CCo01	0.352	0.660	0.534	1.000														
CF01	0.056	0.000	0.571	0.071	1.000													
CF02	0.762	0.621	0.427	0.540	0.033	1.000												
CH01	0.598	0.657	0.491	0.722	0.600	0.582	1.000											
CJ01	0.355	0.628	0.524	0.633	0.054	0.528	0.738	1.000										
CM01	0.457	0.624	0.414	0.548	0.500	0.452	0.460	0.489	1.000									
CM02	0.374	0.580	0.347	0.542	0.500	0.505	0.406	0.500	0.688	1.000								
CN01	0.341	0.559	0.506	0.680	0.054	0.511	0.685	0.731	0.523	0.439	1.000							
CN02	0.236	0.528	0.442	0.580	0.023	0.505	0.589	0.426	0.473	0.472	0.510	1.000						
CN03	0.193	0.462	0.382	0.469	0.041	0.539	0.587	0.473	0.445	0.444	0.506	0.461	1.000					
CP01	0.200	0.440	0.260	0.225	0.133	0.363	0.490	0.203	0.327	0.403	0.225	0.187	0.155	1.000				
CS01	0.524	0.626	0.552	0.809	0.571	0.590	0.742	0.724	0.505	0.464	0.781	0.540	0.555	0.450	1.000			
CT01	0.541	0.659	0.496	0.553	0.060	0.771	0.680	0.549	0.450	0.442	0.454	0.465	0.347	0.290	0.668	1.000		
CW01	0.338	0.483	0.431	0.517	0.000	0.452	0.487	0.489	0.316	0.381	0.476	0.521	0.761	0.147	0.483	0.418	1.000	
CW02	0.336	0.537	0.508	0.518	0.000	0.457	0.497	0.530	0.374	0.428	0.543	0.502	0.663	0.380	0.512	0.431	0.717	1.000

Table-5: Molecular weight of the unique fragments generated in AFLP analysis of eighteen different accessions of *Cymbopogon* species.

Acc. No.	Set A			Set B			Set C			Set D			Set E		
	E-ACT M-CTA	E-AGG M-CTA	E-AGC M-CTA	E-ACT M-CTG	E-AGG M-CTG	E-AGC M-CTG	E-ACA M-CAC	E-AGG M-CAC	E-AAC M-CAC	E-ACT M-CAT	E-AGG M-CAT	E-AGC M-CAT	E-ACT M-CTT	E-AGG M-CTT	E-ACC M-CTT
CCa01	--	186	340	--	--	--	--	--	--	213	444	--	--	--	--
CCa02	--	--	--	--	--	--	--	--	170, 329.	--	--	--	--	--	463
CCi01	--	--	378, 441.	--	151	175, 403, 405, 452.	152, 454.	--	--	--	--	279, 287, 572.	--	--	--
CCo01	--	528, 596	314	--	--	--	--	--	--	--	--	--	--	--	--
CF01	174	--	--	--	475	648	--	--	261	346, 402.	--	494, 651.	--	--	--
CF02	--	--	--	--	--	545	--	--	--	--	--	--	--	--	--
CH01	694	--	--	--	192	251	221	--	--	--	--	--	--	--	--
CJ01	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CM01	351	447, 569.	312	--	225	576	322, 324, 326, 329.	309, 323, 325, 435, 437.	168, 348, 491, 591.	--	--	--	--	100	299
CM02	173, 312	210	479	--	--	179, 233, 319, 620.	140, 427.	571	110, 212.	586	--	--	314	145, 338, 567.	208, 363, 531, 632.
CN01	692	575	--	--	--	--	--	--	--	--	575	--	--	--	--
CN02	--	--	--	--	--	124	407	--	--	--	--	--	--	--	--
CN03	645	--	--	--	149, 731, 759.	283, 799.	--	--	--	--	--	--	--	--	--
CP01	348	103, 118, 270.	300	--	--	--	154, 423.	416, 644.	278	--	262, 365, 446.	--	--	--	549
CS01	--	--	385	568	--	157, 324, 624.	--	--	--	--	--	--	--	--	--
CT01	114	--	412	--	--	--	--	--	--	--	356	--	--	--	--
CW01	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CW02	399	--	307	--	--	--	--	--	258	--	675	--	--	--	--
Total	10	10	10	01	07	18	12	08	11	04	07	05	01	03	07

Table-6: Molecular weight of the monomorphic fragments generated in AFLP analysis of eighteen different accessions of *Cymbopogon* species.

Acc. No.	Set A			Set B			Set C			Set D			Set E		
	E-ACT M-CTA	E-AGG M-CTA	E-AGC M-CTA	E-ACT M-CTG	E-AGG M-CTG	E-AGC M-CTG	E-ACA M-CAC	E-AGG M-CAC	E-AAC M-CAC	E-ACT M-CAT	E-AGG M-CAT	E-AGC M-CAT	E-ACT M-CTT	E-AGG M-CTT	E-ACC M-CTT
CCa01 CCa02 CCi01 CCo01 CF01 CF02 CH01 CJ01 CM01 CM02 CN01 CN02 CN03 CP01 CS01 CT01 CW01 CW02	85, 97, 108, 116, 124, 137, 142, 148, 157, 179, 194, 224, 244, 249, 262, 290, 323, 335, 365, 386, 449.	108, 154, 194, 282, 296, 312, 325, 338, 345, 359, 365, 381, 452.	84, 113, 129, 143, 153, 157, 169, 190, 259, 344, 394, 444.	71	69, 72, 81, 85, 91, 98, 123, 132, 141, 168, 178, 196, 210, 228, 243, 311, 330, 344, 391, 426.	57, 65, 133, 139, 181, 300, 331, 373, 420, 425.	56, 65, 72, 79, 87, 93, 99, 105, 117, 121, 155, 162, 173, 177, 188, 192, 199, 204, 208, 261, 268, 308, 315, 349, 358, 412, 439, 444, 458, 563, 566, 576, 794.	62, 71, 77, 107, 146, 280, 301, 390, 446, 467, 497, 551, 585.	57, 59, 66, 71, 104, 116, 125, 140, 187, 197, 207, 216, 225, 235, 248, 270, 296, 317, 323, 357, 407, 419, 429, 501, 510, 533, 555, 609.	58, 73, 80, 88, 95, 108, 117, 129, 143, 146, 171, 185, 195, 216, 223, 233, 247, 258, 300, 308, 317, 354, 364, 368, 375, 395, 404, 440, 445, 462, 476, 494, 603, 608, 610, 649.	63, 71, 90, 98, 128, 133, 149, 179, 206, 216, 228, 239, 250, 255, 265, 273, 292, 299, 304, 310, 318, 326, 344, 350, 367, 375, 388, 397, 436, 497, 521, 536, 551.	61, 71, 81, 106, 115, 167, 180, 209, 219, 246, 256, 260, 270, 295, 323, 331, 456.	--	61, 63, 70, 103, 168, 171, 197, 206, 210, 237, 240, 368, 379, 421, 438, 453, 544, 548.	63, 77, 88, 100, 110, 119, 126, 133, 136, 153, 163, 172, 189, 195, 204, 223, 229, 239, 257, 286, 292, 315, 321, 350, 367, 379, 399, 414, 427, 456, 517, 519, 529, 556, 596, 617.
Total	21	13	12	01	20	10	33	13	28	36	33	17	00	18	36

Table-7: Average similarity matrix generated from AFLP analysis of eighteen different accessions of *Cymbopogon* species.

	CCa01	CCa02	CCi01	CCo01	CF01	CF02	CH01	CJ01	CM01	CM02	CN01	CN02	CN03	CP01	CS01	CT01	CW01	CW02
CCa01	1.000																	
CCa02	0.450	1.000																
CCi01	0.090	0.158	1.000															
CCo01	0.186	0.213	0.160	1.000														
CF01	0.100	0.155	0.320	0.309	1.000													
CF02	0.255	0.238	0.228	0.133	0.211	1.000												
CH01	0.122	0.345	0.267	0.305	0.245	0.243	1.000											
CJ01	0.173	0.255	0.218	0.311	0.320	0.183	0.500	1.000										
CM01	0.298	0.159	0.104	0.222	0.181	0.099	0.174	0.238	1.000									
CM02	0.301	0.114	0.086	0.132	0.117	0.157	0.120	0.173	0.400	1.000								

CN01	0.200	0.238	0.166	0.529	0.262	0.167	0.365	0.319	0.211	0.161	1.000						
CN02	0.178	0.104	0.161	0.197	0.117	0.155	0.207	0.187	0.127	0.123	0.308	1.000					
CN03	0.120	0.087	0.159	0.182	0.133	0.118	0.204	0.139	0.079	0.107	0.267	0.417	1.000				
CP01	0.094	0.114	0.300	0.023	0.260	0.065	0.082	0.037	0.198	0.114	0.074	0.202	0.153	1.000			
CS01	0.121	0.198	0.188	0.282	0.182	0.164	0.317	0.314	0.243	0.109	0.318	0.229	0.229	0.029	1.000		
CT01	0.298	0.201	0.147	0.393	0.227	0.500	0.191	0.499	0.222	0.107	0.344	0.195	0.181	0.101	0.080	1.000	
CW01	0.134	0.087	0.113	0.181	0.134	0.130	0.159	0.145	0.113	0.089	0.276	0.299	0.316	0.147	0.174	0.225	1.000	
CW02	0.150	0.147	0.131	0.249	0.194	0.180	0.172	0.240	0.184	0.140	0.231	0.230	0.228	0.069	0.197	0.146	0.368	1.000

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REFERENCES

1. Anonymous (1988) In: Ambasta, S.P. (Ed.), Wealth of India, vol. 2. Publication and Information Directorate, CSIR, Hillside Road, New Delhi-110012 (India).
2. Bor NL (1960). The Grasses of Burma, Ceylon, India and Pakistan. Pergamon Press, London. Christopher, J., Sam Raj, P. Karyomorphological and phylogenetic studies of Cymbopogon species from India. J. Cytol. Genet, 1986; 21: 21-34.
3. Christopher J, Sam Raj R Karyomorphological and phylogenetic studies of *Cymbopogon species* from India. J Cytol. and Genet, 1986; 21: 21-34.
4. Das S, Rajagopal J, Bhatia S, Srivastava PS and Lakshmikumaran M (1999). Assessment of genetic variation within *Brassica campestris* cultivars using amplified length polymorphism and random amplification of polymorphic DNA markers. J. Biosc.
5. Dhar AK, Sapru R, Lattoo KS Changes in oil concentration and its constituents, herbage and oil yield in five genotypes of Cymbopogon jwarancusa following foliar application of nitrogen. Indian Perfumer, 1993; 37: 303–310.
6. Ganguly D, Singh KK, Bhagat SD, Upadhyay DN, Chauhan YS, Gupta NK, Singh HS ‘‘RRLJOR-3-1970’’ an improved strain of Java Citronella (Cymbopogon winterianus Jowitt.). Indian Perfumer, 1979; 23: 107–111.
7. Garcia AAF, Benchimol LL, Barbosa AMM, Geraldi IO, Jr. CLS, Souza AP Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Genetics and Mol. Biol, 2004; 27(4): 579-588.
8. Guenther E 91950) The Essential Oils, vol. IV. Van Nostrand, New York.
9. Gupta BK, Jain N Cultivation and Utilization of the genus *Cymbopogon* in India. Indian Perfumer, 1978; 22(2): 55-68.
10. Gupta BK The role of vivpary in the genus *Cymbopogon*. *Curr. Sci,* 1969; 576-577.
11. Gupta B. K., 1970. Studies in the genus *Cymbopogon* Spreng. III. Contribution to the cyto-geography of some Indian species of *Cymbopogon. Proc. Indian Acad. Sci., B, Vol. LXXI, Pl. II.,* 4-13.

12. Hackel E (1887) Graminae in Engler and Prantl Pflanzen Familien Teil II. Anterling 2. Leipzig.
13. Hooker JD (1897) Graminae. Flora of British India, vol. VII. Reeve and Co. Ltd, England.
14. Husain A (1994) Essentials Oil Plants and Their Cultivation. Central Institute of Medicinal and Aromatic Plants, Lucknow, India, pp. 39–48.
15. Ilan P, Ester A, Chen S Variation in *Capsicum annuum* revealed by RAPD and AFLP markers. Euphytica, 1998; 99: 167-173.
16. Jagadish Chandra KS (1975a) Recent studies on Cymbopogon Spreng. With special reference to Indian Taxa. J. Plant Crops 3: 1–5.
17. Jagadish Chandra KS (1975b) Cytogenetical Evolution in Some Species of Cymbopogon Cited in Advancing Frontiers in Cytogenetics. In: Kachroo, P. (Ed.). Hindustan publ. Corp., New Delhi.
18. Jagadish Chandra KS Recent studies on Cymbopogon Spreng. (Aromatic grasses) with special reference to Indian taxa: Taxonomy, Cytogenetics, Chemistry and Scope. Jour. Plantation Crops, 1975; 3(2): 1-5, 43-57.
19. Jain N, Shasany AK, Sundaresan V, Rajkumar S, Darokar MP, Bagchi GD, Gupta AK, Kumar S, Khanuja SPS Molecular diversity in Phyllanthus amarus assessed through RAPD analysis. Curr. Sci, 2003; 85: 1454–1458.
20. Karthikeyan S, Jain SK, Nayar MP, Sanjappa M (1989) Florae Indicae Enumeratio: Monocotyledonae. BSI, Calcutta.
21. Khanuja SPS, Shasany AK, Darokar MP, Kumar S Rapid isolation of PCR amplifiable DNA from the dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils by modified CTAB procedure. Plant Mol. Biol. Report, 1999; 17: 74.
22. Khanuja SPS, Shasany AK, Pawar A, Lal RK, Darokar MP, Naqvi AA, Rajkumar S, Sundaresan V, Lal N, Kumar S. Essential oil constituents and RAPD markers to establish species relationship in *Cymbopogon* Spreng. (Poaceae). Biochem. System. and Eco, 2005; 33: 171-186.
23. Khanuja SPS, Shasany AK, Srivastava A, Kumar S Assessment of genetic relationships in Mentha species. Euphytica, 2000; 111: 121–125.
24. Kulkarni RN, Rajgopal K Broad and narrow sense heritability estimates of leaf yield, leaf width, tiller number and oil content in east Indian lemongrass. J. Plant Breed. Berlin, 1986; 96: 135–139.

25. Kuriakose KP Genetic variability in East Indian lemongrass (Cymbopogon flexuosus Stapf). Indian Perfumer, 1995; 39(2): 76–83.
26. Lal N, Shasany AK, Lal RK, Darokar MP, Rajkumar S, Sundaresan V, Khanuja SPS Diversity analysis of vetiver (Vetiveria zizanioides) gene bank accessions using RAPD and AFLP analysis. J. Med. and Arom. Plant Scs, 2003; 23: 25-32.
27. Maheshwari ML, Sethi KL Selection and improvement in palmarosa. Indian Perfumer, 1987; 31: 17–32.
28. Mignouna HD, Abang MM, Fagbemi SA A comparative assessment of molecular marker assays (AFLP, RAPD and SSR) for yam (*Dioscorea rotundata*) germplasm characterization. Amm. appl. Biol, 2003; 142: 269-276.
29. Nair EGV, Nair KC, Chinnamma NP Field experiments with micronutrients on yield of grass and oil and citral content of east Indian lemongrass, *Cymbopogon flexuosus* variety OD-9. Indian Perfumer, 1979; 23: 55–58.
30. Nandi RP, Chatterjee SK Effect of gibberlic acid on growth, development and essential oil formation in Cymbopogon winterianus jowitt. Indian Perfumer, 1987; 31: 72–77.
31. Narayan KN, Jagadishchandra KS Cytological studies in the species of *Cymbopogon* from South India. Journ. Mysore Univ, 1966; 20: 66-72.
32. Nei N, W Li Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci U.S.A., 1979; 76: 5269-5273.
33. Patra NK, Srivastava RK, Chauhan SP, Ahmed A, Mishra LN Chemical features and productivity of a geraniol rich variety (GRL 1) of *Cymbopogon flexuosus*. Planta Med, 1990; 56: 239–240.
34. Pecchioni N, Faccioli P, Monetti A, Stanca AM, Terzi V Molecular markers for genotype identification in small grain cereals. J. Genet. Breed, 1996; 50: 203–219.
35. Rao BL, Sobti, SN Species status for *C. martinii* var. *motia* and *C. martinii* var. *sofia*. Indian Perfumer, 1987b; 31: 48-53.
36. Rao BL, Sobti SN Breeding of a high oil yielding lemongrass for flavour industry. Indian Perfumer, 1987a; 31: 32.
37. Rao EVSP, Singh M, Rao RSG Effect of NPK fertilizers on yield and nutrient uptake in lemongrass. Indian J. Trop. Agric, 1985; 3: 123–127.
38. Saravanakumar P, Kaga A, Tomooka N, Vaughan DA AFLP and RAPD analyses of intra- and interspecific variation in some *Vigna* subgenus *Ceratotropis* (Leguminoseae) species. Aus. J. Bot, 2004; 52(3): 417-424.

39. Shasany AK, Lal RK, Patra NK, Darokar MP, Garg A, Kumar S, Khanuja SPS Phenotypic and RAPD diversity among *Cymbopogon winterianus* Jowitt. accessions in relation to *Cymbopogon nardus* Rendle. Genetic Resources and Crop Evolution, 1999-2000; 47: 553-559.
40. Shasany AK, Darokar MP, Dhawan S, Gupta AK, Gupta S, Shukla AK, Patra NK, Khanuja SPS Use of RAPD and AFLP Markers to Identify Inter-and Intraspecific Hybrids of *Mentha*. J. Heredity, 2005; 96(5): 542-549.
41. Shasany AK, Lal RK, Darokar MP, Patra NK, Garg A, Kumar S, Khanuja SPS Phenotypic and RAPD diversity among Cymbopogon winterianus Jowitt accessions in relation to Cymbopogon nardus Rendle. Genet. Resour. Crop Evol, 2000; 47: 553–559.
42. Shyalaraj KS, Thomas J Ideal plant type concept of palmarosa (*Cymbopogon martinii* var. *motia*). Indian Perfumer, 1993; 37: 213–217.
43. Sobti SN, Rao BL, Sharma SN, Atal CK Jamrosa - a new geraniol rich *Cymbopogon.* Indian Pefumer, 1981; 25: 116- 118.
44. Soenarko S The genus *Cymbopogon* Spreng. (Gramineae). Reinwardita, 1977; 9(3): 225-375.
45. Sprengel C (1815) Plantarum minus pugillus 2, Halac.
46. Stapf O The oil grasses of India and Ceylon. Kew Bull, 1906; 8: 297–463.
47. Verma, V. & S. N. Sobiti. Karyological studies in the genus *Cymbopogon* Spreng. I. Nucleus (Calcutta), 1982; 25: 165–171.
48. Vos P, Hogers R, Bleeker M, Reijans M, van De Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kulper M, Jabeau M AFLP: a new technique for DNA fingerprinting. Nucleic Acid Res, 1995; 23: 4407–4414.