



DNA barcoding and chemical profiling in medicinal and aromatic grass species, wild *Cymbopogon citratus* (DC) Stapf

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Abstract

Cymbopogon species are high value aromatic grasses accounting for about 140 species worldwide. Globally renowned *Cymbopogon citratus* (*C.citratus*) which is native to South West Asia is occasionally found growing in wild locations of Karnataka. Natural habitat of *Cymbopogon* species, phenotypically appearing as *C. citratus* was sourced out at Thonnur Lake, Mandya district, Karnataka. Fresh leaves were subjected to hydro distillation for essential oil extraction. Qualitative analysis of the essential oil was made by GC-MS analysis. Major constituents in the essential oil were found to be geranial (48%), neral (34%) and beta-myrcene (6.16%). These are the biochemical marker constituents of *C.citratus*. Besides, geranial and neral constituent composition in total was 82% which was found to be higher when compared to the standard references on essential oil composition reported in the cultigen relative, RRL-J (72-75%) developed from Central Institute of Medicinal and Aromatic Plants, Lucknow. In addition, molecular marker based taxonomical identification of the wild *Cymbopogon* species was performed using DNA barcoding technique. Standard DNA barcode primer ITS-2 was used in the study. Morphological observation, cytogenetic, biochemical and molecular analyses validated the wild *Cymbopogon* species as *C.citratus*. Uninhabited aromatic grasses are innately adapted to withstand environmental stress which results in the production of higher amounts of secondary metabolites and diverse components naturally and occasionally, adapt newer pathways and mechanisms to produce novel compounds for sustenance. This presents an opportunity to explore natural compounds for the benefit of mankind. The present study suggests utilization of digitally authenticated native gene pool of *C.citratus* in molecular breeding program and genetic resource management strategies.

Keywords: *Cymbopogon citratus*, essential oil, GC-MS analysis, DNA barcoding

Introduction

Essential oil is a complex chemical mixture of natural ingredients prepared by plants for their own different purposes that include terpenes, alcohols, aldehydes and phenols. The aromatic cells with diverse morphologies are present in different parts of aromatic plants. They are natural biosynthetic factories of essential oils, where these oils are biosynthesized, accumulated and reach the atmosphere by many different reported secretion mechanisms (Rafia Rehman *et al.*, 2016).

Cymbopogon (Poaceae) represents an important natural resource of about 140 species that grow in tropical and subtropical regions around the world (Thara Saraswathi *et al.*, 2014). They exhibit a large variation in morphology and essential oil composition (Rao 1997) [14]. These species are classified into three distinct series based on their chemo types: Citratii (aldehyde type), Rusae (alcohol Type) and Shoeanthi (intermediate type that accumulates both alcohol and aldehydes) (Lavania *et al.*, 2012). Essential oils distilled from *Cymbopogon* species are of immense commercial value on account of their diverse uses in pharmaceutical, cosmetics, food, flavor and agriculture industries (Akhila 2010). Although 45 species of the genus occur in India, only few of them *viz.* *C. flexuosus* (East Indian lemon grass), *C.*

citratus (West Indian lemongrass), *C. pendulus* (North Indian lemongrass), *C.winterianus* (Java citronella), *C. nardus* (Ceylon citronella) and *C. martinii* (Palmarosa) are commercially cultivated for essential oil production (Sangwan *et al.*, 2001) [15]. Many cultivars of these species and their hybrids produce different types of essential oils, *viz.*, lemongrass oil, ginger grass oil, citronella oil, Palmarosa oil, etc. which are significantly utilized in medicinal and aromatic industries (Bishoyi *et al.*, 2017) [4]. *C.citratus* (DC) Stapf. Belonging to Citratii series is reported to have an extensive range of bioactivities and its essential oil widely used as a component of ethno pharmaceuticals. The volatile oil possess strong lemony odour due to the presence of citral in high amounts (Shahi *et al.*, 2005) [12]. Citral (monoterpene aldehyde) and β -Myrcene (oxygenated monoterpene), are reported to be the first and second major components of *C.citratus* oils (Vyshali *et al.*, 2013). Geranial (citral a) and neral (citral b) are geometrical isomers of the citral. Normally, one isomer does not occur without the other. Citral is used for the synthesis of vitamin A and ionones (for example, β - ionone and methyl ionone). Nutritionally, *C.citratus* is a good source of vitamins A and C, folic acid, magnesium, zinc, copper, iron, potassium, calcium and manganese (Shazadi 2017). In addition,

Venugopal *et al.*, (2014) found *C.citratus* as a source of oil for bio-diesel production.

Traditionally, Cymbopogon species are classified by phenotypic markers, aroma of the essential oils and concentration of biologically dynamic complexes present in essential oil matrices which are highly influenced by environment. This presents problem in the exact identification and discrimination at species level. Hence, the identification of Cymbopogon species are increasingly being seen as a major challenge (Muyembe *et. al.*, 2018). Consequently, digital identification through DNA barcoding technique has become an increasingly desirable molecular tool for accurate identification and validation at species level as it is uninfluenced by environment (Armenise *et al.*, 2012, Mahadani & Ghosh, 2014) [2, 13]. The primary goal of DNA barcoding is species recognition of unknown specimens and support to complex taxonomies for the advantage of science and society (Kress & Erickson, 2007). In plants, it was recommended that three gene regions *viz.*, ITS (internal transcribed spacers) 1 & 2, mat K (maturase K) and RbcL (ribulose-1, 5-bisphosphate carboxylase / oxygenase large subunit gene) be efficiently utilized as primary DNA barcoding markers for species identification (Hollingsworth *et al.*, 2009) [9].

C.citratus presents similarity in morphology and odour of essential oil with other member's *C. flexuosus*, *C.pendulus*, *C.winterianus* and *C.nardus* belonging to Citrati series of family Poaceae. This devises an indeterminate identification at species level. This aspect manifests the challenge of identifying and validating the species morphologically. DNA barcoding technique here comes in picture. DNA barcoding technique precisely authenticates the taxonomical class of the species. Digital identification data improves the identification success by overcoming the absence of some seasonal morphological features (Bishoyi *et al.*, 2017) [4] during the present study, an attempt has been made to explore the wild species of Cymbopogon through morphological, cytological, biochemical and molecular characterization and evaluation.

Materials and Methods Morphological studies

The wild species of Cymbopogon was collected from Thonnur Lake, Mandya District, Karnataka and maintained in Biotechnology departmental garden of Bangalore University, Jnanabharathi campus, Bangalore.

Leaf volatiles were primarily assessed for aromatic properties and morphology of the grass was determined by careful observation of the phenotypic features: shape and length of leaf, colour of culm and leaf sheath, length and structure of ligule, number of tillers per bush and inflorescence.

Cytogenetic studies

The cytogenetic investigation was carried out through mitotic study. The chromosomes from the root tip squashes were prepared and the slides were prepared by following the protocol of Thara Saraswathi *et al.* (2014) and Sree Latha *et al.*, (2017). During squashing, excess of stain was blotted out and was provided with uniform pressure to flatten the cells for chromosome spreading. The slides were made semi-permanent by sealing the coverslip with molten paraffin wax. The photomicrographs were taken using Carl Zeiss research microscope with apochromatic objective lens system fitted with MF 35 mm camera. Karyotyping was

conducted to analyse the shape, size and number of chromosomes present and the result schematically represented in the form of an Idiogram.

Essential oil studies Extraction

Fresh leaves of the plant sample were washed with distilled water, weighed (100 gm), hydro distilled in Clevenger's Apparatus (Clevenger 1928) for four hours. The essential oil obtained was distilled and dried over anhydrous sodium sulphate (Na₂SO₄) to remove traces of water associated with essential oil. The oil was preserved in amber bottle at 4° C until further use. The essential oil was then subjected to Gas Chromatography Mass Spectroscopy (GC-MS) analysis for the identification of chemical components present.

Quantitative analysis - Yield

The percentage composition of essential oil obtained was calculated using the formula,

$$\text{Essential oil content (V/W)} = a \times 100 / b$$

Where,

a=volume of the oil (ml) collected through hydrodistillation

b = weight of the sample taken (gm)

Qualitative analysis: GC-MS analysis

The essential oil was analysed by Electron Ionisation (EI) on a Shimadzu GCMS QP-2010 plus instrument equipped with Rtx-5ms capillary column (length: 30m, ID: 0.25 mm, film thickness: 0.25 µm). Helium (1ml/min) was used as carrier gas; oven temperature programmed for 60-2500 C at 300C/min raise; split less ratio 1:25. Sample volume injected was 0.1 µl, injector and detector temperature maintained at 250° C with interface temperature reaching 2800 C. Identification of compounds were made by matching their mass spectra and retention times with those recorded in the National Institute of Standards and Technology 5 (NIST) Mass Library and by comparison with literature data.

Molecular analysis

DNA isolation and amplification

Total cellular DNA was isolated from fresh and young leaves of *C. citratus* through CTAB extraction method optimised by Bishoyi (2017) [4] with slight modifications incorporated in the protocol to suit the sample under study. Pure DNA obtained was quantified by recording absorbance at 260 and 280 nm in a UV/ VIS spectrophotometer. DNA concentration was estimated by measuring the absorbance at 260nm, adjusting the A260 measurement for turbidity, multiplying by the dilution factor, and using the relationship that an A260 of 1.0 = 50µg/ml pure dsDNA. The quality and quantity was estimated against 0.8 % agarose gel through Agarose Gel Electrophoresis (AGE) technique. Polymerase Chain Reaction (PCR) technique was followed for amplification process. PCR reaction mixture contained 1µl DNA template (25 ng), 2µl 10X reaction buffer, 0.5µl MgCl₂ (50pM), 1µl dNTPs mix (10mM), 1µl forward primer (10pM), 1µl reverse primer (10pM), 0.5µl Taq polymerase (5 U/pi) and the final volume 25µl will be adjusted with molecular grade water. PCR conditions were as follows: initial denaturation 950C for 2 min, final denaturation 95°C for 30 sec, annealing at 550C for 30 sec, elongation 720 C for 1 min. The above steps are repeated for 30 cycles, final elongation 72 0C for 10 min and then

hold at 40 C. Standard primer pair ITS-2 was used for barcoding study as mentioned in Table 1. The PCR products were run against 1% agarose gel to separate the amplified bands through AGE technique. The required DNA bands were cut from the agarose gel, solubilised and subjected to Sanger dideoxy sequencing.

Data analysis

Sequences were edited, assembled using Clustal W (codon) and refined manually. The consensus sequence was uploaded to GenBank database, subjected to BLAST (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>) for identification of the barcode sequence at species level. The Phylogenetic tree was constructed by Neighbour- Joining method using MEGA 5.0; the bootstrapping was done with 1000 replications. Sequencing outcome was submitted to GenBank database at NCBI portal.

Table 1: List of DNA barcoding primers used for the study

Primers	Sequence	Annealing temperature
ITS2 F	ATGCGATACTTGGTGTGAAT	530C
ITS2 R	GACGCTTCTCCAGACTACAAT	510C

Results and Discussion Morphological studies

Leaf volatiles assessed for aromatic properties emitted strong lemony odour with bushy appearance of the plant exhibiting characteristic phenotypic features. The leaf showed length of 50-55cm, linearly shaped with parallel venation. The leaf sheath and the culm showed purplish green colour. The ligule was short, stubby and hairy which showed length of 1.0 - 1.5mm. The tillers number per bush consisted of 3-4 shoot branches. There was complete absence of inflorescence. The primary sensory evaluation and observations made on morphological features based on standard references of the plant revealed that the *Cymbopogon* species studied belonged to Citratii series and most likely to be *C.citratus*. The absence of development of inflorescence in the species suggested hexaploid nature.



Fig 1: Morphological identification of *Cymbopogon citratus* A. Habitat of the plant, B. Young plant, C. Mature plant

II Cytogenetic studies

The cytogenetic characterisation in *C.citratus* plants undertaken for the study showed medium to small size chromosomes forming thirty regular bivalents viz. n=30 chromosomes. This confirmed hexaploid nature of the plant. The mitotic study showed the presence 11M (Metacentric chromosome with centromere at the midpoint), 13m (Metacentric chromosome with centromere at the median region) and 6sm (Sub metacentric chromosome). The Karyotype formula derived is 11M+13m+6sm

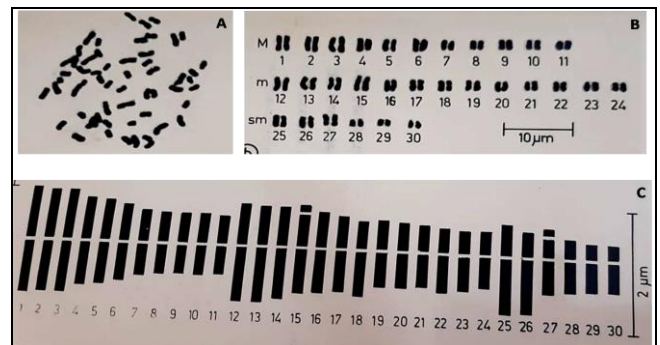


Fig 2: Karyomorphological studies in *Cymbopogon citratus* showing hexaploid nature of the sample A. Mitotic Metaphase, B. Karyotype, C. Idiogram

Essential oil studies Extraction

The essential oil extracted from the leaf sample showed transparent bright yellow colour.

Quantitative analysis - Yield

The quantitative analysis (V/W X 100) of the Essential Oil was found to be 0.8%.

Qualitative analysis- GC-MS analysis

Gas Chromatography Mass Spectroscopic analysis made using essential oil revealed the presence of 35 compounds belonging to monoterpene, alcohol, aldehyde, alkaloid, ketone, phenolic and flavonoid groups. The key compounds detected in the essential oil were geranial (48.04%), neral (34.37%) and β-myrcene (6.16%) which chemically identified the species as *Cymbopogon citratus*. The voucher specimen of the plant was submitted to Regional Ayurveda Research Institute for Metabolic Disorders (RARIMD) where it was validated as *Cymbopogon citratus* (DC) Stapf. With an accession number: RRCBI-mus230.

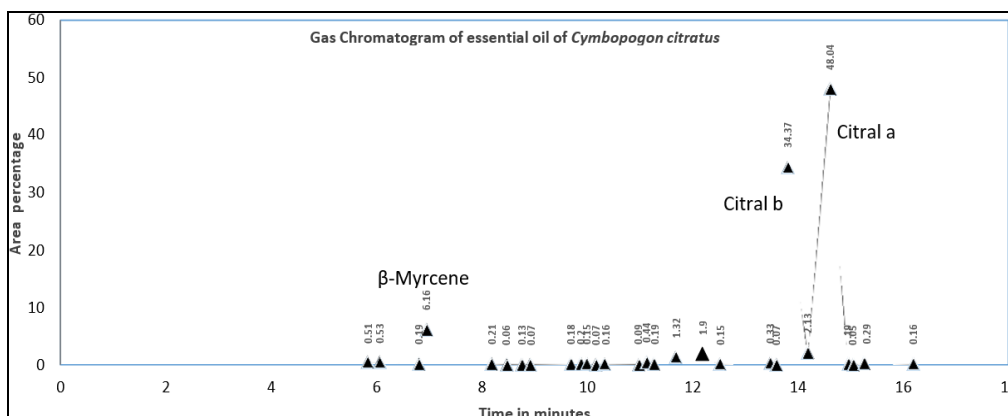


Fig 3: Gas chromatogram of essential oil from *Cymbopogon citratus* (Leaf sample)

Table 2: Chemical composition of essential oil from *Cymbopogon citratus* (Leaf sample)

Sl. No.	Retention Time (RT)	Compound with chemical formula	Molecular Weight (g/mol.)	Composition (%)
01	5.28	1-Ethylbutyl hydroperoxide, C6H14O2	118.17	0.51
02	6.06	1-Methylpentylhydroperoxide, C6H14O2	118.17	0.53
03	6.80	Sulcatone, C8H14O	126.20	0.19
04	6.95	β-Myrcene, C10H16	136.23	6.16
05	8.18	Trans-beta-ocimene, C10H16	136.23	0.21
06	8.47	Cis-beta-ocimene, C10H16	136.23	0.06
07	8.76	5-Isobutylnonane, C10H16	184.36	0.13
08	8.91	5-Butan-2-yl-nonane, C16H34	184.36	0.07
09	9.69	5-Tetradecen-3-yne (Z), C14H24	192.34	0.18
10	9.88	Linalool, C10H18O	154.25	0.20
11	9.99	Phytol, C20H40O	296.50	0.15
12	10.16	4,5-Dimethyl Nonane, C11H24	156.30	0.07
13	10.32	1,2-Epoxy-5,9Cyclododecadiene, C12H18O	178.27	0.16
14	10.97	7-Tetradecyne, C14H26	196.37	0.09
15	11.12	Gamma-IsoGeraniol, C10H18O	154.24	0.44
16	11.26	Alpha-2-dimethyl,1-cyclohexene-1-acetaldehyde, C13H20O	192.30	0.19
17	11.67	4,5-Epoxycarene, C10H16O2	152.23	3.22
18	12.52	3,7-dimethyl, 6-octen-1-ol (R), C10H20O		0.15
19	13.47	Beta-Citronellol, C10H20O	156.26	0.33
20	13.59	3-methyl-3-(4-methyl-3-pentenyl) Oxirane Carboxaldehyde, C10H16O2	168.23	0.07
21	13.80	Beta-citral, C10H16O	152.23	34.37
22	14.19	Nerol, C10H18O	154.24	2.13
23	14.61	Alpha-citral, C10H16O	152.23	48.04
24	14.96	5-(1-Methyl Propyl Nonane), C13H28	184.36	0.19
25	15.05	4-Propenylanisole, C10H14O	148.20	0.05
26	15.26	2-Undecanone, CH3(CH2)8COCH3	170.29	0.29
27	16.19	2,6,11-trimethyldodecane, C15H32	212.41	0.16
28	16.42	2,2-Dimethylcyclohexanol, C8H16O	128.21	0.51
29	18.68	Methylcarveol, C10H16O	166.26	0.10
30	19.38	7,8-Dibromo-4,4,7-trimethyl-hexahydro- benzo (1,3) dioxin-2-one, C11H16BrO3	365.05	0.03
31	20.46	Heneicosane, C21H44	296.60	0.41
32	21.40	Eicosane, C20H42	324.80	0.09
33	21.53	1-Iodoheptadecane, C16H33I	352.34	0.12
34	21.90	Caryophyllene oxide, C15H24O	220.35	0.10
35	22.33	Selina-6-en-4-ol, C15H26O	222.30	0.29
		Total		100

Table 3: Comparative analysis of major constituents in essential oil from *Cymbopogon citratus* (Leaf sample)

	Compound	Essential oil composition (%)	
		wild	cultigen
01	Geraniol	48.04	40.72
02	Nerol	34.37	30.13
03	β-Myrcene	06.16	15.69

Molecular identification studies DNA isolation and amplification

Total cellular DNA was isolated from the leaf sample of wild *Cymbopogon citratus* and DNA concentration was estimated to be 50µg/ml. The efficiency of PCR amplification of ITS-2 region was 99.57%.

Data analysis

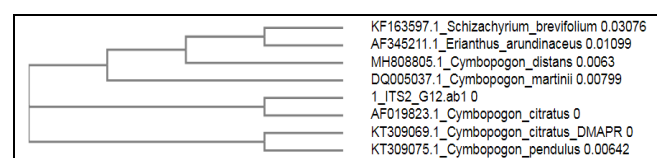
The candidate barcode region of ITS-2 was successfully sequenced by 100%. The digital identification through DNA barcoding technique authenticated the presently studied plant as *Cymbopogon citratus*. The GenBank accession number for DNA barcode sequence of wild *C.citratus* is Acc. No. OK042933

Hit: *Cymbopogon citratus* voucher DMAPR CC 01 Internal Transcribed Spacer (ITS) 2, partial sequence; 5.8S ribosomal RNA

Percentage Identity: 99.57%

E value: 0.0

Query coverage: 91% **Accession number:** KT309069.1

**Fig 2:** Phylogenetic tree showing evolutionary relationship in *Cymbopogon citratus*

Plants in their natural habitats are directly exposed ecological stress conditions such as drought, intense radiation, high temperature, heavy metal contents include abiotic stress and biotic stress such as invasion of microorganisms and herbivore interference. Various forms of secondary metabolites (essential oils in aromatic plants) are produced as part of defence and survival mechanism in plants prone to different stress conditions.

In the present study, quantitative and qualitative traits were assessed in leaf volatile oil from wild *Cymbopogon* species. The amount of essential oil extracted through hydro distillation method was found to be 0.8%. GC-MS analysis

(Figure 3) was performed which works on the principle of elution of different compounds from essential oil complex based on their retention indices. GC-MS analysis also indicates the exact amount of individual compounds present in the essential oil complex. Results revealed the presence of thirty five diverse components (Table 2) and high amount of essential oil (0.8%) in the native gene pool. The biochemical marker constituents of *C.citratus* are geranial, neral and β -myrcene. The presence of these compounds in wild *Cymbopogon* species studied indicated it to be *C.citratus* belonging to Citratii series. The percentage composition of geranial, neral and β -myrcene 48%, 34% and 6.16% respectively in the sample studied. The amount of major constituents present in essential oil of wild *C.citratus* were compared with the same as reported in its cultigen relative, RRL-J. Comparative analysis (Table 3) revealed that the commercially significant component citral (geranial + neral) in wild *C.citratus* was 82% which was found to be higher when compared to its composition as reported in its cultigen relative, RRL-J (72-75%) developed from Central Institute of Medicinal and Aromatic Plants, Lucknow. The richness of *C.citratus* essential oil is well determined by citral content, the aldehyde accountable for lemon odour. (Sangwan *et al.*, 2001) [16]. β - Myrcene, an oxygenated monoterpene contributes to the stability of essential oil in storage. The oil has the tendency to polymerise during storage due to the presence of high amounts of β -Myrcene (8-25 %), thus deteriorating the quality of the oil (Oyen 1999). The amount of β - Myrcene present in currently investigated sample was 6.16% which is lesser when compared to its presence in RRL-J cultigen (15.69%). The presence of low amount of β - Myrcene contributes to the stability of essential oil for long periods during storage. These two attributes in wild *C.citratus* genotype (elevated amounts of geranial and neral and lesser amount of β - Myrcene in comparison with cultigen- RRL-J of *C.citratus*) add value to the native gene pool under study to be considered as potential line for commercial utilization. Natural selection favors prospects for choosing elite lines due to its diverse chemical composition and higher adaptive rate. Morphology of the plant and essential oil produced by plants is influenced by the environment in which they are growing. Though, the plants can be predicted through morphological observation and biochemical marker constituents present in the essential oil, it cannot be discriminated to the species level based on these parameters. The cutting edge molecular marker technologies have been developed to precisely discriminate the plants into species specific identity. Therefore in the present study, efforts were made to evaluate DNA barcode region in wild *Cymbopogon* species for individual discrimination and authentication at species level. Universal barcode region ITS 2 designated for eukaryotes was used as standard primer for molecular identification through DNA barcoding technique. Based on Neighbour Joining (NJ) tree, (Figure 2), *C.citratus* and its relatives belonging to different series could be placed in genus *Cymbopogon* and distinguished from most of the other genera, whereas *C.citratus* and its close relative species belonging to Citratii series could be distinguished from each other based on the sequence divergences. The use of DNA barcoding technique could complete the accurate identification process by compensating the limits of the morphological approach.

Conclusion

During the present study, the chemical and molecular characterization were made in wild *C.citratus* obtained from Thonnur Lake, Mandya district, Karnataka. The study identifies native gene pool of *C.citratus* showing higher concentration of citral compared to the cultigen. The wild relatives of the plant need conservation and utilization for molecular breeding programs. They possess potential agronomic traits naturally and show ability to withstand biotic and abiotic stress, yielding quality essential oil. Morphological, cytological, biochemical, molecular characterization and evaluation serve as the systematic way to explore natural genetic resources.

Acknowledgement

The GC-MS analysis of the essential oil was conducted at IADFAC Laboratories, Bangalore.

Conflict of interest

The authors declare that they have no conflict of interest.

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