

Exploration and Studies of Wild *Cymbopogon martinii* (Roxb.) Wats. Growing in Devarayana Durga Hill, Karnataka

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ABSTRACT

Cymbopogon martinii is an aromatic plant commercially grown for its valuable essential oil, which is used in the pharmaceutical and aromatic sectors. Antimicrobial, antifungal, antiviral, anthelmintic, antioxidant, and chemopreventive medicines are all found in the oil. The widespread growth of *Cymbopogon martinii* was observed in the Devarayana Durga hills of Tumkur, Karnataka, India. The plant was identified using morphological, cytogenetic, and anatomical characteristics (SEM and Microscopic studies). DNA barcoding and flow cytometry analysis was used to confirm ploidy and species specificity. The plant was identified as *Cymbopogon martinii* (Roxb.) Wats. The seeds and the Herbarium (AC-62/2021) were deposited at the ICAR's National Bureau of Plant Genetic Resources (NBPGR), Pusa New Delhi. The morphology of the wild plant differed from that of other *C. martinii* kinds gathered and analyzed from the same wild area, indicating a response to environmental influences. Flow cytometry was used to confirm that the species was tetraploid (*C. flexuosus*). Using NCBI BLAST, the DNA barcoding sequence of *rbcl* revealed close similarities to *C. martinii*, and the sequence was deposited in Gen Bank under the accession number MW538957. As a result, conserving wild *C. martinii* would aid in the development of new breeding lines with potential bioactive chemicals with an economic worth in the future.

Keywords: Wild *Cymbopogon martinii*, Exploration, Authentication, Morphological study, DNA barcoding, Flow cytometry analysis.

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INTRODUCTION

Crop wild relatives (CWRs) are an excellent source of disease resistance, quality, and rootstocks for perennial crops. CWRs had a global benefit of \$115 billion per year by 1997, according to estimates.^[1] Even crops that have been farmed for millennia are typically not genetically distinct from their wild forebears. CWR has provided new genetic diversity that can boost agricultural output and quality, increase pest and disease resistance, and raise the crop's tolerance to harsh environments.^[2]

Cymbopogon martinii Wats. is a fragrant perennial plant belonging to the grass family Poaceae, commonly known as palmarosa or Rosa grass. With a chromosomal number of $2n = 20^{[3-4]}$ It produces an essential oil with a floral, rose-like scent. Geraniol and geranyl acetate are the primary components in the essential oil produced from *C. martinii*. Its leaves and inflorescence (flowering tops) yield an oil that is widely used in soaps, cosmetics, toiletries, and tobacco goods.^[5] Palmarosa oil is said to have antibacterial, mosquito repellent, and pain-relieving effects, making it more important in the pharmaceutical world.^[6] This crop is found in Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, Madhya Pradesh and Uttar Pradesh. In addition to India, it is grown in Brazil, Paraguay, Madagascar, Guatemala, and Indonesia.^[7] During the 2009-2010 fiscal year, India was a major producer and exporter of Palmarosa oil to the global market, exporting approximately 44 tonnes

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to 19 countries, including the United States of America, France, and the Philippines.^[8]

Palmarosa is a widely cross-pollinated crop, thus there are many differences between plants grown in different regions.^[9] These modifications are related to morphological (plant morphology, anatomy, and SEM), ecological (soil and silica), cytogenetics, and molecular plant characterization to study the plant's habitat and ability to thrive in a specific ecotype. During a recent exploration of Devarayana durga hill in Tumkur, Karnataka, palmarosa plants spanning the entire hill were discovered, as was *Cymbopogon martinii*. As a result, plants growing in the wild were collected and investigated for their morphological, cytogenetics, and molecular characteristics for the study.

MATERIALS AND METHODS

Plant Collection and Identification

For the experiments, wild *C. martinii* genotypes were obtained from the hills of Devarayana Durga (planar region), Tumkur, and Karnataka. The plant was identified and validated by the Regional Ayurveda Research Institute for Metabolic Disorders (RARIMD), Ministry of AYUSH, Bengaluru, and an accession number was obtained. The seeds and herbarium were deposited at the Indian Council of Agricultural Research's (ICAR) National Bureau of Plant Genetic Resources (NBPGR) in Pusa, New Delhi.

Ecological Studies

The plant sample was collected from Devarayana Durga hills, Tumkur. Devarayana Durga is a rocky hill surrounded by forest and hilltops and is dotted with several temples with an area of 42.27 sq. km. The habitat and the habit of the Wild Cymbopogons collected were studied and photographed.

Soil Analysis

A sample of soil was taken from the plant's rhizosphere (eight separate sites from the collecting area); it was mixed and air-dried at room temperature before being filtered to remove pebbles and particles. At the Environmental Health and Safety Research and Developmental Centre (EHSRDC) in Bengaluru, Karnataka, the soil sample was analyzed for Macronutrients and Micronutrients (S, N, K, P, Fe, Si, Cu, Bo, Mn, and Zn).

Morphological Studies

The plant was carefully chosen and poised based on its ecological habitat (morphology) and aroma value (8 samples). Morphological characteristics such as the

leaf length, texture and color, length of ligule and inflorescence.

Leaf Anatomy

A transverse section of fresh leaf lamina was used to study the anatomy of the leaf (20 numbers). The section was immersed in freshly prepared Schiff's reagent and incubated at room temperature for 30 min. Sections were then washed three times for ten minutes each in freshly prepared 0.5 percent (w/v) sodium metabisulphite in 0.1 percent HCl and examined at 10X and 40X magnification (Thara Saraswathi *et al.*, 2016).

Scanning Electron Microscopy (SEM) Analysis

The foliar epidermal micro-morphological characters of the ecotype studied were examined using SEM. On both the upper and lower surfaces of the leaf, foliar epidermal structural differences were observed. Scanning Electron Microscopy was used to examine mature, dried leaves of intact grass specimens (SEM). Two leaf pieces were cut and double-coated scotch-taped to the stubs. One leaf was staked on a lower-side stub to expose the upper part, while the other was pointed on upper-side stubs to expose the lower surface in stubs. Before being examined with a JEOL JSM-6490F SEM, the specimens were sputtered with platinum. JEOL Ltd. Field Emission Scanning Electron Microscope.

DNA Barcoding loci studies and Phylogenetic Analysis

Total genomic DNA was extracted from leaf samples using the CTAB method. To amplify the DNA, plant-specific oligo primers (rbcL, matK, and ITS2 spacer region) were used. The PCR reaction mixture contained 50 l of 50 ng gDNA, 100 ng of each forward and reverse primer, two l of 10 mM dNTPs mix, five l of 10X Taq Polymerase buffer, 3U of Taq polymerase enzyme, and PCR grade water.

Five minutes of initial denaturation at 94°C was followed by 35 cycles of one minute at 94°C, annealing temperature normalized at 60°C, two min of extension at 72°C, and min of final extension at 72°C. The PCR product was run on a 1% agarose gel in 1X TAE buffer, and the products were purified with Nucleo-pore, Genetix Biotech PCR clean-up kit, and the purified fragments were sequenced. The Bio edit tool was used to edit the sequenced data. The experiment was performed in triplicate to ensure that the barcode sequence was reproducible.^[10]

The annotated sequence was subjected to BLAST for verification (NCBI domain). The retrieved sequence was submitted for an accession number to the NCBI Gen Bank. A phylogenetic tree was constructed using

the Neighbour Joining method and a Clustal W analysis was performed using a MEGA 6.0 software.

Studies on Genome Content, Size and Ploidy Level Flow Cytometry Analysis

For sample preparation, 50 mg of young leaf tissue was taken, and one ml of buffer solution was added to a Petri dish containing the plant tissue, which was then chopped for 60 sec at 4°C with a sharp razor blade. A 40 m nylon filter was used to filter out large debris from the homogenate. Nuclei were stained with 50 g/ml propidium iodide (PI) and 50 g/ml RNase to prevent staining of double-stranded RNA (Choudhury et al., 2013). Before analysis, samples were placed on ice for 10 min before being studied using the instrument - Cytomics FC500 Flow cytometer, Beckman Coulter, USA with analysis software flow Jo V10.0.7 and was verified for linearity and resolution, as well as being set up for doublet discrimination function using the DNA QC Particles kit from BD Biosciences.^[11]

Genome content is calculated using the formula:

$$2C \text{ DNA content} = \frac{\text{Sample Peak mean}}{\text{Std. Peak mean}} \times \text{Std. 2C DNA content (pg)}$$

RESULTS

Collection and Identification of the Plant

Cymbopogon plants growing in Devarayana Durga hill, Tumkur district, Karnataka, and their habits and habitat were recorded. Plants with a distinct species abundance of up to 80% in the collection site and a unique flowery, rose-like aroma were taken for further study. The plant's morphology and anatomy (SEM and Microscopic) were confirmed as *Cymbopogon martinii* (Roxb.) Wats (Figure 1).

Authentication and Deposition

Herbarium sheet and seeds were deposited in the ICAR National Bureau of Plant Genetic Resources, Pusa, New



Figure 1: Habit and habitat of Wild *Cymbopogon martinii* growing in Devarayana Durga hill, Tumkur district, Karnataka.

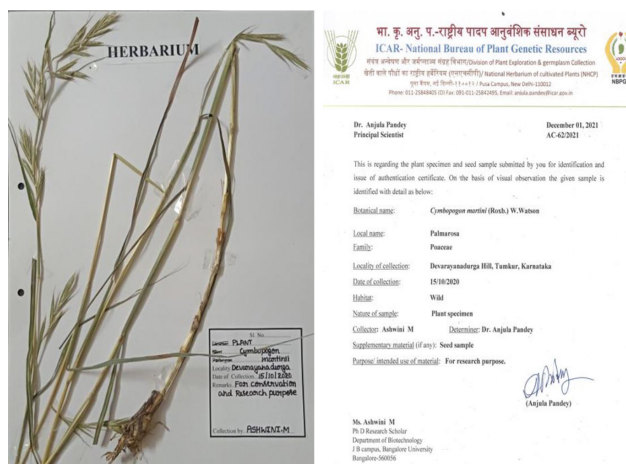


Figure 2: Herbarium and authentication certificate of wild *C. martinii* deposited at ICAR- National Bureau of Plant Genetic Resources (NBPGR).

Table 1: Ecological parameters of plants growing in Devarayana durga hill.

Habitat	Devarayana Durga Hill, Tumkur
Area in sq.mtr	42.27 sq.km
Altitude	1290 m above sea level
Annual rainfall (mm)	1187mm
Vegetation type	Deciduous forest
Latitude	13 23'N 77
Longitude	77.13 ° E
Annual temperature	23°C - 27°C
Precipitation	23%
Wind flow	3 km/h

Delhi, the plant was authenticated under Accession no. AC-25/2021. For conservation (Figure 2).

Ecological studies

Devarayana durga hill is a southern forest of grassland covering an area of 42.27sq. km. The latitude and longitude of the hill are reported as 13.375 and 77.123 respectively. The area has an elevation of 1290 mts above sea level with red sandy soil. The hill receives low rainfall throughout the year with an average temperature of 22.9°C (Table 1). The entire Hill was covered with many types of medicinal plants along with the abundance occurrence of Wild *Cymbopogon* species.

Soil analysis

The plant was found growing in the planar region of a hill. The soil is reddish-brown in color with a PH of 6.8 having a fine texture containing NPK in the soil of about nitrogen (193.80 kg/ha), potassium (81.96 kg/ha),

Table 2: Macro elements and microelements present in the soil.

Parameter	wild <i>C. martinii</i> (planar region)
Macro element	
Quantity	
Available Nitrogen (N)	193.80 kg/ha
Available Phosphorus (P ₂ O ₅)	81.96 kg/ha
Available Potassium (K)	150.23 kg/ha
Available Sulphur (SO ₄)	0.23mg/100g
Micro element	
Boron	0.57mg/kg
Iron	45.22mg/kg
Zinc	24.1mg/kg
Copper	BDL
Manganese	130.3mg/kg
Silica	172.0 mg/kg

and phosphorous (150.23 kg/ha) and silica (172.0 mg/kg) percentages (Table 2).

Morphological Studies

The morphological characteristics of the Wild genotype of *C. martinii* presently studied is the Perennial plant and the characteristics of the different parts of the Plant such as Leaf, Stem Inflorescence etc. were studied and recorded.

STEM (culm)

Stem erect, green, 200 cm high, smooth, glabrous; the lower base of Nodes and Internodes often swollen, Internodes 15 cm high.

LEAF

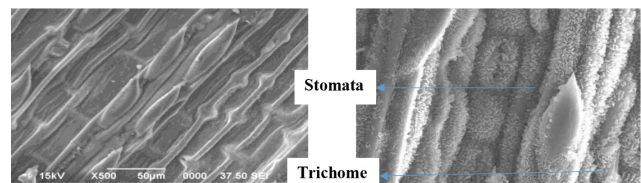
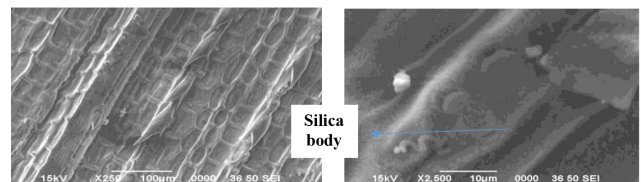
Light green, 50 cm long, 25 mm wide, cordate at the bottom, tapering end often glabrous and smooth. 1.5-4 mm long membranous ligule.

Inflorescence

Large and complex, Pink Panicle, branches slender, Racemes paired with Spatheole and Spikelets, one sessile, other Pedicelled. Sessile spikelets 2-3 mm long Spathe green, 2.5 cm; Spatheole light green, boat-shaped, 1.5 cm long glabrous; Peduncle filiform. Racemes are 20 mm long and heterogamous, with a swollen lower raceme base and lowermost pedicel. Sessile spikelet is 3.5 mm long and glabrous; lower glume is 1 mm wide and broadly winged, with 2 nerves; awn is 12—18 mm long. Pedicellate spikelet is 4 mm long and glabrous; lower glume is lanceolate and 8-nerved. The stamens are two, and the anthers are 1.5-2 mm long. The flowering season is from September to November.

Table 3: Morphological characters of the plant.

Parameter	Wild <i>C. martinii</i>	Cultivar <i>C. martinii</i>
Plant appearance	Bushy nature	Spread type
Plant height	3-3.5m	1.2-2.5m
Root type	Thick and fibrous	Shallow and fibrous
Culm	Erect, nodes swollen	Erect, nodes swollen
Leaf-sheath	Glabrous	Glabrous
Ligule	Membranous	Membranous
Leaf-blade	Linear flat, 50 cm x 25mm cm	Linear flat 120cm x 1.2cm
Inflorescence	Compound panicle	Compound panicle
Inflorescence height	100cm	80cm
Inflorescence color	Dark Purplish pink	Purplish pink
Spatheate	Beneath raceme, 2.5cm ht	Beneath raceme, 1.5cm ht
Raceme	Several spikelet, oblong, elliptic, awned	Several spikelet, oblong, elliptic, awned
Seed	Brown, hairy	Brown, hairy

Abaxial Surface**Adaxial surface****Figure 3: SEM images of leaf surface.**

Abaxial surface: Cell arrangement, stomata, trichome

Adaxial surface: Cell arrangement Silica bodies

Wild *C. martinii* studied showed distinct morphological characteristics when compared to cultivar Trishna from CIMAP which are mentioned in Table 3.

Scanning Electron Microscopy Studies of Leaf

SEM studies revealed the arrangement of cells on adaxial and abaxial surfaces of the leaf. The stomata were localized on abaxial surface nearing midrib region and surrounded by minute micro-hairs and trichome. The adaxial surface contained silica bodies distributed along midrib region (Figure 3).

Leaf anatomy

T. S of leaf

The adaxial layer or upper epidermis consisted of a single layer of large bubble-shaped elongated cells termed bulliform cells. The bulliform cells were followed by the ground tissue having 3-4 layers of cells.

The vascular bundles were oval-shaped conjoint, collateral, having a closed arrangement in the abaxial layer. The vascular bundle consisted of two metaxylem oriented toward the upper epidermis. The phloem, which contained companion cells and sieve tubes, was found close to the lower epidermis. The large vascular bundles were surrounded by two layers of cells that constituted the bundle sheaths, and each bundle was capped by a small group of sclerenchyma fibres on both the upper and lower ends. Single Large vascular bundles in the midrib were connected to the lower epidermis by a group of sclerenchyma cells with lignified walls, which formed 3 to 5 layers. A single layer of small cells formed the abaxial epidermis. and a smaller nine vascular bundles between interspersed the massive vascular bundles. The lower epidermal cells found below the vascular bundles possessed projections and formed Papillae.

Anatomy studies revealed the deposition of essential oils in the lower epidermis by darkly stained cells between and around the vascular bundle.

Molecular identification using DNA barcoding loci

For the molecular identification of plants by using DNA barcoding loci, the three primers (ITS2, matk and rbcL) tested only *rbcL* amplified fragment length of 540 bp. DNA barcode loci sequences were submitted to NCBI's GenBank, and accession numbers were obtained with accession number MW538957, and *rbcL* showed 95.69 percent homology with cultivar *Cymbopogon* sps. MEGA6 software was used to create the phylogenetic tree.

Phylogenetic tree built using neighbor-joining based on *rbcL* gene sequences from all six species. *C. martinii* (KT309057), *C. flexuosus* (KT309054), *C. citratus* (KT309053), *C. nardus* (KT309058), *C. pendulus* (KT309059), and *C. winterianus* were clearly distinguishable from the other five species taken from the NCBI GenBank for studies (KT309060).

The phylogenetic results revealed that wild *C. martinii* and *C. flexuosus* are more related to it has arisen from a common branch point and *C. martinii* (cultivar) were less related as it showed joining with another internode. The phylogenetic tree reflects wild and cultivar *C. martinii* showing divergence in evolutionary relation (Figure 5).

Genome content, size and ploidy studies

Flow Cytometry analysis

It provided data on 2C DNA content and ploidy of wild *C. martinii* against control *C. flexuosus*. 2C DNA content of wild *C. martinii* was estimated to be 1.89 pg over the control *C. flexuosus* (tetraploid) with 2C DNA of 1.56 pg. The nuclear DNA genome size was calculated using the formula and it was found to be 1848Mbp. Based on the histogram alignment and the 2C DNA content the species was found to be tetraploid. Hence, the species undertaken for study belong to the tetraploid race (Table 4 and Figure 4,6).

Wild *C. martinii* (test) sample

Genome content is calculated using the formula:

$$\begin{aligned} 2C \text{ DNA content} &= \frac{\text{Sample Peak mean}}{\text{Std. Peak mean}} \times \text{Std. 2C DNA content (pg)} \\ &= \frac{24204}{20680} \times 1.56 \\ &= 1.89 \text{ pg} \end{aligned}$$

For genome size calculation,

$$1 \text{ pg DNA} = 0.978 \times 10^9 \text{ bp.} = 1.89 \times 0.978 \times 10^9 \text{ bp} = 1848 \text{ Mbp}$$

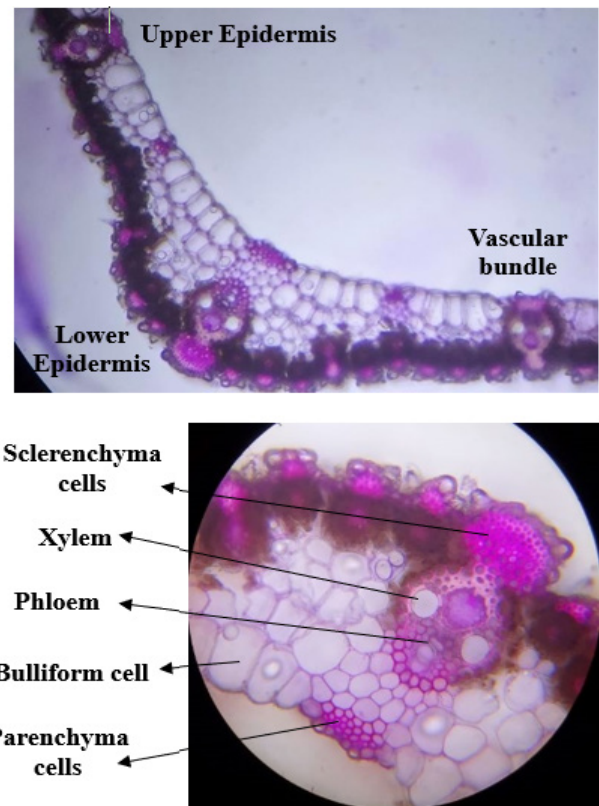


Figure 4: TS of leaf showing the arrangement of vascular bundles.

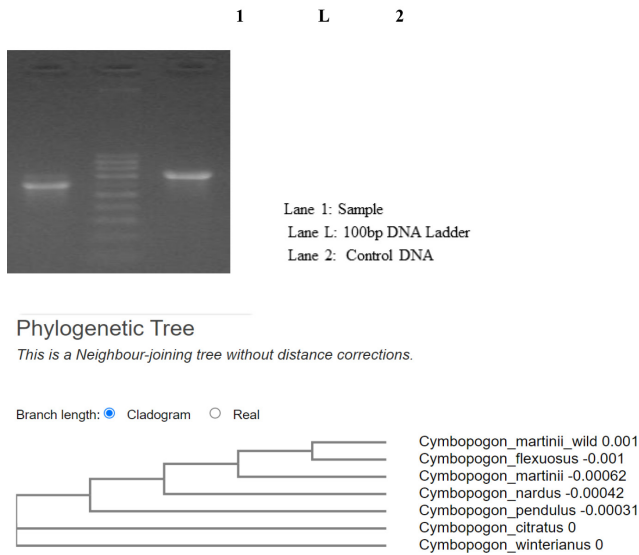


Figure 5: Agarose Gel Electrophoresis of amplified genes (rbcl) and Phylogenetic tree constructed based on nucleotide sequences of rbcl (chloroplast) gene loci.

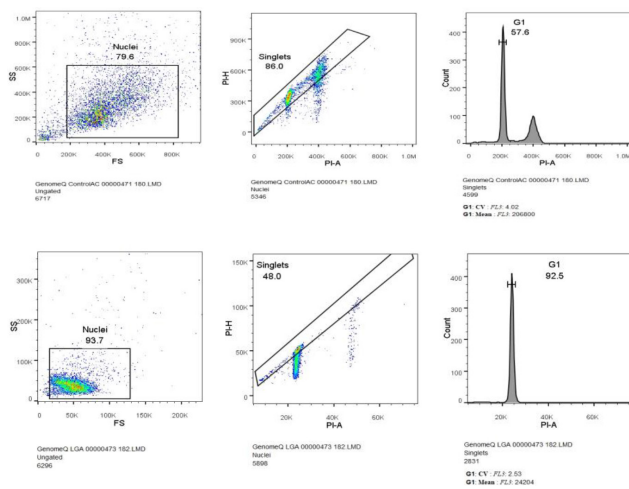


Figure 6: Histograms of cultivar *C. flexuosus* and wild *C. martini*.

Table 4: Total 2C DNA content, genome size and ploidy status of wild *C. martinii* against standard.

Sample	Mean value (M1)	CV	2C DNA Content (pg)	Genome Size (Mbp)	Ploidy
Cultivar <i>C. flexuosus</i> (Control)	20680	3.27	1.56	1516	Tetraploid
wild <i>C. martinii</i> (Test)	24204	2.53	1.89	1848	Tetraploid

DISCUSSION

Cymbopogon is a member of the grass family Poaceae that is grown for its essential oils, which have medicinal and industrial value. *Cymbopogon* species were collected from the wild location of Devarayana Durga

Hill for studies based on species richness and its rose-like aroma since it is commercially important in the production of essential oil. National Bureau of Plant Genetic Resources, Pusa, New Delhi, authenticated and identified the collected plant as *Cymbopogon martinii*. When compared to the NPK ratios of cultivar *C. martinii* of nitrogen (100 kg/ha), phosphorous (50 kg/ha), and potassium (50 kg/ha), soil analysis revealed that plants naturally adopted a high NPK ratio. It implies that the same plant growing in different ecotypic regions needs different quantities of NPK and microelements. to grow and adapt. Morphological characteristics of *C. martinii* plants studied revealed a difference in plant habit by appearance and length of the plant in comparison to the cultivar plant as it appeared bushy with dense fibrous root, the difference in height of plant of about 3-3.5m and inflorescence (100 cm) When compared to *C. martinii* cultivar variety, which appeared to be Spread type with Shallow and fibrous root type, plant height was found to be 1.2-2.5m and inflorescence of 80 cm (CIMAP Source). Anatomical studies of the leaf section showed the deposition of essential oil on lower epidermis which is darkly stained in between the vascular bundle and the presence of bulliform cells and papillae when compared with the *C. martinii* growing on the rock side, bulliform cell helps plants to overcome water loss by helping the folding of leaves.

Molecular identification of plants employing DNA barcoding loci studies indicated evolutionary divergence from cultivar counterparts, and identification is determined primarily by morphological markers, the odor of essential oils, and the composition of bioactive compounds present in oil matrices, which are all strongly influenced by the environment.^[12] Cytogenetic studies employing flow cytometry revealed that the plant is tetraploid it implies that wild *Cymbopogon martinii* growing in the Devarayana Durga is a Sofia variety.^[13]

CONCLUSION

The wild *C. martinii* is explored and studied here to authenticate and characterize the plant growing in the wild location can be used for their commercial importance in the future. It is a hardy, rainfed and drought-tolerant aromatic grass that can be cultivated in marginal and farming lands with a wide range of soil types, which include saline soil (up to pH 9) The wild plant seems to have a reduced prevalence of pests and pathogens as it has evolved to its environment and is resistant to stress and disease. It will aid in the discovery of new cultivated varieties for the production of high-value Palmarosa oil. Through genetic improvement, these explored wild plants would be used for high-

quality palmarosa varieties that are resistant to stress further research was needed to study their importance in the field of aroma and pharmaceutical industries for future applications. These wild plants are explored to study their natural habit and habitats and by few important characteristics hence there is no comparison to discuss here.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CWR: Crop wild relatives; **NBPGR:** National Bureau of Plant Genetic Resources; ***C. martinii*:** *Cymbopogon martinii*; **SEM:** Scanning electron microscopy; **rbcL:** ribulose biphosphate carboxylase; **matK:** Maturase K; **ITS2:** internal transcribed spacer 2; **mts:** Meters; **km:** Kilometer; **Kg/ha:** Kilogram per hectare; **mg:** mili gram; **min:** minute.

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