International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 17-11-2020; Accepted: 01-12-2020: Published: 13-12-2020
www.botanyjournals.com
Volume 5; Issue 6; 2020; Page No. 649-653

Ploidy variation in wild *Cymbopogon martini* (Roxb.) wats of different ecotypes using flow cytometry

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Abstract
Flow cytometry with propidium iodide (P.I.) as the D.N.A. stain was used to estimate their homoploidy genome size (2C) of six *Cymbopogon martinii* species collected from different locations (Ecotypical conditions) in Karnataka, India. Chromosome number was investigated for *C. martinii* and calculated the monoploid genome size of 6 different ecotypes of *Cymbopogon martinii* species. We observed intra-spatial genome size in six ecotypes, with a difference in morphology. The genome size found was lowest in ecotype (1.41 ± 0.006pg). Different genome size (2C) was observed for ecotype 3 (3.06 ± 0.020pg). Among the six ecotypes/varieties, 2.38 fold variation in genome size was observed. The correlation of nuclear D.N.A. content and chromosome number of the studied six ecotypes of *Cymbopogon martinii* species revealed the emergence of the evolutionary history. This work's genome size estimation will provide critical information for breeding and sequencing of *Cymbopogon martinii* species genome.

Keywords: chromosome number, flow cytometry, nuclear d.n.a. content, polyploidy, propidium iodide

Introduction
India is a major center of origin and diversity for aromatic and medicinal plants. It holds an extraordinary significance among the world's top rich countries relating to its abundantly rich land race diversity (Ravichandran and Manimekali, 2001). Due to their economic importance, they need to be conserved in natural needs. Cymbopogon is one of the significant aromatic and medicinal plant genera belonging to the tribe Andropogoneae of the family Poaceae. The plant derives its name from the Greek word kumbe and pogon, meaning boat-shaped beard, which refers to many awned inflorescences and boat-shaped spathes. The genus Cymbopogon is well known for its secondary metabolites which are widely used in perfumery, flavor, and pharmaceutical industries. Accumulation of secondary metabolites is often due to plant exposure to different stress factors, including various elicitors or signal molecules. Secondary metabolites play a significant role in adapting plants to the environment and overcoming stress conditions (Ramakrishna and Ravishankar, 2011). The essential oil consists of a wide array of terpenes, which has a strong aroma and helps in pollination and protection of the plants from deterring parasites and herbivores. Terpenes undergo chemical modification through oxidation or by rearrangement of the carbon skeleton resulting in the formation of terpenoids, also known as isoprenoids.
Genome size (G.S. hereafter) is an important character in the evolution and adaptation of plants (Price, 1976). G.S. estimates are required for large study of taxonomic delineation (Leong-Skornickova *et al*., 2007). Although G.S. varies tremendously across the vascular plants, the knowledge of evolution and diversification of Cymbopogon is still in infancy (Zaveska *et al*., 2011), involving large population size. This tremendous variation is caused by different proportions of non-coding repetitive D.N.A., such as transposable elements, satellite D.N.A., introns, and pseudo genes (Bennett and Leitch, 2005) [4]. Widespread employment of flow cytometry for estimation of G.S. of plants has provided meaningful information for molecular phylogenetic analysis, evaluating the complexity of genomes and understanding the evolutionary pattern of plants (Kron *et al*., 2007) [13]. The mode or reproduction responsible for the genetic diversity patterns in *Cymbopogon martinii* has been shown recently (Zaveska *et al*., 2011). Although genome size estimates of the Cymbopogon species are available widely during the last three decades by flow cytometry (Bharathan *et al*., 1994), Cymbopogon's evolutionary significance *martinii* is not well understood due to the tropical distribution and the taxonomic ambiguity of the family. Current study is an investigation of the knowledge gap among the genome size estimates reported in the studies considering existence of intra spatial G.S. variation of Cymbopogon martini from Karnataka. The study will be helpful in the strategic planning of Cymbopogon martini conservation in Karnataka.

Materials and Methods
Plant material
Six different ecotypes of *Cymbopogon martinii* populations were investigated from Karnataka, India (Table 2). The plant materials were collected during 2016-2019 and were authenticated by Dr. Ramrao, (RARIMD, Bangalore). No specific permissions were required during filed studies involving the collection of the materials from different locations indicated in Table 1, and the Herbarium vouchers were deposited. Furthermore, the field studies did not involve endangered or protected species.

Standards for flow cytometric estimation of G.S.
Leaf of Cymbopogon citratus (2c=) was estimated and obtained before conducting the experimental studies of

sample Cymbopogon martini, with the same instrumentation. The recommended standard reference species of *Cymbopogon citratus* which is standardized with the same instrument. The Standard sample was collected from the Jnanabharathi Campus, Bangalore University, Bangalore.

Isolation and staining of nuclei

The intact nuclear suspension was prepared of young and tender leaves collected from different ecotypes. Chopping of small leaves was performed with a sterile double edged razor blade in 1.0 ml of propidium iodide/hypotonic citrate buffer as described previously (Choudhary *et al*., 2014). Nuclear suspensions were observed for the presence of nuclei using an inverted microscope platform (Applied bio systems, Evos-FL) equipped with filters for propidium iodide. Images were acquired with a C.C.D. camera attached to the microscope (A.M.G., Bothell, WA).

Measurement of nuclear D.N.A. content

Nuclear D.N.A. content was in a FACS Calibre flow cytometer fitted with 15-mW 488-nm argon-ion laser and cell Quest Pro software (B.D. Bioscience, U.S.A.). The acquisition panel was set to collect 10,000 events (nuclei) and co-efficient of variation (CV) using FlowJo v.7.6.5 (FlowJo, Tree Star Inc., Ashland, OR). The experimental procedures for measurement of holoploid genome size (2C) were as described previously (Ramesh *et al*., 2014). The G.S. (2C) was estimated by comparing the M.F.I. of nuclei of the sample materials with that of the reference standard and obtained by multiplying the G.S. (2C) of standard species by the ratio of M.F.I. in internal standardization. Moreover, 1C value refers to the half of G.S. (2C) by not considering the ploidy level of the plant was also taken into account. Conversion from picograms replicates to base pairs (bp) was done as follows: 1 pg D.N.A. + 978Mbp (Dolezel *et al*., 2003) [3]. We considered three plants per population, each with three technical replicates, resulting in a dataset of 1470 GS measurements. The G.S. is reported as average of six technical replicates based on the comparison with the closest internal standards.

Presence of inhibitor is confirmed if mean peak position of reference standard is lower in the presence of the target sample (Price *et al*., 2000). In this study, we first tested for the presence of such inhibitors in the cytosol of *Cymbopogon citratus* (target sample), by comparing the peak positions of Cymbopogon citratus nuclei (reference standard) that were separately processed and those that were simultaneously processed (co-chopped as one sample, with leaves layered on top of each other) with *Cymbopogon citratus in* six replicates. Similar fluorescence of nuclei from standard leaves simultaneously processed with test leaves when compared to nuclei from independently processed leaves was taken as an indicator for the absence of inhibitors in the test samples. Test for the presence of inhibitors were carried out in similar fashion for other species of the family as well. In all experiments, the florescence of at least 10,000 G1-phase nuclei was measured. Three replicate measurements of *Cymbopogon martinii* species were carried out to determine their G.S. (2C).

Hypotonic P.I. solution for live cells was prepared for flow cytometry

1x Hypotonic P.I. Solution (For live cells)- 100ml

100 ml of distilled water
100 mg of Sodium Citrate: 0.1 % Trisodium Citrate
(Keeps the nucleus intact and to
burst open the cells)
4mg of RNAse: 40ug/ml (Removes R.N.A.)
2.5mg P.I.: 25ug/ml (Fluoresces and binds to
30µl NP40/Igepal: 0.03% (Enhances Permeability)
Processing Samples: Take 1 ml of Sample, vortex and
run it at 1500

Chromosome count

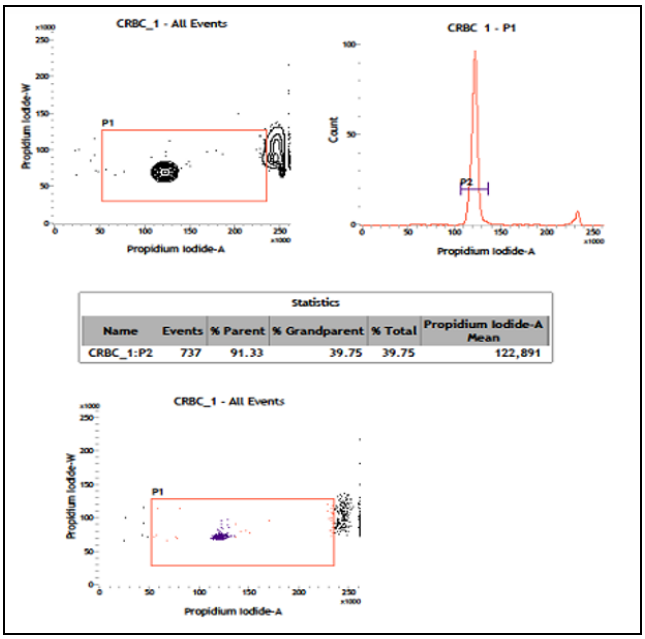
Chromosome numbers were counted in actively growing root tips of the selected Zingiberoideae species. Mature rhizomes (Roots) were collected in the morning hours at different time intervals and were pre-treated with a saturated solution of p-dichlorobenzene (3 h, room temperature), fixed in a freshly prepared 3:1 mixture of ethanol and acetic acid (4h, 4–8 °C), macerated in 1:1 hydrochloric acid/ethanol (30 s, room temperature) and immediately squashed in a drop of lacto-propionic orcein (LeongŠkorničková *et al*., 2007). Metaphase somatic chromosome numbers were determined. A chromosome count was carried out on one individual plant from each of the three to four populations. Two replicates of each individual were tested to avoid the potential errors of chromosome counting.

Results and Discussion

G.S. variation within and among *Cymbopogon martini* species of different Ecotypes.

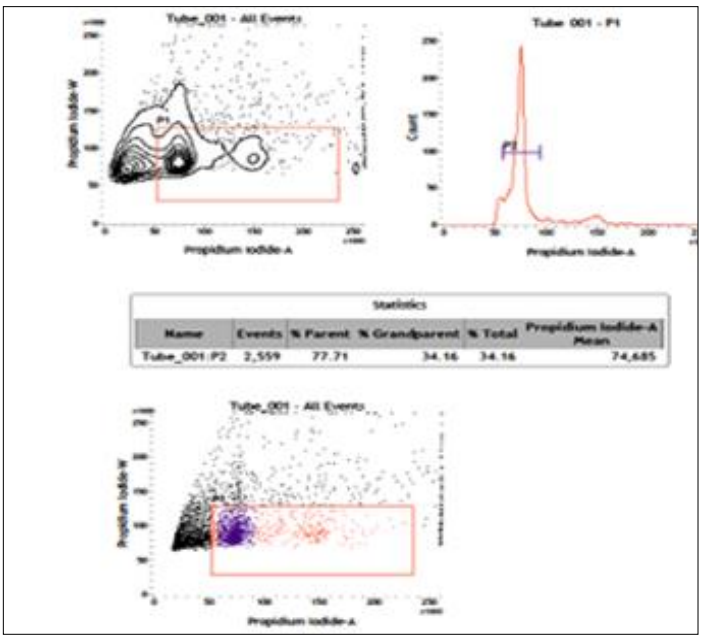
We observed negligible intra-spatial G.S. variation in C. martini of different ecotypes. E1 (1.41), E2 (2.03), E3 (3.06), E4 (2.92), E5 (2.82) and E6 (2.93).

But considerable intra-spatial G.S. variation was investigated in Cymbopogon martini from narrow geographical distribution (E1-E6) (Table 1). Histograms showing relative florescence intensity were obtained with CV of <5% against the closest standard used in current study for interspatial G.S. investigation intra spatial G.S. investigation (fig 2). The intra-spatial, G.S. for six Cymbopogon martini were listed in Table 1 along with their ploidy level for the species for which we have chromosome counts.



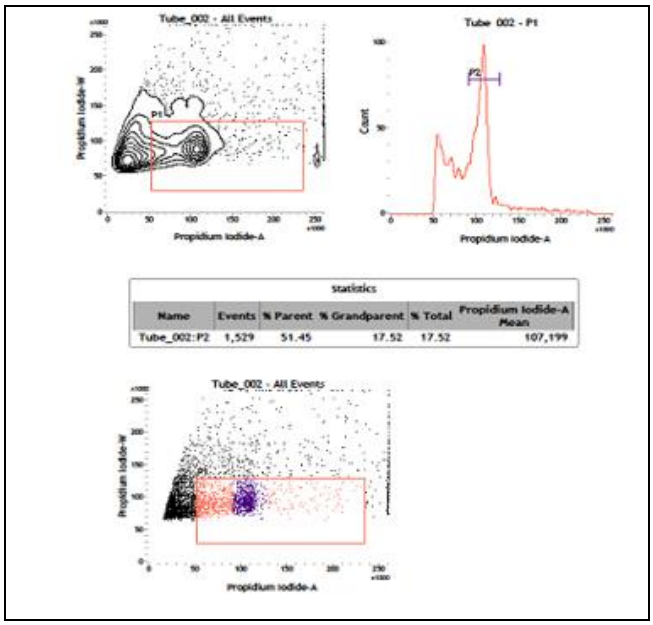
Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
CRBC_1:P2	737	91.33	39.75	39.75	122,891



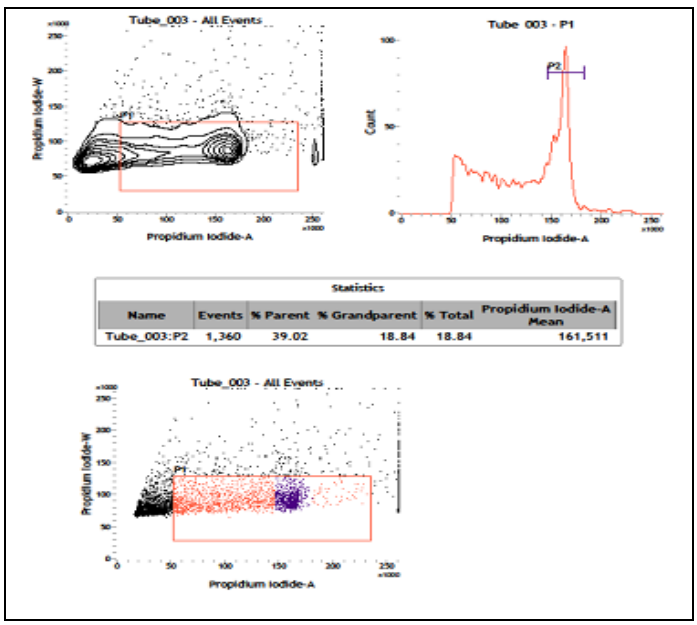
Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
Tube_001:P2	2,559	77.71	34.16	34.16	74,685



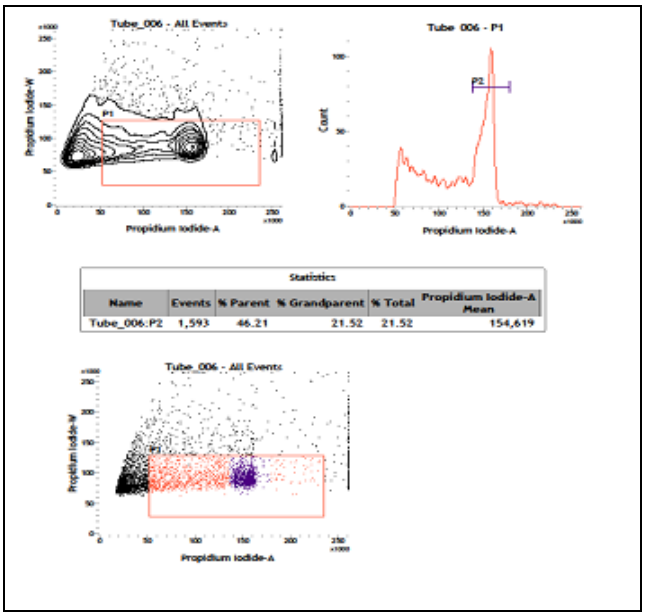
Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
Tube_002:P2	1,529	51.45	17.52	17.52	107,199



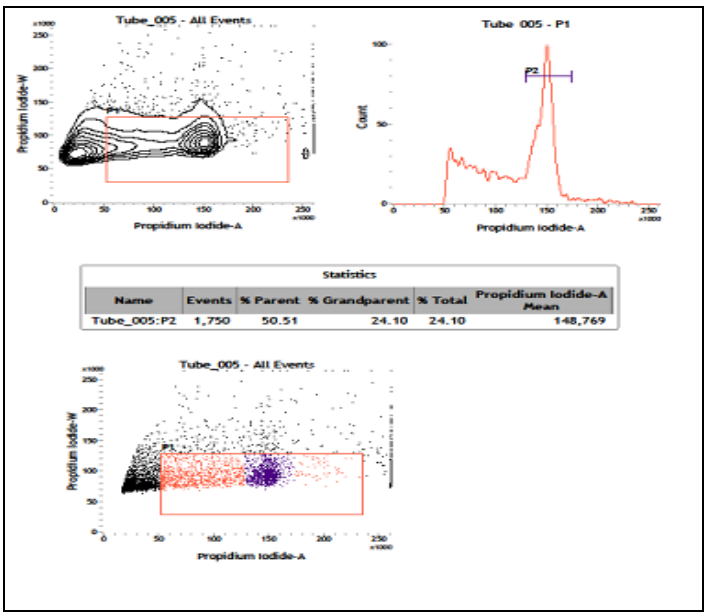
Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
Tube_003:P2	1,360	39.02	18.84	18.84	161,511



Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
Tube_006:P2	1,593	46.21	21.52	21.52	154,619



Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Mean
Tube_005:P2	1,750	50.51	24.10	24.10	148,769

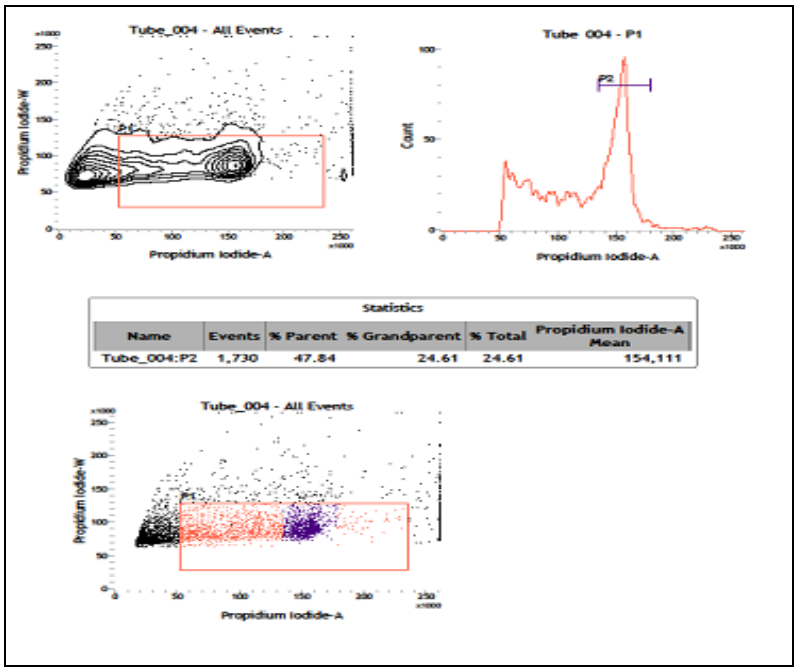


Fig 1: Histogram depicting fluorescent intensity for Cymbopogon species of different E1-E6 using internal standards. The species in the panel are a) E1, b) E2, c) E3, d) E4, e) E5 and f) E6. (Cymbopogon martini)

Somatic chromosome counts in *Cymbopogon martinii*

In this work we report chromosome number for six ecotypes of Cymbopogon martini. For investigating the chromosome number of different ecotypes, the collection time of root tips was first optimized which was found to vary between 07.00 and 11.30 am in the morning. The slides revealed will-spread metaphases in compound light microscope. The intra-spatial differences in chromosome counts were observed among the populations. The chromosome number of E1 (2n=24), E2 (2n=46), E3 (2n=56), E4 (2n=48), E5 (2n=43), E6 (2n=47).

Nuclear D.N.A. content and its correlation with chromosome number

We investigated the correlation between chromosome numbers and nuclear D.N.A. content for six Ecotypes of Cymbopogon martini for which we have the cytological data. The Cn ratio (an estimate of average chromosome size) is a species-specific parameter, which is calculated by dividing chromosomes (2n) (Leitch *et al*., 2010) [14].

Flowering plants exhibit extensive diversity in G.S. (almost 2400 fold; Pellicer *et al*., 2010) and were thus used as model organisms to test selective significance of difference in genome size (Grime,1998) [5]. G.S. is correlated with a number of physiological parameters. Previous investigations demonstrated an association between genome size and cell volume (Bonnett, 1972; Greilhuber, 1995), guard cell length and epidermal cell area (Knight and Beaulieu, 2008), the duration of the mitotic cell cycle (Van't Hof, 1965), minimum generation time (Bennett, 1972), seed size (Bennett, 1972), seed mass (Knight and Beaulieu, 2008) [10] and phenology (Grime et al., 1988) [6]. So investigating G.S., the gross phenotype of different taxa can be understood at a rapid pace. Furthermore, intra-spatial G.S. variation is active area of research for different plants (Biémont, 2008). The understanding of intra-species G.S. variation may well have implications for evolutionary theory, as for example it has been suggested that younger taxa have relatively larger intra-specific G.S. variation than older ones (Šmarda and Bureš, 2010). Moreover intra-spatial G.S. variation provdes evidence for presence of more than one taxonomic entity within a species (Murry, 2005) [19]. The humid climate of the different ecotypical conditions of Karnataka provides ample opportunity for growth of Cymbopogon species in the forest, the study will underpin the evolutionary significance of G.S. variation of Cymbopogon martini.

The prerequisite for estimation of nuclear D.N.A. content is a large population of intact nuclei. The nuclei are protected by the nuclei isolation buffer which not only protects the nucleus from degradation but also provides an appropriate environment for specific and stoichiometric staining of the nucleus, including the minimization of negative effects of some cytosolic compounds on D.N.A. staining (Loureiro *et al*., 2007) [16]. When the species was analysed along with a standard tissue sample, an increment of cellular debris was observed in microscopy and was also reflected by high CV value in the flow cytometric study. Therefore, we calculated the nuclear D.N.A. content for the experiments which produced CV<5%.

In this current work, we investigated intra and inter spatial G.S. variation (2C) and chromosome number for six populations (ecotypes) of Cymbopogon martini occurring in different locations of Karnataka, India. The average intra spatial GS variation in our study was 2.12% (E1-1.41pg), (E2=2.03pg), (E3=3.06), (E4=2.92), (E5=2.82) and (E6=2.93) the mechanism of low intra-spatial GS variation in Cymbopogon martini (average….in our case) is still unknown (Leong-Skornickova *et al*., 2007). This understanding will also advance knowledge of improvement of the species of Karnataka, India and indirectly it will make our economy stronger. Our study also provides the research question despite its high ploidal scale among six ecotypes of Cymbopogon genera investigated. In future, this question need to be investigated which will unravel the mechanism of genome size reduction from *Cymbopogon martinii*.

Table 1: Intra species variation of G.S. among six ecotypes of *Cymbopogon martinii*

Eco types	Location	Intra-species variation	1C (pg)	Ploidy level	Chromo some number
E1	Himavadgopala hill, Chamarajanagar.	2.42	0.7	2x	24
E2	Siddarabetta, Tumkur, Karnataka	2.26	1.01	4x	46
E3	Chikkaballapura	1.48	1.53	6x	56
E4	Dharwad University, Dharwad	3.01	1.46	4x	48
E5	Davanegere	2.43	1.41	4x	43
E6	Koppa, Shivamogga	2.51	1.46	4x	47

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