Estimating Genome Size and Ploidy of Acacia Species in Pakistan Based on Stomata Size and Flow Cytometry

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ABSTRACT

Pakistan is inherently deficient in forests and recently high rate of deforestation, overexploitation, illegal logging, and climate change further exacerbated the forest cover. Arable land is mainly used for crop production therefore forest cover can be increased through afforestation and reforestation of marginal or deserted lands. Acacias are multifunctional and known for a wide adaptation to marginal and deserted lands. However, acacia species are known to naturally hybridize, and reports of natural polyploids have been made. Thus, knowledge of the ploidy of focal species is essential for programs including afforestation, reforestation, polyploidization, hybridization, and mutation breeding. The goal of this work was to identify the ploidy level of acacia species found in Pakistan based on stomatal characteristics and genomic DNA contents and compare both approaches to find the most economical and reliable ploidy detection method. The ploidy of the species under investigation was accurately determined by both approaches, according to the results, when stomata length, width, and genomic DNA were compared to previously published data. It had been shown that the 2C-value for genomic DNA content, stomata length, width, and diploid status of each investigated species varied significantly from one another. All species had stomata present on both adaxial and abaxial leaf surfaces except *A. modesta*. *A. modesta* may have low transpiration losses as compared to other species and therefore may be a preferred species in extreme dry conditions of Pakistan. The stomata's length varied between 7.83 and 31.5 µm, while their breadth varied between 5.08 and 22.67 µm. *Acacia radiana* possessed the longest and widest stomata, while *A. nilotica* had the shortest and narrowest. *A. catechu* had a tiny genome while *A. auriculiformis* had a large genome, with genomic DNA ranging from 1.022 pg to 1.523 pg.

According to this study, both methods for determining the ploidy of acacia species can efficiently be used to identify polyploids. Nevertheless, stomata length and width proved technically less demanding and cheaper method, thus may be a method of choice for ploidy determination in acacia. Flow cytometry, on the other hand, has the greatest advantage of providing a direct and high throughput assessment of DNA but is technically more demanding and expensive.

Keywords: *Acacia., Deforestation., Afforestation., Reforestation., Ploidy., Stomatal characters., Flow Cytometry*

Introduction

Pakistan has always had an energy shortage and is mostly dependent on fossil fuels (Mukhtar, 2023). Pakistan's economy is being troubled as the power-generating sector is coming under more strain due to rising oil prices and increased demand (Mukhtar, 2023). Numerous and protracted power outages prompted the local industry, particularly the textile sector to move to neighboring countries. Local towns experienced a severe economic crisis because of this change. As a result, the Pakistani government committed $340 million to the energy development sector in 2014–2015 to meet the rising demand. Power generation from solar, biomass, and biogas was prioritized (http://www.trust.org/item/20140717081934-6sjf2). While energy sources like wind, solar, and biogas have shown great promise, building the facilities and infrastructure required will be expensive. In Pakistan, biomass is extensively accessible and offers a reasonably priced energy source. Every day, nearly a million tonnes of animal manure, 225,000 tonnes of agricultural residue, and around 50,000 tonnes of solid trash are created. It is thought that adding electricity produced by biomass energy to the national grid can drastically lessen the country's electricity deficit (http://www.reegle.info).

Pakistan is primarily an arid or semi-arid nation where most of the irrigated land is used for crop production. Although it takes up over 5% of the total area, the percentage of forest cover has been steadily declining since 1990, reaching just 1.9 percent in 2015 (World Bank, 2015). The unsustainable use of forests to suit domestic energy requirements is the primary cause of this decline. Degradation and depletion of forests are hence Pakistan's biggest problems. One of the most significant tree species is the acacia, which has a very broad range of adaptation and the capacity to flourish in harsh climates. In addition, they yield pulp and good fuelwood with a very high calorific value. They can grow in marginal areas and in extremely dry

conditions, have a propensity to fix nitrogen, and are essential for the process of afforestation in Pakistan's dry regions (Asif et al., 2017). When tree populations are established in arid and semi-arid areas, the amount of forest area increases, and more vegetation can be grown there than would otherwise be possible. Their strong realization in growing commercial plantations to fulfill fuelwood and pulp demands has made them an important species. But acacias also have some unfavorable traits such as poor biomass, stem forking, disease susceptibility, and high tannin levels (Asif et al., 2017., Asif et al., 2021). It is therefore pertinent to develop genotypes with desired features for afforestation and reforestation programs in Pakistan.

Among the most significant and varied species in Pakistan is *A. nilotica*. Nine subspecies have been identified in the Indian subcontinent, and it is genetically diverse because of spontaneous hybridization (Ali and Faruqi, 1969; Khatoon and Ali 2006; Varghese et al, 1999). Five of these nine subspecies are found in Pakistan (Ali and Faruqi, 1969). Comparably, several exotic species, including *A. senegal*, *A. catechu*, *A. albida*, *A. pendula*, *A. radiana*, and *A. auriculiformis*, have been introduced into Pakistan. It is well known that acacias naturally hybridize, and numerous polyploids have been observed in the field (Ali and Faruqi, 1969; Blakesley et al., 2002; Khatoon and Ali, 2006). For this reason, knowing the ploidy level is crucial for the selection of genotypes with desirable characteristics for afforestation and reforestation programs. Similarly, for polyploidization to help increase biomass, is acknowledged as one of the most useful tools in breeding methods. It has also been used to control invasiveness (Odee et al., 2015).

An efficient chromosome counting technique is crucial if polyploidization and breeding programs are to be utilized to create desired genotypes in the acacia species due to its variable ploidy levels and self-incompatibility (Bennett and Leitch, 1995; Kenrick and Knox, 1989). The traditional method of determining ploidy involves counting the chromosomes in root tip cells that have undergone mitosis. Because tree species have hard root tissues and small chromosomal sizes, this is especially labor-intensive and time-consuming in the case of tree species (Mukherjee and Sharma, 1993). The number of stomata, stomata length, and the number of chloroplasts can be utilized as an indirect technique to determine ploidy (Beck et al., 2003; Tan and Dunn, 1973). In *Acacia mearnsii*, stomatal length has been successfully utilized to differentiate between diploid and tetraploid genotypes (Beck et al., 2003). The most

accurate and straightforward technique for determining ploidy is flow cytometry, which uses a fluorescent dye to label DNA, making it a more robust and sensitive tool (Asif et al., 2001; Dolezel et al., 1992; Habibi et al., 2023). The purpose of this study was to compare the two approaches and then utilize the results to ascertain the genotype ploidy level of several Pakistani acacia species.

Materials and Methods

Plant materials were collected from the Punjab Forest Research Institute (PFRI) arboretum located in Faisalabad, Pakistan. Leaf samples from eight species i.e., *A. modesta, A. auriculiformis, A. pendula, A. radiana, A. senegal, A. catechu, A. albida and A. nilotica* aged between 4 - 5 years were collected in triplicates.

Stomata Observation

For stomata observations, three fully grown leaves were taken from three distinct trees belonging to each species. Using the impression method, stomatal length (SL) and stomatal width (SW) were determined (Wang and Clarke 1993a). Impression stomata were obtained from the midsection of fully grown leaves (Teare et al., 1971). Prior to the stomach examination, a drop of Lugol stain (1:3, Iodine: KI) was applied to a glass slide. At 400 times magnification, ten distinct microscopic fields of view were used to measure the length and width of the stomata. 10 stomata were randomly measured for each impression, and statistical analyses were performed using the mean values of the ten measurements.

DNA Flow Cytometry

Ploidy level was ascertained directly from genomic material using flow cytometry. An analysis using flow cytometry was performed at the Institute of Experimental Botany in the Czech Republic. For the ploidy analysis, three trees from each species were employed.

Determination of Genomic DNA content

A Partec PAS II, Flow cytometer (Partec GmbH, Münster, Germany) fitted with a mercury lamp under high pressure was used to quantify the genomic DNA contents. To calibrate FCM, chicken red blood cells (CRBC) were utilized. Using the fixed CRBC, FCM was calibrated to provide the highest signal amplitude and the lowest CV (1-2 percent) upon staining. Every sample was examined three times. Doležel et al. assessed the content of nuclear DNA (2007). Using a sharp razor blade, whole leaf tissues from both the sample and the internal reference standard (Lycopersicon esculentum; 2C = 1.96 pg) were diced together and placed in a glass petri dish with 500 µl Otto I solution (0.1M Citric acid, 0.5 percent v/v Tween 20) (Doležel et al., 1992). One 50 µm nylon mesh filter was used to filter the crude suspension. 1 ml of Otto II solution (0.4M Na2HPO4) supplemented with 50 µg/ml RNase and 50 µg/ml propidium iodide was added after the mixture had been incubated on ice for 15 minutes (Otto, 1990). After adjusting the flow cytometer's gain, the internal reference standard's G1 peak emerged on channel 100. For every sample, at least 5,000 nuclei were examined. This is how the amount of nuclear DNA in (pg) was estimated:

$$\text{Sample 2C DNA (pg)} = \frac{\text{Peak mean of sample}}{\text{Peak mean of standard}} \times \text{2C DNA content of a standard}$$

The *"agricolae*" package included in "R" was used to analyze the variation in stomata size and genomic DNA content using analysis of variance (ANOVA) and Duncan's multiple range tests (DMR) (R Core Team, 2017).

Results

All acacia species, except for *A. modesta*, have amphistomatous leaves, or stomata on both sides of the leaf. The abaxial, or lower, surface of the leaf of *A. modesta* had stomata. The stomata's

breadth varied between 5.08 and 22.67 µm (Table 2). There was no variability in ploidy level among the species, although the stomata widths of the several species varied significantly (Tables 1 and 2). *A. modesta*, *A. pendula*, *A. auriculiformis*, and *A. radiana* had the largest stomata whereas *A. catechu*, *A. nilotica*, and *A. senegal* had the smallest stomata respectively (Table 2). Length of stomata varied from 7.8 to 31.5 µm (Table 4). In a similar vein, stomata length varied between species but did not change within them (Table 3). Like stomata width, *A. radiana* possessed the longest stomata, with *A. auriculiformis* and A. pendula following suit (Table 4). *A. albida*, *A. nilotica*, *A. catechu*, and *A. senegal* were found to have short stomata (Table 4).

By comparing the relative fluorescence intensity of nuclei obtained from young leaf tissues, the genome sizes of all acacia species were analyzed (Fig. 1). Because of cell wall debris or non-specific staining of other cell components, the first peak that was seen reflected the debris background. Two peaks were seen i.e., the sample peak was located at channel 50, and the internal standard i.e., *Lycopersicon esculentum* with known DNA content was represented by the second peak, which was located at channel 100 (Fig. 1). To improve accuracy, ploidy analysis often uses young, fresh leaf samples. However, *A. nilotica* and *A. albida* leaf samples were not included for additional analysis since they were degraded and produced very high coefficients of variation (CV). Propidium iodide (PI), a fluorescent dye that intercalates to the whole DNA in contrast to AT-specific binding DAPI (3, 5 - dinitro -N, 4, N - dipropylsulphate), was used to label the nuclei. A mean of 5,000 nuclei underwent genetic content screening. More than 90% of the nuclei were in the G1 phase, according to the examination of the distribution of nuclei. The standard deviation varied between 1.6% and 3.5%. The range of the 2C nuclear DNA was 1.02 to 1.52 pg (Table 6). There was significant

heterogeneity in the genetic contents of different species, but little change in the genotypes within a species (Table 6). All species could be divided into five groups: A. *auriculiformis* had the highest 2C DNA content, followed by *A. senegal*; two species had similar DNA content, *A. radiana* and *A. pendula*; *A. modesta* was the fourth; and *A. catechu* had the lowest genomic DNA level (Table 6).

Discussion

Acacia is known for its natural hybridization, and numerous hybrids with varying ploidy levels have been found in *A. nilotica* and *A. senegal* (Blakesley et al., 2002; Diallo et al., 2016; Khatoon and Ali 2006; Odee et al., 2015). Traditionally, the direct measurement of ploidy level is achieved by counting the number of chromosomes in quickly expanding root tips. Nevertheless, this approach is laborious and has a limited throughput in species with tiny chromosomal sizes. As an alternative, highly accurate indirect ways of determining ploidy level have also been used, primarily based on stomata frequency, size, and quantity of chloroplasts (Camargo and Marenco 2011; Diallo et al., 2016; Tripathi and Mondal 2012). There is a documented favorable link between growing ploidy levels and indirect approaches (Blakesley et al., 2002). A direct technique called flow cytometry is based on the precise measurement of the amount of genomic DNA labeled with a fluorescent dye that is directly proportionate to the number of chromosomes (Dolezel et al., 1992). Feulgen densitometry is another technique for determining the ploidy and genomic DNA content, but it is currently rarely utilized due to reports of its decreased accuracy (Das et al., 1998). The results of the stomata size study showed that, except for *A. modesta*, all species had stomata on both the abaxial and adaxial leaf surfaces. Due to its adaptation to a lower transpiration rate, *A. modesta* is primarily found in arid and hot climates, like *A. senegal* and *A. nilotica*. For this reason, it

is a desirable species in Pakistan's arid regions. Both *A. radiana* and *A. auriculiformis*, which have large stomata, are found in tropical or heavy rainfall regions. Although the stomata of different acacia species varied according to their evolutionary background and environmental adaptation, there was no variation in the genotypes of any acacia species, indicating the same ploidy level. The length of the stomata varied between 7.83µm and 31.5µm. Similar stomatal lengths were noted by Tripathi and Mondal (2012) for diploid acacias like *A. auriculiformis* (22.54) and *A. mangium* (33.91), respectively. *A. mearnsii's* ploidy level was ascertained by Beck et al. (2003) using known diploid (2n = 2x = 26) and tetraploid (2n = 2x = 52) DNA. For diploids, the mean stomatal length was 27.17 ± 0.474 µm, but for tetraploids, it was nearly twice as long (40.24 ± 0.521 µm). They found a strong, positive connection between ploidy level and stomatal length. They concluded that *A. mearansii's* ploidy could be reliably determined indirectly using stomatal length and frequency. The study's observations of stomatal length in all acacia species are consistent with the stomatal size previously documented in diploid acacia species (Beck et al., 2003; Tripathi and Mondal 2012). Consequently, it is hypothesized that every genotype examined in our investigation was diploid.

A straightforward technique for figuring out ploidy based on the total amount of genetic material in each cell is flow cytometry. The six species under investigation had total genetic content ranging from 1.02 to 1.52 pg. The 2C value of the genomic content matched that of Bukhari (1997), who proposed that the tetraploid acacia species had a genomic content of 3.309 pg, about twice as much as the diploid species, and that the genomic content of diploid species ranged from 1.17 pg to 1.583 pg. The genomic DNA concentration of six diploid acacia species ranged from 1.15 pg to 1.142 pg, which was close to what Das et al. (1998) observed. Based on the earlier research, it was safe to presume that all genotypes belonging

to various species were diploid. At the interspecific level, notable variations were detected, which was consistent with earlier findings (Bukhari, 1997; Das et al., 1998; Odee et al., 2015). The loss or acquisition of highly repetitive DNA sequences in a genome, which at some threshold level solidify throughout microevolution and progressive selection, has frequently been linked to the genomic DNA variability among different species (Das et al., 1996; Price et al., 1980). Therefore, it can be said that all the species and their genotypes were diploids. This means that they can be used in genetic studies or future breeding programs. It is recommended that stomatal characterization and differentiation be used as a preferred method for ploidy determination in acacia species as it is a simple, cheap, and straightforward method as compared to technically demanding and expensive flow cytometric analysis.

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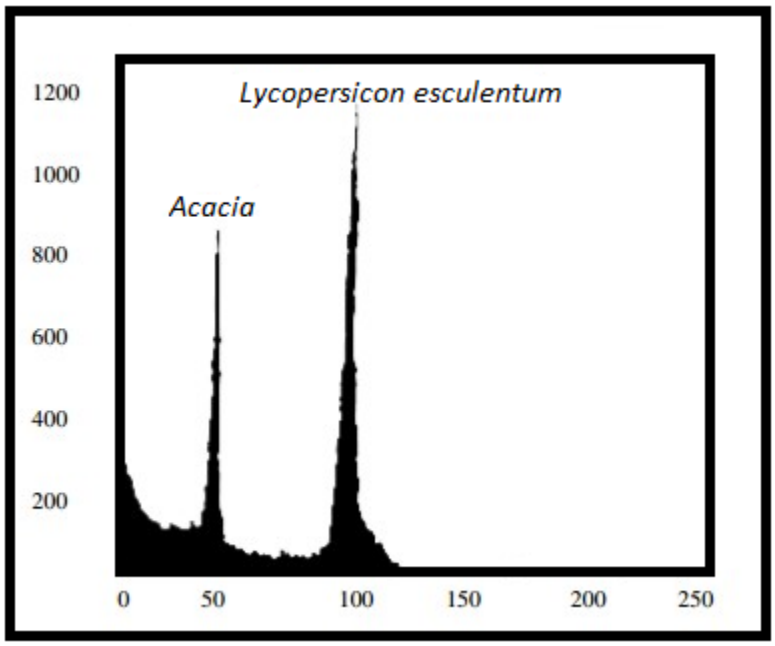

Figure 1. Uniparametric histogram of fluorescence intensities of the nuclei from acacia species and the *Lycopersicon esculentum* an internal standard after staining with propidium iodide. Histogram showing the DNA content of acacia forms a large peak at channel 50 compared to the internal standard (*Lycopersicon esculentum*) that appeared at channel 100.

Table 1. Stomatal width was significantly variable among the *Acacia* species.

Sources of Variation	DF	Mean Sum of Square	F-Value	Prob(>F)
Species	7	737.8	129.6	<2e-16***
Residuals	72	5.7		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Table 2. Comparative mean analysis of Acacia species based on stomatal width. Mean sharing similar letters is not significantly different.

Treatment	Group	Means
A. radiana	a	22.67 ±1.53
A. pendula	b	14.0 ±1.53
A. auriculiformis	b	13.17±1.38
A. modesta	b	12.5 ±1.86
A. albida	c	7.33 ±1.23
A. catechu	d	5.15 ±1.12
A. nilotica	d	5.10±1.35
A. senegal	d	5.08 ±1.2

Table 3. The analysis of variance for stomatal length of acacia species. The stomatal length was significantly different among the species.

Sources of Variation	**Df**	**Mean Sum of Square**	**F-Value**	**Prob(>F)**
Species	7	388.7	199.1	<2e-16***
Residuals	72	2.0		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Table 4. Comparative mean analysis of *Acacia* species based on stomata length. Mean sharing the same letters is not significantly different.

Treatment	Group	Means
A. radiana	a	31.50 ±2.88
A. auriculiformis	b	21.8±2.65
A. pendula	b	21.0±2.62
A. modesta	c	16.0 ±3.61
A. albida	d	10.2±1.47
A. senegal	d	8.33 ±1.76
A. catachu	d	8.33±1.57
A. nilotica	d	7.8±1.58

Table 5. Comparison of 2C nuclear DNA content between different *Acacia* species.

Sources of Variation	**DF**	**Mean squares**	**F-value**	**Pr (>F)**
Species	5	0.093	163.2	1.33e-10 ***
Residuals	12	0.00057		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Table 6. 2C nuclear DNA content, the genome size of Acacia species, and grouping based on DMRT, species sharing the same letter have similar DNA content.

Species	Chromosome number (2n)	Mean	Grouping
A. auriculiformis	26	1.52±0.016	a
A. senegal	26	1.46±0.013	b
A. radiana	26	1.37±0.015	c
A. pendula	26	1.34±0.035	c
A. modesta	26	1.27±0.033	d
A. catechu	26	1.02±0.024	e