Open Access Library Journal, 2014, 1, 1-7
Published Online June 2014 in OALib. http://www.oalib.com/journal
http://dx.doi.org/10.4236/oalib.1100404

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Research on Chromosome Karyotype Analysis of *Plumbago auriculata*

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Received **** 2014

Abstract

In this study, the chromosome karyotype of *Plumbago auriculata* was analyzed by using a chromosome mounting technique with the primary root tip of seed germination. The aim was to find out the most suitable method for *Plumbago auriculata* by comparing the effect of different pretreatment and dissociation time on chromosome. The results showed that the best pretreatment was 0.05% colchicine solution under 0˚C - 4˚C or 2 h and then 1 mol/L HCL under 60˚C water for 6 min. Karyotype analysis of *Plumbago auriculata* showed that the chromosome number was 12 and the karyotype formula was 2n = 2x = 12 = 8 m (4 sat) + 4 sm. The karyotype belonged to 2B and the karyotype asymmetry coefficient was 59.41%. The absolute average length of chromosome was 0.40 μm and the average arm ratio was 1.54. The chromosome of *Plumbago auriculata* belonged to minichromosome, including a couple of SAT-chromosomes.

Keywords

***Plumbago auriculata*, Karyotype Analysis, SAT-Chromosomes, Minichromosome**

1. Introduction

Plumbago auriculata is a sort of perennial evergreen climbing subshrub which is classified to Plumbaginaceae, Plumbago Linn. It also called blue snowflakes. It is native to Southern Africa and widely dispersed in tropical and subtropical areas. Now in China, *Plumbago auriculata* mainly distributed in South China, such as Yunnan, Sichuan, Guangdong, Taiwan [1]. It has light blue flowers crowded and its branches like a ball, which often gives a unique feeling of refreshing and quiet.

Plumbago auriculata has a large amount of annual growth in Sichuan region, and it can blossom in the year of sowing. Its attractive light blue-colored flowers are extremely rare in nature, which are extraordinarily eye-catching in floral design and have a strongly visual impact like a Blue Wizard jumping in the jungle. *Plum-*

bago auriculata has greatly enriched the color system of landscape plant in the southwest of China, which will have a very broad application and the market prospect. Therefore, it seems particularly urgent to study on identification and classification, cultivation and breeding of *Plumbago auriculata* varieties. At present, there is little report about *Plumbago auriculata* which is limited to introductions of garden cultivation technology and ornamental characteristics [2] [3], but its cytogenetics and chromosome karyotype are rarely reported, especially in the genetic mechanism field. This experiment used the primary root of seed of *Plumbago auriculata* as the material to analysis its chromosome karyotype and improve conventional chromosome preparation method, which in order to provide a theoretical basis for the study of its system evolution, genetic trend, cultivation and breeding, as well as the further reveal of the genetic mechanisms of this species.

2. Materials and Methods

2.1. Materials

In this study, the seeds of *Plumbago auriculata* were obtained from the scientific research and teaching base of Sichuan Agricultural University, placed in a petri dish lined with a double layer of filter paper in thermostat under 37˚C and keep the filter paper moist.

2.2. Methods

2.2.1. Pretreatment

When the root tip of seed germinated in 2 - 3 mm, cut off the primary root and put in 0.05% colchicines at 0˚C - 4˚C for 2 h, 4 h, 6 h.

2.2.2. Fixation

Wash the pretreated root tip with double distilled water, then fix them with Carnoy's solution I (V ethanol:V acetic acid = 3:1) for 24 h at 4˚C.

2.2.3. Low Permeability and Dissociation

After the root tip was cleaned, put it in petri dish with double distilled water and hypotonic culture for 3 - 5 min, then dissociate in the water bath at 60˚C using 1 mol/L HCL for 3 min, 6 min, 10 min, respectively. And then, hypotonic culture for 8 min in petri dish with double distilled water after repeated douche, changing water once or twice in the middle of the process.

2.2.4. Staining and Tabletting

Place the root tip on a glass slide, cut down some cells of the root tip with a dissecting needle gently, and stain with carbol fuchsin solution for 3 min. Then, pounding the tissue by dissecting needle and adopted traditional chromosome tabletting technique.

2.2.5. Microscopic Examination

Observe the tabletting with a microscope at 10 × 100 magnification, take pictures and store the better metaphases, and count the metaphase cells in per unit sight area.

2.3. Karyotype Analysis Method

Select 50 metaphase cells which chromosomes were clear and well dispersed from the best tablettings to count its chromosomes number. If there were more than 85% of the cells with constant and consistent chromosome number, which is chromosome number of *Plumbago auriculata* [4]. Choose 5 cells which chromosomes dispersed well and centromere clear to do picture processing with Photoshop CS [5], and measure chromosome length with Image ProPlus software. Using the criterion described by Maoxue Li to perform karyotype analysis. According Levan nomenclature to identify the position of the chromosome centromere [6], chromosome karyotype classification and arm ratio calculation according to Stebbins [7], calculation method of chromosome index of relative length (I.R.L) reference from Kuo [8] and karyotype asymmetrical coefficient (As·K) for Arano [9].

3. Results and Analysis

3.1. Optimization Technology of Chromosome Preparation

3.1.1. Materials

The parts with tillering capacity can be used to do chromosome preparation [10], such as root tips [11] [12], shoot tips [13], plumule [14], tender leaves [15], tissue culture callus [16] and pollen mother cells [17]. As a woody perennial plant, the new roots of mature plant of *Plumbago auriculata* were tender but not appropriate for karyotype analysis. The experiment showed that the new young root tips differentiated many fiber cells and not conducive for chromosome observation.

Drawing materials experiment showed that at 8:00 - 9:00 am, the primary root had metaphase cells more than 80% [18]. Secondly, the optimal length of primary root was 2 - 3 mm, some cotyledons can be cut together to avoid root tip been damaged for improper operation when dissociation later and affect observation on chromosomes.

3.1.2. Pretreatment

The results showed that the optimum pretreatment time was in 0.05% colchicines at 0˚C - 4˚C for 2 h, and can obtain clear and well dispersed chromosomes of metaphase cells. By contrast with 4 h or 6 h pretreatment, the chromosomes were bad dispersed and excessive contraction that had to measure the length of the arms (**Table 1**).

3.1.3. Dissociation

The results revealed that dissolved in the water bath at 60˚C with 1 mol/L HCL for 6 min, cell wall shed off completely and cell dispersity was best. But dissolved for 3 min, root tip was not softened enough, so cells arranged more closely, squash tabletting cannot easily make the cells in the same plane, which bring difficulty to later microscopic examination. However, when dissolved for 10 min, the cells were over softened and achromophilous, chromosomes were over dispersed that not conducive to figure, and affect the accuracy of karyotype analysis (**Table 2**).

3.2. Chromosome Number and Karyotype Analysis of *Plumbago auriculata*

Chromosome Number

Select 50 well dispersed cells of metaphase chromosomes for statistics. There were 44 cells contain 12 chromosomes occupied 88% in total (**Figure 1**), 4 cells contain 13 chromosomes occupied 8% and 2 cells contain 14 chromosomes occupied 4%. Maoxue Li pointed that if more than 85% of the cells with constant and consistent chromosome number, it will be the chromosome number of the plant [4]. According to this, chromosome number of *Plumbago auriculata* is determined as 2n = 12 (**Figure 2**).

Table 1. Comparison of different pretreatment time.

Pretreatment time	Number of clear chromosomes of metaphase cells (in 50 cells)	Dispersity	Contractility
2 h	43	Better	Good
4 h	38	Good	Excessive contraction
6 h	37	Poor (reunion distribution)	Excessive contraction (point shape distribution)

Table 2. Effect of different dissociation time.

Dissociation time	Root tip soften degree	Dyeing effect	Resolving effect
3 min	Cells arranged closely	Remnant dyeing vestige in cell wall, dyed light	Cells were not in the same plane, difficult to observe
6 min	Good	Good	Cell wall shed off, clear
10 min	Over softened, cells scattered	Poor	Chromosome dyed light or not dyed, difficult to observe

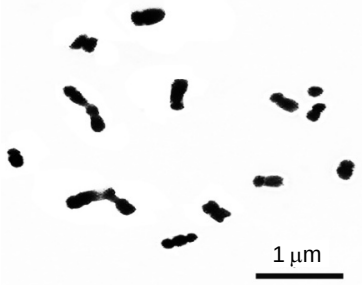

Figure 1. The chromosome morphology in *Plumbago auriculata*.

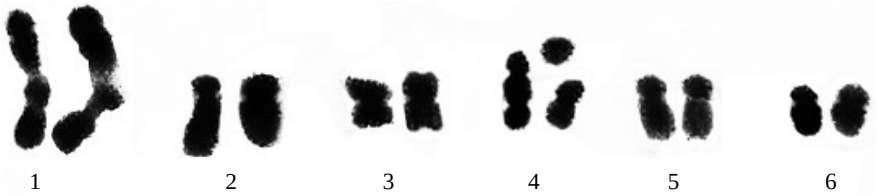

Figure 2. Karyotype of chromosome in *Plumbago auriculata.* Note: The numbers are chromosomes serial number.

3.3. Karyotype Analysis of *Plumbago auriculata*

3.3.1. Karyotype Formula

Based on plant karyotype analysis criteria proposed by Maoxue Li and Ruiyang Chen, choose 5 clear and well dispersed chromosome pictures to accurately measure chromosome average length by using Image ProPlus. Calculate arm ratio (long arm/short arm) of each chromosome, ascertain the position, form and index of the centromere. In most cases, absolute length is not reliable, for different pretreatment conditions may cause varying degrees of chromosome contraction, and sometimes absolute length may be different in the same plant or measured by the same group of laboratory technician [18]. But relative length, arm ratio, centromere index are relatively stable. The calculating methods were as follows: relative length = chromosome length/genome total length × 100%, arm ratio = long arm/short arm, centromere index = short arm length/chromosome length × 100%.

3.3.2. Karyotype Analysis

Table 3 shows that absolute length range of *Plumbago auriculata* chromosome is 0.26 - 0.56 μm, the average length of total genome is 0.40 μm, according to Li Maoxue, it will be minute chromosome if its absolute length of body cell less than 1 μm [4]. And relative length range of chromosome is 10.88% - 23.43%, relative length constitution is 2n = 12 = 4L + 6M1 + 2S, the result indicated that *Plumbago auriculata* is homologous diploid plant. The centromere index range is 30.36% - 48.72%, arm ratio range is 1.05 - 2.29, the ratio of the longest chromosome to the shortest one (Lt/St) is 2.15, between 2:1 and 4:1, the arm ratio of 33.33% chromosomes is over 2, so that the karyotype is 2B and less asymmetrical type according to Stebbins [7], and the chromosome karyotype asymmetry index is As·K% = 59.41, the karyotype formula is 2n = 2x = 8 m (4 sat) + 4s m. Chromosome 2 and 5 are median region(m), chromosome 1,3,4,6 are median point, there are satellites on the chromosome 1 and 4, however, both areintercalary and satellites at the end of short arm are on the chromosome 1 (**Figure 3**).

4. Discussion

4.1. *Plumbago auriculata* Classification, Chromosome Number and Analysis of System Evolution

Plumbago auriculata classified to *Plumbaginaceae*, *Plumbagineae*, *Plumbago Linn*, but it is usually called "*Ceratostigma plumbaginoides bunge*" in Sichuan, Chuan. Actually, *Plumbago auriculata* and *Ceratostigma*

Table 3. Karyotype parameters of chromosome of *Plumbago auriculata.*

Chromosome serial number	Absolute length of chromosome (long arm + short arm) (μm)	Relative length of chromosome (long arm + short arm) (%)	Centromere index (%)	Arm ratio (long arm/short arm)	Type
1*	0.30 + 0.26 = 0.56	12.55 + 10.88 = 23.43	46.43	1.15	m (sat)
2	0.39 + 0.17 = 0.56	16.32 + 7.11 = 23.43	30.36	2.29	sm
3	0.20 + 0.19 = 0.39	8.37 + 7.95 = 16.32	48.72	1.05	m
4*	0.16 + 0.15 = 0.31	6.69 + 6.28 = 12.97	48.39	1.07	m (sat)
5	0.21 + 0.10 = 0.31	8.79 + 4.18 = 12.97	32.36	2.10	sm
6	0.16 + 0.10 = 0.26	6.69 + 4.18 = 10.88	38.46	1.60	m

*The chromosomes that had satellites (the length of satellite was not included); m means metacentric chromosome; sm means submetacentric chromosome.

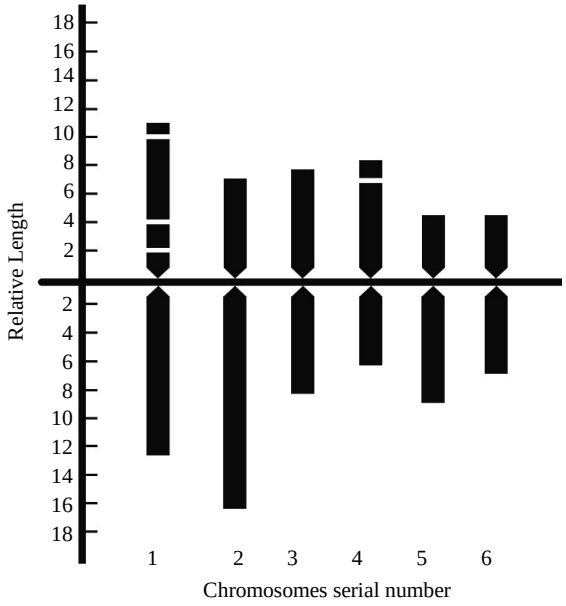

Figure 3. Karyotype pattern of chromosome in *Plumbago auriculata.*

plumbaginoides bunge are the same tribe but different genus. The mainly difference are whether calyx have glands. The calyx of *Plumbago auriculata* covered with glands, and *Ceratostigma plumbaginoides bunge* is glabrous and without glands [1]. But it is difficult to provide a reliable basis for the classification only by the morphological method [10]. The results revealed that the chromosome number of *Plumbago auriculata* was 2n = 12, this was different from the *Plumbago Linn* basic chromosome number x = 7 or x = 8 according to Flora Reipublicae Popularis Sinicae [1], the reason may be related to chromosome morphology and structure.

In this experiments, we found that chromosome of *Plumbago auriculata* was not only micro, but also had complex structure that there were satellites at the end and mediacy on the same chromosome. This brought more difficulty to microscopic examination and measurement under the conventional conditions. During the chromosome preparation, if the chromosome could not unfold completely or fractured in the constriction area, the chromosome number may be considered as 2n = 14 by mistake. That was the reason we observed 13 or 14 chromosomes in metaphase cells many times.

The first chromosome had both terminal and intercalary satellites. When add them to chromosome length, the longest and shortest chromosome ratio was 3.35:1. This indicated that karyotype asymmetry will be greater if consider the length of satellite. Generally, the basic trend of karyotypic evolution is from symmetry to asymmetry, so most plants in ancient or primitive are symmetry, and the asymmetry ones commonly in deuterogenic, specialized, majority evolutionary phytogroup [19]. This also showed that the evolution trend of *Plumbago au-*

riculata was advanced to some extent.

4.2. Organ Shape and Chromosome Karyotype Analysis of *Plumbago auriculata*

Field experiment results indicated that *Plumbago auriculata* existed self-sterile phenomenon. Anatomic observation of the flower showed that it did exist length inconsistency of stamens and styles. At present, there are two main forms of floral organ, one is corolla longer than style and shorter than stamen, the other is corolla shorter than style and longer than stamen. Both of organs can not self-fertilize, this confirmed the statement of Flora Republicae Popularis Sinicae that no mature fruit can be found on *Plumbago auriculata* [1]. But when taken mixed experiment of the different floral organ, we found it fruit well. On the basis of survey and research on heterostyly phenomenon and pollinator, Ferrero [20] believes that different morphology of flower organs (different flower arrangement, style length, etc.) may affect foraging path of the pollinators, thus determines the mating results, and the morphological structure is connected with genotype. Results obtained in karyotype analysis indicated that there was minichromosome on the first chromosome. Generally, minichromosome on chromosome always lead plant abortion [10]. Hongxia Wang [21] in the study of *Lilium casa blanca* explained that structure variation of homologous chromosomes always cause by minichromosome between the secondary constriction and centromere, so we infer that self-sterility of *Plumbago auriculata* may related to chromosome morphology. But in order to reflect differences in genome and chromosome structure accurately, its specific genetic mechanism require experimental result of chromosome banding pattern analysis of different style sample length, gene analysis, pollen vigor and stigma receptivity identification etc. It requires wider and deeper study and discussion.

5. Conclusion

Plumbago auriculata is a diploid plant; its chromosome number is 2n = 12, which is different from the *Plumbago Linn* basic chromosome number x = 7 or x = 8 according to Flora Reipublicae Popularis Sinicae, and its karyotype formula is 2n = 2x = 8 m (4 sat) + 4 sm. Two of the chromosomes exist satellites, and one of them is intercalary and has satellites at the end together, which is its special chromosome morphological characteristic and the basis of species classification and scientific breeding, and will contribute to the judgment and analysis of species genetic relationship, and reveal the genetic evolution process and mechanism. The chromosome of *Plumbago auriculata* belongs to minichromosome, and its karyotype is 2B.

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