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Evaluation of Genome Size, Chromosome Number and Karyotype in *Zygophyllum xanthoxylon*

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Abstract: *Zygophyllum xanthoxylon*, a super-xerophytic shrub in drylands, is widely used for afforestation in the arid and barren mountains of central Asia. Understanding the karyotype and genome size could provide basic information for genome sequencing of species. To date, few data on the DNA content and chromosomal characterization of *Z. xanthoxylon* have been reported. Here, we present both the karyotype analysis and genome size determination of *Z. xanthoxylon* based on the traditional pressing and flow cytometry methods. Chromosome counting showed that *Z. xanthoxylon* is diploid with a chromosome number of 22. Karyotype analysis revealed that the length of chromosomes ranges from 0.88 ± 0.08 μm to 2.36 ± 0.19 μm, the chromosomes are metacentric or submetacentric, and the karyotype formula is 2n = 2x = 22 = 18m + 4sm. Flow cytometry analysis estimated that the nuclear genome size of *Z. xanthoxylon* is 460 ± 7.05 Mbp. Interestingly, our results indicated the seedlings of *Z. xanthoxylon* exhibit endopolyploidy, which may confer better ecological adaptation. Collectively, the present study will provide an important cytological basis for the study of the origin, evolution and utilization of *Z. xanthoxylon*.

Keywords: *Zygophyllum xanthoxylon*, Chromosome Number, Karyotype, Flow Cytometry, Genomic Size, Endopolyploidy

1. Introduction

Zygophyllum xanthoxylon is a super-xerophytic shrub with strong drought tolerance that occurs in deserts and steppe deserts. *Z. xanthoxylon* is one of the pioneer tree species used in barren mountain afforestation in arid areas and can play an important role in soil and water conservation. *Z. xanthoxylon* is an excellent forage in natural pastures, and can provide feed for camels, sheep and other animals. Its leaves, stems, roots and fruits have high medicinal value [1]. Therefore, *Z. xanthoxylon* is of great practical value in terms of environmental recovery and has economic benefits.

Endopolyploidy is defined as the occurrence of different ploidy levels within an organism generated either by endoreduplication, which is predominant in plants, or by endomitosis, which mainly occurs in animals. Endoreduplication occurs via continuous synthesis of DNA in the nucleus without cell division during the nuclear replication of plant cells. Endoreduplication is attributed to the activities of topoisomerase II and topoisomerase VI during the mitotic cycle in plants [2]. This specific strategy of endoreduplication may be adopted to combat internal and/or external stresses, the increase in ploidy levels can influence gene expression, increases the content of secondary metabolites and enzymes, thereby increasing the adaptability of allopolyploidy [3].

In the process of evolution, the number, morphology and size of chromosomes of plants may change, which tends to changes in the size of genomes. Karyotype analysis and genome size are valuable in the studies of taxonomy and systematic evolution [4]. At present, the karyotype and nuclear genome size of *Z. xanthoxylon* have not been studied in detail. What are the characteristics of its genome or karyotype? Does its ploidy change to adapt the environment? These questions need to be answered. Therefore, we determined the genome size and analyzed the chromosome karyotype of *Z. xanthoxylon*. These results may provide useful information for studying the evolution and breeding of *Z. xanthoxylon*.

2. Materials and Methods

2.1. Cultivation of Materials

The seeds of *Z. xanthoxylon* were collected from the desert area of Minqin, Gansu Province. Mature and full seeds of *Z. xanthoxylon* were selected, surface washed with running water, soaked and disinfected for 5 min with 75% alcohol, spread on wet filter paper for seed germination, and cultured in a light incubator at 25°C; the water was refreshed once a day. The root tips of a group of materials were removed for chromosome observation when the roots grew to 1 cm, and another group of materials was cultured for three weeks for determination of the genome size.

2.2. Observation of Chromosomes

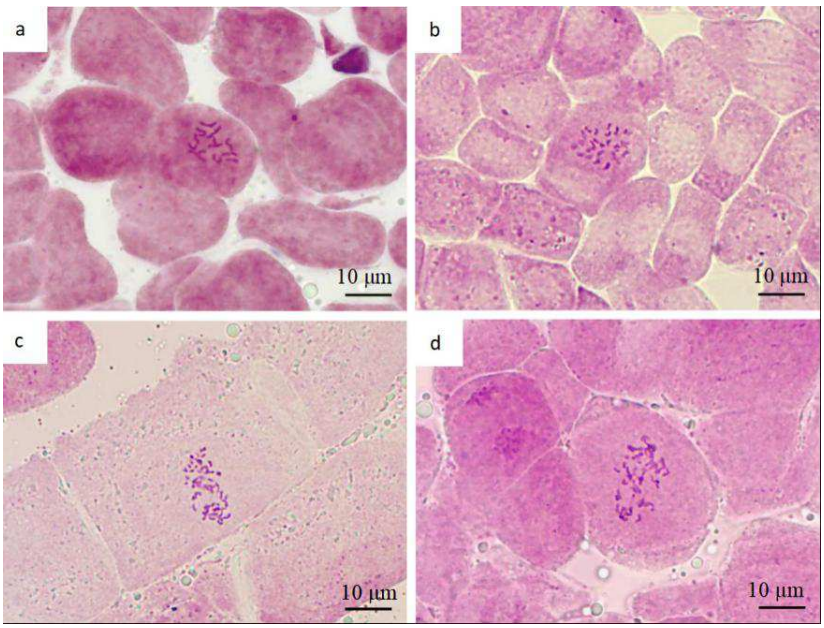
The chromosome number and karyotype of *Z. xanthoxylon* were investigated with the conventional pressing plate method. The root tips of *Z. xanthoxylon* were cut between 10:00 and 11:00, and 8-hydroxyquinoline (2 mmol/L) was selected as the pretreatment solution. The pretreated root tips were washed with distilled water 1-2 times and fixed in Carnot solution for 3 hours. Root tips were removed from the fixation solution, washed with distilled water 1-2 times, and dissociated in 1 mol/L hydrochloric acid in a water bath at 60°C for 10 min. The rinsed root tip was placed on a clean glass slide and stained with carbol fuchsin for 10 min. A cover slide was placed over the slide and gently tapped with the blunt end of the pencil to fully disperse the cells and avoid bubbles and movement of the cover slide. The cells in the mitotic phase and metaphase were observed under a high magnification microscope. More than 20 cells with well-dispersed chromosomes were selected to count the chromosomes and measure the length of each chromosome.

Homologous chromosomes were paired and arranged in order from long to short. The chromosome number, long arm length (L), short arm length (S), total length (TL = L + S) and arm ratio (r = L / S) were measured. The centromere position was determined by the ratio of chromosome arms [5], and karyotype asymmetry was classified according to Stebbins [6]. The results were analyzed by SPSS (version 17; SPSS Inc., Chicago, IL, USA) software.

2.3. Genome Size

Genome size refers to the amount of DNA in a single genome. This study estimated the genome size of *Z. xanthoxylon* by flow cytometry. Estimation of genome size requires internal reference standards [7]. The genome sizes of *Prunus persica* and *Spinacia oleracea* are known [8, 9], so they were chosen as potential reference standards (table 2).

Nuclear isolation buffer was provided by the National Engineering Research Center for Vegetables. The nuclei were stained with propidium iodide (PI). Approximately 1 cm^2 of tender leaves was placed into a petri dish, and then 1 mL of precooled buffer was added to the petri dish. The plant leaves were quickly cut vertically with a sharp blade and mixed, and the extract was filtered through a 42 μm sieve and captured directly into a clean centrifuge tube. The supernatant was then discarded after centrifugation (4°C, 1000 g) for 5 min. Then, 50 μg/mL PI (+ RNase) was added to the prepared nuclear suspension. The nuclear suspension was placed on ice, stained for 10 min, and then observed with a computer. The fluorescence intensity of PI was measured by FACSCalibur flow cytometry (BD, USA) with excitation wavelengths of 488 nm. The fluorescence emitted by PI can be detected by flow cytometry, and the fluorescence intensity can indicate the relative content of genomic DNA. More than 10,000 cells were collected in each sample set, and the coefficient of variation (CV) was controlled to 5%.



a, b Cells with 22 chromosomes; c, d Cells with 44 chromosomes.

Figure 1. *Cells in the metaphase in the root tips, which shows two types of cells in the mitotic stage of Z. xanthoxylon.*

3. Results

3.1. Chromosome Counts

The results suggest that root tip cells of *Z. xanthoxylon* exhibit endopolyploidy (Figure 1). Somatic cells of *Z. xanthoxylon* in metaphase have 22 or 44 chromosomes, the average diameter of cells with 22 chromosomes is 19 ~ 24 μm (Figure 1 a, b), and cells with 44 chromosomes is 40 ~ 50 μm (Figure 1 c, d). The cell size increases but chromosome size decreases with increasing endopolyploidy levels. Images were taken with 10 × 100 magnification. The cell size increased but chromosome size decreased with increasing endopolyploidy levels. The basic number in the genus *Zygophyllum* is x = 11 [10]. Therefore, *Z. xanthoxylon* is diploid (2n = 2x = 22).

3.2. Karyotype Analysis

Diploid metaphase chromosomes and karyotype analysis are shown in Figure 2 and table 3. The length of metaphase chromosomes varies from 0.88 ± 0.08 μm to 2.36 ± 0.19 μm. The karyotype consists of metacentric and submetacentric chromosomes. The karyotype formula of *Z. xanthoxylon* is 2n = 2x = 22 = 18 m + 4 sm, and the karyotype was classified as group 1B.

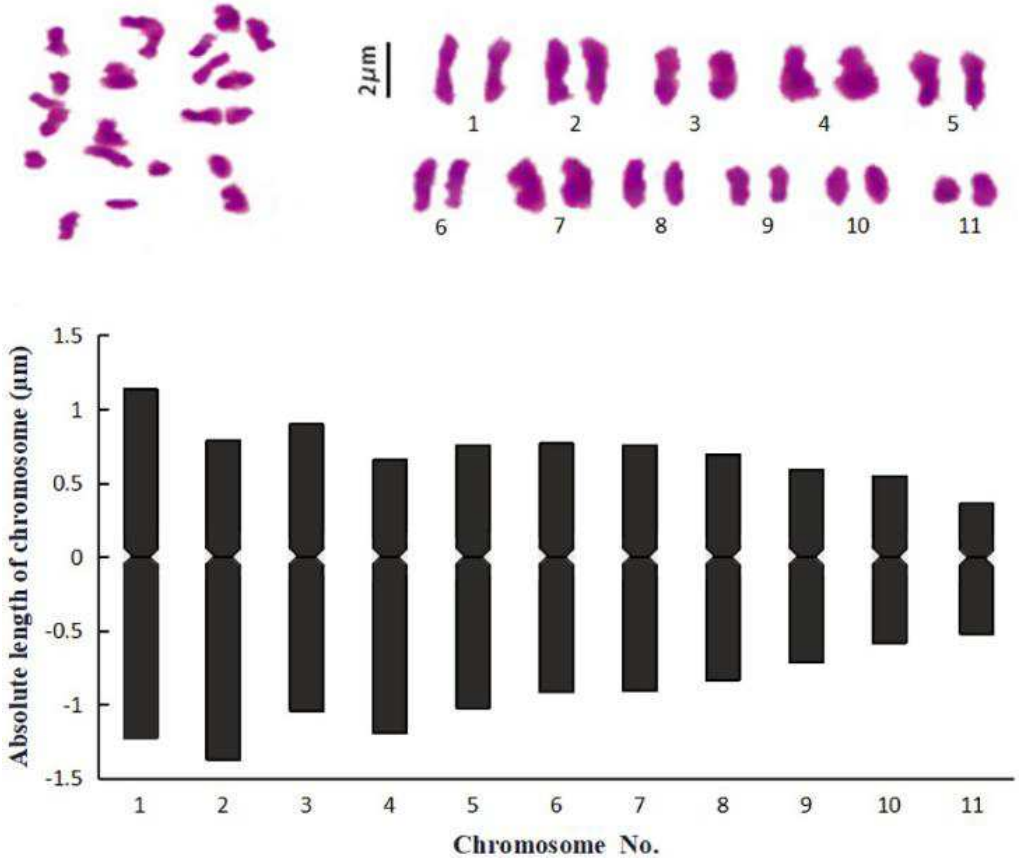


Figure 2. *Karyotype analysis of diploid Z. xanthoxylon.*

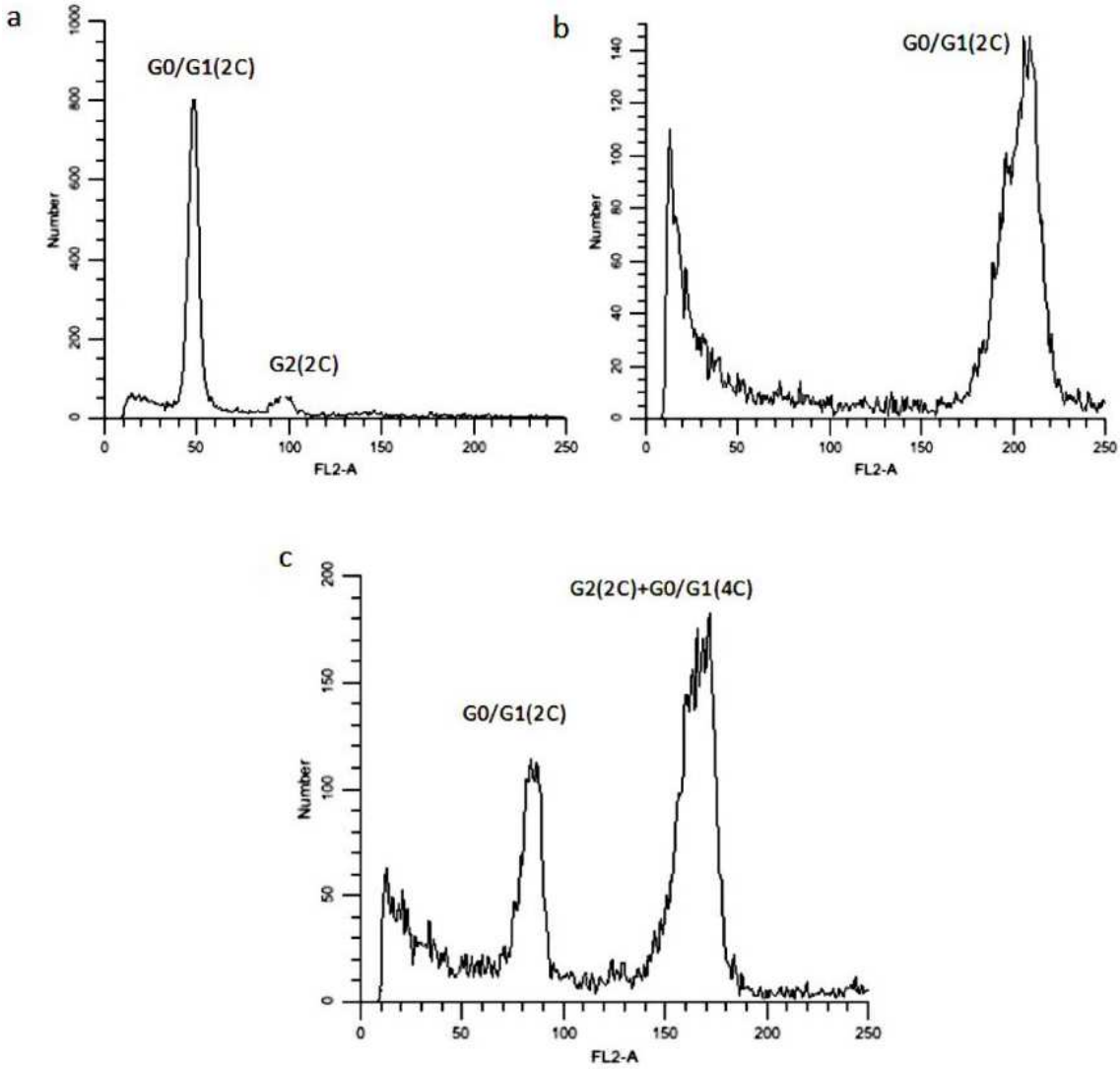
3.3. Flow Cytometric Ploidy Analysis

The histogram of the DNA content was obtained by flow cytometry, which showed the ploidy of plants relative to the position of the G1 peak (Figure 3). Unlike diploid plants, two G1 peaks are shown in the histogram of *Z. xanthoxylon*, one G0/G1 peak (a) appeared on channel 20.43, and another G0/G1 peak (b) appeared on channel 46.81 (Figure 4). The seedlings of *Z. xanthoxylon* are thought to exhibit endopolyploidy based on the results of previous chromosome analysis.

3.4. Determination of Genome Size

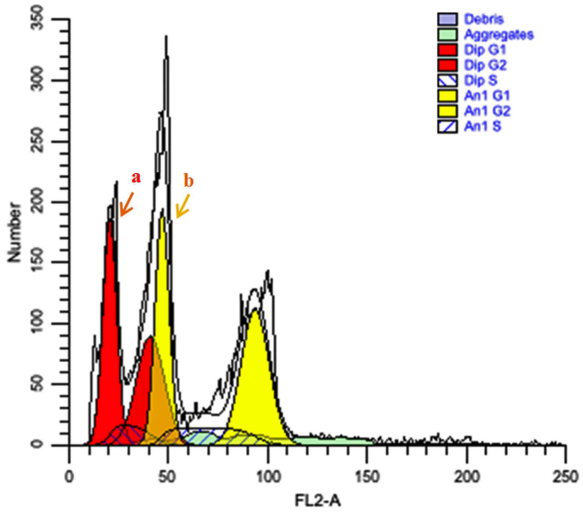
Nuclei of *Z. xanthoxylon* and the internal standard plant (*P. persica* and *S. oleracea*) were stained with PI and tested together (table 1). The reference standard may have a genome size reasonably larger or smaller than that of the unknown sample [7, 11]. There was no overlap between the fluorescence peaks of the tested plants and the two potential internal standard plants, and the degree of differentiation was obvious (Figure 5). The relative fluorescence intensity ratio between 2C *Z. xanthoxylon* (channel number 85.00) and 2C *P. persica* (channel number 48.46) was 1.75, which is in line with the recommendations of Doleže et al. [7]. The ratio of the average relative fluorescence intensity between 2C *S. oleracea* (channel number 202.12) and 2C *Z. xanthoxylon* was 2.38, and the ratios did not satisfy the requirements for internal standardization. Therefore, only *P. persica* was suitable as an internal standard in this study. The reference genome size of *P. persica* is 265 Mbp. Based on the

formula [7], the genome size of *Z. xanthoxylon* is 460 ± 7.05 Mbp.



a P. persica; b S. oleracea (part); c Z. xanthoxylon (part).

Figure 3. *Histograms showing DNA contents.*



a The first G0/G1peak; b The second G0/G1peak.

Figure 4. *Schematic diagram showing endopolyploidy.*

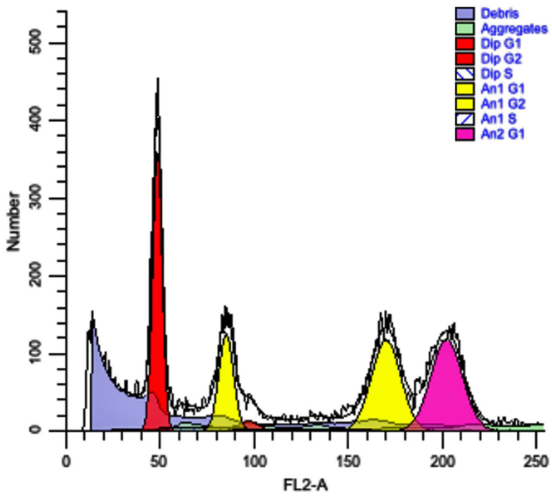


Figure 5. *Histograms of fluorescence intensities of the tested plant Z. xanthoxylon (yellow) with the internal standard plants Prunus persica (red) and Spinacia oleracea (pink).*

Table 1. *Fluorescence intensity in diploids of different species.*

Species	Mean channel number	CV (%)
P. persica	48.49	4.93
S. oleracea	203.08	4.58
Z. xanthoxylon	84.14	4.17

4. Discussion

A more comprehensive understanding of karyotype can help to clarify phylogenetic relationships [12]. Research on two *Zygophyllum* species showed that chromosome numbers of *Zygophyllum eurypterum* (six populations) and *Zygophyllum eichwaldii* (two populations) were diploid (2n = 2x = 22) and tetraploid (2n = 4x = 44), respectively, and all populations were class B, considered a primitive class in the Stebbins classification system [10]. In our study, *Z. xanthoxylon* had 22 chromosomes, which indicates a similar conclusion, and it was classified as group 1B. According to the theory of karyotype evolution proposed by Stebbins [6], karyotype develops from symmetry to asymmetry, which can reflect the evolution of species. Therefore, *Z. xanthoxylon* tend to be a more primitive species, which is consistent with the molecular phylogeny of *Zygophyllum* [13, 14].

The genome includes all the genetic information of a species, and genome size is a useful characteristic for biological research. In addition, it also plays an important role in the evolution and acclimation of plants [15]. No reports of the nuclear genome size of other species in *Zygophyllum* have been found yet. It has been reported that the complete chloroplast genome sequences of *Z. xanthoxylon* and *Zygophyllum. fabago* are 109,577 bp and 108,695 bp in length, respectively [16]. However, we estimated the nuclear genome size of *Z. xanthoxylon* to be approximately 460 Mbp. This result is significantly different from the chloroplast genome, which may provide evidence for the study of the divergence and connection between the nuclear and chloroplast genomes of *Z. xanthoxylon*.

The presence of endopolyploidy as a result of endoreduplication has been characterized in plants. Endopolyploidy occurs preferentially in herbaceous plant species [17]. Here, we found endopolyploidy in seedlings of *Z. xanthoxylon,* a woody angiosperm composed of 2C and 4C cells. Endopolyploidy may be exhibited only at certain stages of ontogeny: in some species, it occurs in seedlings but not in adult plants; in others, it occurs in the radicle but not in the roots of seedlings and adult plants [18]. Since no fresh samples of adult plants of *Z. xanthoxylon* were obtained in our study, whether this condition only exists in a specific period remains to be studied.

In seedlings of *Z. xanthoxylon*, the cell size increases with increasing endopolyploidy levels. A tight correlation between the DNA content and the volume of meristematic tissue cells has been shown [19]. A direct proportionality between DNA content and nuclear and cell volume has also been demonstrated for meristematic cells of 14 herbaceous angiosperms [20]. However, not all plants have a positive correlation between DNA content and chromosome size in the meristem. The chromosome size decreases with increasing endopolyploidy levels in *Z. xanthoxylon*, and this also occurs in *Tradescantia albiflora* (Commelinaceae). In the roots of *T. albiflora*, the chromosome size of induced mitoses decreases stepwise with increasing endopolyploidy levels, but it increases in *Aloe ärborea* (Agavaceae) and *Zebrina pendula* (Commelinaceae) [21].

Cell endoreduplication leads to more extensive cell expansion than in nonendopolyploid plants due to the correlation between nuclear DNA content and cell volume; endopolyploid plant cells avoid the G2 and mitotic phases of the cell cycle, thus shortening the cell cycle duration [19]. Therefore, endopolyploidy indeed represents a means of accelerating plant growth to adapt to habitats that require fast growth and development due to frequent disturbances, and it is also of great significance for *Z. xanthoxylon*.

Endopolyploidy reflects the adaptability of plant growth and development to the environment. Tian et al. [22] identified GaTOP6B in *Gossypium arboreum*, which encodes DNA topoisomerase VI subunit B. They found that GaTOP6B could coordinately regulate plant leaf and root growth via cellular endoreduplication and positively respond to drought stress. The GaTOP6B-silenced plants showed a reduced ploidy level and displayed a compromised drought tolerance phenotype. Ceccarelli et al. [23] found that the probability of endopolyploidy in *Sorghum bicolor* was positively correlated with salinity. In salt-treated plants, the percentages of 8C, 16C, and 32C nuclei in roots in the primary state of growth were significantly higher than those in nonsalinized plants. Therefore, endopolyploidy of *Z. xanthoxylon* may be a part of the adaptive response to arid and salinized environments.

5. Conclusion

Z. xanthoxylon is diploid with a chromosome number of 22, the length of chromosomes ranges from 0.88 ± 0.08 μm to 2.36 ± 0.19 μm, and the karyotype formula is 2n = 2x = 22 = 18m + 4sm. The karyotype of *Z. xanthoxylon* was classified as group 1B. The nuclear genome size of *Z. xanthoxylon* is 460 ± 7.05 Mbp. The study suggested that *Z. xanthoxylon* is a more primitive species.

The seedlings of *Z. xanthoxylon* exhibit endopolyploidy. The somatic cells in metaphase have 22 or 44 chromosomes, and the cell size increases but chromosome size decreases with increasing endopolyploidy levels. This result may reflect the adaptability of the growth and development of *Z. xanthoxylon* to the environment.

Abbreviations

CV	Coefficient of variation
L	Long arm length
m	Metacentric
S	Short arm length
PI	Propidium iodide
r	Arm ratio
sm	Submetacentric
st	Subtelocentric
t	Telocentric
TL	Total length

Author Contributions Statement

LJZ conceived and designed research. YBW, YW and CL conducted experiments. YBW contributed new reagents or analytical tools. YBW analyzed data. YBW wrote the manuscript. LJZ and SDW reviewed and edited the manuscript. All authors read and approved the manuscript.

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Appendix

Table 2. *Genome size of potential reference standards*

Species	Chromosomes	Genome size (Mbp)	References
P. persica	2n = 2x = 16	265	Verde et al. 2013
S. oleracea	2n = 2x = 12	1,009	Xu et al. 2017

Table 3. *Morphometric data for the chromosomes of Z. xanthoxylon*

Chromosome no.	Long arm (μm)	Short arm (μm)	Total length (μm)	Arm ratio	Chromosome type
1	1.22 ± 0.16	1.14 ± 0.09	2.36 ± 0.19	1.07 ± 0.15	m
2	1.37 ± 0.06	0.79 ± 0.04	2.16 ± 0.11	1.72 ± 0.02	sm
3	1.04 ± 0.08	0.90 ± 0.04	1.95 ± 0.08	1.14 ± 0.10	m
4	1.19 ± 0.06	0.66 ± 0.706	1.85 ± 0.13	1.82 ± 0.11	sm
5	1.02 ± 0.02	0.76 ± 0.04	1.78 ± 0.04	1.34 ± 0.07	m
6	0.91 ± 0.16	0.77 ± 0.16	1.68 ± 0.16	1.17 ± 0.06	m
7	0.90 ± 0.15	0.76 ± 0.07	1.66 ± 0.08	1.17 ± 0.09	m
8	0.83 ± 0.03	0.70 ± 0.04	1.52 ± 0.03	1.19 ± 0.10	m
9	0.71 ± 0.06	0.59 ± 0.03	1.30 ± 0.04	1.21 ± 0.16	m
10	0.58 ± 0.06	0.55 ± 0.03	1.13 ± 0.03	1.07 ± 0.18	m
11	0.52 ± 0.04	0.36 ± 0.04	0.88 ± 0.08	1.43 ± 0.07	m

[*] On the basis of centromere position, the arm ratio (r) was used to classify the chromosomes according to Levan et al. (1964) into *m* metacentric (r = 1.05 – 1.69), *sm* submetacentric (r = 1.70 – 2.99), *st* subtelocentric (r= 3.00 – 6.99) and *t* telocentric (r = 7.00 – 39.00) categories.

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