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Original Article

Genome sizes of four important medicinal species in *Kadsura* by flow cytometry

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ABSTRACT

Objective: Dianjixueteng is a geoherb in Yunnan Province, the source plant of which is *Kadsura interior*. However, the formation of this geoherb is not clear in genetic mechanism, in which genome size is the first step that should be known on the genomic level. In this study we aimed to estimate the genome sizes of source plants of *K. interior* and three related herbs *K. heteroclita*, *K. longipedunculata*, and *K. coccinea* by flow cytometry (FCM) and make a comparison.

Methods: The genome sizes of *K. interior*, *K. heteroclita*, *K. longipedunculata* and *K. coccinea*, i.e., the source plants of Dianjixueteng and its relative medicinal materials, were estimated by FCM. The nuclei of *K. interior* were isolated using modified LB01 buffer, for the rest species, by the Galbraith's buffer.

Results: The genome sizes of *K. interior*, *K. heteroclita*, *K. longipedunculata*, and *K. coccinea* were 7.36, 7.12, 7.01, and 5.15 pg/1C, respectively. Genome size of *K. interior* had no significant variation with those of *K. heteroclita* and *K. longipedunculata* ($P = 0.296$), which was significantly larger than that of *K. coccinea*.

Conclusion: Genome size can not distinguish *K. interior* from *K. heteroclita* and *K. longipedunculata*, but could distinguish them from *K. coccinea*, which lays the foundation for future studies on genetic mechanism of the geoherb formation.

1. Introduction

Kadsurae Caulis ("Dianjixueteng" in Chinese), the stems of *Kadsura interior* A. C. Smith, was recorded in *Supplement to Compendium of Materia Medica* ("Ben Cao Gang Mu Shi Yi" in Chinese, published in 1765 CE). It is also listed in the Chinese Pharmacopoeia (2020 edition, volume I) (Chinese Pharmacopoeia Commission, 2020). As the source plant of geoherb Dianjixueteng, *K. interior* shows good efficiency on blood deficiency syndrome and dysmenorrhea. In folk, people call it "Gynecological holy medicine". Meanwhile, *Kadsura heteroclita* (Roxb.) Craib ("Dixuexiang" in Chinese) and *Kadsura longipedunculata* Finet et Gagnep. ("Hongmuxiang" in Chinese) are also used for treating dysmenorrhea (State Administration of Traditional Chinese Medicine of the People's Republic of China, 1996). In our interview, Chinese local pharmacists pointed out *K. interior* showed better efficacy in the treatment of dysmenorrhea in clinical practice. Besides, in *Kadsura*, *Kadsura coccinea* (Lem.) A. C. Smith is used as another popular folk medicinal material ("Heilaohu" in Chinese), which is for treating duodenal ulcer (Fujian Food and Drug Administration, 2006). For the similar efficacy but disability of confusing application of these species, we would like to explore their genetic mechanism of *K. interior* and its relative species expectantly to find their genetic differences.

Research on the formation of geoherb is usually carried out in the aspects like genetic mechanism, chemical constituents, pharmacology, and ecological environment (Huang et al., 2014). Dianjixueteng and its related herbs have been extensively studied in phytochemistry and pharmacological effects. Lignans and triterpenes from those herbs are the two major types of chemical constituents with pharmacological effects such as anti-inflammation, anti-hepatic fibrosis, anti-oxidation and anti-tumor. (Shi et al., 2015; Liu et al., 2018a, 2018b; Yu et al., 2019). However, there are less genetic research on *K. interior* and its related species, other than the study on species identification of *K. interior* and its several relative species by DNA barcode (Guo et al., 2017). With the development of sequencing technology, high-throughput sequencing technology, such as the restriction site associated DNA sequence (RAD-seq) (Cariou et al., 2013), can be used for population genetic

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analysis to explore the authenticity of the geoherb. For genetic studies at the genomic level, it is necessary to estimate the genome size accurately.

The genome size means the total haploid content of nuclear DNA in a cell, i.e., C-value (Bennett, 1972; Gregory, 2001; Soltis et al., 2003). Estimation of genome size could ensure adequate sequencing coverage and provide reference evidences for genome assembly (Kim et al., 2014). Since the 1980s, flow cytometry (FCM) has been used for plant DNA content estimation and it is currently the most accurate technique and best practice for measuring the genome sizes for plants (Sliwinska, 2018; Pellicer & Leitch, 2020). Investigation on genome size requires a reference standard with known genome size (i.e., the sample with known nuclear DNA content). The reference standard can be divided as internal and external reference standard, the internal reference standard is preferred for involving simultaneous isolation, staining, and analysis of nuclei both from the samples and the reference standard (Tiersch & Chandler, 1989; Doležel et al., 2007). In addition, Doležel and Bartoš (2005) suggested that *Zea mays*, *Arabidopsis thaliana*, *Oryza sativa*, *Raphanus sativus*, *Vigna radiata*, *Sorghum bicolor*, *Lycopersicon esculentum*, *Glycine max* could be used as ideal reference standards. Besides, buffers also influence the DNA extraction effects (Loureiro et al., 2006).

This study aims to study the genome sizes of *K. interior* and its related herbs using FCM, with *Zea mays* B73 as reference standard. It will provide evidences for further study on the genome and the genetic mechanism of geoherb Dianjixueteng.

2. Materials and methods

2.1. Plant materials

The leaves of *K. interior* were obtained from the wild individuals in Lincang, Yunnan, China. The leaves of *K. longipedunculata* and *K. heteroclita* were collected from Baojing, Hunan, China; while the leaves of *K. coccinea* were obtained from Nanchuan, Chongqing, China. The leaves of maize (*Zea mays* ssp. mays var. B73) were obtained from Institute of Botany, Chinese Academy of Sciences. A small amount of plant material (typically 20 mg) of young leaf tissues from each accession was used for FCM analysis since young, fast-growing tissues provide the best results.

2.2. Sample preparation and FCM analysis

Nuclear DNA content estimation was performed by FCM with fresh leaf tissues according to the Galbraith's method and improved LB01 method respectively (Doležel et al., 2007). To ensure the accuracy of the results, *Zea mays* B73 (2.3 Gb/1C, diploid) was used as the internal reference standard (Schnable et al., 2009). Fresh leaves from *K. heteroclita*, *K. longipedunculata* and *K. coccinea* were respectively co-chopped with *Zea mays* B73 using a new razor blade in a petri dish containing 0.3 mL ice-cold Galbraith's buffer (45 mmol/L $MgCl_2$, 20 mmol/L MOPS, 30 mmol/L sodium citrate, 0.1% volume percentage Triton X-100, pH = 7.0). Filtered the homogenate through a 48 μm nylon mesh into a labeled sample tube. Fresh leaves of *K. interior* were co-chopped with *Zea mays* B73 using a razor blade in a petri dish containing 0.3 mL improved LB01 lysis buffer (15 mmol/L Tris, 2 mmol/L Na_2EDTA, 0.5 mmol/L spermine tetrahydrochloride, 80 mmol/L KCl, 20 mmol/l NaCl, 0.2% volume percentage Triton X-100. Add β-mercaptoethanol to 15 mmol/L). In addition, Otto buffers (Otto I solution refers to 0.1 mol/L citric acid, 0.5% volume percentage Tween 20. Filter through a 0.22-μm filter and store at 4 °C. Otto II solution refers to 0.4 mol/L Na_2HPO_4 $12H_2O$. Filter through a 0.22-μm filter and store at 18–25 °C) and LB01 buffer (5 mmol/L Tris, 2 mmol/L Na_2EDTA, 0.5 mmol/L spermine tetrahydrochloride, 80 mmol/L KCl, 20 mmol/L NaCl and 0.1% volume percentage Triton X-100. Adjust to pH 7.5 with 1 mol/L NaOH. Filter through a 0.22-μm filter. Add β-mercaptoethanol to 15 mmol/L. Store at − 20 °C in 10 mL aliquots) were also tested on these species. The nuclei suspension was filtered through a 48 μm nylon mesh. Then 2.5 μL RNase (10 mg/mL) and 250 μL propidium iodide (PI) (0.5 mg/mL) solution were added. After incubation for 15 min in darkness, all the homogenates were analyzed based on light scatter and fluorescence signals produced from 20 mW laser illumination at 488 nm using a BD LSRFortessa^{TM} cell analyzer (BD Biosciences, Franklin Lakes, NJ). At least (5×10^3) nuclei were collected in each measurement and each species was repeated at least three times.

Data acquisition and analysis were performed with BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ). The voltage was set to 385 V in the fluorescence channel and the threshold was set based on PI. One parameter PI-A histogram and two-parameter dot plots PI-W versus PI-A, forward scatter (FSC) versus side scatter (SSC) were adopted. Peak channel values and coefficient of variation (CV%) were generated.

2.3. Genome size determination

Species of *Kadsura* were diploids (2C DNA content) (Wu & Huang, 1995; Xiang et al., 2004; Friedman et al., 2003). The cytometric histograms obtained by BD FACSDiva software were used to determine the genome size of each sample, in line with the equation below:

$$\text{Sample DNA content (pg)} = \frac{\text{Sample } P_1 \text{ peak mean}}{\text{Reference } G_0/G_1 \text{ peak mean}} \text{ x Reference DNA content (pg)}$$

The following relationship 1 pg = 978 Mb and 1 Gb = 1024 Mb are used to convert gigabase pair nucleotides (Gb) to DNA content (pg) (Doležel et al., 2003).

2.4. Data analyses

A one-way ANOVA (analysis of variance) was used to compare genome sizes among individuals of the same species and among four sampled species respectively. Tukey-HSD test ($P \leq 0.05$) was used for the multiple comparison.

3. Results

3.1. Selection of nuclei isolation buffers for genome size estimation

In this study, the isolation of nuclei suspensions from four *Kadsura* species was successfully completed with the nuclei isolation buffers including Galbraith's buffer and improved LB01 lysis buffer. In total, we tried Otto's buffers, LB01 lysis buffer, improved LB01 lysis buffer and Galbraith's buffer to extract DNA from fresh leaves of all sampled species. The results showed that all these reagents were suitable for *Zea mays* B73. However, Otto's buffers were not suitable for the leaves of these four species in *Kadsura*. Galbraith's buffer was suitable for the leaves of *K. heteroclita*, *K. longipedunculata*, and *K. coccinea*, but it produced no results with the leaves of *K. interior*. LB01 lysis buffer could not produce a good nuclear isolation effect for *K. interior* either. The improved LB01 lysis buffer, i.e., the proportion of nonionic surfactant Triton X-100 was increased from 0.1% (volume percentage) up to 0.2% (volume percentage), which significantly improved the DNA extraction efficiency of *K. interior*. Probably because of the presence of abundant mucilage and acidity of the leaf tissues of *K. interior* (Salameh, 2014).

3.2. Estimation and comparison of C-values of four Kadsura species obtained by FCM

On average (7×10^3) nuclei were isolated from 20 mg of leaf tissues of each sample. There are many proliferating cells in the young *Zea mays* B73 leaves, the two dominant peaks presented in the FCM histograms represented cells at G_0/G_1 (2C) and G_2 (4C) state. The results indicated that *Zea mays* B73 was an ideal reference standard for all sampled species. In all cases, less than half of the nuclei formed a single peak at channel 10^5 corresponding to the G_2 state of the cell cycle (Fig. 1). In the same instrument settings, the relative fluorescence intensity of samples was all much larger than that of the reference standard, for an instance, the peak of sample *K. interior* went at the right side of those of the reference standard (Fig. 2). Mean fluorescent intensity of singlet G_0/G_1 of *Zea mays* B73 was taken as a basis for calculating the 2C nuclear DNA content of *Kadsura* species. In addition, no significant differences were found among individuals of the same species. With *Zea mays* B73 as internal reference standard, the average C-values of three replicates of each species were found to be 7.36 pg/1C, 7.12 pg/1C, 7.01 pg/1C and 5.15 pg/1C for *K. interor, K. heteroclita*, *K. longipedunculata* and *K. coccinea* respectively. No significant differences were found among the genome sizes of *K. interior*, *K. heteroclita* and *K. longipedunculata* (P = 0.296), and they were all significantly larger than the genome size of *K. coccinea* (P < 0.01). The mean, standard deviation (SD), maximum (Max.) and minimum (Min.) of genome sizes (C-values; 1C/pg), the mean of nuclear DNA contents (2C DNA content; pg), and the number of replicates (N) for each species were shown in Table 1. The coefficient of variation (CV) for peaks of standards and samples were below 5% throughout this study except the CV values of *K. coccinea* were all below 8%. The peak of *K. coccinea* and G_2 peak of *Zea mays* B73 were overlapped, and it led to the CV value higher than 5% (Figs. S1–S4).

4. Discussion

4.1. Is there any relationship between genome size, drug efficacy and plant evolution?

In terms of efficacy, the stems of *K. interior*, *K. heteroclita*, and *K. longipedunculata* all have the effect of treating dispelling wind and dampness, while the stems of *K. coccinea* are mainly for the treatment of gastric and duodenal ulcer (Liu et al., 2012). *K. coccinea* differs from the other three species in genome size and efficacies. We found the genome size of *K. coccinea* is obviously lower than that of *K. interior*, *K. heteroclita*, and *K. longipedunculata*. While the genome sizes of *K. interior*, *K. heteroclita*, and *K. longipedunculata* are similar. This phenomenon inspired us that the relationship between efficacies of other traditional Chinese medicines and the genome sizes of their source plants is worth further studying.

The previous phylogenetic tree established by *psb*A-*trn*H, *mat*K, *rbc*L and internal transcribed spacer (ITS) sequences showed that *K.*

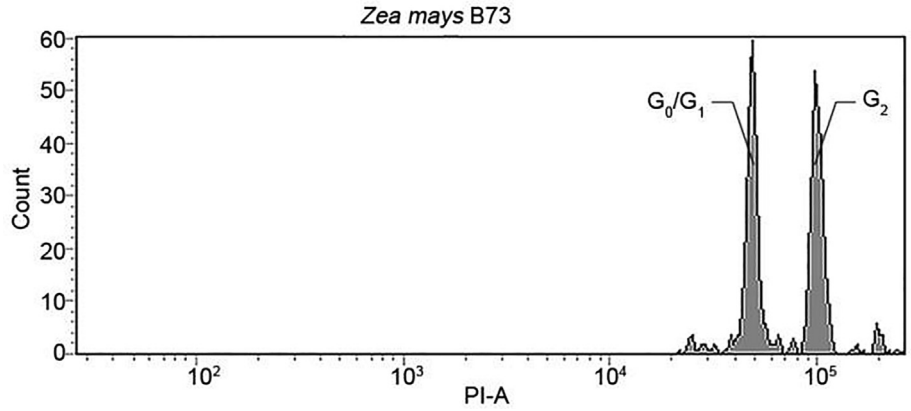

Fig. 1. Flow cytometric histogram of relative fluorescence intensity of *Zea mays* B73. The left peak: 2C-values of *Zea mays* B73 at G_0/G_1 state; The right peak: 2×2 C-values of *Zea mays* B73 at G_2 state.

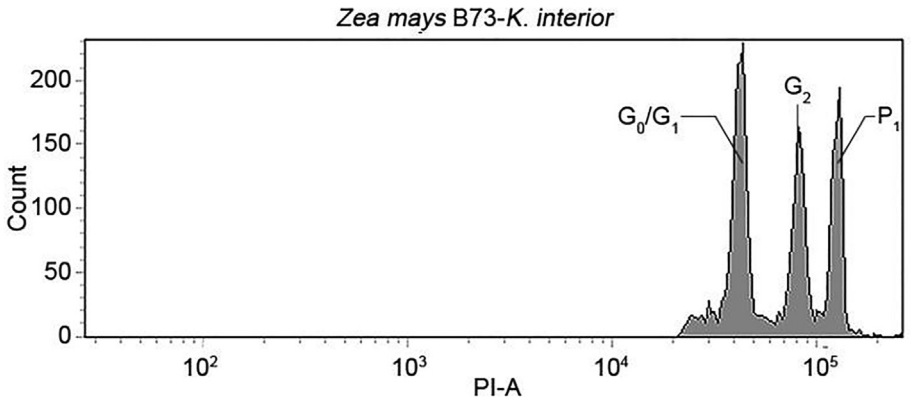

Fig. 2. Flow cytometric histogram of relative fluorescence intensity of *K. interior* with *Zea mays* B73. The left peak: 2 C-values of *Zea mays* B73 at G_0/G_1 state; The middle peak: 2×2 C-values of *Zea mays* B73 at G_2 state; P_1 = 2 C-values of *K. interior*.

Table 1
Four plant material and genome size analysis.

Species (collection number)	1C value / pg				2C DNA content/ pg	N[B]
	Mean[A]	SD	Min	Max		
K. interior (Y190925-1)	7.36[a]	0.29	7.11	7.53	14.72	3
K. heteroclita (H190924-3)	7.12[a]	0.90	7.06	7.22	14.24	3
K. longipedunculata (H190924-1)	7.01[a]	0.10	6.91	7.11	14.02	3
K. coccinea (C190924-2)	5.15[b]	0.26	4.99	5.45	10.30	3

[A] Means followed by the same letter were not statistically different according to the multiple comparison Tukey-HSD tests at $P \leq 0.05$.
[B] Means replication numbers.

interior, *K. heteroclita*, and *K. longipedunculata* formed one cluster which separated from *K. coccinea* (Guo et al., 2017). The results of this study showed a similar pattern, the genome sizes of *K. interior*, *K. heteroclita*, and *K. longipedunculata* are relatively similar and significantly different from that of *K. coccinea.*

In addition, the enormous differences in the genome size of species within genus also exist in other genera, for example, in *Ocimum* L., the DNA contents of *O. selloi* Benth. are 3.05 pg/2C while the DNA contents of *O. campechianum* Mill. are 1.18 pg/2C. The differences in genome size of species within the same genus might result from genome evolution through polyploidization aneuploidy, sequence insertions or deletions, and chromosome rearrangements (Rewers & Jedrzejczyk, 2016). In addition, whole genome duplications, proliferating transposable elements, tandem repeats and polyploidy events might lead to larger genome sizes (Michael, 2014). In *Kadsura*, the above events might occur in their common ancestor, thus resulted in the genome sizes of *K. interior*, *K. heteroclita* and *K. longipedunculata* being significantly larger than that of *K. coccinea*.

Correlation between the efficacy of geoherb, genome size, and the evolutionary relationship of the source plants is complex and still need more evidences.

4.2. Estimation of genome size is the foundation of genomic research

K. interior, *K. heteroclita*, *K. longipedunculata* and *K. coccinea* are all wild sources without large-scale artificial cultivation (Deng et al., 2008). Since these four medicinal plants are respectively recorded in the Chinese Pharmacopeia (2020 edition, volume I) (Chinese Pharmacopoeia Commission, 2020) and local pharmacopeias, there is a high demand for the collection of these plants, but over time, over harvest may lead to the extinction of these species (Chen et al., 2016). Especially for *K. interior*, its geographical distribution is relatively narrow. Currently, some experts classify *K. interior* as a vulnerable species. For these species at risk of extinction, it is necessary to carry out molecular breeding to improve their survival. And the whole genome sequencing based on the genome sizes should be conducted (Zhang et al., 2016; Kole et al., 2015).

In order to clarify the genetic mechanism of geoherb formation of *K. interior*, we have obtained genome sizes, based on which we will further explore the restriction site associated DNA sequence (RAD-seq) analysis and population genetic diversity (Cariou et al., 2013). Also, the estimation of genome size lays the foundation for whole genome sequencing which will help to conduct a more comprehensive and in-depth study of the origin and evolution of plants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2021.05.002.

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