A rapid method for identifying ploidy level in *Isatis indigotica* Fortune and *Isatis tinctoria* Linnaeus

Yong Su (✉ 18119845526@163.com)

Nanjing Agricultural University

Man Zhang

Nanjing Agricultural University - Weigang Campus: Nanjing Agricultural University

Qiao Sheng Guo

Nanjing Agricultural University - Weigang Campus: Nanjing Agricultural University

Tao Wang

Nanjing Agricultural University - Weigang Campus: Nanjing Agricultural University

Chang Liu

Nanjing Agricultural University - Weigang Campus: Nanjing Agricultural University

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Abstract

The genus *Isatis* is widely distributed throughout the world. In this work, thirty-two *Isatis indigotica* Fortune germplasms, collected from different regions and geographical locations in China, were analyzed the ploidy levels by flow cytometry. *I. indigotica* Fort. and *Isatis tinctoria* Linnaeus distinguished with root tip chromosome compression staining and cell flow cytometry. Microscopic observation showed that the chromosome numbers of *I. indigotica* Fort. and *I. tinctoria* L. were $2n = 14$ and $2n = 28$, respectively. In order to establish a flow cytometric nuclear experiment system suitable for *I. indigotica* Fort., the leaves of *I. indigotica* Fort. were used to prepare nuclear suspension with LB01, OTTO, Tris·MgCl₂, patent of Luochang and Galbraith extraction buffer. It was found that the histogram generated by LB01, OTTO, Tris·MgCl₂ and Patent of Luochang dissociation solution has poor peak shapes and large CV values, and Galbraith dissociation solution extraction buffer was suitable for extracting nuclei from most germplasms. Flow cytometry proved to be a simple, rapid, and highly accurate method for identifying ploidy levels *Isatis* species.

Introduction

Isatis indigotica Fort., a biennial herbaceous plant of the family Cruciferae (Chen et al. 2014), it was used as traditional Chinese medicine with roots (*Isatidis radix*) and leaves (*Isatidis folium*)(Sun et al. 2021). As a medicinal plant for more than 2000 years from Shen Nong's Materia Medica, *Isatidis radix* has a widely useful activities including anti-virus, anti-bacterial, anti-endotoxic, anti-inflammatory, and immune regulatory effects(Ding and Zhu 2020; Kong et al. 2008). However, *I. indigotica* Fort. has been merged into *Isatis tinctoria* Linnaeus in Flora of China (2001) (Zhou et al. 2001). However, there is a transition between *I. indigotica* Fort. and *I. tinctoria* L., and the two have big differences in morphology. Due to the chaos of the original plants of *I. indigotica* Fort., the use of medicinal materials has also been restricted(Muzayyinah et al. 2016). This may lead to a series of inconsistent treatment effects and quality problems in the herbal medicine industry. Clarifying the germplasms source of medicinal materials is an important prerequisite to ensure that the medicinal materials are safe and effective(Xu et al. 2011). Accurating identification plays an important role in the first key step for developing quality security assurance measures for Traditional Chinese Medicine(Chen et al. 2021). The development of accurate and reliable method for the identification *I. indigotica* Fort. appears to be extremely urgent.

The DNA encoding the genetic information of most organisms is located in the nucleus, and the detection technology of DNA content is one of the earliest developed, most widely used, and most basic flow cytometry(Bennett and Leitch 2005). Galbraith and the Hare developed a method for DNA content by flow cytometry plant cell nucleus, thus promoting botany unprecedented development(Galbraith et al. 2001; Hare and Johnston 2011). The principle of DNA content detection by flow cytometry is to use special fluorescent dyes (propidium iodide, 4',6-diamidino-2-phenylindole, Acridine Orange, etc.) in series with the DNA inside the cell, enter the flow chamber of the flow cytometer, and emit after laser irradiation fluorescence, to be collected through the respective filter sheets fluorescence intensity per cell, and then the fluorescence intensity of the stained cells to calculate the cellular DNA content (Ddg et al. 2021).

Flow cytometry provides powerful tools and detection methods for plant cell research(Olszewski et al. 2015). It can not only count plant cells, detect genome size, DNA content and ploidy level, but also sort cells in different periods and cycles, and construct chromosome DNA library. The use of flow cytometry to detect plant nuclear

DNA content and ploidy is very important for the description of species characteristics, classification and genetic research. We often encounter a lot of interference in the detection of DNA fragments *Isatis* samples, mainly due to the plant cell structure (cell wall central vacuole) and component (secondary metabolites) particularity and complexity. At present, there are not many researches on the identification methods of *I. indigotica* Fort.(Kang et al. 2021). The chromosomes of *I. indigotica* Fort. are small and difficult to observe, which hinders ploidy analysis. Flow cytometry technology can solve this problem.

Materials And Methods

Plant material, culture medium and conditions

The seeds consisted of 32 *I. indigotica* Fort. germplasms collected from different locations (Table 1). The seeds were surface-sterilized with 70% ethanol for 1 min followed by 2.5% sodium hypochlorite for 8 min. The sterilized seeds were rinsed with double distilled water five times and then dried by blotting on sterilized paper prior to testing.

Murashige and Skoog (MS) medium was used at full strength for callus induction and shoot regeneration and at half-strength for in vitro rooting(Duan et al. 2018). The culture medium contained 3% (w/v) sucrose and 0.7% agar or 0.3% phytagel. The pH of each medium was adjusted to 5.8 using potassium hydroxide (KOH) prior to autoclaving (103 kPa and 121°C for 20 min). Unless otherwise noted, cultures were maintained at 25±1°C under a 16 h/8 h light/dark photoperiod, provided by cool-white fluorescent light with an irradiance of 35 µmol photons/(m²/s), and at 60% relative humidity. The sucrose used was the products of Solarbio (Beijing, China).

The seedling after seed germination become strong in 7 d. Planted in 30 cm pots in a greenhouse under natural light at 25°C (16 h/8 h of light/dark).

Sample preparation

Take about 0.5 g of the young leaf sample, chop it with a sharp scalpel blade in a petri dish dripped with 1.5 mL extraction buffer (Table 2), then filter with a 500 mesh nylon mesh(Yan and Shao 2014). The purified nuclei were stained with 250 µL propidium iodide (PI; 50 µg/mL; Fluka, Buchs, Switzerland). RNase (50 µg/mL; Fluka) was then added to the nuclear suspension to prevent staining of double-stranded RNA. The samples were incubated on ice in the dark for 10 min before analysis by flow cytometry. All stages of extraction and staining were performed at 4°C. Young leaf sample from *I. indigotica* Fort. (Altay, Xinjiang, China) were used as a diploid reference. 10,000 nuclei were analyzed per sample.

Flow cytometric analysis

Ploidy identification: using CytoFLEX-flow cytometry sorter (Beckman Coulter, S.Kraemer Boulevard Brea, CA , USA), excitation light source is 15 argon ions, excitation wavelength is 488 nm, irradiated by the excitation light source can excite the fluorescent dyed molecules to promote fluorescence, and measure the fluorescence intensity and signal intensity. It is directly proportional to the DNA content. At the same time, it is theoretically believed that as the number of chromosomes doubles, the DNA content of the cell nucleus must also double, so the DNA content can be used to estimate the ploidy of the cell chromosomes(Stanisic et al. 2015).

For each measurement, PI fluorescence area signals (phycoerythrin area, PE-A) from 10,000 nuclei were collected using CytoFLEX. A gate was set using the PE-A and FSC parameters, allowing the fluorescence measurements from nuclei to generate a histogram of PE-A. Acquisition parameter settings (gain): FSC, 700; SSC, 250; FITC, 126; PE, 250; PB450, 101; KO525, 51. The mean values PE-A of the G1/G2 (nuclei) peak for the sample and the external standard were determined by analyzing the data using CytExpert 2.3 software. To estimate ploidy level, the position of the G1 peak on the histogram obtained for each individual plant was compared with that of *I. indigotica* Fort. (Altay, Xinjiang, China).

Result

Sample preparation

The young and fresh leaf samples can be used in Flow cytometer (De et al. 2010). After aseptic operation, the seeds are inoculated on MS medium and can be detected by Flow cytometry. Then the leaf bases of 7 d seedlings were used as initial samples (Fig. 1). At this time, the leaves were relatively fresh, chopped with a double blade, mixed with the dissociation solution, and then added PI staining, which will show a better experimental results.

Determination of blank control

In the MS medium, the seedlings of *I. indigotica* Fort. were well developed. The meristem of the root tip of the 7 d seedling was used for chromosome counting (Liu and Zhang 2001). The chromosome number of *I. indigotica* Fort. and *I. tinctoria* L. were determined to be 14, 28 (Fig. 2), indicating that *I. indigotica* Fort. was diploid, and *I. tinctoria* L. was tetraploid. This was consistent with the description in the literature (Tuo et al. 2012).

Screening extraction buffer

Excellent preparation of nuclei is crucial for flow cytometry analysis (Rainer et al. 2014). However, obtaining nuclei from different plant species requires different extraction buffers suited to a particular plant tissue and metabolic components. We chose the five extraction buffers including LB01, OTTO, Tris·MgCl₂, Galbraith and Patent of Luo chang (Bhardwaj et al. 2014). Not enough cell nuclei can be detected in the sample, and no obvious DNA peaks can be generated in the histogram including LB01, OTTO, Tris·MgCl₂ and Patent of Luo chang. We found that Galbraith extraction buffer was suitable for examining most germplasms (Galbraith 2004), resulting in a complete histogram of the peak shape (Fig. 3).

Data analysis

In the flow cytometer, the Mean PE-A of 32 germplasms of *I. indigotica* Fort. ranges from 927837~1026530, and the CV value ranges from 3.50%~4.74%, which is less than 5%, indicating that the data is true and reliable. The Mean PE-A±SD of *I. tinctoria* L. is (1863935±6150), and the CV±SD is (2.53±0.19)%, which is 1.5-2.0 times that of *I. indigotica* Fort. (Fig 4). The results showed that the 32 germplasms were *I. indigotica* Fort. instead of *I. tinctoria* L..

Discussion

The yield of cell nuclei in flow cytometry samples depends on the leaf age. The nucleus yield of old leaves is lower than that of young leaves. In addition, some plant leaves are prone to produce sticky substances when shredded, which adsorbs cell nuclei, resulting in a small number of cell nuclei (Pfosser et al. 2010). Therefore, the seedlings grown on the 7d after seed germination have young leaves and are applicable to this experiment.

Obtaining nuclei from different plant species requires different extraction buffers. Inappropriate dissociation fluid will accelerate the rupture of the nucleus, resulting in excessive fragments of the nucleus suspension, poor quality, and failure to form peaks or poor peak shapes in the histogram. This can explain the fact that the samples prepared by Tris·MgCl₂, LB01, OTTO and Luochang patent extraction buffer have too few nuclei and poor quality, resulting in poor histogram peak shapes (Galbraith 2004; Galbraith et al. 2001).

The CV value of the DNA peak is an important indicator to measure the reliability of the measurement result (Backović et al. 1992). Generally, the recognized CV value of plants is about 3%~5% (Dolezel et al. 1998); and for the plants whose cytoplasm is rich in staining inhibitors, the recognized CV is not more than 7% (Ca L et al. 2005). Using the Galbraith extraction buffer, the measured CV value is 3–5%, indicating that the measured data is credible, and it also shows that the Galbraith extraction buffer is suitable for the analysis of these 32 germplasms of cell nuclei in *I. indigotica* Fort.

The experimental results show that the ploidy of *I. indigotica* Fort. was different from *I. tinctoria* L.. By Root tip chromosome compression staining and cell flow cytometry, distinguish *I. indigotica* Fortune and *I. tinctoria* Linnaeus. Root tip chromosome compression shows that the number of chromosomes in *I. indigotica* Fort. is $2n = 14$, the number of chromosomes in *I. tinctoria* L. is $2n = 28$. In flow cytometry 32 germplasms of *I. indigotica* Fort. all showed the same ploidy level. And different from *I. tinctoria* L. There were no significant differences in the different germplasms within the specie of *I. indigotica* Fort. collected from different locations with ploidy level, which indicates that ploidy level is not associated with geographic location.

In conclusion, we successfully used flow cytometry to determine ploidy levels in *I. indigotica* Fort. The high sensitivity and accuracy of flow cytometry enabled us to rapidly estimate the ploidy levels of *Isatis* species. This efficient technique helps to further elucidate the genetic diversity of *I. indigotica* Fort. and *I. tinctoria* L.

Declarations

Acknowledge

I. indigotica Fort.(2) and *I. tinctoria* L.(33) were supplied by Resources Sanjiu Co., Ltd of China.

Conflict-of-interest

Authors declare no conflict of interests.

Authors Contribution Statement

Yong Su researched the relevant literature and wrote the body of the article.

Qiaosheng Guo provided overall direction for the program of work and edited the article.

Tao Wang and Chang Liu provided some suggestions for the article.

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Tables

Table1 The information of *I. indigotica* Fort. seeds

No. ^a	Color	Location	Origin
1	brown	N35°42', E110°84'	Wanrong, Shanxi, China
2	brown	N43°80', E87°63'	Altay, Xinjiang, China
3	brown	N34°70', E110°70'	Ruicheng, Shanxi, China
4	brown	N36°09', E111°53'	Linfen, Shanxi, China
5	brown	E33°37', E115°74'	Ruanqiao, Anhui, China
6	brown	E33°85', E115°78'	Bozhou, Anhui, China
7	brown	E38°88', E115°47'	Baoding, Hebei, China
8	brown	E34°09', E119°32'	Ruicheng, Shanxi, China
9	brown	E36°52', E118°55'	Linqu, Shanxi, China
10	brown	E34°62', E112°46'	Luoyang, HeNan, China
11	brown	E35°22', E113°25'	Jiaozuo, Henan, China
12	brown	E35°07', E113°95'	Yuanyang, Henan, China
13	brown	E34°55', E114°79'	Qixian, Henan, China
14	brown	E34°15', E113°50'	Yuzhou, Henan, China
15	brown	E34°17', E112°85'	Ruzhou, Henan, China
16	brown	E34°52', E110°90'	Lingbao, Henan, China
17	brown	E38°88', E116°46'	Wenan, Hebei, China
18	brown	E38°05', E114°52'	Shijiazhuang, Hebei, China
19	brown	E33°88', E109°92'	Shangluo, Shanxi, China
20	brown	E35°63', E106°12'	Longde, Ningxia, China
21	brown	E38°93', E100°46'	Zhangye, Gansu, China
22	brown	E35°01', E104°64'	Longxi, Gansu, China
23	brown	E28°06', E115°55'	Zhangshu, Jiangxi, China
24	brown	E25°35', E100°50'	Midu, Yunnan, China
25	brown	E37°12', E79°94'	Hetian, Xinjiang, China
26	brown	E32°90', E115°82'	Fuyang, Anhui, China
27	brown	E33°85', E115°78'	Bozhou, Anhui, China
28	brown	E46°59', E125°11'	Daqing, Heilongjiang, China
29	brown	E37°08', E114°51'	Xingtai, Hebei, China
30	brown	E35°01', E104°64'	Longxi, Gansu, China

31	brown	E31°24', E121°48'	Shanghai, China
32	brown	E34°28', E108°96'	Shanxi, China

aNumber

Table2 Main components of five nuclei extraction solutions

Type of buffer	Components
LB01	15 mmol·L ⁻¹ Tris-HCl 80 mmol·L ⁻¹ KCl 80 mmol·L ⁻¹ KCl 20 mmol·L ⁻¹ NaCl 2 mol·L ⁻¹ EDTA-Na ₂ 15 mmol·L ⁻¹ Mercaptoethanol 0.05% (v/v) Triton X-100; at 4 °C
OTTO	100 mmol·L ⁻¹ citric acid monohydrate; 0.5 % (v/v) Tween20; pH 2.0-3.0, at 4 °C
Tris·MgCl ₂	200 mmol·L ⁻¹ Tris 4 mmol·L ⁻¹ MgCl ₂ ·6H ₂ O 0.5 % (v/v) X-100; pH 7.5, at 4 °C
Galbraith	45 mmol·L ⁻¹ MgCl ₂ ·6H ₂ O 30 mmol·L ⁻¹ Sodium citrate 20 mmol·L ⁻¹ MOPS 0.1% (v/v) Triton; pH 7.0, at 4 °C
Patent of Luochang	15 mmol·L ⁻¹ MOPS 2 mol·L ⁻¹ EDTA-Na ₂ 0.5 mmol·L ⁻¹ Spermine tetrahydrochloride 80 mmol·L ⁻¹ KCl 20 mmol·L ⁻¹ NaCl 0.1% (v/v) Triton 0.1% (v/v) Mercaptoethanol 1% pvp-10; pH 7.5, at 4 °C

Table3 Analysis of ploidy level in the *I. indigotica* Fort. and *I. tinctoria* L.

No. ^a	Species	Location	Average Mean PE-A±SD	Average CV(%) ±SD	Chromosome Number ^b (2n)	Ploidy level ^c
1	<i>I. indigotica</i> Fort.	Wanrong, Shanxi, China	1011413±9624	4.52±0.11	14	2×
2	<i>I. indigotica</i> Fort. (blank)	Altay, Xinjiang, China	1006376±3554	4.64±0.24	14	2×
3	<i>I. indigotica</i> Fort.	Ruicheng, Shanxi, China	1014782±6218	4.67±0.11	14	2×
4	<i>I. indigotica</i> Fort.	Linfen, Shanxi, China	1008644±10494	4.55±0.35	14	2×
5	<i>I. indigotica</i> Fort.	Ruanqiao, Anhui, China	981743±20665	4.71±0.26	14	2×
6	<i>I. indigotica</i> Fort.	Bozhou, Anhui, China	1025877±13121	4.60±0.37	14	2×
7	<i>I. indigotica</i> Fort.	Baoding, Hebei, China	999456±11023	4.50±0.27	14	2×
8	<i>I. indigotica</i> Fort.	Ruicheng, Shanxi, China	975757±10045	4.51±0.22	14	2×
9	<i>I. indigotica</i> Fort.	Linqu, Shanxi, China	1009730±3391	4.74±0.20	14	2×
10	<i>I. indigotica</i> Fort.	Luoyang, HeNan, China	978036±12134	4.53±0.21	14	2×
11	<i>I. indigotica</i> Fort.	Jiaozuo, Henan, China	1014391±8995	3.84±0.25	14	2×
12	<i>I. indigotica</i> Fort.	Yuanyang, Henan, China	1022565±2897	3.95±0.35	14	2×
13	<i>I. indigotica</i> Fort.	Qixian, Henan, China	960283±2384	3.78±0.12	14	2×
14	<i>I. indigotica</i> Fort.	Yuzhou, Henan, China	1026530±1628	4.79±0.06	14	2×
15	<i>I. indigotica</i> Fort.	Ruzhou, Henan, China	1026258±7391	4.77±0.13	14	2×
16	<i>I. indigotica</i> Fort.	Lingbao, Henan, China	1020022±4168	4.56±0.35	14	2×
17	<i>I. indigotica</i> Fort.	Wenan, Hebei, China	963273±25308	4.51±0.42	14	2×
18	<i>I. indigotica</i> Fort.	Shijiazhuang, Hebei, China	940127±5515	4.49±0.70	14	2×
19	<i>I. indigotica</i> Fort.	Shangluo, Shanxi, China	933833±1135	3.95±0.31	14	2×

20	<i>I. indigotica</i> Fort.	Longde, Ningxia, China	929686±4069	4.60±0.27	14	2×
21	<i>I. indigotica</i> Fort.	Zhangye, Gansu, China	963229±4509	3.50±0.09	14	2×
22	<i>I. indigotica</i> Fort.	Longxi, Gansu, China	969801±3102	4.59±0.08	14	2×
23	<i>I. indigotica</i> Fort.	Zhangshu, Jiangxi, China	947062±7803	4.61±0.16	14	2×
24	<i>I. indigotica</i> Fort.	Midu, Yunnan, China	927837±6702	4.23±0.27	14	2×
25	<i>I. indigotica</i> Fort.	Hetian, Xinjiang, China	978566±16399	4.75±0.25	14	2×
26	<i>I. indigotica</i> Fort.	Fuyang, Anhui, China	929192±7031	4.56±0.09	14	2×
27	<i>I. indigotica</i> Fort.	Bozhou, Anhui, China	972003±17165	4.68±0.28	14	2×
28	<i>I. indigotica</i> Fort.	Daqing, Heilongjiang, China	957537±15546	4.45±0.39	14	2×
29	<i>I. indigotica</i> Fort.	Xingtai, Hebei, China	957419±9679	4.67±0.12	14	2×
30	<i>I. indigotica</i> Fort.	Longxi, Gansu, China	950492±3675	4.44±0.14	14	2×
31	<i>I. indigotica</i> Fort.	Shanghai, China	1011776±12628	4.74±0.30	14	2×
32	<i>I. indigotica</i> Fort.	Shanxi, China	938794±2597	4.45±0.22	14	2×
33	<i>I. tinctoria</i> L.	Altay, Xinjiang, China	1863935±6150	2.53±0.19	28	4×

^a Species Number

^b Chromosome numbers found in the experiment

^c The ploidy level detected according flow cytometry

Figures

Figure 1

Morphological Characteristics of *I. indigotica* Fort. and *I. tinctoria* L. A: The seeds of *I. indigotica* Fort.; B: The seeds of *I. tinctoria* L.; C: The seedling of *I. indigotica* Fort. (7 d); D: The seedling of *I. tinctoria* L. (7 d).

Figure 2

Chromosomes of *I. indigotica* Fort. and *I. tinctoria* L. A: Diploid of *I. indigotica* Fort. (Aletai, Xinjiang, China) (2×); B: Tetraploid of *I. tinctoria* L. (Aletai, Xinjiang, China) (4×).

Figure 3

Examples of flow cytometric histograms showing the relative PI fluorescence intensity in nuclei from the different extraction buffer in *I. indigotica* Fort. (Aletai, Xinjiang, China). A: Diploid (LB01); B: Diploid (OTTO); C: Diploid (Tris.MgCl₂); D: Diploid (Galbraith); E: Diploid (Patent of Luochang).

Figure 4

Examples of flow cytometric histograms showing the relative PI fluorescence intensity in nuclei from the leaves of different *I. indigotica* Fort. and *I. tinctoria* L. germplasms.(1~32 is diploid of *I. indigotica* Fort. (2 $\times$);33 is tetraploid of *I. tinctoria* L.(4 $\times$)).