

Research Article**2C DNA Value of Persian Poppy (*Papaver bracteatum* Lindl.) Medicinal Plant As Revealed By Flow Cytometry Analysis; A Quick Effective Criteria for Distinguishing Unidentified *Papaver* Species****Saeed Tarkesh Esfahani¹, Ghasem Karimzadeh^{1,*},
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ABSTRACT:

Papaver bracteatum Lindl. ($2n = 2x = 14$) commonly known as Persian poppy is an Iranian endemic medicinal plant mainly known for containing valuable amounts of the pharmaceutically important alkaloid of thebaine. The C-value index is a species-specific characteristic highly useful in systematics, genome size estimation and many other biological fields related to eukaryotic organisms. It is also considered as a reliable criterion for clear identification of ambiguously classified species. In this study, calculation of 2C DNA value of the Persian poppy, using original plants derived from the most evidently known natural habitat of the species in Northern Iran is being reported. The 2C DNA value of *P. bracteatum* was determined by flow cytometry technique using *Pisum sativum* (2C DNA = 9.09) as the internal standard. The 2C DNA value for the *P. bracteatum* was determined to be 6.15 ± 0.05 pg. The calculated 2C DNA value for Persian poppy differs from two previously reported values most likely because of their lack of access to reliable accurate estimation methods as well as possible misidentification of locally available *Papaver* sp. seed lots for *P. bracteatum*. These results clearly indicated the effectiveness of flow cytometry analysis as a rapid and reliable strategy for discriminating *P. bracteatum* from other identified or unidentified *Papaver* species with similar morphological traits.

Keywords: *Papaver bracteatum*, Seed, Flow Cytometry, 2C DNA value, diploid**[I] INTRODUCTION**

Persian poppy (*Papaver bracteatum* Lindl.) is a valuable medicinal plant generally endemic to mountainous regions in Northern and Western Iran [10, 20]. Alborz Mountains in northern Iran is considered as the most evidently known origin and diversity center of this species [20, 21]. Persian poppy is one of the three species classified as section *Oxytona* in *Papaveraceae* family. It is closely related to *P. orientale* L. ($2n = 4x = 28$) and

P. pseudoorientale (Fedde) Medw ($2n = 6x = 42$) and highly resembles them in morphological characteristics [1, 21]. All species in section *Oxytona* however are perennial and readily distinguishable from other *Papaver* species [21]. Thus, much confusion has been made during botanical identification as well as taxonomic classification of these three species. However they are known to possess distinct phytochemical

characteristics where the predominant alkaloid of the Persian poppy is thebaine and that of the latter two species are oripavine and isothebaine respectively [22]. Thebaine is readily convertible to the most pharmaceutically used alkaloid of Codeine [22]. It is also used as a precursor of other pharmaceutically important medicines and morphine antagonists and for many other medicinal purposes [17]. Therefore, it is important to distinguish *P. bracteatum* from other species of the Papaveraceae by simple and low-expense criteria.

Cell DNA plays central role in heredity. The term C-value was coined by Swift (1950) as the DNA content of an unreplicated haploid chromosome complement i.e. a gamete [11]. C-value refers to the constant amount of DNA per species and is usually expressed as 2C DNA content in each organism [5, 8]. It is known as a highly useful index in many biological fields such as estimation of genome size and taxonomical studies. Data regarding to the plants C-value should be readily available for reference and application in study purposes [2]. The 2C DNA content of an organism can be determined either by analyzing DNA extracted from a large number of cells or by analyzing individual nuclei. Flow cytometry is known as the most reliable method based on single nuclei analyzing for determination of 2C DNA content [6].

In this study, the 2C nuclear DNA content of *P. bracteatum* was determined for the first time by using plant material originally sampled from the most evidently known origin of the species and by application of highly reliable flow cytometry analysis (based on *Pisum sativum* cv. Citrad as internal standard). The contradiction of calculated C-value with previously reported values is clarified and possible causes for the observed contradiction are discussed.

[II] MATERIALS AND METHODS

2.1. Plant material

Seeds of mature *Papaver bracteatum* plants were collected from three locations representing different growing elevations and habitats consisting of Polour, Rineh and Baladeh regions (Table 1) in

Alborz Mountains in Northern Iran. The seeds of individual plants were collected separately and enveloped in small air-tight plastic bags. Seeds were sterilized by immersing in ethanol 70% (v/v) for three times 30 s each followed by sodium hypochlorite 5% (v/v) for 7 min followed by rinsing by distilled water for 5 min. The sterilized seeds were then transferred on two layers of moisturized filter papers in glass petri dishes and irrigated regularly by distilled water until germination. After about 12 days, the newly germinated seeds with a root about 3-5 mm length were transferred to 250-ml glass baby food jars containing 40 ml of ½ Murashige and Skoog medium [18] with 1 g l⁻¹ charcoal. The seedlings were re-cultured once a month on new medium with the above-mentioned composition and conditions. After three months, the plants with 6-7 true developed leaves were transferred to 500 g pots containing peat moss, cocopeat and perlite as the main components composed with an orderly ratio of 5:4:1.

Location	Latitude	Longitude	Altitude
Plour	35°51' 51.2"	52°03' 24.5"	2505
Rineh	35°53' 5.7"	52°08' 45.6"	2564
Baladeh	36°13' 44.3"	51°26' 2.0"	2887

Table 1: Location name and geographical coordinates of collection sites for seeds of *Papaver bracteatum* plants within the natural habitat of the species in Northern Iran.

2.2. Flow cytometry (FCM) analysis

About one cm² of young, healthy and fully developed leaf from each Persian poppy plant together with about 1/3-1/2 in area of leaf material from *Pisum sativum* cv. Citrad (2C DNA = 9.09 pg) [9] as internal reference standard, were chopped into small pieces by a sharp razor blade in a 100 mm glass petri dish, containing one ml of Woody Buffer Plant buffer (WPB) [14]. The buffer was used in order to release the nuclei from the cells and making the intact nuclei exposed. The resultant nuclear suspension was filtered through a 30 µm-nylon mesh followed by treating with 50 µg ml⁻¹

RNase (Sigma-Aldrich Corporation, MO, USA) to prevent staining of double-stranded RNA and 50 $\mu\text{g ml}^{-1}$ of Propidium Iodide (PI, Fluka) as DNA staining agent and incubated for two min at room temperature. The nuclei suspension was loaded into and analyzed by a BD FACSCanto II flow cytometer (BD Biosciences, Bedford, MA, USA), using BD FACSDiva™ Software. Data were transferred to a Flowing Software version 2.5.0 (Cell Imaging Core, Turku Centre for Biotechnology) in order to be editable in Partec FloMax ver. 2.4e. Gating region range was accomplished on FCM histograms using the latter software.

2.3. Chromosome counting

Root tips of examined plants were pretreated with α -monobromonaphthalene for 1 h at 24°C followed by rinsing with distilled water for 3 \times 3 min. The pretreated roots were then fixed in Carnoy solution (3:1 ethanol:glacial acetic acid) and stored at 4°C followed by washing in distilled water, hydrolyzing with 1 N HCl for 8 min at 65°C and staining with 1% (w/v) aceto-orcein for 1 h. Treated root tips were excised 1-2 mm long and squashed on slide glass, with a drop of 45% (v/v) acetic acid, and protected with a cover slip. Chromosome counts were analyzed by observation under a BX51 Olympus light microscope (Olympus Optical Co., Tokyo, Japan) and high resolution digital photographs were taken from the best metaphasic plates, using a DP12 (Olympus Optical Co., Ltd., Tokyo, Japan) digital camera.

2.4. Data Analysis

The absolute DNA amount of a sample was calculated based on the values of the G_1 peak means [6, 7, 8] as follows:

Sample 2C DNA (pg) = (Sample G_1 peak mean/Standard G_1 peak mean) \times Standard 2C DNA (pg). Data obtained from flow cytometry analysis were further analyzed through a multi-observation Completely Randomized Design (CRD) with three replications. All statistical analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

[III] RESULTS

3.1.2. C DNA variation

The ANOVA results revealed that there was no significant difference between the Persian poppy plants (Table 2) originated from different locations within the natural habitat of the species in northern Iran (Table 1, Figure 1). It indicates no correlation between 2C DNA content and geographical coordinates particularly growing elevation within the natural habitat of the species.

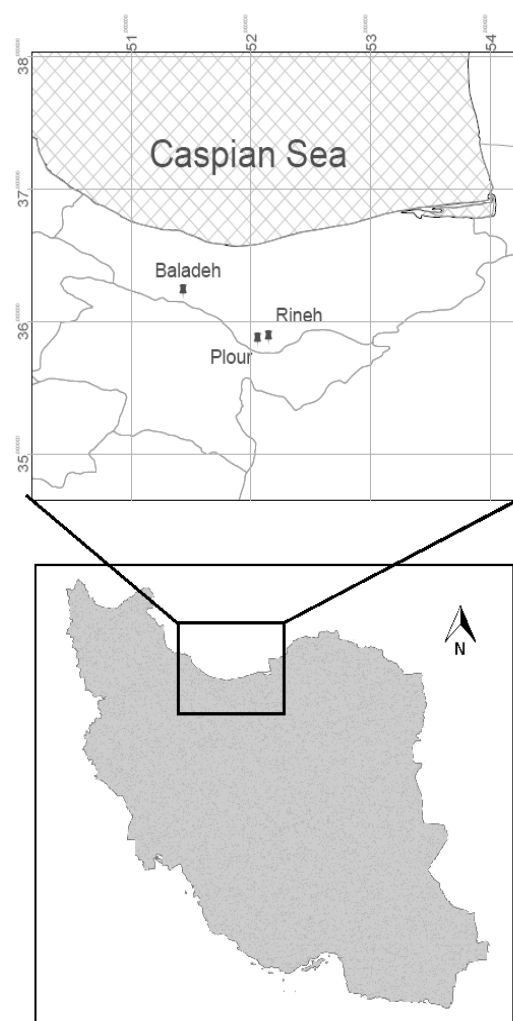


Fig. 1. Collection sites of the seed samples of *Papaver bracteatum* plants illustrated on the map of Iran.

In the present study, the average 2C DNA content of all examined Persian poppy plants were estimated as 6.15 ± 0.03 (Figure 2). The calculated 2C DNA values for all examined

plants varied from 5.81 to 7.16 pg, indicating a relatively narrow variation.

Source of variation	Degree of freedom	Mean square
Location	2	0.17 ^{ns}
Observation	8	1.65 ^{**}

Table 2: Analysis of variance for multi-observation data of the effect of geographical origin on the nuclear DNA content in Persian poppy (*Papaver bracteatum*)

^{ns}, ^{**} non-significant and significant at $P < 0.01$, respectively

3.2. Ploidy level variation

All plants with apparently normal growth and development were determined to be as diploids ($2x$) based on their calculated $2C$ DNA contents. As shown in related histograms (Figure 2), diploid plants revealed a peak about the position of channel 40 of the relative fluorescent intensity.

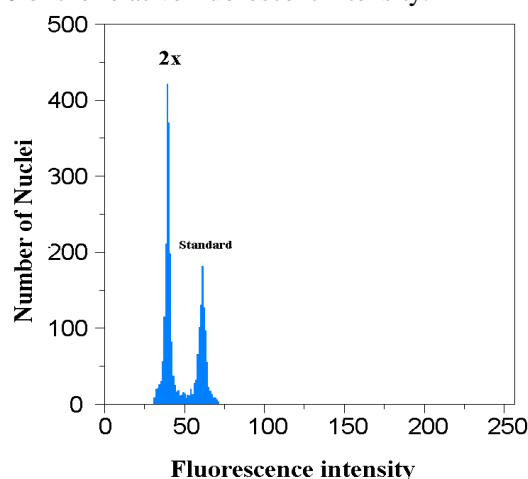


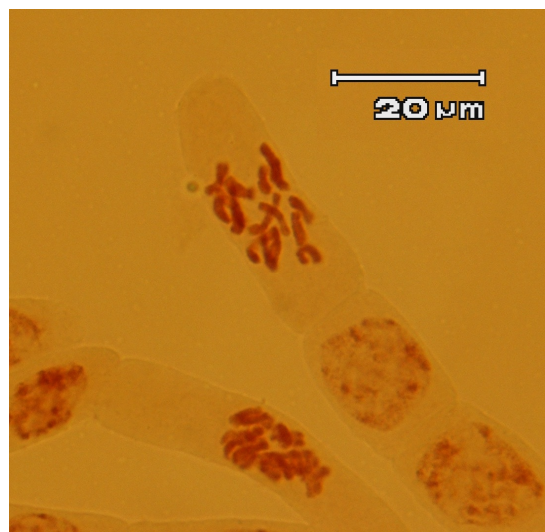
Fig. 2. Flow cytometric histogram of the relative fluorescence intensity of nuclei isolated from *P. bracteatum* plants. The standard peak indicates the peak resulted by the cells of the *Pisum sativum* cv. Citrad ($2C$ DNA = 9.09 pg) used as the internal standard

3.3. Chromosome number count

The ploidy state of the examined plants were further confirmed through traditional chromosome counting techniques (Figure 3) where the chromosome number of all examined plants were determined as $2n = 2x = 14$.

The *P. bracteatum* has been reported as a naturally diploid plants with the chromosome number of $2n = 2x = 14$ [16, 17, 20, 23].

A



B

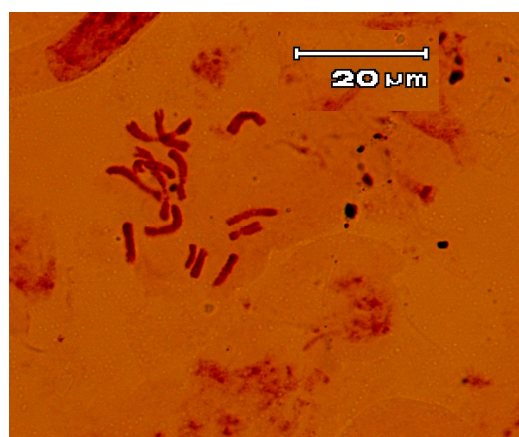


Fig. 3. Chromosome numbers of two diploid *P. bracteatum* plants originated from Plour (A) and Baladeh (B) both with the same chromosome number ($2n = 2x = 14$).

[IV] DISCUSSION

The correlation between the amount of nuclear DNA content and different geographical coordinates is previously reported in various wild and cultivated plant species such as *Corchorus olitorius* [3] *Echinocereus* [4], *Lathyrus sativus* [12], *Cucumis melo* [13], *Thymus* [15] and *Consolea* [19]. Our present results however show that the plants belonging to different locations throughout the known natural habitat of the *P. bracteatum* species tend to have the same nuclear DNA content where the

calculated C-value of three evaluated populations do not seem to be statistically different and may not be affected by the evolutionary changes. However it remains to study the nuclear DNA variation through a wider range of geographical and climatic condition related to *P. bracteatum* original habitat. The C-value index defined as the DNA content of an unreplicated haploid chromosome complement (i.e. a gamete) is highly useful in systematics, genome size estimation and many other biological fields related to eukaryotic organisms [5, 6, 7]. It is often expressed as 2C DNA content which refers to the nuclear DNA content in G₁ phase of the cell cycle in terms of pictograms (pg). The 2C DNA content of an organism can be determined with a high precision by FCM technique [6, 11].

To the best of our knowledge, there are two reports about 2C DNA content estimation for *P. bracteatum* reporting two different estimated values of 9.80 pg [23] and 2.17 pg [1]. In our present study however, the calculated 2C DNA value for *P. bracteatum* differs from both previously reported values. The inconsistency between obtained results with those of previous authors might be explained by their lack of access to reliable accurate estimation methods in older studies as well as some imperfections in their several technically important considerations. Furthermore, both previous studies were conducted on plant material which were available out of the geographical origin of *P. bracteatum* but solely identified as belonging to the species [1, 23]. The former reported C-value (9.80 pg) was measured based on the inaccurate criteria of calculated correlation between haploid chromosome length and the global 2C DNA amount in Papaveraceae [23]. Hence, the method could be highly prone to error and the resulted 2C DNA amount is highly likely to be misestimated. Moreover, though the more recent authors [1] applied reliable flow cytometry technique for 2C DNA estimation, some differences in their technical considerations such as using a less commonly applied and less scientifically validated plant (*Trifolium repens* L.) as an internal standard in flow cytometry procedure, could play a role in obtaining

contradictory results for 2C DNA amount of *P. bracteatum*. On the other hand possible misidentification of locally available *Papaver* sp. seed lots for *P. bracteatum* could act as a potential cause of deviation in their obtained 2C DNA value. Indeed, since *P. bracteatum* was considered as an illegal narcotic plant in their country [1], the authors intended to apply Flow cytometry analysis and 2C DNA index as a criteria for quick discrimination of this species from its two morphologically similar relatives namely *P. orientale* ($2n = 4x = 28$) and *P. pseudo-orientale* ($2n = 6x = 42$) [1]. Two latter species were reported legal to be cultivated as ornamental plants and must therefore be rapidly and reliably discriminated from illegal known of *P. bracteatum* [1]. However, they used seed lots which were sold as oriental poppies for screening them based on their taxonomic differences. They are likely to have misidentified seeds of other ambiguous species for *P. bracteatum*.

[V] CONCLUSION

Our present study is being the first report on the calculation of 2C DNA content of *P. bracteatum* and estimation of its variation by using plants which were directly sampled from the most evidently known natural habitat of the species in Northern Iran. Furthermore, the high capability of flow cytometry-based analysis and C-value index as rapid and reliable strategies for discriminating *P. bracteatum* from other identified or unidentified *Papaver* species with similar morphological traits was showed. These results indicated the effectiveness and reliability of combining the potent flow cytometry technique with reliably known plant material for estimation of 2C DNA content in plant species and providing less ambiguous information for future applications in taxonomical studies.

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