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SCIENCE DIRECT
Plant Science 170 (2006) 859–866
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Genetic characterization of selected *Trifolium* species as revealed by nuclear DNA content and ITS rDNA region analysis

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Received 29 September 2005; received in revised form 12 December 2005; accepted 12 December 2005
Available online 18 January 2006

Abstract

Thirty-one clover species with significant agronomic value, originating from Eurasia, Africa and America, were analyzed to reveal their nuclear DNA content and propidium iodide/4,6-diamidino-2-phenylindole (PI/DAPI) ratio. Chromosome numbers of the same accessions were determined for calculation of 1Cx genome size values, and the relationships between species were studied based on ITS-rDNA analysis. The nuclear DNA content of the majority of species was determined for the first time and revealed large variations among investigated species. The somatic nuclear DNA content ranged from 0.688 pg (*T. ligusticum*) to 7.375 pg (*T. burchellianum*), exhibiting a 10.7-fold difference. Since polyploidy is characteristic of several species of the genus *Trifolium*, 1Cx values provided the most informative measure of interspecific DNA content variation. Analysis of nuclear DNA content based on 1Cx-values within the *Lotoidea* section revealed that the basic genome size values of six African and American species were twice those of the six Eurasian species of the same section. Measurements of nuclear DNA content using propidium iodide/DAPI staining highly correlated ($r = 0.99$), the average PI/DAPI ratio of 29 species was 1.043. Clustering based on ITS polymorphisms showed high relationship with botanical sections with the only exemption in the section *Lotoidea*, which was divided mainly according to its origin (American, African or Eurasian). Genome size data and the ITS clustering were highly correlated in sections *Chronosemium*, *Involucrarium* and *Vesicaria* and varied approximately two-fold within sections *Trifolium*, *Lotoidea* (African origin) and *Lotoidea* (Eurasian origin).

Keywords: *Trifolium* species; Genome size; Flow cytometry; Chromosome number; Internal transcribed spacer (ITS)

1. Introduction

The genus *Trifolium* L. comprises approximately 240 species belonging to the tribe Trifolieae, family of Leguminosae (Fabaceae). Closely related genera include *Trigonella*, *Medicago* and *Melilotus*, which all belong to Trifolieae tribe [1]. The very large *Trifolium* genus is the only one of this tribe with a cosmopolitan distribution. It is distributed throughout the temperate and subtropical region of the globe and can exceptionally be found in tropical regions of W. Africa and S. America, where it is restricted to the mountain zones. Zohary and Heller [2] indicated two regions of interest for genus evolution: the Mediterranean region with 110 species belonging to seven sections being one of the main centres of distribution of the genus and also the centre of domestication and breeding, and the Californian region as an additional centre of distribution, which includes a smaller number of species. The centre of origin of this genus is still a matter of controversy. Zohary [3] considered the Californian region to be the primary centre of speciation and speculated that clovers spread from there into Asia and hence to Europe and Africa. Taylor [4], in contrast, suggested that the Mediterranean area may be the true centre of origin because of the high diversity in chromosome numbers and forms that have been found there.

Several attempts have been made to divide this genus into natural groups. Boissier [5] recognized seven sections. Hossain [6] divided the genus into eight subgenera. The most up-to-date revision, based on morphological and chromosomal characteristics of the genus, was proposed by Zohary and Heller [2]. The authors divided the *Trifolium* species into eight sections: *Lotoidea*, *Paramesus*, *Mistyllus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum* and *Involucrarium*.

Despite the importance of the genus, cytological characterization of several species is either absent or incomplete in nature. Pritchard [7] collected data and partly additionally established chromosome numbers for 129 *Trifolium* species,

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0168-9452/$ – see front matter © 2006 Published by Elsevier Ireland Ltd.
doi:10.1016/j.plantsci.2005.12.007

while later studies [8–10] reported additional chromosome counts for 11, 2 and 13 species of *Trifolium*, respectively. The chromosome numbers differed for some species, and the effect was particularly obvious for those species with higher chromosome numbers. There is general agreement that the basic chromosome numbers in *Trifolium* form an aneuploid series of $x = 5$, 6, 7 and 8, while the most common number, present in about 80% of species is $x = 8$ [2]. The great majority of species are diploids, a few are tetraploids, while higher ploidy levels are very rare.

Chromosome counts are helpful in taxonomy and may suggest evolutionary processes, while data on nuclear DNA content and base composition provide supporting evidence. Molecular phylogenetics data are observed as the most convincing data sets, which can be used for identifying phylogenetic relationships. A combination of all data sets gives even more valuable information on genetic similarity and taxonomic position. Such data can be also used as guidance to plant breeders, especially when inter-specific hybrids are involved.

The evaluation of nuclear DNA content of a species is an important tool in genetic diversity studies, since it can contribute to species identification and also reveals divergence within a genus, as illustrated in *Alstroemeria* [11], *Lathyrus* [12], *Hydrangea* [13], *Asparagus* [14], *Lupinus* [15,16] and many other genera. In closely related species, it is important to know the origin of DNA variation, the direction of changes and the fraction of DNA involved in these changes [17]. Nuclear DNA content may also be an important parameter for the improvement of agricultural and horticultural features. Correlations, for instance, have been found between nuclear DNA content and seed size, leaf width and leaf length in soybean [18].

Despite the high agronomic value of the genus, nuclear DNA content has been reported for a surprisingly limited number of *Trifolium* species. In an early study, Grime and Mowforth [19] obtained data for five *Trifolium* species using Feulgen microdensitometry and determined: *T. arvense* (1.4 pg), *T. campestre* (0.9 pg), *T. medium* (6.7 pg), *T. pratense* (1.3 pg) and *T. repens* (3.0 pg). Arumuganathan and Earle [20] performed flow cytometric measurements using propidium iodide staining and revealed 2C genome size estimations for *T. repens* (2.07 pg) and *T. pratense* (0.97 pg). In an additional study of intra-specific variability, Campbell et al. [21] analyzed seven populations of *T. repens* (by Feulgen densitometry) and found a considerable variation among populations, ranging from 2.20 to 2.67 pg.

Modern genetic evaluation studies of related species are based on various biochemical and DNA molecular marker techniques. Seed protein electrophoretic patterns of 20 *Trifolium* species [22] were discussed in relation to chromosome criteria and found to be informative and useful for determining sectional and subsectional delimitations.

RAPD markers were implemented to determine the potential roles of diploid ancestral taxa in the evolution of allotetraploid white clover [23], through comparison of *T. repens* accessions with those from 19 related species. Several Old World *Trifolium* species have been studied using analysis based on nucleotide sequence data of the internal transcribed spacer (ITS) of nuclear ribosomal DNA and chloroplast DNA restriction sites [24].

The aim of the present study was to obtain basic genetic data on 31 *Trifolium* species with notable agronomic value, originating from Eurasian, African and American centres of divergence and belonging to seven major taxonomic sections of the genus *Trifolium*.

The major objectives of our study were:

- to determine nuclear DNA content of the investigated species;
- to obtain the PI/DAPI ratio and to calculate 1Cx genome size based on chromosome counts of accessions—as general descriptors of the nuclear status of individual species;
- to assess the interspecies relationships among analyzed species on the basis of sequence polymorphism of nuclear ITS region of rDNA;
- to compare and discuss these sets of data in relation to the proposed taxonomic classification.

2. Material and methods

2.1. Plant material

Seeds of 31 *Trifolium* species were obtained from five gene banks and were cultured in the greenhouse of the Biotechnical Faculty, University of Ljubljana. The species studied, their origin and accession numbers are listed in Table 1. The sections in Table 1 are arranged according to Ref. [2].

2.2. Determination of chromosome number

Somatic chromosome numbers were counted from root tips of in vitro germinated seeds. Germination was performed in growth chambers at 24 °C on wet paper in Petri dishes. The root tips of germinating seeds were collected and pretreated with icy water for 16–18 h, fixed in Carnoy's solution 2 (alcohol–chloroform–acetic acid 6:3:1, v/v/v) for at least 24 h at 4 °C. Roots were hydrolyzed in 1N HCl for 12 min at 60 °C, stained with Shiff's reagent (Sigma) for 30 min and squashed in 2% acetocarmine. Slides were examined with a Zeiss Jenalumar 250 microscope and photographs were taken with a Zeiss MC 80 camera.

2.3. Determination of nuclear DNA content by flow cytometry

Young clover leaves were collected from several week old seedlings. The total DNA amount in the leaf nuclei was assessed by flow cytometry using *T. repens* L. cv. Milo (2C = 2.07) [20] as an internal standard. *T. pratense* L. (genome size estimated using *T. repens* as standard was 2C = 0.85 pg) served as an intermediate standard for DNA estimation of clover species in those instances in which *T. repens*-derived peaks overlapped with those the species under measurements.

Leaf tissues of the analyzed samples together with leaf tissues of standard species were chopped with a razor blade in plastic Petri dishes in cold LB01 buffer, according to a technique