A Computerized Image Analysis System to Characterize Small Plant Chromosomes

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Keywords: Medicago sativa, cytogenetics, karyotype, video enhancement, genome

INTRODUCTION

Alfalfa, Medicago sativa ssp. sativa (L.) L. & L., has very small somatic chromosomes (1.7 - 2.5 µm in length) which are almost identical in morphology. It is an autotetraploid (2n = 4x = 32) with four nearly identical genomes. Because of these characteristics, preparing good chromosome spreads for identifying all eight sets of homologous chromosomes has been extremely difficult.

Investigations of somatic chromosomes of alfalfa have been previously reported [1-4]. Agarwal and Gupta [1] karyotyped several *Medicago* species with chromosome measurements made using an ocular micrometer, thus the accuracy of the measurements is questionable. Falistocco [2] and Falistocco et al. [3] measured alfalfa chromosomes from photomicrographs to arrange the chromosomes for the development of a karyotype. Falistocco [2] measured chromosomes from photomicrographs, resulting in much larger chromosome measurements, e.g. the total length ranged between 9 and 12 µm, than what has been reported for the genus *Medicago*. Schlarbaum et al. [4] analyzed the chromosomes from alfalfa plants that had been regenerated from tissue culture. It is generally known that plants regenerated from a tissue culture system potentially can have structurally altered chromosomes [5].

Image analysis of plant chromosomes is rather a recent development. Fukui [6] was the first to develop an image analysis system CHIAS (CHromosome Image Analysis System) which characterized plant chromosomes. This system uses a large main frame computer based on the IBAS (Zeiss/Kontron) system and a CCD video camera with a resolution of 512 x 512 pixels. Since then, Fukui and his colleagues have improved their system and have utilized it to explore the chromosomes of several plant species: *Atriplex rosea* L. [7]; *Brassica* ssp., mustard [8]; *Glycine* ssp., soybeans [9]; *Oryza sativa* L., rice [10]; and *Zea mays* L., corn [11]. The CHIAS system is based on a large main frame computer, although a PC-based version (CHIAS-mini) is available [12], which is generally too expensive for the average cytogenetic laboratory budget.

The exponential growth of the computer industry has made it possible for PC-based computers to accomplish the tasks which formerly only main frame computers could accomplish. An NIH image analysis system (http://zippy.nimh.nih.gov/pub/nih-image) based on a PC computer has been developed by Wayne Rasband at the National Institutes of Health (Bethesda, MD) for biomedical use. We have developed an image analysis system specifically for analyzing chromosomes, which utilizes a PC computer and a high resolution camera. An earlier version of this system was used to analyze the somatic chromosomes of diploid alfalfa (2n = 2x = 16), *M. sativa* ssp. *coerulea* (L. & L.) Schmalh. [13].

This study was conducted to determine the efficacy of the image analysis system to differentiate between the homologous sets of chromosomes of autotetraploid alfalfa and develop a karyotype for cultivated alfalfa.

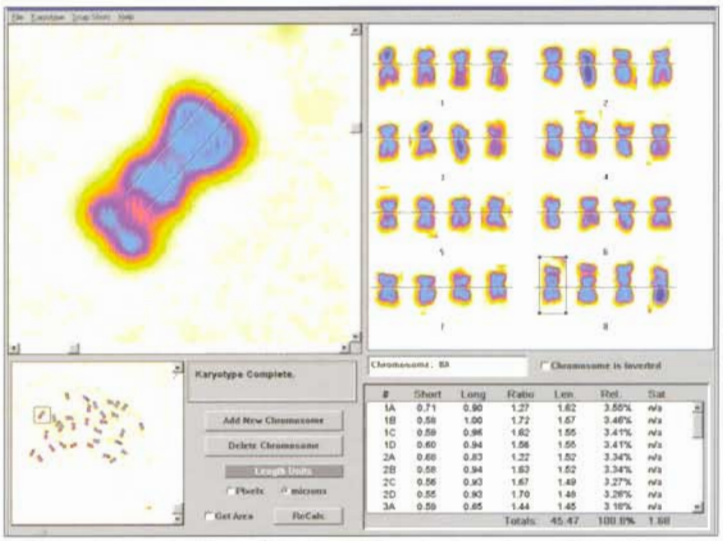

Figure 1: Image analysis computer screen. The lower left window contains the original chromosome spread, the upper left window displays an enlarged image for critical measurements of the individual chromosomes, upper right window displays the karyotype, and the lower right window contains the morphometric measurement data.

MATERIALS AND METHODS

Germplasm Source and Cytological Technique

Seeds of *M. sativa* ssp. *sativa* cv. 'Saranac' and the 'African' germplasm source (PI 536539) were obtained from the U.S. Plant Introduction Station in Pullman, WA. This cultivar and germplasm source were studied to represent alfalfa's diversity in dormant ('Saranac') and nondormant ('African') types. 'African' is one of the nine historically distinct sources of alfalfa germplasm introduced into different regions of the U.S. between 1850 and 1947 [14]. Seeds were scarified and germinated in Petri dishes at room temperature on filter paper. Root tips obtained two to three days after germination were pretreated in an ice bath for 18 h before fixation in Farmer's Fixative (3:1 v/v, 95% ethanol:glacial acetic acid). Root tips were hydrolyzed in 1N HCl for 10 min at 60o C, placed in Feulgen's stain for at least one hour and squashed in 1% acetocarmine. Twenty-five cells from 20 different plants containing well-spread chromosomes were observed in each germplasm. Observations were made using a Zeiss Axiophot Microscope using a 100X objective with a computerized image analysis system attached to the microscope.

Description of the Image Analysis System

A Karyotyper software module was developed for the morphometric measurement of chromosomes utilizing the Loats Associates Inquiry© Image Analysis System (201 East Main St., Westminster, MD 21157 USA, www.loats.com/index.html). The image analysis system is based on a Gateway 2000 PC with a Pentium III microprocessor running at 600 MHz clock speed. The computer has 128 MB SDRAM, 20.4 GB hard drive, 1.44 MB floppy drive, an IOmega 100 MB Zip drive, Philip's read-write CD, 53.34 cm Sony Trinitron Multiscan HG RGB analog color monitor, Microsoft

two button mouse, Polaroid CI-5000 Digital Palette, and Epson Color Stylus 800 ink jet printer.

The video digitizing components consist of a specially configured black and white Dage MTI 81 video camera with a resolution of 1600 TV lines. Image acquisition is enabled by a high resolution frame grabber (digitizing board) with a digitizing speed of 34 MHz, a memory buffer of 256 kB with a maximum resolution of 1024 x 960 pixels - 8 bit gray scale resolution (983,040 pixels per video image). The 8-bit information for each pixel represents one of 256 possible gray levels which can be enlarged 18X so that an individual chromosome can fill an entire video screen. Various colors can be assigned to each pixel based upon the densotometric measurement to present a pseudocolorized image. The standard output is analog-RGB and is displayed on a high-resolution (1280x1024 pixels) analog color monitor. Each image is approximately 1 MB, thus data are stored on either a 100 MB Zip drive or 600 MB CD. Data can be exported to Excel (Microsoft) for data analysis and images can be exported to Powerpoint (Microsoft) or Photoshop (Adobe) for production of slides or prints. Good quality prints can be produced using the Epson printer set at 1440 dpi on Epson glossy photography paper.

Karyotyper Software Program

The process of karyotyping was basically the same as described by Bauchan and Campbell [13]. The Karyotyper software program was co-developed by Loats Associates, Inc. in cooperation with Dr. Gary Bauchan. The major difference, besides the improvements in the PC computer (80386 processor with a clock speed of 20 MHz [13] versus Pentium III processor with a clock speed of 600 MHz PC computer), in this upgraded system is the increased resolution of the digitized image (512 x 480 vs 1024 x 960 pixels) and the output of data is compatible with Windows programs.

Briefly, all the measurements are made interactively using a mouse while viewing the images on the screen. The Windows environment makes it possible for all the information to be displayed simultaneously. Separate windows display the original chromosome spread, (Fig 1, lower left), the enlarged image for critical measurements of the individual chromosomes (Fig 1, upper left), the karyotype (Fig 1, upper right), and the measurement data (Fig 1, lower right). The centromere for each chromatid is identified and each chromatid is measured starting with the short arm and then the long arm. As each chromosome is measured, a copy of the chromosome image is rotated, short arms up, and placed into a template which has been preselected to accommodate the specified number of chromosomes into the karyotype window (Fig 1, upper right). The chromosome arm lengths are averaged together and the arm ratio (long arm/short arm average), total chromosome length (short arms plus long arms), and relative chromosome lengths (length of the individual chromosome/total length of all chromosomes in the genome) are calculated and displayed in the data window (Fig 1, lower right). The chromosome homologs are determined by sorting the data based on relative chromosome length and the chromosomes are arranged from longest to shortest with the SAT chromosomes following the shortest chromosome. Chromosomes in the karyotype window can be manually moved and arranged if necessary. The chromosomes can be numbered on the original chromosome spread in the order in which the chromosomes were measured to facilitate the identification of individual chromosomes following karyotyping.

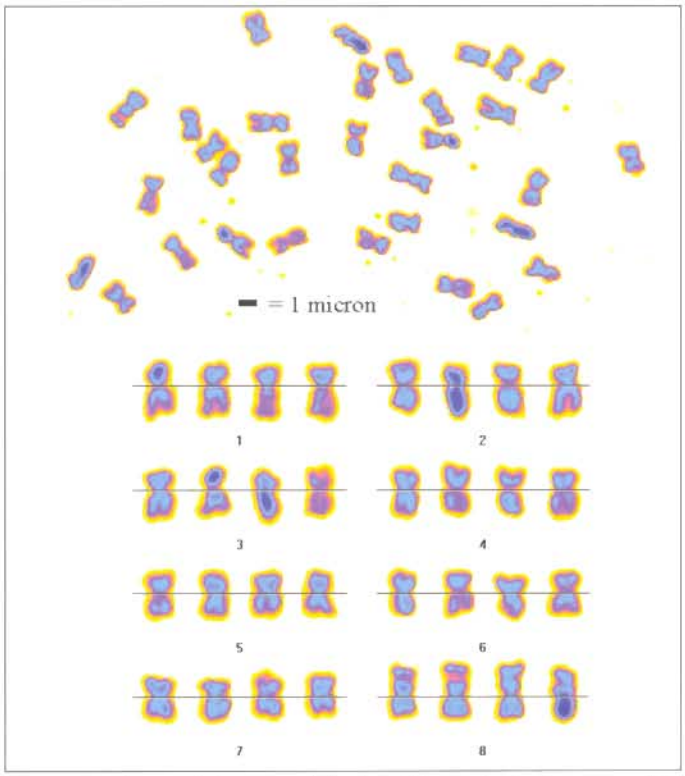

Figure 2:
Chromosomes of tetraploid M. sativa ssp. sativa using a computerized image analysis system. The bar represents 1 µm.

The efficacy of the image analysis system to differentiate between alfalfa chromosomes was tested on 50 cells, 25 cells from cv. 'Saranac' and 25 cells from the 'African' germplasm source. Statistical analysis of the data was done using SAS [15].

RESULTS AND DISCUSSION

FChromosomes in the past have been characterized manually by taking photographs, measuring the photographs with a ruler, cutting out the chromosomes, arranging the chromosomes from longest to shortest chromosome, pasting the chromosomes to a piece of paper and photographing the final product. This takes considerable time and effort for this analytical process. Furthermore, the final judgement as to how the chromosomes are arranged depends entirely on the researcher's judgement which requires long-term training and experience.

The Karyotyper software module is a fast, efficient and accurate method of analyzing chromosomes. The image analysis system is a proficient method of obtaining quality images of chromosomes due to its superior enhancement capability. Enhancement of the chromosomes by pseudocoloration and enlargement of the images enable the edges of the chromosomes to be distinguished for easy identification and measurement. The image analysis system is also a rapid method of obtaining large amounts of data on chromosome morphology. The karyotypic analysis of both the 'Saranac' and 'African' alfalfa germplasm sources gave very similar results, thus the data were combined. The efficacy of the image analysis system for discriminating among chromosome sets showed that only the relative chromosome length can be used to distinguish homologous groups. Based on Tukey's test this was the only measurement which could be used reliably to distinguish the chromosomes (Table 1). Coefficients of variation indicate that parameter estimation was reasonably precise (Table 1).

The analysis of the chromosomes developed from this study (Fig 2) is comparable to the karyotypes presented by Schlarbaum et al. [4] and Falistocco et al. [3]. However, in both of the previous studies the total chromosome length measurement and the arm ratio was utilized to discern the homologous chromosomes. We have shown that only the relative chromosome length measurement can be used to reliably distinguish the chromosomes. The tetraploid genome of alfalfa consists of eight sets of four homologous chromosomes with one set of satellited (SAT) chromosomes (chromosome 8), four sets of submetacentric (sm) chromosomes (chromosomes 1 - 4) and three sets of metacentric (m) chromosomes (chromosomes 5 - 7) (Table 1). Distinctive features include the large SAT chromosome (chromosome 8), the large submetacentric chromosome (1), and occasionally a tertiary constriction in chromosome 4.

Despite the difficulties of cytogenetically studying tetraploid alfalfa due to similar chromosome morphology, very small chromosome

Table 1. Efficacy of the image analysis system for differentiating among chromosome sets in alfalfa for 50 cells. Measurements are in micrometers (± SD).

Chrom set	Short Arm	Long Arm	Arm Ratio	Total Length	Relative. Length (%)	SAT. Length	Centro-mere position[b]
1	0.88 ± 0.03 a	1.36 ± 0.05 a	1.55 ± 0.01e	2.24± 0.07 a	13.83 ± 0.03 a		sm
2	0.87 ± 0.03 a,b	1.28 ± 0.05 a	1.47 ± 0.01d,e	2.15 ± 0.06 a	13.27 ± 0.02 b		sm
3	0.84 ± 0.03 b,c	1.20 ± 0.05 b	1.42 ± 0.01c,d	2.04 ± 0.06 b	12.59± 0.01 c		sm
4	0.83±0.03b,c,d	1.14 ± 0.04 b,c	1.37 ± 0.01b,c	1.97 ± 0.05 b,c	12.16± 0.01d		sm
5	0.83 ± 0.03 c,d	1.09 ± 0.04 c,d	1.31 ± 0.01a,b	1.92 ± 0.06 c,d	11.85± 0.01 e		m
6	0.80 ± 0.03 d	1.03 ± 0.04 d,e	1.29 ± 0.01a	1.83 ± 0.06 d	11.30± 0.01 f		m
7	0.73 ± 0.02 e	0.99 ± 0.03 e	1.36± 0.01a,b,c	1.72 ± 0.05 e	10.62± 0.02 g		m
8	0.66 ± 0.03 f	0.99 ± 0.25 e	1.51 ± 0.01e	2.33 ± 0.09 a	14.38± 0.04 a	0.68 ±0.03	SAT
CV[c]	19.6	20.0	19.8	16.9	7.8		

[a]Means not followed by the same letter are significantly different at the 0.05% level on Tukey's test.

[b]Centromere designation. SAT = satellited; sm = submetacentric and m = metacentric

[c]CV = coefficient of variation.

size, high number of chromosomes and autotetraploid nature, an image analysis system has been developed to characterize and identify the individual alfalfa chromosomes. This computerized image analysis system has become an integral part of our modern cytogenetics laboratory. This PC-based system is a less expensive system (approximately US$60,000) than typical main frame computer systems and is particularly effective for critically measuring plant chromosomes which have been difficult to analyze due to their similar chromosome morphology and small size.

CONCLUSIONS

A computerized image analysis system has been developed to analyze the vary small chromosomes of alfalfa (M. sativa ssp. sativa). Alfalfa chromosomes average between 1.7-2.5 µm in length. The image analysis system is able to characterize and identify the individual chromosomes of cultivated tetraploid (2n = 4X = 32) alfalfa. Two germplasm sources were studied, the cultivar 'Saranac', a dormant type of alfalfa and the 'African' germplasm, a non-dormant type of alfalfa. The computerized image analysis system was utilized to quantify the measurements of the chromosomes. The analysis revealed that alfalfa possess four nearly identical sets of chromosomes based on chromosome morphology. The tetraploid genome of alfalfa consists of eight sets of four homologous chromosomes: one set of satellited (SAT) chromosomes (chromosome 8), four sets of submetacentric (sm) chromosomes (chromosome 1 - 4) and three sets of metacentric (m) chromosomes (chromosomes 5 - 7). Distinctive features include the large SAT chromosome (chromosome 8), the large submetacentric chromosome 1, and occasionally depending on the quality of the preparation a tertiary constriction in chromosome 4. This computerized image analysis system is an efficient method of obtaining quality enhanced images and a rapid method of obtaining large amounts of chromosomal morphometric data. This PC-based system has become an integral part of our modern cytogenetic investigations, because it is less expensive than the typical main frame computer systems and is particularly effective for critically characterizing plant chromosomes which have been difficult to analyze due to their very similar chromosome morphology and small size.

ACKNOWLEDGEMENTS

Mention of a trade name or proprietary product does not constitute a guarantee, warranty, or recommendation of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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