Physical mapping of the 45S rDNA on the chromosomes of *Triticum turgidum* and *T. aestivum* using fluorescence *in situ* hybridization for chromosome ancestors

(Received: 20.10.2006; Accepted: 05.11.2006)

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ABSTRACT

Four amphiploid varieties of wheat in Egypt belonging to two species (Triticum turgidum and T. aestivum) were analyzed by fluorescence in situ hybridization (FISH) using 18S, 5.8S and 26S ribosomal genes (45S rDNA), which were applied to mitotic chromosome spreads. Tetra-amphiploids of Bani Suief (AABB genome; 2n=28) showed four subterminal FISH yellow signals and the hexa-amphiploids of Sids (AABBDD genome; 2n=42) showed six signals of which four were subterminal as those of the tetraploid (AABB) and two signals were terminal that might be related to the D genome. This rDNA mapping was also able to reveal differences in the genome composition that were mainly in the NOR. The FISH signals of the 45S rDNA sites of metaphase chromosomes were confirmed at interphase of all varieties studied. FISH signals were 12 sites of the 45S rDNA in each chromosome complements of the polyploidy species of Chrysanthemum indicum (tetraploid; 2n=36), Ch. occidentali-japonense (hexaploids; 2n=54) and Ch. vestitum (hexaploids; 2n=54). The ratio of FISH signal numbers per chromosome complement were decreasing with increasing the chromosome numbers in Chrysanthemum species, while they were stable in wheat varieties.

Key words: *Amphiploids, Chrysanthemum, fluorescence in situ hybridization, rDNA, wheat.*

INTRODUCTION

Chromosomes of the higher eukaryotes are composed of a number of repetitive DNAs, which may have played an important role in constructing the chromosome shape and may have affected the divergence of chromosomes by mutation (Harding *et al.*, 1992). Such DNAs appear to have big variation in size and number of loci in the plant species that have polyploid chromosomes. Therefore, the polyploidy might play an important role in the evolution of higher plants (Leich and Bennett, 1997; Soltis and Soltis, 2000). An appreciation of the types of changes in chromosomes-/genomes that have occurred during species evolution, has a direct impact on the basic and applied botany and to the aims of plant researchers and breeders in understanding genome evolution and genetics makes this area worthy of further investigation (Heslop-Harrison, 2000). Several studies on the genus *Triticum* that is characterized by polyploid series were important to justify and clarify the species origin and evolutionary process (Zohary and Feldman, 1962; Caldwell and Kasarda, 1978; Kerby and Kuspira, 1988;

Dvorák and Zhang, 1990; Friebe *et al.*, 1993; Talbert *et al.*, 1995; Daud and Gustafson, 1996; Maestra and Naranjo, 1998; Zhang *et al.*, 2002). There is extensive genetic evidence that *Triticum aestivum* L. (genomes AABBDD), ssp. *Aestivum*; bread wheat) originated by hybridization of tetraploid *T. turgidum* L. (AABB genome) with diploid *Aegilops tauschii* Coss. (DD genome) (Dvorak *et al.*, 1998). Because the D genome does not recombine in such crosses, its genetic relationships with the gene pool of *Ae. tauschii* can potentially provide valuable information on the relationships among the various forms of hexaploid wheat of *T. aestivum*.

As a pioneer among molecular cytogenetic techniques, FISH allows identification of specific sequences in a structurally preserved cell, in metaphase or interphase. This technique, based on the complementary double-stranded nature of DNA, hybridizes labeled specific DNA (probe). The probe, bound to the target, will be developed into a fluorescent signal. The fact that the signal can be detected clearly, even when fixed in interphase, improves the accuracy of the results, since in some cases it is extremely difficult to obtain mitotic samples (Mühlmann, 2002). FISH is a valuable method for studying the chromosomal distribution of DNA sequences and copy numbers at different sites, and to follow evolutionary changes in their physical organization in the genome (Harrison and Heslop-Harrison, 1995). FISH makes chromosomes of plant species providing the information of molecular characters of nucleolar organizing region (NORs) (Heslop-Harrison *et al.*, 1991).NOR loci are chromosome regions most intensively studied, in terms of both their structure and function. Several molecular evidences indicated that 45S rDNA is located on NOR-bearing chromosomes (Kondo *et al.*, 1996; Ørgaard and Heslop-Harrison, 1994; Kondo and Abd El-Twab, 2002; Abd El-Twab and Kondo, 2003). Analyses of NOR distribution and organization in rDNA carrying chromosomes are important to understand the events of divergence and hybridization of species. FISH mapping of rDNA would be a simple and effective way to characterize diverse collections of germplasm materials and breeding lines correctly. The mapping of rDNA location and number has detected apparent differences among the polyploidy relatives of wheat (Leitch *et al.*, 1992). Investigating the number and location of rDNA loci is important to gain a better understanding of evolution and phylogeny of various species in higher plant (Adams *et al* 2000; Abd El-Twab and Kondo, 2003).

The aim of the present study was to (1) determine the number and location of the 18S–5.8S–26S rDNA loci in the chromosomes of the investigated species, (2) inferring evolutionary changes in number and location of rDNA loci and (3) mapping the 45S rDNA on the chromosomes of the Egyptian wheat varieties in comparison with some polyploidy species of *Chrysanthemum*.

MATERIALS AND METHODS

Wheat varieties (*T. turgidum* var. Bani Suif-1 and -3; AABB genome and *T. aestivum* var. Sids-1 and -6; AABBDD genome) were kindly received from the Sids Agriculture Research Center, Bani-Suif, Egypt. The materials of *Chrysanthemum indicum*, *Ch. occidentali-japonense and Ch. vestitum* species were obtained from the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Hiroshima, Japan.

Chromosome preparations

Following Kondo *et al.* (1996), growing root tips were collected and pretreated in 0.002 M 8-hydroxyquinolin at 18°C for 1.5 hr. They were fixed in the 1: 3 glacial acetic acid and ethanol at 4°C for 2 hr. Fixed roots were excised and washed in distilled water many times to remove the fixative. Five to ten tips (2-5 mm long) each from root tips were placed in 1.5 ml microcentrifuge tube containing the enzymatic buffer of 5% cellulase (Yakult), and 2% pectolyase Y-23 (Kikkoman). They were incubated at 37°C for 20 min, then, the soft meristematic tissues were washed in distilled water to remove the enzymatic solution and were squashed in 45% acetic acid. The cover-slip was removed by the dry-ice freezing method and the preparation was dried at room temperature.

Ribosomal DNA probe

The probe pTa71 (kindly offered by Prof. Katsuhiko Kondo, Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Hiroshima, Japan) consisted of a 9 kb *Eco RI* fragment of rDNA derived from *Triticum aestivum* L. (Gerlach and Bedbrook, 1979), which was recloned into pUC19 plasmid. It was comprised of the coding sequence for the 18S, 5.8S and 26S genes and the non-transcribed spacer sequences.

Labeling of the pTa71 rDNA probe

Biotinylated nicktranslation labeling of the probe was carried out according to the manufacturer's protocol (Gibco BRL) with biotin-14-dATP nick translation kit. One and half □g of pUC19 plasmid DNA were added with 5 □l of 10X dNTP mixture, 34 □l of distilled water and 5 □l of 10X enzyme mixture to the final volume of 45 □l in 500 □l microcentrifuge tube. The mixture was nicktranslated for three hours at exactly 16°C. After the nicktranslation, 5 □l of stop buffer, 5 □l of 5 M NaCl and 300 □l of 99% alcohol were added and kept in the dry-ice chamber for 15 min. Then, the mixture was ultracentrifuged for ten minutes at 4°C in 15,000 rpm. Then, the supernatant was carefully removed from the tube, 300 □l of 70% ethanol were added to the pellet and ultracentrifuged for ten min at 4°C in 15,000 rpm. The supernatant was removed and the pellet was air-dried for 20 min. Finally 100 □l formamide and 100 □l of 10% dextran sulphate in 2XSSC (w/v), was added to the pellet and kept in -20°C.

FISH method

The procedure of FISH and the detection of the probe followed the methods described by Heslop-Harrison *et al.* (1991) and Kondo *et al.* (1996) with minor modifications. Slides were pretreated with 100 □l/ml of DNase-free RNase in 2XSSC for one hr at 37°C. Subsequently, the slides were washed twice in 2XSSC for ten min each. Then, the slides were postfixed in 4% depolymerized paraformaldehyde solution at room temperature for ten min and then, washed twice in 2XSSC for ten min each. After that, the slides were dehydrated in the graded ethanol series of 70, 80 and 100% for five minutes each and air-dried. Thirty micro liters of denatured hybridization mixture were added to each slides and covered with parafilms in humid chamber at 80°C for 15 min, which slowly cooled down up to hybridization at 37°C for 12 hr. For post hybridization, the slides were washed twice in 2XSSC for ten min each at room temperature. Then, the slides were washed in 2XSSC, in 50% (v/v) formamide in 2XSSC, 2XSSC and 4XSSC at 42°C for ten min each respectively. For the detection of hybridization signals, 4 □l of Avidin-FITC was added to 500 □l of 1% Bovine Serum Albumin in 4XSSC and

100 □l of this mixture was added to each slide, which were incubated at 37°C for one hr. After incubation, the slides were washed in 4XSSC, 0.2% of Tween 20 in 4XSSC and 2XSSC at room temperature for ten min each. Then, the slides were either stained with 0.1 □l/ml of DAPI or with 0.4 □l/ml of propidium iodide for ten min, and were mounted in DABCO. The fluorescence signals were examined with an epifluorescence microscope with Nikon B-2A filter cassette and microphotographs were taken on CCD camera (Pixera Pengium 600CL). Analysis of hybridization signals was produced using Adobe Photoshop 7.

RESULTS AND DISCUSSION

Variation in chromosome number and karyotype has proved to be an important source of information for understanding plant evolution (Stebbins, 1966; Kondo *et al.* 1992 and 1994). Physical maps of genes by fluorescent *in situ* hybridization represent a potentially new source of chromosomal characters that may be phylogenetically informative. In plants, physical mapping has focused on highly repetitive DNA or multigene families because technical difficulties remain for mapping low-copy genes (Jiang and Gill, 1994; Abd El-Twab and Kondo, 2003). The most frequently mapped gene is the 18S–5.8S–26S ribosomal RNA gene (rDNA). Evolutionary implications of variation in the number and location of rDNA loci have been explored in various plant groups (e.g., Maluszynska and Heslop-Harrison, 1993; Jiang and Gill, 1994; Thomas *et al.*, 1997). In the present study, Fluorescence *in situ* hybridization with the biotin labelled probe of pTa71 of 45S rDNA produced strong signals at the secondary constriction of the satellited mitotic metaphase chromosomes of the four wheat varieties. FISH signals of the 45S rDNA sites, which hybridized with the probe were four signals on the NOR- regions of the short arms of four somatic metaphase chromosomes in the chromosome complement of *T. turgidum* (Bani Suif-1 and -3; AABB genome; Fig.1 A, C and E) and six signals were on the short arms of six somatic metaphase chromosomes in the chromosome complement of *T. aestivum* (Sids-1 and -6; AABBDD genome; Fig.1 B, D and F). The interphase nuclei of the varieties of Bani Suif of *T. turgidum* (A) and Sids of *T. aestivum* (B) showed four and six yellow-FISH signals of the 45S rDNA sites respectively. The metaphase chromosomes of the two varieties of Bani Suif of *T. turgidum* had four subterminal signals of the 45S rDNA sites (Fig. 1 C and E), while the two Sids varieties of *T. aestivum* had six signals of which four were subterminal and two terminal signals (Fig. 1 D and F). Abd El-Baki and Abd El-Twab (2004) could not count the exact sat-chromosome numbers that carried the NOR, which varied between one pair to two pairs in both the tetraploid and hexaploids cultivars. The present results showed that the two and three chromosome neither pairs carrying NOR could be detected by FISH analysis. The discrepancies observed could be due to that not all conventionally stained mitotic squash preparations resolve secondary constrictions either at the NOR or elsewhere.This could be due to differential contraction of the chromosomes during pre-treatments (Kondo *et al.*, 1996; Irigoyen *et al.*, 2001). Consequently, the distribution and FISH signals of rDNA loci in tetraploid species observed in this work suggest that rRNA genes in the short arms of what chromosome number are associated with NORs. The comparison of homologous genome-specific repetitive DNA sequences from closely related organisms not only indicates the evolution of the sequences themselves, but also highlights relationships

among species. The present examination of pTa71 hybridization patterns in tetra-amphiploid and hexa-amphiploid varieties of the species reveals some interesting features. This marker band reflects the differentiation of both the tetraploid and hexaploid genomes in the genotypes analyzed. The FISH signals could confirm that the wild type species of *Aegilops speltoides* is impossible to be an ancestor of the tetraploid or even the hexapoloid varieties of the Egyptian wheat since the FISH signals of the 45S rDNA sites in the chromosome complement of *Ae. speltoides* were 4-6 sites after using the probe of pTa71 (Raskina *et al.*, 2004).

Table (1): FISH signals of 45S rDNA in mitotic chromosomes of Triticum species.

Species	Varieties	Ploidy	Genome	No .of cells exanimed/ FISH photos at metaphase/plant	No of FISH signals at			
					metaphase chromosomes			interphase
					terminal	subterminal	total	
T. turgidum	Bani-1	Tetraploid (2n=28)	AABB	5/5	—	4	4	4
	Bani-3	Tetraploid (2n=28)	AABB	3/3	—	4	4	4
T. aestivum	Sids-1	Hexaploid (2n=42)	AABBDD	3/3	2	4	6	6
	Sids-6	Hexaploid (2n=42)	AABBDD	6/6	2	4	6	6

Table (2): FISH signals of 45S rDNA sites in somatic chromosomes of several species in wheat and Chrysanthemum.

Species	2n	FISH signals of pTa71 of 45S rDNA probe		References of 45S rDNA signals in wheat and *Chrysanthemum*
		No.	%	
Triticum turgidum	28	4	14.3	present study
T. aestivum	42	6	14.3	present study
Expected diploid ancestors of polyploidy in *Chrysanthemum*	18	2, 4, 6 & 8	11.1,22.2, 33.3 &44.4	Honda *et al.* 1997; Khaung *et al.* 1997; Abe El-Twab and Kondo 2003
Ch. indicum	36	8	22.2	Khaung *et al.* 1997
	36	12	33.3	Abe El-Twab and Kondo 2003
	36	12	33.3	present study
Ch. occidentali-japonense	54	10	18.5	Abe El-Twab and Kondo 2003
		12	22.2	present study
		18	33.3	Abe El-Twab and Kondo 2003
Ch. vestitum	54	10	18.5	Abe El-Twab and Kondo 2003
		12	22.2	present study

Because of the possibility of random hybridization between tetraploid and hexaploid wheats, the A and B genomes are less reliable sources of information on the relationships among the hexaploid forms of wheat than the D genome. Because the D genome does not recombine in such crosses, its genetic relationships with the gene pool of *Aegilops tauschii* can potentially provide valuable information on the relationships among the various forms of hexaploid wheat and on the various scenarios of the origin of *T. aestivum* (Dvorak *et al.*, 1998). Clarification of the number and location of rDNA loci, might provide additional insights into the mechanisms of concerted evolution of rDNA in plants (Zhang *et al.*, 2002).

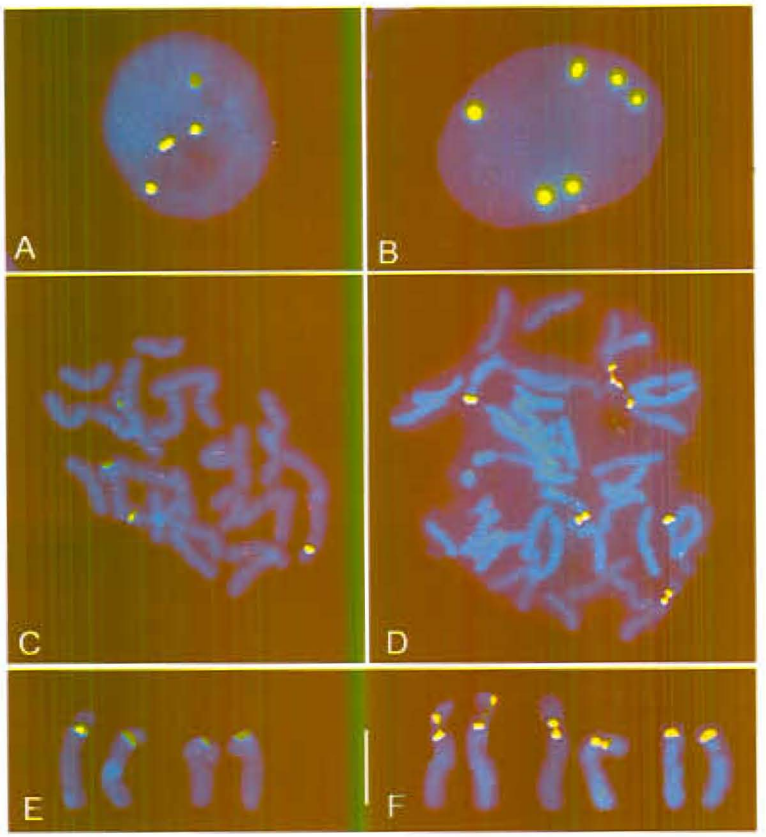

Fig. (1A-F): FISH signals of the 45S rDNA sites hybridized with biotin labelled pTa71 probe on mitotic metaphase chromosomes in T. turgidum (Beni Suif line-1; four subterminal signals, AABB genome; A, C & E), T. aestivum (Sids line-6; AABBDD genome;six signals:four subterminal and two terminal signals, B, D & F). A-B. Interphase. C-D. Metaphase chromosomes. E-F. FISH signals of the 45S rDNA carrying chromosomes of the species. Yellow-color was fluoresced and visualized by FITC and blue-color by DAPI. Bar=10 μm.

FISH signals of the 45S rDNA were 12 yellow sites in the chromosomes of the polyploidy species of *Chrysanthemum indicum* (Fig. 2A-B), *Ch. occidentali-japonense* (Fig. 2 C-D) and *Ch. vestitum* (Fig. 2E-F) after using the biotin labelled probe of pTa71. All the FISH signals of the 45S rDNA sites in the chromosome complement of *Chrysanthemum* were at terminal regions in all the species under investigation. The number of FISH signals of the 45S rDNA sites at interphase were confirmed the signal numbers of the metaphase chromosomes, since the signals were 12 of the 45S rDNA sites in the chromosome complements of each *Chrysanthemum indicum*, *Ch. occidentali-japonense* and *Ch. vestitum* (Fig. 2A, C and F; Table 2). In the present FISH study on the tetraploid and hexaploid *Chrysanthemum* (Fig. 2), Abd El-Twab and Kondo (2003) investigated the distribution patterns of the 45S rDNA in the chromosomes of various polyploidy species and found that all the FISH signals were terminal and very similar in shape and size, but the signal numbers per chromosome complement were different in each ploidy level Such kind of similarity of FISH size and position supported the speculation of the monophyletic origin of the species, while the difference in signal numbers per chromosome complement (Table 2), supported the allopolyploid origin of those species. In the present study on wheat, there was a terminal signal in the hexaploid cultivars and subterminal signals were found in both of the tetra- and hexa-amphipolid cultivars; the size of the signals were found to be different (Fig. 1E). Length of polymorphism of FISH rDNA hybridization sites is usually associated with differences in the number of repeats per site (Schubert and Wobus, 1985; Zurita *et al.*, 1997). This may be responsible for the interspecific variations in the length of rDNA sites found in wheat, as well as the heteromorphism in each chromosome pair exhibited by cultivars. The large size of rDNA sites observed in the present varieties is probably due to their high number of rDNA repeats. The shape of each FISH pair of signals on each chromosome pair carrying the NOR, was found to be different from each other, since in the tetra-amphiploid of Bani Suif, the first pair had a large signal and the other pair have two large sub-terminal FISH signals. Comparing the subterminal signals of both the tetraploid and hexaploids, it was found that both the two chromosome pairs have two large signals different from those of the tetraploids. Such kind of differentiation might be due to different genome origin or chromosomal rearrangements due to translocations that might increase the signal size or change the gene positions and structures. The diploid genomes like A genome, which had two chromosomal sites for rRNA genes (Gerlach and Dyer, 1980) had undergone a change after incorporation into hexaploid (6x) bread wheat. Also, the terminal signals of wheat chromosomes showed a very tiny FISH signal of the rDNA sites in the hybrid chromosome complement of wheat and barley (Cuadrado *et al.*, 1997), while the present FISH results showed quite large signals of the 45S rDNA sites. The third chromosome pair in the hexaploid genome that carries the terminal signals might be related to the D genome.

FISH opens the possibility of obtaining molecular cytogenetic results from interphase cells, which was impossible to achieve prior to the FISH technique and its colorful results are easy to interpret, explain and understand. Abd El-Twab and Kondo (2003) found that FISH signals of the 45S rDNA in the interphase were attached to the edge of the nucleoli. In the present study, the interphase FISH signals

of the 45S rDNA are found attached to the edges of the interphase nucleoli in each nucleus. The FISH signals of the 45S rDNA sites of metaphase chromosomes were confirmed at interphase of all varieties studied. In this, FISH can complement the information provided by both conventional and molecular cytogenetics analysis. The methods of FISH used here were effective in identifying the sat-chromosomes of each species and differentiating breeding cultivars. The rDNA markers are specific, as they could differentiate individual Nor-bearing chromosomes and are therefore the key markers in the identification of parental species of amphiploids in the present study. The rDNA FISH would be a simple and effective way to characterize diverse collections of germplasm materials and breeding lines correctly. All the amphiploids examined here are stable in signal numbers as hexaploids (Table 1; 2n=42). The mapping of the ribosomal genes has detected apparent differences among these amphiploids in their rDNA location and number (Fig. 1 and Table 1), indicating that such variation is tolerated at the genetic level. Indeed the ribosomal genes in these amphiploids appear to be active essentially in the wheat genomes, as indicated by extended constriction and the dispersed *in situ* pattern of rDNA expression in interphase nucleoli typical of wheat ribosomal genes (Leitch and Heslop-Harrison, 1992). The chromosome complement of the hexaploid wheat (*Triticum aestivum*) consists of three genomes,designated A, B, and D. Inferences on *T. aestivum* genome organization and evolution are therefore directly applicable to diploid relatives of wheat (Akhunov *et al.*, 2003).

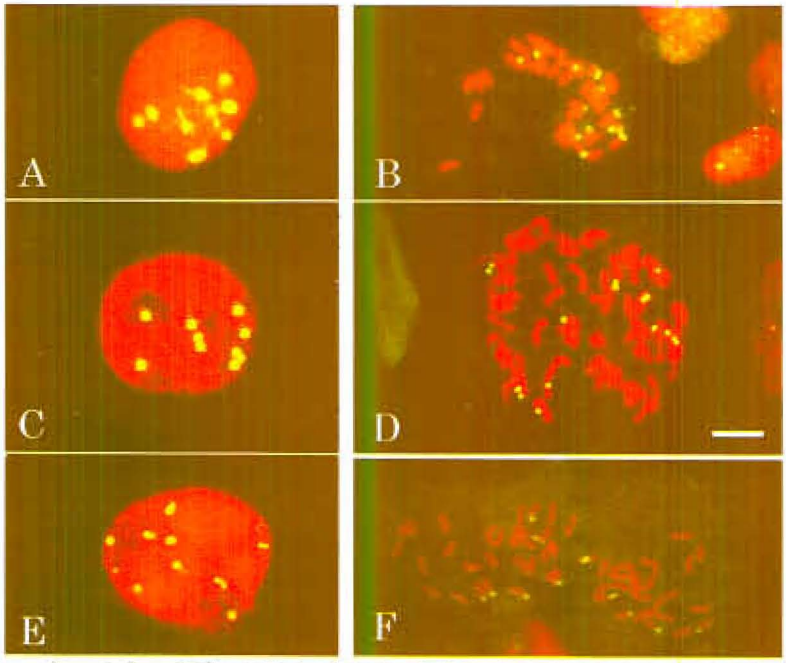

Fig. (2A-F): FISH signals of the 45S rDNA sites hybridized with biotin labelled pTa71 probe on mitotic metaphase chromosomes in Chrysanthemum indicum (A-B), Ch. occidentali-japonense (C-D) and Ch. vestitum (E-F). A, C & E. Interphase. B, D &F. Metaphase chromosomes. Yellow-color was fluoresced and visualized by FITC and red color by PI Bar=10 m.

genome chromosomes that have terminal signals (Fig. 1 B, D and F) and confirmed identification of chromosome arms bearing NOR sites. The molecular cytogenetic evidence here suggested that these annual, fully fertile amphiploids that originated from long years ago became genetically differentiated and fixed in stable forms. It seems that all amphiploids have contributed equally to the gene pool after a single amphi-hexaploid established. The type of knowledge attainable from this approach is expected to have a significant impact on both theoretical and applied research concerning wheat and related Triticeae. Further the FISH signal numbers of the 45S rDNA could be used to speculate the allopolyploid origin of wheat and *Chrysanthemum*. In case of wheat it was possible to support the amphiploid origin while in case of *Chrysanthemum* it was impossible to detect the exact

When the improvements in cytogenetic techniques seemed to have reached a plateau, a combination of cytogenetics and molecular biology gave rise to fluorescence *in situ* hybridization, opening many new opportunities in the field. Once an appropriately labelled DNA probe is available, FISH becomes the technique of choice.Further study might be applied on the chromosomes of the tetra- and hexa-amphiploid varieties in comparison with their diploid ancestors, using the probes of 5S rDNA genes, the telomeric sequence repeats as well as total genomic in situ hybridization might increase our knowledge about the genome characterization, ancestors and evolution of the Egyptian species

ACKNOWLEDGEMENTS

The author wishes to thank Prof. Dr. Katsuhiko Kondo for offering the labelled probe for this study during my Post Doc in his laboratory, autumn 2005, and Prof. Dr. Abd El-Twab Professor of Genetics, Faculty of Agriculture El-Minia University, El-Minia, Egypt, for his valuable discussion and critical reading of the manuscript.

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الملخص العربي

خريطه طبيعيه لل ٤٥ اس أر دي إن إيه علي كروموزومات تريتيكم تورجيدم وتريتيكم استيفام باستخدام الفلوريسنس انسيتيو هايبردايزيشن لمعرفة أسلاف كروموزومات

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استخدمت اربعه سلالات من القمح تنتمي إلي جنس تريتيكم والتي حللت بواسطة الفلوريسنس انسيتيو هايبردايزيشن والتي طبقت علي الكروموزومات المايتوزيه مستخدما جين ال ١٨ اس و٥،٨ اس و٢٦اس أر دي إن إيه (٤٥ اس أر دي إن ايه). كوموزومات القمح الرباعي لسلالة بني سويف (لجينوم: ا ا ب ب, ٢ إن=٢٨) أظهرت أربع علامات تحت طرفيه للفيش والقمح السداسي لسلالة سدس (لجينوم: ا ا ب ب دد, ٢ إن=٤٢) اظهر ست علامات أربعه تحت طرفيه كما في الرباعي وعلامتين كانت طرفيه والتي تنتمي إلي دي جينوم. استطاع ال ار دي ان ايه أن يظهر اختلافات في تركيب الجينوم والذي كان أساسي في إل إن أو أر .علامات الفيش لاماكن إل ٤٥ اس أر دي إن إيه لكروموزومات الطور الميتافيس امكن تاكيدها بواسطه طور الانترفيس في كل الأنواع المدروسة. بمقارنة علامات الفيش لأنواع الكريزانثيمم انديكم (رباعي: ٢ ان =٣٦), والكريزانثيمم اوكسيدنتالي جابونينس (سداسي: ٢ان =٥٤) و الكريزانثيمم فيستتم (سداسي: ٢ان =٥٤) والتي كانت ١٢ علامه لكل نوع لاماكن ال ٤٥ اس أر دي إن إيه: وجد أن الرقم النسبي لعلامات الفيش لكل مجموعه كروموزوميه كانت تتناقص مع زيادة العدد الكروموزومي في أنواع الكريزانثيمم بينما كانت ثابتة في أنواع القمح.

مفاتيح الكلمات: فلوريسينس انسيتيو هايبريدايزيشن، ار دي ان ايه، القمح, كريزانثيمم.