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Cytomolecular characterization of cultivars and landraces of wheat tolerant and sensitive to aluminum toxicity

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ABSTRACT: Karyotyping and chromosome complement knowledge are a prerequisite for cytogenetic mapping. The aim of this study was to characterize 1 common wheat cultivar (BH 1146) and 4 durum wheat genotypes (IAC 1003, Langdon, P19 and P33) from the breeding program of the Agronomic Institute of Campinas (IAC), using karyotype analysis together with Chromomycin A₃/4'-6-Diamidino-2-phenylindole (CMA/DAPI) banding techniques for longitudinal characterization of chromosomes and fluorescence *in situ* hybridization (FISH) in the mapping of the 45S rDNA region. Durum wheat chromosome complement number was $2n = 4x = 28$ with the following karyotype formula: $22m + 6sm$. Common wheat chromosome complement number was $2n = 6x = 42$ with the following karyotype formula: $34m + 8sm$. Tetraploid and hexaploid genomes presented 2 pairs of

chromosomes with secondary constriction and terminal satellites. All 5 wheat genotypes exhibited CMA and DAPI bands and signals in the 45S rDNA regions (FISH-probe pTa71) which varied in number and location on the chromosome complement. All lines showed greater amount of DAPI bands than CMA bands. The FISH-rDNA 45S *in situ* hybridization signals, related to the nucleolar organizing regions (NORs), were observed in common wheat, presenting signals for the 45S rDNA sequence in 3 pairs of chromosomes. Durum wheat presented signals in 2 pairs. The CMA bands coincided with the location of 45S rDNA region in the chromosomes of durum wheat. The techniques were efficient for chromosomal characterization of all 5 genotypes.

Key words: *Triticum aestivum*, *Triticum turgidum* ssp. *durum*, karyotype, CMA/DAPI banding, FISH-rDNA, cytomolecular mapping.

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INTRODUCTION

After maize, wheat is the world's second most produced cereal. The most commonly grown types with good commercial potential are common wheat (also called 'bread wheat' because it has a large amount of starch) and durum wheat, which contains higher protein content (gluten) and is used in the production of pasta (Felicio et al. 1999; Awika 2011).

In Brazil, cultivation of durum wheat has limitations because this type of wheat has little tolerance to acidic soils with Al^{3+} and H^+ ions. Acidic soils are common in different regions of Brazil, mainly in the North and Midwest, followed by the Southeast and South regions (Fageria and Stone 1999). Wheat cultivation is prevalent in the South, Southeast and Midwest Brazil, and several studies have been carried out in the last decades in order to enable large-scale cultivation of durum wheat in Southeast Brazil, particularly in the State of São Paulo (Camargo et al. 1992; Camargo et al. 1999; Camargo et al. 2006; Del Guercio and Camargo 2011). However, besides the lack of tolerance to aluminum toxicity, durum wheat in Brazil also has no grain dormancy, exhibits low productivity and is largely susceptible to diseases such as wheat leaf rust (*Puccinia triticina* = *P. recondite* f. sp. *tritici*), leaf spot (*Bipolaris sorokiniana*, *Drechslera tritici-repentis*, *Stagonospora nodorum* and *Septoria tritici*), wheat head blight fungus (*Gibberella zeae*), powdery mildew (*Blumeria graminis* f. sp. *tritici*) and blast (*Pyricularia grisea*).

Common wheat belongs to the *Triticum aestivum* L. species and has 3 genomes, called A, B and D, with 7 chromosome pairs each ($x = 7$). There are reports that genetic traits for tolerance to aluminum toxicity are mainly present in chromosomes of the D genome. However, genes for tolerance to aluminum toxicity have also been identified in the chromosomes of A and B genomes in some common wheat cultivars (Lagos et al. 1991; Luo and Dvorak 1996; Ryan et al. 2009). According to Camargo et al. (2006), the aluminum tolerance of cultivar BH 1146 might be controlled by a pair of dominant genes, whereas Aniol (1984) and Ryan et al. (2009) have reported that tolerance to Al^{3+} is controlled by multiple genes in other common wheat cultivars. In contrast to common wheat, durum wheat (*Triticum turgidum* ssp. *durum* Desf) has only A and B genomes, with no reports of cultivars presenting tolerance

to aluminum toxicity (Lagos et al. 1991; Camargo et al. 1992; Luo and Dvorak 1996; Camargo et al. 2006).

Leymus mollis (NsNsXmXm, $2n = 28$) is a wild relative of wheat and has agronomic characteristics such as resistance to abiotic stress, fungi and bacteria that are possible to transfer to wheat in breeding programs by chromosome segment substitution lines (CSSLs) (Pang et al. 2014). Cytogenetic mapping by fluorescence *in situ* hybridization (FISH) is a tool that helps localize directly on the chromosome complement specific DNA sequences and genes (Lukaszewski et al. 2012; Zhang et al. 2012; Pang et al. 2014; Tang et al. 2014; Zhang et al. 2015). This technique is common in breeding programs in order to detect and track the introgression of genes via chromosomal mapping. Pang et al. (2014), using genomic *in situ* hybridization (GISH) and FISH, determined the karyotypic constitution of a wheat — *Leymus mollis* 3D (3Ns#1) disomic substitution line, which was designated as 10DM57 ($2n = 42$). By employing hybridization via GISH, it was possible to identify that 10DM57 was a line with 40 chromosomes from wheat and 2 of the Ns chromosomes from *L. mollis*. The FISH technique showed pattern with probe pAs1 bands of chromosome 3D and the Ns chromosome. The authors deduced that the Ns chromosome was 3Ns and that chromosome 3D was replaced by 3Ns in 10DM57. This study demonstrated that the line 10DM57 was resistant to leaf rust when compared with common wheat and recommend it as an intermediate material in wheat genetic and breeding programs.

Karyotyping or the knowledge of the chromosome complement is a prerequisite for cytogenetic mapping. In genotypes in which the karyotype is well known, it is possible to identify genes or DNA sequences on specific chromosomes. Mapping also allows recognizing and monitoring the presence of chromosomes or chromosome segments during introgression in breeding programs.

Karyotype analysis based on rDNA regions with the 45S region probe, known as pTa71, and 5S has been employed in several groups of plants such as Leguminosae, Poaceae and Rubiaceae (Nardy et al. 2010; Cuadrado et al. 2013; Iacia and Pinto-Maglio 2013). The amplification of the 45S rDNA region is related to the Nucleolus Organizer Region — NOR (Carpeta et al. 2002), and the species must have at least 1 pair of 45S rDNA signals corresponding to the pair of nucleolar chromosomes of its chromosome complement.

The objective of this study was to characterize 1 common wheat cultivar (BH 1146) and 4 durum wheat cultivars/landraces (IAC 1003, Langdon, P19 and P33) from the breeding program of the Agronomic Institute of Campinas (IAC - Campinas, São Paulo, Brazil), using karyotyping and 4'-6-Diamidino-2-phenylindole/Chromomycin A₃ (DAPI/CMA) banding techniques for longitudinal characterization of chromosomes and FISH of nucleic acids for mapping 45S rDNA sites. Knowing these karyotypes will allow mapping sequences related with tolerance to aluminum toxicity in the studied wheat chromosome complement. This will enable monitoring the introgression of specific chromosomes bearing sequences related to aluminum tolerance through introgression lines in breeding programs.

MATERIAL AND METHODS

Biological material

Seeds of 5 wheat genotypes (BH 1146, IAC 1003, Langdon, P19 and P33) available at the Bank of Wheat Seeds from the IAC (BAG-IAC WHEAT) were used to obtain the meristems in cytological preparations. The BH 1146 common wheat cultivar belongs to *Triticum aestivum*, whereas the IAC 1003, Langdon, P19 and P33 durum wheat genotypes belong to *Triticum turgidum* ssp. *durum*. Using crosses of BH 1146 and Langdon genotypes followed by 2 backcrosses with IAC 1003 cultivar (BH 1146/Langdon//2*IAC1003), the gene from BH 1146 common wheat that confers tolerance to aluminum toxicity was transferred to the durum wheat landraces P19 and P33 (Silva et al. 2010).

Pretreatment, fixation and preparation of chromosomes

Metaphase chromosomes were obtained using a pretreatment of root tips from common and durum wheat, germinated as described in the following protocol. Common wheat seeds were germinated on moist cotton in Petri dishes and then kept in the dark in a controlled temperature of 25 °C for 48 h. After this period, the roots were collected and submitted to a pretreatment in cold water (4 °C) for 32 h. After pretreating, roots were fixed in Carnoy solution (absolute ethanol:glacial acetic acid — ratio 3:1, respectively) and stored at -20 °C.

Durum wheat seeds were subjected to thermal shock to break dormancy. Seeds were germinated in Petri dishes with moist cotton and then kept in the dark for 24 h at 25 °C. Then seeds were transferred to 4 °C for 48 h and then maintained at 25 °C for 24 h (Brammer et al. 2009). Germinated roots were collected and submitted to pretreatment with the PDB antimitotic (Paradichlorobenzene) for 25 h. After pretreating, the roots were fixed in Carnoy solution (absolute ethanol:glacial acetic acid — ratio 3:1, respectively) and stored at -20 °C.

Slides were prepared using the “SteamDrop” method used by Kirov et al. (2014) with slight modifications. Cell digestion or hydrolysis was performed in 30 µL enzyme solution with a final concentration of 6%, containing a mixture of 0.03% Pectolyase, Cellulase and Cytohelicase (Sigma-Aldrich). The roots on this enzyme solution remained at 45 °C for 45 min.

Analysis of chromosome morphology

For the analysis of chromosomal morphology, slides were mounted with 15 µL of 45% acetic acid. Slides were covered with coverslip and examined under phase microscopy.

Chromomycin A₃ banding

Banding with the fluorochrome Chromomycin A₃ with Distamycin (CMA/DA) was performed following the procedure described by Schweizer (1976) with some adjustments. It was applied a quantity equal to 40 µL of buffer containing CMA on the dry slides. Slides were covered with coverslips and kept in the dark at room temperature for 1h30min. Then slides were rinsed with distilled water and dried at room temperature for 1 h in the dark. After adding 30 µL of 0.1 mg·mL⁻¹ distamycin solution, the slides were covered with coverslips and kept in the dark at room temperature for 20 min. Then slides were rinsed with distilled water and dried for 1 h in the dark at room temperature. The slides were mounted with 40 µL of 1 M magnesium chloride solution, McIlvane buffer and Vectashield (5 µL, 1 mL and 1 mL, respectively), covered with coverslip and incubated at 37 °C for 4 days.

4'-6-Diamidino-2-phenylindole banding

For banding with DAPI/AMD (4'-6-Diamidino-2-phenylindole/Actinomycin D) fluorochrome, the procedure described by Schweizer (1976) was employed,

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and a quantity equal to 40 μL of a diluted Actinomycin D solution ($0.2 \text{ mg}\cdot\text{mL}^{-1}$ in deionized water) was applied to dried slides. Slides were then covered with coverslips and maintained in the dark for 20 min then washed again with distilled water and kept in the dark at room temperature during 1 h for drying. After drying, a quantity equal to 30 μL DAPI solution (2 μL stock DAPI plus 198 μL water) was applied. These preparations were covered with coverslips and maintained in the dark for 30 min. Then, slides were rinsed with distilled water and kept drying at room temperature and in the dark. The slides were mounted with a drop (10 μL) of Vectashield (Vector Laboratories) and covered with a coverslip.

Fluorescence *in situ* hybridization

The protocol based on Pendás et al. (1993) and modified as described by Pinto-Maglio et al. (2001)³ and Iacia and Pinto-Maglio (2013) was used for FISH. The probe (pTa71) was the 45S sequence of ribosomal DNA (45S rDNA) isolated from the wheat genome (*Triticum aestivum*), which was labeled with digoxigenin-11-dUTP (Roche) using the nick translation reaction according to manufacturer's instruction. Detection was with anti-digoxigenin conjugated to fluorescein isocyanate (FITC). Cytological preparations were completed with Vectashield mounting medium for fluorescence containing the DAPI counterstain.

Fluorescence images

Images of the chromosomes were analyzed and captured using an Olympus BX50 epifluorescence microscope coupled to a computer containing the Image-ProPlus software, version 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). The following Olympus filters for fluorescence were used: U-MWU for DAPI (BLUE/UV — excitation at 330/385 nm and emission at 420 nm), U-MWBV to CMA (GREEN/UV — excitation at 400/440 nm and emission at 475 nm) and U-MNB2 to FITC (GREEN/UV — excitation at 450 nm and emission at 570 nm).

Image analysis

Images of chromosomes resulting from the application of different techniques were edited with Adobe Photoshop

CS6 (64-bit) and Image-ProPlus 6.0 — Media Cybernetics. Nomenclature to establish the centromere position was the one suggested by Levan et al. (1964). The karyotype asymmetry was determined by the total form (TF, in %) according to Huziwara (1958; 1962). The TF takes into account the sum of the short arms and the total length of the chromosomes, and the percent values range from 0 to 50%.

For each genotype was measured the chromosomes of ten cells at metaphase using the MicroMeasure 3.3 program version (MM) (Reeves 2001) and idiograms were built using Microsoft® PowerPoint 2010.

Statistical analysis

The results from the morphological analysis of chromosomes with the banding patterns (DAPI/AMD and CMA/DA) and the hybridization signals for the 45S rDNA sequence were subjected to multivariate analysis, via Principal Component Analysis (PCA), in order to compare the degree of correlation among the morphological characteristics, the main banding patterns and the number of rDNA signals that determine the similarity and/or differentiation between genotypes. The statistical software “Program GENES” (http://www.ufv.br/dbg/genes/Genes_EUA.htm) was used to perform the multivariate analysis (Cruz 2013).

RESULTS AND DISCUSSION

Numerical and morphological chromosome analysis

Mitotic analysis of durum wheat genotypes (IAC 1003, Langdon, P19 and P33) allowed to observe complements with $2n = 4x = 28$ chromosomes and with karyotype formula of $22m + 6sm$. The common wheat cultivar (BH 1146) presented $2n = 6x = 42$ chromosomes and karyotype formula of $34m + 8sm$ (Tables 1, 2). Chromosomal complements with $2n = 28$ and $2n = 42$ chromosomes have been recorded previously in durum wheat and common wheat respectively by other authors such as El-Twab (2006) and Endo et al. (2014).

The Langdon durum wheat cultivar is one of the cultivars that originated P19 and P33 landraces. According to Joppa and Williams (1988), the Langdon was an aneuploid line that had a pair of chromosomes homoeologous replaced

³Pinto-Maglio, C. A. F., Barbosa, R. L., Cuéllar, T., Maluf, M. P., Pierozzi, N. I., Silvarolla, B. and Orsi, C. H. (2001). Chromosome characterization in *Coffea arabica* L. using cytomolecular techniques. Proceedings of the 14th International Chromosome Conference; Würzburg, Alemanha.

Table 1. Total length average of chromosome pairs, mean coefficient of variation, average of short arms, centromeric index and classification for common wheat (BH 1146) and durum wheat (IAC 1003, Langdon, P19 and P33).

BH 1146					
Pairs	CP	CV%	SA	CI	CL
1	12.58 ± 1.87	14.90	5.54 ± 0.82	1.27	M
2	11.39 ± 2.18	19.20	4.81 ± 1.10	1.36	M
3	10.88 ± 1.96	18.07	3.91 ± 1.36	1.78	S
4	10.65 ± 2.07	19.43	4.96 ± 0.85	1.14	M
5	10.34 ± 1.90	18.41	3.99 ± 0.84	1.58	M
6	10.13 ± 1.94	19.16	4.19 ± 0.79	1.41	M
7	10.11 ± 2.20	21.81	4.58 ± 1.09	1.20	M
8	9.58 ± 1.91	19.99	3.73 ± 0.85	1.56	M
9	9.25 ± 1.69	18.37	3.81 ± 0.79	1.42	M
10	9.05 ± 1.72	19.05	3.70 ± 0.78	1.44	M
11	8.85 ± 1.68	19.03	3.56 ± 0.87	1.48	M
12	8.68 ± 1.61	18.60	3.90 ± 0.89	1.22	M
13	8.41 ± 1.52	18.14	3.33 ± 0.64	1.52	M
14	8.28 ± 1.46	17.72	3.76 ± 0.81	1.19	M
15	8.08 ± 1.38	17.10	3.69 ± 0.78	1.18	M
16	7.89 ± 1.41	17.93	2.66 ± 0.49	1.96	S
17	7.54 ± 1.34	17.81	3.37 ± 0.61	1.23	M
18	7.30 ± 1.25	17.15	3.16 ± 0.68	1.30	M
19	7.30 ± 1.49	20.45	2.51 ± 0.56	1.90	S
20	6.86 ± 1.26	18.49	2.26 ± 0.49	2.03	S
21	5.88 ± 0.77	13.16	2.47 ± 0.30	1.37	M

IAC 1003						Langdon					
Pairs	CP	CV%	SA	CI	CL	Pairs	CP	CV%	SA	CI	CL
1	11.05 ± 1.41	12.79	4.49 ± 0.59	1.46	M	1	11.19 ± 2.30	20.61	4.71 ± 0.83	1.37	M
2	10.06 ± 1.32	13.19	4.62 ± 0.68	1.17	M	2	10.57 ± 1.86	17.66	4.49 ± 0.87	1.35	M
3	9.64 ± 1.30	13.55	4.13 ± 0.62	1.33	M	3	10.12 ± 1.76	17.45	4.73 ± 1.00	1.14	M
4	9.46 ± 1.17	12.38	3.83 ± 0.68	1.46	M	4	9.72 ± 1.70	17.57	3.97 ± 0.70	1.44	M
5	9.28 ± 1.12	12.07	4.05 ± 0.61	1.29	M	5	9.31 ± 1.72	18.53	3.12 ± 0.45	1.97	S
6	9.11 ± 1.10	12.15	3.64 ± 0.33	1.49	M	6	9.04 ± 1.54	17.11	4.11 ± 0.91	1.20	M
7	8.94 ± 1.10	12.29	3.91 ± 0.51	1.28	M	7	8.92 ± 1.58	17.75	3.49 ± 0.65	1.55	M
8	8.76 ± 0.99	11.40	3.76 ± 0.47	1.32	M	8	8.60 ± 1.51	17.63	3.63 ± 0.88	1.36	M
9	8.49 ± 0.89	10.51	3.00 ± 0.43	1.82	S	9	8.36 ± 1.52	18.19	3.74 ± 0.63	1.23	M
10	8.28 ± 0.87	10.59	3.80 ± 0.63	1.18	M	10	8.16 ± 1.51	18.50	3.10 ± 0.69	1.62	M
11	8.00 ± 0.83	10.45	2.69 ± 0.39	1.96	S	11	7.62 ± 1.44	18.94	2.55 ± 0.50	1.98	S
12	7.69 ± 0.89	11.68	2.77 ± 0.30	1.77	S	12	7.37 ± 1.30	17.67	2.73 ± 0.73	1.69	M
13	7.49 ± 1.02	13.66	3.08 ± 0.41	1.42	M	13	6.96 ± 1.18	16.96	2.74 ± 0.50	1.54	M
14	6.82 ± 0.93	13.73	2.97 ± 0.64	1.29	M	14	6.19 ± 1.18	19.22	2.19 ± 0.44	1.82	S

...continue

Table 1. Continuation...

P19						P33					
Pairs	CP	CV%	SA	CI	CL	Pairs	CP	CV%	SA	CI	CL
1	12.13 ± 1.79	14.82	6.46 ± 0.96	1.22	M	1	14.09 ± 1.87	13.30	6.10 ± 1.08	1.31	M
2	11.52 ± 1.86	16.19	4.81 ± 0.79	1.39	M	2	12.54 ± 1.68	13.47	4.24 ± 0.74	1.95	S
3	10.73 ± 1.68	15.66	4.53 ± 1.16	1.36	M	3	12.28 ± 1.62	13.21	5.24 ± 0.67	1.34	M
4	10.70 ± 1.71	16.01	4.88 ± 0.81	1.19	M	4	11.86 ± 1.50	12.68	4.93 ± 0.63	1.40	M
5	10.34 ± 1.59	15.40	3.38 ± 0.52	2.05	S	4'	8.27 ± 0.82	9.94	3.85 ± 0.38	1.14	M
6	10.09 ± 1.46	14.48	4.03 ± 0.67	1.50	M	5	11.53 ± 1.41	12.24	4.74 ± 0.53	1.43	M
6'	8.07 ± 1.11	13.81	3.98 ± 1.17	1.02	M	6	11.22 ± 1.46	13.03	4.54 ± 0.77	1.47	M
7	9.81 ± 1.33	13.60	4.17 ± 0.72	1.34	M	7	10.92 ± 1.58	14.54	5.10 ± 1.01	1.14	M
8	9.60 ± 1.43	14.92	4.29 ± 0.65	1.23	M	8	10.69 ± 1.45	13.58	4.45 ± 0.52	1.40	M
8'	6.74 ± 1.21	17.94	2.88 ± 0.61	1.34	M	9	10.35 ± 1.47	14.26	4.61 ± 0.67	1.24	M
9	9.28 ± 1.45	15.61	3.20 ± 0.58	1.89	S	10	10.23 ± 1.49	14.62	3.48 ± 0.45	1.93	S
10	9.22 ± 1.47	15.93	4.12 ± 0.82	1.23	M	11	9.89 ± 1.45	14.70	4.37 ± 0.68	1.26	M
11	8.84 ± 1.29	14.67	3.75 ± 0.75	1.35	M	12	9.21 ± 1.49	16.24	3.09 ± 0.44	1.97	S
12	8.29 ± 1.25	15.15	2.74 ± 0.29	2.02	S	13	8.84 ± 1.32	15.00	3.94 ± 0.72	1.24	M
13	8.21 ± 1.25	15.29	3.69 ± 0.35	1.22	M	14	7.85 ± 1.20	15.35	3.20 ± 0.44	1.45	M
14	7.52 ± 1.32	17.57	3.26 ± 0.60	1.30	M	-	-	-	-	-	-

The heteromorphic pairs of chromosomes in P19 (6, 6', 8 and 8') and P33 (4 and 4') are represented. CP = Chromosome pairs; CV% = Mean coefficient of variation; SA = Short arms; CI = Centromeric index; CL = Classification; M = Metacentric chromosome; S = Submetacentric chromosome.

Table 2. Comparative data of karyotype formulas and mean of chromosome total length, length of short arms, total form, the larger and smaller chromosome lengths in common wheat (BH 1146) and the 4 durum wheat genotypes (IAC 1003, Langdon, P19 and P33).

Genotype	Karyotype formulas (2n)	Total average (µm)	Short arm (µm)	Total form (%)	Larger chromosome (µm)	Smaller chromosome (µm)
BH 1146	34m + 8sm	9.00 ± 1.65	3.71 ± 0.83	41.23	12.58 ± 1.87	5.88 ± 0.77
IAC 1003	22m + 6sm	8.79 ± 1.10	3.62 ± 0.62	41.25	11.05 ± 1.41	6.82 ± 0.93
Langdon	22m + 6sm	8.72 ± 1.41	3.52 ± 0.81	40.38	11.19 ± 2.30	6.19 ± 1.18
P19	22m + 6sm	9.44 ± 1.46	3.95 ± 0.75	41.84	12.13 ± 1.79	7.52 ± 1.32
P33	22m + 6sm	10.65 ± 1.69	4.39 ± 0.80	41.26	14.09 ± 1.87	7.85 ± 1.20

The average values of the total chromosome lengths, short arms, long arms, larger and smaller chromosomes refer to the average of 10 meristematic root cells in metaphase stage.

by a pair of D genome chromosomes of the Chinese Spring common wheat cultivar. However, in the analyzed seeds and in the IAC samples, the meristematic cells presented euploid number of chromosomes from durum wheat, i.e., $2n = 4x = 28$. The multiplication and renewal of seeds are necessary for maintaining germplasm banks and although these procedures may tend to promote, over time, a trend toward normalization of the characteristic number of the species. Such normalization likely occurred with Langdon cultivar when selfing with normal gametes.

According to the analysis of the chromosome morphology of the 5 genotypes, the highest values for chromosome lengths average were observed in P33 ($10.65 \pm 1.69 \mu\text{m}$) and

P19 ($9.44 \pm 1.46 \mu\text{m}$) and the averages with smaller values in Langdon ($8.72 \pm 1.41 \mu\text{m}$), IAC 1003 ($8.79 \pm 1.10 \mu\text{m}$) and BH 1146 ($9.00 \pm 1.65 \mu\text{m}$) cultivars. The TF values obtained for common and durum wheat ranged from 40.38 to 41.84% (average 41.19%) (Table 2). Thus, according to the TF values for karyotypes, all 5 genotypes were considered asymmetrical. Asymmetrical karyotype was also observed in hexaploid wheat by Arabbeigi et al. (2011).

The highest and lowest chromosomal length values observed in genotypes were: BH 1146 ($12.58 \pm 1.87/5.88 \pm 0.77$); IAC 1003 ($11.05 \pm 1.41/6.82 \pm 0.93$); Langdon ($11.19 \pm 2.30/6.19 \pm 1.18$); P19 ($12.13 \pm 1.79/7.52 \pm 1.32$); and P33 ($14.09 \pm 1.87/7.85 \pm 1.20$) (Table 1). According to Stebbins

(1971), the karyotypes of wheat genotypes analyzed in this study may be also considered asymmetric once they present chromosome pairs quite different in length, that is, these karyotypes are considered heterogeneous regarding the length of their chromosomes.

The highest chromosome length was identified in the landrace P33 ($14.09 \pm 1.87 \mu\text{m}$), followed by BH 1146 and P19 ($12.58 \pm 1.87 \mu\text{m}$ and $12.13 \pm 1.79 \mu\text{m}$, respectively), while the smallest chromosome length was identified in the genotypes BH 1146 and Langdon ($5.88 \pm 0.77 \mu\text{m}$ and $6.19 \pm 1.18 \mu\text{m}$, respectively) (Table 1). As expected, the common wheat cultivar BH 1146 had complement with the smallest chromosomal lengths when compared to lengths of the chromosomal complements of durum wheat genotypes. One explanation that could justify the smaller chromosomes is the presence of the D genome chromosomes in the complement of BH 1146, because, as shown by Jahan and Vahidy (1989) and Gill et al. (1991), the common wheat has the AABBDD genome while durum wheat has the AABB genome, and the D genome chromosomes are smaller when compared with chromosomes of A and B genomes.

All analyzed genotypes in this study, either tetraploid or hexaploid, showed the presence of 2 pairs of chromosomes carrying satellites (SAT) on the short arms. That is a common feature among the *T. turgidum* ssp. *durum* and *T. aestivum* species. Chromosomes that have satellites (SAT) normally are carriers of nucleolar organizing regions (NORs). There is 1 pair of this kind of chromosome per genome. Common wheat (BH 1146) has 3 genomes (ABD) but it presents only 2 pairs of these chromosomes in its complement. This fact leads to the conclusion that a third pair in the complement may have been suppressed because of a dominance interaction in hybrid genomes, as mentioned by Pikaard (2000).

Two pairs of homologous chromosomes, heteromorphic in length, were found in P19 (pairs 6 and 8) and 1 in P33 (pair 4) (Figure 1). Differences among homologous pairs of heteromorphic chromosomes with different lengths were complemented by comparing the banding patterns obtained with DAPI fluorochrome. As described previously by Schubert (2007), chromosomes can be modified within a group of organisms by chromosome rearrangements. Thus, the difference in morphology between heteromorphic homologous pairs can be explained by problems during the replication process when changes such as insertions

or deletions may promote the shrinkage or expansion of chromosomes.

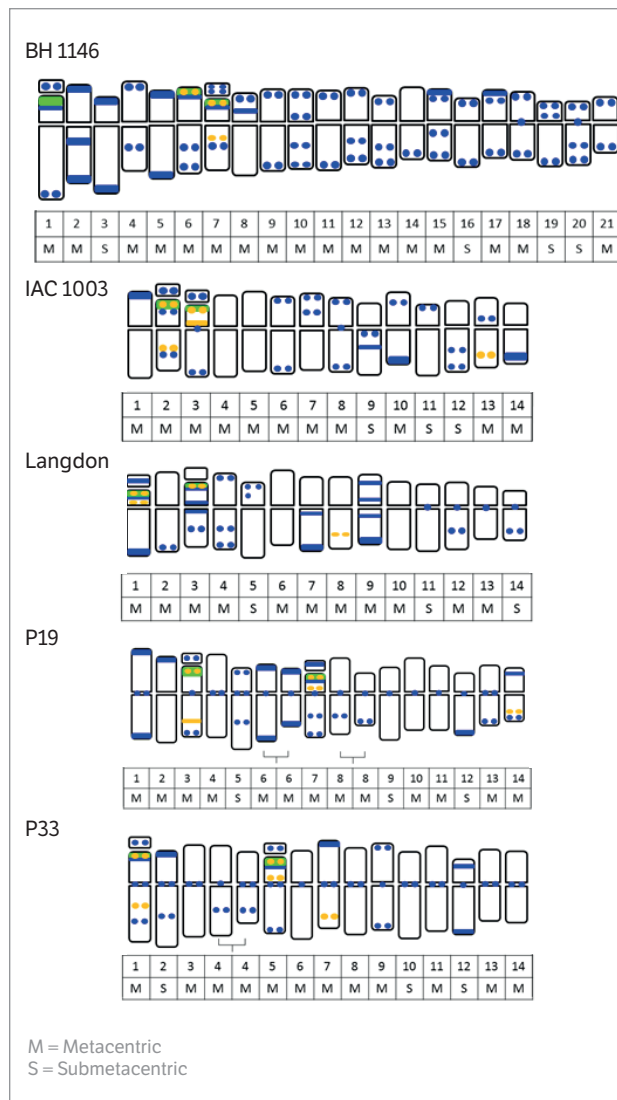


Figure 1. Idiograms of karyotypes of *Triticum* sp. BH 1146, IAC 1003, Langdon, P19 and P33. Banding with 4'-6-Diamidino-2-phenylindole/Actinomycin D (DAPI/AMD; blue), Chromomycin A₃ with Distamycin (CMA/DA; orange) and FISH-45S rDNA - pTa71 probe hybridization sites (green). Heteromorphic pairs of chromosomes are shown for genotypes P19 (chromosomes 6 and 8) and P33 (chromosome 4).

Distribution of 4'-6-Diamidino-2-phenylindole, Chromomycin A₃ bands and location of 45S rDNA

All 5 wheat genotypes were positive for banding with DAPI and CMA fluorochromes and for *in situ* hybridization signals in the 45S rDNA region (FISH probe-pTa71). The CMA and DAPI bands and the FISH-rDNA 45S hybridization signals varied in number and position in

the chromosome complements of different genotypes (Figure 2). The DAPI/CMA techniques assisted in linear characterization of the homologous chromosome pairs and FISH assisted in the identification of chromosomes with NORs. Regarding the banding with fluorochromes, it was found that all accesses complements had chromosomes with larger amount of heterochromatin containing AT (DAPI positive bands) than CG bases (CMA positive bands) (Figure 1). Chromosomes showing no clearly defined DAPI and CMA positive bands, probably harbor heterochromatin with AT or GC bases evenly distributed and not in specific regions (Table 3).

4'-6-Diamidino-2-phenylindole banding

Considering the 5 accesses, positive DAPI heterochromatic regions were viewed either as bands or spots. The common wheat BH 1146 cultivar showed a typical heterochromatic pattern, with DAPI bands prevalent in the terminal regions of both short and long arms, and also interspersed in the chromosome. The durum wheat landraces P19 e P33, on the contrary, showed heterochromatin with a more concentrated pattern of positive DAPI bands in the proximal or centromeric region of chromosomes. The 2 other durum wheat cultivars, IAC 1003 and Langdon, presented dispersed bands along the length of the chromosomes.

The durum and common wheat genotypes also showed, in addition to differences in the pattern and location of DAPI bands along the chromosomes for each genotype, differences in the resulting number of the total sum of such bands for each complement. The BH 1146 cultivar showed higher amount of DAPI bands (total of 114), when compared to the other four durum wheat genotypes, with a variation in their number, from 46 to 60 positive DAPI

bands among different chromosome complements. The durum wheat landraces P19 and P33 had similar values for total quantity of DAPI bands per complement (60 and 58, respectively) and for equal number of total bands with short arms (16 bands). The IAC 1003 and Langdon cultivars, in turn, revealed a total of 46 and 50 bands, respectively, in the chromosome complement (Table 3). Except for the Langdon cultivar, that showed positive DAPI band in the SAT in just one pair of nucleolar chromosomes, all other genotypes exhibited both pairs of chromosomes with DAPI bands located in the SATs (Figure 1). The SATs regions of chromosomes were AT-rich.

Chromomycin A₃ banding

The CMA fluorochrome banding presented six bands for complement of BH 1146 common wheat. These bands were restricted to 2 chromosome pairs (6 and 7), and one of these pairs is a SAT carrier (pair 7).

Durum wheat genotypes (IAC 1003, P19 and P33) showed 10 bands, except for the Langdon complement that presented seven bands for this fluorochrome. Positive CMA bands in all wheat genotypes were located in short and long arms in three chromosome pairs of each genotype, 2 of these chromosome pairs as SAT carriers. Genotypes showed bands in the following chromosome pairs: 'IAC1003' in 2, 3 (with SAT) and 13; 'Langdon' in 1, 3 (with SAT) and 8 (only 1 chromosome in pair 8 presented the CMA band); P19 in 3, 7 (with SAT) and 14; and P33 in 1, 5 (with SAT) and 7 (Figure 1, Table 3).

Durum wheat (tetraploid) accessions present a smaller number of chromosomes ($2n = 28$) and 2 genomes (AABB), and common wheat (hexaploid) has a higher chromosome number ($2n = 42$) and 3 genomes (AABBDD), nevertheless

Table 3. Total number and arms (short and long) of 4'-6-Diamidino-2-phenylindole with Actinomycin D; Chromomycin A₃ with Distamycin bands and FISH-rDNA 45S hybridization signals on chromosome complement of common wheat (BH 1146) and durum wheat (IAC 1003, Langdon, P19 and P33).

Genotype	DAPI/AMD				CMA/DA			FISH (pTa71)	
	Total	Short arm	Centromere	Long arm	Total	Short arm	Long arm	Total	Short arm
BH 1146	114	56	4	54	6	4	2	6	6
IAC 1003	46	22	4	20	10	6	4	4	4
Langdon	50	18	8	24	7	6	1	4	4
P19	60	16	24	20	10	6	4	4	4
P33	58	16	28	14	10	6	4	4	4

DAPI/AMD = 4'-6-Diamidino-2-phenylindole with Actinomycin D; CMA/DA = Chromomycin A₃ with Distamycin; FISH = Fluorescence *in situ* hybridization.

durum wheat showed greater number of GC-content regions (8 or 10 bands) and common wheat had only 6 bands in its complement. Thus, there is an inversely proportional relationship between the amount of CMA positive bands and the number of chromosomes in the complements. The greatest amount of bands rich in GC-content in durum wheat may be related to a duplication process and in common wheat the smallest amount of positive CMA bands may be related to deletion of chromosomal segments rich in GC-content.

Location of 45S rDNA region

The results of the *in situ* hybridization technique for the location of 45S rDNA regions showed strong hybridization signals of chromosome pairs in the terminal and subterminal regions of short arms. Cultivars showed signals in the following chromosome pairs: 1, 6 and 7 in BH 1146 cultivar; 2 and 3 in IAC 1003 cultivar; 1 and 3 in Langdon cultivar; 3 and 7 in P19 landraces; and 1 and 5 in P33 landraces (Figures 1,2). All durum wheat genotypes ('IAC 1003', 'Langdon', P33 and P19) that are tetraploid (AABB) revealed 4 45S rDNA signals in 2 pairs of chromosome carriers of SAT. The BH 1146 common wheat cultivar, a hexaploid with AABBDD genomes, showed 6 signs located in 3 pairs of chromosomes (pairs 1, 6 and 7), 2 of them carriers of SAT (1 and 7). Similar results for number and location of 45S rDNA sites were also reported by El-Twab (2006), with 4 signals in durum wheat and 6 in common wheat located in the terminal and subterminal regions in short arms of chromosomes of *Triticum aestivum* and *Triticum turgidum*.

The amount of 45S rDNA signals found in durum wheat species that are tetraploid (4 signals) and common wheat species that are hexaploid (6 signals) is directly related to genomic allopolyploid constitution of the analyzed genotypes.

The size and intensity of 45S rDNA region hybridization signals differed within the complement in all genotypes. The BH 1146 cultivar revealed better signal on chromosome pair 1; IAC 1003 in pair 2; Langdon in pair 1 and lines P19 and P33 in pairs 3 and 5, respectively. It is not possible to conclude whether the difference observed in the intensity and size of the signals are directly related to the ability of NOR expression. No technique was used to confirm whether the difference in signal intensity was related to

the expression of NOR. However, it would be possible to consider these differences in the number of 45S rDNA sequence repeats in different chromosomes.

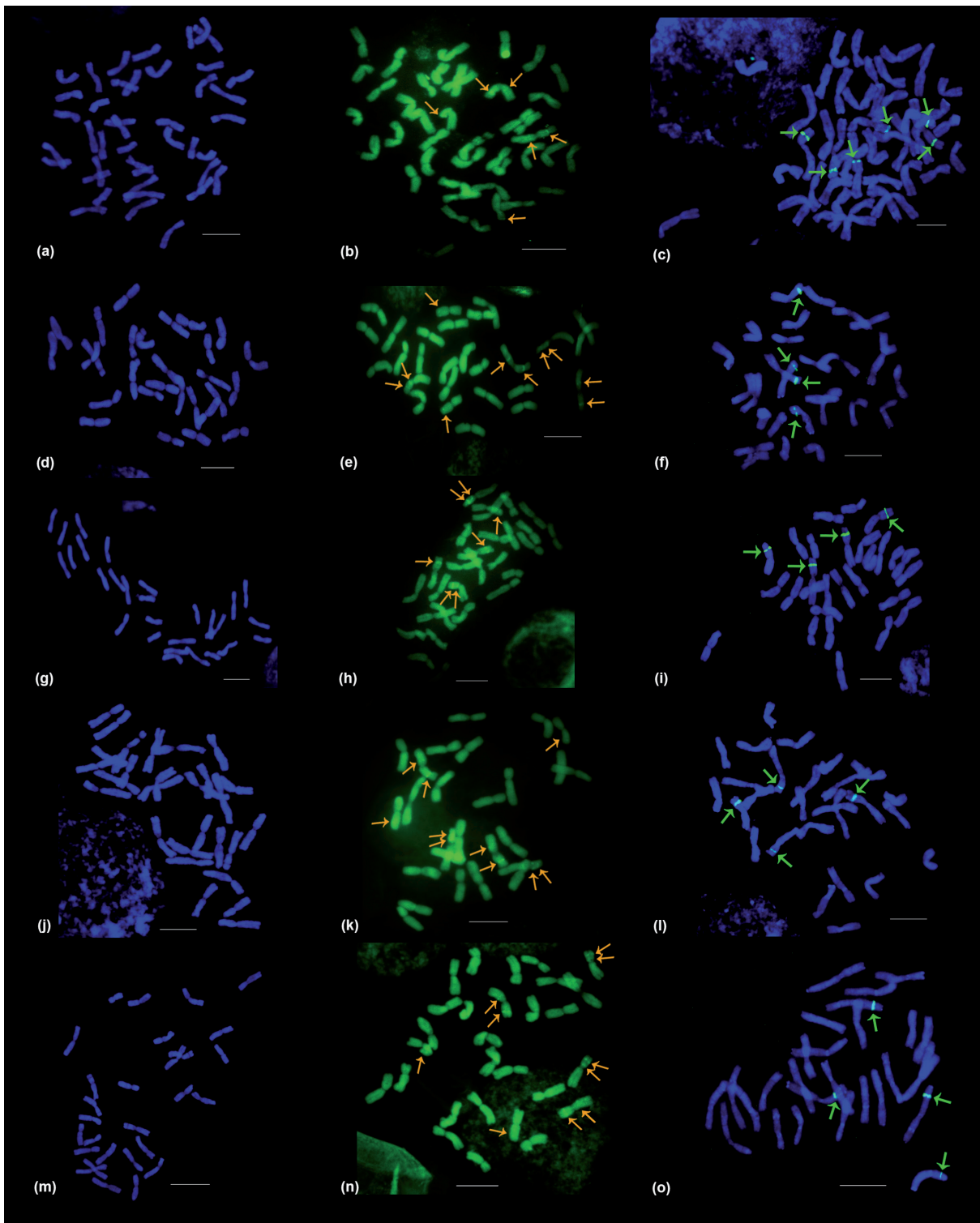
It is known that hybrid organisms that contain multiple NORs have a dominant control mechanism for the expression of ribosomal genes such as DNA methylation and histone modification (Pikaard 1999; 2001; Carpeta et al. 2002). The existence of dominance relationships between NORs in hybrid genomes, like in tetraploid and hexaploid cultivars, could be confirmed by comparing the results of FISH-rDNA and the results of the impregnation with silver (AgNOR) technique and immunolocalization techniques for detecting DNA methylation patterns and histone modifications. The AgNOR technique, for example, would demonstrate only the number of NORs that were active or expressed in the last interphase, while the FISH-rDNA technique indicates the total number of NORs, both active and inactive. Some studies (Siljak-Yakovlev et al. 2002; Garcia et al. 2007; Oliveira et al. 2012) have shown that NOR matches the CMA banding in some species and thus such regions are GC-rich.

The location of the 45S rDNA signals in durum wheat genotypes ('IAC 1003', 'Langdon', P19 and P33) also co-located with the positive CMA bands in the short arm subterminal region of 2 chromosome pairs, carriers of SAT. However, in common wheat (BH 1146) from the 3 pairs of 45S rDNA signals present in the complement, only 2 co-located with the CMA bands present in the subterminal region in the short arm of chromosome pair number 7 (with SAT) and the terminal region of chromosome pair number 6 (without SAT). The third signal of 45S rDNA is located on chromosome pair number 1 (with SAT), which showed no CMA banding.

Multivariate analysis

The Principal Components Analysis (PCA) included 17 variables, such as the numerical and morphological analysis, amount of DAPI/CMA bands and 45S rDNA region signals (Tables 2,3). The 6 variables that showed greater relevance to graphic composition were: a) average of the largest chromosome; b) amount of CMA bands in the long arm of chromosomes; c) amount of metacentric chromosomes; d) average of the smallest chromosome; e) amount of submetacentrics chromosomes; f) amount of CMA bands on the short arm of chromosomes. The





a, b and c = Common wheat BH 1146 cultivar; d, e and f = Durum wheat IAC 1003 cultivar; g, h and i = Langdon cultivar
j, k and l = P19 line; m, n and o = P33 line; Orange arrow = CMA/DA; Green arrow = FISH-rDNA signals; Bar = 10 μ m.

Figure 2. Metaphase chromosomes of *Triticum* sp.: 4'-6-Diamidino-2-phenylindole with Actinomycin D (DAPI/AMD); Chromomycin A₃ with Distamycin (CMA/DA) bands and FISH-rDNA 45S *in situ* hybridization signals (pTa71 probe with digoxigenin), from left to right, respectively, in common wheat and durum wheat accesses.

cumulative percentage of the total variance given the first 2 main components explained 99.43% of the total sample variance. According to Johnson and Wichern (2007), this percentage means that the variance of the sample is already well reported if summarized by only 2 main components. The cumulative percentage provided high reliability in the grouping and similarity between genotypes provided in the graph (Figure 3).

Few studies with karyological data have been submitted to multivariate analysis and this analysis allowed the formation of groups with varying degrees of similarity. The PCA established 2 main groups, separating the genotypes between species. One group contained *T. aestivum* (BH 1146) and the other *T. turgidum* ssp. *durum* (IAC 1003, Langdon, P19 and P33). This grouping showed chromosomal variability, indicating the distinction of access between species and the genetic proximity of the accesses of the same species (Figure 3).

With the PCA analysis, it was possible to reveal that the P19 and P33 landraces from the crossing between BH 1146/Langdon//2*IAC 1003 showed greater similarity to each other when compared with other genotypes within the

group that contains the lines of durum wheat. Considering the parental of durum wheat species, Langdon cultivar was closer to P19 and P33 lines than to IAC 1003. This multivariate analysis allowed to observe that many of the chromosomal characteristics derived from Langdon remained preserved even after backcrossing with IAC 1003. As for the P19 and P33 landraces, in general, the data suggest that they inherited more chromosomal regions of Langdon and IAC 1003 durum wheat than BH 1146 common wheat.

Based on the results obtained in the clustering analysis, it can be concluded that morphological examination by phase contrast, chromosomal banding patterns obtained with the DAPI and CMA fluorochrome, as well as the location and the number of hybridization sites of the 45S rDNA sequences characterize the chromosomes and consequently differentiate the 5 genotypes.

CONCLUSION

Chromosomal characterization obtained by numerical and morphological analysis and the aforementioned techniques allow the localization and association of gene regions responsible for specific characteristic to their chromosomes, such as the gene or sequence that confers tolerance to aluminum toxicity, present in the BH 1146 cultivar. Thus, chromosomal characterization of P33 and P19 lines, for example, allow their use in the generation of introgression lines (addition or substitution lines) and in their use thereof in breeding programs. The application of the CMA/DAPI banding and FISH-rDNA 45S techniques in the progenies will assist in the identification and monitoring of the transfer of chromosome with the gene sequence that confers tolerance to aluminum toxicity.

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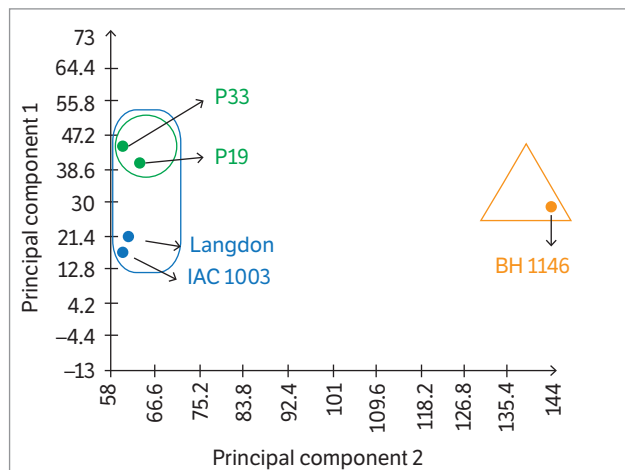


Figure 3. Principal Component Analysis in 4 genotypes of durum wheat (IAC 1003, Langdon, P19 and P33) and 1 common wheat genotype (BH 1146) based on chromosome morphology, number of bands generated with 4'-6-Diamidino-2-phenylindole/Actinomycin D (DAPI/AMD), Chromomycin A₃ with Distamycin (CMA/DA) banding techniques and the amount of FISH-rDNA 45S (pTa71 probe) signal. Genes program (Cruz 2013).

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