VIVIAN TORRES BANDEIRA TUPPER

PHYSICAL MAPPING OF HAPLOID-INDUCING GENES IN *Zea mays* L.

Dissertation submitted to the Genetic and Breeding Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Wellington Ronildo Clarindo

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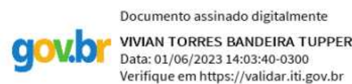

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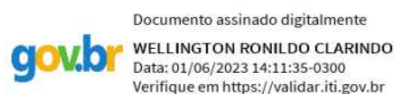

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À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

“There’s no lemon so sour that you can’t make something resembling lemonade.”

(This is us)

ABSTRACT

TUPPER, Vivian Torres Bandeira, M.Sc., Universidade Federal de Viçosa, March, 2023. **Physical Mapping of Haploid-Inducing Genes in *Zea mays* L.** Advisor: Wellington Ronildo Clarindo.

Cytogenomics in *Zea mays* L. have been provided physical maps, which gather data from cytogenetics, genetic maps and genome sequencing. Since McClintock's data about the basic chromosome number (x = 10), *Z. mays* karyotype has been characterized, evidencing the dynamism of its genome. Haploid induction is the initial step to produce double haploid lines, which are genetically homozygous for all *loci* in up to two generations. Haploid induction is a quantitative trait related to different genes, such as *r-navajo* (*r1*), *colored plant1* (*b1*), *matrilineal1* (*mtl*) and *membrane protein domain1* (*dmp1*). The physical loci of these genes are still unknown. We aimed to map the physical loci of the *r1*, *b1*, *mtl* and *dmp1* genes related to *Z. mays* haploid induction. For this, seeds of *Z. mays* 'Krasnodar' haploid inducer and of different genitors were used. We co-hybridizated probes for rDNA *18S + r1*, *18S + b1*, *18S + mtl*, and *18S + dmp1*. Two hybridization signals for each gene were identified in five interphase nuclei for all lines. The mapping of these sequences in *Z. mays* metaphase chromosomes evidenced specific *locus* for each gene. *18S rDNA* is located on chromosome 6 short arm, *mtl* on chromosome 1 short arm, *b1* on chromosome 2 short arm, dmp*1* on chromosome 9 short arm, and *r1* on chromosome 10 long arm. Due to specific *locus*, these genes can also be used as chromosome-specific cytogenetic markers for *Z. mays* and the *18S* rDNA. We conclude that the construction of physical maps contributes to understanding the structure and evolution of *Z. mays* genome, increasing the cytogenomic data about this species.

Keywords: Cytogenetics. Double haploid. FISH. Plant breeding. Maize.

RESUMO

TUPPER, Vivian Torres Bandeira, M.Sc., Universidade Federal de Viçosa, Março de 2023. **Mapeamento físico de genes de indução de haploidia em *Zea mays* L.** Orientador: Wellington Ronildo Clarindo.

Abodagens citogenômicas em *Zea mays* L. fornecem mapas físicos, os quais reúnem dados de citogenética, mapas genéticos e sequenciamento do genoma. Desde os primeiros relatos de McClintock sobre o número básico de cromossomos (x = 10), o cariótipo de *Z. mays* foi caracterizado, evidenciando também o dinamismo de seu genoma. A indução de indivíduos haploides é o passo inicial para produzir linhagens duplo-haploides. A indução haploide é uma característica quantitativa expressa a partir de diferentes genes, como *r-navajo* (*r1*), *coloured plant1* (*b1*), *matrilineal1* (*mtl*) e *membrane protein domain1* (*dmp1*). Apesar do conhecido papel desses genes na indução da haploidia, as localizações físicas no genoma de *Z. mays* ainda são desconhecidas. Portanto, nosso objetivo foi mapear a localização física dos genes *r1*, *b1*, *mtl* e *dmp1* relacionados à indução da haploidia em *Z. mays*. Sementes de *Z. mays* de diferentes linhagens de genitores e do indutor 'Krasnodar haploid inducer' (KHI) foram usadas para obtenção de núcleos interfásicos e cromossomos metafásicos. As sondas rDNA *18S + r1*; *18S + b1*; *18S + mtl* e *18S +* dmp1 foram co-hibridizadas nos núcleos interfásicos e cromossomos metafásicos. Dois sinais de hibridização de cada gene foram identificados em cinco núcleos interfásicos para todos os genótipos. O mapeamento dessas sequências em cromossomos metafásicos de *Z. mays* evidenciou *locus* específicos para cada gene. O *18S* rDNA está localizado no braço curto do cromossomo 6, *mtl* está localizado no braço curto do cromossomo 1, *b1* no braço curto do cromossomo 2, *dmp1* no braço curto do cromossomo 9, e o *r1* no braço longo do cromossomo 10. Em virtude do locus específico, esses genes também podem ser usados como marcadores citogenéticos cromossomo-específicos para *Z. mays*, assim como o *18S* rDNA. Concluímos que a construção de mapas físicos contribui para o entendimento da estrutura e evolução do genoma de *Z. mays*, aumentando os dados citogenômicos sobre esta espécie.

Palavras-chave: Citogenética. Duplo haploide. FISH. Melhoramento. Milho.

SUMMARY

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1. INTRODUCTION

Z. mays (2n = 2x = 20, Kuwada, 1925) was one of the first plant species in which individual chromosomes were cytologically identified (Mcclintock, 1929). Since that discovery, *Z. mays* karyotype description (structure, composition and organization) has increasingly become robust and detailed with the advent of fluorescent *in situ* hybridization (FISH) (Kato et al., 2004; Wang et al., 2006). Four types of maps have been constructed for *Z. mays* based on genetic *loci*: cytological map (Davis et al., 1999), genomic physical map (Wei et al., 2007), genetic linkage map (Schnable et al., 2009), and physical map using FISH (Figueroa et al., 2012). *Z. mays* physical maps provide data about the *loci* of different DNA sequences (mainly repeatome sequences), which can be previously mapped in genetic maps and/or sequencing. Concerning the identification and mapping of single-copy genes, the physical maps have been considered more complex due to intraspecific variation of *Z. mays* genome size (Wang et al., 2006).

The variation of the *Z. mays* genome is due to mainly to the mobile elements (Schnable et al., 2009; Silva et al., 2018). *Z. mays* genome is composed of ~85% of mobile elements, being ~77.1% of retroelements and ~6.7% of transposons (Yang et al., 2019; Stitzer et al., 2021). *Z. mays* mobile elements promote genomic changes (as mutations and new allelic forms), interfering in gene expression and phenotypic diversity (May & Dellaporta, 1998).

Haploid induction *in vivo* represents the initial step to produce double haploid (DH) plants (Maqbool et al., 2020). DH method generates homozygous lines (Prasanna et al., 2012). DH method was responsible to replace the conventional method of self-fertilization in some plant breeding programs (Gilles et al., 2017). Haploid induction in *Z. mays* is a spontaneously phenomenon (Chaikam et al., 2019; Maqbool et al., 2020) that naturally occurs in a low frequency (~0,1%) (Coe, 1959). Thus, some lines were developed to increase the rate of haploid induction in *Z. mays*, improving the rate to 10% (Zhao et al. 2013).

Haploid and, consequently, DH induction are a quantitative traits influenced by many genes (Lashermes and Beckert, 1988). *R-Navajo* (*r1*) and *colored plant1* (*b1*) genes act, respectively, in the kernel control (Melchinger et al., 2016) and in anthocyanin biosynthesis regulation, which are essential in color expression of haploid seeds (Andorf et al., 2019). *matrilineal1* (*mtl1*) and *domain membrane protein1* (*dmp1*) genes are responsible to induce the haploidy in *Z. mays*. Some

allele forms of these genes are related to increase the haploid induction rate (Kelliher et al., 2017; Zhong et al., 2019). In *Z. mays*, starting from the haploids, chromosome set duplication is induced mainly by treatment with colchicine, resulting in homozygous fertile diploids (Kelliher et al., 2017).

Despite the potential of these genes in the induction of haploids in *Z. mays* (Maqbool et al., 2020), physical loci in the genome are still unknown (Wang et al., 2006). Considering the remarkable data about haploid induction to provide DH plants, we aimed to construct the physical map from *r1*, *b1*, *mtl* and *dmp1* genes, using *18S* rDNA as FISH control, in genitors and inductor lines of *Z. mays*. We also answered the following questions: (1) What is the copy number of these genes? (2) What is the physical location of these genes? (3) These genes can be used as chromosome-specific cytogenetic markers?

2 MATERIAL AND METHODS

2.1 Plant material

Root meristems were obtained from seeds of ‘Krasnodar haploid inducer’ (KHI) and genitors (‘UFV-MP3 – 18-176-5’, ‘Viçosa – 20-2027’, ‘Viçosa – 20-2064’ and ‘Beija-Flor – 20-2010’, ‘Beija-Flor – 20-2009’) of *Z. mays,* which were germinated at 30˚C in a Petri dish containing dH_2O.

2.2 Cytogenetic preparations

Z. mays root meristems were incubated at 30°C for 18h in solution containing 0.20 g L^{-1} Murashige and Skoog Basal Salt Mixture (Sigma®) and 1.75 mM hydroxyurea (inhibitor of ribonucleotide reductase, Sigma®). The roots were washed in dH_2O four times for 15 min and treated with 4 μM dinitroaniline (inhibitor of microtubule polymerization, Sigma®) + 60 μM dimethyl sulfoxide (Sigma®) for 4 h at 30°C (Silva et al., 2020). Root meristems were excised about 1 cm in length, and then fixed three times in 3:1 (v/v) methanol:acetic acid solution (Merck®) and stored at -20°C for 24 h. After that, the roots were washed three times in dH_2O for 30 min and macerated for 2 h at 36°C in enzymatic mix (4% cellulase + 0.4% hemicellulase + 1% pectolyase diluted in pectinase solution, Sigma®) in a proportion of 1:8 (enzyme:dH_2O). Then, the roots were washed in dH_2O, fixed in methanol:acetic acid 3:1 (v/v) (Merck®) and stored at -20°C for 24 h. Finally, the slides were prepared using the cell dissociation and air-drying techniques (Carvalho et al., 2011). The slides were chosen for FISH based on metaphasic index and occurrence of morphologically preserved chromosomes.

2.3 Primer selection, sequencing and probe construction

Using MAIZEGDB database, genes involved in the induction and/or identification of haploids were filtered. Primers were constructed based on similarity analysis obtained from the National Center for Biotechnology Information (NCBI) database. For this, we used the sequence of reference genome of *Z. mays* ‘B73’ (GCA_902167145.1). We considered the exon regions of each gene, namely: 7th exon for *r1* (100126972), 5th exon for *b1* (103646088), 4th exon for *mtl* (103635924) and 1st exon for *dmp1* (100277972) as described in NCBI accession (Table 1). The

tool Primer 3 Plus software was used to design primers. The primers were validated *in silico* using Oligo Analyzer Tool from Integrated DNA Technologies by performing multiple Basic Local Alignment Search (BLAST) searches in the *Z. mays* genome.

Table 1. Primers designed for DNA sequence amplification and probe construction of *18S rDNA* and four haploid induction genes in *Z. mays*.

Sequence name	Primers forward and reverse	Tm (°C)	Exon region
18S rDNA	F: 5'- TAATTCCAGCTCCAATAG -3' R: 5'- CCACCCATAGAATCAAGA -3'	50°C	-
R-navajo (r1)	F: 5'- GGCAGGAGGAGGAGCTAAGA -3' R: 5'- CCTGTGAATGGACGGAAGCA -3'	50°C	7° - 765 pb
DUF679 domain membrane protein1 (dmp1)	F: 5'- ACGCTGCTAACCTTCGAGATG -3' R: 5'- GTTACACTGCCGTACTCTCTGT -3'	50°C	1° - 700 pb
matrilineal (*mtl*)	F: 5'- CGATGACGCAGATCACCAAA -3' R: 5'- GCCTTGTTCTCCTCTCCTCG -3'	40°C	4° - 588 pb
colored plant1 (b1)	F: 5'- GGAGCCGCAATGTCCGATAT -3' R: 5'- TTTCCTGGGCTGTTACCGTC -3'	45°C	5° - 729 pb

Probes of the *18S* rDNA and haploid induction genes of *Z. mays* were constructed through PCR. For this, *Z. mays* genomic DNA was extracted using the GenElute Plant Genomic DNA Miniprep (Sigma®) (Silva et al., 2020). The sequences were amplified according to respective annealing temperature for each F and R primers (Table 1). The amplification reaction mix consisted of 0.5 µM of each primer (Table 1), 100 ng genomic DNA, 200 µM of each dNTP (Promega®), 1X reaction buffer (Invitrogen®), 2 mM $MgSO_4$ (Invitrogen®), and 2 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen®) for a final volume of 50 µL. PCR conditions were: 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min; annealing at respective primer temperature (Table 1) for 1 min; extension at 68°C for 1 min and 30s; and final extension at 68°C for 5 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gel stained with Gel Red®. For labeling, the PCR conditions were the same as mentioned before: 2.5 U of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen®), 1X of the enzyme reaction buffer (Invitrogen®), 1.8 mM of $MgSO_4$ (Invitrogen®), 0.2 mM of dATP, dCTP and dGTP and 0.15 mM of dTTP 0.05 mM de Chromatide® Alexa Fluor® 488-5-dUTP for *r1*, *matl*, *dmp1* and *b1* and 0.05 mM of Tetramethylrhodamine 5-dUTP for 18S rDNA; 0.5 µM of each primer and 100 ng for 50 µL volume of each reaction. Amplification products were also analyzed by 1.5% agarose gel electrophoresis.

The same PCR amplifications were performed for sequencing. The amplification products were purified using the kit Wizard® SV Gel and PCR Clean-Up System (Promega®). The concentration and purity of the PCR products were determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific®). The purified PCR products were sequenced by the Sanger method at the ACTGene Análises Moleculares Ltda. Sequence similarity was performed using Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11, available at: https://www.megasoftware.net/). Subsequently, the sequences were compared with those deposited in GenBank using the BLASTn tool.

2.4 Physical mapping of the *18S rDNA* and *r1*, *b1*, *mtl*, *dmp1* genes

Selected slides containing prometaphases/metaphases and interphase nuclei of *Z. mays* roots were aged for at least three days at 37°C. The slides were washed in 1X phosphate-buffered saline (PBS) for 5 min, fixed in 4% formalin for 12 min, and washed again in 1X PBS for 5 min. After that, denaturation of chromosomes was

performed in 70% formamide/2X saline-sodium citrate (SSC) buffer for 3 min at 68°C in a water bath, subsequently dehydrated an ice-cold ethanol series (70% – 85% – 100%) for 5 min each and dried at room temperature. The hybridization mixture consisted of 50% formamide (Sigma®) + 2X SSC (Sigma®) and 200 ng of the probe, with denaturation at 85°C for 5 min in the Thermocycler. The mixture was immediately placed on ice for at least 5 min. Slides were incubated with 35 µL hybridization mixture, covered by plastic coverslip HybriSlip (Sigma®) and sealed with Rubber Cement (Elmer's).

We conducted the FISH from co-hybridization with the probes: *r1 + 18S, dmp1 + 18S, mtl + 18S,* or *b1 + 18S.* Co-hybridization was used because the *18S* rDNA is already used as a specific cytogenetic marker for *Z. mays* (Ananiev et al., 2009). The hybridization was carried out with Thermobrite™ equipment at 37°C for 24 h. Following hybridization, slides were subjected to stringency washes in 2x SSC at 40°C for 5 min. Subsequently, slides were counterstained with 60 µL of 40% glycerol/PBS + 6-diamidino-2-phenylacetic acid (DAPI) (Soares et al., 2020; Silva et al., 2020).

Nuclei, prometaphases and metaphases were captured with a digital video camera 12-bit CCD (Olympus®) coupled to a photomicroscope Olympus BX-60 equipped with epifluorescence and immersion objective (100X, NA = 1.4). The frames were captured at 1.8 exposure and processed using Image ProPlus 6.1 software (Media Cybernetics®).

Prometaphases and metaphases were selected for the genitors and 'KHI' inducer, and karyograms were assembled for each gene. After that, we proposed an ideogram, mapping the physical loci of the *18S rDNA*, *mtl*, *b1*, *dmp1* and *r1*, as well as other chromosome-specific markers (Braz et al., 2020).

3 RESULTS

The probes generated from *Z. mays* genomic DNA and primers (Table 1) were sequenced and the similarity was verified in MEGAX and the NCBI GenBank database using BLAST function. The alignment performed in MEGAX evidenced the similarity between the sequencing data and the sequences available at the NCBI. The similarity was equivalent to 98.52% for *r1*, 94.17% for *b1*, 97.12 % for *dmp1* and 94.00% for *mtl*.

So, we used a co-hybridization mix for FISH, on each slide containing interphase nuclei, prometaphases and metaphases of each genitor or 'KHI' inducer. Interphase nuclei were used to identify the copy number of *r1*, *b1*, *mtl* and *dmp1* genes in *Z. mays*. From this, two hybridization signals of each gene were identified in five interphase nuclei for all lines (Fig. 1).

There were no differences between the hybridization signals and their respective *loci* among the lines. *Z. mays* genome has one copy of the *r1*, *b1*, *mtl* and *dmp1* genes in all lines. Instead, the *18S* rDNA exhibited tandem copies for all lines showing a different hybridization pattern in relation to the haploid-inducing genes. The *18S* rDNA fluorescence signals resulted in an expressive signal (red) due to the tandem repeat copy number. Differently, *r1*, *b1*, *dmp1* and *mtl* resulted in a spot and small signal (green) in nuclei and chromosomes.

The mapping of the sequences on *Z. mays* karyotype evidenced specific chromosome *loci* for each gene, which corroborates with the linkage groups reported in MAIZEGDB. *18S* rDNA was mapped in the terminal portion of the short arm of the chromosome 6. *mtl* (Fig. 2), *b1* (Fig. 3) and *dmp1* (Fig. 4) genes were mapped in the short arm of the chromosomes 1, 2 and 9, respectively. Meanwhile, *r1* gene was mapped on the long arm of chromosome 10 (Fig. 5). Therefore, as well as the *18S* rDNA, *r1*, *mtl*, *b1* and *dmp1* genes occur in all lines in a single copy, evidencing the presence of these genes in the '*Z. mays* genitors and 'KHI' inducer.

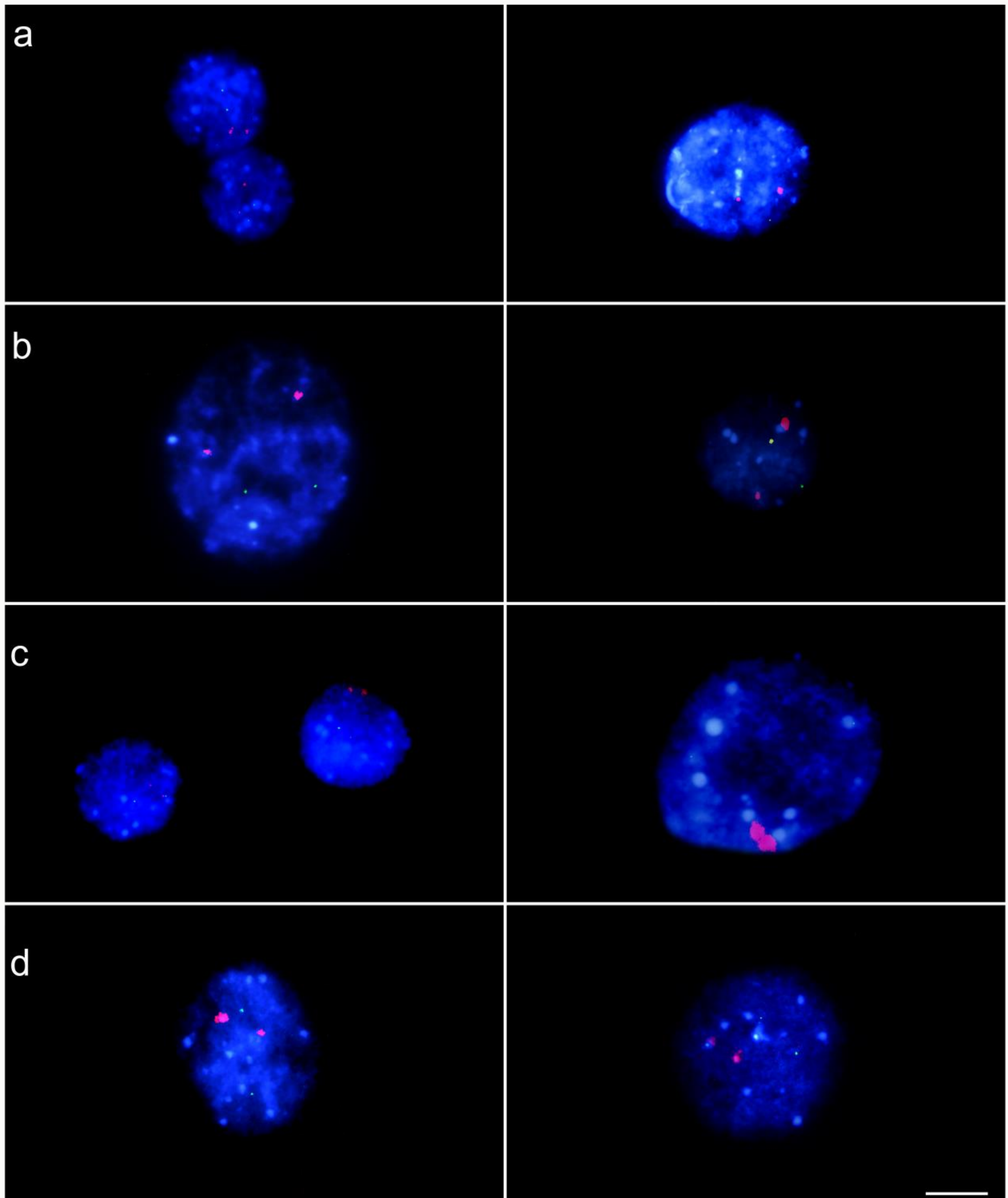

Figure 1. Gene copy number determined in interphase nuclei for the haploid-inducing genes (green) and *18S rDNA* (red) in *Z. mays* lines. Genitor (a – 'Beija-Flor – 20-2010', b – 'Viçosa – 20-2064', c – 'Viçosa – 20-2064', d – 'Beija-Flor – 20-2010') nuclei are represented on the left and 'KHI' inductor on the right. (a) *mtl* and *18S* rDNA, (b) *b1* and *18S* rDNA*,* (c) *dmp1* and *18S* rDNA, and (d) *r1* and *18S* rDNA. Scale bar = 10 μm**.**

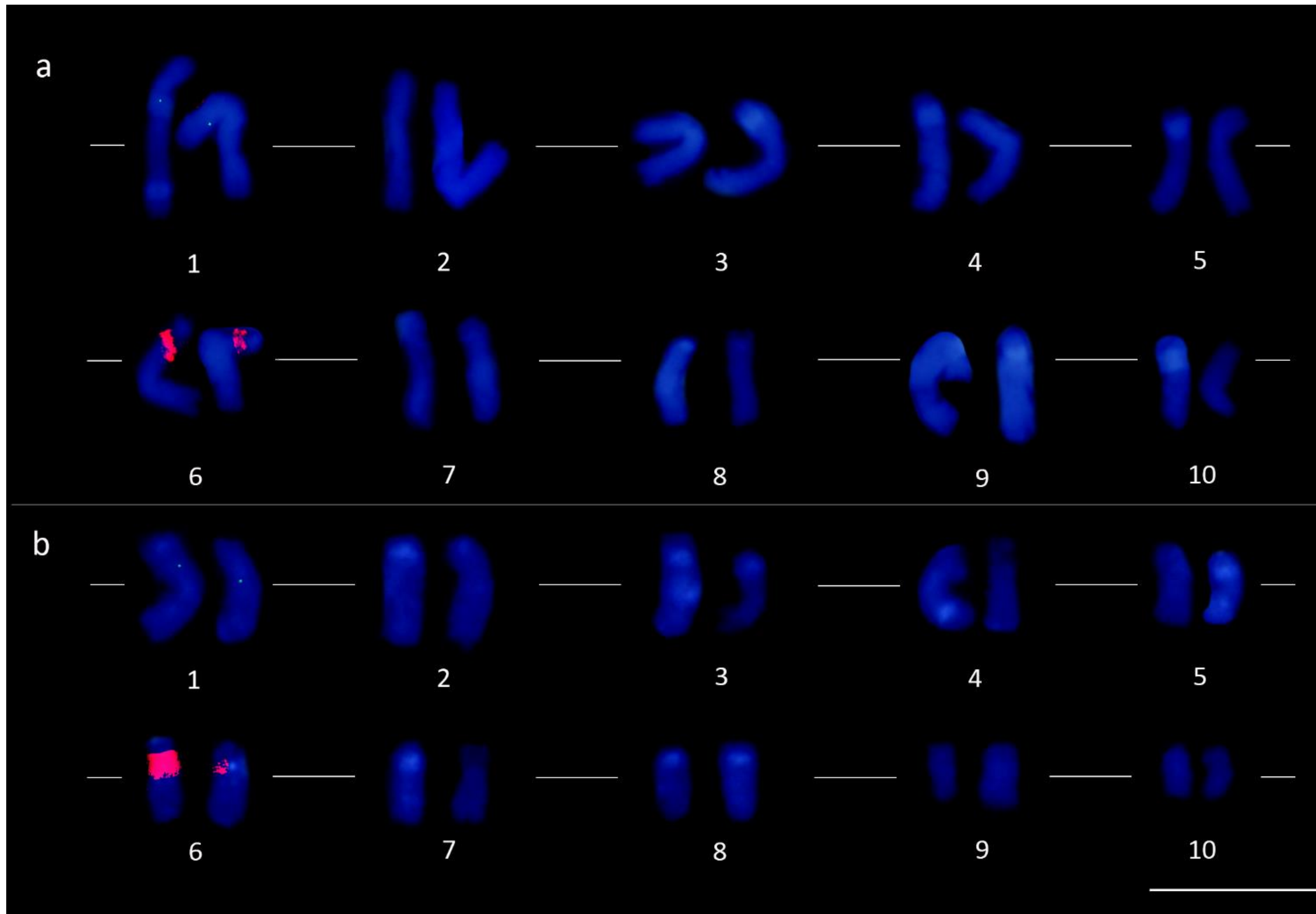

Figure 2. Mapping of the *18S rDNA* and *mtl* in *Z. mays* metaphase chromosomes. The *18S rDNA* (red) and *mtl* gene (green) *loci* in short arm of chromosome 6 and short arm of chromosome 1, respectively. (a) 'KHI' inductor, (b) 'Beija-Flor – 20-2010' genitor. Scale bar = 10 μm.

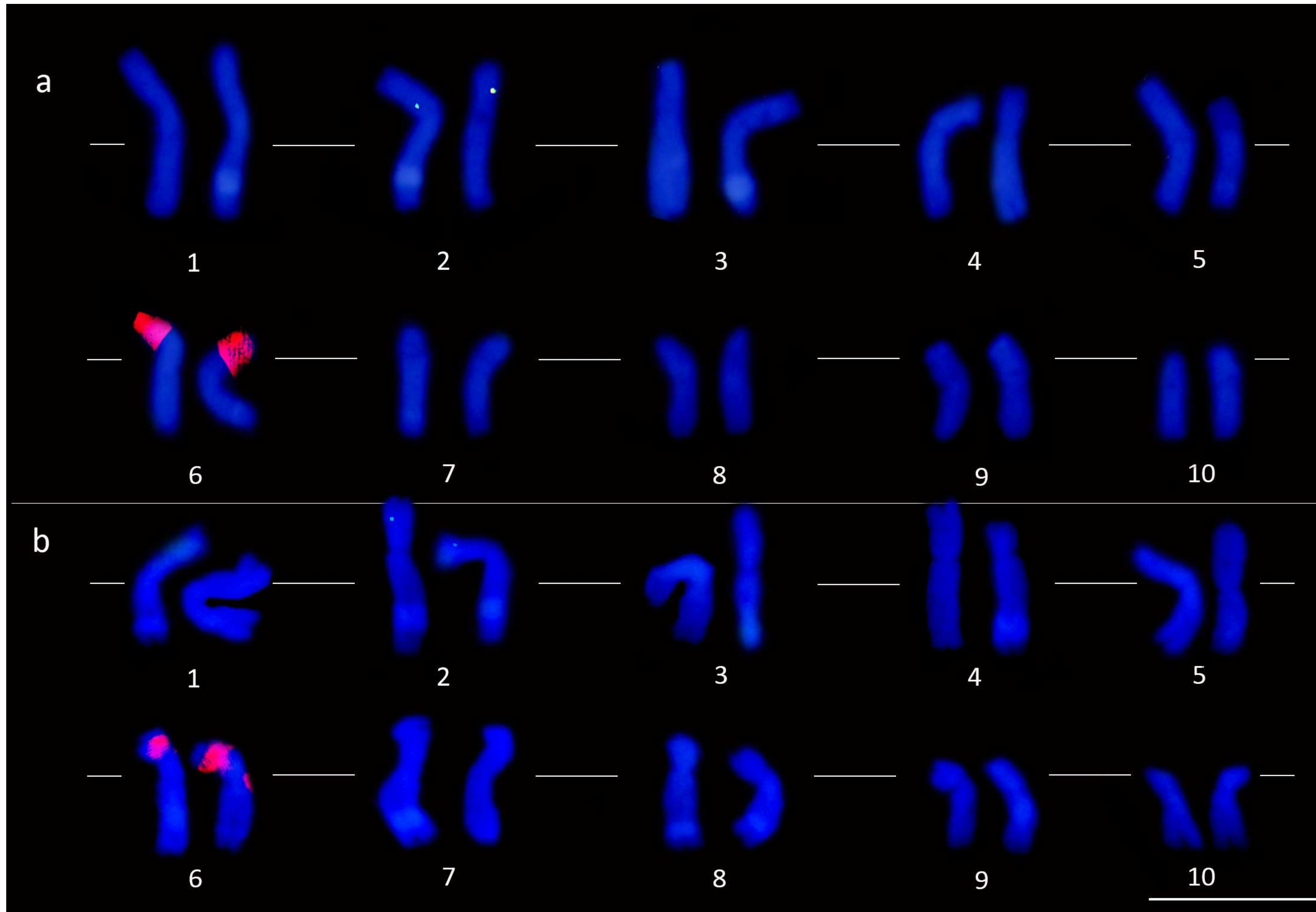

Figure 3. Mapping of the *18S rDNA* and *b1* in *Z. mays* metaphase chromosomes. The *18S rDNA* (red) and *b1* gene (green) *loci* in short arm of chromosome 6 and short arm of chromosome 2, respectively. (a) 'KHI' inductor, (b) 'Beija-Flor – 20-2010' genitor. Scale bar = 10 μm.

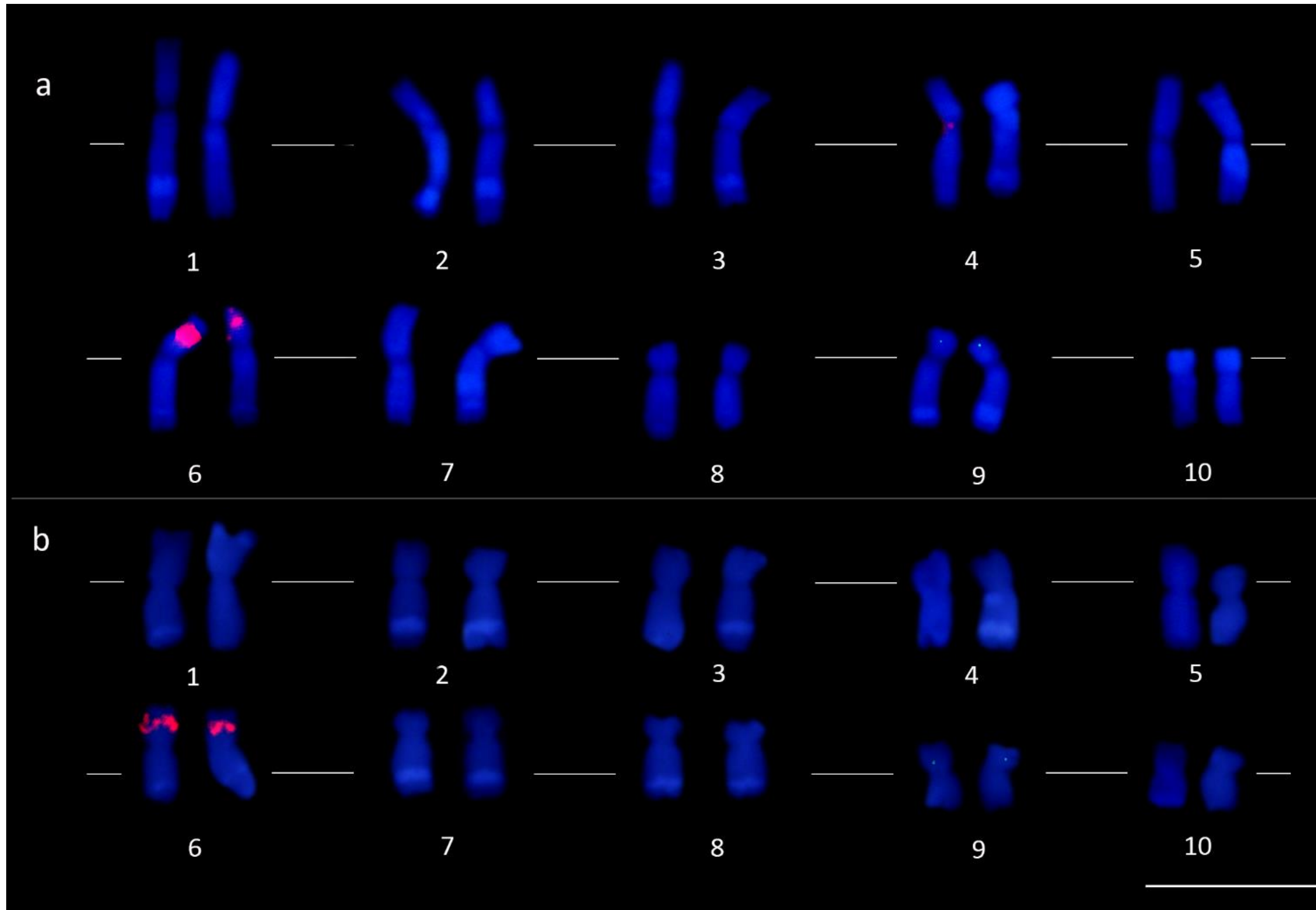

Figure 4. Mapping of the *18S rDNA* and *dmp1* in *Z. mays* metaphase chromosomes. The *18S rDNA* (red) and *dmp1* gene (green) *loci* in short arm of chromosome 6 and short arm of chromosome 9, respectively. (a) 'KHI' inductor, (b) 'Beija-Flor – 20-2010' genitor. Scale bar = 10 μm.

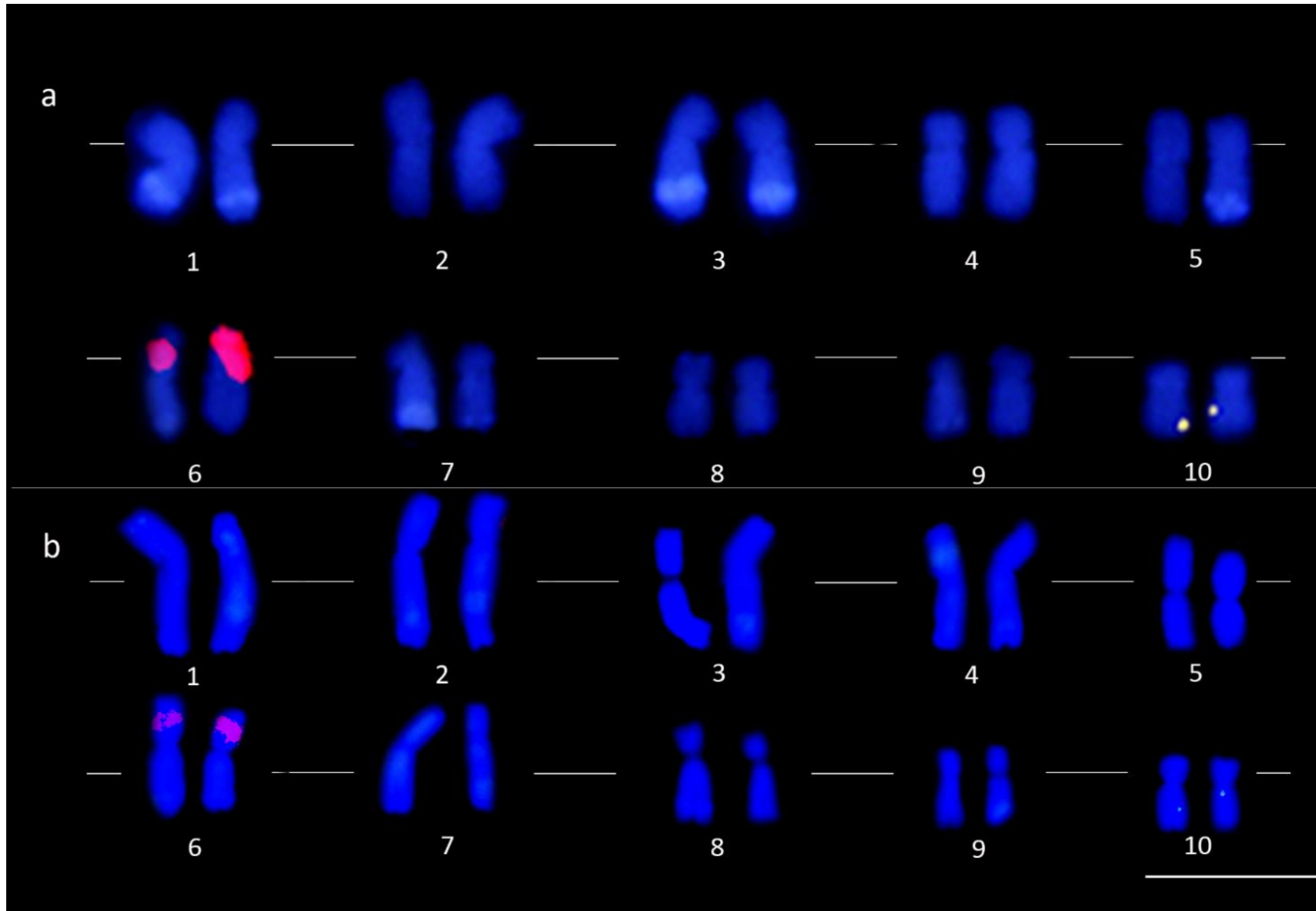

Figure 5. Mapping of the *18S rDNA* and *r1* in *Z. mays* metaphase chromosomes. The *18S rDNA* (red) and *r1* gene (green) *loci* in short arm of chromosome 6 and long arm of chromosome 10, respectively. (a) 'KHI' inductor, (b) 'Viçosa – 20-2027' genitor. Scale bar = 10 µm**.**

4 DISCUSSION

Based on the deposited sequences and the FISH mapping, we constructed physical maps for the genes *r1*, *mtl*, *b1* and *dmp1* in *Z. mays* mitotic metaphases. Since the FISH development, a collection of probes for repeatome sequences have been developed for *Z. mays*, such as *18S* and *5S* rDNA genes, and 180-bp knob (Lamb et al., 2007; Bennetzen, 2009; Braz et al., 2020). These sequences are highly conserved and repetitive, and, for these, considered a valuable marker for cytogenomics to construct physical maps (Sattler et al., 2019). *18S* rDNA consists of repetitive tandem arrangements (Kato et al., 2004) and their physical mapping is appointed a chromosome-specific cytogenetic marker for *Z. mays* chromosome 6 (Ananiev et al., 2009).

We adapted the FISH procedure, allowing the co-hybridization of probes of different bp size (*18S + r1*, *18S + b1*, *18S + mtl*, or *18S + dmp1*) mainly single-copy sequences. Repeatome sequences, as *18S* rDNA, are usually used for probe construction and cytogenomics in plants due to frequency of these sequences in the genomes. So, repeatome probes are often applied for chromosome identification, karyotype characterization, and evolutive comparison (Jiang and Gill, 2006). These probes are advantageous in terms of consistent probe quality and reduced time and cost for probe preparation compared to probes traditionally prepared from replicates of cloned satellites (Jiang, 2019). Based on our data, the single-copy sequences can successfully applied for the cytogenomics in plants.

The routine detection of single-copy sequences on chromosomes by FISH has been difficult in plants. In *Z. mays*, two single-copy loci were localized*, umc105a* and *csu145a*, on chromosome 9 using cosmid clones to construct the probes (Sadder and Weber, 2002). Cytomarks have been established from specific FISH signals (Lamb et al. 2007; Braz et al. 2020) using genes as *18S* rDNA in *Z. mays*, cDNAs, CentC microsatellites and BACs for the probe constructions (Fig. 6). A new class of FISH probes was developed based on clustered single-copy oligonucleotides (oligo) (Jiang et al., 2019; Braz et al., 2020). Oligo-FISH probes generate specific hybridization patterns on individual chromosomes (Braz et al., 2020). Oligo-BAC probes were identified in the karyotype of *Z. mays* in all chromosome pairs (Braz et al., 2020), differing only in the amount of signals between the chromosome arms of each pair. This chromosome identification strategy can also be used in other species, such as *Oryza sativa* (Liu et al., 2020). These specific hybridization patterns can be

used as a chromosome identification system in *Z. mays* based on signal intensity and position. However, some FISH signal patterns are highly polymorphic between different *Z. mays* lines. This polymorphism can make it challenging to correctly identify the chromosomes. Approximately 11% of *Z. mays* genes contain repetitive sequences in their introns (Haberer et al., 2005). Therefore, single-copy genes are candidates for chromosome-specific markers even with repetitive elements.

Based on the karyograms (Figs. 2 – 5), we structured an ideogram (Fig. 6) about the *Z. mays* basic chromosome number to represent the cytomarks. In addition, the ideogram presents the cytogenomic data of the physical *loci* of 24 signals (12 red/12 green) oligo-FISH (Braz et al., 2020). The *mtl* loci is between two BAC-R sequences, while the *b1* and *r1* are linked to one BAC-G and one BAC-R sequence, respectively. *dmp1* in chromosome 9 and *18S* in chromosome 6 did not link any oligo-BAC signal in the same arm.

r1 was mapped to the long arm of chromosome 10 (Fig. 1) and is an anthocyanin biosynthesis regulator gene responsible by phenotypic expression of colour grain in *Z. mays* (Wu et al., 2022). The colour expression attributed to *r1* has been adopted as a phenotypic marker to differentiate and identify haploid seeds. Thus, the inhibition of pigmentation in *Z. mays* lines has been attributed to the absence of the allelic form (Geiger & Gordillo, 2009; Chaikam et al., 2015; Milani et al., 2016). However, the *r1* gene is present in both inducer and genitor lines of *Z. mays*. In this sense, the phenotypic variation is related to the distinct genotypes (allelic forms) that lead to different expression patterns in *Z. mays* lines. Gene expression variations, and consequently distinct phenotypes, can be explained by the interaction with other genes and their allelic forms. For example, *c1* gene acts as a transcriptional repressor of *r1*, inhibiting the anthocyanin biosynthesis pathway (Chase, 1969; Paz-Ares et al., 1990; Geiger and Gordillo, 2009). Besides that, several allelic forms of the *r1* genes (such as *r1-sc:m3*, *R1-st*, *r1-sc::m3*, *R1-sc:124* and *R1-mb*) are associated with the Ac/Ds system of transposable elements (Wu et al., 2022). These allelic forms can be activated or inactivated in the genome, influencing the phenotypes associated to anthocyanin pigmentation in *Z. mays* seeds.

b1 gene was mapped to the short arm of chromosome 2 (Fig. 2). This gene also acts in the anthocyanin biosynthetic pathway in *Z. mays* (Dooner et al.,, 1991) and regulates the transcription of *r1*. The *b1* and *r1* genes have 85% – 90% of

identity (Kermicle, 1980; Chandler et al., 1989). Furthermore, LTR-retrotransposons represent about 65% of DNA sequence of *b1* and *r1* genes (Swigoňová et al., 2005). The presence of these elements may be associated with sequence rearrangement and acquisition of new expression patterns of the gene (May & Dellaporta, 1998; Wu et al., 2022). Despite the high percentage of similarity between these sequences, no unspecific signals were found between the *r1* and *b1* genes in the FISH mapping, resulting in specific hybridization signals for both genes. This is possibly due to the construct of primers' specifics for each sequence (Supplementary Table 2), from specific exon regions of the respective genes.

mtl and *dmp1* genes were mapped in the chromosomes 1 and 9, respectively. These genes show different allelic forms (Kelliher et al., 2017; Zhong et al., 2019), which are related to the haploid induction. A rare mutation in the *mtl* gene of the of chromosome 1 is responsible for triggering the haploid induction in *Z. mays*, since the insertion of four bp in exon 4 leads to premature termination of the gene transcription and consequently to function loss of the translated polypeptide (Kelliher et al., 2017; Gilles et al., 2017; Liu et al., 2017). Some allelic forms of the *mtl* are responsible to the increase the haploid induction rates (4.0 – 12.5%) in *Z. mays* (Kelliher et al., 2017). Moreover, *dmp1* and *mtl* genes have been together reported to increase the induction rate up to 3-fold in *Z. mays* (Zhong et al., 2019). Allelic forms, as *zmdmp* or *zmdmp-ko* mutant alleles, of *dmp1* edited from CRISPR/Cas9 significantly improve the haploid induction rate (Kelliher et al., 2017; Zhong et al., 2019). This *in vivo* induction mechanism can be extended to other species, such as *Oryza sativa* (rice), *Triticum aestivum* (wheat) and *Sorghum bicolor* (sorghum) (Yao et al., 2018; Wang et al., 2022), since the sequences of *mtl* and *dmp1* genes are highly conserved in cereals (73% – 91% identity) (Kelliher et al., 2017; Zhong et al., 2019). High rates of haploid induction have proven application potential in double-haploid breeding programs.

r1, *mtl*, *b1* and *dmp1* are present in all *Z. mays* lines. The phenotype diversity associated with these genes is due to the variation of allelic forms and genotypes. This data regarding the physical mapping of haploid inducer genes and *18S* rDNA in *Z. mays* mitotic chromosomes are unprecedented. In this study, the results obtained expand knowledge about the copy number and physical location of these genes in interphase nuclei and metaphase chromosomes of the lines of *Z. mays*. Our results

corroborate with data from linkage groups as reported on MAIZEGDB for *Z. mays* genome.

Physical maps contribute to understand the structure and evolution of the *Z. mays* genome, increasing the cytogenomics. Thus, they can also be associated with genetic maps and sequencing, resulting in more data reliability. Furthermore, these genes are also cytomolecular markers because they have a specific hybridization signal in only one chromosome pair. The results expand the knowledge about the physical *loci* of single-copy genes.

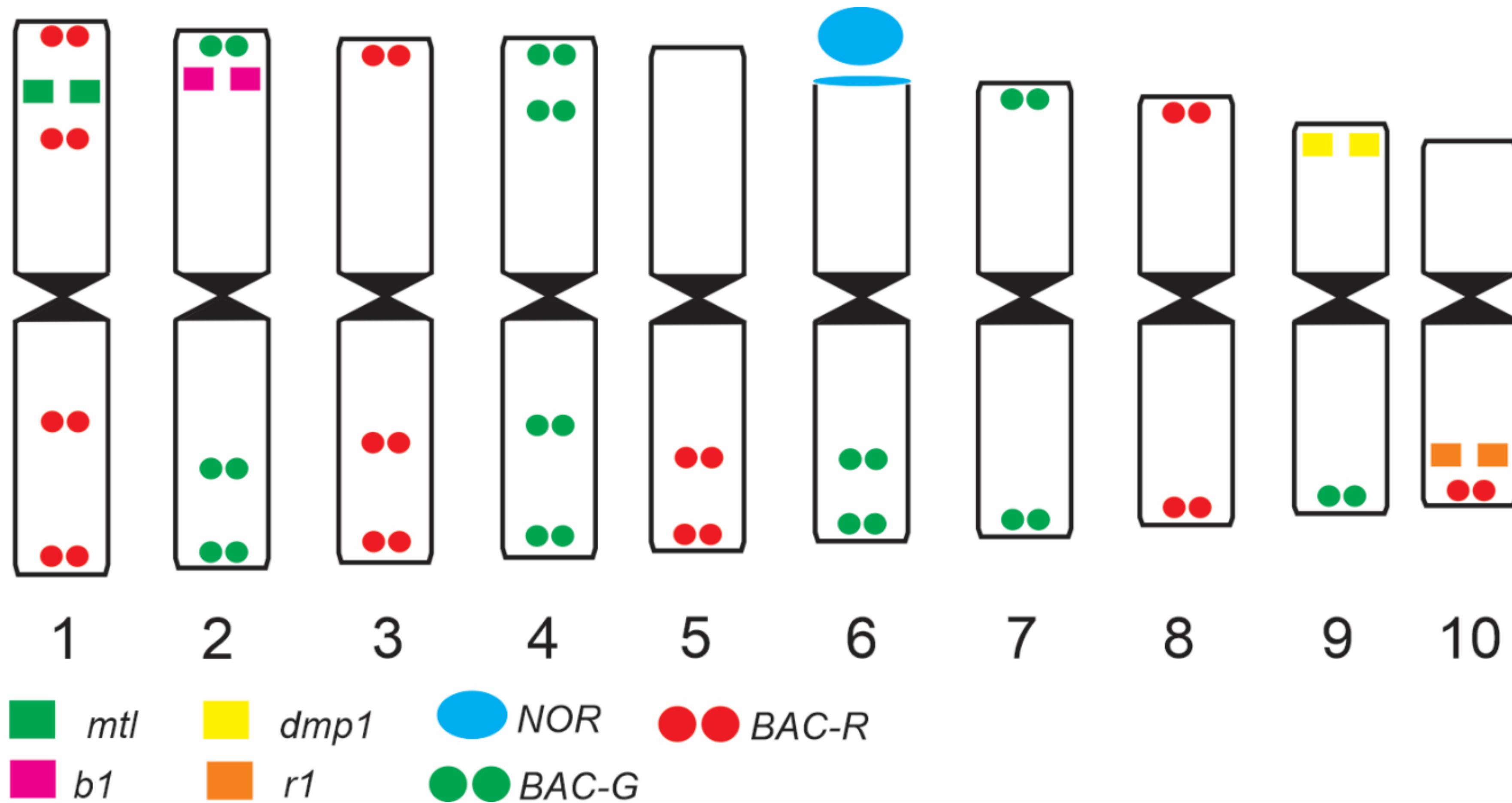

Figure 6. Ideogram assembled based on chromosome morphometry, representing *mtl* (chromosome 1 short arm – green), *b1* (chromosome 2 short arm – pink), *18S* rDNA (chromosome 6 terminal short arm – NOR region), *dmp1* (chromosome 9 short arm – yellow) and *r1* (chromosome 10 long arm – orange). Signals from BAC-R and BAC-G oligo-FISH probes are represents in red and green circles, respectively. These probes were designed from single-copy sequences along the 10 chromosomes of *Z. mays* (Braz et al., 2020) and are used as specific cytomarkers.

5 CONCLUSION

For the first time, we physically mapped *r1*, *b1*, *dmp1* and *mtl* genes related to haploid induction in *Z. mays*. *mtl* loci is on the short arm of chromosome 1, *b1* on the short arm of chromosome 2, *dmp1* on the short arm of chromosome 9, and *r1* on the long arm of chromosome 10. *Z. mays* has one copy of the *r1*, *b1*, *mtl* and *dmp1* genes in all lines mapped here. We also conclude that these genes can be used as chromosome-specific cytogenetic markers for *Z. mays* . Furthermore, these results corroborate with data from genetic maps and linkage groups of *Z. mays*. So, our physical map results contribute to understand the structure and evolution of the *Z. mays* genome, increasing cytogenomics of this species.

6 ACKNOWLEDGMENTS

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7 SUPPLEMENTARY INFORMATION

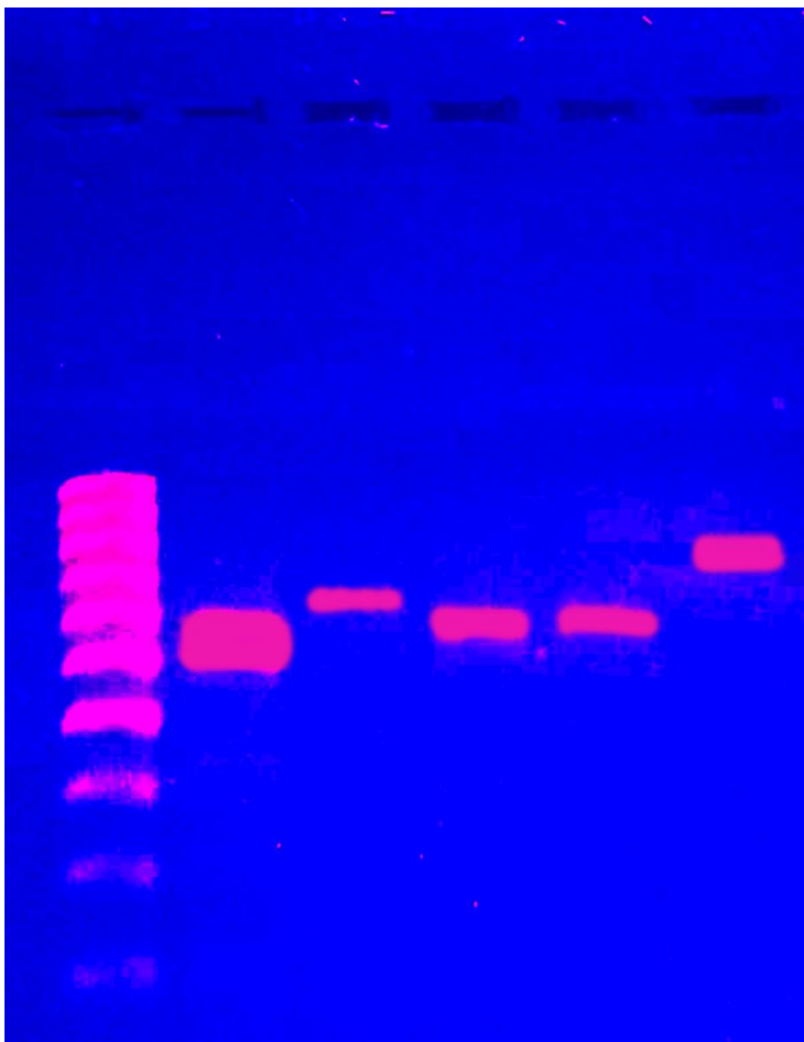

Figure S1. PCR products resulting from the amplification of *Z. mays* genomic DNA using molecular marker and specific primers for the *r1*, *b1*, *dmp1* genes and *18S rDNA*, respectevely, analyzed on a 1.5% agarose gel.

Table S1. Exon sequence of *r1* and *b1* genes in *Z. mays* genome for primer construction

r1	*b1*
GTGCCGGAGGCCCCGGACTTGGTCAGCCGAGCAACCG CGGCTTTCTGGGAGCCGCAGTGCCCGACGTACTCGGA AGAGCCGAGCTCCAGCCCGTCAGGACGAGCAAACGAG ACCGGCGAGGCCGCAGCAGACGACGGCACGTTTGCGT TCGAGGAACTCGACCACAATAATGGCATGGACATAGA GGCGATGACCGCCGCCGGGGGACACGGGCAGGAGGAG GAGCTAAGACTAAGAGAAGCCGAGGCCCTGTCAGACG ACGCAAGCCTGGAGCACATCACCAAGGAGATCGAGGA GTTCTACAGCCTCTGCGACGAAATGGACCTGCAGGCG CTACCACTACCGCTAGAGGACGGCTGGACCGTGGACG CGTCCAATTTCGAGGTCCCCTGCTCTTCCCCGCAGCC AGCGCCGCCTCCGGTGGACAGGGCTACCGCTAACGTC GCCGCCGACGCCTCAAGGGCACCCGTCTACGGCTCTC GCGCGACGAGTTTCATGGCTTGGACGAGGTCCTCGCA GCAGTCGTCGTGCTCCGACGACGCGGCGCCCGCAGCA GTAGTGCCGGCCATCGAGGAGCCGCAGAGATTGCTGA AGAAAGTGGTGGCCGGCGGCGGTGCTTGGGAGAGCTG TGGCGGCGCGACGGGAGCAGCACAGGAAATGAGTGGC ACTGGCACCAAGAACCACGTCATGTCGGAGCGAAAGC GACGAGAGAAGCTCAACGAGATGTTCCTCGTCCTCAA GTCACTGCTTCCGTCCATTCACAGG	GTGCCGGAGGACCCGGACTTGATCAACCGAGCAACCG CAGCCTTTCGGGAGCCGCAATGTCCGATATACTCGGA ACAGCCGAGCTCCAACCCGTCAGCAGACGAAACCGGC GAGGCCGCAGACATAGCTGTGTTCGAGGGCCTCGATC ACAATGCCATGGACATGGAGACCGCAGGCATAGCTGT GTTCGAGGGCCTCGATCACAATGCCATGGACATGGAG ACGGTGACTGCCGCCGCCGGGAGACACGGAACCGGAC AGGAGCTAGGAGAAGCCGACAGCCCGTCAAATGCAAG CCTGGAGCACATCACCAAGGGGATCGACGAGTTCTAC AACCTCTGCGAGGAAATGGACGTGCAGCCGCTAGAGG ATGCCTGGATAATGGACGGGTCTAATTTCGAAGTCCC CTCGTCAGCGCTCCCGGTGGATGGCTCAAGCGCACCC GCTGATGGCTCTCGCGCGACCAGTTTCGTGGCTTGGA CGAGGTCATCGCAGTCCTGCTCCGGTGAAGCGGCGGC TGTGCCGGTCATCGAAGAGCCGCAGAAATTGCTGAAG AAAGCGGTGGCCGGCGGCGGTGCTTGGGCGAACACGA ACTGCGGTGGCGGGGGCACGACGGTAACAGCCCAGGA AAACGGCGCCAAGAACCACGTCATGTTAGAGCGAAAG CGCCGGGAGAAGCTCAACGAGATGTTCCTCGTTCTCA AGTCGTTGGTTCCCTCCATTCACAAG

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