

Article

A dual-color Tyr-FISH method for visualizing genes/markers on plant chromosomes to create integrated genetic and cytogenetic maps

Natalya Kudryavtseva^{1,2,#}, Aleksey Ermolaev^{2#}, Gennady Karlov³, Ilya Kirov^{4,5}, Masayoshi Shigyo⁶, Shusei Sato⁷, Ludmila Khrustaleva^{1,2,8*}

- 1 Plant Cell Engineering Laboratory, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskaya 42 str., 127550 Moscow, Russia; natalia.kudryavtseva92@gmail.com; ludmila.khrustaleva19@gmail.com;
- 2 Center of Molecular Biotechnology, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy, Timiryazevskaya 49 str., 127550 Moscow, Russia; ermol-2012@yandex.ru;
- 3 Laboratory of Applied Genomics and Crop Breeding, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskaya 42 str., 127550 Moscow, Russia; karlovg@gmail.com;
- 4 Laboratory of Marker-assisted and genomic selection of plants, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskaya 42 str., 127550 Moscow, Russia; kirovez@gmail.com
- 5 Kurchatov Genomics Center of ARRIAB, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskaya 42 str., 127550 Moscow Russia
- 6 Graduate School of Sciences and Technology for Innovation, Yamaguchi University, 1677-1 Yoshida, Yamaguchi, Yamaguchi 753-8515, Japan; shigyo@yamaguchi-u.ac.jp;
- 7 Graduate School of Life Science, Tohoku University, 2-1-1 Katahira, Sendai, Miyagi 980-8577, Japan; shuseis@m.tohoku.ac.jp;
- 8 Department of Botany, Breeding and Seed Production of Garden Plants, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy, Timiryazevskaya 49 str., 127550 Moscow Russia;

These authors contributed equally to this work

*correspondence: ludmila.khrustaleva19@gmail.com; Tel: +7 (915) 051-38-66;

Abstract: *In situ* imaging of molecular markers on a physical chromosome is an indispensable tool for refining of genetic maps and validation genome assembly at the chromosomal level. Despite tremendous progress in genome sequencing the plant genome assembly at chromosome level still remain a challenge. Recently developed optical and Hi-C mapping aim to assist in genome assembly. For high-confidence in the genome assembly at chromosome level more independent approaches will be required. The present study aimed to refined an ultrasensitive Tyr-FISH technique and to develop a reliable and easy method for *in situ* mapping of a short unique DNA sequences on plant chromosomes. We have carefully analyzed the critical steps of the Tyr-FISH technique to find out the reasons for the failures of using the method. It has been shown that successful visualization of marker/gene depends significantly on method of chromosome slide preparation, probe design and labelling, high stringency washing. Appropriate adjustment of these steps allowed us to detect a short DNA sequence of 1.7Kb with a frequency of 51.6%. Based on our results, we developed a reliable and simple protocol for dual-color Tyr-FISH visualization of short unique DNA sequences on plant chromosomes. New protocol allows more accurate determination of the physical distance between markers and can be applied for faster integration of genetic and cytogenetic maps.

Keywords: Tyr-FISH, plant chromosome preparation, recombinant and cytogenetic maps, transcript-based markers, genome assembly, *Allium cepa*

1. Introduction

Genetic linkage maps that have been used for over 100 years aided greatly in *de novo* assembly of plant genomes [1-4]. Linkage map based on recombination rate between markers can anchor *de novo* sequences and order small fragments into chromosome-scale sequences. However, linkage maps, which are most accurate in regions of the genome with high rates of recombination, can result in mistakes in arranging scaffolds in region of suppressed recombination e.g., pericentromeric, knob and subtelomeric heterochromatin [5-7]. The cytogenetic map can compensate for the discrepancy between the real position of the DNA sequence and its position on the genetic linkage map. Moreover, the cytogenetic map is useful in synteny and collinearity comparison between relative genomes [8,9], especially for complex-genome organisms which has large amounts of repetitive DNA, such as wheat [10] or onion [11]. Third generation sequencing technologies such as PacBio and Oxford Nanopore offer vast improvements over Sanger and Illumina sequencing for long reads. Now whole genomes can be quickly sequenced, but genome assembly is much slower and more labor and computational. Whole genome sequence is much more informative when linked and oriented to chromosomes than unlinked and disordered scaffolds. Hi-C and optical mapping methods aim to assist in genome assembly at chromosome level. Hi-C is a sequencing-based assay originally designed to inquire the 3D structure by measuring the contact frequency between all pairs of loci in the genome [12, 13]. However, the Hi-C assembly data contains a significant number of contig orientation errors when the contig is in the correct position but not in the correct orientation [14]. Optical mapping has been used for arranging the scaffolds of pseudo chromosomes in *de novo* assembly of plant genomes [15]. Rearrangements such as translocations involving the ends of pseudomolecules in *Spirodela polyrhiza* genome assembly were more easily detected by the optical mapping, while inverted orientation of a sequence within a scaffold was possible to detect only via a cytogenetics approach [16].

It is obvious that the creation a reference genome at the chromosome level of resolution will probably require more independent approaches for high-confidence in the genome assembly at chromosome level. *In situ* imaging of molecular markers on a physical chromosome for integration of chromosomal and genetic maps is an indispensable tool for refining of genetic maps and validation genome assembly at the chromosomal level. BAC-FISH mapping was successfully used for genome assembly in plant species with small genomes such as tomato [6, 17], grass species *Brachypodium distachyon* [18], duckweed *Spirodela intermedia* [19]. Though BAC-FISH mapping is less applicable for large-genome species that contain a huge amount of dispersed, repetitive DNA sequences. Integrated genetic and cytogenetic maps are a necessary step for the assembly of large genomes possessing a huge number of repetitive sequences, like *A. cepa* (1C = 16.3 Gb of which 98% is repetitive DNA). An excellent solution to this problem can be direct *in situ* visualization of molecular markers from genetic maps used to guide genome assembly. However, here we are faced with such an obstacle as the sensitivity of the FISH method. The sensitivity threshold of common FISH is 10Kb, while the size of markers is much smaller, often several hundred base pairs. Tyramide (Tyr)-FISH is an approach to increase the sensitivity of FISH [20] and has been applied to plant chromosome [11, 21-25]. However, the method has not become routine because of indistinct *in situ* hybridization signals for some probes, low detection frequency and low repeatability among different laboratories [26].

Taking all together, development of a reproducible method for visualizing relatively short target DNA sequences on a physical chromosome to create integrated genetic and cytogenetic maps is a timely task in the rapid progress in sequencing of numerous plant genomes for validation of genome assembly at chromosomal level. In this paper we describe a reliable Tyr-FISH method for two-color detection of short DNA sequences (1.7-3.2Kb) on compact plant chromosomes using a detailed study of all steps of the protocol, including troubleshooting analysis.

2. Results

2.1 Probe preparation and labeling: reducing background and increasing signal-to-noise ratio

The method of tyr-FISH signal amplification based on the combination of the advantages of enzymatic deposition of many tyramide molecules and sensitive fluorescence detection requires a special approach in the creation of DNA probe. The probe should be (i) extended for specific target sequences - design an exon-intron sequences, (ii) lack repetitive DNA, (iii) proper size after labelling to easy access the target sequences.

Increasing amount of data in different databases makes possible to design exon-intron probes without additional sequencing of target genes via primer construction and producing genomic amplicon. Advances in genome and transcriptome sequencing and assembly provide an opportunity to detect introns and exons boundaries and positions in target gene. The presence of unique intron sequences within a transcript-based probe having conserved exon sequences increases both the specificity and the length of the target chromosomal sequences. For the design of the probe, we selected two markers from the transcriptome map assigned to chromosome 2, which were mapped at a distance on recombinant map [27]. To design an exon-intron probe we blasted Unigene572 and Unigene5305 marker sequences against TSA (Transcriptome Shotgun Assembly) databases of *Allium cepa* in order to find the full transcript sequences. To identify exon-exon junctions in transcript and exon-intron boundaries in the genome sequence the draft genome assembly of *A. cepa* was used (the draft genome assembly of *A. cepa* received from Dr Shigyo). The repetitive DNA sequences within designed probe DNA and subsequently cloned and sequenced genomic DNA amplicons can be located precisely in draft and complete genomic sequence contigs by computational methods (Figure 1). To detect fragments of probe that has identity to repeats and transposable elements (TE) target sequence was annotated using CENSOR [28]. Information about coordinates and lengths of introns and fragments of repeats and TEs were used to design primers and obtaining an exon-intron PCR-product (Table 1). The exon-intron PCR-products were cloned and sequenced.

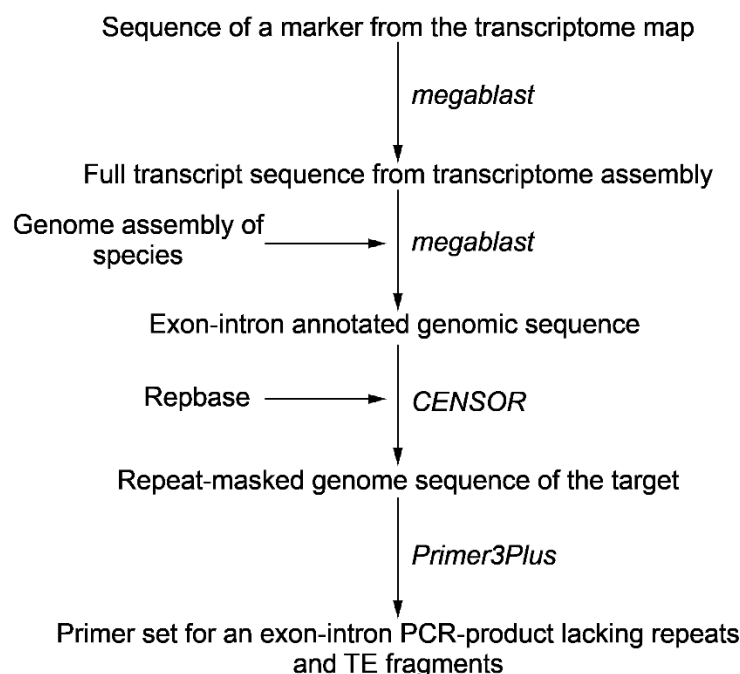


Figure 1. Pipeline in order to design an exon-intron probe for Tyr-FISH.

Nick-translation is the most popular method to create a labeled probe for ISH so far [29]. The result of Nick-translation is a set of fragments of different lengths. The lengths distribution is an important factor for decreasing the background and increasing the probe access to target DNA. The normal range of Nick-translation fragments is from 200 bp to 500 bp. Probes above this range usually results in high background and noisy signals due

to nonspecific sticking to the slide surface. Smaller probes will be hybridized at chromosomes non-specifically and result in spotty background. On the Figure 2 are shown the results of the Nick-translation of plasmids containing genomic amplicons of Unigene572 and Unigene5305 labelled with both biotin and digoxigenin after 90 minutes of reaction. All probes demonstrated a full fragmentation and proper fragments size distribution (Figure 2).

Table 1. Sequences of primers used to produce genomic amplicons for Tyr-FISH.

Probe name	Direction	Primer sequence (5' to 3')	Size
Unigene572	F	AGTGGTGCAGTTCCTCAGCA	3.2Kb
	R	AACCGATTGGCAGGGAAGTT	
Unigene5305	F	TGTTTCACAAACGCTTCGTCC	1.7Kb
	R	TGTCGCCACCACTCATTCAA	

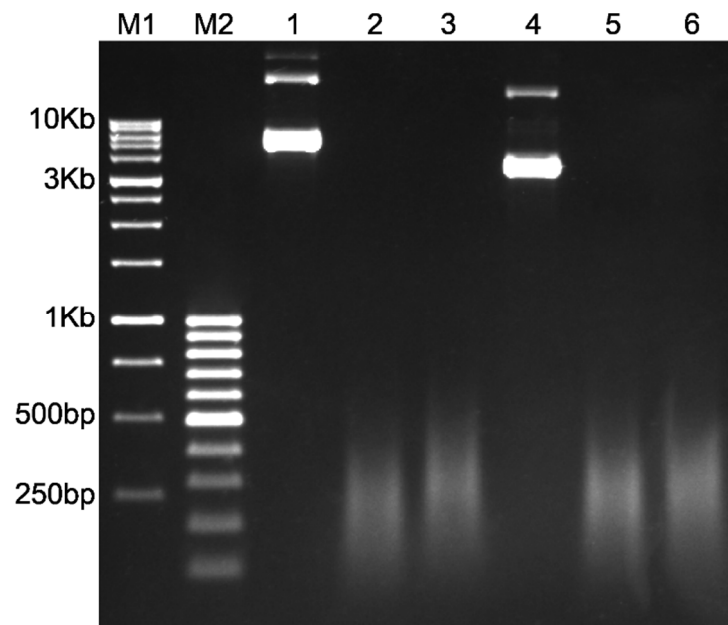


Figure 2. The results of the Nick-translation of the plasmid DNA containing insert of the genomic amplicon in 1% agarose gel: M1 – DNA Ladder 1Kb; M2 – DNA Ladder 100bp+; lines 1-5 – Unigene572 (insert size is 3.2Kb): line 1 – the untreated plasmid DNA, line 2 – labelling with Dig-11-dUTP, line 3 – labelling with Biotin-16-dUTP; lines 4-6 – Unigene5305 (insert size is 1.7Kb): line 4 – the untreated plasmid DNA, line 5 – labelling with Dig-11-dUTP, line 6 – labelling with Biotin-16-dUTP.

2.2 Chromosome preparation is a key step in successful Tyr-FISH

Our many years of experience with the Tyr-FISH method have shown that one of the key steps in obtaining a reliable fluorescent signal is proper chromosome preparation. We examined how the method of preparing chromosome slides affects the frequency and sensitivity of signal detection using Tyr-FISH. We carried out a comparative analysis of two methods of plant chromosome preparation: (1) squashing and (2) dropping.

In the squashing method, chromosomes are spread by squashing the plant material in 45% acetic acid between the slide and cover slip, freezing the material to the slide in liquid nitrogen, and then removing the cover slip (see Materials and Methods).

For dropping method, we used “SteamDrop” method that we early developed [30]. The method is based on obtaining a cell suspension from meristematic tissue, dropping

on the slide and using steam for better chromosome spreading. We minimized the washing steps to reduce the chromosome damage and cell loss. For dropping we use cell suspension in 96% ethanol. Then we add ethanol-acetic acid fixative directly to the slide. In this case, the ratio of ethanol and acetic acid in the fixative is selected depending on the chromosome spreading and the amount of the cytoplasm in the first preparation from a sample of cell suspension. This allows customization of the degree of chromosome spreading and the amount and transparency of the cytoplasm around chromosome. Moreover, long-term storage of the cell suspension in 96% ethanol does not affect the quality of the chromosome preparations.

We have implemented differential interference contrast (DIC) microscopy for the analysis of chromosome preparations. The technique was developed by Polish and naturalized-French physicist Georges Nomarski in 1952 [31]. The DIC microscopy allows to determine the optical density of the object under study on the basis of the principle of interference and thus see the details inaccessible to the eye. DIC creates a volumetric relief image corresponding to the change in the optical density of the sample, accentuating the lines and borders.

DIC microscopy revealed differences in the structure of chromosomes depending on the method of the slide preparations. The dropped chromosome slides showed more pronounced relief structure with clear edge contours of chromosomes (Figure 3a) while the squashed chromosome slides were flatter and denser (Figure 3b). We can assume that the target DNA of the chromosomes prepared by “SteamDrop” method is more preserved and accessible for the unique DNA probe than the chromosomes prepared by squashed method.

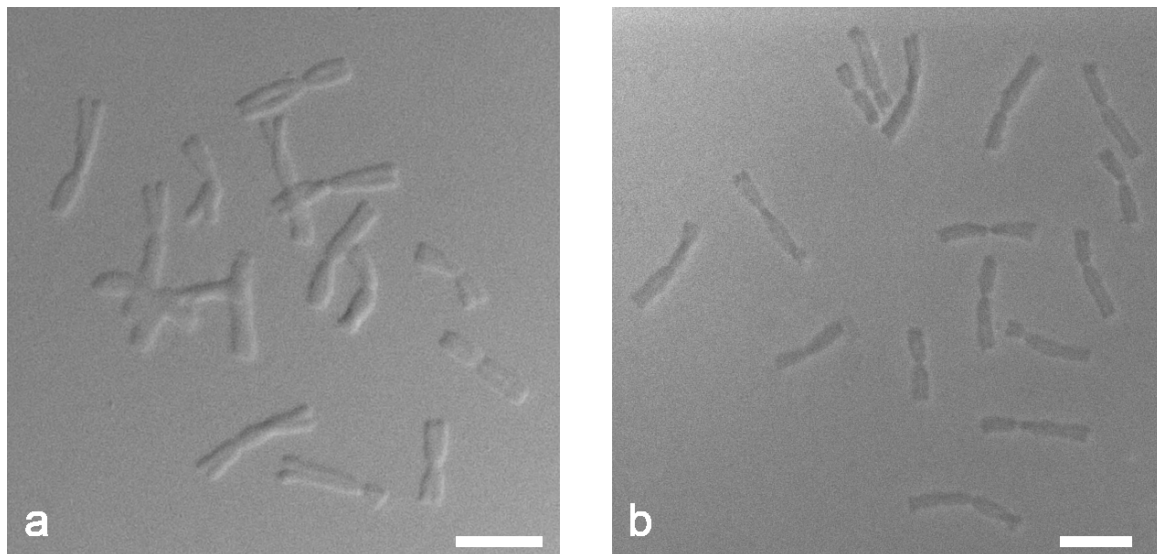


Figure 3. Differential interference contrast (DIC) microscopy of mitotic metaphase chromosomes of *Allium cepa*: (a) “SteamDrop” method of chromosome slide preparation; (b) Squashed method of chromosome slide preparation. Scale bar – 10 μm .

2.3 Quenching endogenous peroxidase and horseradish peroxidase (HRP)

Tyr-FISH method is based on the use of enzyme HRP which catalyzes the deposition of many molecules of a fluorophore-labelled tyramide adjacent to the immobilized HRP (Figure 4).

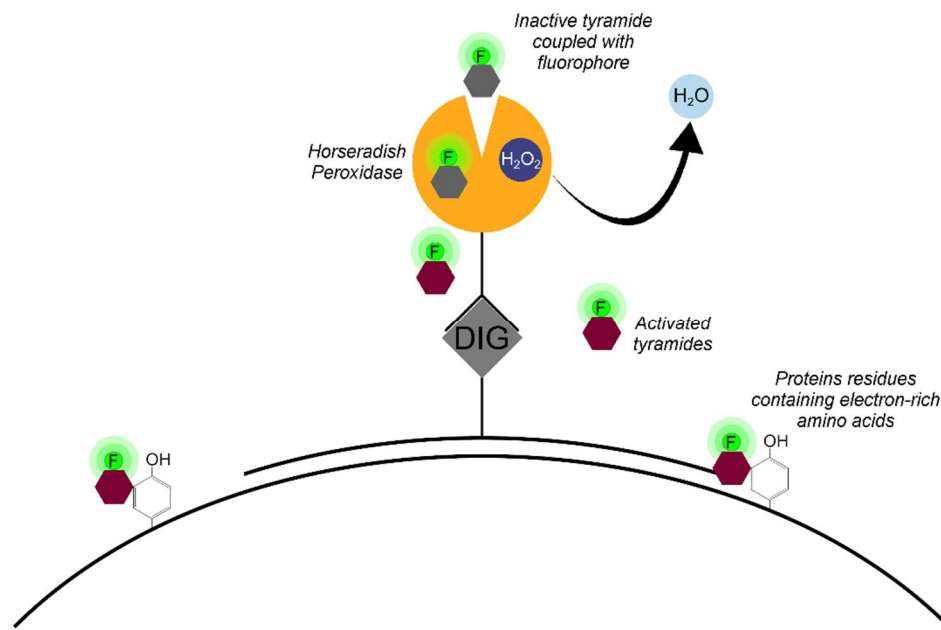


Figure 4. Principle of signal detection on physical chromosomes using horseradish peroxidase (HRP) and fluorophore-labeled tyramides.

The principle of labelled tyramide deposition is via free radical formation, and reaction with electron-rich moieties such as tyrosine, tryptophane, etc. [32]. HRP, in the presence of low concentrations of H₂O₂, converts tyramides into an oxidized form with highly reactive free radical [33]. These activated tyramides then covalently bind to tyrosine or other nuclear and cytoplasmic protein residues on the slide in close proximity to the HRP, thus depositing many of the labeled tyramides. (Figure 5).

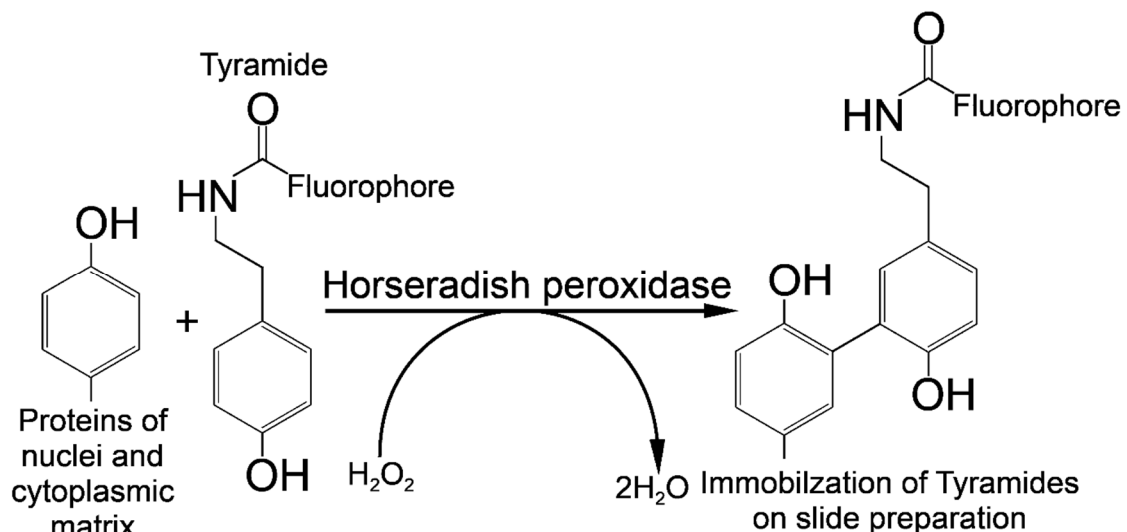


Figure 5. Horseradish peroxidase is a hydrogen peroxide (H₂O₂) decomposing enzyme concomitant with the oxidation of phenolic substrate (tyramide) and reaction with electron-rich moieties of proteins of nuclear and cytoplasmic matrices.

We used two probes, namely, Unigene572 and Unigene5305 genomic amplicon clones, which were labeled by haptens: biotin and digoxigenin. Haptens are small, relatively inert organic molecules that can be attached to DNA without disrupting the DNA's hybridization properties. Labelled Unigene572 and Unigene5305 probes were added together into one hybridization mixture and left overnight to hybridize with the complementary DNA sequences of chromosomes. In the common FISH, the probe detection is

carried out simultaneously with fluorescently labeled reporter molecules such as avidin (streptavidin) for biotin and anti-digoxigenin antibody. In contrast to FISH method, in Tyr-FISH it is impossible to carry out simultaneous detection of two probes, since in both cases the same enzyme HRP is used: streptavidin-HRP and anti-digoxigenin-HRP. In Tyr-FISH method, the detection of probes is carried out sequentially. Once HRP is introduced, the Fluorophore-Tyramide should be added to identify the first probe, for instance, Biotin labeled probe detected by Streptavidin-HRP and visualized by deposition of tyramide-Cy3. Quenching HRP of the first layer of detection must be performed prior to detection of the next probe: anti-DIG-HRP followed by tyramide-FITC deposition.

For quenching HRP we used hydrogen peroxide (H_2O_2). In the active catalytic site of HRP, namely the ferric heme domain, heterologous cleavage of H_2O_2 occurs [34]. However, with a large excess of H_2O_2 , iron in the HRP domain is oxidized to the state of ferrous iron, as a result of which the activity of the enzyme is quenched [35]. In order to estimate the activity of H_2O_2 in quenching HRP, we used 0.3% and 3% H_2O_2 in PBS on "SteamDrop" slides. At a lower concentration (0.3% for 30 minutes), enzyme activity was not inhibited because a hybridization site for the biotin-labeled Unigene572 probe was found along with digoxigenin-labeled Unigene5305 after the second round of detection with anti-DIG-HRP + tyramide-FITC on the filter FITC (Figure 6c). Slide treatment with 3% H_2O_2 for the same incubation time 30 minutes resulted in complete quenching of peroxidase activity (Figure 7).

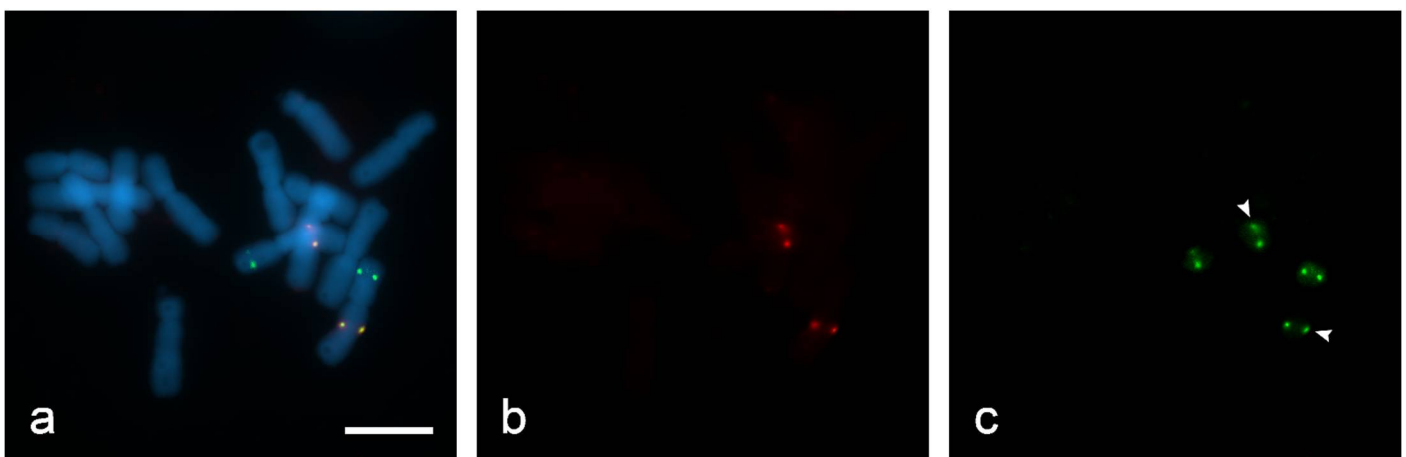


Figure 6. Evaluation of quenching of horseradish peroxidase (HRP) in dual-color sequential Tyr-FISH "SteamDrop" slide. Quenching with 0.3% H_2O_2 for 30 min.: (a) visualization of two probes - merged image; (b) visualization of Unigene572 detected with tyramide-Cy3 using Cy3-filter (red); (c) visualization of Unigene5305 detected with Tyr-FITC using FITC-filter (green), arrows indicate signals arising from Unigene572 because HRP activity after the first detection step was not quenched.

Tyr-FISH super-sensitivity demand that undesirable background be effectively quenched. Peroxidases are ubiquitous in nature being found in bacteria, fungi, algae, plants and animals. The plant peroxidases, belonging to Class III peroxidase, are involved in various vital processes of plant [35]. To quench endogenous peroxidase, we used 0.3% H_2O_2 for 30 minutes. Then slides were pretreated with 4% paraformaldehyde for 10 min and dehydrated with 70%, 90% and 100% ethanol. After washing in TNT buffer, the tyramides were added to the slides for 15 min. To test the presence of endogenous peroxidase activity the chromosome preparations were incubated with both tyramide-FITC and Tyramide-Cy3 at a 1:50 dilution. The chromosome preparations were stained with DAPI and analyzed with epifluorescence microscope Axiolmager M2, Zeiss. Microscopic analysis of chromosomal preparations treated with 0.3% H_2O_2 and in the control variant (non-treated with H_2O_2 slides) did not reveal fluorescent signals in both variants. Based on the results of this experiment, we removed the quenching of endogenous peroxidase from the proto-

col. Although the presence of endogenous peroxidase in other plant species is not excluded. Therefore, we recommend that such a test for endogenous peroxidase be carried out before starting work with other plants.

2.4 A dual-color Tyr-FISH visualization of markers

We performed the dual-color sequential Tyr-FISH on “SteamDrop” and squashed chromosome preparations. The Unigene572 genomic amplicon (3.2Kb) was labeled with biotin-16-dUTP and detected with tyramide-Cy3 (red fluorescence) and Unigene5305 genomic amplicon (1.7Kb) was labeled with digoxigenin-11-dUTP and detected with tyramide-FITC (green fluorescence).

The dual-color Tyr-FISH revealed twin-signals arising from two sister chromatids on the long arm of chromosome 2 probing with Unigene5305 and on the short arm of the chromosome 2 probing with Unigene572 (Figure 7). On “SteamDrop” slide preparations the frequency of Unigene572 detection was 77.4%, and Unigene5305 was 51.6% (Table 2). In contrast on squashed chromosome preparations the frequency of Unigene572 detection was 35.3% and Unigene5305 was only 14.7%. Moreover, the frequency of signal detection on the chromosomes of both homologs within the same metaphase on “SteamDrop” preparations was 41.9% for Unigene572 and 12.9% for Unigene5305. For comparison, on squashed preparations, the signal on both homologous chromosomes was detected only in 2.9% of metaphases for Unigene572, and for Unigene5305, no such metaphase was found at all.

Table 2. Influence of the method of chromosome slide preparation on the frequency of signal detection

Method	Probe	Number of analyzed metaphases	Number of metaphases with signal			Signal detection frequency, %		
			total	on one homolog	on both homologues	total metaphases with signal	on one homolog	on both homologues
Squashed	Unigene572 (3.2Kb)	34	12	11	1	35.3	32.4	2.9
	Unigene5305 (1.7Kb)		5	5	0	14.7	14.7	0.0
SteamDrop	Unigene572 (3.2Kb)	62	48	22	26	77.4	35.5	41.9
	Unigene5305 (1.7Kb)		32	24	8	51.6	38.7	12.9

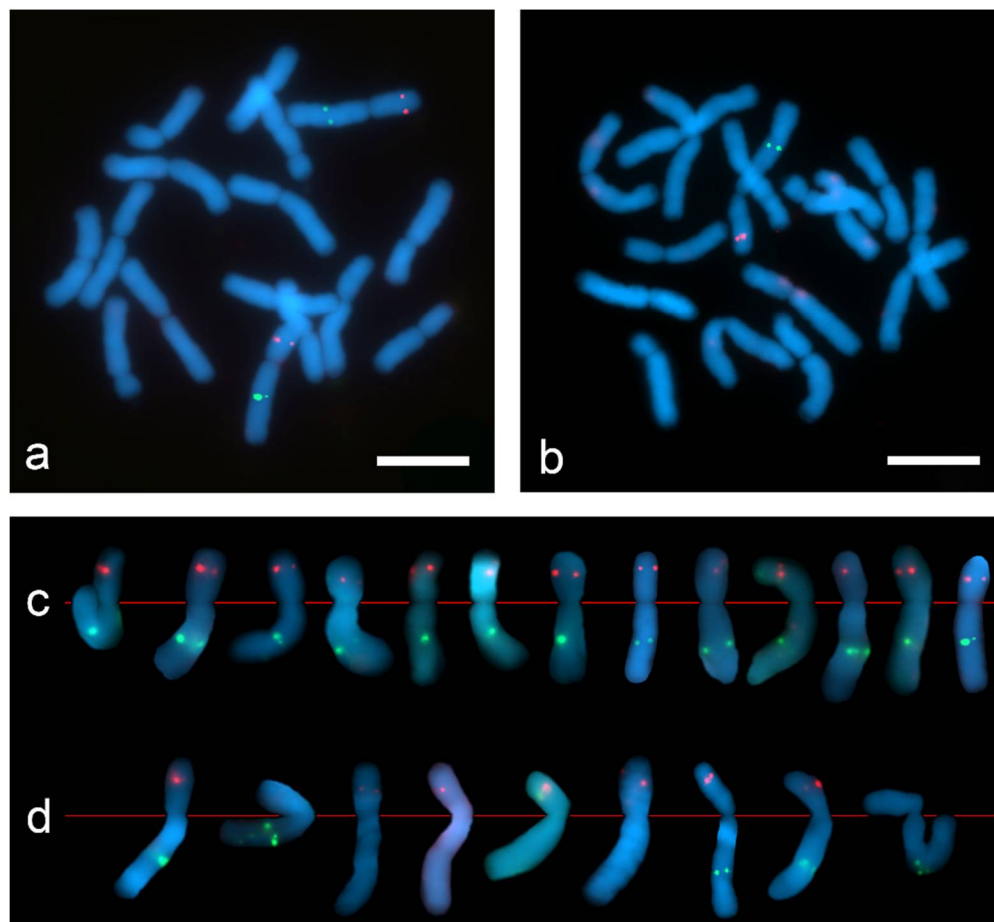


Figure 7. Dual-color Tyr-FISH on mitotic metaphase chromosomes of *Allium cepa* probing with Unigene572 (red) and Unigene5305 (green): (a) “SteamDrop” chromosome preparation; (b) squashed chromosome preparation; (c) extracted chromosome 2 from metaphases of “SteamDrop” chromosome preparations; (d) extracted chromosome 2 from metaphases of squashed chromosome preparation.

We also analyzed the frequency of detecting signals from two probes together per metaphase (Figure 8). On “SteamDrop” preparations, signals arising from two probes on the chromosomes of both homologs within the same metaphase were detected in 12.9% of metaphases and in the case of squashed chromosome preparations, such metaphases were not found. Metaphases with signals from both probes on one homologous chromosome accounted for 36.1% (combined variants 1 and 2, Figure 8) for “SteamDrop” preparations and 8.8% for squashed preparations.

Taking all together, dual-color Tyr-FISH allowed a reliable visualization of two amplicons of unique genes with high detection frequency on dropped chromosome slides. Visualization of two probes on the same chromosome was on almost every second analyzed metaphase (49.1%, combined variants 1, 2 and 3 for “SteamDrop”, Figure 8). The frequency of signal detection depended on the method of preparing chromosome slides.

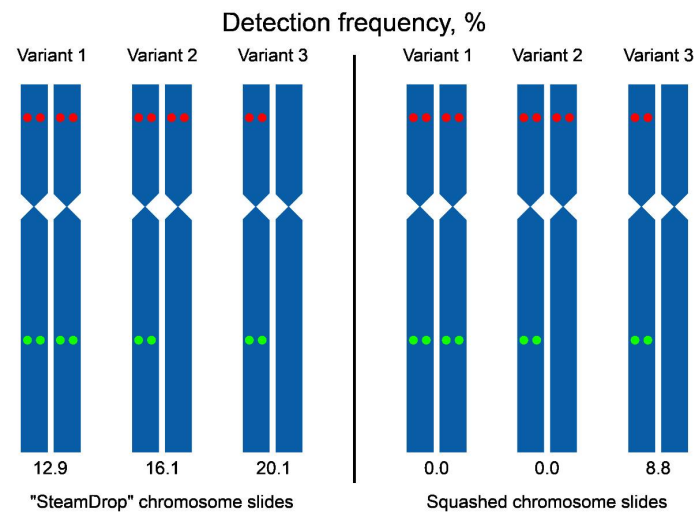


Figure 8. Scheme of variants of a signal detection on a pair of homologous chromosomes 2 using chromosome preparations obtained by “SteamDrop” and squashed methods.

2.5. The integration of recombination and cytogenetic maps

We aligned the chromosomal positions of Unigene 572 and Unigene 5305 with their position on genetic map [27]. Using the DRAWID program [36] the position of the Tyr-FISH signals arising from Unigene 572 on the short arm and Unigene 5305 on the long arm were measured. Only non-overlapping chromosomes 2 were used for measurement. The relative positions of hybridization sites on chromosome arm (RPHC) were calculated as the ratio of the distance between the site of hybridization and the centromere to the length of the chromosome arm. The position of signals on “SteamDrop” and squashed chromosome preparations were compared. The RPHC of Unigene572 on “SteamDrop” chromosomes was 60.63 ± 2.55 and on squashed chromosomes 57.61 ± 3.19 . The RPHC of Unigene 5305 on “SteamDrop” chromosomes was 52.32 ± 1.46 and on squashed chromosomes 50.92 ± 5.57 . Thus, the relative position of the markers on the chromosomes remains unchanged in the preparations obtained by the “SteamDrop” method and the common squashed method. Our observation agrees with results obtained by Wang et al. [37] on maize chromosomes. The authors reported that squashed chromosome preparation produces longer chromosomes than that observed in 3-D fixed cells, but relative positions of genes on chromosomes prepared by different methods remain the same.

The genetic positions of the markers were anchored with their position on the chromosome 2. The physical position of the markers was expressed as a percentage of fractional length (FL) from the end of short arm of chromosome 2 and corresponded with their position on genetic map (Figure 9).

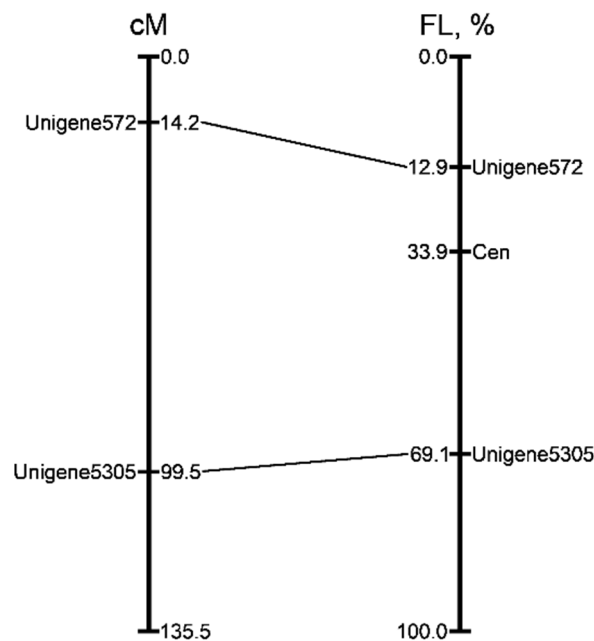


Figure 9. Alignment of the genetic and cytogenetic maps of onion chromosome 2. The genetic map (*left figure*) described by Fujito et al. [27]. Distances in centimorgans are shown on the right of linkage group. The physical positions (*right figure*) of two mapped markers are expressed as percentage of the fractional length (distance from the end of the short arm to the signals divided by the length of the entire chromosome). Corresponding positions on the genetic map are indicated with lines.

3. Discussion

The dual-color Tyr-FISH visualization of two short DNA sequences on a single individual chromosome has a number of advantages: (1) more accurate determination of the physical distance between markers due to the simultaneous detection of two markers on the same chromosome; (2) short procedure of mapping; (3) high detection frequency (4) coverage of regions with suppression of recombination; (5) the mapped sequences are located in the context of major chromosomal landmarks, e.g. centromeres, telomeres, heterochromatin.

Method of chromosome preparation strongly influences on signal detection with tyramide-fluorophore

Trying to figure out why the sensitivity of Tyr-FISH method is significantly reduced on chromosomes prepared by squashed method compared to "SteamDrop" we analyzed key difference between these two methods. Squashed method based on squashing a cell-wall digested root tips on a drop of 45% acetic acid, but in "SteamDrop" method, the enzyme digested cell suspension is dropped onto slide in a fixative containing ethanol and acetic acid in a 3:1 ratio. Considering that the deposition of tyramides occurs via free radical formation and reaction with electron-rich moieties of protein residues of nucleus and cytoplasm, we may suggest that in the variant of "SteamDrop" method, the ability of these electron-rich moieties to form covalent bonds with tyramides is much higher than in the variant of squashed method. Side chains of electron-rich aromatic amino acids are hydrophobic and they behave differently in water and ethanol solutions: in water solutions the hydrophobic amino acids become buried inside the protein whereas the hydrophobic conditions of the ethanol solution are triggering proteins to unfold [38] and transition from compact to extended state. As a result, hydrophobic amino acids forming the "core" of protein become more surfaced [39]. Slide pretreatment with paraformaldehyde, which causes covalent crosslinks between molecules, effectively gluing them together into an

insoluble meshwork [40], preserves this structure of proteins for subsequent Tyr-FISH detection. Hydrophobic conditions created by ethanol in “SteamDrop” method make side chains of electron-rich amino acids in proteins more easily accessible for the deposition of tyramides compared to squashed method. This may explain difference in detection frequency of Tyr-FISH probe on slides obtained by dropped and squashed methods. Moreover, the higher availability of labeled probe DNA for annealing with short target DNA on dropped chromosomes compared to squashed chromosomes also contributes to the high signal detection rate on “SteamDrop” slides. Low chromatin accessibility may result in weak or no Tyr-FISH signals. The chromatin accessibility depends on chromosome compaction, presence of a layer of cytoplasm covering chromosomes as well as on chromatin damage during the preparation of chromosome slides. These parameters may cause low frequency of probe hybridization and the signal-to-noise ratio. To increase the chromatin accessibility less compact pachytene chromosomes [41], interphase nuclei [42] and DNA fiber [26] were used. Fiber-FISH and FISH on interphase nuclei provide superior accessibility but do not allow to assign signals to specific chromosomes. We assessed how the method of slide preparation can affect the structure of chromatin in terms of its accessibility. DIC microscopy clearly showed that the chromosomes prepared by “SteamDrop” method retain their three-dimensional structure providing better sample accessibility compared to the squashed chromosomes, which are denser and may have chromatin damage.

Most studies on tyr-FISH visualization of short single DNA sequences have been done on animal or human chromosomes [43–48]. Typically, slides of human and animal chromosomes are prepared by dropping a cell suspension in a fixative containing methanol (or ethanol) and acetic acid in a 3:1 ratio. In contrast, common method for plant chromosome preparation is the squashed method. Perhaps this explains the failure of using the Tyr-FISH method on plant chromosomes.

A dual-color tyr-FISH for integration of recombination and cytogenetic maps and genome assembly

We applied the dual-color Tyr-FISH method to visualize a short target DNA sequence on high compacted onion chromosomes (250 Mb μm^{-1} , [21]). Study in details of each step of the protocol allowed us to optimize the protocol and to propose the reliable and shortened runtime method. Slide pre-treatment is shortened and includes only para-formaldehyde crosslinking to preserve chromatin structure and dehydration in ascending concentrations of ethanol. The pipeline of the exon-intron probe design using database and bioinformatics tools was developed. The method allowed to detect the transcript marker (1.7Kb) on physical chromosomes in 51.6% of analyzed metaphases. However, high sensitivity of the method based on the deposition of many fluorescent molecules limits the resolution of the method on compacted plant chromosome. To resolve the colocalized Tyr-FISH probes on mitotic metaphase chromosomes, pachytene chromosomes [49] and even extended pachytene chromosomes [50] can be used.

Despite tremendous progress in genome sequencing, the assembly of the plant genome at the chromosome level is still a challenge. According to the NCBI data, about 200 edible plant genomes were assembled at the chromosome level [51]. There are 6000 plant taxa that are considered crops by various cultures [52]. This means that the vast number of crop genomes that support the world's plant-based food production still remain unassembled and not even sequenced.

A good genome assembly does not only rely on assembly of contigs into scaffolds but also the placement and contiguity of scaffolds on chromosomes to form the physical map. For instance, *Allium cepa* genome assembly (GCA_905187595.1) has total length ~15Gbp which is close to the total genome size of *A. cepa* (~16Gbp) but only portion are placed into placed sequences. Vast majority (~12.7Gbp) of assembly is in unplaced scaffolds. In our previous study we physically mapped marker ACM082 from the genetic map

of *A. cepa* on chromosome 4 [11]. This marker is not presented in placed sequences of assembly. Alignment the sequence of this marker against unplaced scaffolds of *A. cepa* assembly showed its presence in one of the unplaced scaffolds. This unplaced scaffold has length ~677Kb and can be used to improve assembly.

Therefore, a reproducible method for visualizing short DNA sequences can serve as a convenient tool for validation bioinformatics genome assembly using cytogenetic anchor points that provide reliable support for the integration of sequence data. Fifteen years ago, J. Jiang and B. Gill prophetically wrote: "In the foreseeable future, whole-genome sequencing will no longer be a hurdle for any plant species. However, this does not spell the end of FISH as a physical mapping tool" [26].

4. Materials and methods

4.1 Plant materials

Allium cepa L., var. 'Haltsedon' ($2n = 2x = 16$) were grown in pots in a greenhouse under controlled conditions: 14-h photoperiod (REFLUX lamp 400 watts; light intensity: 8000 lx) at a temperature of 22 °C.

4.2 Chromosome preparations

In order to arrest the chromosomes at metaphase stage young roots were submerged in a saturated aqueous solution of α -bromonaphthalene (1:1000, v/v) overnight at 4°C. The root tips were fixed in freshly-prepared 3:1 (v/v) ethanol:acetic acid mixture for 1h at RT and stored at -20°C.

"SteamDrop" method

Chromosome preparations were made according to the "SteamDrop" protocol [30]. The fixed roots were washed in water for 30 minutes following by washing in citrate buffer (10 mM sodium citrate, 10mM citric acid) pH 4.8 for 5 min. Dissected meristems (1–5 meristems) were transferred to 0.5 ml tubes with 20–30 μ L of 0.1% enzyme mixture (1: 1: 1) pectolyase Y-23 (Kikkoman, Tokyo, Japan), Cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) and Cytohellicase (Sigma-Aldish Co.LLC, France) for 120 min at 37° C. After proper digestion of cell wall in enzyme mixture (see video [55]) the tube was plugged into ice. 600 μ L of ice-cold TE were added and gently mixed to wash the root sections, centrifuged at 6,000 rpm for 45 sec. A supernatant was removed and 600 μ L of 96% ethanol was added and mix. Cell suspension can be stored at -20°C for at least 6 months or slide preparation can be continued immediately. The cell suspension was centrifuged at 6,000 rpm for 30 sec. The supernatant was discarded by inverting the tube. The pellet was suspended in 20–100 μ L of 96% ethanol, depending on cell concentration. 4 μ L of cell suspension was dropped onto a slide and when the surface became granule-like and the layer of fixative became thin (10–15 sec) 30 μ L of ethanol:acetic acid fixative (3:1) was added. When the surface became granule-like (25–35 sec) the slides were putted upside down under the steam from a water bath at 55°C (10–15 cm from water surface of the water bath) for 3–5 sec. Then 30 μ L of ethanol:acetic acid fixative (1:1) with higher proportion of acetic acid was dropped on the slide, after the surface becomes granule-like (25–35 sec) the slides were putted upside down under the steam for 1 sec. only and immediately were dried with air flow (e.g. a tabletop fan).

Squash method

Mitotic metaphase chromosomes were prepared from young root tips. The fixed roots were washed in water for 30 minutes following by washing in 10 mM citrate buffer (pH 4.8) for 5 min. The roots were transferred to Petri dish (diameter 35mm) containing 0.5 ml of 0.1% enzyme mixture (see above) and incubated for 75 min at 37 °C. The macerated root tips were spread by being dissected and squashed in a drop of 45% acetic acid. The permanent slides were prepared using liquid nitrogen. After removing the cover slip with a razor blade the slides were rinsed briefly in 98% ethanol and air-dried at least for one hour.

4.3 Probe preparation

Primers for probe preparation (Table 1) was design to obtain an exon-intron PCR-product. 25 µL of PCR mixture contained 2.5 uL of 10x Taq Turbo buffer (Evrogen, 25 mM MgCl₂, pH=8.6), 0.2 mM of each dNTP, 0.2 mM of each primer, 2.5U of Taq DNA polymerase (Evrogen) and 100 ng of genomic DNA of *Allium cepa* L. var. Haltsedon. Amplification was performed using following PCR program: 5 minutes of initial denaturation at 95 °C, 35 cycles of 95 °C – 30 seconds, 60 °C – 30 seconds, 72 °C – 150 seconds and 5 minutes of final elongation at 72 °C. PCR-product was checked using electrophoresis in 1% agarose gel (0.5x TBE, 5 V/cm). PCR-product was precipitated using ethanol and 3M potassium acetate accordingly to Wallace et al. [56] with modifications. 20 µL of PCR-product was mixed with 2 µL of 3M potassium acetate and 66 µL (3 volumes) of 96% ethanol (-20 °C), mixed using vortex and incubated 1 hour at -20 °C. After that mixture was centrifuged in benchtop centrifuge (14000 rpm, 30 minutes, RT). Supernatant was carefully discarded, replaced with 1 mL of 70% ethanol (-20 °C), mixed and centrifuged (14000 rpm, 10 minutes, RT). Supernatant was gently removed, pellet was air-dried and resuspended in 10 µL of ddH₂O. Concentration of precipitated PCR-product was measured using NanoDrop ND-1000 (Thermo Fisher Scientific). AT-cloning of PCR-product was performed into pAL2-T vector (Evrogen) and inoculated into *E. coli* strain XL1-Blue (Evrogen) using electroporation accordingly to a manufacturer protocol. 10-times excess of PCR-product (insert) to vector was used in ligation reaction. Blue-white screening was used to select colonies containing plasmid with an insert of interest. Selected colonies were screened using PCR with insert-specific primers and inoculated into 5 mL of LB medium (10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl) for overnight culture and following up plasmid DNA isolation. Plasmid DNA was isolated using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) accordingly to a manufacturer protocol. Concentration of isolated plasmid DNA was measured using NanoDrop ND-1000 (Thermo Fisher Scientific). 1 uL of isolated plasmid DNA was checked using electrophoresis in 1% agarose gel (0.5x TBE, 5 V/cm). 5'-end and 3'-end of insert was Sanger sequenced using standard M13 primer set. Plasmid DNA was labeled with digoxigenin-11-dUTP or biotin-16-dUTP using DIG or Biotin-Nick Translation Mix respectively (Roche, Mannheim, Germany). Determination of fragment length of labeled probe was checked using electrophoresis accordingly to a manufacturer protocol.

4.4 Dual-color sequential Tyr-FISH

Protocol of Dual-color sequential Tyr-FISH

Pre-treatment

1. Incubate the slides in 4% paraformaldehyde at RT for 10 min (USE THE FUME HOOD!).
2. Wash slides in 2xSSC at RT three times for 5 min.
3. Dehydrated slides for 2 min each 70%, 90% and 100% ethanol and air-dry.

Hybridization

4. Prepare the hybridization mixtures (40 µl per slide) *
 - 20 µl formamide
 - 8 µl 50% dextranulphate
 - 4 µl 20x SSC
 - 1 µl 10% SDS
 - x µl probe DNA (50 ng/slide)
 - z µl probe DNA (50 ng/slide)
 - y µl water
5. Denature hybridization mix at 75°C for 5 min and directly plunge into ice for 3 minutes.

6. Add the appropriate hybridization mix to each slide and cover with 24x25 coverslip.
7. Denature the slides at 75°C for 5 minutes.
8. Place the slides in prewarmed humid chamber and incubate overnight at 37°C.

Stringency washing

Stringent of washing – 82%

9. Wash slides in 2xSSC at 42°C twice for 5 min (with agitation).
10. Wash slides in 0.1x SSC at 55°C twice for 7 min (without agitation).
11. Wash slides in 2xSSC at 42°C for 3 min (with agitation).
12. Take Coplin jar out of water bath and leave to cool to RT for 20-25 minutes.
13. Wash slides in 4x SSC at RT for 3 minutes.
14. Wash slides in 2xSSC at RT for 2 minutes (with agitation).

Detection

15. Washing slides in fresh TNT (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5, 0.05% Tween 20) buffer at RT for 5 min with agitation.
16. Block slides with 100 µl TNB buffer (1% Blocking reagent in TN buffer) and place a coverslip (25x50 mm) to reduce evaporation. Incubate the slides in a humid chamber at RT for 15 minutes.
17. Drain off TNB buffer. Add 100 µl of STREPTAVIDIN-HRP (PerkinElmer, USA) (1:500 diluted in TNB) to each slide and place a coverslip (25x50 mm) on top to reduce evaporation. Incubate the slides in a humid chamber at RT for 40 min.
18. Wash slides three times in fresh TNT at RT with agitation for 5 min.
19. Pipet 200 µL of the TSA PLUS Cy3 Reagent (Akoya Biosciences) (1:50 in 10% dextranulphate in 1x Amplification) onto each slide. Incubate the slides at RT for 3 to 10 min*. (per one slide: 4 µL stock Tyramide-CY3+ 40 µL 50% dextranulphate + 156 µL 1xAmplification Diluent).
20. Wash slides three times in fresh TNT at RT with agitation for 5 min.
21. Deactivate the remaining HRP activity by adding 100 µL 3% H₂O₂ in 1xTN (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) for 30 minutes.
22. Wash the slides three times for 5 min each in TNT at RT with agitation.
23. Block slides with 100 µL 1% TNB buffer and place a coverslip (25x50 mm) to reduce evaporation. Incubate the slides in a humid chamber at RT for 15 minutes.
24. Drain off 1% TNB buffer. Add 100 µL of ANTI-DIGOXIGENIN HRP (Akoya Biosciences) (1:500 diluted in 1% TNB) to each slide and place a coverslip (25x50 mm) on top to reduce evaporation. Incubate the slides in a humid chamber at RT for 40 min.
25. Wash slides three times in fresh TNT at RT with agitation for 5 min.
26. Pipet 200 µL of the TSA PLUS Fluorescein Reagent (Akoya Biosciences) (1:50 in 10% dextranulphate in 1x Amplification) onto each slide. Incubate the slides at RT for 3 to 10 min. (per one slide: 4 µL stock Tyramide-FITC+ 40 µL 50% dextranulphate + 156 µL 1xAmplification Diluent).
27. Wash slides three times in fresh TNT at RT with agitation for 5 min.
28. Counterstaining
29. Wash slides in 2xSSC at RT three times for 5 min.
30. Dehydrated slides for 2 min each 70%, 90% and 100% ethanol and air-dry in dark place.
31. Prepare freshly 1:20 dilution of DAPI in Vectashield. Add 20 µL per slide. Apply a 24x50 mm coverslip.

Note

* - mix all components of the hybridization mixture without adding DNA probes. Mix well by vortex to homogeneous mixture. Add probes in homogeneous mixture and mix briefly on vortex and centrifuge.

** - incubation time with tyramides depends on target size. For 13.3Kb target site enough 3 minutes [21]. For 2-3Kb target site enough 7 - 10 minutes (in this paper). If the incubation times was longer it will look like strong band instead of two spots on both sister chromatids.

4.5. Microscopy

For differential interference contrast (DIC) microscopy air-dried chromosome preparations obtained by both "SteamDrop" and squashed methods were directly examined under the Imager.D1 microscope, (<http://www.zeiss.com>), using an immersion objective x100 equipped with DIC.

For fluorescent microscopy slides were examined under a Zeiss AxioImager M2 microscope (<http://www.zeiss.com>). The selected images were captured using a digital Hamamatsu camera C13440-20CU (<http://www.hamamatsu.com>). Image processing and thresholding were performed by Zen 2.6 (blue edition) image analysis software. The captured images of the chromosomes and position of Tyr-FISH signals were measured using the program DRAWID [36]. Only non-overlapping chromosomes were used for the measurement of the positions of Tyr-FISH signals. The relative position of hybridization sites on chromosomes (RPHC) was calculated as the ratio of the distance between the site of hybridization and the centromere to the length of the chromosome arm. Karyotype analysis were performed according to the standard onion nomenclature system proposed by Kalkman [57] and confirmed by the Fourth Eucarpia *Allium* Symposium [58].

Author Contributions: LK – conceptualization, data analysis and writing of original draft of the paper; NK and AE – performing of experiments, data analysis, text processing, design of figures and writing of original draft of the paper; GK and IK - review and editing; SS and MS – transcripts and genome sequencing.

Funding: This research was funded by Russian Scientific Foundation, grant number 20-46-07005.

Acknowledgments: The authors are grateful to Alexander Voronkov, K.A. Timiryazev Institute of Plant Physiology RAS for excellent technical assistance in the DIC microscopy and Katerina Danko, St. Petersburg State University, for helpful discussion of biochemical aspects of slide preparation.

References

1. Sturtevant, A.H. The Linear Arrangement of Six Sex-linked Factors in *Drosophila*, as Shown by their Mode of Association. *J Exp Zool* **1913**, 14, 43-59.
2. Wu, H.; Yao, D.; Chen, Y.; Yang, W.; Zhao, W.; Gao, H.; Tong, C. *De novo* genome assembly of *Populus simonii* further supports that *Populus simonii* and *Populus trichocarpa* belong to different sections. *G3* **2020**, 10(2), 455-66. doi: 10.1534/g3.119.400913.
3. Maccaferri, M.; Harris, N.S.; Twardziok, S.O.; Pasam, R. K.; Gundlach, H.; Spannagl, M.; Ormanbekova, D.; Lux, T.; Prade, V. M.; Milner, S. G; et al. Durum wheat genome highlights past domestication signatures and future improvement targets. *Nat Genet* **2019**, 51, 885–895. doi: 10.1038/s41588-019-0381-3.
4. Chen, Y.; Zhang, Y.; Wang, Y.; Zhang, L.; Brinkman, E. K.; Adam, S. A.; Goldman, R.; van Steensel, B.; Ma, J.; Belmont, A. S. Mapping 3D genome organization relative to nuclear compartments using TSA-Seq as a cytological ruler. *J Cell Biol* **2018**; 4025-4048. doi: 10.1083/jcb.201807108.
5. Koo, D. H.; Jo, S. H.; Bang, J. W.; Park, H. M.; Lee, S.; Choi, D. Integration of cytogenetic and genetic linkage maps unveils the physical architecture of tomato chromosome 2. *Genetics* **2008**, 179(3), 1211-1220. doi: 10.1534/genetics.108.089532. Epub 2008 Jul 13.
6. Szinay, D.; Chang, S.-B.; Khrustaleva, L.; Peters, S.; Schijlen E.; Bai, Y.; Stiekema, W. J.; van Ham, R. C. H. J.; de Jong, H.; Lankhorst, M. R. K.; High-resolution chromosome mapping of BACs using multi-colour FISH and pooled-BAC FISH as a backbone for sequencing tomato chromosome 6. *Plant J* **2008**, 56, 627–637. doi: 10.1111/j.1365-313X.2008.03626.x.
7. Ren, Y.; Zhao, H.; Kou, Q.; Jiang, J.; Guo, S.; Zhang, H.; Hou, W.; Zou, X.; Sun, H.; Gong, G.; Levi, A. A high resolution genetic map anchoring scaffolds of the sequenced watermelon genome. *PLoS One* **2012**, 11;7(1), 627-637. doi: 10.1111/j.1365-313X.2008.03626.x.
8. Yang, L.; Koo, D. H.; Li, D.; Zhang, T.; Jiang, J.; Luan, F.; Renner, S. S.; Hénaff, E.; Sanseverino, W.; Garcia-Mas, J. Next-generation sequencing, FISH mapping and synteny-based modeling reveal mechanisms of decreasing dysploidy in *Cucumis*. *Plant J* **2014**, 77(1), 16-30. doi: 10.1111/tpj.12355.

9. Bielski, W.; Książkiewicz, M.; Šimoníková, D.; Hřibová, E.; Susek, K.; Naganowska, B. The Puzzling Fate of a Lupin Chromosome Revealed by Reciprocal Oligo-FISH and BAC-FISH Mapping. *Genes* **2020**, *11*, 1489. doi: 10.3390/genes11121489.
10. Danilova, T. V.; Friebe, B.; Gill, B. S. Development of a wheat single gene FISH map for analyzing homoeologous relationship and chromosomal rearrangements within the Triticeae. *Theor Appl Genet* **2014**, *127*, 715-730. doi: 10.1007/s00122-013-2253-z.
11. Khrustaleva, L.; Kudryavtseva, N.; Romanov, D.; Ermolaev, A.; Kirov, E. Comparative Tyramide-FISH mapping of the genes controlling flavor and bulb color in *Allium* species revealed an altered gene order. *Sci Rep* **2019**, *9*, 12007. doi: 10.1038/s41598-019-48564-9.
12. Lieberman-Aiden, E.; Van Berkum, N.L.; Williams, L.; Imakaev, M.; Ragoczy, T.; Telling, A.; Amit, I.; Lajoie, B. R.; Sabo, P. J.; Dorschner, M. O. et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* **2009**; 326(5950), 289–293. doi: 10.1126/science.1181369.
13. Burton, J.N.; Adey, A.; Patwardhan, R.P.; Qiu, R.; Kitzman, J.O.; Shendure, J. Chromosome-scale scaffolding of de novo genome assemblies based on chromatin interactions. *Nat Biotechnol* **2013**, *31*(12), 1119–1125. doi: 10.1038/nbt.2727.
14. Dudchenko, O.; Batra, S. S.; Omer, A. D.; Nyquist, S. K.; Hoeger, M.; Durand, N. C.; Aiden, E. L. De novo assembly of the *Aedes aegypti* genome using Hi-C yields chromosome-length scaffolds. *Science* **2017**, *356*, 92-95. doi: 10.1126/science.aal3327.
15. Tang, H.; Lyons, E.; Town, C. D. Optical mapping in plant comparative genomics. *GigaScience* **2015**, *4*(1), 13742-015. doi: 10.1186/s13742-015-0044-y.
16. Hoang, P.N.T.; Michael, T.P.; Gilbert, S.; Chu, P.; S Motley, T.; Appenroth, K. J.; Schubert, I.; Lam, E. Generating a high-confidence reference genome map of the Greater Duckweed by integration of cytogenomic, optical mapping and Oxford Nanopore technologies. *Plant J.* **2018**, *96*, 670–684. doi: 10.1111/tjp.14049.
17. Shearer, L. A.; Anderson, L. K.; de Jong, H.; Smit, S.; Goicoechea, J. L.; Roe, B. A.; Hua, A.; Giovannoni, J. J.; Stack, S. M. Fluorescence *in situ* hybridization and optical mapping to correct scaffold arrangement in the tomato genome. *G3* **2014**, *4*(8), 1395-1405. doi: 10.1534/g3.114.011197.
18. Febrer, M.; Goicoechea, J. L.; Wright, J.; McKenzie, N.; Song, X.; Lin, J.; Bevan, M. W. An integrated physical, genetic and cytogenetic map of *Brachypodium distachyon*, a model system for grass research. *PLoS One* **2010**, *5*, e13461. doi: 10.1371/journal.pone.0013461.
19. Hoang, P. T.; Fiebig, A.; Novák, P.; Macas, J.; Cao, H. X.; Stepanenko, A.; Schubert, I. Chromosome-scale genome assembly for the duckweed *Spirodela intermedia*, integrating cytogenetic maps, PacBio and Oxford Nanopore libraries. *Sci. Rep* **2020**, *10*(1), 1-14. doi: 10.1038/s41598-020-75728-9.
20. Raap, A.; Van De Corput, M.; Vervenne, R.; Van Gijlswijk, R.; Tanke, H.; Wiegant, J. Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides. *Hum Mol Genet* **1995**, *4*(4): 529–534. doi: 10.1093/hmg/4.4.529.
21. Khrustaleva, L.I.; Kik, C. Localization of single copy T-DNA insertion in transgenic shallots (*Allium cepa*) by using ultra-sensitive FISH with tyramide signal amplification. *Plant J.* **2001**, *25*, 699–707. doi: 10.1046/j.1365- 313x.2001.00995.x.
22. Stephens, J.L.; Brown, S.E.; Lapitan, N.L.; Knudson, D.L. Physical mapping of barley genes using an ultrasensitive fluorescence *in situ* hybridization technique. *Genome* **2004**, *47*(1), 179-89. doi: 10.1139/g03-084. PMID: 15060614.
23. Pérez, R.; de Bustos, A.; Jouve, N.; Cuadrado, A. Localization of Rad50, a single-copy gene, on group 5 chromosomes of wheat, using a FISH protocol employing tyramide for signal amplification (Tyr-FISH). *Cytogenet Genome Res* **2009**, *125*(4), 321-328. doi: 10.1159/000235938.
24. Sanz, M. J.; Loarce, Y.; Ferrer, E.; Fominaya, A. Use of Tyramide-Fluorescence *in situ* Hybridization and Chromosome Microdissection for Ascertaining Homology Relationships and Chromosome Linkage Group Associations in Oats. *Cytogenet Genome Res* **2012**, *136*, 145-156. doi: 10.1159/000335641.
25. Kirov, I.; Van Laere, K.; De Riek, J.; De Keyser, E.; Van Roy, N.; Khrustaleva, L. Anchoring linkage groups of the Rosa genetic map to physical chromosomes with Tyramide-FISH and EST-SNP markers. *PloS one* **2014**, *9*. e95793. doi: 10.1371/journal.pone.0095793.
26. Jiang J.; Gill, B. S. Current status and the future of fluorescence *in situ* hybridization (FISH) in plant genome research. *Genome* **2006**, *49*, 1057-1068. doi: 10.1139/g06-076.
27. Fujito, S.; Akyol, T. Y.; Mukae, T.; Wako, T.; Yamashita, K.; Tsukazaki, H.; Hirakawa, H.; Tanaka, K.; Mine, Y.; Sato, S.; Shigyo, M. Construction of an ultrahigh-density linkage map and graphical representation of the arrangement of transcriptome-based unigene markers on the chromosomes of *Allium cepa* L. *BMC Genomics* (under review).
28. Kohany, O.; Gentles, A. J.; Hankus, L.; Jurka, J. Annotation, submission and screening of repetitive elements in Repbase: RepbaseSubmitter and Censor. *BMC bioinformatics* **2006**, *7*(1), 1-7. doi: 10.1186/1471-2105-7-474.
29. Morrison, L. E.; Ramakrishnan, R.; Ruffalo, T. M.; Wilber, K. A.; Labeling fluorescence *in situ* hybridization probes for genomic targets. *Methods Mol Biol* **2002**, *204*, 21-40. doi: 10.1385/1-59259-300-3:21.
30. Kirov, I.; Divashuk, M.; Van Laere, K.; Soloviev, A.; Khrustaleva, L. An easy “SteamDrop” method for high quality plant chromosome preparation. *Mol Cytogenet* **2014**, *7*(21), doi: 10.1186/1755-8166-7-21.
31. Murphy, D. Differential interference contrast (DIC) microscopy and modulation contrast microscopy. In *Fundamentals of Light Microscopy and Digital Imaging*, 2nd ed.; Wiley, J.; Sons, I.; Wiley-Liss: New York, 2001; pages 153-168.
32. Bobrow, M.N.; Harris, T. D.; Shanghnessy, K.J.; Litt, G.J. Catalized reporter deposition, a novel method of signal amplification. Application to immunoassays. *J. Immunol. Meth* **1989**, *125*:279-285. doi: 10.1016/0022-1759(89)90104-x.
33. Gross, R. A.; Kumar, A.; Kalra, B. Polymer synthesis by *in vitro* enzyme catalysis. *Chem. Rev.* **2001**, *101*(7), 2097-2124. doi: 10.1021/cr0002590.

34. Nielsen, K. L.; Indiani, C.; Henriksen, A.; Feis, A.; Becucci, M.; Gajhede, M.; Smulevich, G.; Welinder, K. G. Differential activity and structure of highly similar peroxidases. Spectroscopic, crystallographic, and enzymatic analyses of lignifying *Arabidopsis thaliana* peroxidase A2 and horseradish peroxidase A2. *Biochemistry* **2001**, *40*(37), 11013-11021. doi: 10.1021/bi010661o.
35. Pandey, V. P.; Awasthi, M.; Singh, S.; Tiwari, S.; Dwivedi, U. N. A comprehensive review on function and application of plant peroxidases. *Biochem Anal Biochem* **2007**, *6*(1), 2161-1009. doi: 10.4172/2161-1009.1000308.
36. Kirov, I.; Khrustaleva, L.; Van Laere, K.; Soloviev, A.; Meeus, S.; Romanov, D.; Fesenko, I. DRAWID: user-friendly java software for chromosome measurements and idiogram drawing. *Comp. Cytogenet.* **2017**, *11*, 747-75. doi:10.3897/compcyto-gen.v11i4.20830.
37. Wang, C. J. R.; Harper, L.; Cande, W. Z. High-resolution single-copy gene fluorescence *in situ* hybridization and its use in the construction of a cytogenetic map of maize chromosome 9. *The Plant Cell* **2006**, *18*(3), 529-544. doi: 10.1105/tpc.105.037838.
38. Mohanta, D.; Santra, S.; Jana, M. Conformational disorder and solvation properties of the key-residues of a protein in water-ethanol mixed solutions. *Phys Chem Chem Phys* **2017**, *19*(48), 32636-32646. doi: 10.1039/c7cp06022j.
39. Halder, R.; Jana, B. Exploring the role of hydrophilic amino acids in unfolding of protein in aqueous ethanol solution. *PRO-TEINS* **2021**, *89*, 116-125. doi: 10.1002/prot.25999.
40. Solovei, I.; Cavallo, A.; Schermelleh, L.; Jaunin, F.; Scasselati, C.; Cmarko, D.; Cremer, C.; Fakan, S.; Cremer, T. Spatial preservation of nuclear chromatin architecture during three-dimensional fluorescence *in situ* hybridization (3D-FISH). *Ex Cell Res* **2002**, *276*(1), 10-23. doi: 10.1006/excr.2002.5513.
41. De Jong, J. H.; Fransz, P.; Zabel, P. High resolution FISH in plants—techniques and applications. *Trends Plant Sci* **1999**, *4*, 258-263. doi: 10.1016/s1360-1385(99)01436-3.
42. Jiang, J.; Gill, B. S.; Wang, G. L.; Ronald, P. C.; Ward, D. C. Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial artificial chromosomes. *PNAS* **1995**, *92*, 4487-4491.
43. Raap, A. K. Advances in fluorescence *in situ* hybridization. *Mutat Res* **1998**, *400*, 287-298. doi: 10.1016/s0027-5107(98)00029-3.
44. Schriml, L. M.; Padilla-Nash, H. M.; Coleman, A.; Moen, P.; Nash, W. G.; Menninger, J.; Jones, G.; Ried, T.; Dean, M. Tyramide signal amplification (TSA)-FISH applied to mapping PCR-labeled probes less than 1Kb in size. *Biotechniques* **1999**, *27*, 608-613. doi: 10.2144/99273pf01.
45. Yamato, K. T.; Ishizaki, K.; Fujisawa, M.; Okada, S.; Nakayama, S.; Fujishita, M.; ... & Ohyama, K. (2007). Gene organization of the liverwort Y chromosome reveals distinct sex chromosome evolution in a haploid system. *Proceedings of the National Academy of Sciences*, *104*(15), 6472-6477. doi: 10.1073/pnas.0609054104.
46. Seifertova, E.; Zimmerman, L. B.; Gilchrist, M. J.; Macha, J.; Kubickova, S.; Cernohorska, H.; Zarsky, V.; Owens, N.D.L.; Sesay, A.K.; Tlapakova, T.; Krylov, V. Efficient high-throughput sequencing of a laser microdissected chromosome arm. *BMC genomics* **2013**, *14*(1), 1-9. doi: 10.1186/1471-2164-14-357.
47. Chen, Y.; Zhang, Y.; Wang, Y.; Zhang, L.; Brinkman, E. K.; Adam, S. A.; Belmont, A. S. Mapping 3D genome organization relative to nuclear compartments using TSA-Seq as a cytological ruler. *J Cell Biol* **2018**, *217*, 4025-4048. doi: 10.1083/jcb.201807108.
48. Kint, S.; Van Criekeing, W.; Vandekerckhove, L.; De Vos, W. H.; Bomsztyk, K.; Krause, D. S.; Denisenko, O. Single cell epigenetic visualization assay. *Nucleic Acids Res.* **2021**, gkab009. doi: 10.1093/nar/gkab009.
49. Kirov, I. V.; Van Laere, K.; Khrustaleva, L. I. High resolution physical mapping of single gene fragments on pachytene chromosome 4 and 7 of *Rosa*. *BMC genetics* **2015**, *16*, 1-10. doi: 10.1186/s12863-015-0233-9.
50. Khrustaleva, L.; Jiang, J.; Havey, M. J. High-resolution tyramide-FISH mapping of markers tightly linked to the male fertility restoration (Ms) locus of onion. *Theor. Appl. Genet.* **2016**, *129*, 535-545, doi: 10.1007/s00122-015-2646-2.
51. National Center for Biotechnology Information (NCBI). <https://www.ncbi.nlm.nih.gov/genome/browse#!/eukaryotes/plants>
52. Jackson, S.; Rounsley, S.; Purugganan, M. Comparative Sequencing of Plant Genomes: Choices to Make. *Plant Cell* **2006**, *18*, 1100-1104. doi: 10.1105/tpc.106.042192.
53. Prescott-Allen, R.; Prescott-Allen, C. How many plants feed the world? *Conserv Biol* **1990**, *4*(4), 365-374. doi: 10.1111/j.1523-1739.1990.tb00310.x.
54. Raven, P.; Fauquet, C.; Swaminathan, M.S.; Borlaug, N.; Samper, C. Where next for genome sequencing? *Science* **2006**, *311*, 468. doi: 10.1126/science.311.5760.468b.
55. YouTube. <https://www.youtube.com/watch?v=UiXIPJDtN2E>
56. Wallace, D. M. Precipitation of nucleic acids. *Methods Enzymol* **1987**, *152*, 41-48. doi: 10.1016/0076-6879(87)52008-0.
57. Kalkman, E.R. Analysis of the C-banded karyotype of *Allium cepa* L. standard system of nomenclature and polymorphism. *Genetics* **1984**, *65*: 141-148. doi: 10.1007/BF00135278.
58. De Vries J.H. Onion chromosome nomenclature and homoeology relationships –workshop report. *Euphytica* **1990**, *49*, 1-3. doi: 10.1007/BF00024124.