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Full Length Article

Association of Sub-Telomeric Satellite DNA with 45S rDNA in Cultivated Onion (*Allium cepa*) with Denaturing and Non-Denaturing (ND-) FISH

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Abstract

Onion (*Allium cepa* L.) is a major vegetable crop, widely cultivated throughout the world. The very large genome of onion (16.35 Gbp) is not sequenced and chromosome structural analysis can assist in studies of the large genome, showing the nature and locations of major repetitive DNA families, including telomeric, ribosomal rDNA and satellite DNA sequences. This study investigates the telomeric tandem repeat sequences, telomeric satellite DNA sequences and their association with 45S rDNA using non-denaturing (ND-) and denaturing fluorescence *in situ* hybridization (FISH). In the present study, two termini of short arm of nuclear organizing region (NOR) of chromosome 6 were detected, without any signal from the sub-telomeric satellite DNA in both ND-FISH and denaturing (simple) FISH, while the terminal part of the short arm of chromosome 8 has a signal for sub-telomeric tandem repeats. Three chromosome termini (two from the short arm of chromosome 6 and one from short arm of chromosome 8) hybridize only with 45S rDNA, except in one plant where one homologue of chromosome 8 hybridized with both 45S rDNA and apparently the non-Allium telomeric DNA motif, thus showing heterozygosity, and perhaps indicating presence of a satellite related to TTTAGGG. ND-FISH is a useful tool for plant cytogenetic to study less exploited repeat sequences and to compare related species or accessions. It also assists with exploitation of related species for inter-specific hybridization notwithstanding their genome complexity and size. © 2020 Friends Science Publishers

Keywords: 5S and 45S rDNA; *Allium cepa* L.; ND-FISH; Satellite DNA; telomeric repeats

Introduction

The genome of onion is yet to be sequenced due to its large and repetitive genome (~16.35 Gbp). Chromosome structural analysis can assist in studies of the large genome, showing the nature and locations of major repetitive DNA families, such as telomeric, sub-telomeric, centromeric, and ribosomal rDNA, including dispersed repetitive and satellite DNA sequences. Understanding the range and nature of variation in a small number of repetitive DNA sequence is valuable to understand genome relationships and chromosome evolution in the many species with complex and large genomes, such as the genus *Avena* (Katsiotis *et al.* 2000; Liu *et al.* 2019), where there is as yet no complete DNA sequence Available. Furthermore, chromosomal recombination and genome stability can be assessed efficiently using both genomic and repetitive DNA probes within germplasm pools and wild relatives of cultivated species such as *Arachis* (Nascimento *et al.* 2018).

Most eukaryotic chromosomes end with a specialized repetitive DNA sequence, the telomere. The term was introduced by Müller (1938) and used by McClintock (1941) in maize as the terminal element of the chromosomes that caps the chromosome, enables replication of the full length, and inhibits end-joining, ensuring stability of the genome (Siroky *et al.* 2003). The terminal regions of plant chromosomes, internal to the true telomere, often contain repetitive DNA motifs, likely to relate to genome stability and packaging (Vershinin *et al.* 1995). The true terminal telomeric sequences are added by an enzyme including an RNA template to chromosome ends, rather than by semi-conservative replication (Watson *et al.* 2010).

Most plant species are similar to *Arabidopsis* in having a telomeric repeat comprised of 7 nucleotides

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(5′-TTTAGGG-3′) (Richards and Ausubel 1988), while most animal species have a six-nucleotide repeat (O'Connor 2008) occurring in arrays from 10 kb (human) to 150 kb (mice), and 2–5 kb in plants (*Arabidopsis thaliana*) (Riha *et al.* 1998), to more than 100 kb in barley (*Hordeum vulgare*) and tobacco (*Nicotiana tabacum*) (Kilian *et al.* 1995; Schwarzacher and Heslop-Harrison 1991). Cuadrado *et al.* (2009) while studying non-denaturing FISH (ND-FISH) on different species of cereals observed the brighter telomeric signal with shorter repeat sequences as compared to species with high repeat sequences. Dysfunctional telomeres, reduction or unavailability of the telomere leads to massive instability in the genome of crop plant (Siroky *et al.* 2003). Fuchs *et al.* (1995) showed that chromosomes in some Amaryllidaceae species, particularly in Allioideae group, did not terminate with the 7 bp repeat of other plant species. There was speculation about the nature of the chromosome termini, including the stabilization of chromosome ends by amplification of mobile elements, of satellite DNA (such as a 375 bp repeat found near the ends of *Allium* chromosomes, Barnes *et al.* 1985) and rDNA (Pich *et al.* 1996; Pich and Schubert 1998). It is now clear that *Allium* telomeres consist of the unusual telomeric sequence $(CTCGGTTATGGG)_n$, synthesized and added to the ends of chromosomes by a different telomerase from those found in most plant species (Fajkus *et al.* 2016). Many other repetitive DNA sequence can also be useful to identify chromosomes and genomic variation (Kubis *et al.* 1997).

The aim of the current study was to use fluorescent staining and *in situ* hybridization methods to generate a karyotype and to assess the presence of DNA sequences with simple FISH (involving heat denaturation of the chromosomal DNA to make it single-stranded) and FISH with no specific step to make the chromosomal DNA single-stranded, termed non-denaturing (ND-) FISH in *A. cepa.* Tandemly repeated satellite DNA repeats and 45S rDNA were used as probes. By this study we will also confirm the usefulness of ND-FISH in plant cytogenetics. ND-FISH can be effective and fast, of value for genome analysis in species with very large genomes.

Materials and Methods

Plant material

The seeds of twelve diverse accessions of cultivated onions were obtained from Magnus Kahl Seeds (MKS) Pakistan and commercial seed source in Pakistan. The seed were germinated to collect roots for mitotic chromosome preparation and young leaf tissues used for extraction of DNA for amplification of genes to be used for probe development for FISH analysis.

Mitotic chromosome preparation

Young roots (~ 1.5 to 2.0 cm) from different accessions of onion were collected in 1.5 mL Eppendorf tubes containing drinking water. These roots were immediately shifted into a container pressurized by Nitrous Oxide (N_2O) for around 50 min as a pre-treatment to get good metaphase index followed by fixation in freshly prepared ethanol : acetic acid (3:1) Solution. The root tips were then subjected to enzymatic digestion (cellulase @ 20U/mL and pectinase @ 20U/mL diluted in 1x enzyme buffer solutions) for around 35 min in at 37°C. Mitotic chromosome preparation was carried out by following the methodology of Schwarzacher and Heslop-Harrison (2000).

DNA extraction and probe development

DNA extraction was performed by the CTAB method (Doyle and Doyle 1990) from fresh onion leaves. The extracted DNA was further used in amplification of telomeric tandem repeats (314 bp), telomeric satellite DNA (375 bp) and 18S rDNA gene (1.1 kb).

The sub-telomeric tandem repeat sequence pAc074 with a sequence ID # AF227152.1 (https://www.ncbi.nlm.nih.gov/nuccore/AF227152.1/) initially reported by Do *et al.* (2001) was used as probe for ND-FISH analysis based on the sequence of pAc074. An oligo-nucleotide probe, named Oligo-pAc074 of 59bp sequences (5′-GACATCGATTATTCGGACGGCCATAACTGTTGCCTCGTTTAGAGTTACGGGAGCCATAA -3′) was synthesized and 5′ labeled with 6-carboxyfluorescein (6-FAM) (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China). The oligo-nucleotide probes Oligo-5SrDNA (TCAGAACTCCGAAGTTAAGCGTGCTTGGGCGAGAGTAGTAC) and Oligo-18SrDNA (GACGGGCGGTGTGTACAAAGGGCAGGGACGT) for targeting 5S rDNA and 18S rDNA in *Allium* were synthesized. The sequences of Oligo-5SrDNA were partially homologous to the last part of the *Allium cepa* gene for 5S rRNA clone pAc5S-15 (NCBI gene bank number AB056586, Hizume *et al.* 2002). Primers were designed using sequence of telomeric satellite DNA repeats having sequence ID # X02573.1 (https://www.ncbi.nlm.nih.gov/nuccore/X02573.1/) of cultivated onion reported by Barnes *et al.* (1985). Briefly, to amplify the fragment through PCR, a pre-denaturation was done for 4 min at 95°C followed by 30 cycles of denaturation at 95°C for 45 s, annealing of primers at 59°C for 40 s, extension for 1 min at 72°C before final extension at 72°C for 10 min. The amplified DNA product from *A. cepa* L. (Fig. 1a) was then labeled with biotin-11-dUTP by random priming (Roche, Diagnostics, Basel, Switzerland) and used as telomeric satellite probes. For 45S rDNA a gene sequence of 18S rDNA (1.1 kb) was amplified from *A. cepa* L. (Fig. 1b) using a pair of primer P1:5´ CGAACTGTGAAACTGCGAATGGC-3´ and P2:5´-TAGGAGCGACGGGCGGTGTG-3´desigend by Chang *et al.* (2010). PCR condition for 45S rDNA, was with an initial

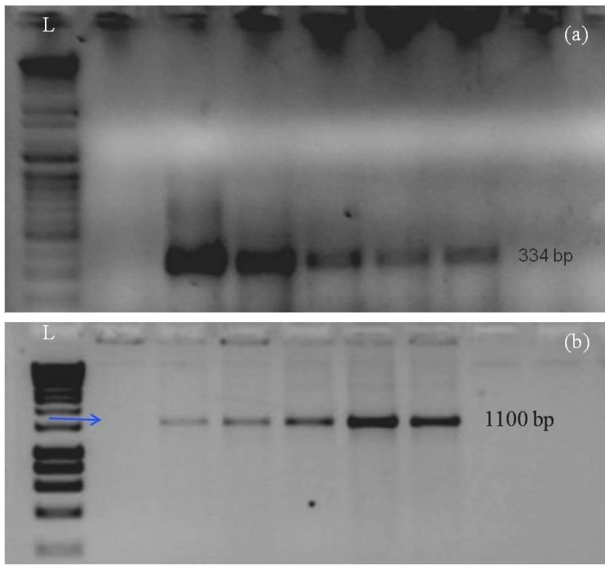

Fig. 1: PCR amplification results of (**a**) sub-telomeric satellite DNA, (**b**) 45S rDNA amplified from *A. cepa*

denaturation at 95°C for 4 min followed by 30 cycles of amplification - denaturation at 95°C for 45 s, annealing of primers at 55°C for 40 s, DNA extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified product was then purified and labelled with digoxigenin-16-dUTP according to manufacturer's instruction (Roche, Germany) and used as a probe to localize 45S rDNA sites.

In situ hybridization

The prepared slides with good metaphase index were selected and re-fixed in ethanol: acetic acid (3:1) followed by RNase treatment (100 μg/mL) for 1hr at 37°C followed by a wash with 2x SSC. The slides were incubated with HCl (0.01N) followed by pepsin treatment (0.05 μg/mL). Application of paraformaldehyde was done for 10 min followed by a wash with 2x SSC. The slides were then dehydrated with a series (70, 85 and 90%) of ethanol for two min each. Hybridization mixture (50% formamide, 2x SSC, 10% dextran sulphate, 0.025 μg/mL salmon sperm DNA, 1.25 mM EDTA, 0.125% SDS and probes) was denatured at 85°C for 7 min and placed in ice for 10 min. About 40 μL of hybridization mixture was applied to each slide and covered with plastic cover slip. Slides were then shifted to a hybridization chamber and denaturation of chromosomes was performed for 6 min at 70°C before cooling to allow hybridization overnight at 37°C. The slides were removed from hybridization chamber and washings were performed with 2x SSC, 0.1x SSC for 12 min and in detection buffer for 5 min. Hybridization site detection was performed with FITC-antidigoxigenin for digoxigenin labelled probe and streptavidin-alexa-594 was used for biotin-labelled probes. For the detection of physical localization of synthesized Oligo-pAc074 on onion chromosomes, the protocol of non-denaturing FISH (ND-FISH) was employed as described by Lang *et al.* (2019), similar to the above procedure but without the 70°C step. After post-hybridization washes, counterstaining of whole chromosomes was performed with 4', 6-diamidino-2-phenylindole (DAPI) and slides were mounted in Vectashield. The slides were analyzed on Nikon Eclipse N80i fluorescent microscope equipped with a DS-QiMc monochromatic camera (Nikon, Japan) and appropriate filter set. Adobe Photoshop cc was used to process the images.

Individual chromosome lengths were measured using ImageJ software. The chromosome analysis was performed by the classification system proposed by Levan *et al.* (1964). On the basis of arm ratios, chromosomes were further subdivided into four different classes (Adonina *et al.* 2015; Malik and Srivastava 2009) and homology of chromosomes were assigned by centromeric positions and length similarities (Gomez-Rodriguez *et al.* 2013).

Results

Chromosome characterization and analysis

Chromosome preparations were arranged on the basis of chromosome arm length and arm ratios by measuring long and sort arm of five randomly selected metaphases and taking the averages of these values. The arm ratios were calculated by dividing value of long arm to short arm. The eight pairs of chromosomes were arranged in decreasing length order (Fig. 2e and Table 1). The chromosomes formula for onion is $2n = 2x = 2\ m + 2\ m + 2\ m + 2\ m + 2\ m + 2st^{45s\ rdna} + 2\ m + 2\ sm^{45s\ rdna}$.

ND-FISH for sub-telomeric tandem repeat and repetitive DNA sequences

ND-FISH by using labelled probe Oligo-pAc074 representing a sub-telomeric tandem repeat sequence in different accessions of cultivated onion produced strong signal strength on almost all onion chromosomes (Fig. 3). The ND-FISH signals by Oligo-pAc074 (Fig. 3) are similar to denaturing FISH results obtained by telomeric satellite DNA repeats (accession no. X02573.1; Fig. 4). There were two termini where signals were absent on the short arm of chromosome 6 (red arrows, Fig. 2a, b, c, d). On one terminal region of chromosome 8, the signal was weak (green arrows, Fig. 2a, b), or under detection limit on the other homologue of chromosome 8 in different accessions of onion (white arrows, Fig. 2c, d). ND-FISH was also performed for identification of signals for 5S and 45S rDNA. Probes designed included Oligo-5S rDNA labeled red to tag 5S rDNA and Oligo-18S rDNA labeled green to tag 45S rDNA as indicated in Fig. 3a, b. The difference for the results of ND-FISH and simple FISH was chromosome were more precise and in good shape in ND-FISH

Table 1: Chromosome analysis of *Allium cepa* hybridize with sub-telomeric satellite DNA, 5S rDNA and 45S rDNA

Chromosome number	Mean chromosome length (μm)			Arm ratio	Chromosome type	FISH results			
	Short arm	Long arm	Total length			5S rDNA	45S rDNA	Telomeric repeats	
								Short arm	Long arm
1	7.60	10.64	18.24	1.40	m	-	-	+	+
2	6.45	10.42	16.87	1.62	m	-	-	+	+
3	5.63	9.16	14.79	1.63	m	-	-	+	+
4	6.35	8.24	14.59	1.30	m	-	-	+	+
5	5.69	7.44	13.13	1.31	m	-	-	+	+
6	2.40	10.5	12.90	4.38	st	-	S	-	+
7	5.07	6.59	11.66	1.30	m	S	-	+	+
8	3.94	7.15	11.09	1.82	sm	-	S	-,+	+

m= metacentric; st= subtelomeric; sm= submetacentric; S= site for 5S rDNA and 45S rDNA on short arm of chromosome. += present; -= absent

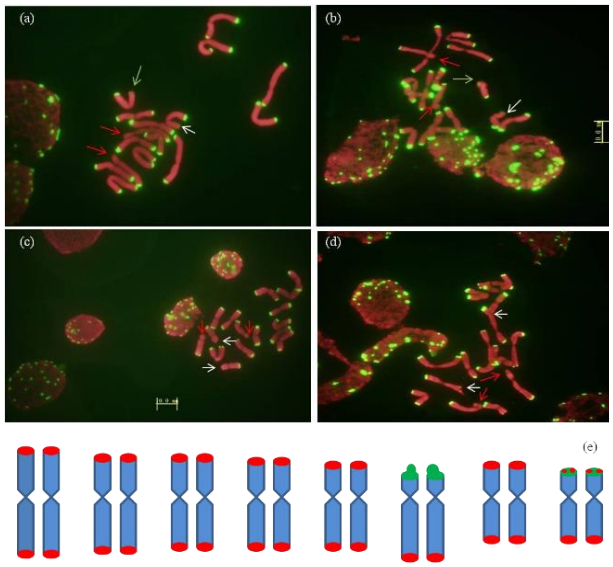

Fig. 2: Non-denaturing Fluorescence *in situ* hybridization for sub-telomeric repeat DNA (Oligo-pAc074) with the labelled with5'FAM (green signals). Red arrows indicated for absence of signals on terminal part of chromosome, white arrows indicated the termini with week signals (**a-d**). **a**) metaphase of MKS-57165, **b**) metaphase and prophase of Saryab Red, **c**) metaphase and interphase of Super Sarhad, **d**) metaphase and prophase of Phulkara. **e**) Ideogram of *A. cepa* chromosome in descending order

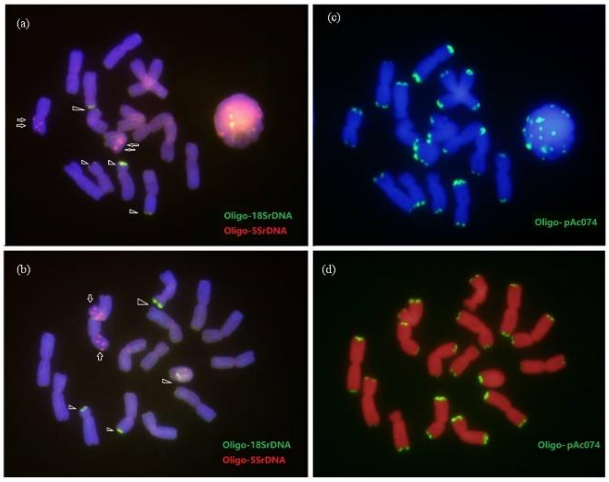

Fig. 3: Non-denaturing Fluorescence *in situ* hybridization for sub-telomeric repeat DNA with the Oligo-pAc074 probe (**c**, **d**) that is labelled with5'FAM (green signals). Oligo-5S rDNA labeled red (indicated white arrows) and Oligo-18S rDNA labeled green (indicated by white arrow heads) in Fig-3a, b. Chromosome spread of MKS-57165 with 5s and 45S rDNA signals (**a**) and Oligo-pAc074 (**c**). Chromosome spread of Saryab Red with 5s and 45S rDNA signals (**b**) and Oligo-pAc074 (**d**). Counter staining was performed by Propidium iodide (red)

hybridization (Fig. 2 and Fig. 3) compared to the results of simple FISH in which hybridizations were taken place after chromosome denaturation (Fig. 4).

FISH for telomeric satellite DNA Repeats and 45S rDNA

In situ hybridization with probes for the sub-telomeric satellite DNA repeat and 45S rDNA (Fig. 4) show sub-terminal hybridization of the satellite (red signals) on all chromosomes except for the short arm of chromosome 6 where 45S rDNA (green) is hybridized. On the short arm of chromosome 8, there were strong signals of 45S rDNA (white arrow, Fig. 4a, b, c, d) on one chromosome, while the other homologue of chromosome 8 hybridized with satellite (green arrows, Fig. 4a, b); thus co localization of 45S rDNA and satellite DNA was observed (white arrow, Fig. 4d, e and green arrow, Fig. 4d; also seen in pro-metaphase, green arrow Fig. 4c; and interphase, green arrow Fig. 4d). The 28 signals for the telomeric satellite DNA repeats are also seen in interphase nuclei (Fig. 4c, d). Hybridization of satellite DNA showed almost the same pattern of hybridization sites as indicated in sub-telomeric tandem repeats above (Fig. 2), and showed that the chromosomal locations in chromosomes 6 and 8 which are not hybridized either with telomeric repeats or satellite DNA repeats hybridized with 45S rDNA. It was found that signals for 45S rDNA and sub-telomeric repeats with ND-FISH (Fig. 3) were similar to the signals observed by simple or denaturing FISH (Fig. 4).

Discussion

Using an *in situ* hybridization protocol without a step for denaturing DNA, termed non-denaturing or ND-FISH,

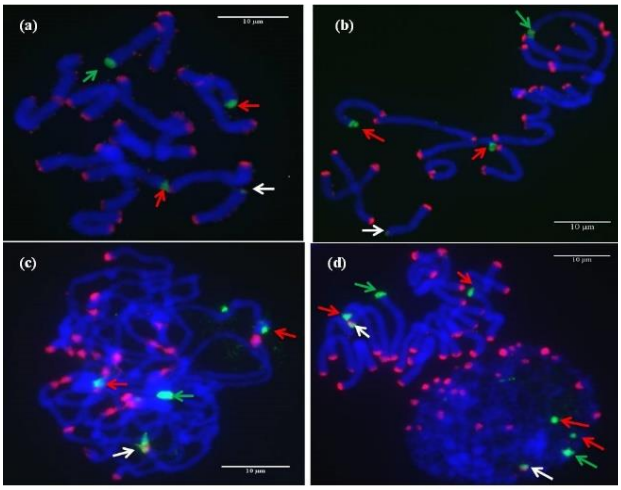

Fig. 4: Dual colour FISH for telomeric satellite DNA and 45S rDNA. Telomeric satellite DNA labeled with biotin-11-dUTP (red signals) and 45S rDNA are labelled with digoxigenin-16-dUTP (green signals). Counter staining was performed by DAPI. **a**) metaphase chromosomes of Saryab Red **b**) metaphase of MKS-57165, **c**) prophase of Super Sarhad, **d**) metaphase and prophase of Phulkara

a comparative study of sub-telomeric tandem repeats (Oligo-pAc074) a sub-telomeric satellite DNA clone, and the 45S rDNA sequence as probes showed their locations at the sub-terminal regions (seen in micrographs Fig. 1–4) of chromosomes of accessions of onion. Surprisingly similar pictures were obtained with and without the chromosomal denaturation step, indicating that other steps in the protocol were making the DNA accessible to the probes, and/or the probes were displacing sequences on chromosomes. The chromosome analysis and karyotype formula for onion (variety Eumjinara) are similar to those in variety Sinseonhwang (Mancia *et al.* 2015). Nevertheless, one significant polymorphism was found here (Fig. 3), perhaps surprising in commercial seed where inbreeding would be expected. Polymorphism for microsatellite was also observed in *A genome* (*Triticum dicoccoides*) of wheat by Adonina *et al.* (2015).

As for as results of the sub-telomeric satellite DNA and its association with 45S rDNA is concerned, in all the studied metaphases and interphases of *A. cepa* chromosomes it was observed that ends of short arm of chromosome 6 (NOR chromosome) did not show signals for satellite DNA (Fig. 4, with red arrows). A similar observation was found in case of sub-telomeric tandem repeats (Fig. 1). However, these ends of short arms hybridized and showed strong signals for 45S rDNA as indicated with red arrows (Fig. 3a, b and Fig. 4).

As from the studies of Peška *et al.* (2019), there was similarity between the sequences of Oligo-pAc074 and the sub-telomeric satellite DNA repeats with some deletions, a few insertions and having differences in some single nucleotide positions. The present study revealed that there are no sub-telomeric tandem or satellite repeats on the distal end of short arm of chromosome 6, supporting the results of Pich and Schubert (1998) and Do *et al.* (2001) by using GC rich telomeric satellite DNA repeats and tandem repeat sequences. They found signals at all termini except for nuclear organizing (NOR) chromosome 6, whose short arm did not hybridize with the telomeric satellite. They also concluded that this sub-telomeric satellite also intermixed with 45S rDNA. In the present study very weak signals of sub-telomeric satellite DNA were observed on terminal of the short arm of one homologue of chromosome 8 (Fig. 2, white and green arrows), however, in interphase these signals are comparatively prominent (Fig. 4d), most likely due to the elongation of the chromosomes and separation of arrays of rDNA from the satellite. Similar results were observed for the telomeric sequence in potato by Torres *et al.* (2011). Weak signals are attributed to the low copy number of available sequences that might be beyond the detection limit of routinely used FISH techniques, although can also be due to lower homology of the probe to a high-copy target. Torres *et al.* (2011) also found a great variability for copy number of these telomeric repeats at different ends of chromosomes in potato. Fajkus *et al.* (2005) demonstrated that all termini in *Allium* are not same and there is a variable distribution of rDNA sequences at these positions. Barnes *et al.* (1985) identified this abundant sub-telomeric satellite DNA sequence (375 bp) in cultivated onion and found similar results with 30 termini (ends of 15 chromosomes) hybridizing with this satellite DNA. Mancia *et al.* (2015) studied dual colour FISH and observed that all 30 termini were hybridized with sub-telomeric satellite repeats with very low copy number on chromosome 8, except for 2 termini of short arm of NOR-Chromosome (chromosome 6) that hybridized with 45S rDNA. All these studies verified our results for tandem repeats or sub-telomeric satellite DNA and 45S rDNA using ND-(FISH) methodology. Li *et al.* (2012) studied the linkage of telomeres and rDNA with chromosome in *Chrysanthemum segetum* L. by double-target FISH. It was found that sub-telomeric repeats are interspersed with 45S rDNA sequences. Lakshmanan *et al.* (2015) studied karyotypes of members of Araceae and found that 45S rDNA was located near intercalary position of short arm of chromosome. Pich *et al.* (1996) concluded that instead of protecting the integrity of DNA these rDNA loci developed 'bridges' between both the chromosome of nuclear organization region (sometimes with the satellite distant from the remaining chromosome, so it is wrongly considered a supernumerary or B-chromosome) and can produce recombination for terminal sequences. Dvořáčková *et al.* (2015) suggested that both telomeres and rDNA are a source to maintain the integrity of genome and its stability, although it is now clear that the stability of chromosome termini in *Allium* comes from enzymatic addition of a modified telomeric oligomer (Fajkus *et al.* 2016). Detailed

investigation of sub-telomeric tandem repeats, telomeric satellite DNA coupled with 45S rDNA is useful to understand the complexity and organization of the large onion genome, and the maintenance of genome integrity.

ND-FISH is a fast, simple and efficient technique as it reduces hybridization time up to one hour and also potentially maintains chromosome morphology by omitting the denaturation step (Fu *et al.* 2015; Zhu *et al.* 2017) and variation in the time and temperature of denaturation that may need to be optimized, often depending on length and conditions of storage of fixations and preparations. ND-FISH for physical location of repetitive sequences was relatively successful on cytogenetic investigation among wheat and related species (Tang *et al.* 2016; Lang *et al.* 2019). Lang *et al.* (2019) also mentioned in their studies that the results that were unobtainable by simple FISH probes were obtained by ND-FISH by quoting the results of Oligo-3A1 signals on 3A chromosome of wheat. After successful comparison between FISH and ND-FISH in current study it was found that the ND-FISH results for Oligo-5S rDNA, Oligo-18S rDNA (Fig. 3a, b) and Oligo-pAc074 (Fig. 2, Fig. 3c, d) were similar to that of FISH patterns of telomeric satellite DNA probe and 45S rDNA (Fig. 4), suggesting that ND-FISH methods coupled with oligonucleotides can replace the use of cloned or amplified repetitive sequence probes for FISH in onion chromosome identification. Oligonucleotide probes may be developed for high-throughput and precise chromosome identification and evolutionary studies (Jiang 2019), but require large tracts of chromosomal DNA sequence that are not available in onion. ND-FISH is convenient and reliable, with the fluorochrome labeled oligonucleotides being extremely convenient and relatively cheap to purchase (Fu *et al.* 2015) compared to labelled nucleotides, labelling enzymes and probe purification systems. PCR labelling is less reliable, although in combination with oligonucleotide probes in ND-FISH is clearly beneficial for plant cytogenetics, providing new information about some less explored repeats from the species of interest with huge genomes.

Conclusion

ND-FISH results, omitting a denaturation step for chromosomes before hybridizations, for Oligo-pAc074 (Fig. 2), Oligo-5S rDNA and Oligo-18S rDNA (Fig. 3a, b) were similar to that of FISH patterns following denaturation, showing the sub-telomeric satellite DNA and NOR (45S rDNA) locations (Fig. 4), suggesting that ND-FISH methods by oligonucleotides can be used for FISH in onion chromosome identification. The fast and low-cost ND-FISH for physical location of repetitive sequences was successful for cytogenetic investigations among wheat and related species (Fu *et al*. 2015; Tang *et al*. 2016; Lang *et al*. 2019). As in many other species (Galasso *et al*. 1995; Osuji *et al*. 1998), even in recent years with availability of high-throughput DNA sequences, particularly in work with species like *Allium* with larger genomes, *in situ* hybridization enables chromosome identification, including discovery of heteromorphisms (Fig. 4) and evolutionary rearrangements between species of potential value in making wide hybrids for introduction of novel genetic variation from wild species into cultivated onion. It is likely that the fast, simple ND-FISH with oligonucleotide probes can be effectively used for *Allium* germplasm identification in larger collections and populations.

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Author Contributions

J. S. (Pat) Heslop-Harrison, Zujun Yang, M. Kausar Nawaz and Nadeem Khan design the study. Rafiq Ahmad and Guangrong Li performed the experiments. J. S. (Pat) Heslop-Harrison, Rafiq Ahmad and Nadeem Khan analyzed the data. Rafiq Ahmad, Mahmood Ul Hassan, Sadia Saeed and Danish Ibrar wrote the manuscript. All authors have read and approved the manuscript.

References

Adonina IG, NP Goncharov, ED Badaeva, EM Sergeeva, NV Petras, EA Salina (2015). $(GAA)_n$ microsatellite as an indicator of the A genome reorganization during wheat evolution and domestication. *Compar Cytogenet* 9:533–547

Barnes SR, AM James, G Jamieson (1985). The organisation, nucleotide sequence, and chromosomal distribution of a satellite DNA from *Allium cepa*. *Chromosoma* 92:185–192

Chang KD, SA Fang, FC Chang, MC Chung (2010). Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant *Oryza* species. *Genomics* 96:181–190

Cuadrado Á, H Golczyk, N Jouve (2009). A novel, simple and rapid nondenaturing FISH (ND-FISH) technique for the detection of plant telomeres. Potential used and possible target structures detected. *Chromosome Res* 17:755–762

Do GS, BB Seo, M Yamamoto, G Suzuki, Y Mukai (2001). Identification and chromosomal location of tandemly repeated DNA sequences in *Allium cepa*. *Genes Genet Syst* 76:53–60

Doyle JJ, JL Doyle (1990). Isolation of plant DNA from fresh tissue. *Focus* 12:39–40

Dvořáčková M, M Fojtová, J Fajkus (2015). Chromatin dynamics of plant telomeres and ribosomal genes. *Plant J* 83:18–37

Fajkus J, E Sýkorová, AR Leitch (2005). Telomeres in evolution and evolution of telomeres. *Chromosome Res* 13:469–479

Fajkus P, V Peška, Z Sitová, J Fulnečková, M Dvořáčková, R Gogela, E Sýkorová, J Hapala, J Fajkus (2016). *Allium* telomeres unmasked: The unusual telomeric sequence (CTCGGTTATGGG) n is synthesized by telomerase. *Plant J* 85:337–347

Fu S, L Chen, Y Wang, M Li, Z Yang, L Qiu, B Yan, Z Ren, Z Tang (2015). Oligonucleotide probes for ND-FISH analysis to identify rye and wheat chromosomes. *Sci Rep* 5:1–7

Fuchs J, A Brandes, I Schubert (1995). Telomere sequence localization and karyotype evolution in higher plants. *Plant Syst Evol* 196:227–241

Galasso I, T Schmidt, D Pignone, JS Heslop-Harrison (1995). The molecular cytogenetics of *Vigna unguiculata* (L.) Walp: The physical organization and characterization of 18s-5.8s-25s rRNA genes, 5s rRNA genes, telomere-like sequences, and a family of centromeric repetitive DNA sequences. *Theor Appl Genet* 91:928–935

Gomez-Rodriguez VM, B Rodriguez-Garay, G Palomino, J Martínez, R Barba-Gonzalez (2013). Physical mapping of 5S and 18S ribosomal DNA in three species of Agave (Asparagales, Asparagaceae). *Compar Cytogenet* 7:191–203

Hizume M, F Shibata, Y Matsusaki, Z Garajova (2002). Chromosome identification and comparative karyotypic analyses of four *Pinus* species. *Theor Appl Genet* 105:491–497

Jiang J (2019). Fluorescence *in situ* hybridization in plants: Recent developments and future applications. *Chromosome Res* 9:153–165

Katsiotis A, M Loukas, JS Heslop-Harrison (2000). Repetitive DNA, genome and species relationships in *Avena* and *Arrhenatherum* (Poaceae). *Ann Bot* 86:1135–1142

Kilian AN, C Stiff, A Kleinhofs (1995). Barley telomeres shorten during differentiation but grow in callus culture. *Proc Natl Acad Sci* 92:9555–9559

Kubis S, JS Heslop-Harrison, T Schmidt (1997). A family of differentially amplified repetitive DNA sequences in the genus *Beta* reveals genetic variation in *Beta vulgaris* subspecies and cultivars. *J Mol* 44:310–320

Lakshmanan PS, KV Laere, T Eeckhaut, JV Huylenbroeck, EV Bockstaele, L Khrustaleva (2015). Karyotype analysis and visualization of 45S rRNA genes using fluorescence *in situ* hybridization in aroids (Araceae). *Compar Cytogenet* 9:145–160

Lang T, G Li, H Wang, Z Yu, Q Chen, E Yang, S Fu, Z Tang, Z Yang (2019). Physical location of tandem repeats in the wheat genome and application for chromosome identification. *Planta* 249:663–675

Levan A, K Fredga, AA Sandberg (1964). Nomenclature for centromeric position on chromosomes. *Hereditas* 52:201–220

Li J, S He, L Zhang, Y Hu, F Yang, L Ma, J Huang, L Li (2012). Telomere and 45S rDNA sequences are structurally linked on the chromosomes in *Chrysanthemum segetum* L. *Protoplasma* 249:207–215

Liu Q, X Li, X Zhou, M Li, F Zhang, T Schwarzacher, JS Heslop-Harrison (2019). The repetitive DNA landscape in Avena (*Poaceae*): Chromosome and genome evolution defined by major repeat classes in whole-genome sequence reads. *BMC Plant Boil* 19; Article 226

Malik A, A Srivastava (2009). Karyotypic analysis of different populations of *Carthamus tinctorius* Linnaeus (Asteraceae). *Compar Cytogenet* 3:51–55

Mancia FH, SH Sohn, YK Ahn, DS Kim, JS Kim, YS Kwon, CW Kim, TH Lee, YJ Hwang (2015). Distribution of various types of repetitive DNAs in *Allium cepa* L. based on dual color FISH. *Hortic Environ Biotechnol* 56:793–799

McClintock B (1941). The stability of broken ends of chromosomes in *Zea mays*. *Genetics* 26:234–282

Müller HJ (1938). The remaking of chromosomes. *Collect Net* 13:181–198

Nascimento EFD, BVD Santos, LO Marques, PM Guimarães, AC Brasileiro, SC Leal-Bertioli, DJ Bertioli, AC Araujo (2018). The genome structure of *Arachis hypogaea* (Linnaeus, 1753) and an induced *Arachis* allotetraploid revealed by molecular cytogenetics. *Compar Cytogenet* 12:111–140

O'Connor C (2008). Telomeres of human chromosomes. *Nat Educ* 1:166

Osuji JO, J Crouch, G Harrison, JS Heslop-Harrison (1998). Molecular cytogenetics of Musa species, cultivars and hybrids: Location of 18S-5.8 S-25S and 5S rDNA and telomere-like sequences. *Ann Bot* 82:243–248

Peška V, T Mandáková, V Ihradská, J Fajkus (2019). Comparative Dissection of Three Giant Genomes: *Allium cepa*, *Allium sativum*, and *Allium ursinum*. *Intl J Mol* 20:733–757

Pich U, I Schubert (1998). Terminal heterochromatin and alternative telometric sequences in *Allium cepa*. *Chromosome Res* 6:315–322

Pich U, J Fuchs, I Schubert (1996). How do Alliaceae stabilize their chromosome ends in the absence of TTTAGGG sequences? *Chromosome Res* 4:207–213

Richards EJ, FM Ausubel (1988). Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* 53:127–136

Riha K, J Fajkus, J Siroky, B Vyskot (1998). Developmental control of telomere lengths and telomerase activity in plants. *Plant Cell* 10:1691–1698

Schwarzacher T, JS Heslop-Harrison (1991). *In situ* hybridization to plant telomeres using synthetic oligomers. *Genome* 34:317–323

Schwarzacher T, P Heslop-Harrison (2000). Practical *in situ* hybridization. BIOS Scientific Publishers Ltd.

Siroky J, J Zluvova, K Riha, DE Shippen, B Vyskot (2003). Rearrangements of ribosomal DNA clusters in late generation telomerase-deficient *Arabidopsis*. *Chromosoma* 112:116–123

Tang S, L Qiu, Z Xiao, S Fu, Z Tang (2016). New Oligonucleotide Probes for ND-FISH Analysis to Identify Barley Chromosomes and to Investigate Polymorphisms of Wheat Chromosomes. *Genes* 7:118–117

Torres GA, Z Gong, M Iovene, CD Hirsch, CR Buell, GJ Bryan, P Novák, J Macas, J Jiang (2011). Organization and evolution of subtelomeric satellite repeats in the potato genome. *G3 Genes Genomics Genet* 1:85–92

Vershinin AV, T Schwarzacher, JS Heslop-Harrison (1995). The large-scale genomic organization of repetitive DNA families at the telomeres of rye chromosomes. *Plant Cell* 7:1823–1833

Watson, J Matthew, K Riha (2010). Comparative biology of telomeres: Where plants stand. *FEBS Lett* 584:3752–3759

Zhu M, P Du, L Zhuang, C Chu, C Zhao, Z Qi (2017). A simple and efficient non-denaturing FISH method for maize chromosome differentiation using single-strand oligonucleotide probes. *Genome* 60:657–664