GUILHERME TOMAZ BRAZ

CHROMOSOME IDENTIFICATION AND COMPARATIVE MOLECULAR CYTOGENETIC MAPPING BASED ON oligo-FISH IN MODEL PLANTS

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, área de concentração em Citogenética, para a obtenção do título de Doutor.

Profa. Dra Giovana Augusta Torres
Prof. Dr. Jiming Jiang
Orientadores

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CHROMOSOME IDENTIFICATION AND COMPARATIVE MOLECULAR CYTOGENETIC MAPPING BASED ON oligo-FISH IN MODEL PLANTS

INDENTIFICAÇÃO CROMOSSÔMICA E MAPEAMENTO CITOGENÉTICO MOLECULAR COMPARATIVO USANDO oligo-FISH EM PLANTAS MODELOS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, área de concentração em Citogenética, para a obtenção do título de Doutor.

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À Deus,

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RESUMO

Desde a descoberta dos cromossomos e a associação de seu comportamento com os “fatores” de Mendel, diferentes estratégias têm sido usadas para estudar a organização e a função dessa estrutura. Nesse sentido, o desenvolvimento de um sistema preciso de identificação de cromossomos é crucial para o sucesso da pesquisa citogenética. No início, dados morfológicos como comprimento cromossômico, posição do centrômero e da constrição secundária foram usados como marcas para distinguir os cromossomos uns dos outros. Métodos de bandeamento cromossômico representaram um grande avanço, mas um dos mais informativos, o bandeamento G, nunca forneceu resultados consistentes em plantas. Após o desenvolvimento da técnica de hibridização *in situ* fluorescente, foi produzida uma variedade de sondas que permitiram que os citogeneticistas criassem uma grande quantidade de marcas cromossômicas e identificassem os cromossomos homólogos. Recentemente, sondas de FISH baseadas em oligonucleotídeos demonstraram ser uma estratégia poderosa, barata e replicável para a identificação de cromossomos em citogenética de mamíferos e plantas. No presente estudo foi desenvolvido o mapeamento citogenético comparativo baseado em sondas de oligo-FISH. Basicamente, selecionamos regiões específicas de cromossomos de batata e milho para criar um sistema de “código de barras” que combina duas cores (verde e vermelho). Esta estratégia permitiu distinguir todos os cromossomos uns dos outros em batata, milho e em espécies relacionadas a ambos usando apenas uma preparação de FISH. Este mostrou ser um método poderoso para a identificação de cromossomos e estudos de evolução cariotípica. Em batata, cada um dos 12 cromossomos de espécies diploides e poliploides foi identificado com precisão, bem como de espécies de *Solanum* distantemente relacionadas, como tomate e berinjela. Duas translocações cromossômicas recíprocas foram identificadas em *Solanum etuberosum* e *S. caripense*, essas foram validadas utilizando a pintura cromossômica baseada em oligonucleotídeos. Em milho, identificamos cada um dos 10 cromossomos em preparações mitóticas e em paquíteno. As sondas baseadas em oligo foram utilizadas nas espécies de "teosinte", permitindo a identificação dos cromossomos homeólogos dessas espécies. Os cromossomos dessas espécies são semelhantes, exceto pela distribuição e tamanho dos knobs. Uma inversão paracêntrica homozigota foi identificada em *Zea luxurians*. A oligo-FISH mostrou ser um método poderoso para identificação de cromossomos e estudos de evolução cariotípica.

Palavras-chave: FISH. Oligonucleotídeo. Pintura cromossômica. Evolução cariotípica.

ABSTRACT

Since the discovery of chromosomes and the association of their behavior with Mendel's "factors", different strategies have been used to study their organization and function. In this way, the development of an accurate system of chromosome identification is crucial for the success in cytogenetic research. At the beginning, morphological data like chromosome length, centromere and secondary constriction position were used as marks for distinguishing chromosomes from each other. Chromosome banding methods like G-band were a great advance, but the most informative, the G-banding, never produced consistent results in plants. After the development of fluorescent *in situ* hybridization technique a variety of probes were developed which allowed cytogeneticists to create a vast amount of chromosome marks and identify homologous chromosomes. Recently developed, oligo-based FISH probes showed to be a powerful, cheap and repeatable strategy for chromosome identification in mammals and plants cytogenetics. Here we developed a comparative cytogenetics mapping based on oligo-FISH probes. Basically, we selected specifics regions of potato and corn chromosomes to create a "barcode" system combining two colors (green and red). This strategy allowed us to distinguish all individual chromosomes from each other of potato, corn and their relatives using only one round of FISH preparation. In potato each of 12 chromosomes from diploid and polyploid species were accurately identified, as well as from distantly related *Solanum* species like tomato and eggplant. Two reciprocal chromosomal translocations were identified in *Solanum etuberosum* and *S. caripense*, which were validated using oligo-based chromosome painting. In corn, we identified each of 10 chromosomes in mitotic and pachytene preparations. We used our oligo-based probes in "teosinte" species which allowed us to identify all homeologous chromosomes of these species. The chromosomes of those species are similar except by the knob distribution and size. A homolozygous paracentric inversion was identified in *Zea luxurians*. Oligo-based FISH showed to be a powerful method for chromosome identification and karyotype evolution studies.

Key words: FISH. Oligonucleotide. Chromosome Painting. Karyotype evolution.

SUMÁRIO

SECTION I – REVIEW

1 INTRODUCTION

Different strategies have been developed for chromosome identification in the course of cytogenetics history. Firstly, the utilization of morphological data and later the development of chromosome banding techniques gave cytogenetics tools to create chromosome marks and distinguish the chromosomes from each other. The combination of cytogenetics and molecular biology allowed the development of *in situ* hybridization technique and later fluorescent *in situ* hybridization. The variety of probes created increased the power of cytogeneticists to generate new chromosome marks, which gave them higher resolving power for distinguishing the homologous chromosomes.

FISH probes based on large-insert genomic DNA clones like BAC have been very useful for chromosome identification. Unfortunately, this methodology has a limitation for species with big and complex genome since intense cross-hybridization occurs due to the difficulty in blocking the repetitive sequences. Alternatively, a pool of probes based on repetitive DNA elements located in specific chromosome domains, as well as single copy gene probes, were used to generate FISH signals on individual chromosomes. Even though they are two powerful strategies, limitations associated with time-consuming, labor-intensive and difficulty to repeat in different laboratories restrict applicability of these methodologies.

The development of new strategies of genome analysis and DNA synthesis allowed cytogeneticists to design probes, bioinformatically, based on oligonucleotides from any entire chromosome or chromosome domain and use them as chromosome paint probes. This methodology was developed recently (BELIVEAU et al., 2012; BOYLE et al., 2011; YAMADA et al., 2011) and showed to be an efficient, repeatable and relatively cheap FISH variation method for chromosome identification and karyotype evolution studies. Using this methodology, chromosome-specific DNA markers were generated in different species like cucumber (*Cucumis sativus* L.) (HAN et al., 2015), woodland strawberry (*Fragraria vesca* L.) (QU et al., 2017), wheat (*Triticum aestivum* L.) (DU et al., 2017) and its relatives, rice (*Oryza sativa* L.) (HOU et al., 2018) and *Populus* species (*Populus tomentosa* C.K. Schneid. and *Populus deltoides* Marshall) (XIN et al., 2018).

However, none of these approaches focused on the simultaneous identification of all individual chromosomes, a challenge for karyotype studies. Using oligo-FISH based probes could accelerate the process and improve accuracy of chromosome identification in species

with published genome sequencing. In this way, we developed a “barcode system” based on two colors oligo-FISH signals distribution to distinguish all individual chromosomes of corn (*Zea mays* L. - 2n=2x=20; 2.3 Gb) and potato (*Solanum tuberosum* L. - 2n=2x=24; 670 Mb). The successful methodology allowed unambiguous chromosome identification of these two model plants, with different genome complexity and size, and also of their relatives, even distantly related, in only one round of FISH. The methodology showed to be a powerful method for chromosome tracking in cytogenetic and evolutionary studies.

2 REVIEW

2.1 Classic cytogenetics era

Cytogenetic studies have made landmark contributions to the knowledge in biology. The history of chromosome research begins with the discovery of a thread-like structure in the nuclei of plant cells by Karl Wilhelm von Nägeli in 1842 (SINGH, 2017). This structure was called “transitory cytoblasts” and later, in 1888, Waldeyer using staining techniques coined the term “chromosome”, chromos = Greek for colour; soma = Greek for body (SINGH, 2017). In 1900, Mendel’s law (Principles of Heredity) was rediscovered by DeVries, Tschermak and Correns and after the association of Mendel’s factors and chromosome behavior (BOVERI, 1904; SUTTON, 1903), cytologists were turned into cytogeneticists (FERGUSON-SMITH, 2015). The interest in decipher the structure and function of chromosomes in plants and animals quickly led to numerous extensions of genetic information and interpretation and after more than a century of the establishment of Sutton & Boveri theory, cytogenetics approaches still are essential to resolve questions in different areas in biology.

For the success in cytogenetics field, an accurate chromosome identification is crucial (JIANG; GILL, 2006). Barbara McClintock was one of the pioneers on chromosome research and have had a deep influence on cytogenetics development. A combination of chromosomal features like relative length and the positions of centromere, secondary constriction, and knob allowed her to distinguish homologous chromosomes in corn (MCCLINTOCK, 1929). Her method for individual chromosome identification permitted discoveries about structure and dynamic behavior of corn genome (CREIGHTON; MCCLINTOCK, 1931; MCCLINTOCK, 1930, 1938, 1941) and showed to be useful for cytogenetic map development in other plant species (FIGUEROA; BASS, 2010). Even though morphological data are still usable, in species with small and similar-sized chromosomes additional techniques were required for an accurate chromosome identification.

The development of chromosome banding techniques in the 1970’s decade opened new frontiers on classical cytogenetics research. These techniques emerged with the utilization of fluorescent dye quinacrine mustard to produce banding patterns, Q-bands, on plant (CASPERSSON et al., 1968) and human chromosomes (CASPERSSON; ZECH; JOHANSSON, 1970). Using this technique, several human chromosomes (3, 13-15, Y) were identified based on the distribution of fluorescent signals correlated with heterochromatic

domains. However, because the fluorescent staining quickly quenched, this technique was not optimal for routine studies. Alternatively, other chromosome banding techniques were developed like C-, NOR- and G-banding, CMA (chromomycin A3) and DAPI (4´,6-diamidino-2-phenylindole) staining, each one with specific properties producing differential banding pattern along metaphase chromosomes, being usable for chromosome identification.

C-banding (PARDUE; GALL, 1970; VOSA; MARCHI, 1972) have been used intensively in plant cytogenetics research since 1970's to distinguish individual chromosomes and karyotyping. This technique produces intensive stained chromosome domains related with constitutive heterochromatin (A-T or G-C rich) after a series of chemical treatment steps (HCl, barium hydroxide) and Giemsa staining. The resolving power of this technique is not enough to distinguish homologous chromosomes accurately, requiring a combination with others techniques to create extra chromosome marks. NOR-banding is a procedure that stains the nucleolar organizing region of chromosomes (MATSUI; SASAKI, 1973). These chromosome domains are known as ribosomal RNA genes regions and their differentiated staining can be used to study their dynamics along cell cycle and as chromosome mark as well.

G-banding was the most valuable band technique created for chromosome routine analyze due to its stability, sensitivity and simplicity (YUNIS; SANCHEZ, 1973). G-band is useful for human clinical cytogenetics and comparative cytogenetic mapping. Using this method, cytogeneticist can produce a pattern of bands that allow them to easily distinguish chromosomes and detect numerical and structural aberrations (translocations, inversions, deletions and duplications). Unfortunately, this methodology does not produce a band pattern in plant chromosomes being useless for plant cytogenetic research.

CMA and DAPI are two fluorochrome with greatest affinity for GC- and AT-rich sequences, respectively. Using the combination of them, cytogeneticists can identify different types of heterochromatin as GC-rich (DAPI − /CMA), AT-rich (DAPI / CMA −) or AT/GC-neutral (DAPI 0 /CMA 0). This technique produces a pattern of bands that can be used to identify some chromosomes (SUMMER, 2003).

Classical cytogenetics methods showed to be valuable for chromosome identification and characterization, but the combination of cytogenetics and molecular biology increased significantly the power of cytogeneticists to identify chromosomes.

2.2 Molecular Cytogenetics era - Fluorescent in situ Hybridization (FISH)

The molecular cytogenetics era opened up the opportunities for analyze any species regardless of its chromosome morphology (FIGUEROA; BASS, 2010). Using different kinds of probes, cytogeneticists have been creating a variety of chromosomes specific marks that improved the power to distinguish homologous chromosomes. This new era of cytogenetics emerged with the development of *in situ* hybridization (ISH) technique (GALL; PARDUE, 1969; PARDUE; GALL, 1970) that provided the opportunity to detect DNA sequences in cytological preparation using isotopic probes. Because its resolution limitation, radioactive nature and long exposure time for detection, the utilization of this kind of probe was limited (JIANG; GILL, 1994). As an alternative, radioactive labeling was replaced by fluorescent molecules (LANGER-SAFER; LEVINE; WARD, 1982).

Fluorescent *in situ* hybridization (FISH) allowed significant advances in resolution, speed and safety. These technical advantages associated to applications in structural, comparative and functional genomics increased the popularity of cytogenetics. The chromosome identification based on FISH is a versatile methodology since a variety of probes can be used. In human and animal cytogenetics entire individual chromosomes can be isolated by flow-sorting or microdissection technique and used as probe for chromosome painting. This is a powerful methodology for karyotype evolution studies in mammal's species. In plants, due to the inefficiency in blocking dispersed repetitive sequences, cross-hybridizations are observed, and unfortunately, this methodology is not applicable (FUCHS et al., 1996).

As an alternative, Lysak et al. (2001) painted the entire chromosome 4 of *Arabidopsis thaliana* using bacterial artificial chromosome (BAC) during mitotic and meiotic divisions as well as in interphasic nuclei. Basically, they selected pools of contiguous specific BACs clones spanning 2.6-13.3 Mb from the short and long arms of chromosome 4. These probes were used to paint chromosomes in related species, which showed to be a powerful method to study karyotype evolution in Brassicaceae species (LYSAK et al., 2005, 2006; MANDAKOVA et al., 2010). Because *A. thaliana* has very small (125 Mb) (The Arabidopsis Genome Initiative 2000) and largely euchromatic genome it was possible to select BACs with single or low copies sequences. Whereas in species with big and more complex genomes, the chromosome painting based on BAC-FISH is not usable because the probes tend to label all chromosomes nonspecifically.

Dong et al. (2000) also used fluorescence in situ hybridization (FISH) signals derived from bacterial artificial chromosomes (BACs). They screened a potato BAC library from

genetically mapped restriction fragment length polymorphism (RFLP) and identified a set of 12 chromosome-specific BAC clones. These clones were labeled as FISH probes that allowed them to identify all 12 chromosomes of potato. Using these cytological marks, they mapped the 5S rRNA genes, the 45S rRNA genes, and a potato late blight resistance gene to three specific potato chromosomes.

In corn, repetitive sequences (microsatellites, sub-telomeric, 5S rRNA, centromeric satellite 4, centromeric satellite C, knob, nucleolus-organizing region, tandemly repeated DNA sequence 1 (TR-1) and pMTY9ER telomere-associated) located at specific chromosomal regions were selected and used as multicolor fluorescence *in situ* hybridization probes (KATO; LAMB; BIRCHLER, 2004). This technique allowed the authors to identify all individual chromosomes and construct the karyotype of corn and its relatives. Although this is a powerful method, the pattern of FISH signals is variable even between lines from the same species (ALBERT et al., 2010), requiring the characterization of hybridization pattern of each line. As an alternative, single copy genes were used as FISH probes for chromosome identification of corn and its relatives (LAMB et al., 2007). These chromosome domains generally are more conserved, which allowed the authors to construct the karyotype without prior line characterization. Although usable, this technique is limited by the difficulty to detect single-copy DNA sequences on plant chromosomes due to debris from the cell wall and cytoplasm.

2.2.1 **Oligo-FISH:** the future of chromosome identification and comparative cytogenetic mapping

New FISH probes, based on thousands of oligonucleotides synthesized independently, have been successfully used to paint individual chromosomes in mammalian and *Drosophila* species (BELIVEAU et al., 2012; BOYLE et al., 2011; YAMADA et al., 2011). Using bioinformatics approach, the authors filtered out all repetitive DNA of the genome and selected oligonucleotides located in desired chromosomal region. This oligos were labeled and used as FISH probes for individual chromosome painting.

In plants, oligo-based FISH probes were first used by HAN *et al.*, 2015. They developed three probes containing 23000-27000 oligos each for paint specifics regions of three different chromosomes (1, 3 and 7) of cucumber (*Cucumis sativum* L., 2n = 2x = 24; 203 Mb). The authors hybridized the bulked oligo probes in related diploid (2n = 2x = 24) and polyploid (2n = 4x = 48; 2n = 6x = 72) species diverged from cucumber for up to 12 million

years, showing that this methodology is usable for phylogenetic studies. Furthermore, they tracked the homeologous chromosome pairing in early meiotic stage.

The power of this FISH variation technique cannot be estimated. Using a bioinformatic pipeline, cytogeneticists can identify any entire chromosome and/or chromosome domains for a variety of approaches. In woodland strawberry (*Fragaria fresca* L., 2n = 2x = 14; 240 Mb), the small-sized and high similar chromosomes turn their identification almost impossible using traditional methods. QU *et al.* (2017) used different mixes of oligo libraries painting probes in successive hybridizations to identify all seven chromosomes in a single cell of *F. fresca*. For the first time FISH-based molecular cytogenetic karyotype was constructed for this specie and according to the authors, the oligo-based probes will enable studying karyotype evolution among *Fragaria* and facilitate the application of breeding technologies as well.

Wheat (*Triticum aestivum* L., 2n = 6x = 42, genome AABBDD) alien chromosome introgression lines have been used as a strategy to increase genetic diversity in wheat breeding programs. Different classical (JIANG; FRIEBE; GILL, 1994) and molecular cytogenetic (MUKAI; NAKAHARA; YAMAMOTO, 1993) methods have been used to identify and track those alien chromosomes. However, a new methodology was necessary for a simple, fast, and efficient chromosome identification to accelerate the introgression process. DU *et al.* (2017) designed oligo multiplex probes that allowed them to identify and distinguish chromosomes of wheat and *Thinopyrum bessarabicum* (Savul. & Rayss) Á.Löve (2n = 2x = 14, genome JJ), an important genetic resource for wheat improvement. Using this technique, they quickly identified the chromosomes in wheat–*Th. bessarabicum* alien chromosome introgression lines. Moreover, they used this probe to reveal the genetic diversity among wheat cultivars.

Another approach was the development of oligo-based painting probes to follow accurately the pairing of chromosome 19 at pachytene stage of *Populus tomentosa* C.K. Schneid (2n = 2x = 38) and *Populus deltoids* Marshall (2n = 2x = 38) (XIN et al., 2018). These species are known as dioecious and the sex-determining locus was previously mapped to distal ends of chromosome 19. They observed unpainted distal ends of the two chromosomes 19, which means that the DNA sequences in this region have not been associated to the painting probe developed. Moreover, they observed that in 22–24% of the pachytene stage this region did not pair. According to the authors, the reduction in pairing frequency suggest a structural divergency of the two copies of the sexual chromosomes 19.

The pool of oligos showed to be a useful tool for chromosome painting in rice (*Oryza sativa* L., 2n = 2x = 24; 372 Mb) as well (HOU et al., 2018). Chromosome 9 specific probes

containing 25,000 oligos were developed based on the genome sequence of *japonica* rice (*Oryza sativa* L. subsp. *japonica*, 2n = 2x = 24). The authors easily identified this chromosome in *indica* rice (*Oryza sativa* L. subsp. *indica*, 2n = 2x = 24). A reciprocal translocation between chromosomes 9 and 11 and two new aneuploids associated with chromosome 9 were identified in a line from *indica* rice. Moreover, the chromosome 9 was identified in a wild rice (*O. eichingeri* Peter, 2n = 2x = 24), diverged approximately 5.7 million years ago from *O. sativa*. The pool of oligos showed to be a useful technique to study the structural and numerical variations in rice and in related species, even distant ones.

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SECTION II – SCIENTIFIC PAPERS

PAPER 1 - COMPARATIVE OLIGO-FISH MAPPING: AN EFFICIENT AND POWERFUL METHODOLOGY TO REVEAL KARYOTYPIC AND CHROMOSOMAL EVOLUTION

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GENETICS HIGHLIGHTED ARTICLE | INVESTIGATION

Comparative Oligo-FISH Mapping: An Efficient and Powerful Methodology To Reveal Karyotypic and Chromosomal Evolution

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ABSTRACT Developing the karyotype of a eukaryotic species relies on identification of individual chromosomes, which has been a major challenge for most nonmodel plant and animal species. We developed a novel chromosome identification system by selecting and labeling oligonucleotides (oligos) located in specific regions on every chromosome. We selected a set of 54,672 oligos (45 nt) based on single copy DNA sequences in the potato genome. These oligos generated 26 distinct FISH signals that can be used as a "bar code" or "banding pattern" to uniquely label each of the 12 chromosomes from both diploid and polyploid (4× and 6×) potato species. Remarkably, the same bar code can be used to identify the 12 homeologous chromosomes among distantly related *Solanum* species, including tomato and eggplant. Accurate karyotypes based on individually identified chromosomes were established in six *Solanum* species that have diverged for >15 MY. These six species have maintained a similar karyotype; however, modifications to the FISH signal bar code led to the discovery of two reciprocal chromosomal translocations in *Solanum etuberosum* and *S. caripense*. We also validated these translocations by oligo-based chromosome painting. We demonstrate that the oligo-based FISH techniques are powerful new tools for chromosome identification and karyotyping research, especially for nonmodel plant species.

KEYWORDS chromosome identification; karyotype; oligo-FISH; chromosome painting; translocation

THE karyotype of a eukaryotic species represents the number, size, and shape of all chromosomes in the nucleus. Karyotype has long been used as the most general description of the basic genetic makeup of individual eukaryotic species. In most lineages, closely related species share a similar karyotype. For example, gorilla (*Gorilla gorilla*) diverged from the human/chimpanzee (*Pan troglodytes*) lineages >10 MYA and human and chimpanzee have been separated by 7–8 MY (Langergraber *et al.* 2012). These three species, however, have maintained a similar karyotype, except that human chromosome 2 was fused from two different chromosomes, resulting in the reduction of chromosome number from $2n = 48$ in chimpanzee and gorilla to $2n = 46$ in humans (Jauch *et al.* 1992).

Karyotype analysis relies on the identification of individual chromosomes and has been a challenge for most nonmodel plant and animal species, especially those with polyploidy and/or those with a large number of small chromosomes. Chromosome banding and fluorescence *in situ* hybridization (FISH) were two milestone techniques in the history of chromosome identification and karyotype analysis. Unfortunately, only a few plant species with large chromosomes have benefited from the chromosome banding techniques (Friebe *et al.* 1996). G-banding, which is commonly used in

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karyotyping in mammalian species, does not generate bands on chromosomes from most plants (Greilhuber 1977; Anderson *et al.* 1982); while FISH can be universally applied in plant species (Schwarzacher *et al.* 1989; Lim *et al.* 2000; Mandakova *et al.* 2010; Szinay *et al.* 2012; Weiss-Schneeweiss and Schneeweiss 2013). Various types of DNA probes can be used in FISH, including repetitive DNA sequences (Mukai *et al.* 1993; Fransz *et al.* 1998; Kato *et al.* 2004) and large-insert genomic DNA clones (Jiang *et al.* 1995; Dong *et al.* 2000; Kulikova *et al.* 2001; Kim *et al.* 2002). However, it is often a major challenge to establish a FISH-based chromosome identification system in a nonmodel species because of the lack of chromosome-specific DNA probes. Although karyotypes have been described in many plant species, individual chromosomes were not identified in most of these reported karyotypes. Such karyotypes, therefore, are not comparable among related species and cannot be used for evolutionary studies.

The Solanaceae is an important plant family comprising >3000 species. One of the genus, *Solanum*, contains several major food crops, including potato, tomato, and eggplant. Solanaceae species were derived ~40 MYA from an ancestral diploid species with $2n = 24$ chromosomes. Nearly all diploid family members have maintained this chromosome number (Wu *et al.* 2006). However, this identical basic chromosome number does not indicate maintenance of genomic synteny of the 12 homeologous chromosomes among the solanaceous species. Although both potato and tomato genomes have been sequenced (The Potato Genome Sequencing Consortium 2011; The Tomato Genome Consortium 2012), the karyotypes, genomes, and their evolution in other solanaceous species are largely unknown.We developed a novel chromosome identification system using solanaceous species as a model. We selected a set of 54,672 oligonucleotides (oligos) from the single copy sequences associated with 26 specific chromosome regions in the potato genome. These oligos were massively synthesized *de novo* in parallel and were labeled as FISH probes (Han *et al.* 2015). The pooled oligos produced 26 distinct FISH signals, which can be used as a "bar code" or a "banding pattern" to identify all 12 potato chromosomes. Strikingly, this bar code has been maintained among distantly related *Solanum* species, including tomato and eggplant, which diverged from potato ~5–8 and 15 MYA, respectively (Y. Wang *et al.* 2008; Wu and Tanksley 2010; Sarkinen *et al.* 2013). Modifications to this bar code in different species can be inferred as potential rearrangements of the associated chromosome(s) during evolution. We demonstrate that the oligo-FISH-based techniques are powerful new tools for chromosome identification and karyotyping research in nonmodel species.

Materials and Methods

Plant materials

Seven diploid species were used in FISH mapping, including the doubled monoploid *Solanum tuberosum* Group Phureja clone DM1-3 516 R44 [doubled monoploid (DM)], *S. bulbocastanum* (PI 498223; Oaxaca, Mexico), tomato (*S. lycopersicum*) variety Micro Tom, *S. etuberosum* (E genome, PI 558306; O'Higgins, Chile), *S. melongena* (eggplant) (PI 665010, cultivar Black Beauty), *S. caripense* (PI 243342, Costa Rica), and pepper (*Capsicum annuum* var. annuum ACE F1). Tetraploid potato cultivar "Katahdin" and hexaploid species *S. demissum* (PI 225711; Boyaca, Colombia) were also used in FISH mapping.

Oligo-FISH probe design

The oligo probes were designed using Chorus software (https://github.com/forrestzhang/Chorus) with only minor modifications (Han *et al.* 2015). Briefly, the repetitive sequences in the potato genome (The Potato Genome Sequencing Consortium 2011; Hardigan *et al.* 2016) were filtered and remaining sequences were then divided into oligos (45 nt) in a step size of 5 nt. Each oligo was aligned to the potato genome to filter out those with duplicates in the genome (>75% similarity over all 45 nt). Oligos within the centromeric regions (Gong *et al.* 2012) were also excluded. Oligos with dTm >10 [dTm = melting temperature (Tm) − hairpin Tm] were kept to build a probe database. Oligo sequences that were homologous to the tomato genome were preferentially selected for chromosome painting probes. We adjusted the number of oligos across the chromosomes to ensure that the painting probes produce uniform signals on the entire chromosomes. For the bar code oligo probes, we first selected target regions with a relatively high density of oligos based on the density distribution profile on the entire chromosome. We then selected oligos that show >90% homology with tomato sequences. The oligos were synthesized by Arbor Biosciences (Ann Arbor, MI) were labeled following published protocols (Han *et al.* 2015).

Oligo-FISH

To prepare mitotic metaphase chromosomes, root tips were harvested from greenhouse-grown plants and treated with nitrous oxide at a pressure of 160 psi (~10.9 atm) for 20–50 min. The root tips were then fixed in fixative solution (3 ethanol:1 acetic acid) and kept at −20°. An enzymatic solution with 3% cellulase (Yakult Pharmaceutical, Tokyo, Japan), 1.5% pectinase (Plant Media), and 1% pectolyase (Sigma Chemical, St. Louis, MO) was used to digest the root tips for 50 min at 37°, and slides were prepared using a stirring method. Briefly, root tips were put on a microscope slide and macerated with a needle in 20 μl of 45% acetic acid. Then, the suspension was spread with a needle on a hot plate at 50° for 2 min. Chromosomes were fixed by adding 200 μl of ethanol:acetic acid (3:1) fixative solution on a hot plate at 50° for 10 sec. Afterward, an additional 200 μl of ethanol: acetic acid (3:1) fixative solution was dropped on the tilted slide, which was dried at room temperature. Slides were also prepared using the dropping method (Kato *et al.* 2004) for chromosome painting experiments.

FISH was performed following published protocols (Dong *et al.* 2000). The hybridization mixture (500 ng of each labeled probe of single-stranded DNA, 50% formamide, 10% dextran sulfate, 2× SSC) was applied directly to denaturated chromosome slides and incubated for 2 days at 37°. Approximately 2000 ng of sheared genomic DNA (with average size of 100 bp) prepared from *S. etuberosum* and *S. caripense* was used as blocking DNA in chromosome painting experiments. The hybridization mixture for chromosome painting was denatured at 95° for 8 min and incubated at 37° for 2 hr before being applied to denatured chromosome slides. Biotin- and digoxigenin-labeled probes were detected by anti-biotin fluorescein (Vector Laboratories, Burlingame, CA) and anti-digoxigenin rhodamine (Roche Diagnostics, Indianapolis, Indiana), respectively. Chromosomes were counterstained with DAPI in VectaShield antifade solution (Vector Laboratories). FISH images were captured using a QImaging Retiga EXi Fast 1394 CCD camera attached to an Olympus BX51 epifluorescence microscope. Images were processed with Meta Imaging Series 7.5 software. The final contrast of the images was processed using Adobe Photoshop CS3 software.

Karyotyping

The short (*S*) and long (*L*) arms of individual chromosomes were measured from 10 complete metaphase cells for each species using the computer application MicroMeasure version 3.3 (Reeves and Tear 2000). The chromosomal arm measurements were used to calculate the total length of each chromosome ($tl = S + L$), total length of entire set of chromosomes ($TL = \sum tl$), arm ratio ($AR = L/S$) of each chromosome, and relative length of each chromosome ($RL = tl/TL \times 100$).

Synteny analysis of potato and tomato DNA sequence

Potato genome (V404) (The Potato Genome Sequencing Consortium 2011) and tomato genome (SL3.0) (The Tomato Genome Consortium 2012) were aligned using MUMmer 3 -(Kurtz *et al.* 2004). The parameters used for mummer were "-mum -n -c -b -l 30" and the parameters used for gaps were "-l 60 -f .12 -s 1000." Synteny blocks between potato and tomato genome were identified using DAGchainer (Haas *et al.* 2004) with parameters "-o -0f -e-2f -A 10." The positions of potato and tomato centromeres were determined as the major peaks of CENH3 chromatin immunoprecipitation-sequencing reads for each chromosome. For chromosomes with unassembled centromeric/pericentromeric sequences, the centromere positions were determined by analyzing the distribution of centromeric repeats, transposable elements, and sequencing gaps in the chromosomes.

Data availability

Supplemental Material, Table S1 in File S1 contains all information about the number and locations of oligos associated with each of the 26 individual FISH signals generated by the two bar code FISH probes. The Chorus software used for oligo-FISH probe design is freely available (https://github.com/forrestzhang/Chorus).

Results

Development of oligo-based FISH probes for chromosome identification in Solanum species

We developed two oligo-FISH probes: PB9446 (green) and PB8495 (red). These two probes contain 27,306 and 27,366 oligos (45 nt), respectively, and are derived from 26 different regions on the 12 potato chromosomes (Table S1 in File S1). These two probes were designed to produce 26 distinct FISH signals, which can be used as a bar code or banding pattern to uniquely label each of the 12 potato chromosomes (Figure 1). Each chromosomal region is covered by 2000–2250 oligos (Table S1 in File S1) that were selected using our oligo-FISH probe development pipeline (Han *et al.* 2015). The oligos were selected from single copy sequences in the potato genome (The Potato Genome Sequencing Consortium 2011; Hardigan *et al.* 2016). The oligos associated with each of 26 FISH signals spanned a genomic region ranging from 184 to 707 kb (Table S1 in File S1). Some chromosomal arms contained two signals, which were separated by at least 7 Mb (Table S1 in File S1) to ensure the separation of the two signals on the same arm.

A total of 54,672 oligos were included in the two probes. Sequence analysis showed that 33,911 oligos (62%) are associated with annotated potato genes, including 16,489 with coding sequences, 13,354 with introns, and 4068 with 5′ and 3′ UTRs. The remaining oligos were derived from intergenic regions. We analyzed the sequence similarity of these potato oligos with the tomato genome sequence (The Tomato Genome Consortium 2012). Only 3023 oligos (11%) were identical to the corresponding tomato sequences. In addition, 19,033 oligos (35%) showed one to four mismatches (>90% homology) with the tomato sequences.

Chromosome identification in diploid and polyploid potato species

The two oligo-FISH probes were labeled and hybridized to the somatic metaphase chromosomes prepared from *S. tuberosum* Group Phureja clone DM1-3 516 R44 ($2n = 2x = 24$) (DM), which is a homozygous clone and has been fully sequenced (The Potato Genome Sequencing Consortium 2011). The green and red FISH signals derived from the two probes (Figure 2A) matched to the predicted patterns (Figure 1). The signals formed a bar code that uniquely labels the 12 chromosomes. Chromosome 2 is the only nucleolus organizer (Nor) chromosome in the potato genome (Dong *et al.* 2000). The 45S ribosomal RNA genes were located at the distal end of the short arm, which is distinctly decondensed and stained faintly by DAPI (Figure S1 in File S1). Karyotyping analysis revealed that most potato chromosomes are metacentric or submetacentric (except for chromosome 2) with an arm ratio ranging from 2.67 to 1.19 (Table 1). Chromosomes 1 and 2 (without

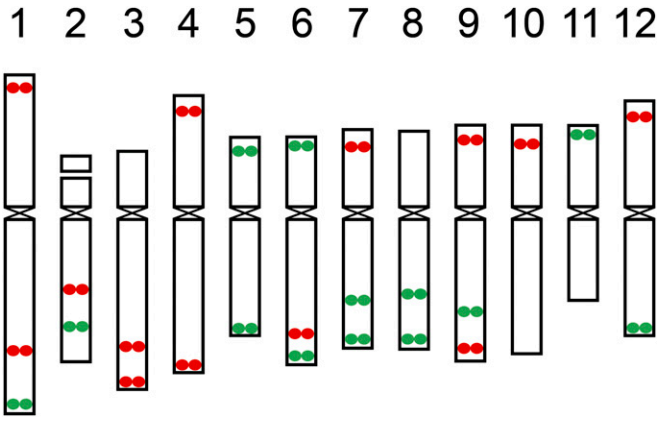

Figure 1 Predicted locations of the oligo-FISH signals on 12 potato chromosomes. Oligos were selected from a total of 26 chromosomal regions (13 red regions and 13 green regions). The 12 chromosomes can be distinguished from each other based on number and location of the red/green signals. The centromere positions on the 12 chromosomes in the potato reference genome were based on the locations of sequences associated with CENH3 nucleosomes (Gong *et al.* 2012).

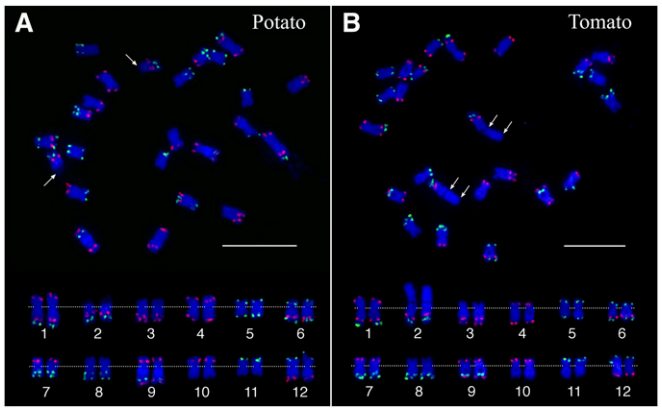

Figure 2 FISH mapping of potato and tomato chromosomes using two oligo-FISH probes. (A) FISH mapping of DM potato. Arrows point to the 45S rDNA regions associated with chromosome 2 (FISH mapping of the 45S rDNA on the same metaphase cell is shown in Figure S1 in File S1). The rDNA region is distinctly decondensed compared to the rest of the chromosome. (B) FISH mapping of tomato. The double arrows indicate the extent of the 45S rDNA regions (FISH mapping of the 45S rDNA on the same metaphase cell is shown in Figure S1 in File S1). The rDNA region is similarly condensed compared with the rest of the chromosome. The top panels show a complete metaphase cell from potato and tomato, respectively. Homologous chromosomes in the bottom panel were digitally excised from the same cells and paired. The centromeres of the chromosomes are aligned by a dotted line. Bar, 10 μm.

including the 45S rDNA region) represent the largest and smallest chromosomes, respectively (Table S2 in File S1).

We then performed FISH on cultivated potato (*S. tuberosum*, $2n = 4x = 48$), an autotetraploid species. We observed four identical copies of each of the 12 chromosomes from potato cultivar Katahdin (Figure 3A). *S. demissum* ($2n = 6x = 72$) was recognized as an allohexaploid species based on traditional chromosome pairing analyses of hybrids between *S. demissum* and various *Solanum* species (Matsubayashi 1991). The consensus conclusion from traditional cytogenetic studies was that *S. demissum* contains two similar genomes that differ from the third genome (Matsubayashi 1991). We identified 6 copies of each of the 12 potato chromosomes in *S. demissum* (Figure 3B). The FISH signal patterns from the six homologous/homeologous chromosomes were identical to those from DM potato. Interestingly, two of the six copies of chromosome 2 lack the 45S ribosomal gene arrays (Figure S2 in File S1).

Comparative karyotyping of potato and tomato

DNA sequence-based analysis suggested that tomato and potato have diverged for ~5–8 MY (Y. Wang *et al.* 2008; Sarkinen *et al.* 2013). Chromosome synteny between the potato and tomato has been well maintained based on comparative genetic linkage mapping and comparative cytogenetic mapping (Tanksley *et al.* 1992; Iovene *et al.* 2008; Tang *et al.* 2008; Gaiero *et al.* 2017). We conducted DNA sequence-based synteny analyses between the 12 pairs of pseudomolecules from potato and tomato genomes. Multiple inversions in different sizes were found to be associated with all 12 homeologous chromosome pairs (Figure S3 in File S1), which revealed abundant intrachromosomal rearrangements, but no interchromosomal arrangement, occurred during the divergence of these two species.

The two oligo-FISH probes generated an identical signal bar code on tomato and potato chromosomes (Figure 2). Two tomato chromosomes showed distinct morphology compared to the potato homeologues. The tomato 45S ribosomal RNA genes were also located at the distal region of the short arm of chromosome 2 (Figure 2B and Figure S1 in File S1). However, the 45S ribosomal DNA (rDNA) region was as condensed as the rest of the tomato chromosome 2 (Figure S1 in File S1), which was consistently observed in all metaphase cells. This unique condensation pattern of the 45S rDNA region makes chromosome 2 the longest chromosome in tomato (Figure 2B). Chromosome 4 from the two species showed a distinct difference in arm ratios. Potato chromosome 4 is a submetacentric chromosome with an arm ratio of 1.50; while tomato chromosome 4 appeared to be a subtelocentric (or acrocentric) chromosome with an arm ratio of 2.21 (Figure 2 and Table 1). At least two inversions in the long arms, each spanning several megabases of DNA, distinguished the two chromosomes (Figure S3 in File S1). By contrast, no inversion was detected in the short arms of the two chromosomes. It is not clear whether the different arm ratios of these two chromosomes were caused by an inversion that spanned the centromere of the chromosome in one species or by some other chromosomal rearrangement events.

Comparative karyotyping of Solanum species that are distantly related to potato

To reveal the karyotype evolution of the *Solanum* species, we performed comparative oligo-FISH in five additional species using the two probes developed in potato. These species have diverged variously from potato, including *S. bulbocastanum*

Table 1 Arm ratio of individual chromosomes in six *Solanum* species

Chromosome	*S. tuberosum* (potato)	*S. bulbocastanum*	*S. lycopersicum* (tomato)	*S. etuberosum*	*S. caripense* (tzimbalo)	*S. melongena* (eggplant)
1	1.80 ± 0.46	2.20 ± 0.38	1.57 ± 0.25	1.71 ± 0.63	2.56 ± 0.60	1.44 ± 0.27
2[a]	3.63 ± 0.61	3.94 ± 0.73	3.31 ± 1.37	2.89 ± 0.60	3.32 ± 1.13	2.58 ± 0.76
3	2.67 ± 0.49	2.29 ± 0.64	2.96 ± 0.49	1.76 ± 0.31	2.83 ± 0.68	1.37 ± 0.18
4	1.50 ± 0.22	1.64 ± 0.25	2.21 ± 0.37	1.21 ± 0.14	1.43 ± 0.43	1.46 ± 0.24
5	1.30 ± 0.13	1.32 ± 0.22	1.17 ± 0.12	1.25 ± 0.14	1.23 ± 0.24	1.35 ± 0.21
6	1.98 ± 0.29	1.78 ± 0.49	2.11 ± 0.34	1.63 ± 0.27	2.53 ± 0.64	1.63 ± 0.25
7	1.85 ± 0.32	1.75 ± 0.21	1.67 ± 0.32	1.27 ± 0.28	2.40 ± 0.69	1.20 ± 0.13
8	1.90 ± 0.25	2.04 ± 0.49	1.84 ± 0.38	2.37 ± 0.54	2.61 ± 0.55	1.14 ± 0.11
9	1.96 ± 0.29	1.55 ± 0.18	1.81 ± 0.29	1.26 ± 0.22	1.58 ± 0.48	1.47 ± 0.20
10	1.38 ± 0.18	1.50 ± 0.33	1.44 ± 0.20	1.51 ± 0.19	1.52 ± 0.32	1.48 ± 0.20
11	1.19 ± 0.15	1.16 ± 0.10	1.55 ± 0.17	1.17 ± 0.11	1.50 ± 0.34	1.22 ± 0.28
12	1.43 ± 0.27	1.28 ± 0.21	1.32 ± 0.21	1.53 ± 0.32	1.49 ± 0.65	1.12 ± 0.09

Measurement was conducted on each chromosomal arm in 10 metaphase cells.
[a] The 45S rDNA on the short arm of chromosome 2 was not included in the measurement.

(a wild species closely related to potato), *S. etuberosum*, *S. caripense* (tzimbalo), *S. melongena* (eggplant), and *C. annuum* (pepper), which are more distantly related to potato than tomato is to potato (Lou *et al.* 2010).

S. bulbocastanum: The FISH signals generated on *S. bulbocastanum* chromosomes were identical to those from potato (Figure 4). The arm ratio (Table 1) and relative length (Table S2 in File S1) of individual *S. bulbocastanum* chromosomes were also highly similar to the homeologous potato chromosomes.

S. etuberosum: *S. etuberosum* is a nontuberizing wild species that has been used in potato breeding due to its resistance to various potato diseases (Dong *et al.* 1999; Novy *et al.* 2002, 2007). Phylogenetically, *S. etuberosum* is more distantly related to potato than tomato is to potato (Lou *et al.* 2010). The FISH signals on most *S. etuberosum* chromosomes were identical to those on potato chromosomes. Chromosome 2 is the sole Nor chromosome (Figure S4 in File S1). However, signal modifications were observed on chromosomes 2 and 7. The long arm of chromosome 2 lost its distal green signal and gained an additional red signal ("b" in Figure 4). By contrast, the short arm of chromosome 7 lost its distal red signal but gained a green signal ("c" in Figure 4). A reciprocal translocation between chromosomes 2 and 7 would explain the observed FISH signal pattern changes (Figure 5A). The distal red signal on *S. etuberosum* chromosome 2 is more close to the end of the chromosome compared to the distal green signal on potato/tomato chromosome 2 (Figure 4). This can be explained by the fact that the green signal on chromosome 2 is 9.3 Mb away from the end, while red signal on chromosome 7 is only 4 Mb away from the end (Figure 5A). The other 10 *S. etuberosum* chromosomes showed a similar arm ratio and relative length to the homeologous potato chromosomes (Table 1 and Table S2 in File S1).

S. caripense: *S. caripense*, also known as tzimbalo, is an evergreen shrub native to South America and is grown for its edible fruit. The *S. caripense* chromosomes were visibly larger than potato chromosomes. Phylogenetically, *S. caripense* is more distantly related to potato than *S. etuberosum* is to potato (Lou *et al.* 2010). Overall *S. caripense* showed a similar karyotype as potato and tomato. However, we observed distinct FISH signal patterns on chromosomes 4 and 11, respectively. The red signal on the long arm of chromosome 4 was replaced by a green signal ("d" in Figure 4). On the other hand, the green signal on the short arm of chromosome 11 was replaced by a red signal ("e" in Figure 4). A reciprocal translocation between chromosomes 4 and 11 would explain this signal pattern change (Figure 5B). The rest of the *S. caripense* chromosomes showed a similar arm ratio and relative length to the homeologous potato chromosomes (Table 1 and Table S2 in File S1).

Eggplant (S. melongena): Eggplant diverged from a common ancestor of potato/tomato ~15.5 MYA (Wu and Tanksley 2010). The two oligo-FISH probes generated uniform but generally weak background signals on all eggplant chromosomes. Surprisingly, the patterns derived from the major FISH signals matched those from potato and tomato chromosomes (Figure 4). Eggplant chromosome 8 is a metacentric chromosome with an arm ratio of 1.14. However, chromosome 8 from the other five *Solanum* species have subtelocentric morphology with an arm ratio ranging from 1.84 to 2.61 (Figure 4 and Table 1). Since the two green signals on the long arm of chromosome 8 of *S. melongena* were clearly closer to the centromere than those on chromosome 8 of other *Solanum* species ("f" in Figure 4), chromosome 8 of *S. melongena* likely resulted from an inversion spanning the centromere, and a large fragment from the long arm was moved to the short arm due to the inversion. Similarly, a pericentric inversion is also likely involved in chromosome 10, which would explain the red signal at the distal region on the long arm ("g" in Figure 4), which is located on the short arms of chromosome 10 in other species (Figure 4).

Pepper (C. annuum): Pepper diverged from a common ancestor of potato/tomato ~19.6 MYA (Wu and Tanksley

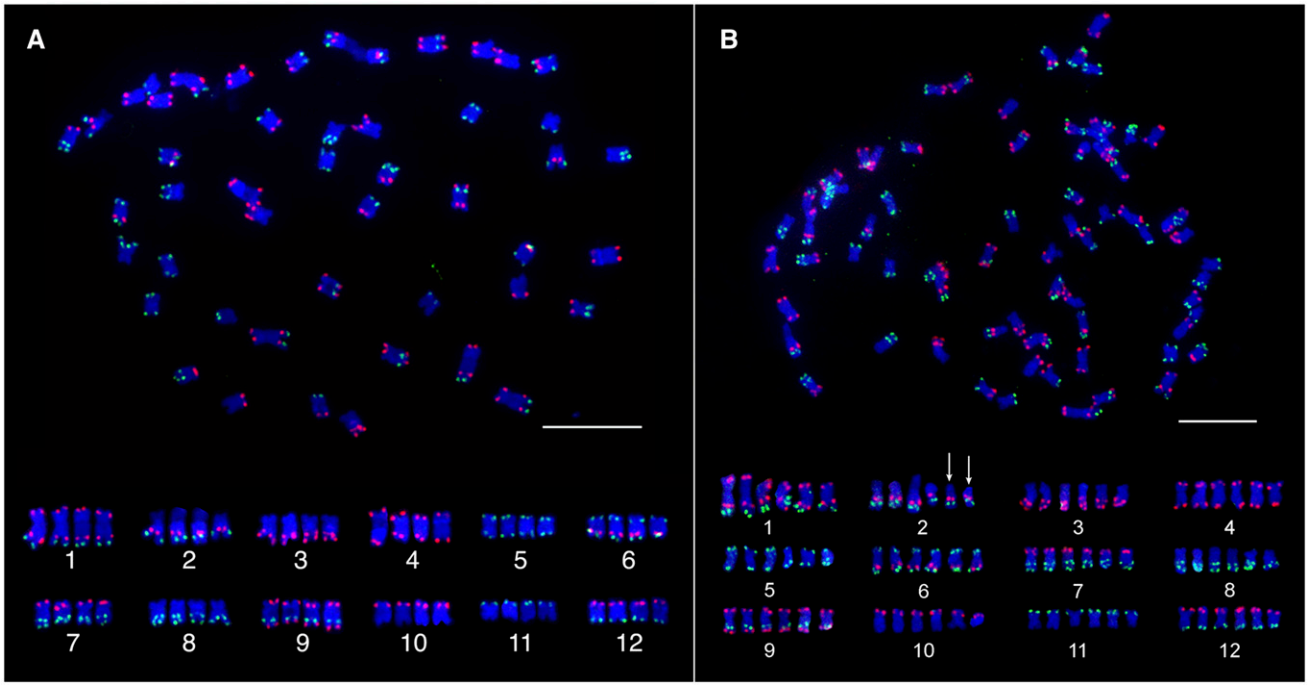

Figure 3 Chromosome identification in polyploid *Solanum* species. (A) Chromosome identification of potato cultivar Katahdin. The top panel shows a complete metaphase cell hybridized with two oligo-FISH probes. The bottom panel shows the 4 homologous chromosomes of each of the 12 potato chromosomes digitally excised from the same cell. (B) Chromosome identification in the hexaploid species *S. demissum*. The top panel shows a complete metaphase cell hybridized with two oligo-FISH probes. The bottom panel shows the 6 homologous chromosomes of each of the 12 potato chromosomes digitally excised from the same cell. The two arrows indicate the two copies of chromosome 2 that are not associated with 45S rDNA (FISH mapping of the 45S rDNA is showed in Figure S2 in File S1). Bar, 10 μm.

2010). The two oligo-FISH probes produced massive background signals on pepper chromosomes (Figure S5 in File S1). Punctuated major signals were observed on every chromosome. However, most of the pepper chromosomes cannot be unambiguously identified based on the signal patterns on potato chromosomes, suggesting that major structural arrangements have occurred between most potato and pepper chromosomes. The sizes of the pepper chromosomes appeared to be at least twice that of potato chromosomes. The current sequence assemblies estimate 3000 Mb for the pepper genome (https://www.ncbi.nlm.nih.gov/assembly, GCA_000512255.1), which is significantly larger than the potato genome (~800 Mb). These results suggest that the pepper genome has undergone major expansion and rearrangements during evolution.

Confirmation of interchromosomal translocation by oligo-based chromosome painting

We developed oligo-based chromosome painting probes to validate the interchromosomal translocations in *S. etuberosum* and *S. caripense*, which were predicted based on bar code FISH signal modifications relative to potato chromosomes (Figure 5). Oligos unique to a single potato chromosome were computationally identified and synthesized in parallel (Han *et al.* 2015). We selected 27,392 oligos for both potato chromosomes 2 and 7. The chromosome 7 probe generated uniform FISH signals on DM chromosome 7 (Figure 6A2). However, the chromosome 2 probe generated weak signals on the short arm and the proximal region on the long arm of DM chromosome 2 (Figure 6A3). These two probes, especially that for chromosome 2, generated very weak signals in the pericentromeric regions of chromosome 2 and 7 of *S. etuberosum* (Figure 6B3). This is likely caused by divergence of the DNA sequences located in the pericentromeric regions. Nevertheless, chromosome painting clearly showed that a small chromosome 7 segment was translocated to chromosome 2 (2^7). In contrast, a relatively large chromosome 2 segment was translocated to chromosome 7 (7^2) (Figure 6, B1 and B4). Thus, the chromosomal painting results matched to the predicted reciprocal translocation based on the modification to the bar code (Figure 5A).

Similarly, we developed painting probes for potato chromosomes 4 and 11, each containing 27,392 oligos. Both probes generated uniform FISH signals on DM chromosomes with only limited hybridization background (Figure 6, C2 and C3). The painting probes, however, produced unambiguous hybridization signals only at the distal ends of chromosomes 4 and 11 of *S. caripense* (Figure 6, D1 and D4). Only background-level FISH signals were detected in the pericentromeric regions of the homeologous chromosomes in *S. caripense* (Figure 6D3). Nevertheless, chromosome painting in *S. caripense* clearly revealed the reciprocal translocation between chromosome 4 and 11, resulting in chromosomes 4^{11} and 11^4, respectively (Figure 6, D1 and D4). The

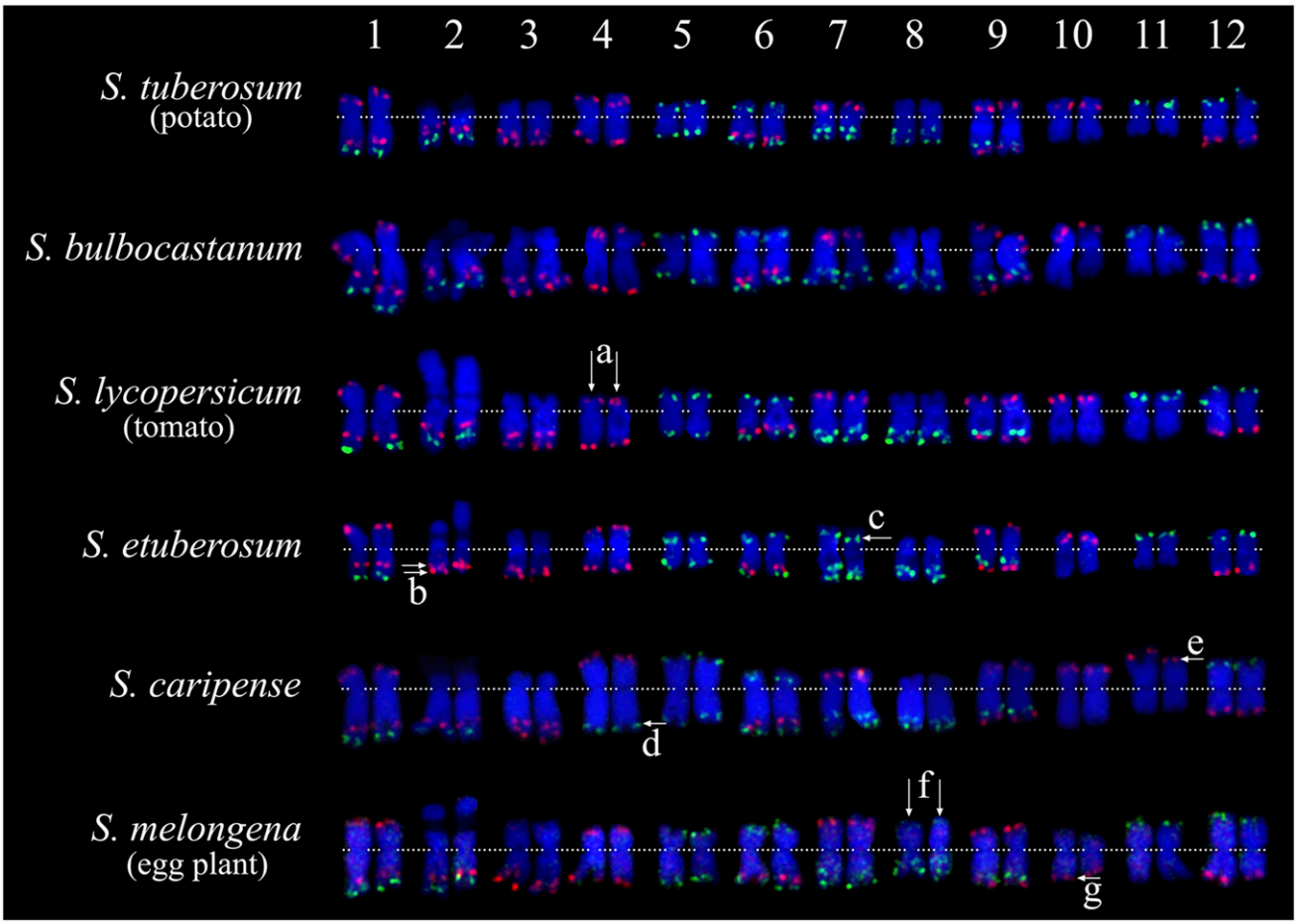

Figure 4 Comparative karyotyping of six diploid *Solanum* species. Chromosomes 1–12 from each species are arranged from left to right. Karyotypes of potato and tomato were developed from the same metaphase cells in Figure 2. Karyotypes of the remaining four species are developed from the same metaphase cells in Figure S4 in File S1. (a) Double arrows point to the two copies of tomato chromosome 4, which have a distinct arm ratio compared to chromosome 4 from other species. (b) Double arrows point to two closely linked red signals on *S. etuberosum* chromosome 2, the bottom red signal is predicted to be derived from the short arm of chromosome 7. For comparison, we used the karyotype of potato as our reference, see switches between red and green signals among these two species. (c) Arrow indicates the green signal on the short arm of *S. etuberosum* chromosome 7, which is predicted to be derived from the long arm of chromosome 2. (d) Arrow points to the green signal on the long arm of *S. caripense* chromosome 4, which is predicted to be derived from the short arm of chromosome 11. (e) Arrow points to the red signal on *S. caripense* chromosome 11, which is predicted to be derived from the long arm of chromosome 4. (f) Double arrows point to the two copies of eggplant chromosome 8, which have a distinctly large short arm compared to chromosome 8 from other species. (g) Arrow indicates the location of the red signal on the long arm of eggplant chromosome 10. This signal is located at the short arm of chromosome 10 from other species.

exchanged chromosomal segments from the two chromosomes showed a similar size (Figure 6D4). Thus, the chromosomal painting results in *S. caripense* also matched the predicted reciprocal translocation based on the modifications to the oligo-FISH bar code (Figure 5B).

Discussion

Oligo-FISH bar code: a new chromosome identification methodology

FISH is the most important technique for chromosome identification in plants (Jiang and Gill 1994, 2006). Repetitive DNA sequences were commonly used as probes in FISH-based chromosome identification (Mukai *et al.* 1993; Kato *et al.* 2004). However, it is often challenging to find a repeat that would produce distinct FISH signals on individual chromosomes in a plant species. More importantly, the FISH signals from repetitive DNA probes can potentially be highly polymorphic among different varieties and accessions, which may prevent consistent identification of individual chromosomes (Jiang and Gill 2006). Alternatively, large-insert genomic DNA clones, such as bacterial artificial chromosome (BAC) clones, can be used as FISH probes for chromosome identification (Jiang *et al.* 1995). However, this approach is dependent on the availability of a large-insert genomic DNA library as well as a major effort to isolate clones specific to every chromosome (Dong *et al.* 2000; Cheng *et al.* 2001; Kulikova *et al.* 2001; Kim *et al.* 2002; K. Wang *et al.* 2008). In addition, BACs from plant species with large and complex genomes often contain high proportions of repetitive DNA

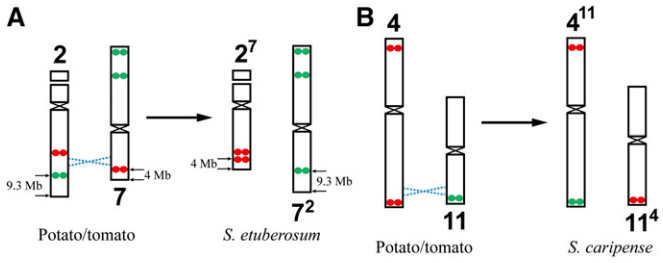

Figure 5 Predicted reciprocal chromosomal translocations identified in *Solanum* species. (A) A reciprocal translocation between chromosomes 2 and 7 in *S. etuberosum*. Chromosomes 2 and 7 from potato/tomato are hypothesized to be the ancestral types. A reciprocal translocation (dashed blue lines) is predicted based on the modifications to the oligo-FISH bar code, which result in the two translocation chromosomes 2^7 and 7^2, respectively, in *S. etuberosum*. (B) A reciprocal translocation between chromosomes 4 and 11 in *S. caripense*. The chromosomes 4 and 11 from potato/tomato are hypothesized to be the ancestral types. A reciprocal translocation (dashed blue lines) is predicted based on the modifications of the oligo-FISH bar code, which result in the two translocation chromosomes 4^{11} and 11^4, respectively, in *S. caripense*.

sequences and do not produce chromosome-specific FISH signals (Zhang *et al.* 2004; Janda *et al.* 2006).

We demonstrate that oligo-FISH bar codes provide a powerful and efficient technique for plant chromosome identification. It has several major advantages compared to the repeat- or BAC-based FISH probes: (1) Oligo-based FISH probes can be designed in any species with a sequenced genome, which has been demonstrated in several animal and plant species (Boyle *et al.* 2011; Yamada *et al.* 2011; Beliveau *et al.* 2012; Han *et al.* 2015). Thus, a single or few oligo pools can be designed to identify all chromosomes in a plant species with a sequenced genome. If the majority of oligos are associated with genic sequences, the same bar code can be expected from different varieties and accessions in the same species. (2) We demonstrate that a bar code probe can potentially be used to identify homeologous chromosomes among distantly related species, which allow for evolutionary studies. (3) Oligos can be selected from multiple regions from the same chromosome. Such a cocktail oligo probe will generate a unique hybridization pattern that resembles FISH signal patterns generated from multiple BACs derived from a single chromosome (Iovene *et al.* 2008; Szinay *et al.* 2008, 2012; Tang *et al.* 2008). An unlimited number of possible patterns can be designed for each chromosome. (4) Each oligo-based probe can be used for nearly 1,000,000 FISH experiments (Han *et al.* 2015). Thus, such bar code oligo-FISH probes are cost effective and can be maintained as a permanent resource.

The total number of FISH signals will be the most important factor in designing an oligo-FISH bar code. Oligos spanning 30–50 kb of single copy sequences can generate a strong FISH signal on metaphase chromosomes. However, it may be difficult to identify such long stretches of single copy sequences in some plant genomes. If multiple signals are designed on a single chromosome arm, the groups of oligos should be separated by a sufficient distance to ensure separate FISH signals. We demonstrate that 7 Mb is sufficient to consistently separate two FISH signals on potato metaphase chromosomes. However, a longer distance (>10 Mb) should be considered for plant species with chromosomes much larger than those of potato.

Chromosomal inversion and translocations in Solanum species

Chromosomal evolution of the solanaceous species has been investigated traditionally using pairwise comparative genetic linkage mapping (Wu and Tanksley 2010). Since genetic linkage maps and DNA markers were best developed in tomato (Tanksley *et al.* 1992), most of the pairwise mapping was performed between tomato and other solanaceous species, including potato, eggplant, pepper, and *Nicotiana* species (Bonierbale *et al.* 1988; Tanksley *et al.* 1992; Livingstone *et al.* 1999; Doganlar *et al.* 2002; Wu *et al.* 2009, 2010). Comparative FISH mapping has also been conducted among *Solanum* species using common sets of BACs isolated from potato or tomato (Iovene *et al.* 2008; Tang *et al.* 2008; Lou *et al.* 2010; Szinay *et al.* 2012; Gaiero *et al.* 2017). These comparative studies showed that inversions were the most common cause of chromosomal rearrangements among the solanaceous species. Translocations were also reported in some comparisons, for example, tomato and eggplant were found to differ by 24 inversions and 5 translocations based on eggplant linkage mapping using a set of 232 tomato-derived DNA markers (Wu *et al.* 2009).

The resolution of linkage mapping is restricted by the number of markers used. Genotyping or mapping errors, caused by wrong marker order or population size, may result in misidentified chromosomal rearrangements, such as inversion. In addition, population-based linkage mapping is an expensive and time-consuming approach; it has mostly been conducted in crops or economically important plant species. Although translocations were reported in some of the comparative mapping investigations among *Solanum* species, no cytological evidence was provided for any of the predicted translocations. For example, linkage mapping suggested that eggplant chromosome 5 is an equivalent of a fusion of the short arm of chromosome 5 with the long arm of chromosome 12 in tomato. Similarly, eggplant chromosome 11 is an equivalent of a fusion of the short arm of chromosome 11 with the short arm of chromosome 4 in tomato (Wu *et al.* 2009). However, our comparative oligo-FISH does not indicate whole-arm translocations associated with eggplant chromosomes 4, 5, 11, and 12 (Figure 4). We cannot exclude the possibility that the interchromosomal translocations are specific to the eggplant accession used by Wu *et al.* (2009). Thus, application of additional eggplant genotypes in oligo-FISH mapping may explain the discrepancy of results based on genetic linkage mapping and comparative oligo-FISH mapping.

It is intriguing that chromosomal inversions are highly common among the *Solanum* species (Figure S3 in File S1). By contrast, chromosomal translocations are relatively rare. Interestingly, we discovered reciprocal translocations in

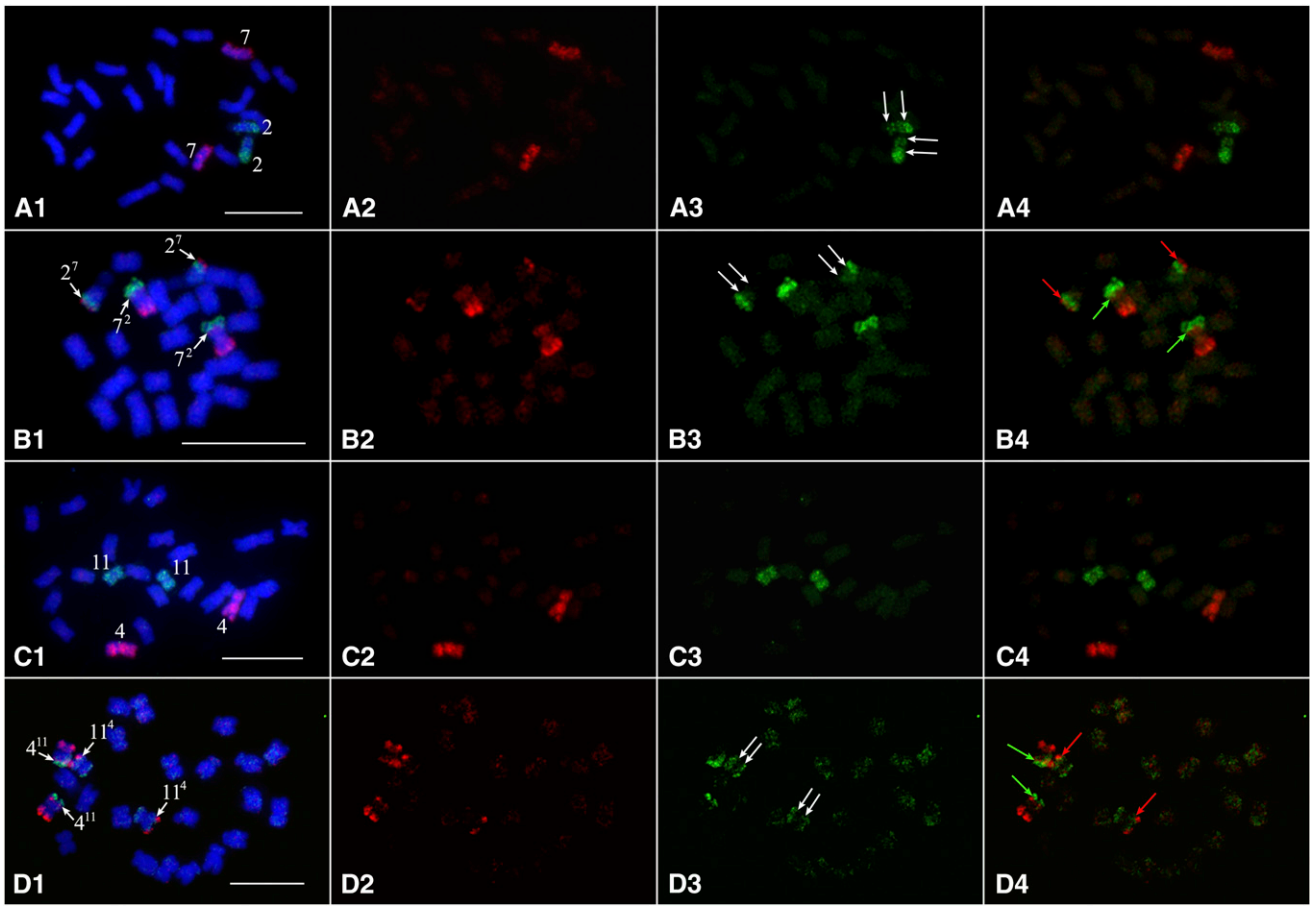

Figure 6 Validation of chromosomal translocations by chromosome painting. (A1–A4) Painting of chromosomes 2 (green) and 7 (red) of DM potato. Red (A2), green (A3), and both red and green (A4) fluorescence signals were digitally separated from (A1). Double white arrows in (A3) indicate relatively weak FISH signals that span the short arm and proximal region of the long arm of chromosome 2. (B1–B4) Painting of chromosomes 2 (green) and 7 (red) in *S. etuberosum*. Red (B2), green (B3), and both red and green (B4) fluorescence signals were digitally separated from (B1). Double white arrows in (B3) indicate very weak or background level FISH signals that span the short arm and proximal region of the long arm of chromosome 2. Red arrows in (B4) point to the breakpoint where a small chromosome 7 fragment attached to chromosome 2 (2^{7}). Green arrows in B4 point to the breakpoint where a large chromosome 2 fragment attached to chromosome 7 (7^{2}). (C1–C4) Painting of chromosomes 4 (red) and 11 (green) of DM potato. Red (C2), green (C3), and both red and green (C4) fluorescence signals were digitally separated from (C1). (D1–D4) Painting of chromosomes 4 (red) and 11 (green) in *S. caripense*. Red (D2), green (D3), and both red and green (D4) fluorescence signals were digitally separated from (D1). Double white arrows indicate background level FISH signals that span pericentromeric region of chromosome 11. Red arrows in (D4) point to the breakpoint where a chromosome 4 fragment attached to chromosome 11 (11^{4}). Green arrows in (B4) point to the breakpoint where a chromosome11 fragment attached to chromosome 4 (4^{11}). Bar, 10 μm.

S. etuberosum and *S. caripense*, and both are wild species. Strikingly, the oligo-FISH probes generated nearly identical signal patterns on chromosomes from potato and eggplant (Figure 4), which have diverged for ~15.5 MY (Wu and Tanksley 2010). A recent study in humans showed that a translocation can change the spatial position of the translocated chromosome fragment in the nucleus and, thus, alter the expression of the associated genes (Harewood *et al.* 2010). Since potato, tomato, and eggplant are crop species, selection in breeding practice may have eliminated chromosomal variants that may have negatively affected the fitness of the species due to the altered gene expression associated with the chromosomal rearrangement. Translocations have previously been reported to be rare in wheat cultivars but common in their wild ancestors (Badaeva *et al.* 1995). Analysis of the presence of the translocations in multiple populations of *S. etuberosum* and *S. caripense* will reveal if these chromosomal variants have been fixed in these wild species.

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Author contributions: J.J. conceived the research, G.T.B. and L.H. conducted FISH experiments. T.Z. and H.Z. designed oligo-FISH probes. K.S. and J.-M.R. synthesized probes and provided reagents. G.T.B., L.H., G.A.T., and J.J. analyzed data. J.J. wrote the article.

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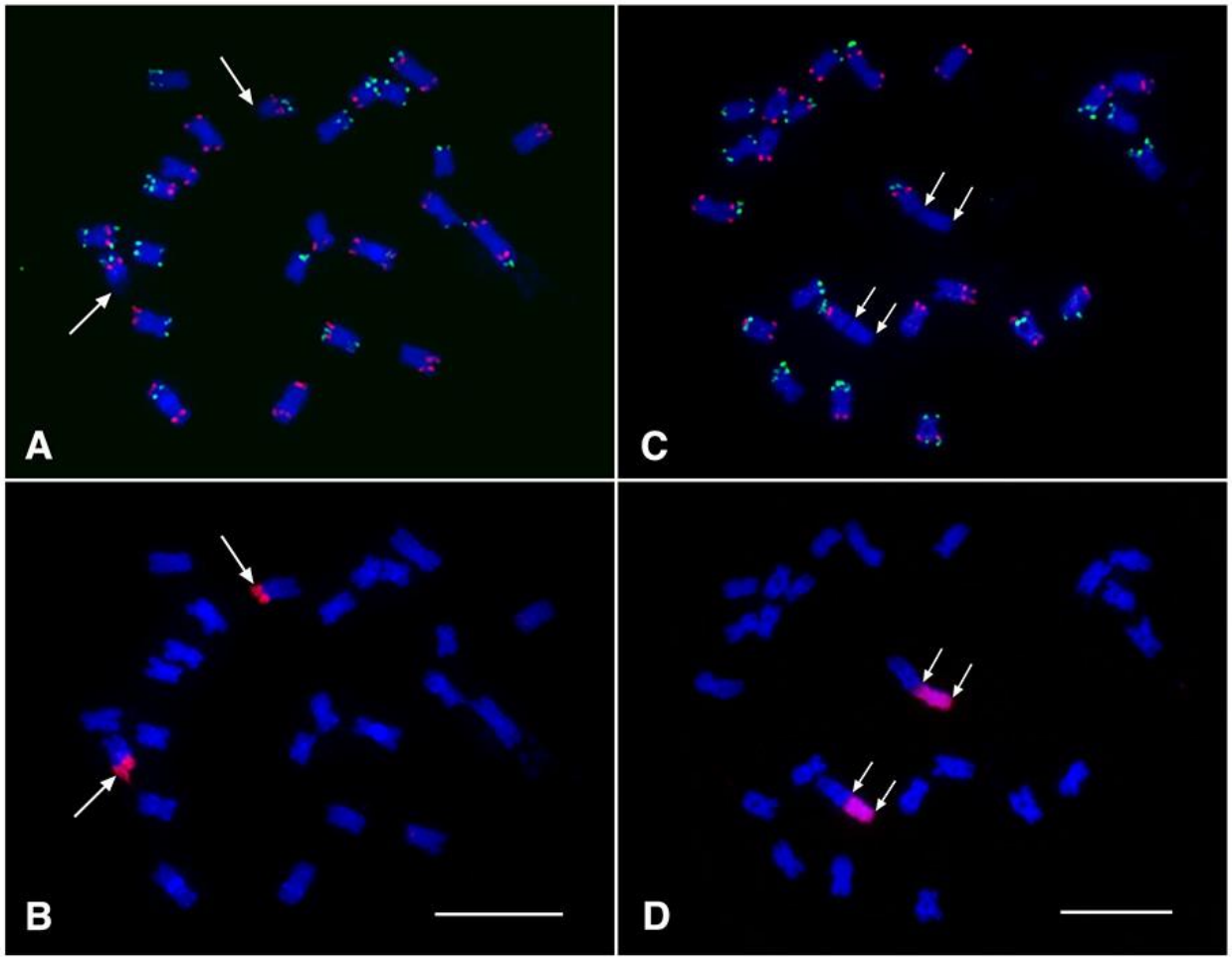

Figure S1. FISH mapping of the 45S ribosomal RNA genes in potato (A and B) and tomato (C and D). (**A**) A metaphase cell (same as Figure 2A) hybridized with two oligo-FISH probes. Arrows indicate the 45 rDNA loci associated with chromosome 2. (**B**) The same metaphase cell was hybridized to a 45 rDNA probe. (**C**) A metaphase cell (same as Figure 2B) hybridized with two oligo-FISH probes. Double arrows indicate the 45 rDNA loci associated with chromosome 2. (**D**) The same metaphase cell was hybridized to a 45 rDNA probe. Bars = 10 µm.

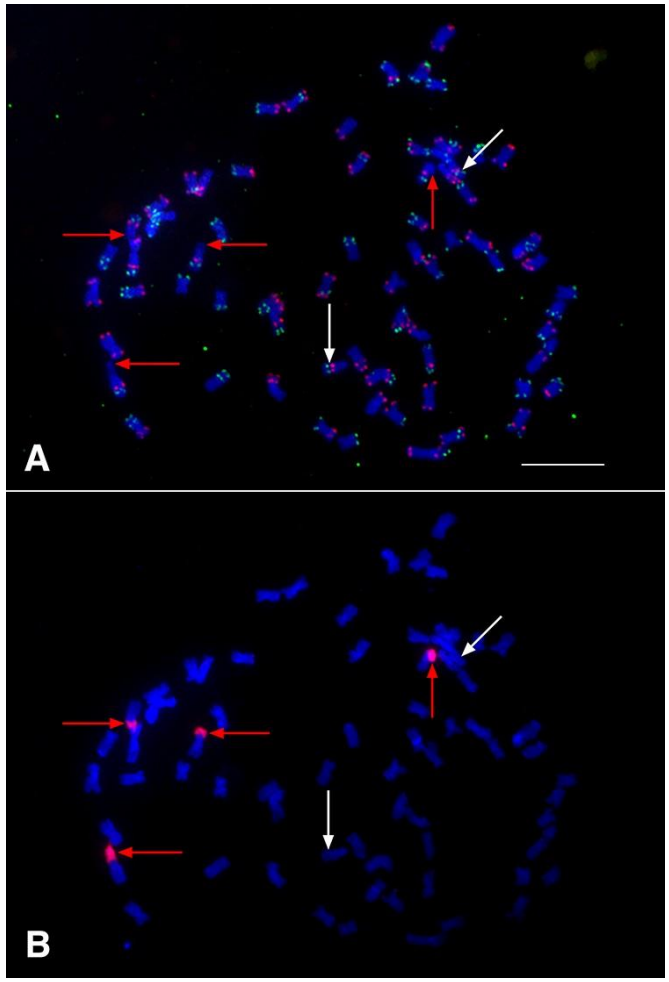

Figure S2. FISH mapping of the 45S ribosomal genes in *S. demissum*. (**A**) A metaphase cell (same as Figure 4) hybridized with two oligo-FISH probes. (**B**) The same metaphase cell was hybridized to a 45S rDNA probe. Red arrows indicate the four copies of chromosome 2 associated with 45S rDNA. White arrows indicate the two remaining copies of chromosome 2 that are do not associated with 45S rDNA. Bar = 10 µm.

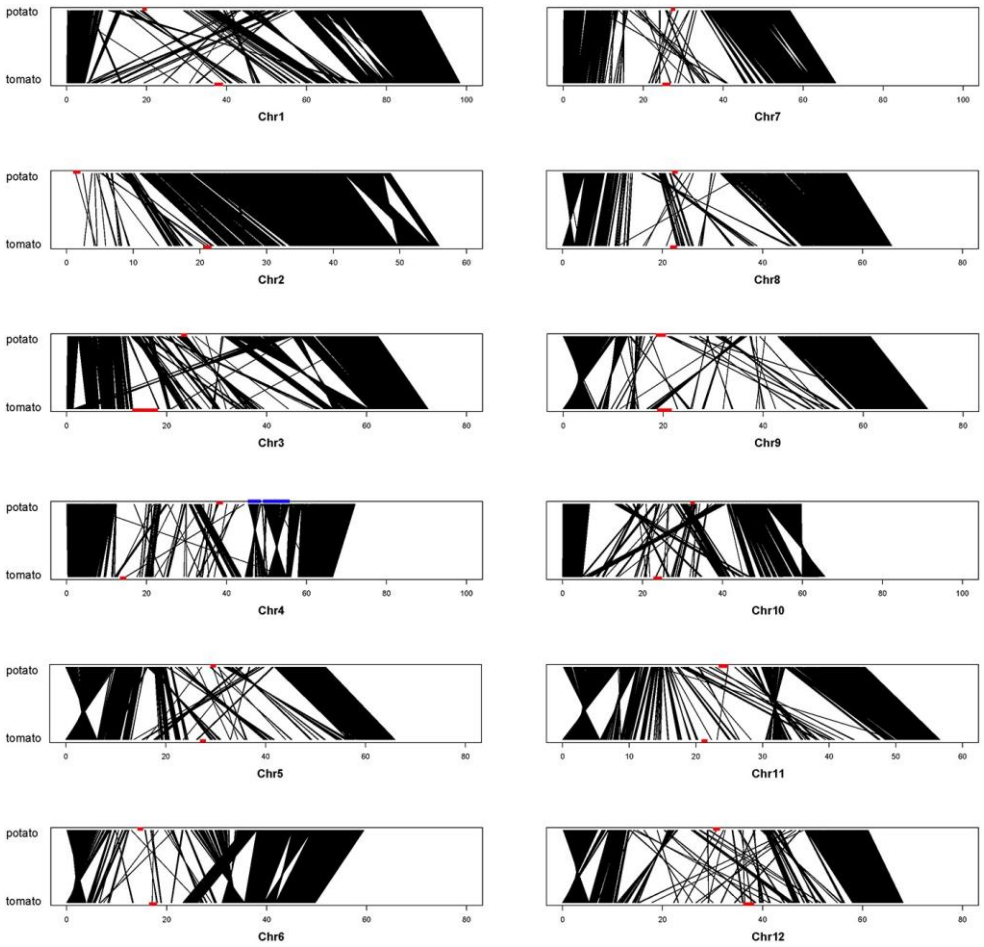

Figure S3. Syntenic relationship between the 12 pairs of potato/tomato pseudomolecules. The red blocks mark the positions of the centromeres on the pseudomolecules. The centromeric positions were determined according to the distribution of centromere-specific repeats (Gong et al. 2012) and sequence reads derived from chromatin immunoprecipitation using CENH3 antibodies followed by Illumina sequencing (ChIP-seq) in potato (Gong et al. 2012) and tomato (unpublished data). The two blue blocks on top of potato chromosome 4 indicate the positions of two inversions located in the long arms of potato/tomato chromosome 4.

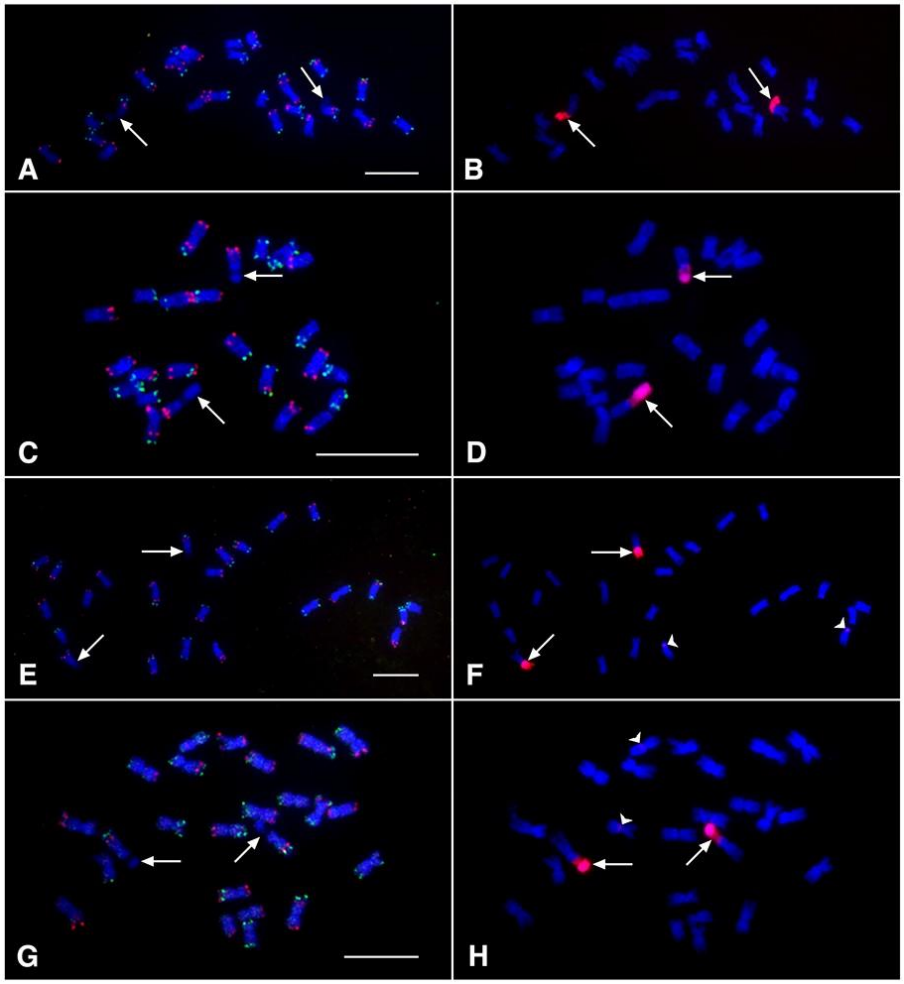

Figure S4. FISH mapping of the 45S ribosomal genes in four diploid *Solanum* species. (**A**) A metaphase cell of *S. bulbocastanum*, (**C**) of *S. etuberosum*, (**E**) of *S. caripense*, and (**G**) of *S. melongena* hybridized with two oligo-FISH probes. Nearby cells (**B**, **D**, **E**, and **H**) in each row show the same metaphase hybridized with 45S rDNA probe. Arrows indicate the two copies of chromosome 2 in A, C, E, and G, and point to the 45S rDNA signals in B, D, F, and H. Arrowheads in F and H indicate the additional minor signals derived from the 45S rDNA probe. Bar = 10 µm.

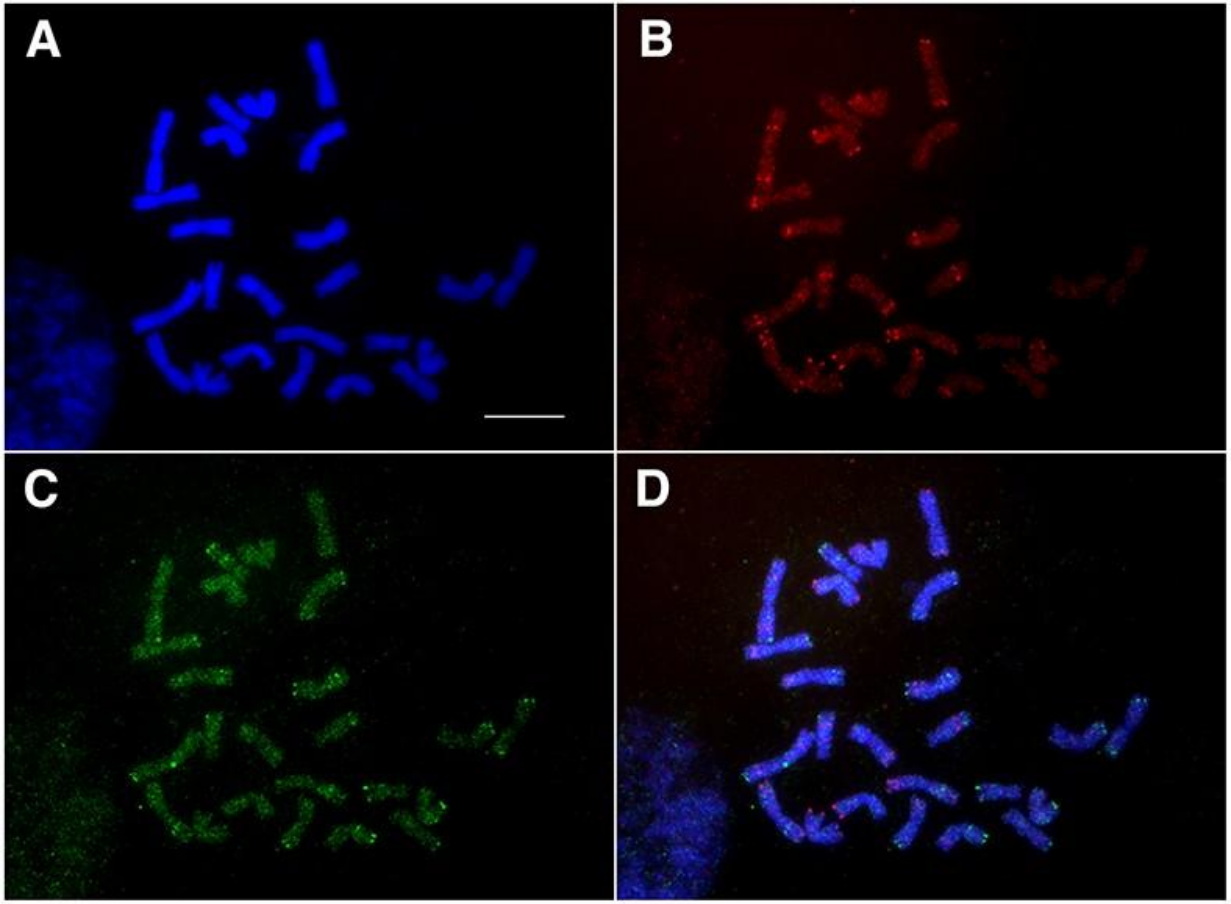

Figure S5. FISH mapping of pepper using two oligo-FISH probes from potato. (**A**) A complete metaphase cell. Bar = 10 µm. (**B**) FISH signals derived from probe PB8495 (red signals). (**C**) FISH signals derived from probe PB9446 (green signals). (**D**) Merged image of chromosomes and FISH signals. Note: strong and dispersed background signals were generated by both probes. The signal patterns on most chromosomes, based on the major punctuated signals, did not match to those from potato and tomato.

Supplemental Table S1. Numbers of oligos and chromosomal positions of 26 regions selected for oligo-FISH probe development

Chromosome	Start position (bp)	End position (bp)	Number of oligos	Covered length (bp)	Region length (bp)	Probe color
1	3056208	3546018	2100	100161	489811	Red
1	71173457	71514869	2100	98085	341413	Red
2	28664037	28976914	2100	99262	312878	Red
3	50340028	50693000	2100	98378	352973	Red
3	61013042	61310686	2100	100075	297645	Red
4	3717797	4164095	2016	96033	446299	Red
4	71469010	71772247	2200	101794	303238	Red
6	51203627	51575645	2200	103146	372019	Red
7	4000037	4464083	2100	100187	464047	Red
9	3811637	4196989	2000	93331	385353	Red
9	58214023	58701462	2000	92859	487440	Red
10	4400078	4856331	2200	104412	456254	Red
12	4100098	4483818	2150	101756	383721	Red
1	86260923	86572070	2100	97821	311148	Green
2	39020107	39342890	2100	98158	322784	Green
5	3660055	4033978	2200	103691	373924	Green
5	51633733	51899798	2200	103681	266066	Green
6	2493429	3199965	2006	94151	706537	Green
6	58371050	58680920	2000	93377	309871	Green
7	44300545	44688011	2100	99261	387467	Green
7	55265047	55558074	2000	92672	293028	Green
8	42710043	43123069	2100	99329	413027	Green
8	54822018	55105812	2100	98160	283795	Green
9	48200828	48772150	2100	98982	571323	Green
11	2320168	2695985	2250	107048	375818	Green
12	59800274	60114954	2050	95942	314681	Green

Supplemental Table S2. Relative length of individual chromosomes in six *Solanum* species[a]

Chr.	***S. tuberosum*** (potato)	***S. bulbocastanum***	***S. lycopersicum*** (Tomato)	***S. etuberosum***	***S. caripense*** **(**Tzimbalo)	***S. melongena*** (Eggplant)
1	12.23 ± 0.65	11.61 ± 0.69	12.08 ± 0.58	10.97 ± 0.67	10.14 ± 0.44	10.08 ± 0.34
2	6.34 ± 0.41	7.04 ± 0.57	7.78 ± 0.68	6.01 ± 0.39	8.98 ± 0.33	7.95 ± 0.75
3	8.49 ± 0.49	9.17 ± 0.81	8.70 ± 0.42	8.71 ± 0.41	8.65 ± 0.24	9.34 ± 0.37
4	9.56 ± 0.49	9.02 ± 0.42	8.31 ± 0.52	9.20 ± 0.60	9.05 ± 0.55	7.86 ± 0.52
5	7.46 ± 0.44	7.36 ± 0.37	7.90 ± 0.28	6.26 ± 0.43	8.62 ± 0.60	7.57 ± 0.44
6	8.25 ± 0.40	8.13 ± 0.53	7.70 ± 0.25	8.71 ± 0.53	8.51 ± 0.59	8.66 ± 0.33
7	7.95 ± 0.53	7.75 ± 0.67	8.55 ± 0.62	10.01 ± 0.52	7.83 ± 0.27	9.45 ± 0.54
8	8.04 ± 0.46	7.82 ± 0.32	8.17 ± 0.37	8.24 ± 0.31	7.65 ± 0.39	8.06 ± 0.24
9	9.55 ± 0.44	8.96 ± 0.24	8.22 ± 0.44	9.06 ± 0.42	7.63 ± 0.31	8.32 ± 0.44
10	7.85 ± 0.38	7.63 ± 0.90	7.52 ± 0.30	7.66 ± 0.42	7.90 ± 0.33	6.80 ± 0.38
11	6.53 ± 0.37	6.76 ± 0.53	7.23 ± 0.42	6.68 ± 0.47	7.84 ± 0.42	7.57 ± 0.53
12	7.75 ± 0.32	8.75 ± 0.30	7.83 ± 0.36	8.49 ± 0.49	7.22 ± 0.47	8.34 ± 0.69

[a] Measurement was conducted on each chromosome in 10 metaphase cells.

PAPER 2 - CHROMOSOME IDENTIFICATION OF CORN (*Zea mays mays* L.) AND ITS RELATIVES USING OLIGO-BASED FISH BARCODE

The manuscript will be submitted to Chromosome Research Journal.

Chromosome identification of corn (*Zea mays* subsp. *mays* L.) and its relatives using oligo-based FISH barcode

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Abstract

The accurate chromosome identification is the most important fundament for the success on cytogenetic research. Different strategies based on conventional and molecular cytogenetics analyses have been used to create chromosome-specific marks to unambiguously distinguish homologous chromosomes pairs from each other. A variable fluorescent *in situ* hybridization probes were generated based on large genomic insertions, single DNA repeats and single copy genes. Even though these strategies are useful, they are time-consuming, labor-intensive and have low repeatability across different laboratories. Oligonucleotides-based FISH probes, recently developed, showed to be a repeatable, cheap and easy methodology for chromosome identification. In our current study, we showed that a "cytogenetics bar code system" based on oligo-FISH probes can be used to identify all 20 chromosomes of corn (*Zea mays mays* L.) in metaphase chromosome spreads and meiotic pachytene stage. The probes designed for corn generated a similar FISH signals pattern in its relatives chromosomes, showing that this is a powerful method for evolutionary studies.

Key words: oligonucleotide, oligo-probes, *Zea*, karyotype, pachytene, knob

Introduction

The interest on chromosome research increased significantly after the association of the transmission of characteristics across generations with chromosomes behavior in cell division by Sutton (1903) and Boveri (1904). Associated with this, many techniques in cytogenetics and genome research field have been developed for a better understanding of chromosome organization, structure and function.

The success in cytogenetic research relies on a robust method for chromosome identification (Jiang and Gill 2006)**.** Different strategies based on classical and molecular cytogenetics have been developed to generate markers on chromosomes for this purpose. After the development of *in situ* hybridization (Pardue and Gall 1970) and fluorescent *in situ* hybridization (Langer-Safer et al. 1982), a wide range of probes was created, increasing the power of cytogeneticists to generate new chromosome markers and, therefore, improving significantly the resolving power for distinguish chromosomes from each other.

FISH probes based on large-insert genomic DNA clones like BAC (Dong et al. 2000; Lysak et al. 2001) showed to be very useful for chromosome identification and phylogenetic studies, but unfortunately, it was not usable for species like corn (*Zea mays* subsp. *mays* L.) (Zhang et al. 2004; Lamb and Birchler 2006; Lamb et al. 2007b). Due to its huge (2.3 Gb) and complex genome (85% composed by repetitive elements dispersed nonuniformly) (Schnable et al. 2009), FISH signals tend to label all chromosomes making impossible to distinguish them. Alternatively, repetitive DNA elements-based probes (Kato et al. 2004) and single copy gene probes (Lamb et al. 2007a) have been used to generate FISH signals on individuals chromosomes of corn and its relatives. Those are powerful methodology but are time-consuming, labor-intensive and difficult to repeat in different laboratories. Furthermore, the pattern of signals of probes based on repetitive DNA elements are variable even between different lines from *Z. mays* (Kato et al. 2004; Albert et al. 2010), limiting the application of this method.

Recently, a new methodology based on oligonucleotide (oligo) probes have been developed (Beliveau et al. 2012). It showed to be an efficient, repeatable and cheap FISH variation method for chromosome identification and karyotype evolution studies. Using this methodology, chromosome-specific FISH probes were designed to paint specific chromosomes in different species and its relatives like cucumber (Han et al. 2015), *Fragraria vesca* (Qu et al. 2017), wheat (Du et al. 2017), potato (Braz et al. 2018) and *Populus sp.* (Xin et al. 2018). Furthermore, Braz et al. (2018) created a “bar code” system combining two

colors of FISH signals to distinguish all 12 chromosomes of potato and perform the comparative mapping in species diverged from 7 to 15.5 million years from potato.

We used this oligo-based FISH barcode system to generate chromosome-specific FISH signals and identify all 20 chromosomes of corn and its relatives in only one round of FISH and easily construct their karyotypes. This methodology will support different areas of corn genetic research.

Material and Methods

Plant Materials

We used seeds from *Zea mays* L. subsp. *mays* (cultivar B73, USA) and "teosinte" including *Z. mays* subsp. *parviglumis* Iltis & Doebley (Ames 21826, Guerrero, Mexico), *Z. mays* subsp. *huehuetenangensis* (Iltis & Doebley) Doebley (PI 441934, Huehuetenango, Guatemala), *Z. luxurians* (PI 422162, Guatemala, via Florida) and *Z. diploperennis* (PI 462368, Jalisco, Mexico). The accessions with PI and Ames numbers were obtained from Germplasm Resource Information Network (GRIN), the National Genetics Resource Program (Ames, Iowa), respectively. All species and subspecies used are diploid with 2n=20 chromosomes.

Oligo-FISH probes design

The oligo-based FISH barcode probes were designed following Han et al. (2015), with some modifications, using an updated version of Chorus software (https://github.com/forrestzhang/Chorus).

All repetitive sequences were eliminated from corn (B73) reference genome (Schnable et al. 2009) using k-mer method, which showed to be more effective than RepeatMasker (http://www.repeatmasker.org). Then, we divided the maize genome sequence in oligos (45 nt) in step size of 3 nt and the short sequence reads were mapped back using BWA (Burrows–Wheeler Alignment tool) (Li and Durbin 2009). Oligos mapped in two or more locations (with 70% of homology) and dTm < 10°C were eliminated. Pandas (http://pandas.pydata.org/) and Matplotlib (https://matplotlib.org/) packages were used to visualize the distribution of selected oligos in pseudomolecules.

The oligo libraries designed were synthesized by Arbor Bioscience, formerly MYcroarray (Ann Arbor, MI).

Mitotic chromosome spread and pachytene preparation

Root tips harvested from plants grown in the green house were treated with nitrous oxide at a pressure of 160 psi (~10.9 atm) for 2hs and 20 min (Kato 1999), fixed in fixative solution (3 ethanol:1 acetic acid) and kept at -20°C. Root tips were digested using an enzymatic solution composed of 4% cellulase (Yakult Pharmaceutical, Japan), 2% pectinase (Sigma-Aldrich Co., USA) and 2% pectolyase (Plant Media, USA) for two hours at 37°C and slides were prepared using a stirring method (Ross et al. 1996; Schubert et al. 2001).

For pachytene preparation we harvested anthers from young flower buds, fixed them in fixative solution (3 ethanol:1 acetic acid) at room temperature for 24 h and stored under -20°C until use. The developmental stage of pollen mother cells (PMCs) was determined using one anther from each flower bud. Then, we digested the remaining anthers from selected buds in 3% cellulase (Yakult Pharmaceutical, Japan), 2% pectolyase (Plant Media, USA), and 1.5% cytohelicase (Sigma, USA) solution at 37°C for 30 minutes. The slides were prepared stirring method (Ross et al. 1996; Schubert et al. 2001).

Oligo-FISH

Two oligo-based FISH probes were labeled following Han et al. (2015). FISH was performed following published protocol (Jiang et al. 1996). Biotin- and digoxygenin-labeled probes were detected by anti-biotin fluorescein (Vector Laboratories, Burlingame, California) and anti-digoxygenin rhodamine (Roche Diagnostics, Indianapolis, Indiana), respectively. Chromosomes were counterstained with 4,6-Diamidino-2-phenylindole (DAPI) in VectaShield antifade solution (Vector Laboratories).

FISH images were captured using a QImaging Retiga EXi Fast 1394 CCD camera attached to an Olympus BX51 epifluorescence microscope. Images were processed with Meta Imaging Series 7.5 software. The final contrast of the images was processed using Adobe Photoshop CS3 software.

Karyotyping

We used 10 and 4 complete metaphases of corn and its relatives, respectively, to measure the short (S) and long (L) arms of individual chromosomes using DRAWID software version 0.26 (Kirov et al. 2017). These measurements were used to determine the total length of each chromosome ($tl = S + L$), total length of entire set of chromosomes ($TL = \sum tl$), arm ratio ($AR = L/S$) of each chromosome, and relative length of each chromosome ($RL = tl/TL\ x100$). The chromosomes were classified following Levan et al. (1964). Knobs were identified as DAPI-positive bands.

To construct karyotype of pachytene the chromosomes were digitally straighten using the software ImageJ (Schneider et al. 2012).

Results

Identification of corn (*Zea mays mays*) chromosomes using Oligo-based FISH barcode

All 20 mitotic metaphase chromosomes of corn were identified simultaneously using oligo-based FISH barcode method (Braz et al. 2018). In total, we selected 50082 oligonucleotides (45nt each) in corn reference genome (Schnable et al. 2009) to design two oligo-FISH probes, identified as 12773 (green) and 12774 (red). Each probe was composed by a library of 25023 and 25059 oligos, respectively, that produced a set of 24 specific marks (12 red and 12 green) distributed across all corn chromosomes in a way to work as a barcode system (Figure 1a). Each signal was composed by 1978-2282 oligonucleotides spanning from 1.30 to 3.3 Mb.

From the total of oligonucleotide used, 24888 oligos (49.69%) were located in annotated genes, with 11600 oligos (46.6%) and 13288 oligos (53.4%) related to green and red signals, respectively.

After the library amplification and probe labeling process, we checked the distribution of our probes on corn chromosome spreads using only one round of FISH analysis (Figure 1b). This methodology, as predicted, allowed us to identify accurately all 20 chromosomes and to construct the molecular cytogenetic karyogram based on single copy oligonucleotide distribution (Figure 1c).

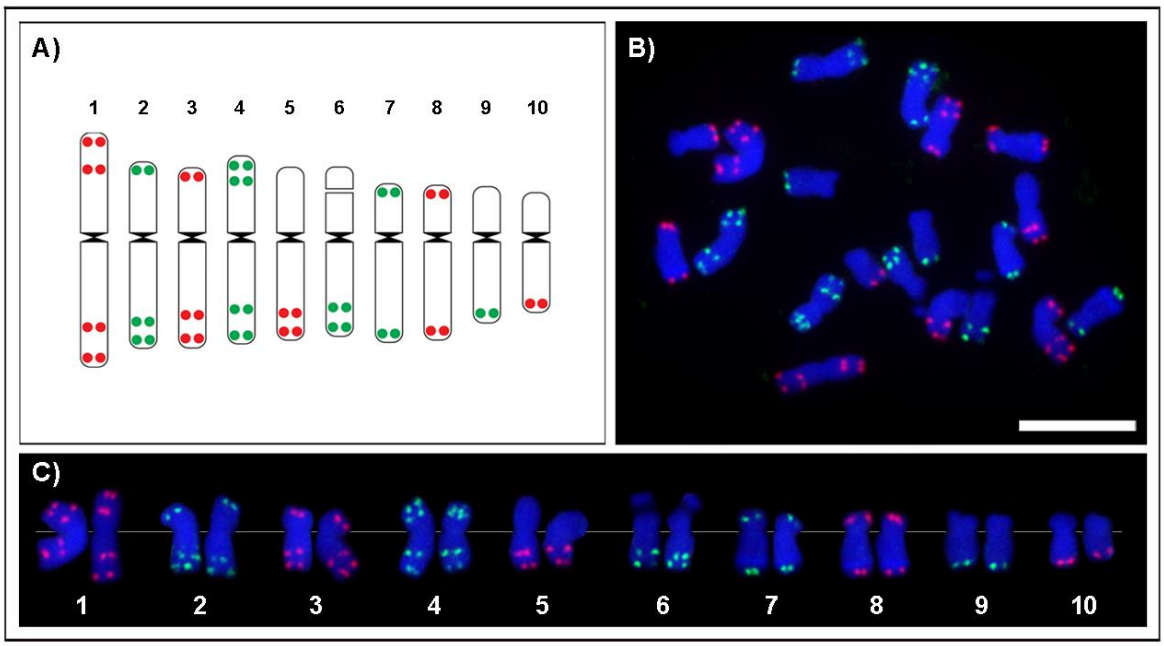

Fig 1 Chromosome identification and karyotype of corn using oligo-based FISH barcode. A) Predicted distribution of the oligo-FISH signals on 10 corn chromosomes. B) Distribution of oligo-FISH signals on metaphase chromosomes. C) Karyogram of corn based on single copy oligonucleotide distribution. Scale bar = 10 µm.

Even though the resolution of FISH signals in mitotic metaphase chromosomes was enough for chromosome identification, we showed that those probes can be used in pachytene stage as well (Figure 2). This system allowed us to isolate the bivalents from each other and to construct the karyogram at pachytene stage from corn.

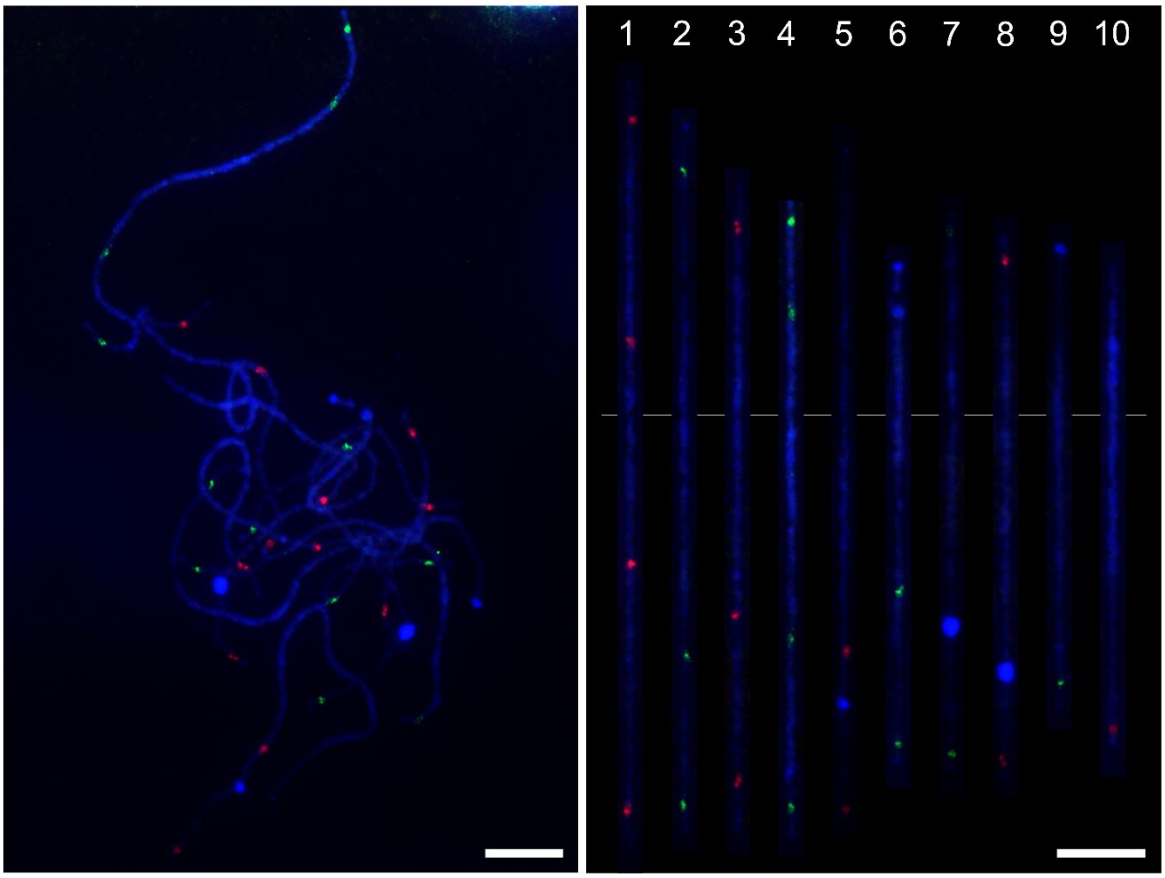

Fig 2 Chromosome identification and karyogram of corn at the pachytene stage. The meiotic cells were hybridized using the two oligo-based FISH barcode probes (left). Pachytene chromosomes were digitally straightened (right). Scale bar = 10 μm.

Comparative karyotyping in corn and its relatives

We used our oligo-FISH probes to identify the chromosomes of corn relatives (Figure 3). We observed a conserved distribution of all FISH signals, which allowed us to identify unambiguously all 20 chromosomes in the other *Zea* species and subspecies and construct the karyogram (Figure 4).

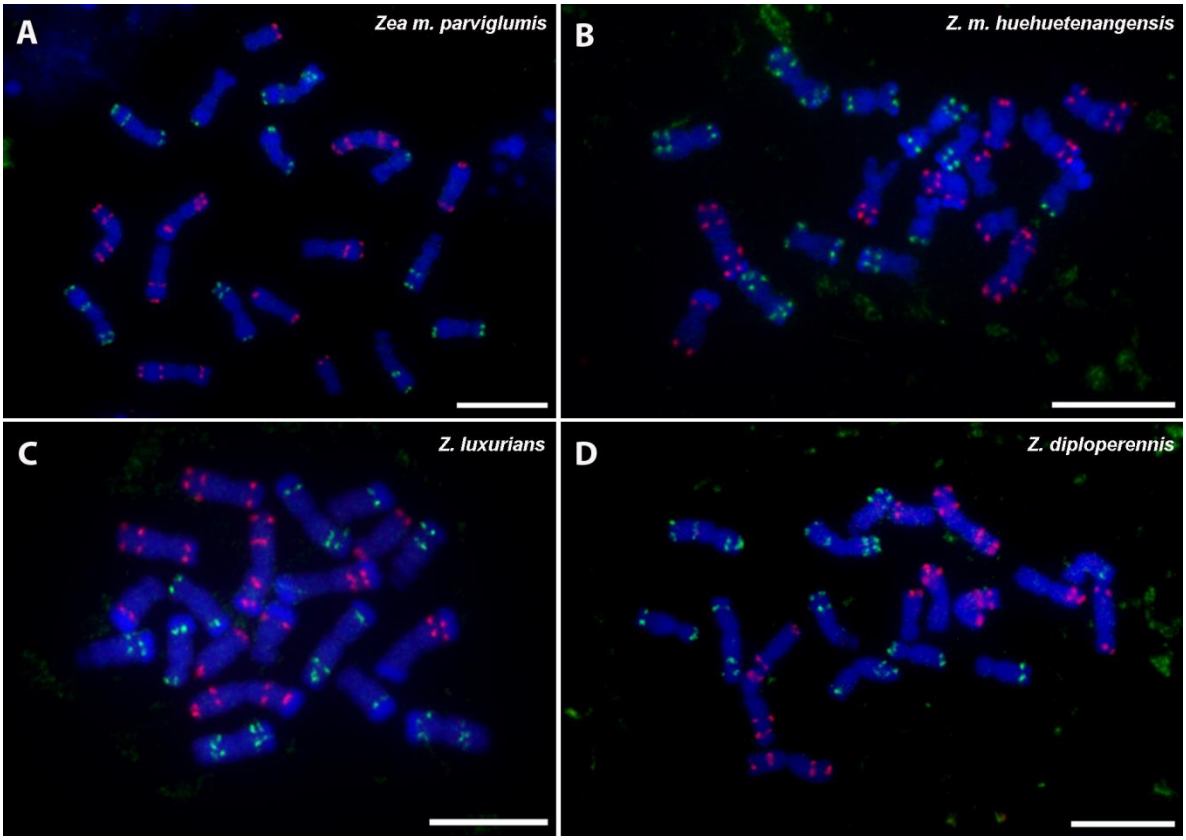

Fig 3 Chromosome identification of relatives of corn using oligo-based FISH barcode probes. Scale bar = 10 µm.

We observed that the relative length and arm ratio of corn and its relatives homeologous chromosomes are similar (Table 1). In the other hand, the unambiguous identification of all chromosomes allowed the detection of some karyotype variability. One source of variation is the number and distribution of knobs among species and subspecies (Figure 4, S1). In *Zea mays* subsp. *mays* the knobs are interstitials and located in the chromosomes 5, 7, 8 and 9. In *Z. mays* subsp. *parviglumis* the knobs are either terminals or interstitials and located in all chromosomes except 1 and 10. In Z. *mays* subsp. *huehuetenangensis* the knobs are terminals and located in all chromosomes except 10. In *Z. luxurians* the knobs are terminals and located in all chromosomes except 8. In *Z. diploperennis* the knobs are terminals and located in the chromosomes 2, 3, 7 and 9.

In *Z. luxurians*, the knobs in the long arm of chromosome 4 are located in the terminal position whereas in the other species they are between the two green signals (Figure 4). In *Z. mays* subsp. *parviglumis*, there is a huge difference in the length of the homologous of chromosome 2, 3 and 9 (Figure 4).

Table 1 Relative length and arm ratio of individual chromosomes in corn and its relatives.

Chr.	*Z. m mays*	*Z. m parviglumis*	*Z. m huehue* [1]	*Z. luxurians*	*Z. diploperennis*
Relative length (%)					
1	14.26 ± 1.22	12.44 ± 0.37	13.43 ± 0.16	13.00 ± 0.89	13.69 ± 0.48
2	11.19 ± 0.28	11.45 ± 0.35	11.48 ± 0.55	11.71 ± 0.57	12.34 ± 0.36
3	10.76 ± 0.54	11.67 ± 0.37	11.20 ± 0.12	10.45 ± 0.35	11.32 ± 0.20
4	11.28 ± 0.53	12.00 ± 0.27	10.81 ± 0.62	10.68 ± 0.62	12.13 ± 0.37
5	10.36 ± 0.22	10.84 ± 0.38	10.32 ± 0.40	12.61 ± 0.45	10.47 ± 0.40
6	8.51 ± 0.34	8.96 ± 0.37	8.66 ± 0.31	8.88 ± 0.77	8.05 ± 0.49
7	9.44 ± 0.50	8.76 ± 0.48	9.13 ± 0.22	8.64 ± 0.35	8.43 ± 0.47
8	9.18 ± 0.33	8.82 ± 0.28	9.11 ± 0.52	7.87 ± 0.54	8.63 ± 0.27
9	8.03 ± 0.36	8.78 ± 0.29	8.92 ± 0.40	8.33 ± 0.34	8.07 ± 0.37
10	6.99 ± 0.46	6.27 ± 0.16	6.94 ± 0.22	7.83 ± 0.30	6.88 ± 0.02
Arm ratio					
1	1.26 ± 0.14	1.23 ± 0.11	1.34 ± 0.38	1.13 ± 0.09	1.27 ± 0.15
2	1.52 ± 0.18	1.46 ± 0.34	1.42 ± 0.21	1.25 ± 0.16	1.30 ± 0.12
3	1.65 ± 0.28	1.54 ± 0.28	1.44 ± 0.20	1.53 ± 0.18	1.79 ± 0.22
4	1.34 ± 0.17	1.49 ± 0.24	1.24 ± 0.16	1.20 ± 0.11	1.55 ± 0.09
5	1.54 ± 0.45	1.52 ± 0.24	1.26 ± 0.20	1.09 ± 0.07	1.24 ± 0.17
6	2.40 ± 0.46	3.44 ± 0.90	2.45 ± 0.35	3.33 ± 0.50	2.64 ± 0.52
7	2.11 ± 0.46	2.54 ± 0.48	1.79 ± 0.19	2.40 ± 0.30	1.97 ± 0.22
8	2.13 ± 0.48	2.83 ± 0.39	1.62 ± 0.38	2.17 ± 0.26	2.22 ± 0.35
9	1.80 ± 0.34	1.56 ± 0.24	1.30 ± 0.22	2.48 ± 0.33	2.34 ± 0.37
10	1.78 ± 0.40	1.98 ± 0.22	1.97 ± 0.34	2.64 ± 0.20	2.14 ± 0.39
Chr. Morphology	5m:5sm	6m:3sm:1a	7m:3sm	5m:4sm:1a	4m:6sm

*Each chromosomal arm was measured in 10 metaphase cells of corn and in 4 of its relatives. m = metacentric, sm = submetacentric, a = acrocentric. [1] *Z. m huehuetenangensis*

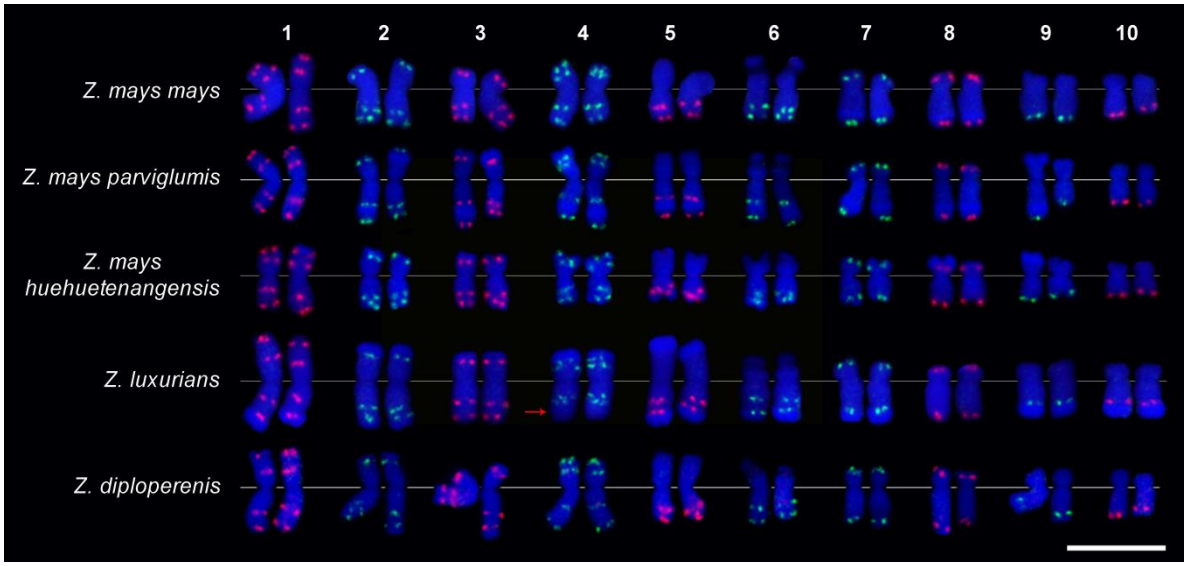

Fig 4 Comparative oligo-based FISH mapping in *Zea mays mays* and its relatives. Red arrow indicates a putative inversion in chromosome 4 of *Z. luxurians*. Scale bar = 10 µm.

Discussion

The identification of homologous chromosome pairs is critical for many aspects in cytogenetics and genome research. Recently developed (Beliveau et al. 2012; Han et al. 2015), FISH probes based on oligonucleotides showed to be a powerful methodology to support the chromosome research in different areas. Braz et al. (2018) created oligo-based FISH barcode probes to distinguish all 12 chromosomes of potato (genome sized = 670 Mb) and related species diverged up to 15.5 My, an efficient methodology for chromosome identification and karyotype evolution studies.

Differently from potato, corn has a huge (2.3 Gb) and complex genome with about 85% composed by repetitive elements dispersed nonuniformly (Schnable et al. 2009). In this work, we showed that oligo-based FISH barcode method can be used in species with big chromosomes and complex genomes, as an accurate and relatively cheap method for chromosome research in corn and its relatives.

Fluorescent *in situ* hybridization have been used as support for different genetics and genome research fields like in genome assembly projects (Stack et al. 2009; Chamala et al. 2013), validating genetics mapping works (Sadder and Weber 2002; Ohmido et al. 2018) and for the chromosomal localization of transgenic vectors inserted (Svitashev and Somers 2001, 2002; Partier et al. 2017). For those kind of projects, the pachytene stage is usable due the higher resolving power comparing with mitotic metaphase chromosomes. We showed that

our probe can generate clear FISH signals on pachytene preparations being this a powerful method.

The hybridization of our oligo-based FISH probes in the relatives of corn allowed us to compare accurately the same chromosome in different species and subspecies. In this way, we observed that relative length and arm ratio of all 10 chromosomes were very conserved features. Only few chromosome morphology variations were observed and are probably related to knobs distribution and length and thus to repetitive sequence content, which are evident in the chromosomes 2, 3 and 9 of *Z. mays* subsp. *parviglumis*, species proposed as ancestor of maize.

Knobs are defined as large chromosomal heterochromatic blocks visible in cytologic preparations (McClintock 1929). Located in more than 30 different sites on 17 chromosome arms of corn and teosintes (Buckler et al. 1999) they are dramatically variable in number, location and size even between lines from the same specie (Albert et al. 2010). As we observed, Albert et al. (2010) showed that knobs are located in interstitial chromosome position in corn whereas in more distant species they are terminal.

Detailed analysis have showed that these chromosome structures are colocalized with DAPI (4',6-diamidino-2-phenylindole) positive bands (González and Poggio 2011) and organized in multi-megabase tandem repeats arrays composed primarily by 350-bp TR-1 element or 180-bp knob repeats (Ananiev et al. 1998), or a mixture of both (Ananiev et al. 1998; Hiatt 2002; Kato et al. 2004). These repeats represent 21% of the genome, being an important chromosomal feature for the genome evolution of corn (Dawe et al. 2018).

Knobs are known as functionally inert. However, they are converted in neocentromeres in the presence of abnormal chromosome 10 (Ab10), a chromosome 10 with an extended long arm composed by four knobs. This Ab10 confer a preferential chromosome transmission to egg cells for all knobbed chromosomes, process known as "meiotic drive" (Rhoades 1942). Dawe et al. (2018) showed that the neocentromere activation occur in the presence of "kinesin driver" (*Kindr*), a cluster of eight kinesin genes located on the distal position of the long arm of Ab10 haplotype. All this complex together drives the emergence, accumulation and maintenance of knobs repeats, affecting the genome architecture (Buckler et al. 1999).

The difference in the pattern of FISH signals distribution in the long arm of chromosome 4 of *Z. luxurians* suggest a homozygous paracentric inversion. Chromosomal rearrangements are integral part of genome evolution. Paracentric inversion is a kind of chromosome alteration that involve two breaks at the same chromosome arm with a

reinsertion of the fragment in opposite orientation. Then, the gene order along the chromosome will be changed and, as consequence, gene regulatory networks can be disrupted (Goidts et al. 2005) and genes with new functions can be promoted (Korneev and O'Shea 2002). The paracentric inversion that our results suggest can be related with the divergency of Z. *luxurians* in the genus. Confirmation of this hypothesis requires additional information with higher resolution in the region.

Even though the molecular biology and bioinformatics tools went through improvements in past 20 years and new technologies have emerged, FISH is still the only way to anchor a DNA sequence to specific location on chromosome. Using oligo-based FISH probes for chromosome identification is a powerful strategy to support different areas of genetics and genome research and help, in a near future, to answer different and complex biological questions.

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SUPPLEMENTAL - Chromosome identification of corn (*Zea mays* subsp. *mays* L.) and its relatives using oligo-based FISH barcode

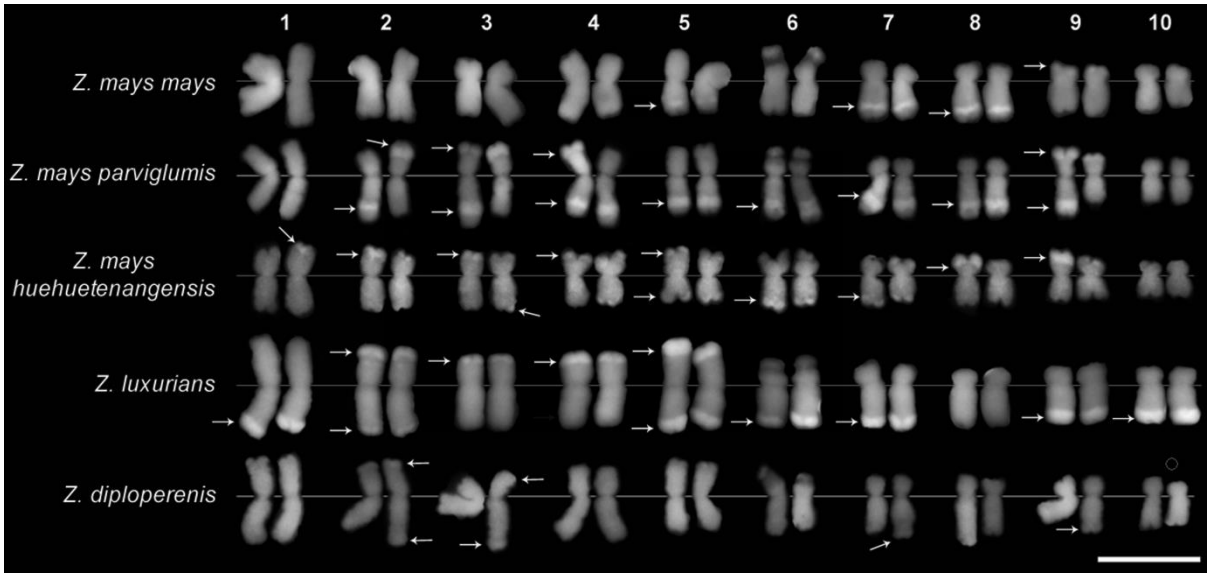

Figure S1. Knobs (arrow) distribution in chromosomes of corn and its relatives. Scale bar = 10µm.