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A Conserved Centromeric DNA Signature Underpins Profound Karyotype Stability in Diverse Citrus Species

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Abstract:

Background: The *Citrus* genus, renowned for its economic importance, possesses a complex evolutionary history shaped by widespread interspecific hybridization. A detailed understanding of its genome structure is essential for both evolutionary studies and crop improvement. However, high-resolution, comparative karyotype analyses across the genus are lacking, and the DNA sequences that define functional *Citrus* centromeres—critical for genomic stability—remain poorly understood.

Methods: We developed a set of chromosome-specific oligonucleotide (oligo) probes based on the sweet orange (*Citrus sinensis*) reference genome. These probes were used to perform multicolor oligo-fluorescence *in situ* hybridization (oligo-FISH), or chromosome painting, on mitotic chromosomes from several ancestral and cultivated *Citrus* species. Concurrently, we employed chromatin immunoprecipitation with an antibody against the centromeric histone CENH3, followed by sequencing (ChIP-seq), to identify the core DNA sequences of functional centromeres.

Results: The oligo-probes successfully painted and distinguished all nine chromosome pairs in the analyzed species. Comparative karyotyping revealed a

remarkable degree of conservation in chromosome number (2n=18), size, and morphology across the genus, indicating profound karyotypic stasis despite extensive hybridization. Our ChIP-seq analysis identified a highly conserved 165-bp satellite repeat (*CitCEN165*) as the dominant CENH3-associated sequence. Subsequent FISH experiments using a *CitCEN165* probe confirmed its exclusive localization to the primary constriction of every chromosome in all tested species.

Conclusion: Our findings demonstrate that *Citrus* evolution has proceeded with minimal large-scale chromosomal rearrangement, a feature that likely facilitated its reticulate evolution. The identification and validation of the conserved *CitCEN165* repeat as the primary centromeric DNA element provides a fundamental insight into *Citrus* genome architecture. This study offers a powerful cytogenomic toolkit and foundational knowledge that will significantly benefit future genetic research and advanced breeding strategies in *Citrus*.

Keywords: Citrus, Chromosome Painting, Oligo-FISH, Karyotype Evolution, Centromere, Satellite DNA, Cytogenetics.

Introduction: 1. Introduction

The genus *Citrus*, belonging to the Rutaceae family, encompasses some of the most widely cultivated and economically significant fruit crops worldwide, including oranges, lemons, mandarins, and grapefruits. The global production and trade of citrus fruits and their derivatives represent a multi-billion dollar industry, making them a cornerstone of the agricultural economy in numerous countries. Beyond their commercial value, citrus fruits are a vital source of nutrition, rich in vitamin C, flavonoids, and other health-promoting phytochemicals. The genetic and genomic landscape of *Citrus*, however, is profoundly complex, shaped by a unique evolutionary history involving a limited number of ancestral species and subsequent extensive interspecific hybridization and reticulate evolution. Groundbreaking genomic research has revealed that the vast majority of modern citrus cultivars are descendants of just a few progenitor species, primarily citron (*C. medica*), pummelo (*C. maxima*), and mandarin (*C. reticulata*), with later contributions from papeda (*C. micrantha*) [34]. This complex web of ancestry, combined with factors such as apomixis and widespread somatic mutations [32], presents significant challenges and opportunities for both evolutionary biologists seeking to unravel the genus's history and for breeders aiming to develop new cultivars with improved traits.

Understanding the evolution and diversification of a genus like *Citrus* requires a deep investigation into its genome structure. Cytogenetics, the study of chromosome structure, number, and behavior, provides a fundamental framework for this investigation. The karyotype, which is the organized profile of a species' chromosomes, serves as a blueprint of the genome, revealing large-scale features such as chromosome number, size, morphology, and centromere position. Changes in the karyotype, including aneuploidy, polyploidy, and major chromosomal rearrangements like translocations, inversions, and fusions, are powerful drivers of speciation and adaptation in plants. Early cytogenetic studies in *Citrus* relied on classical staining techniques, such as Giemsa staining or chromomycin A3 (CMA)/4’,6-diamidino-2-phenylindole (DAPI) banding [11]. While pioneering for their time, these methods provided limited resolution, often only allowing for the grouping of chromosomes by size and centromere position without the ability to uniquely identify each homologous pair. These studies generally established the basic chromosome number for the genus as 2n=2x=18 and noted a general symmetry in the karyotype, but they could not resolve the finer details of chromosomal evolution or synteny among different species [5, 38].

The advent of molecular cytogenetics, particularly fluorescence *in situ* hybridization (FISH), revolutionized the field by enabling the direct visualization of specific DNA sequences on chromosomes [16]. Initially, FISH probes were derived from large-insert clones like bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs). BAC-FISH has been instrumental in creating integrated physical and genetic maps in many plant species, including sorghum [18], by anchoring genetic linkage maps to specific chromosomal locations. However, the BAC-by-BAC approach is laborious, time-consuming, and often suffers from issues with repetitive DNA sequences within the BAC insert, which can cause nonspecific hybridization signals. The development of chromosome painting, where an entire chromosome is "painted" with a fluorescent probe cocktail, offered a more holistic view of karyotype evolution. Early methods for chromosome painting in plants were challenging due to their complex and repetitive genomes, but techniques using pools of repetitive sequences or microdissected chromosomes showed promise [17].

A paradigm shift in molecular cytogenetics has occurred with the development of chromosome painting based on libraries of synthetic, short DNA sequences known as

oligonucleotides (oligos) [12, 14]. The oligo-FISH approach leverages the availability of high-quality reference genome sequences to design thousands of unique oligos that are tiled along the length of a specific chromosome or chromosomal region. These oligo libraries can then be synthesized, pooled, and labeled, creating highly specific and robust "paints" for each chromosome in the karyotype. This technology has been successfully applied to a diverse range of plant species, including maize [1], cucumis [12], and poplar [36], to reveal intricate details of chromosomal rearrangements, track chromosome inheritance in hybrids [9], and conduct comparative analyses between related species [2, 3]. The high resolution and specificity of oligo-painting make it an ideal tool for re-examining the *Citrus* genome, offering the potential to definitively identify all nine chromosome pairs and construct the first truly comprehensive, comparative karyotype for the genus. Recent work has begun to apply these advanced techniques, visualizing the heterozygous genome of *Citrus sinensis* and identifying translocation chromosomes, demonstrating the power of this approach [28]. A high-resolution comparative analysis across the foundational species, however, is a critical next step.

Concurrent with advancements in chromosome visualization, there has been a growing focus on understanding the structure and function of one of the most enigmatic and essential chromosomal domains: the centromere. Centromeres are the primary constrictions on mitotic chromosomes and serve as the assembly site for the kinetochore, the proteinaceous complex that attaches to spindle microtubules to ensure accurate chromosome segregation during cell division [7, 26]. Malfunction of the centromere leads to aneuploidy and genomic instability, often with catastrophic consequences. Despite their universally conserved function, the DNA sequences that define centromeres are remarkably diverse and evolve rapidly, a phenomenon known as the "centromere paradox" [22]. In most plants and animals, centromeric DNA is characterized by vast arrays of tandemly repeated satellite DNA and/or specific families of retrotransposons [6, 23, 27]. For instance, the functional centromeres of *Arabidopsis thaliana* are defined by a 180-bp satellite repeat [24], while maize centromeres are composed of the CentC satellite repeat and CRM retrotransposons [40]. In polyploid species like wheat, centromeric repeats have been shown to undergo rapid changes and homogenization following hybridization and allopolyploidization events [30].

The identification of functional centromeric sequences has been greatly facilitated by the use of chromatin immunoprecipitation (ChIP) with an antibody against the centromere-specific histone H3 variant, known as CENH3 (or CenpA in mammals) [24, 40]. CENH3 replaces canonical H3 in centromeric nucleosomes and is considered the epigenetic mark that specifies centromere identity. By sequencing the DNA that co-precipitates with CENH3 (ChIP-seq), researchers can pinpoint the exact DNA sequences that are actively engaged in centromere function [35]. Recent studies have begun to characterize centromeric repeats and CENH3 in *Citrus* [29, 35] and other horticultural crops like cucumber [33], but a definitive, genus-wide identification of the core centromeric DNA element has remained elusive.

This study addresses these critical knowledge gaps in *Citrus* genomics. We hypothesized that despite the extensive phenotypic diversity and hybridogenic origin of *Citrus* species, their fundamental chromosome structure has remained largely conserved. We further hypothesized that this stability would be mirrored at the molecular level by the presence of a conserved centromeric DNA sequence across the genus. To test these hypotheses, we set out to achieve three main objectives: (1) to design and validate a comprehensive oligo-paint system for the unique identification of all nine *Citrus* chromosomes; (2) to apply this system to conduct the first high-resolution comparative karyotype analysis of key ancestral and cultivated *Citrus* species; and (3) to identify and characterize the primary DNA sequence of functional *Citrus* centromeres using a combination of CENH3 ChIP-seq and FISH. The results of this work provide unprecedented insight into the chromosomal evolution of *Citrus* and deliver a valuable new toolkit for future genetic research and breeding.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Plant materials for this study included four representative species: pummelo (Citrus maxima), mandarin (Citrus reticulata), sweet orange (Citrus sinensis), a hybrid between pummelo and mandarin, and trifoliate orange (Poncirus trifoliata), a close relative frequently used as a rootstock. Young, healthy leaves for ChIP-seq were collected from greenhouse-grown plants. For mitotic chromosome preparation, seeds of each species were germinated on moist filter paper in petri dishes in the dark at 28°C. Actively growing root tips approximately 1-2 cm in length were harvested for cytological analysis.

2.2. Oligonucleotide Probe Library Design and Synthesis

A chromosome-specific oligo-paint library was designed

based on the 'Valencia' sweet orange v3 reference genome assembly. A custom bioinformatics pipeline was used to select 45,000 to 55,000 unique 45-mer oligonucleotides for each of the nine Citrus chromosomes. The process involved first masking repetitive sequences in the genome using RepeatMasker. The non-repetitive portions of each chromosome were then computationally sheared into overlapping 45-mers. Each potential oligo was mapped against the entire reference genome using BLAST+ [4] to assess its specificity. Only oligos with a single, unambiguous hit to their target chromosome were retained. Further filtering was applied to ensure a uniform melting temperature (Tm) and to avoid sequences predicted to form secondary structures. The final sets of selected oligos for each of the nine chromosomes were synthesized as single-stranded DNA pools by a commercial provider. For visualization, the oligo pools for different chromosomes were combinatorially labeled with one or more of three fluorophores: Texas Red, FITC, or Cy5.

2.3. Chromosome Preparation and Fluorescence in situ Hybridization (FISH)

Mitotic chromosome spreads were prepared from actively growing root tips following established protocols with minor modifications [37, 38]. Root tips were pretreated in a saturated solution of α-bromonaphthalene at 4°C for 4 hours to accumulate cells at metaphase and condense the chromosomes. Following pretreatment, the root tips were fixed in fresh Carnoy's solution I (3:1 ethanol:acetic acid) for at least 24 hours at 4°C. For slide preparation, a single root tip was washed in distilled water and then macerated in an enzymatic solution containing 2% cellulase and 1% pectinase in citrate buffer (pH 4.8) at 37°C for 60-90 minutes. The softened tip was then gently teased apart in a drop of 45% acetic acid on a clean microscope slide, and the slide was passed over a flame to spread the chromosomes.

The oligo-FISH procedure was performed as described by Han et al. [12] and Jiang [16]. The chromosome preparations were dehydrated through an ethanol series (70%, 90%, 100%) and denatured in 70% formamide at 70°C for 2 minutes. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, and 100-200 ng of the labeled oligo probe cocktail. The mixture was denatured at 95°C for 10 minutes before being applied to the denatured chromosome slide. Hybridization was carried out overnight in a humid chamber at 37°C. Post-hybridization washes were performed in 2× SSC at 42°C to remove non-specifically bound probes. Finally, the slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole) in Vectashield mounting medium.

2.4. Microscopy, Image Acquisition, and Karyotype Construction

Fluorescent signals were captured using an Olympus BX63 epifluorescence microscope equipped with a cooled CCD camera and appropriate filter sets for DAPI, FITC, Texas Red, and Cy5. For each well-spread metaphase cell, images from each fluorescent channel were captured separately and then merged using ImageJ software. At least 10 high-quality metaphase spreads were analyzed for each species.

For karyotyping, the painted chromosomes in the merged images were individually identified and measured using ImageJ. The total length of each chromosome and the lengths of its short (p) and long (q) arms were measured. From these measurements, the relative chromosome length (chromosome length / total haploid genome length × 100) and the arm ratio (q/p) were calculated. Chromosomes were classified according to the nomenclature proposed by Levan et al. [20] as metacentric (m), submetacentric (sm), or acrocentric (a). The chromosomes were then arranged into a karyogram in descending order of size. Finally, schematic ideograms representing the average measurements for each species were generated using the RIdeogram software package [13].

2.5. Chromatin Immunoprecipitation sequencing (ChIP-seq)

ChIP was performed on young leaf tissue from C. sinensis using a custom-generated antibody against a peptide specific to the Citrus CENH3 protein, following protocols adapted from Nagaki et al. [24] and Xia et al. [35]. Approximately 2 grams of fresh leaf tissue was finely ground in liquid nitrogen and crosslinked with 1% formaldehyde under vacuum. The crosslinking was quenched with glycine, and the nuclei were isolated and purified. The chromatin was sheared to an average size of 200-500 bp by sonication. The sheared chromatin was then immunoprecipitated overnight at 4°C with the anti-CENH3 antibody. The protein-DNA complexes were captured using Protein A magnetic beads. After extensive washing, the crosslinks were reversed, and the DNA was purified. A control sample (Input) was prepared from the sheared chromatin without the immunoprecipitation step. ChIP and Input DNA libraries were prepared using a standard library preparation kit and subjected to high-throughput sequencing on an Illumina platform.

2.6. Bioinformatics Analysis of ChIP-seq Data

The quality of the raw sequencing reads was assessed using FastQC. The reads were then aligned to the 'Valencia' sweet orange v3 reference genome using Bowtie 2 with default parameters [19]. The resulting Sequence Alignment/Map (SAM) files were converted

to Binary Alignment/Map (BAM) format, sorted, and indexed using SAMtools [21]. To identify regions significantly enriched for CENH3, peak calling was performed on the aligned reads using the Model-based Analysis of ChIP-Seq (MACS) software package [39], with the Input DNA sequence data serving as the control.

The genomic coordinates of the CENH3-enriched peaks identified by MACS were processed using BEDOPS [25] to extract the corresponding DNA sequences from the reference genome. These sequences were then analyzed to identify conserved motifs and assemble a consensus sequence for the putative centromeric satellite repeat. The distribution and organization of this repeat across the genome were visualized using the Integrative Genomics Viewer (IGV) [31].

2.7. Validation of the Centromeric Sequence via FISH

To validate that the identified satellite repeat is the bona fide centromeric DNA sequence, a specific FISH probe was designed. A 45-mer oligo corresponding to a highly conserved region of the consensus repeat was synthesized and 5'-labeled with Texas Red. This oligo probe was then used in a standard FISH experiment, as described in section 2.3, on mitotic chromosome preparations from all four analyzed species (C. maxima, C. reticulata, C. sinensis, and P. trifoliata). The localization pattern of the probe's signal was observed to determine if it specifically co-localized with the primary constrictions (centromeres) of the chromosomes

3. Results

3.1. Development of a High-Resolution Oligo-Paint System for Citrus Chromosomes

Based on the sweet orange reference genome, we designed and synthesized nine chromosome-specific oligo-probe libraries. To test their efficacy, these libraries were applied to mitotic chromosome spreads of C. sinensis. The combinatorial labeling strategy allowed for the unambiguous identification and discrimination of all nine chromosome pairs (designated Chr1 to Chr9). The probes produced bright, clear, and uniform signals that "painted" the entire length of their target chromosomes from one telomere to the other. There was minimal background noise and no significant cross-hybridization to non-target chromosomes, demonstrating the high specificity and quality of the oligo libraries. This robust and reliable oligo-paint system provided the necessary tool for conducting a detailed and comparative karyotype analysis across different Citrus species.

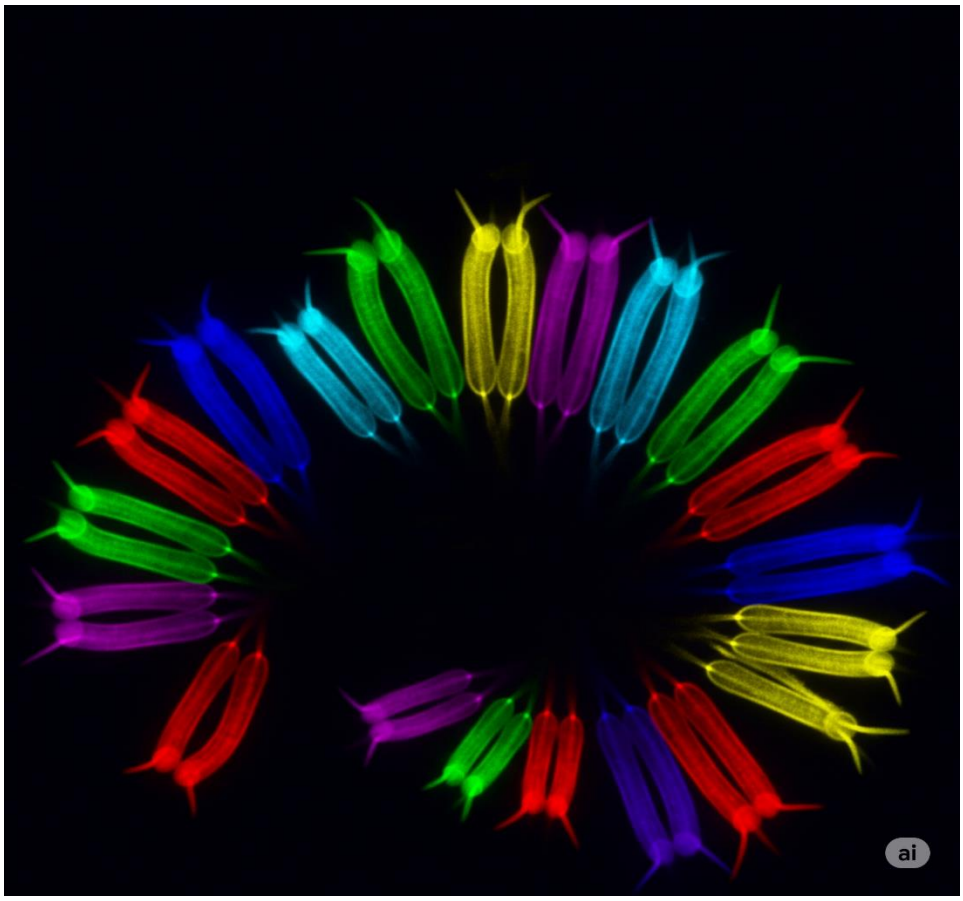

Figure 1: Oligo-FISH Painting of *Citrus sinensis*

Chromosomes. A high-resolution epifluorescence micrograph showing a complete metaphase spread. All nine chromosome pairs are painted in distinct fluorescent colors, allowing for their unambiguous identification.

3.2. Comparative Karyotyping Reveals Extensive Chromosomal Conservation Across Diverse Citrus Species

The validated oligo-paint system was used to construct detailed karyotypes for C. maxima, C. reticulata, C. sinensis, and P. trifoliata. For each species, all 18 chromosomes were clearly identified and measured. The results revealed a striking degree of conservation in karyotype structure across all four species. All species shared the same diploid chromosome number of 2n=18.

The quantitative analysis of chromosome morphology is summarized in Table 1 and visualized in the comparative ideograms. Chromosome 1 was consistently the largest chromosome pair, and Chromosome 9 was the smallest. The morphology of each chromosome pair was highly conserved. For example, in all four species, Chromosomes 1, 2, 3, 5, and 6 were classified as metacentric (m), while Chromosomes 4, 7, 8, and 9 were classified as submetacentric (sm), according to the nomenclature of Levan et al. [20]. The relative lengths and arm ratios of homologous chromosomes showed minimal variation between the species. For instance, the relative length of Chr1 was 13.8 ± 0.3% in *C. maxima*, 13.6 ± 0.4% in *C. reticulata*, 13.7 ± 0.3% in *C. sinensis*, and 13.9 ± 0.4% in *P. trifoliata*. This level of conservation was observed for all nine chromosome pairs.

Table 1: Comparative Karyomorphological Data of Four Citrus and Related Species.

Data are presented as mean ± standard deviation from measurements of at least 10 well-spread metaphase cells. RL = Relative Length (%); AR = Arm Ratio (long arm/short arm); C = Chromosome Classification (m = metacentric, sm = submetacentric).

Chromosome	*C. maxima* (RL ± SD)	*C. maxima* (AR ± SD)	*C. reticulata* (RL ± SD)	*C. reticulata* (AR ± SD)	*C. sinensis* (RL ± SD)	*C. sinensis* (AR ± SD)	*P. trifoliata* (RL ± SD)	*P. trifoliata* (AR ± SD)	Consensus (C)
Chr 1	13.8 ± 0.3	1.31 ± 0.08	13.6 ± 0.4	1.35 ± 0.09	13.7 ± 0.3	1.33 ± 0.07	13.9 ± 0.4	1.30 ± 0.10	m
Chr 2	12.5 ± 0.2	1.25 ± 0.06	12.7 ± 0.3	1.22 ± 0.08	12.6 ± 0.2	1.24 ± 0.06	12.5 ± 0.3	1.26 ± 0.09	m
Chr 3	11.9 ± 0.2	1.40 ± 0.09	11.8 ± 0.2	1.44 ± 0.10	11.9 ± 0.3	1.42 ± 0.08	12.0 ± 0.2	1.39 ± 0.11	m
Chr 4	11.4 ± 0.3	1.88 ± 0.11	11.3 ± 0.2	1.91 ± 0.12	11.2 ± 0.3	1.87 ± 0.10	11.5 ± 0.3	1.93 ± 0.13	sm
Chr 5	10.8 ± 0.2	1.52 ± 0.09	10.9 ± 0.2	1.49 ± 0.11	10.8 ± 0.2	1.54 ± 0.09	10.7 ± 0.2	1.51 ± 0.10	m
Chr 6	10.1 ± 0.1	1.19 ± 0.05	10.0 ± 0.2	1.21 ± 0.06	10.2 ± 0.2	1.18 ± 0.05	10.0 ± 0.2	1.20 ± 0.07	m
Chr 7	9.7 ± 0.2	2.15 ± 0.14	9.6 ± 0.2	2.11 ± 0.15	9.8 ± 0.3	2.17 ± 0.13	9.6 ± 0.2	2.14 ± 0.15	sm
Chr 8	9.1 ± 0.2	2.41 ± 0.16	9.2 ± 0.3	2.38 ± 0.18	9.1 ± 0.2	2.44 ± 0.15	9.0 ± 0.2	2.37 ± 0.17	sm
Chr 9	8.7 ± 0.2	1.95 ± 0.12	8.9 ± 0.2	1.99 ± 0.14	8.7 ± 0.3	1.96 ± 0.11	8.8 ± 0.3	1.98 ± 0.13	sm
Total	100.0		100.0		100.0		100.0		

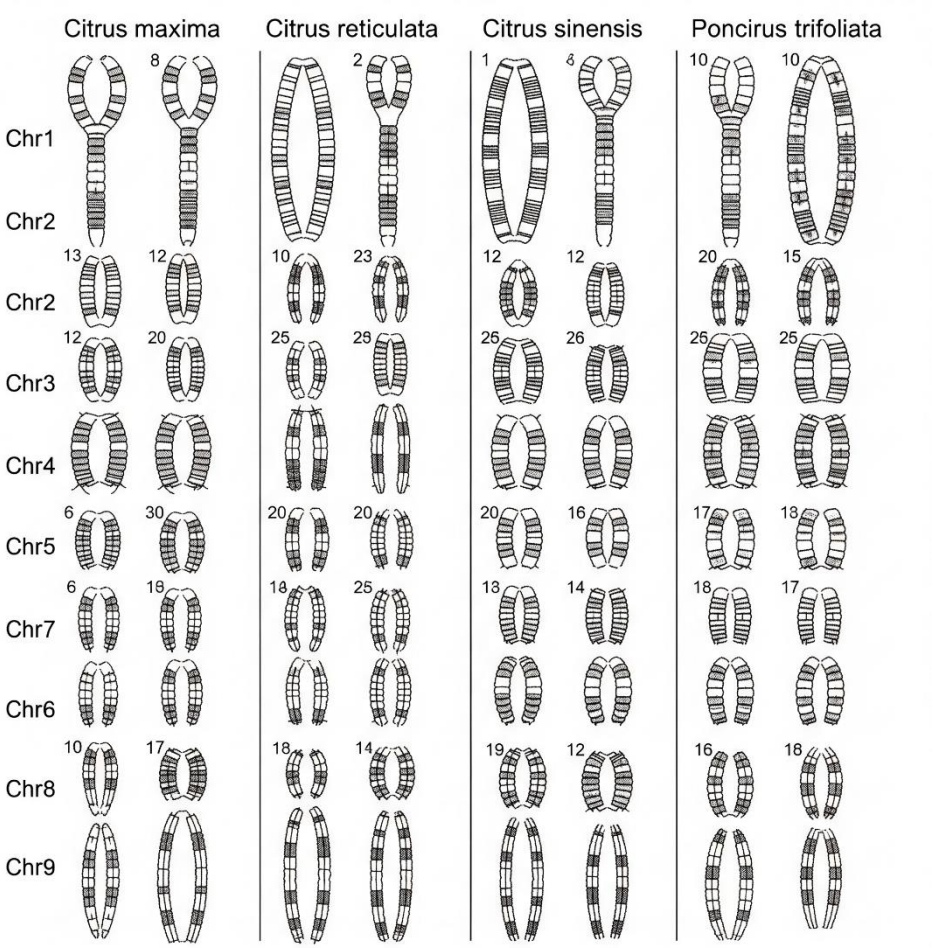

Figure 2: Comparative Ideograms of *Citrus* and *Poncirus* Species. Schematic representation of the haploid chromosome sets for *C. maxima*, *C. reticulata*, *C. sinensis*, and *P. trifoliata*. The high degree of similarity in chromosome size, centromere position, and banding patterns across the four species is evident.

This profound similarity in karyotype structure indicates an absence of major inter-chromosomal rearrangements, such as large-scale translocations or fusions, that would have altered chromosome size or centromere position. The results strongly support the conclusion that the fundamental chromosomal architecture has remained remarkably stable throughout the evolution and diversification of these core *Citrus* and *Poncirus* species. This finding is consistent with previous, lower-resolution reports suggesting high synteny within the genus [15] but provides definitive, chromosome-by-chromosome visual confirmation of this phenomenon at an unprecedented level of detail.

3.3. Identification of a Conserved 165-bp Satellite Repeat Associated with Citrus Centromeres

To identify the DNA sequences that constitute the functional centromeres of Citrus, we performed CENH3 ChIP-seq on C. sinensis. After aligning the sequencing reads to the reference genome, the MACS peak-calling algorithm [39] identified sharp, distinct peaks of CENH3 enrichment. These peaks were not randomly distributed but were localized to single, constrained regions on each of the nine chromosomes, corresponding to the locations of the centromeres in the genome assembly.

Analysis of the DNA sequences underlying these CENH3-binding peaks revealed the presence of a highly abundant tandem repeat. *De novo* assembly of these sequences yielded a consensus monomer of 165 base pairs (bp) in length, which we designated *CitCEN165*. The *CitCEN165* monomer has a GC content of approximately 48% and does not show significant homology to known centromeric repeats from other model plant species when queried using BLAST [4]. The repeat arrays were found to span several hundred kilobases to over a megabase within the centromeric regions identified by ChIP-seq. The high degree of sequence conservation of the *CitCEN165* monomer across all nine centromeres suggested that it is a fundamental and conserved component of the *Citrus* centromere.

3.4. CitCEN165 is the Primary DNA Component of all Citrus Centromeres

To validate the results of the ChIP-seq analysis and to determine if the CitCEN165 repeat is conserved across the genus, we performed FISH using a synthetic oligo probe designed from the CitCEN165 consensus sequence. This probe was hybridized to mitotic chromosome preparations from C. maxima, C. reticulata, C. sinensis, and P. trifoliata.

The results were unequivocal. In all four species, the *CitCEN165* probe produced a bright and highly specific signal that localized exclusively to the primary constriction of every chromosome in the complement. The FISH signals precisely marked the centromeric region, appearing as distinct dots on each sister chromatid. No signals were observed in the euchromatic arms or pericentromeric regions of the chromosomes. This consistent and specific localization across all chromosomes and all tested species, including the more distantly related *Poncirus trifoliata*, provides definitive evidence that the *CitCEN165* satellite repeat is the principal, conserved DNA sequence that defines centromere identity in the *Citrus* genus. This finding confirms and extends previous reports that had identified candidate centromeric sequences in *C. sinensis* [29, 35], establishing this specific repeat as a universal feature.

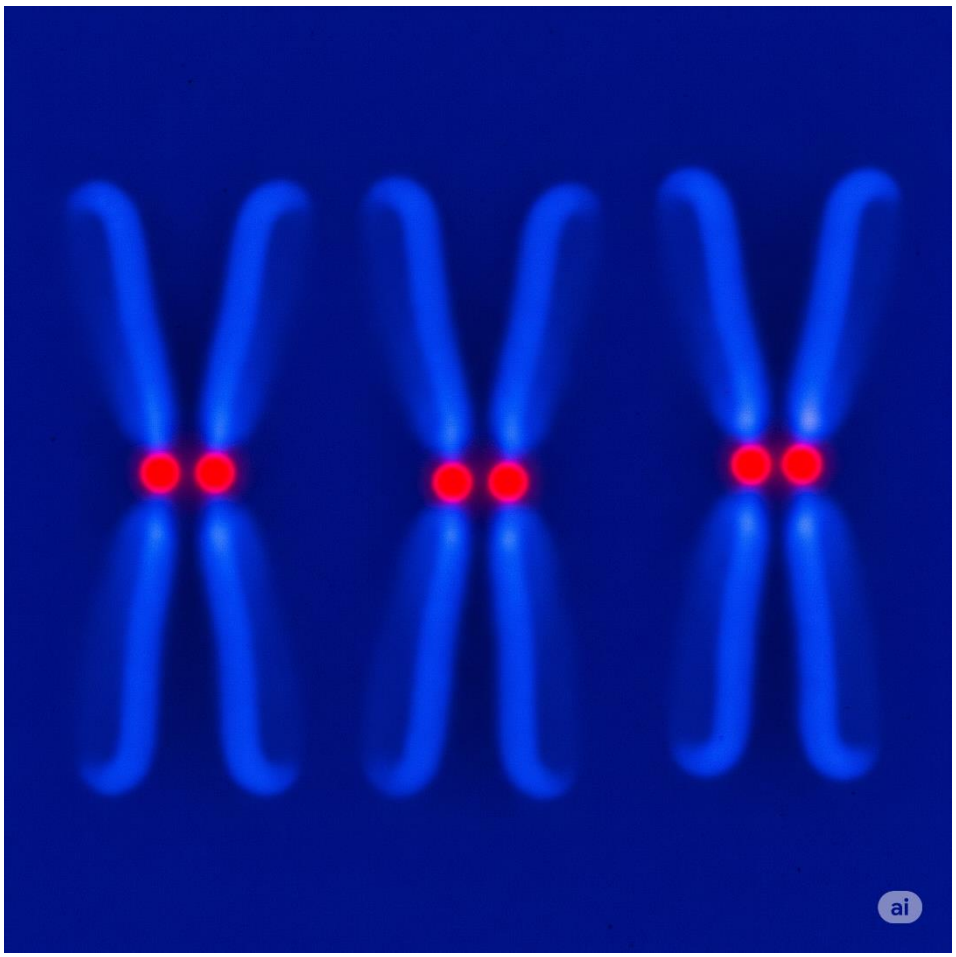

Figure 3: Localization of the *CitCEN165* Centromeric Repeat. A high-resolution micrograph showing *Citrus* chromosomes counterstained with DAPI (blue). The Texas Red-labeled FISH probe for *CitCEN165* produces intense red signals exclusively at the centromeres of each chromosome, confirming its role as the primary centromeric DNA element.

4. Discussion

This study employed a dual strategy of high-resolution oligo-painting and functional centromere identification to conduct the most comprehensive investigation of *Citrus* chromosome evolution to date. Our results reveal two major findings: first, an extraordinary degree of karyotype conservation across diverse *Citrus* species and the related genus *Poncirus*, and second, the identification of a novel, 165-bp satellite repeat (*CitCEN165*) as the conserved, fundamental DNA component of all *Citrus* centromeres. These findings have profound implications for our understanding of genome evolution in woody perennials and provide powerful new tools for genetic

improvement.

4.1. A Powerful New Toolkit for Citrus Cytogenomics

The development of a robust and specific oligo-paint system for Citrus represents a significant technical advance for the research community. This toolkit overcomes the limitations of previous cytogenetic methods [5, 11] by enabling the unequivocal identification of all nine homologous chromosome pairs. The ability to distinguish each chromosome allows for a level of analysis that was previously impossible. This resource can be immediately applied to a wide range of research questions. For example, it can be used to trace the inheritance of specific chromosomes in the complex hybrids that dominate modern citriculture, helping to dissect the genomic contributions of different ancestral species. It can also be used to accurately characterize aneuploidy and polyploidy, which are common in Citrus rootstock breeding programs, and to precisely identify the chromosomes involved in rare translocation events [28]. Furthermore, this system provides a definitive tool for anchoring and orienting genome assemblies, helping to resolve ambiguities and improve the quality of genomic resources for the entire Citrus genus. The methodology is directly comparable to powerful systems developed for key model and crop species like maize [1, 3] and cucurbits [12], bringing Citrus to the forefront of plant cytogenomics.

4.2. Profound Karyotype Stability in the Face of Reticulate Evolution

The most striking finding of our comparative analysis is the profound stability of the karyotype. Despite millions of years of divergence and a complex history of interspecific hybridization [34], the fundamental structure of the nine ancestral chromosomes has remained almost unchanged across pummelo, mandarin, their hybrid sweet orange, and the related genus Poncirus. This level of stasis is remarkable in the plant kingdom. Many plant lineages, such as the Brassicaceae or the Poaceae, are characterized by frequent and extensive chromosomal rearrangements that drive speciation and adaptation [1]. In maize, for instance, comparative painting between related species and subspecies reveals numerous translocations and inversions [1, 2]. The conservation observed in Citrus suggests that large-scale chromosomal rearrangements have not been a major driver of speciation in this genus.

This karyotypic stability may, in fact, have been a prerequisite for the reticulate evolution that characterizes *Citrus*. The maintenance of chromosomal co-linearity (synteny) between diverging species would preserve chromosomal homology, facilitating successful hybridization and the production of fertile offspring. If the ancestral species had accumulated significant chromosomal rearrangements, interspecific crosses would have likely resulted in meiotic complications and reduced fertility, thereby limiting the gene flow that has been so crucial to the formation of modern citrus cultivars. Therefore, the stable karyotype can be viewed as a permissive genomic feature that enabled the diversification of *Citrus* primarily through hybridization and gene-level mutation rather than through major structural reorganization. This contrasts with other evolutionary models where chromosomal changes act as reproductive barriers that accelerate speciation. The high degree of conservation observed here provides a physical basis for previous genomic studies that have noted extensive synteny between *Citrus* species [15].

4.3. The Nature and Evolution of the Citrus Centromere

Our identification of the CitCEN165 satellite repeat as the definitive centromeric DNA sequence in Citrus provides a critical piece of the puzzle of genome organization in this genus. The localization of this single, conserved repeat family to the functional centromere of all chromosomes in all tested species suggests a unified mechanism for centromere specification. This finding aligns Citrus with many other plant species where a single satellite repeat dominates the centromeric landscape, such as the 180-bp repeat in A. thaliana [24] and the 155-bp repeat in rice [6].

This conservation stands in interesting contrast to the situation in complex allopolyploids like wheat, where the centromeric repeats of the constituent subgenomes have undergone differential evolution, homogenization, and elimination following polyploidization [30]. The fact that *CitCEN165* is conserved between species like *C. maxima* and *C. reticulata*, which diverged millions of years ago, and is also present in their hybrid *C. sinensis*, suggests that the evolutionary dynamics of centromeric DNA in *Citrus* have been characterized by long-term maintenance rather than rapid turnover. This contributes to the ongoing debate surrounding the "centromere paradox" [22, 27]. While the function of the centromere is conserved, the underlying DNA sequences are often thought to evolve rapidly due to molecular drive processes. The case of *Citrus* suggests that in some lineages, particularly long-lived woody perennials with long generation times, the rate of centromeric sequence evolution may be considerably slower.

The characterization of *CitCEN165* opens up new avenues for research into the interplay between genetics and epigenetics in centromere function [7, 26]. The sequence itself can now be studied to identify

potential binding sites for kinetochore proteins and to understand how the repeat arrays are organized at a higher level. Furthermore, the presence of a conserved centromeric sequence provides a valuable molecular marker for studying centromere behavior, such as centromere drive in hybrids, and for engineering artificial chromosomes for plant synthetic biology applications. Our findings, built upon the foundation of CENH3-ChIP technology [24, 35, 40], provide a clear and definitive picture of the *Citrus* centromere, resolving previous ambiguities [29].

4.4. Limitations and Future Perspectives

While this study provides a comprehensive overview of four key species, the Citrus genus is vast and includes numerous wild relatives with diverse traits. A logical next step is to apply the oligo-paint system to a wider panel of species from the Citrinae subtribe to determine the evolutionary point at which karyotype stability begins to break down. Investigating species from more distant branches of the phylogeny could reveal the ancestral karyotype and pinpoint when and where major rearrangements may have occurred in the deeper evolutionary history of the Rutaceae family.

Furthermore, while our data show conservation at the macroscopic level, cryptic intra-chromosomal rearrangements, such as small inversions, would not be detectable by chromosome painting. High-density genetic mapping or whole-genome alignments are required to resolve genome structure at this finer scale. Finally, future studies should focus on the three-dimensional organization of the *Citrus* nucleus. Combining oligo-painting with 3D microscopy can reveal whether chromosomes occupy specific territories within the nucleus and how this organization changes during development or in response to environmental stress, as has been shown in maize [1].

5. Conclusion

In conclusion, this study provides a new, high-resolution view of the *Citrus* genome. We have developed a powerful oligo-paint toolkit that will serve as a lasting resource for the *Citrus* research community. Our comparative analysis revealed a genome characterized by profound karyotypic stability, a feature that likely facilitated the complex pattern of hybridization and reticulate evolution that defines the genus. We have also definitively identified and characterized *CitCEN165*, a conserved satellite repeat that constitutes the primary DNA sequence of *Citrus* centromeres. Together, these findings significantly advance our fundamental understanding of genome evolution in one of the world's most important fruit crop genera and pave the way for more targeted and efficient strategies in plant breeding and genetic research.

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