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„Evolution of selected satellite DNAs in cultivated *Capsicum* species"

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Introduction

1. Plant genome

The evolution of all organisms requires the ability to store and transmit hereditary information to the next generation. Hereditary information of eukaryotic organisms is organized in linear chromosomes, located inside the nucleus (Youssef et al. 2019). The eukaryotic chromosomes are composed of chromatin – DNA and associated proteins. Two chromatin states can be distinguished: loosely packed euchromatin and tightly condensed heterochromatin (Park et al. 2000). The latter can be further divided into constitutive heterochromatin that is condensed in all cells and has a high content of repetitive DNA and facultative heterochromatin that is condensed in most cells but can be decondensed, as it may contain genes that are required at certain ontogenetic stages or in response to certain biotic or abiotic factors (Weiss-Schneeweiss & Schneeweiss 2013).

Plant genomes vary greatly in their chromosome number, genome size, as well as composition and the organization of chromosomes (Schubert 2007). The high levels of genome size variations are caused by two major processes: polyploidization and repetitive DNA copy number changes via amplification and repeat removal. Polyploidy, or whole genome doubling, is one of the main driving forces in the evolution of plants, especially in the diversification of angiosperms. It is very common and occurs repeatedly in cycles throughout the evolution of various plant lineages. The estimates of the frequency of polyploid ancestry in plants lie between 35% and 70% (Wendel et al. 2016).

Two major types of polyploidy are recognized: autopolyploidy resulting from multiplication of whole chromosome sets within one individual/taxon and allopolyploidy which, in addition to whole genome multiplication, involves also hybridization between closely related taxa (Wendel 2015). Polyploidization is followed by diploidization whereby the excess of DNA is removed and genomes are rearranged to assume diploid-like behavior in meiosis before next polyploidization round can occur (Dodsworth et al. 2016).

The second process that strongly affects plant genome size and structure is the dynamics of repetitive DNA elements. Swift proposed for the first time the term “C-value” in reference to the haploid unreplicated (1C) DNA amount (Swift, 1950). Twenty years later Thomas coined the terms “C-value paradox”, referring to the enigma that different organisms, even closely related ones, can show considerable differences in their C-values (Eddy, 2012). The largest angiosperm genome recorded up to now has been found in *Paris japonica* (Melanthiaceae) with 1C = 152.23 pg, whilst *Genlisea aurea* (Lentibulariaceae) was on the other end of the scale with 1C = 0.06 pg - showing a 2500 fold variation

between these two extremes (Pellicer et al. 2010; Greilhuber et al. 2006). Coincidentally, both species have the same chromosome number *2n* = 40 (Fleischmann, 2018; Yang et al. 2019). The genome sizes of plants do not correlate with the number of chromosomes into which their DNA is organized, and these two characters evolve independently (Weiss-Schneeweiss & Schneeweiss 2013).

The components of the genome responsible for genome size variation across plant species, in the absence of polyploidy are repeats (Vitales et al. 2019), which are DNA elements or sequence motifs present in multiple copies across the genome. The commonly accepted hypothesis states, that repetitive DNAs have accumulated during the evolution of most of the angiosperms lineages, since ancestral angiosperms have been inferred to have had small genome sizes (Mehrotra & Goyal 2014).

Initially, repetitive DNA sequences were considered to be "junk DNA" or "selfish elements" simply because they were thought to not carry any coding information. Recent years have brought new data to light and it is now commonly accepted that repetitive DNA is a fast evolving part of the genome, is often involved in chromosomal as well as genomic rearrangements and has at least some regulatory functions (Knoll et al. 2014). Repetitive DNA has been shown to be involved in movement, pairing and recombination of chromosomes, sister chromatid cohesion, chromosome association with the mitotic spindle, determination of chromosomal structure and the regulation of gene expression in response to environmental changes, among others (Mehrotra & Goyal 2014).

The total repetitive DNA of the genome is often referred to as repeatome. It consists of two major types of repeats, the tandem repeats and the dispersed repeats (Heslop-Harrison & Schmidt 1998). Dispersed repeats are represented by mobile genetic elements also called transposable elements (TEs). Plant genomes vary greatly in the content of transposable elements, which ranges from 3% in the genome of *Utricularia gibba* up to 85% in polyploid plants, e.g., wheat (Krasileva 2019).

TEs can be divided into two classes: class I retrotransposons and class II DNA transposons (Weiss-Schneeweiss et al. 2015). Retrotransposons use copy and paste transposition mechanism to amplify in the genome via RNA intermediates that are reverse transcribed and new copies inserted in new genomic locations. They can be further divided into long terminal repeat retrotransposons (LTRs) and non-LTR elements (Weiss-Schneeweiss et al. 2015). DNA transposons, in contrast, move in the genome using cut and paste transposition mechanism with the help of transposase encoded by the elements themselves. They are excised from one genomic location and reinserted at another locus (Galindo-González et al. 2017). Retrotransposons are frequently found in centromeric regions and are known to also accumulate in sex or B chromosomes (Weiss-Schneeweiss & Schneeweiss 2013). The most dominant transposable elements in higher plant genomes are the LTR-retrotransposons.

The second group of repeats are tandem repeats. Since the focus of this thesis lies on the tandem repeats evolution, a more detailed overview of the subclasses and the characteristics of tandem repeats will be provided in the following sections.

1.1. Tandem repeats

Tandem repeats (TRs) owe their name to the fact that they consist of DNA motifs (monomers), arranged tandemly (as two or more copies) in direct orientation. Tandem arrays may occur as large sized homogenous arrays in heterochromatin, in interstitial, pericentric/centromeric or subtelomeric regions of chromosomes, or as smaller arrays dispersed in multiple smaller loci along the chromosomes (Zakrzewski & Schmidt 2017).

The function of tandem repeats is still unknown except for two specific types of tandem repeats – rRNA gene arrays that are the only tandemly repeated coding sequences in the genome and telomeres which protect chromosome ends from being shortened as well as from being processed (Garcia et al. 2012; Chan et al. 2004). Tandem repeats are not essential for the formation of functional centromeres, although they are often present in centromeric regions (Melters et al. 2013). It is hypothesized, that they serve as placeholders for epigenetic signals, that guide heterochromatin formation and function in the repair of double strand breaks (Bilinski et al. 2017).

The repetitive sequences show concerted evolution, which implies that the repeatomes of diverging species change non-independently, resulting in sequence similarity of the repeating units being higher within than among species (Belyayev et al. 2019). Homogenization of monomers of a tandem repeat family in a genome allows rapid spread and fixation of mutations throughout the majority of monomers (Pavlek et al. 2015).Tandem repeats are very dynamic in the genome. They originate, spread, amplify and reduce their copy numbers very rapidly in the evolution, compared to the coding part of the genome. Polymerase slippage is proposed to be the major mechanism generating simple tandem repeat sequences whilst generation of longer tandem sequences is thought to occur through other processes, often using small regions of already repeated nature that are further amplified (Lower et al. 2018). Already existing arrays of tandemly repeated sequences can undergo unequal recombination resulting in rapid changes of repeat copy numbers in the arrays (Plohl et al. 2008). Another mechanism that potentially leads to the amplification and sequence homogenization of tandem repeats is the recombination-based formation of extrachromosomal circular DNAs (from existing tandem repeat arrays), which then serve as a template for rolling circle replication followed by reintegration of the products into the genome at a different genomic location (Cohen & Segal 2009). Since the amplification

as well as the elimination of repeats can occur either before or after species divergence, they are useful tools in the analyses of genome evolution accompanying speciation (Waminal et al. 2018). Due to their dynamics, tandem repeats often show a unique distribution in chromosomal regions of related taxa or even individuals and thus, they are excellent tools for individual chromosome identification.

1.1.1. Satellite DNAs

The satellite DNAs (satDNA) are highly repetitive noncoding DNA sequences located in the constitutive heterochromatin, that form arrays by lining up one repetitive sequence after the other (Louzada et al. 2020). Based on the monomer length, tandem repeats are usually divided into three types: microsatellites, minsisatellites and satellite DNAs. Whilst the microsatellites consist of tandem repeats of 2-6bp in length (typically in arrays of less than 1kb), minisatellites are characterized by monomers sizes of 10 to 100 bp (Garrido-Ramos 2017). Although the satellite DNAs are present in all eukaryotic genomes, their sequence composition is highly variable and most families are specific to one genus, or even a species (Vondrak et al. 2019).

SatDNAs are tandemly repeated units (monomers) arranged in long arrays (up to several megabases) in one to many chromosomal locations. The monomer lengths typically were hypothesized to predominantly be between either 150-180 bp or 300-360 bp in size (Meštrović et al. 2015), but recent studies using novel technologies suggest that monomer lengths can range from 100 bps to thousands of bps (Weiss-Schneeweiss et al. 2015). These arrays often form blocks of condensed chromatin with specific structural and epigenetic properties. Since the recombination between and within the arrays may result in the loss of some arrays, the chance of satellite DNA preservation in heterochromatic regions is higher, because of low recombination levels (Lower et al. 2018).

The origin of satellite DNAs typically requires *de novo* duplication of a sequence motif at a genomic site, subsequent amplification and spreading throughout the genome as well as local amplification (Duílio et al. 2017). Several different origins of satellite repeats have been demonstrated. These include the generation of satellites by tandem amplification of various genomic sequences (Gong et al. 2012), repair of static replication forks (Kuzminov 2016) as well as by extension of already existing short tandem repeats within e.g. rDNA spacers or hypervariable regions of LTR retrotransposons (Vondrak et al. 2020).

Related species often share a set of satDNA families inherited from their common ancestor. This “library hypothesis” (Fry et al. 1977) proposes that related organisms share a common set of satellite DNAs. In each of the descendant species, certain variants of these sequences may be amplified while others might be reduced in copy numbers or even be entirely removed, generating unique but to some extent overlapping, sets of satellite DNAs in closely related species (Fry et al. 1977). The appearance of a

satellite DNA in a taxon might therefore also result from amplification of already pre-existing, albeit in very low copy number, satellite DNA from such library (Duílio et al. 2017). However, novel families of satellite DNAs can also arise *de novo* after species divergence.

1.1.2. rRNA genes

Ribosomal RNAs (rRNAs) and ribosomal proteins are the molecular building blocks of the small (40S) and the large (60S) ribosomal subunits (Salim & Gerton 2019). Together, these subunits form ribosomes, which are macromolecular machines whose major task is the production of proteins in a process called translation. Since ribosomes are essential for protein synthesis, all of the living organisms possess at least one locus of tandemly repeated 5S and 35S rRNA monomers that contain the genes and various spacers. The 35S ribosomal RNA (rRNA) genes together with the 5S ribosomal RNA genes are the only existing coding tandem repeats in the transcriptome of the organism. Each eukaryotic genome carries at least one chromosome pair with a secondary constriction or satellite, in which 35S rRNA genes are located. These structures are also called nucleolus organizer regions (NORs).

35S rDNA (also referred to as 45S rDNA) comprises three rRNA genes (18S-5.8S-25/28S), separated by two noncoding internal transcribed spacers called ITS1 and ITS2 as well as ETS (external transcribed spacer) and a complex intergenic spacer (IGS) between the adjacent repeats (Roa & Guerra, 2015). The 35S rDNA is transcribed by RNA polymerase I (Garcia & Kovařík 2013). The 5S rDNA monomers are composed of 121 bp of 5S rRNA gene and adjoining non-transcribed spacer of different length (Weiss-Schneeweiss & Schneeweiss 2013). The gene is transcribed by RNA polymerase III (Abascal-Palacios et al. 2018).

The ribosomal DNA repeats may occur at one or many loci in the genome. In most cases the 35S rRNA and 5S rRNA genes are localized separately on the chromosomes (S-type arrangement; Garcia et al. 2009). In several plant groups, however, they are physically linked in the same unit, with secondary type monomers that include both 5S rDNA monomers inserted within the IGS region of 35S rDNA. This “L-type arrangement” is found in some clades of Asteraceae (Garcia et al. 2009), in Gymnosperms (Garcia & Kovařík 2013) and in some early land plants (Sone et al. 1999; Weiss-Schneeweiss & Schneeweiss 2013). However, even in linked configuration, 5S and 35S rDNAs are transcribed independently and by different polymerases. The ribosomal RNA genic sequences within an array are very homogenous, though their non-coding regions do diverge and thus differ even between closely related species (Eickbush & Eickbush 2007).

All these described features make both satellite DNAs and rDNA loci (especially when combined) perfect tools for the analysis of karyotype evolution accompanying and following diversification and speciation in plants. They also provide tools to identify at least some chromosome pairs in analyzed species for comparative evolutionary analyses.

2. *Capsicum*

The genus *Capsicum*, commonly referred to as "chili" or "pepper belongs to the family of the *Solanaceae* that comprises around 100 genera and 2500 species (Olmstead et al. 2007). The genus comprises approximately 35 species distributed in tropical and temperate Central and Southern Americas distributed from Mexico through Brazil and Paraguay to Central Argentina (Carrizo García et al. 2016). All species of the genus are subdivided into eleven clades: Annuum clade, Baccatum clade, Tovarii clade, Pubescens clade, Purple Corolla clade, Longidentatum clade, Bolivian clade, Flexuosum clade, Antlantic Forest clade, Caatinga clade and Andean clade (Barboza et al. 2019; Figure 1)

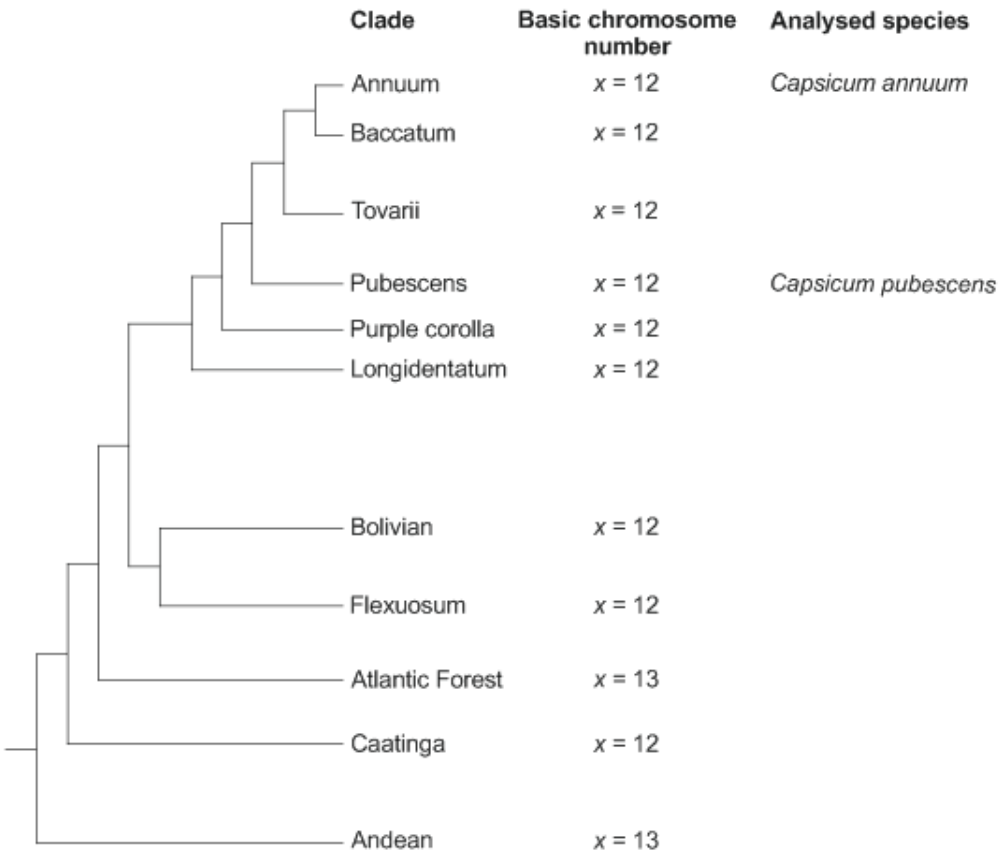

Figure 1: Phylogenetic relations of *Capsicum* clades, the basic chromosome numbers of clades and the analyzed species of the Annuum and Pubescens clades (modified from Garcia et al. 2016).

Nearly all *Capsicum* species produce unique secondary metabolites named capsaicinoids. They are responsible for the typical "hot" taste of the fruits (Stewart et al. 2007). The main compounds of this alkaloid group are capsaicin and dihydrocapsaicin (Aza-González et al. 2011). They are synthesized via complex reactions involving branched chain fatty acid pathways (Mazourek et al. 2009). The biosynthesis and the later accumulation of capsaicin take place in the placenta of the fruits and, in some exceptionally hot taxa, in the pericarp (Bosland et al. 2015). It has been shown that the enrichment of capsaicin in the fruits protects the plants from herbivores but does not ward off birds. Whilst mammals feel a hot sensation as capsaicin binds to their pain receptors, birds do not - due to a genetic divergence in the transient receptor potential vanilloid 1 (TRPV1) (He et al. 2019). Thus, birds facilitate seed dispersal of peppers (Tewksbury et al. 2001). Capsaicinoids are known to have antifungal properties and the ability to deter invertebrate pests (Romero-da Cruz et al. 2017). They are also thought to have beneficial effects on the human body and are used to treat a variety of medical conditions. Historically they were used as analgesics but nowadays their application ranges from treatment of neurological disorders, bladder and digestive syndromes to cancer (Hayman & Kam 2008; Chapa-Oliver & Mejia-Teniente 2016).

The pungency of chilis is measured using an organoleptic scale called the "Scoville scale".The Scoville heat units (SHU) indicate the amount of capsaicin that can be tasted in a given pepper species or variety. The hotness of the fruits, a unique feature of most of the *Capsicum* species, is thought to be the major reason for *Capsicum* domestication. They were used as first spices as early as 6000 years ago (Hill et al. 2013) leading to the present worldwide cultivation of five species.

Three of the cultivated species belong to the *Capsicum annuum* complex – *Capsicum annuum, Capsicum chinense* and *Capsicum frutescens*. The remaining two domesticated species, *Capsicum baccatum* and *Capsicum pubescens* are mainly cultivated locally in Latin America (Carrizo García et al. 2016). Over the whole domestication history of the peppers, human-assisted selection favored several specific traits of the fruits, including their levels of pungency, color, shape, flavor and desiccation rate (Paran et al. 2004). Very specific selection of traits in the domesticated *Capsicum* species also proves to be disadvantageous, because domesticated species often lose their ability to grow in the wild.

In this study, the analyzed species are *Capsicum annuum* and *Capsicum pubescens*. Therefore, the next paragraphs are dedicated to their more detailed description.

2.1. *Capsicum annuum* (Figure 1)

Capsicum annuum var. *annuum* is the worldwide most commonly cultivated *Capsicum* species and was the first *Capsicum* species brought to Europe by Columbus and other new world explorers (Hill et al. 2013). Hundreds of varieties of *Capsicum annum* exist with a broad variation in fruit shape, leave size and overall plant structure. The wild form of *Capsicum annuum* var. *annuum* is its progenitor *C. annuum* var. *glabriusculum*, which has small, round, erect, highly pungent and soft-fleshed fruits (Romero-da Cruz et al. 2017).

2.2. *Capsicum pubescens* (Figure 1)

Whilst the other four domesticated *Capsicum* species, *C. annuum, C. chinense, C. frutescens,* and *C. baccatum*, possess mostly white flowers, glabrous leaves and stems, *Capsicum pubescens* has purple flowers as well as pubescent leaves and stems (Park et al. 2000). Furthermore, it can grow taller than the other domesticated species, reaching a maximum height of nine meters. In contrast to the other domesticated species, no wild progenitor is known.

3. *Capsicum* genomes

All *Capsicum* species are diploid with basic chromosome numbers of either $x = 12$ or $x = 13$ (Moscone et al. 1995). Only the Andean and the Atlantic Forest clade *Capsicum* species possess a basic chromosome number of $x = 13$ (Carrizo García et al. 2016), whereas all other species have $x = 12$.
Comparison of different genera of the *Solanaceae* family has shown that the *Capsicum annuum* and *Capsicum chinense* genomes are about four times larger than the tomato genome (also family Solanaceae) due to accumulation of repetitive sequences in heterochromatic as well as euchromatic regions (Kim et al. 2014). Within the *Capsicum* genus, species vary nearly 10-fold in genome sizes from 0.8pg to 7.5pg (1C) mostly due to the differential accumulation of repeats (H. Schneeweiss, unpubl.), in the complete absence of polyploidy. Whole genome sequencing of several cultivated species revealed that around 60-83% of the genomes consist of repeats, with Ty3-*gypsy* retrotransposons dominating (de Assis et al. 2020).

3.1. rDNA in *Capsicum*

35S and 5S rDNA loci have been mapped in various *Capsicum* species and an extensive variation in loci number has been reported, both among and within species. In the following paragraphs, an overview of the obtained results is presented.

In all *Capsicum* species one 5S rDNA locus per haploid genome (= two signals per diploid genome) has consistently been reported (Aguilera et al. 2016; Romero-da Cruz et al. 2017; Zhou et al. 2019). This 5S rDNA locus is usually positioned on a metracentric chromosome pair at a conserved and, in most species, intercalary position (Scaldaferro & Moscone 2019). The 35S rDNA loci in *Capsicum* are much more variable, ranging in numbers from one locus to more than 30 loci. They often coincide with GC-rich heterochromatic regions, especially if the loci numbers are high. Therefore, it has been hypothesised that non-coding parts of 35S rDNA could have given rise to novel satellite DNA and thereby have an effect on the total genome size variation. The active 35S rDNA loci numbers might thus be much lower in some of the species (e.g. *Capsicum pubescens* - Scaldaferro et al. 2006).

3.1.1. rDNA in *Capsicum annuum*

The number of reported 35S rDNA loci is highly variable between different *Capsicum annuum* varieties and even individuals. The cultivar “Ají balita” possessed four major signals, “Bolivian rainbow” two major and 12 minor signals and “Ají quitucho grande” two major and 14 minor signals (Romero-da Cruz et al. 2017). One pair of major and two pairs of minor signals of 35S rDNA were reported in accession number IT032384 (Zhou et al. 2019). One major and two small loci of 35S rDNA were reported in *C. annuum* L. var. *annuum* (cytotype 1) and two major and two small loci in *C. annuum* L. var. *annuum* (cytotype 2) (Scaldaferro & Moscone 2019). Between one to six loci of 35S rDNA were found in six different analyzed cytotypes of *C. annuum* L. var. *glabriusculum* (Scaldaferro & Moscone 2019). Only one locus of 5S rDNA has been found in all analyzed varieties.

3.1.2. rDNA in *Capsicum pubescens*

Different numbers of 35S rDNA loci have also been reported for *Capsicum pubescens*. As many as 26 distal rDNA loci were reported in the cultivar “Locoto rojo” (Grabiele et al. 2018), 14 loci (12 major plus two small) were reported in other accessions (Scaldaferro & Moscone, 2019). This variability in the number and location of 35S rDNA in cultivated *Capsicum* species suggests that rDNA evolution involves dynamic and ongoing loci loss and/or gain or amplification of novel rDNA-related satellite DNAs.

3.2. Satellite DNAs in *Capsicum*

Analysis of intergenic-spacer sequences (IGSs) regions positioned between the 25S and 18S rDNAs in five *Capsicum* species revealed, that it consists of a central repeated region, which is variable in number of subrepeats between species and two flanking regions (Jo et al. 2011). It has also been suggested, that part of IGS region gave rise to satellite DNA in *C. annuum*, *C. frutescens, C. baccatum*, C. *chacoense* and *C. chinense* (Jo et al. 2011). Major repeats in the genome of *Capsicum annuum* have been analyzed using RepeatExplorer (Novak et al. 2013). One putative tandem repeat, a 167-bp long satellite DNA repeat called "Ca167TR", has been tested as a cytogenetic marker. Two pairs of Ca167TR signals have been detected in subtelomeric regions of two chromosomes in *C. annuum* (Zhou et al. 2019). No other data on tandem repeats in the genus *Capsicum* are available to date.

4. Aims of the study

The levels of reported variation in number and location of 35S rDNA loci, as well as the lack of information about the genomic abundance and chromosomal location of satellite DNAs in *Capsicum* species underline the importance of further investigation. The recent developments and further improvements of next generation DNA sequencing, as well as genomic and novel analytic bioinformatics tools offer the unprecedented possibility to broaden our knowledge about types, distribution, variability and evolution of repetitive DNAs in any plant genome.

Thus, the aims of this study are to analyze the abundance and localization of selected satellite DNAs as well as 5S and 35S rDNAs in two cultivated *Capsicum* species, *C*. *annuum* and *C. pubescens*. The analysis of the localization of three satellite DNAs in three individuals of *C. annuum* and two individuals of *C. pubescence*, all belonging to different cultivars/varieties, together with localization of 5S and 35S rRNA genes allowed for:

(1) comprehensive characterization of the basic set of chromosomes of these two species,

(2) analyses of intraspecific variation of rDNA and satDNA loci and

(3) identification of at least some chromosome pairs in both species providing chromosomal markers for the chosen cultivars.

Thereby this study aims to understand the dynamics and variation of the selected tandem repeats during the evolution of peppers accompanied by domestication. It also provides further first detailed insights into the diversity of the repeatome in genus *Capsicum*.

Material and Methods

1. Plant Material

Two cultivated species of *Capsicum, Capsicum annuum* and *Capsicum pubescens,* were analyzed. Five individuals in total, one individual per each of three varieties of *C. annuum* and two varieties of *C. pubescens*, were used for analysis. All of the plants were cultivated in the Botanical Garden of the University of Vienna.

Table 1: List of used *Capsicum* species, variety and individuals with their common names and origin of the cultivated plants

Species	**Accession/Individual**	**Common name/variety**	**Origin**
C. annuum	50/2	Ají Balita	local market, Salta, Argentina
C. annuum	61/3	Dwarf Chiltepin	La Palma Seeds Company, Spain
C. annuum	20141/2	-	-
C. pubescens	3858/2	Locoto	Apa Apa, La Paz, Bolivia
C. pubescens	56/1	Locoto	Guatemala

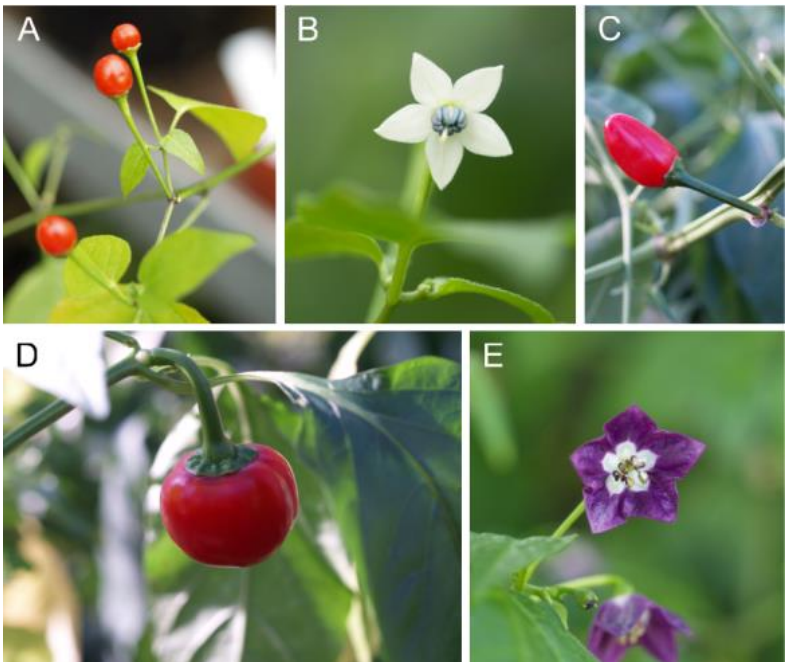

Figure 2: Flowers and fruits of used *Capsicum* cultivars; Fruits (A) and flower (B) of *C. annuum* variety *Dwarf Chiltepin*; (C) Fruit of *C. annuum* variety *Ají Balita*; (D) Fruit of *C. annuum* accession 20141 ; (E) Flowers of *C. pubescens* accession 3858;

2. Root pre-treatment and fixation

Actively growing root tip meristems were transferred into small bottles filled with tap water. Soil particles were washed off the roots using a pipette. This step was repeated several times until the roots were completely clean. The material was then transferred into bottles filled with 0.002M 8-hydroxyquinoline and incubated for 2.5 hours at room temperature and 2.5 hours at 4°C. The 8-hydroxyquinoline was removed from the bottles and the root tips were gently but thoroughly washed with tap water. After the last wash, the water was removed and the material was fixed in freshly prepared fixative (3 parts of ethanol: 1 part of acetic acid) for a few hours with occasional mixing. The fixed material was stored at -20°C in the freezer until use.

3. Fluorescence *in situ* hybridization (FISH)

The enzymatic digestions of the roots, chromosome preparation as well as FISH were performed following the protocol of Jang & Weiss-Schneeweiss et al. (2015) with some modifications.

3.1. Enzymatic digestion and chromosome preparation

The fixed roots were removed from the fixative, transferred into the embryo glass filled with citric buffer (pH 4.8) and incubated at room temperature for 16 minutes to wash off all fixative. Subsequently the material was incubated for 16 minutes in an enzyme mixture containing 0.4% pectolyase (Sigma-Aldrich, Vienna, Austria), 0.4% cytohelicase (Sigma-Aldrich, Vienna, Austria) and 1% cellulase (Serva, Heidelberg, Germany) in citric buffer (pH 4.8), in an incubator (Pol-Eko-Aparatura, Poland) at 37°C. After the removal of the enzyme mixture, the roots meristems were washed in citric buffer at room temperature for 16 minutes and then transferred into a drop of 45% acetic acid on glass slides. Digested material was fragmented, root caps were removed and a coverslip was added, before the material was squashed. The slides were then examined for quality (at least 15 well spread complete metaphase plates) under the phase contrast microscope (Carl Zeiss, Vienna, Austria). The good quality slides were frozen on a cold plate (-80°C) and the coverslips were removed. The slides were dried at room temperature and stored at 4°C until use.

3.2. Fluorescence *in situ* hybridization

Slides were refixed in ethanol: acetic acid (3:1) for 30 minutes, washed with 100% ethanol for 30 minutes to dehydrate the material and air dried for 1 hour at room temperature. To remove all single-stranded cytoplasmic and nuclear RNA, the slides were treated with RNase A in 2x SSC buffer (100 µg/ml) for 60 minutes at 37°C, followed by washing three times with 2xSSC for 5 minutes each. Slides were then incubated for 20 minutes in pepsin (10 mg/ml, pH 2) at 37°C, in order to digest all unwanted proteins. Subsequently, the slides were washed three times in 2xSSC for 5 minutes each, to remove pepsin, incubated for 5 minutes in 4% paraformaldehyde to crosslink chromosomal proteins (histones), and washed three times in 2xSSC for 5 minutes each wash. The slides were then incubated for 3 minutes in 70% ethanol and for 3 minutes in 90% ethanol (both at 4°C) to dehydrate the material and dried at room temperature for at least 30 minutes to 1 hour. The hybridization mix consisted of 0.02xSSC, 10% dextran sulphate (Promega, Madison, USA), salmon sperm (100ng/µl, Sigma-AldrichGmbH, Vienna, Austria), sterile water and 25-200ng of each of the labelled probes. Labelled satellite DNA probes were commercially synthesized (IBA, Germany). The rDNA probes were either directly labelled with biotin by PCR (5S rDNA) or labelled with digoxygenin using nick-translation kit (Roche, Vienna, Austria). The hybridization mix was denatured on a thermoblock (Eppendorf, Vienna, Austria) for 4 minutes at 96°C and immediately cooled on ice, to stabilize the single stranded DNAs. 8 µl of hybridization mix were added to each of the slides, covered with coverslip, and sealed with Fixogum. Slides with hybridization mix were denatured on a PCR *in situ* block (PeqlabGMBH, Erlangen, Germany) for 4 minutes at 72°C, followed by 1 minute at 65°C, 1 minute at 55°C and 1 minutes at 45°C. Preparations were then transferred into the incubation box and incubated overnight in an incubator (Pol-Eko-Aparatura, Wodzisław, Poland) at 37°C. Fixogum and coverslips were removed in 2xSSC at room temperature. In order to remove the remaining hybridization mix, unbound and weakly (unspecifically) bound probes, stringent washes in 2xSSC, 0.1xSSC and 2xSSC+0.2% Tween20 (each for 5 minutes at 39°C) followed. Subsequently, the blocking solution containing 5% bovine serum albumin (Sigma-Aldrich GmbH, Vienna, Austria) in 2xSSC+2% Tween20 was added to each of the slides and slides were incubated for 20 minutes at 37°C. The blocking solution was then replaced by detection solution (blocking solution with appropriate antibodies conjugated with fluorochromes). Digoxygenin was detected using anti-digoxygenin conjugated with FITC (5 µg/ml; Roche, Vienna, Austria) and biotin was detected using streptavidin conjugated with Cy3 (2 µg/ml; Sigma-Aldrich, Vienna, Austria). The detection was carried out at 37°C for 1 hour. Two washes in 2xSSC and one wash with 2xSSC+0.2%Tween20 were performed for 5 minutes, each at 42°C, to wash off the excess of the detection solution. The drained, but still wet slides, were then mounted in antifade buffer Vectashield (Vector Laboratories, Burlingame, California)

containing 2 µg/ml DAPI (Sigma-AldrichGmbH, Vienna, Austria) and covered with fresh coverslips. Ready slides were stored at 4°C at least overnight for the fluorescence to stabilize. Slides were analyzed using an AxioImager M2 epifluorescent microscope (Zeiss, Vienna, Austria) and the images were acquired with a CCD camera and AxioVision 4.8 software (Zeiss, Vienna, Austria).
Images were contrasted in Adobe Photoshop using only those functions that equally applied to whole images. Chromosomes were cut out in Corel Photo Paint X7, and figures arranged in CorelDraw X7 (Corel Corporation, Ottawa, Canada).

4. Flow Cytometry

The isolation of the nuclei and the propidium iodide staining were performed following the protocol of Temsch (2003), based on Baranyi and Greilhuber (1996) with minor modifications (E.M. Temsch, unpubl.). 25 mg of young leaves were collected from each of the five *Capsicum* individuals as well as from the reference plant (*Solanum pseudoCapsicum,* 1C = 1.2946 pg; Temsch et al. 2010) used as internal standard. The material of each individual was co-chopped (Galbraith et al. 1983) together with material of *S. pseudoCapsicum* in 1 ml isolation buffer (pH 1.5) containing 0.1 M citric acid monohydrate and 0.5 % Triton® X-100 (both Merck, Darmstadt, Germany). One additional preparation per experiment contained only leaf material of the standard and was used in order to identify the standard G_1 peak. The chopped material was filtered through a 30 µm nylon mesh (Saatilene Hitech, Sericol, Germany), to remove any large cellular fragments, that could lead to blockage of the flow chamber. The filtered isolate was incubated with RNase A (0.15 mg/ml; Sigma-Aldrich GmbH, Vienna, Austria) for 30 minutes at 37°C, to eliminute extant RNAs. Propidium iodide (Sigma-Aldrich GmbH, Vienna, Austria) was dissolved in disodium hydrogen phosphate buffer to a final concentration of 50 µg/ml (pH ~ 9.5). Each sample was aliquoted into two test tubes and 2 ml of PI solution was added to each tube. The samples were incubated in the refrigerator (4°C) for 60 minutes to stain the DNA before the final measurement. This preparation protocol using one acidic and one basic buffer during preparation, followed the method published by Otto et al. (1981). Each round of measurement with the CyFlow ML flow cytometer (Partec, Muenster, Germany; green laser equipped: 532nm, 100mW, Cobolt Samba, Cobolt AB, Stockholm, Sweden) consisted of one run of the standard, followed by three runs per sample (3333 particles detected by each run). FloMax software (Partec) was used to measure the fluorescence intensity of each particle and a Gaussian model was applied on the linear scale, to calculate the mean fluorescence intensity of the sample.

Calculation of the 1C-value was performed using the formula:

$$1C - value\ of\ sample\ (pg) = \frac{(mean\ G_1\ peak\ position\ of\ sample)}{(mean\ G_1\ peak\ position\ of\ standard)} \times\ standard\ 1C\ value\ (pg)$$

5. Next- generation sequencing and satellite DNAs identification

Genomic DNAs, of both cultivated *Capsicum* species analyzed in the study (*Capsicum annuum* and *Capsicum pubescens*), were isolated. Leaf material was harvested from plants grown in the Botanical Garden of the University of Vienna and the isolation was performed following a CTAB protocol (Jang & Weiss-Schneeweiss 2015) with minor modifications. The quality of the extracted genomic DNAs was checked on agarose gel, and the DNA concentration measured using NanoDrop - fluorospectrophotometer and Quant-iT Picogreen dsDNA assay kit (PeqLab, Austria). The fragmentation of the DNAs (into 600-800 bp fragments) as well as the library construction were performed by CSF-NGS (Vienna Biocenter, Austria). DNAs were sequenced using 150 nt paired-end Illumina HiSeq2500 technology (Illumina, San Diego, CA, USA).Two libraries were separately prepared and independently sequenced for each of the analyzed individuals. The read pairs were checked for their quality, adaptors, *PhiX* spike-in DNA (Illumina), and all organellar DNAs were filtered out. Analysis of the data was performed using the default settings in RepeatExplorer (Novák et al. 2013; http://repeatexplorer.org) The clustering was done separately for each species, but the reads of the separately sequenced libraries of each species were pooled together, to alleviate differences in the library preparations. RepeatExplorer performs a blast comparison of all-to-all reads. The resulting graphs are thus composed of similar DNA sequence reads connected by edges reflecting their similarity (Novák et al. 2010). The automatic annotation (implemented in RepeatExplorer) of clusters was verified manually and resulted in the identification of several tandem repeats per species, among other repeat types. Several of these tandem repeats were then selected for FISH experiments.

Results

1. Genome size measurements of the two analyzed *Capsicum* species

Genome sizes of the five analyzed individuals were measured by flow cytometry. The DNA content of the unreplicated haploid nuclei (1C - value in picograms [pg]), the coefficients of variations (CV) and the standard deviations (SD) are shown in Table 2.

Table 2 – List of analyzed *Capsicum* individuals with measured genome sizes in pg, the corresponding coefficients of variations and standard deviations.

Species	Population	Means of genome size (pg)	CV% of measurements	SD
C. annuum	50	3.4894	0.0545	0.0019
C. annuum	61	3.4837	0.0431	0.0015
C. annuum	20141	3.4585	0.0520	0.0018
C. pubescens	3858	4.4968	0.0867	0.0039
C. pubescens	56	4.4787	0.3260	0.0146

The genome size (1C value) of the *Capsicum annuum* individual representing population 50 was 3.49 pg, the individual from population 61 had a genome size of 3.48 pg and the individual from population 20141 had a genome size of 3.46 pg (Table 2). The unreplicated, haploid nuclei of the *Capsicum pubescens* individual representing population 3858 had a genome size of 4.50 pg and that of population 56 had a genome size of 4.48 pg (Table 2). Thus, the genome size of *Capsicum annuum* individuals was on average 3.48 pg, whereas *Capsicum pubescens* individuals had an average genome size of 4.64 pg per haploid unreplicated nucleus.

The coefficients of variation varied between 0.04 in *C. annuum* accession 61 and 0.33 in *C. pubescens* accession 56. All the CVs of the G0/G1 peaks fulfilled the requirement to be below 3% and were therefore accepted.

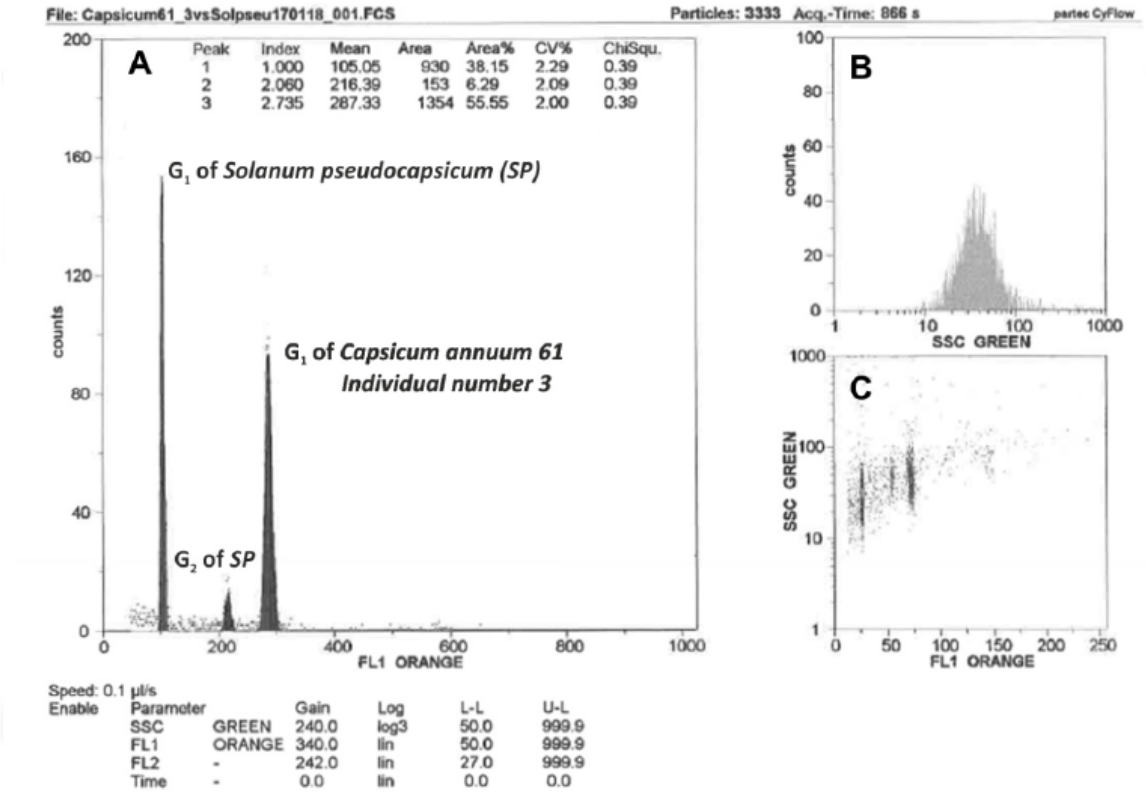

Figure 3 – An example of flow cytometry histogram: (A) DNA content of *C. annuum* 61/3 and the internal standard of *S. pseudocapsicum* (B) side scatter histogram (C) cytogram fluorescence (FL1) vs side scatter.

2. Identification of tandem repeats in the two analyzed species (NGS data)

The RepeatExplorer pipeline was used to analyze the repetitive DNA profiles of *Capsicum annuum* and *Capsicum pubescens.* Among other types of repeats, three relatively abundant and shared tandem repeats were identified (H. Schneeweiss, unpubl.). Satellite DNA 1, from now on referred to as CapSat1, had monomer length of 37 bp and was found in both analyzed species. Satellite DNA 4 (referred to as CapSat4) had a monomer sequence length of 33 bp. Satellite 5 (CapSat5) had basic monomer length of 75 bp. These three satellite DNAs were selected for more detailed analyses and were mapped in chromosomes of five selected individuals of these two *Capsicum* species, using FISH.

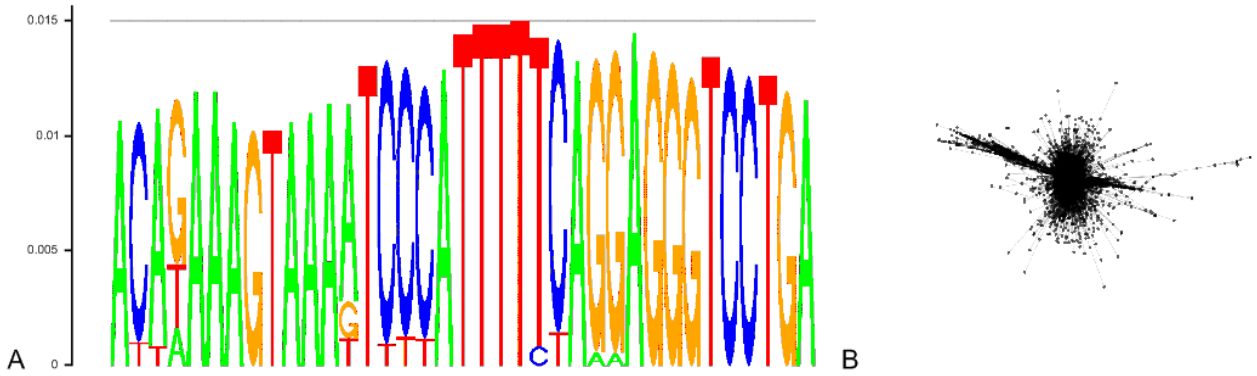

Figure 4 – Monomer characterization of CapSat1: (A) monomer sequence logo (B) graph layout identified by RepeatExplorer

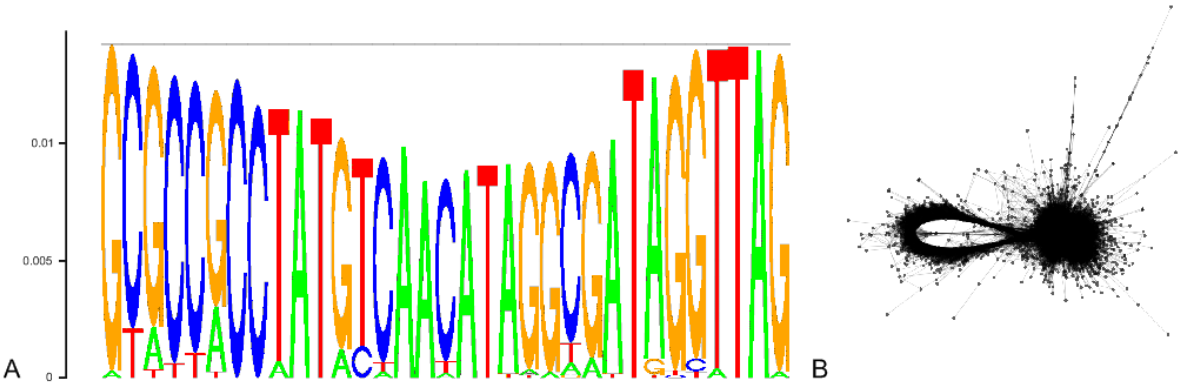

Figure 5 – Monomer characterization of CapSat4: (A) monomer sequence logo (B) graph layout identified by RepeatExplorer

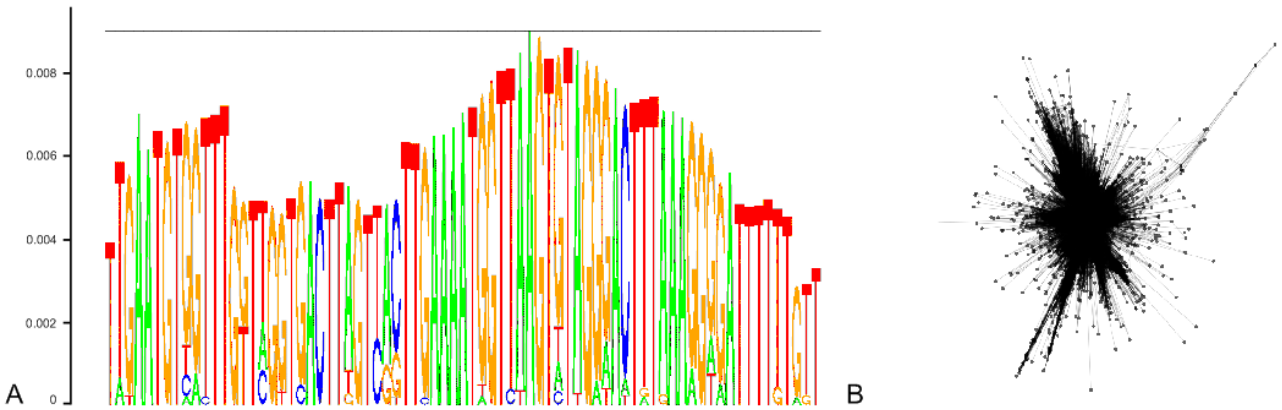

Figure 6 – Monomer characterization of satellite CapSat5: (A) monomer sequence logo (B) graph layout identified by

Table 3 – The characterization of three satellite DNAs used for FISH analysis

Satellite DNA name	Cluster	Genome proportion (%) in *C. annuum*	Genome proportion (%) in *C. pubescens*	Consensus monomer length (bp)	Consensus monomer sequence
CapSat1	CL78	0.18	0.60	37	ACAGAAAGTAAAATCCCATTTT TCAGGAGGGTCCTGA
CapSat4	CL151	0.02	0.43	33	GCGCCGCCTATGTCAACATAGG CGATAGGTTAG
CapSat5	CL72	0.10	0.23	75	TTGAATGTGGTTTGGTTGGTGAC TTAGTTACTTGAAAATGGTTAAG TGTAGGGACTTTAAAGGGGATTT TTTGTT

3. Mapping of tandem repeats in chromosomes of two selected cultivated *Capsicum* species using fluorescence *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization was used to map two rDNA probes (5S and 35S rDNA) as well as three chosen satellites (CapSat1, CapSat4 and CapSat5) in chromosomes of three individuals of *C. annuum* representing three populations and two individuals of *C. pubescens* representing two populations. In order to analyze relative positions of all the probes in the chromosomes within each karyotype, different combinations of the rDNAs and the satellite DNAs (Table 3) were used (Figs. 7-31).
Because of the similar size of all chromosomes and difficulty to unequivocally identify short and long arms, the signals were mapped in chromosomes without indicating the position of their centromeres. The order of the chromosomes was guided by the relative localization of the probes, and therefore did not necessarily correspond to numbering of the pairs of chromosomes in other papers.

3.1. *Capsicum annuum*

3.1.1. *C. annuum* accession 20141 individual 2

One 5S rDNA locus was found at a subterminal position of chromosome 5 (Figures 7A, 11) and one subterminal 35S rDNA locus was located in one of the arms of chromosome 11 (Figures 7, 8, 11).

In total, eleven loci of CapSat1 per haploid set of chromosomes were found in this individual, nine major ones, consistently found in all the cells and two minor ones, variable and detectable in only some cells (Figures 7B, 9, 11). Major loci were found on chromosomes 1, 2, 3, 4, 5, 8, and 11. Chromosomes 1, 3 and 4 each carried one small CapSat1 locus at a subterminal position within one of the arms. Another small subterminal CapSat1 locus was found in one of the arms of chromosome 8 (Figures 7B, 9). Chromosome 2 possessed two larger (one subterminal and one interstitial) loci of CapSat1 located in close proximity to one another within the same chromosomal arm and one small locus in subterminal position in the other chromosomal arm. One large locus of CapSat1 was found in the subterminal region of one of the arms of chromosome 5. Chromosome 11 possessed a large, subterminal CapSat1 locus in close proximity to and within the same chromosomal arm as the more subterminally located 35S rDNA locus. The two minor CapSat1 loci were found on chromosomes 5 and 6. On chromosome 5, the minor locus was found subterminally and in close proximity to the 5S rDNA locus. The second minor CapSat1 locus was located subterminally within one arm of chromosome 6.

Overall ten loci of CapSat4 were observed in this individual in eight out of the twelve chromosomes (Figures 8C, 9E, 10G, 11). Five of these loci were major and the other five were minor. The major loci were found on chromosomes 3, 4, 5, 9 and 11. Chromosomes 3 and 4 each had one small subterminal CapSat4 locus next to the CapSat1 locus. On chromosome 5 one small CapSat4 locus was found in the subterminal region of one arm between the minor CapSat1 locus and the major CapSat5 locus. Another small, major and subterminally located CapSat4 locus was observed on one arm of chromosome 9. Chromosome 11 had one large CapSat4 locus in interstitial position within the same arm as the subterminally located CapSat1 and 35S rDNA loci. The five minor CapSat4 loci were found within chromosomes 2, 8 and 12. Chromosomes 2 and 8 each showed two minor loci (one subterminal and one interstitial) within the same arm as the subterminally located CapSat1 locus. One small minor CapSat4 locus was found within the interstitial region of one arm of chromosome 12.

Altogether, five major loci of CapSat5 have been found in *C. annuum* accession 20141 individual 2 (Figures 8D, 9F, 10G, 11). These loci were observed on chromosomes 1, 2, 5 and 11. Chromosome 1 possessed two major CapSat5 loci within the same arm. One larger subterminally located locus and one smaller interstitial locus. Chromosome 2 carried one large CapSat5 locus close to the centromere (Figures 8D, 9F, 10G). One small CapSat5 locus was found on chromosome 5 within the same arm that also carried the more subterminally located 5S rDNA, CapSat1 and CapSat4 loci. Chromosome 11 had a subterminally located CapSat5 locus within the arm not carrying the 35S rDNA, CapSat1 and CapSat4 loci.

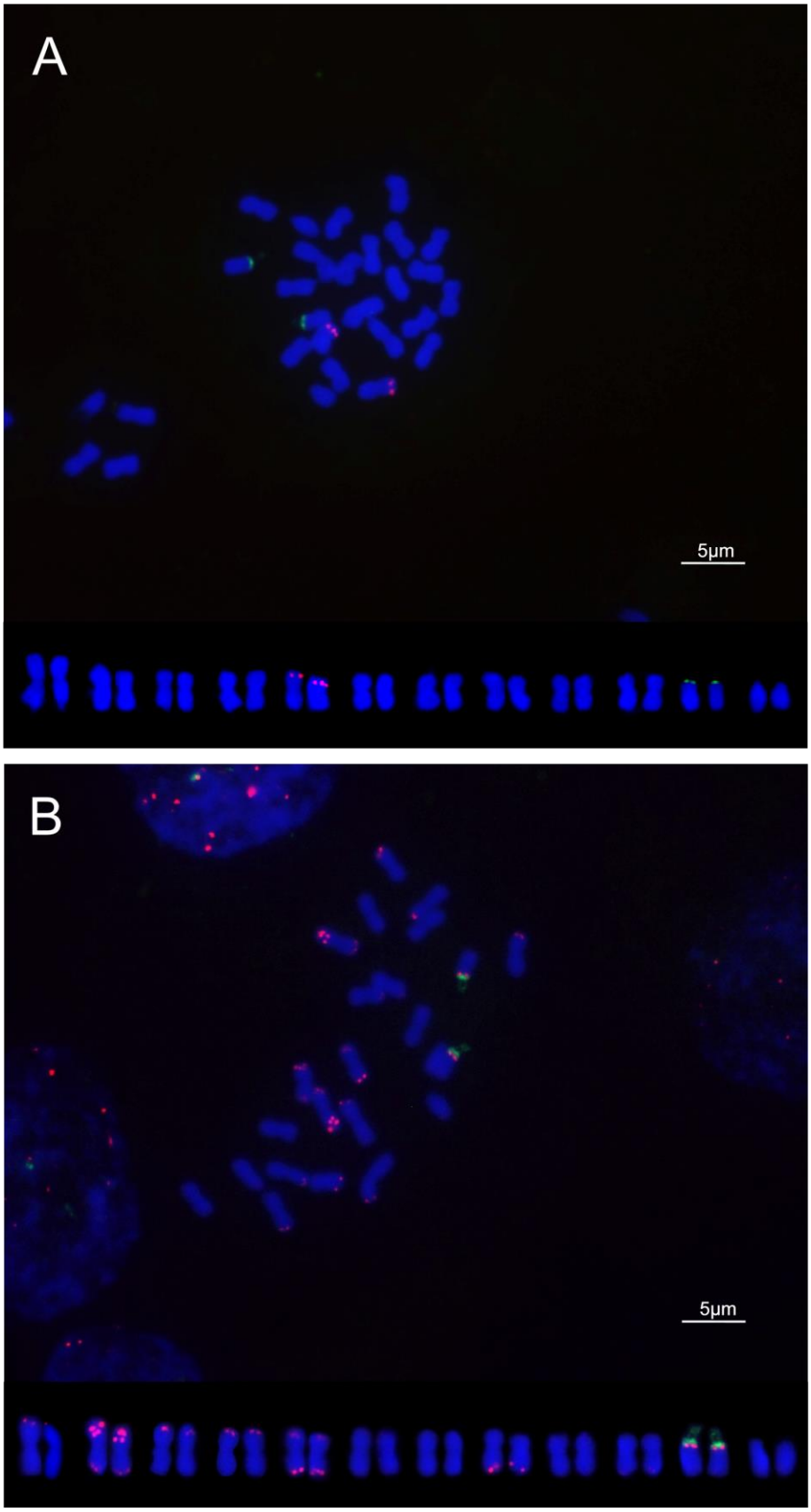

Figure 7 - Localization of rDNAs, and satellite DNAs in chromosomes of *C. annuum* accession 20141/2 .Cut-out chromosome pairs are arranged by size and probe localization across Figures 7-11 to allow for comparative analysis among individuals. Probe combinations: (A) 35S rDNA (green) and 5S rDNA (red), (B) 35S rDNA (green) and CapSat1 (red).

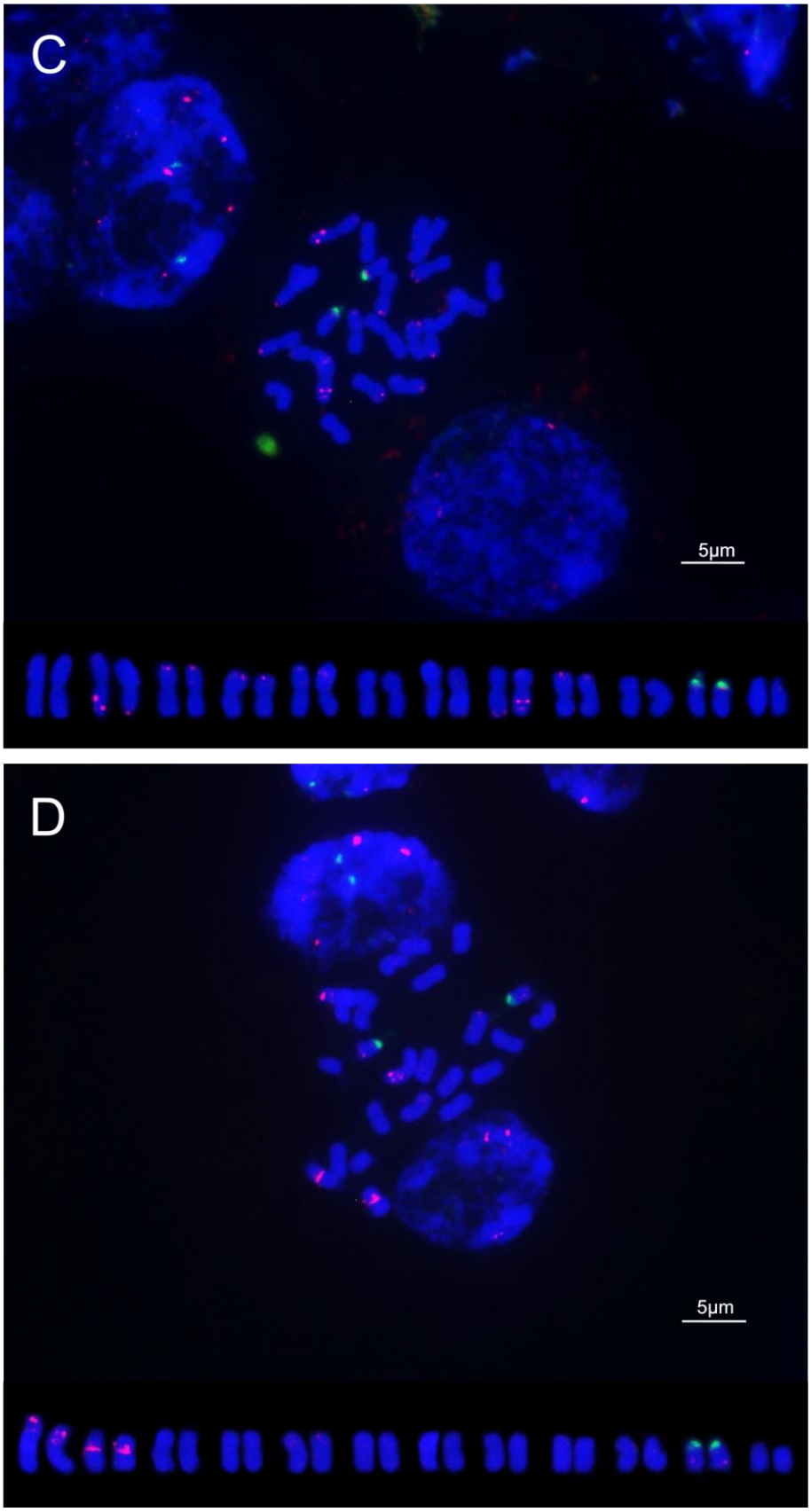

Figure 8 - Localization of 35S rDNA, and satellite DNAs in chromosomes of *C. annuum* accession 20141/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 7-11 to allow for comparative analysis among individuals. Probe combinations: (C) 35S rDNA (green) and CapSat4 (red), (D) 35S rDNA (green) and CapSat5 (red).

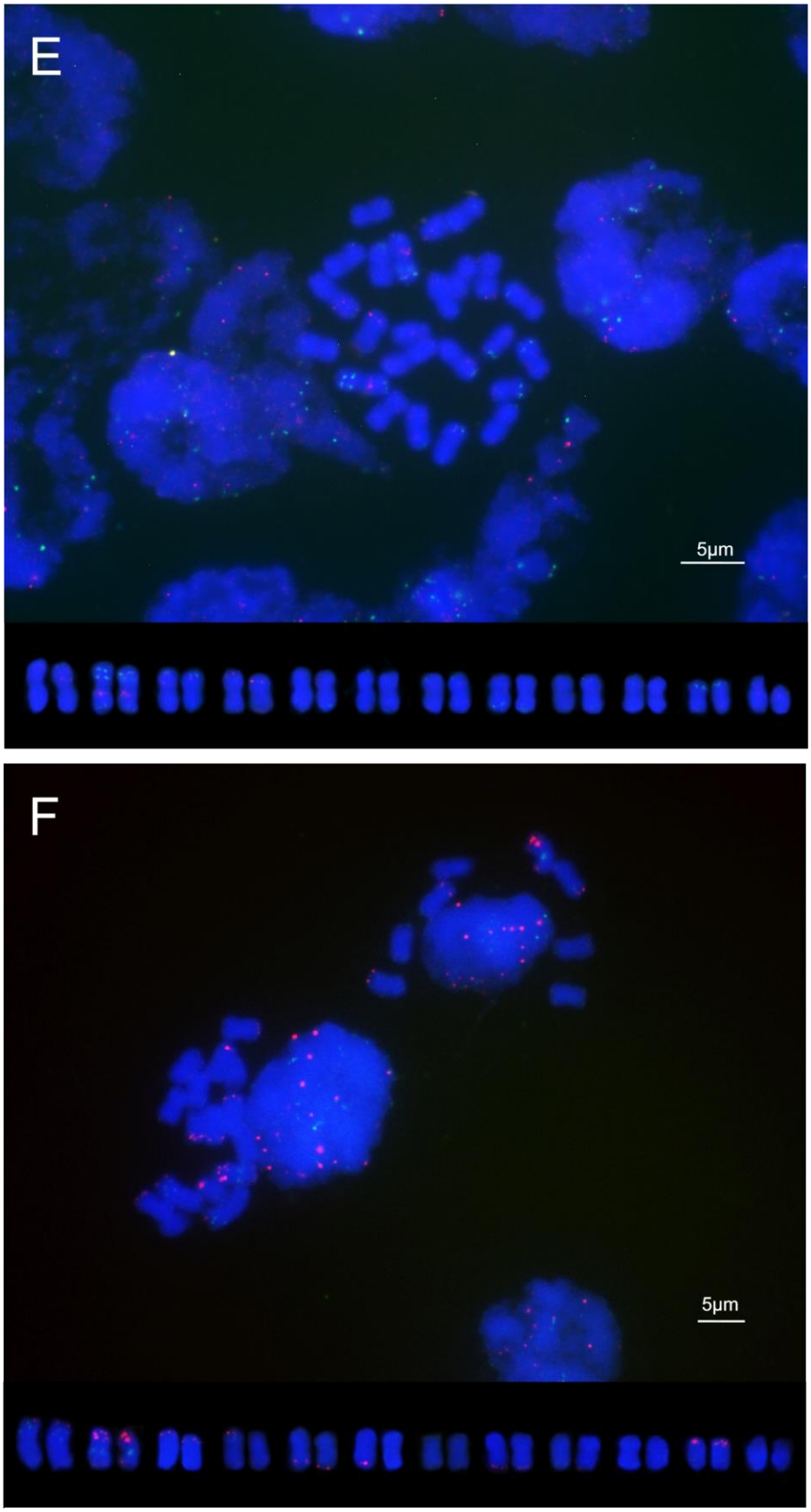

Figure 9 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 20141/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 7-11 to allow for comparative analysis among individuals. Probe combinations: (E) CapSat1 (green) and CapSat4 (red), (F) CapSat5 (green) and CapSat1 (red).

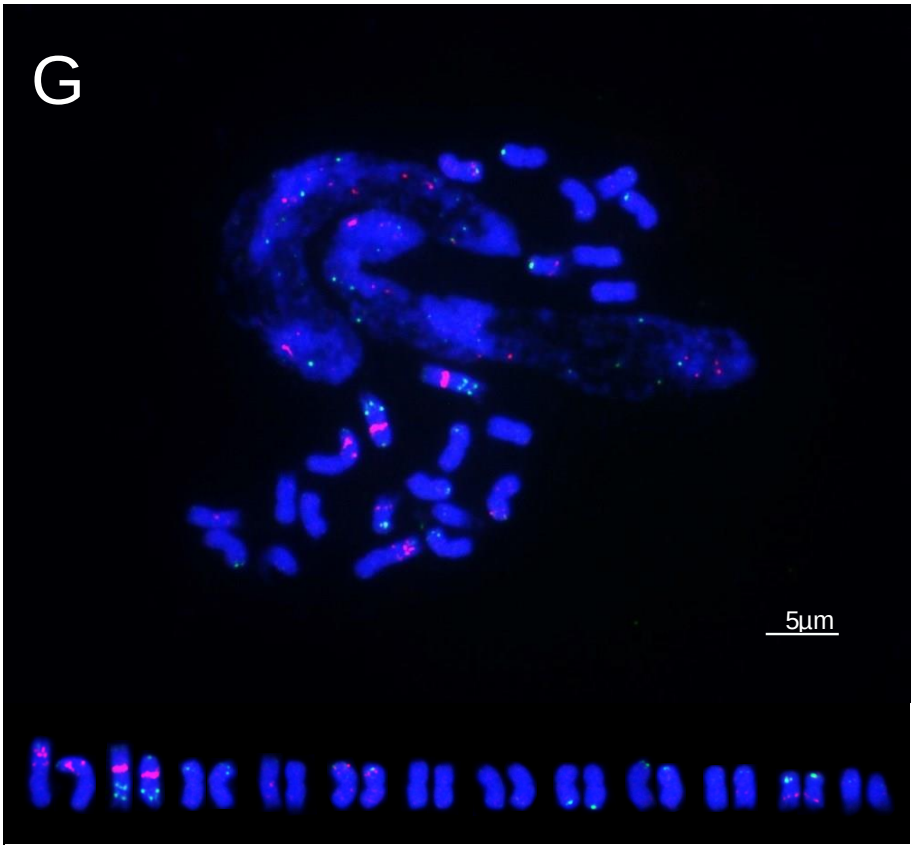

Figure 10 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 20141/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 7-11 to allow for comparative analysis among individuals. Probe combinations: (G) CapSat4 (green) and CapSat5 (red).

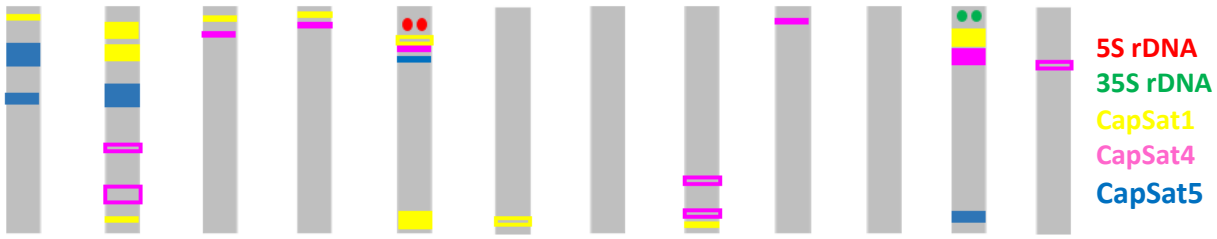

Figure 11 – Localization of all five tandemly repeated DNA probes in the haploid set of chromosomes (ideogram) of individual 2 of *C. annuum* accession 20141. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes in Figures 7-10. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 7-10. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

3.1.2. *C. annuum* accession 50 individual 2

A single 5S rDNA locus was found at a subterminal position of chromosome 5 (Figures 12A, 16) and two 35S rDNA loci were located in subterminal position in one of the arms of chromosomes 11 and 12 (Figures 12, 13, 16).

Altogether thirteen loci of CapSat1 per haploid set of chromosomes were found in this individual, nine major ones and four minor ones (Figures 12B, 14, 16). The major loci were found on chromosomes 1, 2, 3, 4, 6, 7, 8 and 11. Chromosomes 1, 3, 4 and 8 each carried one small CapSat1 locus at a subterminal position within one of the arms. Chromosome 2 possessed two large - one subterminal and one interstitial - loci of CapSat1 located in close proximity to one another within the same chromosomal arm. One large locus of CapSat1 was found in the subterminal region of one of the arms of chromosomes 6 and 7. A small subterminal CapSat1 locus was found in the subterminal region of one of the arms of chromosome 11, close to the 35S rDNA locus. Minor CapSat1 loci were observed in chromosomes 2, 4, 5 and 12. Chromosome 2 had one small minor CapSat1 locus in the subterminal region of the arm not carrying the two major CapSat1 loci. One small minor and subterminally located CapSat1 locus was found in chromosome 4 on the arm not carrying the major CapSat1 locus. Another minor locus of CapSat1 was observed in subterminal position on one arm of chromosome 5. Chromosome 12 carried one small minor CapSat1 locus within the same arm and in close proximity to the 35S rDNA locus.

Eight loci of CapSat4 were observed in this individual in seven out of the twelve chromosomes (Figures 13C, 14E, 15G, 16). Five of these loci were major and the other three were minor. The major loci were found on chromosomes 1, 2, 3, 9 and 11. Chromosomes 1 and 3 each had one small subterminal CapSat4 locus next to the CapSat1 locus. Chromosome 2 showed one major interstitial CapSat4 locus on the same arm that carried the minor CapSat1 locus. Chromosome 9 did not have any satellite DNA loci besides one small major and subterminally located CapSat4 locus on one arm. Another small major CapSat4 locus was found close to the CapSat1 locus in subterminal position on chromosome 11. The three minor CapSat4 loci were found on chromosomes 2, 4 and 8. Chromosome 2 carried a minor CapSat4 locus on the same arm as the major CapSat4 locus, but in a more subterminal position. Chromosomes 4 and 8 each had one minor, subterminal CapSat4 locus located within the same arm as the CapSat1 locus.

Four major loci of CapSat5 have been found in *C. annuum* accession 20141 individual 2 (Figures 13D, 14F, 15G, 16). These loci were observed on chromosomes 1, 2 and 11. Chromosome 1 carried two major CapSat5 loci within the same arm, one larger subterminally located and one smaller interstitial locus. Chromosome 2 carried one large CapSat5 locus, close to the centromere within the same arm as the

minor CapSat1 locus and the two CapSat4 loci. Chromosome 11 had a subterminally located CapSat5 locus within the arm not carrying the 35S rDNA, CapSat1 and CapSat4 loci.

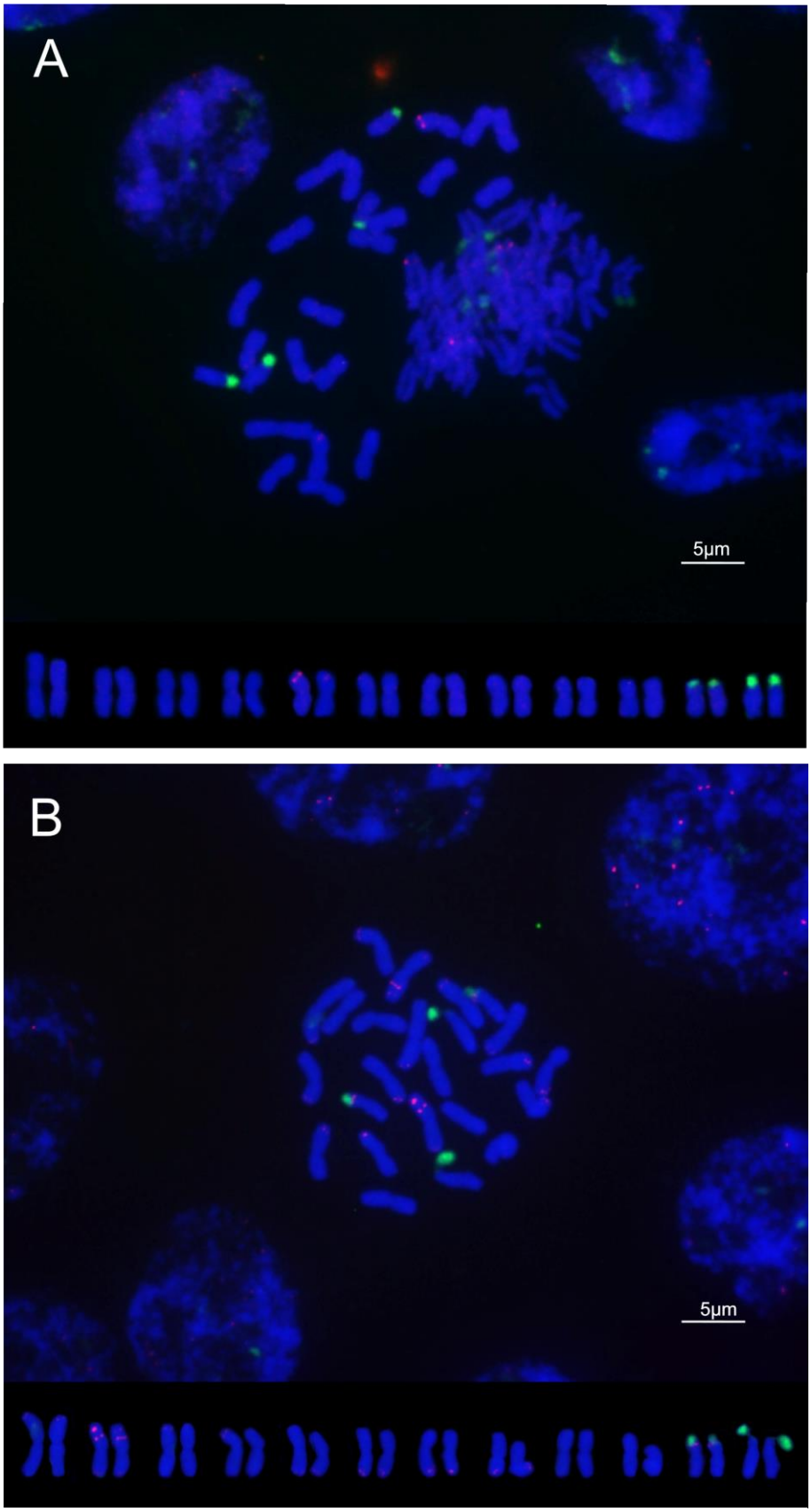

Figure 12 - Localization of rDNAs, and satellite DNAs in chromosomes of *C. annuum* accession 50/2 .Cut-out chromosome pairs are arranged by size and probe localization across Figures 12-16 to allow for comparative analysis among individuals. Probe combinations: (A) 35S rDNA (green) and 5S rDNA (red), (B) 35S rDNA (green) and CapSat1 (red).

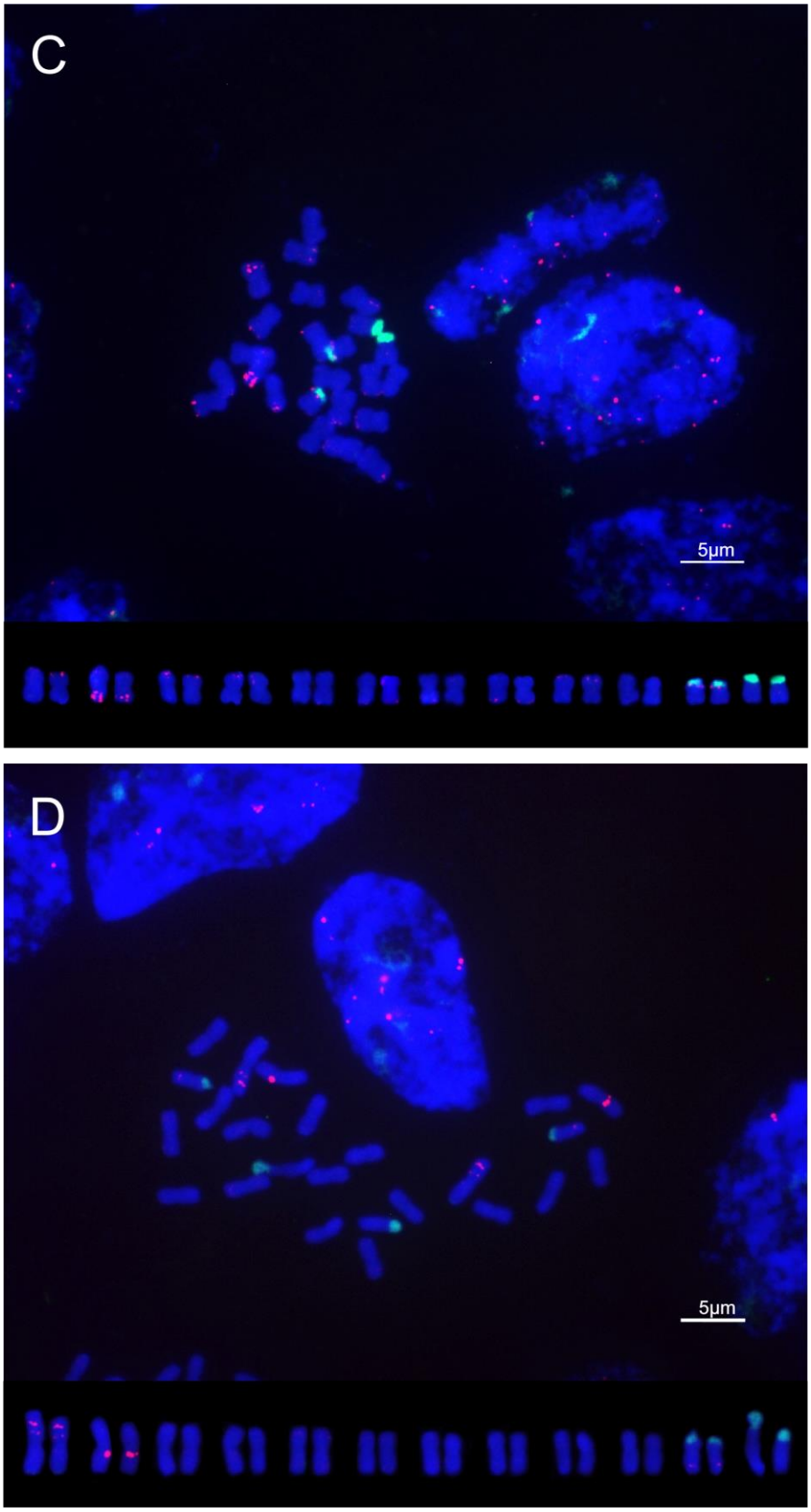

Figure 13 - Localization of 35S rDNA, and satellite DNAs in chromosomes of *C. annuum* accession 50/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 12-16 to allow for comparative analysis among individuals. Probe combinations: (C) 35S rDNA (green) and CapSat4 (red), (D) 35S rDNA (green) and CapSat5 (red).

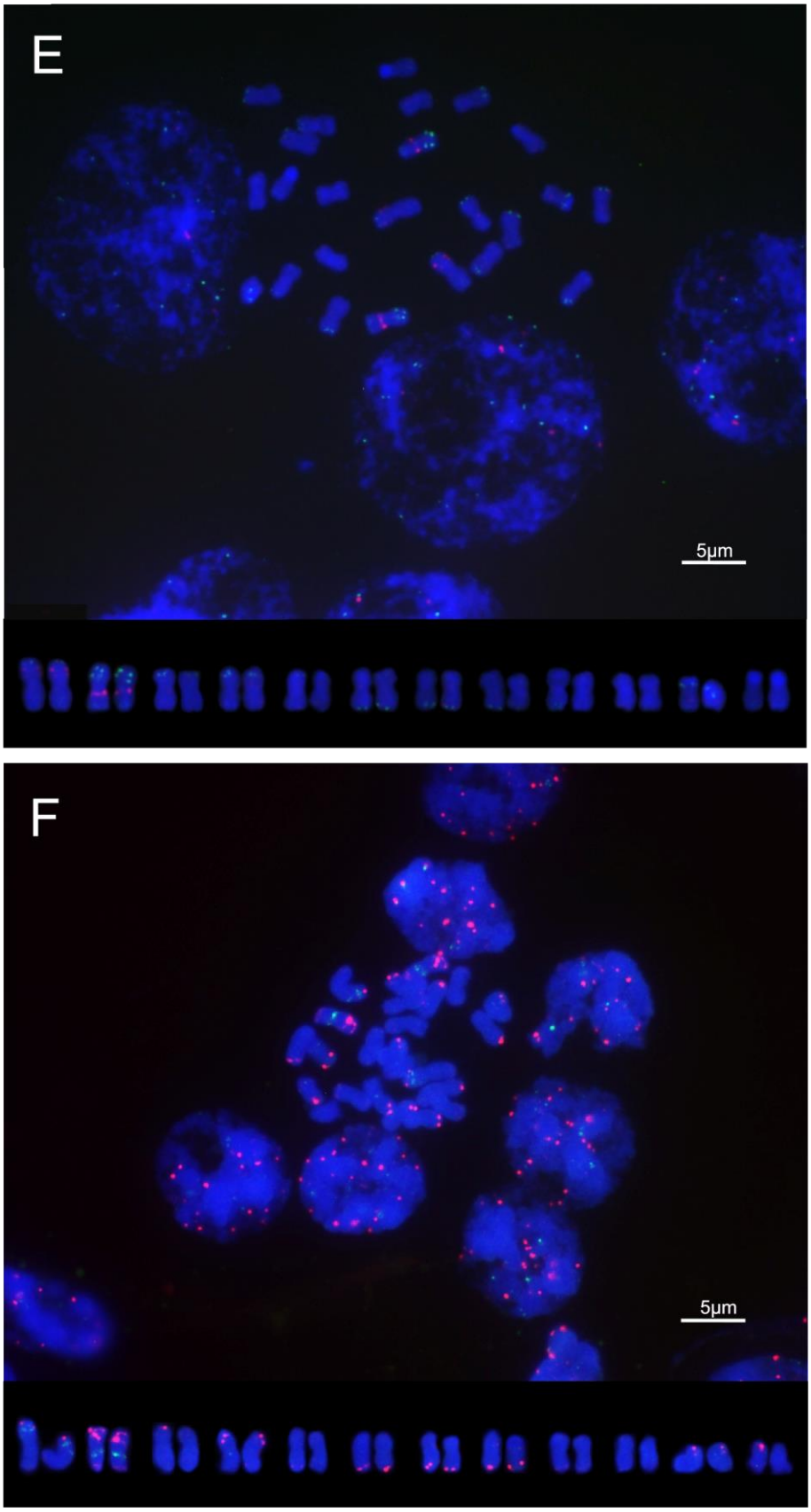

Figure 14 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 50/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 12-16 to allow for comparative analysis among individuals. Probe combinations: (E) CapSat1 (green) and CapSat4 (red), (F) CapSat5 (green) and CapSat1 (red).

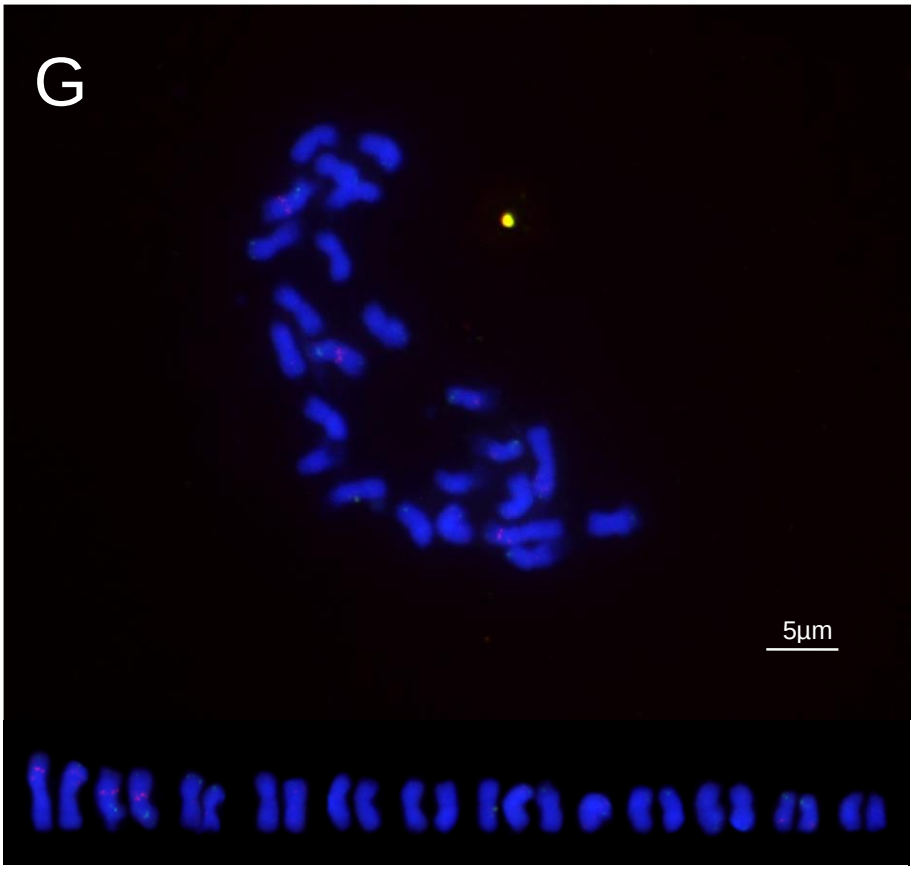

Figure 15 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 50/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 12-16 to allow for comparative analysis among individuals. Probe combinations: (G) CapSat4 (green) and CapSat5 (red).

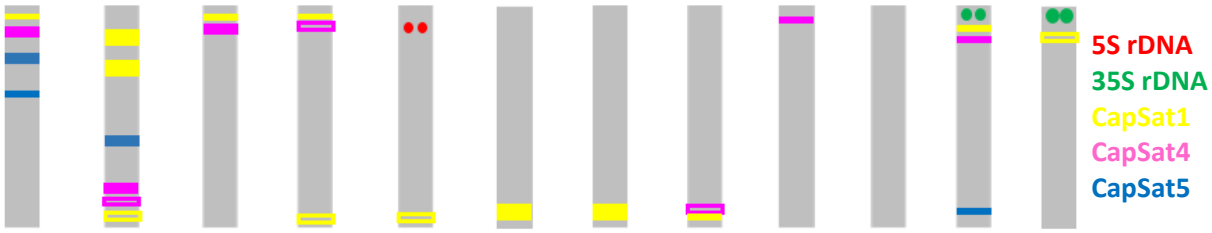

Figure 16 – Localization of all five tandemly repeated DNA probes in the haploid set of chromosomes (ideogram) of individual 2 of *C. annuum* accession 50. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes in Figures 12-15. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 12-15. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

3.1.3. *C. annuum* accession 61 individual 3

The 5S rDNA locus was found at a subterminal position of chromosome 5 (Figures 17A, 21) and two 35S rDNA loci were located subterminally in one of the arms of chromosomes 8 and 11 (Figures 17, 18, 21).

Eleven loci of CapSat1 were found in this individual. Ten were major loci and one was a minor locus. (Figures 17B, 19, 21). Major loci were found on chromosomes 1, 2, 3, 4, 5, 6, 11 and 12. Chromosome 1 carried one large major locus of CapSat1 in an interstitial position in one arm. Chromosome 2 possessed in total three major loci of CapSat1. Two loci were in close proximity to each other in the interstitial region of the same arm and one smaller subterminal CapSat1 locus was found on the other arm. Chromosomes 3, 4 and 6 each had a small major CapSat1 locus in subterminal position on the arm that also carried CapSat4 locus. Another small subterminal CapSat1 locus was found on chromosome 5 within the arm not carrying the 5S rDNA. In one of the arms of chromosome 11 a major subterminal CapSat1 locus was found close to the 35S rDNA locus. Chromosome 12 had one small, major and subterminally located CapSat1 locus. The only minor CapSat1 locus was found on chromosome 1, on the same arm as the larger major CapSat1 locus, but was located more subterminally.

In total, thirteen loci of CapSat4 were observed in this individual in ten out of the twelve chromosomes (Figures 18C, 19E, 20G, 21). Eleven of these loci were major and the other two were minor. All chromosomes carried at least one major CapSat4 locus except for chromosomes 7 and 12. Chromosome 1 had two major interstitial loci (one smaller and one bigger) on the same arm. Chromosomes 2, 3, 4 and 6 each had one small subterminal CapSat4 locus on the same arm as and located next to the CapSat1 locus. Another small, major and subterminal CapSat4 locus was found close to the 5S rDNA locus on chromosome 5. Chromosome 8 possessed one small subterminal CapSat4 locus on the arm not carrying the 35S rDNA locus. Chromosome 9 had one small, major and subterminally located CapSat4 locus on one arm. Chromosome 11 showed two major small CapSat4 loci within the same arm, one interstitial and one pericentric. The minor CapSat4 loci were found on chromosomes 1 and 10. The minor CapSat4 locus on chromosome 1 was located on the other arm than the two major CapSat4. The other minor subterminal CapSat4 locus was found on chromosome 10 next to the two major CapSat5 loci.

Overall, seven loci of CapSat5 have been found in this accession (Figures 18D, 19F, 20G, 21). The only two major CapSat5 loci were located in subterminal position within the same arm of chromosome 10, in close proximity to the more subterminal CapSat4 locus. The five minor CapSat5 loci were found on chromosomes 1, 2 and 11. Chromosome 1 possessed two minor CapSat5 loci of the same size. One pericentric locus was found within the arm that also carried the CapSat4 locus and two CapSat1 loci, whereas the other CapSat5 locus was found on the other arm between the two major CapSat4 loci.

Chromosome 11 carried two minor CapSat5 loci in close proximity to each other, in the interstitial region of the same arm.

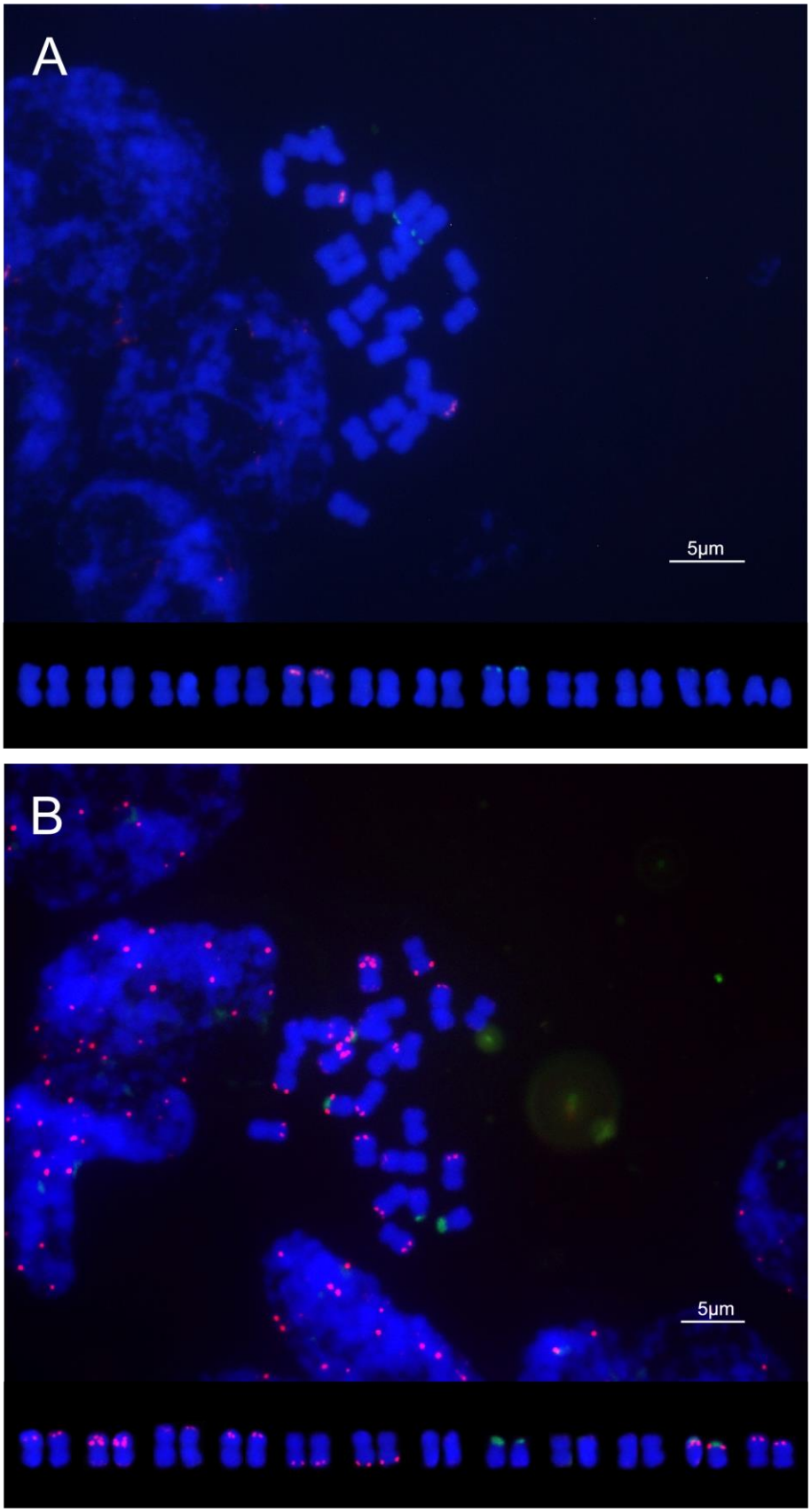

Figure 17 - Localization of rDNAs, and satellite DNAs in chromosomes of *C. annuum* accession 61/3 .Cut-out chromosome pairs are arranged by size and probe localization across Figures 17-21 to allow for comparative analysis among individuals. Probe combinations: (A) 35S rDNA (green) and 5S rDNA (red), (B) 35S rDNA (green) and CapSat1 (red).

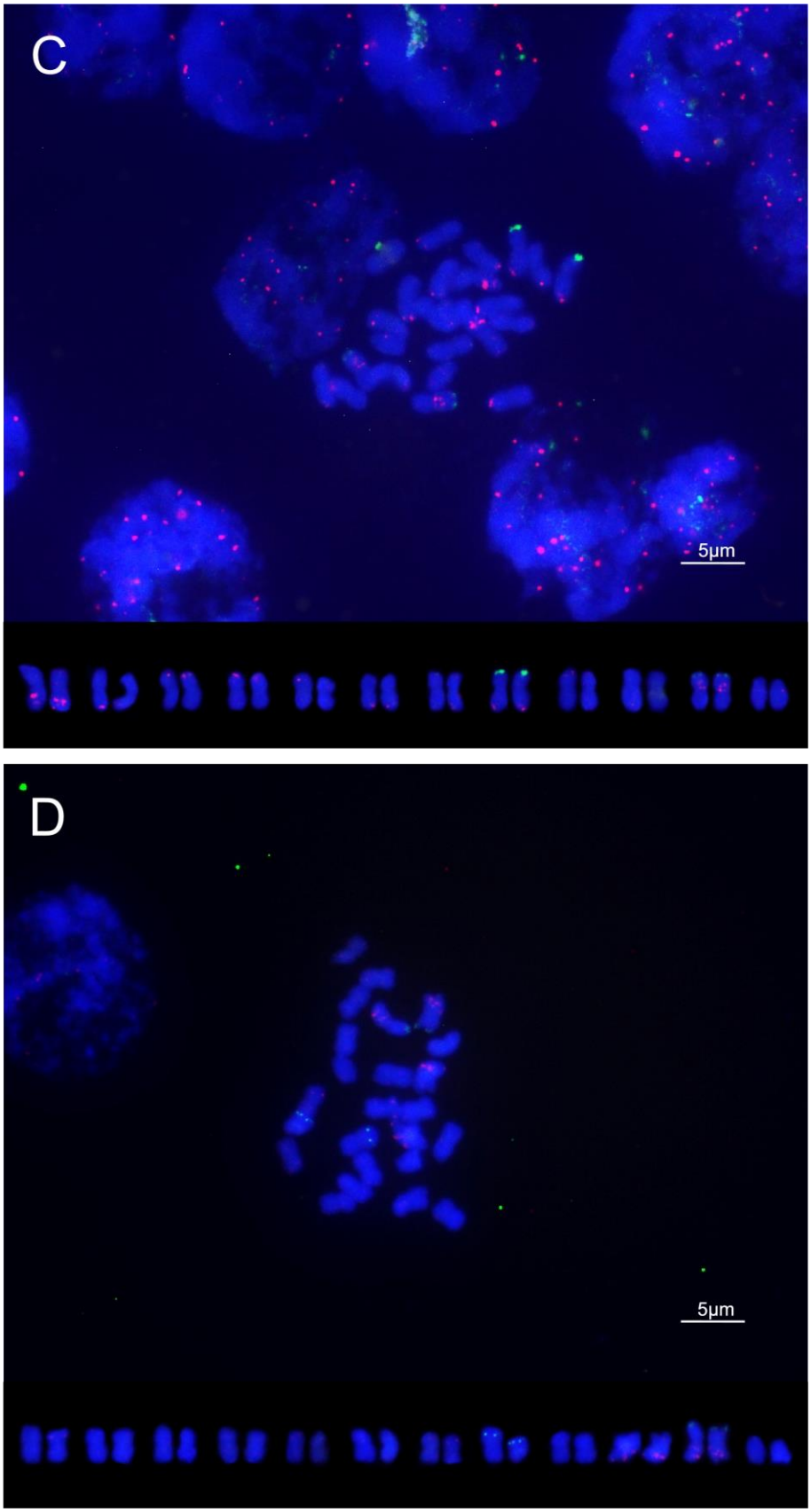

Figure 18 - Localization of 35S rDNA, and satellite DNAs in chromosomes of *C. annuum* accession 61/3. Cut-out chromosome pairs are arranged by size and probe localization across Figures 17-21 to allow for comparative analysis among individuals. Probe combinations: (C) 35S rDNA (green) and CapSat4 (red), (D) 35S rDNA (green) and CapSat5 (red).

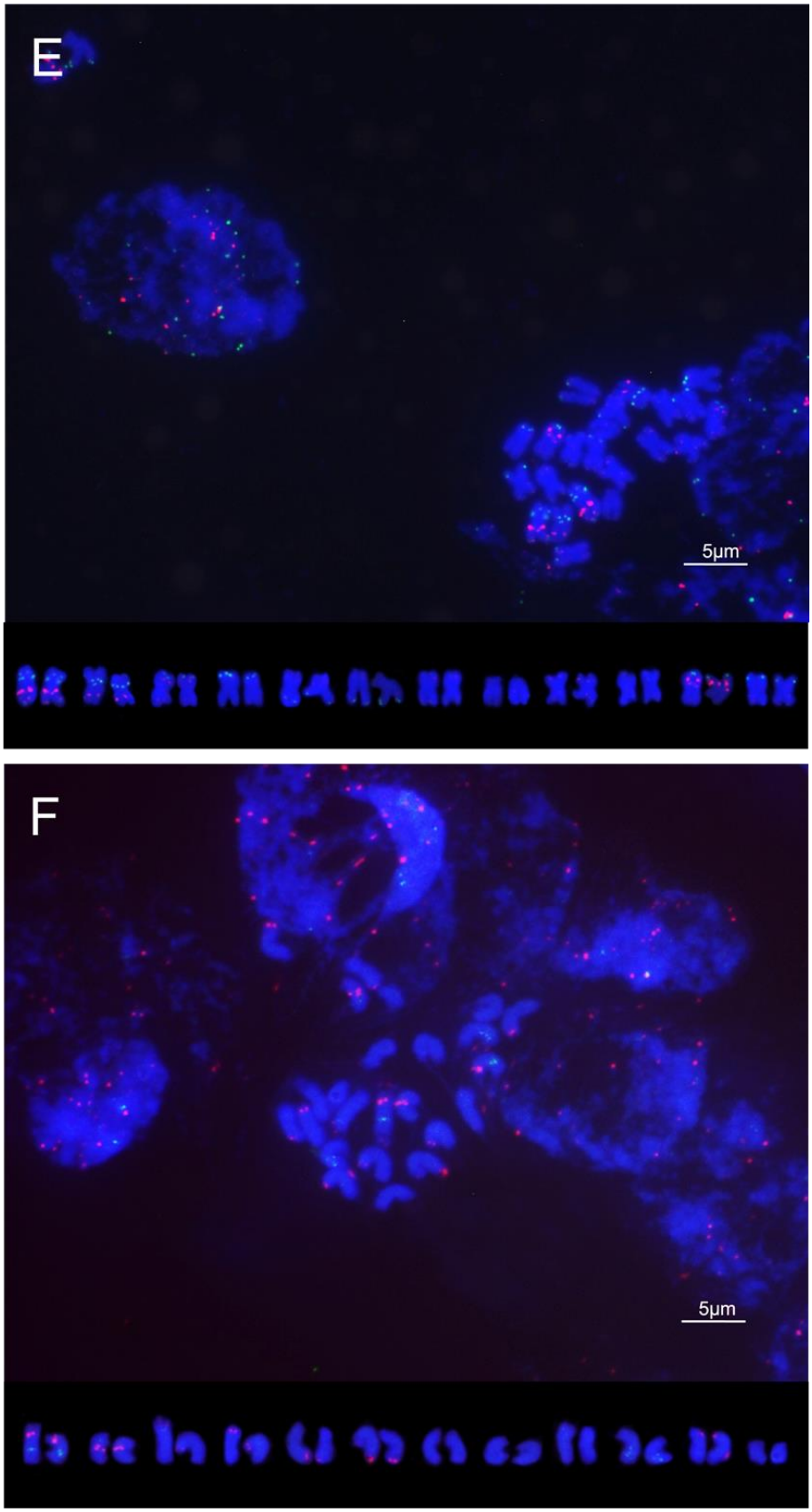

Figure 19 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 61/3. Cut-out chromosome pairs are arranged by size and probe localization across Figures 17-21 to allow for comparative analysis among individuals. Probe combinations: (E) CapSat1 (green) and CapSat4 (red), (F) CapSat5 (green) and CapSat1 (red).

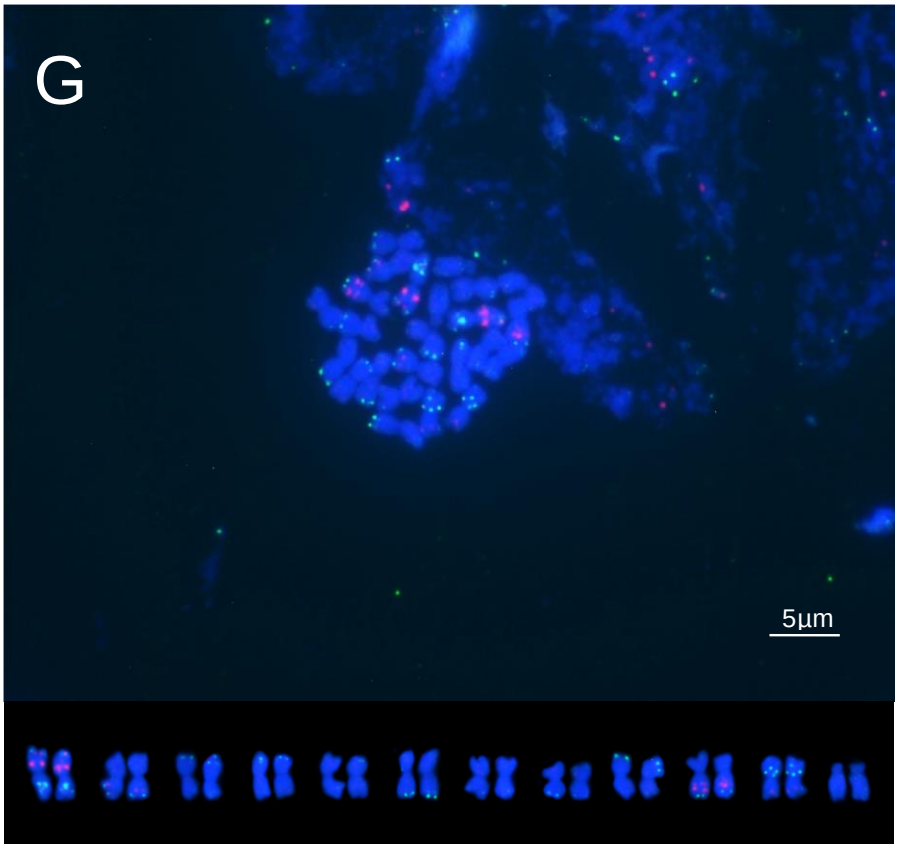

Figure 20 - Localization of satellite DNAs in chromosomes of *C. annuum* accession 61/3. Cut-out chromosome pairs are arranged by size and probe localization across Figures 17-21 to allow for comparative analysis among individuals. Probe combinations: (G) CapSat4 (green) and CapSat5 (red).

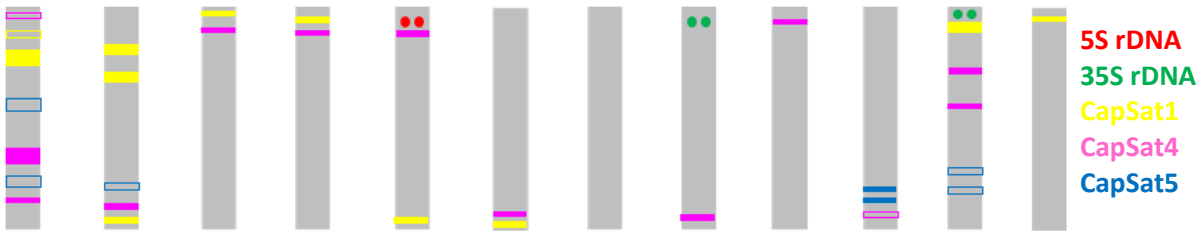

Figure 21 – Localization of all five tandemly repeated DNA probes in the haploid set of chromosomes (ideogram) of individual 3 of *C. annuum* accession 61. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes in Figures 17-20. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 17-20. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

3.2. *Capsicum pubescens*

3.2.1. *C. pubescens* accession 3858 individual 2

One 5S rDNA locus was found at a subterminal position of chromosome 3 (Figures 22A, 26) and as many as twelve subterminal 35S rDNA loci in total were found located in chromosomes 1, 2, 3, 4, 6, 8, 9, 10, 11 and 12 (Figures 22, 23, 26). All chromosomes had one locus of 35S rDNA except for chromosomes 3 and 6, both of which had two 35S rDNA loci, one in each chromosomal arms.

Seventeen loci of CapSat1 were found in this individual, fifteen major ones and two minor ones (Figures 22B, 24, 26). Major loci were found on all chromosomes except for chromosomes 3 and 6. Chromosome 1 carried two major loci of CapSat1, one large, interstitial and one small subterminal within the same arm. Chromosome 2 had two major loci of CapSat1, one in close proximity to the more subterminally located 35S rDNA locus and one larger subterminal CapSat1 locus in the other chromosomal arm. The chromosomes 4 and 9 both had a major CapSat1 locus in subterminal position within the same arm as the 35S rDNA locus. One subterminal CapSat1 locus was found in the subterminal regions of chromosomes 5 and 8. Chromosome 7 possessed three major CapSat1 loci, one larger subterminal and one smaller interstitial, both located within the same chromosomal arm. The third locus was found in the subterminal region of the other arm. Chromosome 10 carried two small interstitial CapSat1 loci within the same arm. A large major interstitial CapSat1 locus was found within one of the arms of chromosome 11. Chromosome 12 carried one small major subterminal CapSat1 locus in the chromosomal arm that did not carry the 35S rDNA locus. The two minor CapSat1 loci were found on chromosomes 5 and 11, the former in subterminal and the latter in interstitial position.

Ten loci of CapSat4 were observed in this individual in nine out of the twelve chromosomes (Figures 23C, 24E, 25G, 26). Eight of these loci were major and the other two were minor. The major loci were found on chromosomes 1, 3, 4, 6, 7, 8, 9 and 10. Chromosomes 1 and 8 both possessed one small, major and subterminal CapSat4 locus in close proximity to the 35S rDNA locus. One larger major CapSat4 locus was found close to an rDNA locus in both chromosome 3 and 6. The CapSat4 locus on chromosome 3 was located close to the 5S rDNA locus and that on chromosome 6 was located close to the 35S rDNA locus. One small major subterminal CapSat4 locus was found on both chromosomes 4 and 9 within the chromosomal arms that did not carry the 35S rDNA loci. Chromosome 7 possessed a small major CapSat4 locus in the interstitial region of the chromosomal arm that also carried the CapSat1 locus. One large

major subterminal CapSat4 locus was found on chromosome 10 within the same chromosomal arm as the two CapSat1 loci. Chromosomes 9 and 11 each carried one small minor subterminal CapSat4 locus in close proximity to the 35S rDNA locus.

In total, ten loci of CapSat5 have been found in *C. pubescens* accession 3858 individual 2 (Figures 23D, 24F, 25G, 26). Two major CapSat5 loci were found on chromosomes 7 and 11. Chromosome 7 had one small major interstitial CapSat5 locus on the same arm as the two CapSat1 loci and the CapSat4 locus. The other large major interstitial CapSat5 locus was located on chromosome 11, on the same arm as the minor CapSat4 locus and the major CapSat1 locus. The remaining eight minor CapSat5 loci were found on chromosomes 1, 4, 5, 6, 8 and 10. Chromosomes 1 and 10 both had a small minor interstitial CapSat5 locus on the same arm as the 35S rDNA locus. One small minor CapSat5 locus on chromosome 4 was located within the same arm as the 35S rDNA locus albeit in a more pericentric position. Another small minor interstitial CapSat5 locus was found in one chromosomal arm of chromosome 5. Chromosomes 6 and 8 both had two interstitial minor CapSat5 loci, one locus in each of the arms. The loci in chromosome 8 were both small, whereas one smaller and one larger minor CapSat5 locus were present in chromosome 6.

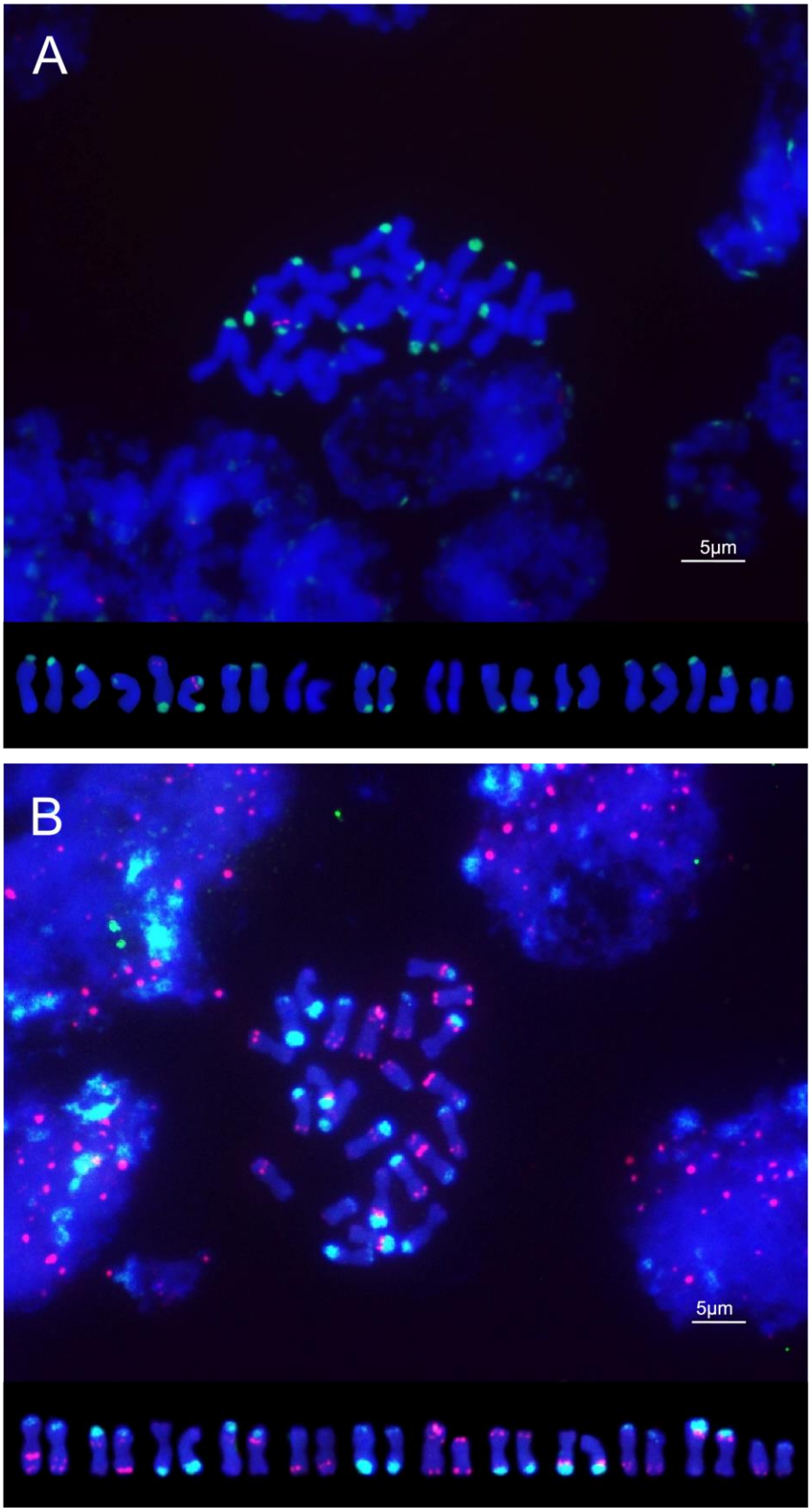

Figure 22 - Localization of rDNAs, and satellite DNAs in chromosomes of *C. pubescens* accession 3858/2 .Cut-out chromosome pairs are arranged by size and probe localization across Figures 22-26 to allow for comparative analysis among individuals. Probe combinations: (A) 35S rDNA (green) and 5S rDNA (red), (B) 35S rDNA (green) and CapSat1 (red).

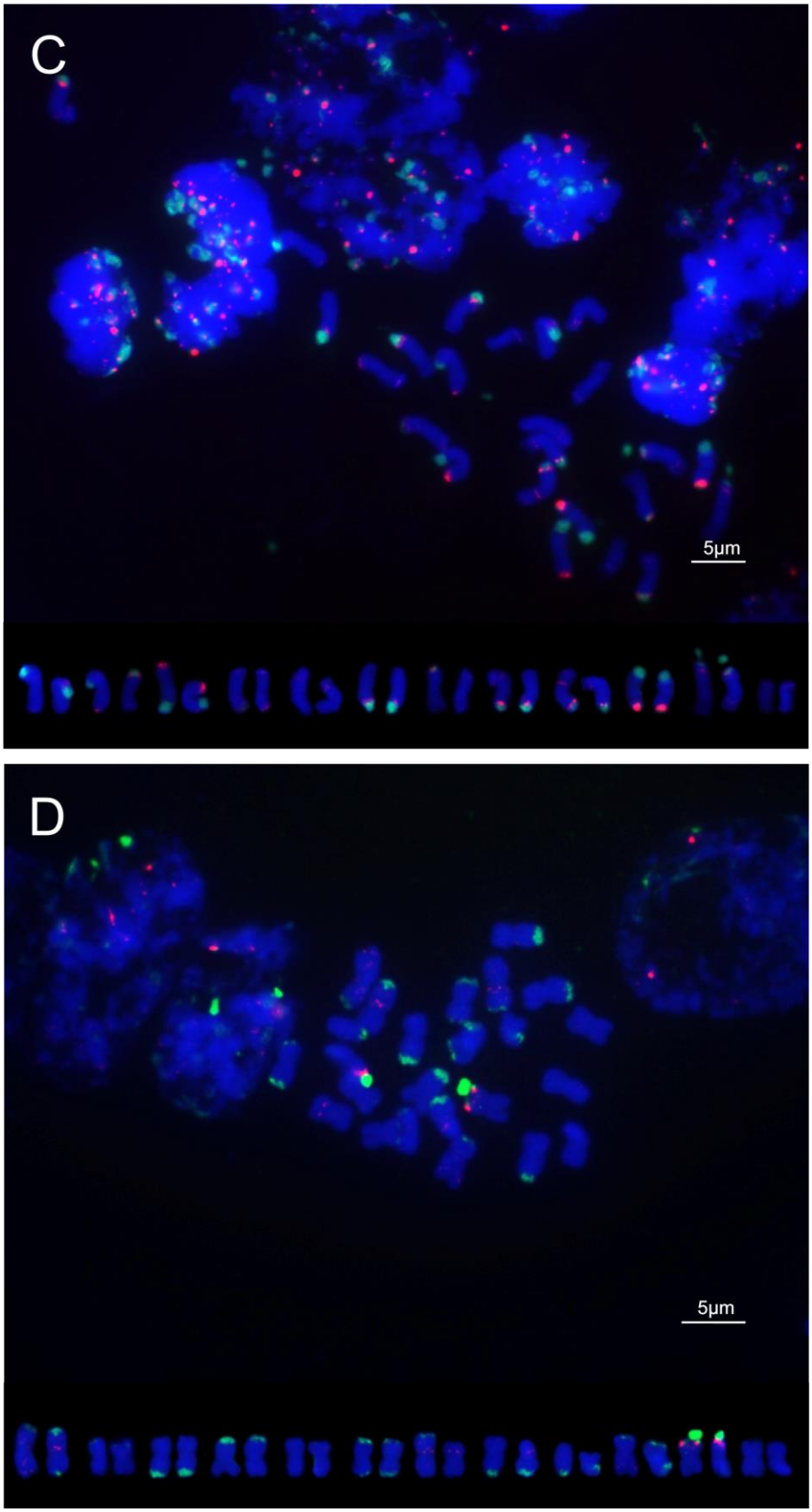

Figure 23 - Localization of 35S rDNA, and satellite DNAs in chromosomes of *C. pubescens* accession 3858/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 22-26 to allow for comparative analysis among individuals. Probe combinations: (C) 35S rDNA (green) and CapSat4 (red), (D) 35S rDNA (green) and CapSat5 (red).

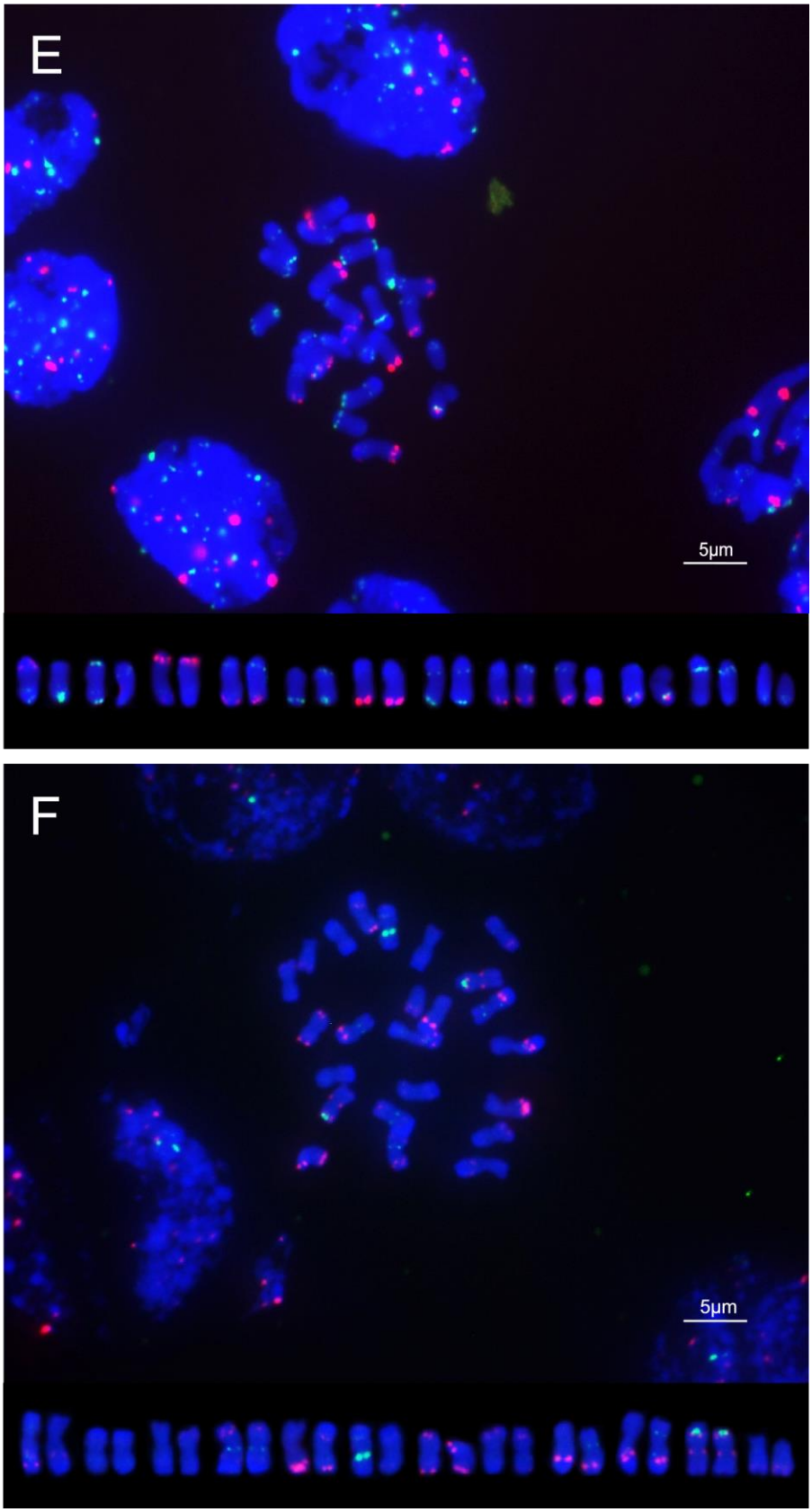

Figure 24 - Localization of satellite DNAs in chromosomes of *C. pubescens* accession 3858/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 22-26 to allow for comparative analysis among individuals. Probe combinations: (E) CapSat1 (green) and CapSat4 (red), (F) CapSat5 (green) and CapSat1 (red).

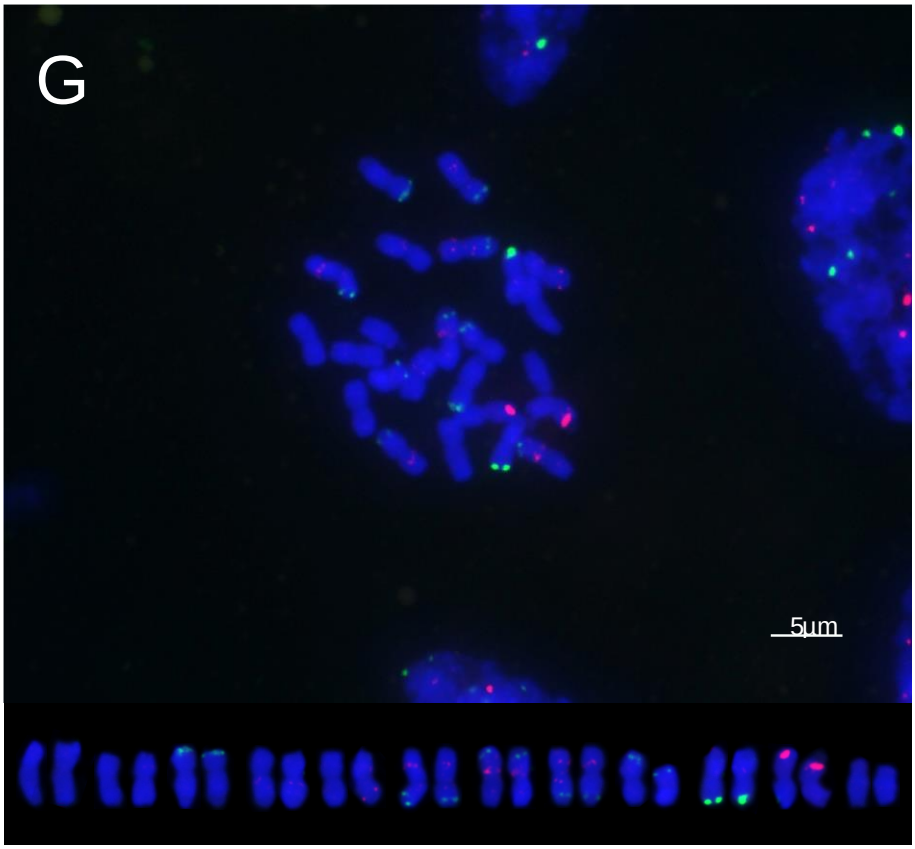

Figure 25 - Localization of satellite DNAs in chromosomes of *C. pubescens* accession 3858/2. Cut-out chromosome pairs are arranged by size and probe localization across Figures 22-26 to allow for comparative analysis among individuals. Probe combinations: (G) CapSat4 (green) and CapSat5 (red).

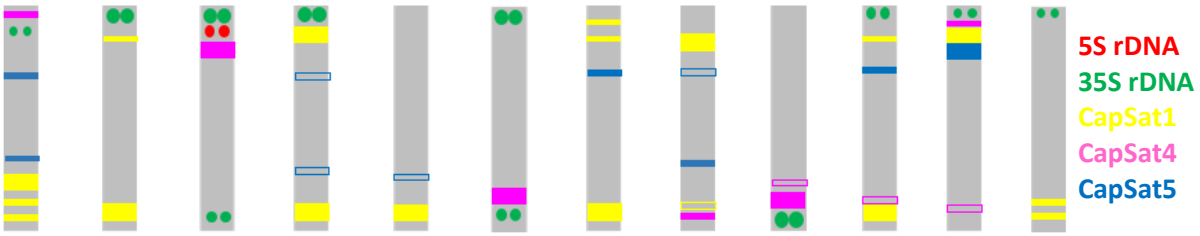

Figure 26 – Localization of all five tandemly repeated DNA probes in the haploid set of chromosomes (ideogram) of individual 2 of *C. pubescens* accession 3858. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes in Figures 22-26. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 23-26. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

3.2.2. *C. pubescens* accession 56 individual 1

One subterminal 5S rDNA locus was found in chromosome 3 (Figures 27A, 31), whereas eleven subterminal 35S rDNA loci were found in total in chromosomes 1, 2, 3, 4, 6, 9, 10, 11 and 12 (Figures 27, 28, 31). All of those chromosomes carried one 35S rDNA locus in one of their arms, whereas chromosomes 3 and 6 additionally carried a second 35S rDNA locus within the other chromosomal arm.

Eighteen loci of CapSat1 were found in this individual, seventeen of which were major and one was minor (Figures 27B, 29, 31). Major loci were found on chromosomes 1, 2, 4, 5, 7, 8, 10, 11 and 12. Chromosome 1 carried three major loci within one chromosomal arm. One locus was large and interstitial and two loci were small and subterminal. Chromosomes 2 and 10 both possessed two major loci of CapSat1, one smaller and one larger, each located in a different chromosomal arm. One of these loci in both chromosomes was smaller and located in close proximity to the more subterminal 35S rDNA locus. Chromosome 4 had two major larger subterminal CapSat1 loci, one per each of the arms. One of these loci was adjacent to the 35S rDNA locus. One subterminal CapSat1 locus in chromosome 5 was located within the same chromosomal arm as a minor CapSat5 locus. Chromosome 7 possessed three major CapSat1 loci. Two smaller loci, one subterminal and one interstitial, were found on the same arm. The third locus was larger and found in subterminal position on the other chromosomal arm. One large major interstitial CapSat1 locus was found in chromosome 8 in the chromosomal arm that also carried a small minor CapSat5 locus. A large major interstitial CapSat1 locus was found on chromosome 11. Chromosome 12 carried one small major subterminal CapSat1 locus on the arm not carrying the 35S rDNA. The only minor CapSat1 locus was found on chromosome 8 located in the chromosomal arm not carrying major CapSat1 locus.

Nine loci of CapSat4 were observed in this individual in seven out of the twelve chromosomes (Figures 28C, 29E, 30G, 31). Six of these loci were major and the other three were minor. The major loci were found on chromosomes 1, 3, 8, 9, 10 and 11. Chromosome 1 possessed one small, major and subterminal CapSat4 locus in close proximity to the 35S rDNA locus. Chromosomes 3 and 6 each had one larger major CapSat4 locus close to rDNA locus. CapSat4 locus on chromosome 3 was located close to the 5S rDNA locus and the one on chromosome 6 was adjacent to the 35S rDNA locus. One small major subterminal CapSat4 locus was found on chromosome 8 within the same chromosomal arm that carried the minor CapSat1 locus. Chromosomes 9 and 11 each had one major CapSat4 locus in proximity to the 35S rDNA locus. Three minor CapSat4 loci were found in total. They were located on chromosomes 9, 10 and 11. Small minor CapSat4 locus was located interstitially and close to the more subterminally located major

CapSat4 locus on chromosome 9. One small subterminal minor CapSat4 locus was found on each of the chromosomes 10 and 11, in the arms that did not carry the 35S rDNA.

Ten loci of CapSat5 were found in *C. pubescens* accession 56 individual 1 (Figures 28D, 29F, 30G, 31). The six major CapSat5 loci were found on chromosomes 1, 7, 8, 10 and 11. Chromosome 1 had two interstitial small major CapSat5 loci, one in each of the chromosomal arms. Chromosome 7 had one small major interstitial CapSat5 locus on the same arm as the two CapSat1 loci. Another small major interstitial CapSat5 locus was found on chromosome 8 within the arm that carried one major CapSat4 locus. Chromosome 10 additionally had a small interstitial major CapSat4 locus within the arm that also carried the 35S rDNA locus. The large major CapSat5 locus on chromosome 11 was located interstitially between the major CapSat4 locus and the major CapSat1 locus. Chromosome 4 had two interstitial minor CapSat5 loci, one in each of the chromosomal arms. Another small interstitial minor CapSat5 locus was found on chromosome 5 within the chromosomal arm that carried also major CapSat1 locus. Chromosome 8 had a small interstitial minor CapSat5 locus on the other arm not carrying the major CapSat5 locus.

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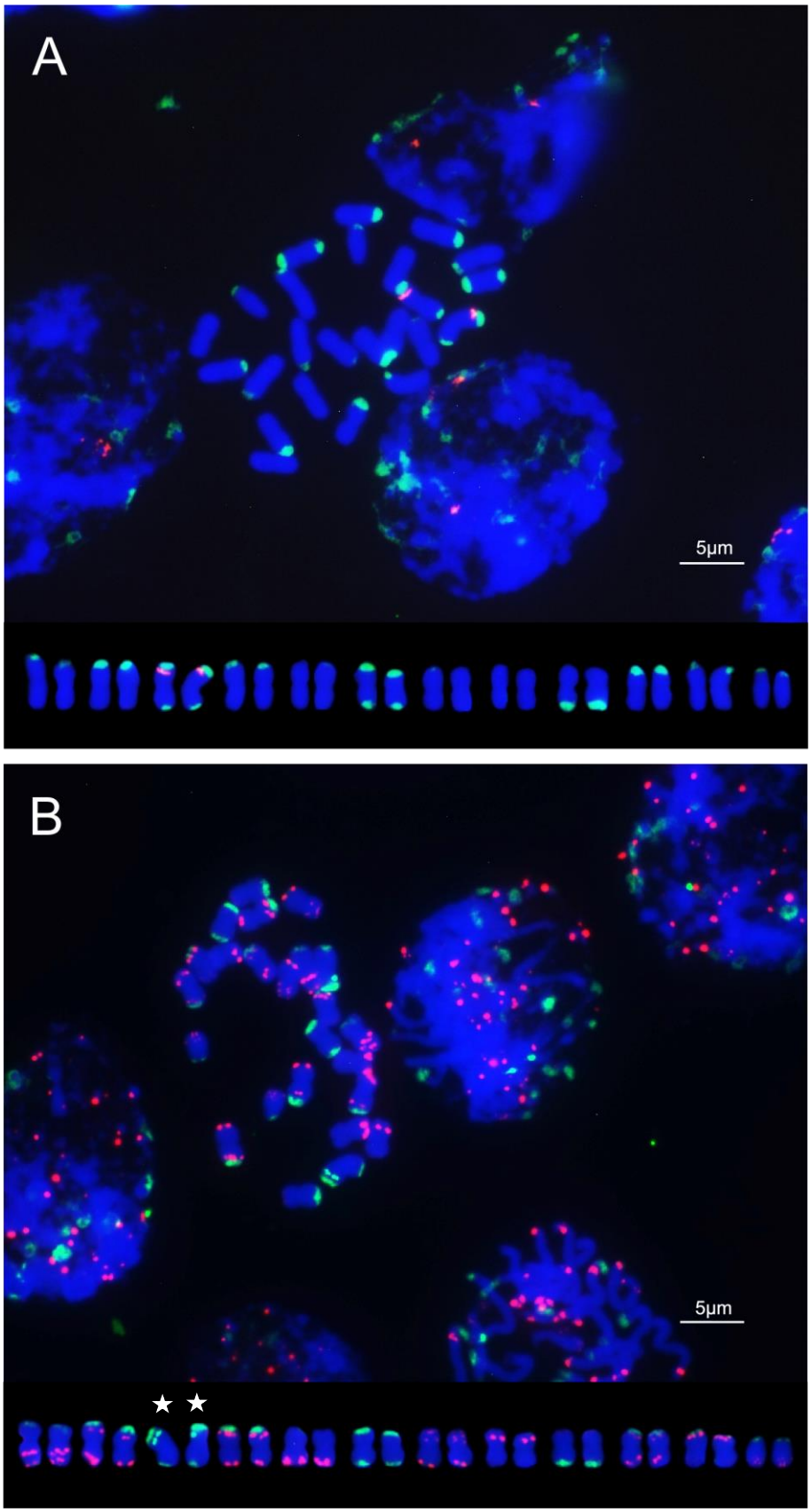

Figure 27 – Localization of rDNAs, and satellite DNAs in chromosomes of *C. pubescens* accession 56/1 .Cut-out chromosome pairs are arranged by size and probe localization across Figures 27-31 to allow for comparative analysis among individuals. Probe combinations: (A) 35S rDNA (green) and 5S rDNA (red), (B) 35S rDNA (green) and CapSat1 (red).White stars mark chromosomes in which additionally the 35S rDNA is colored in green.

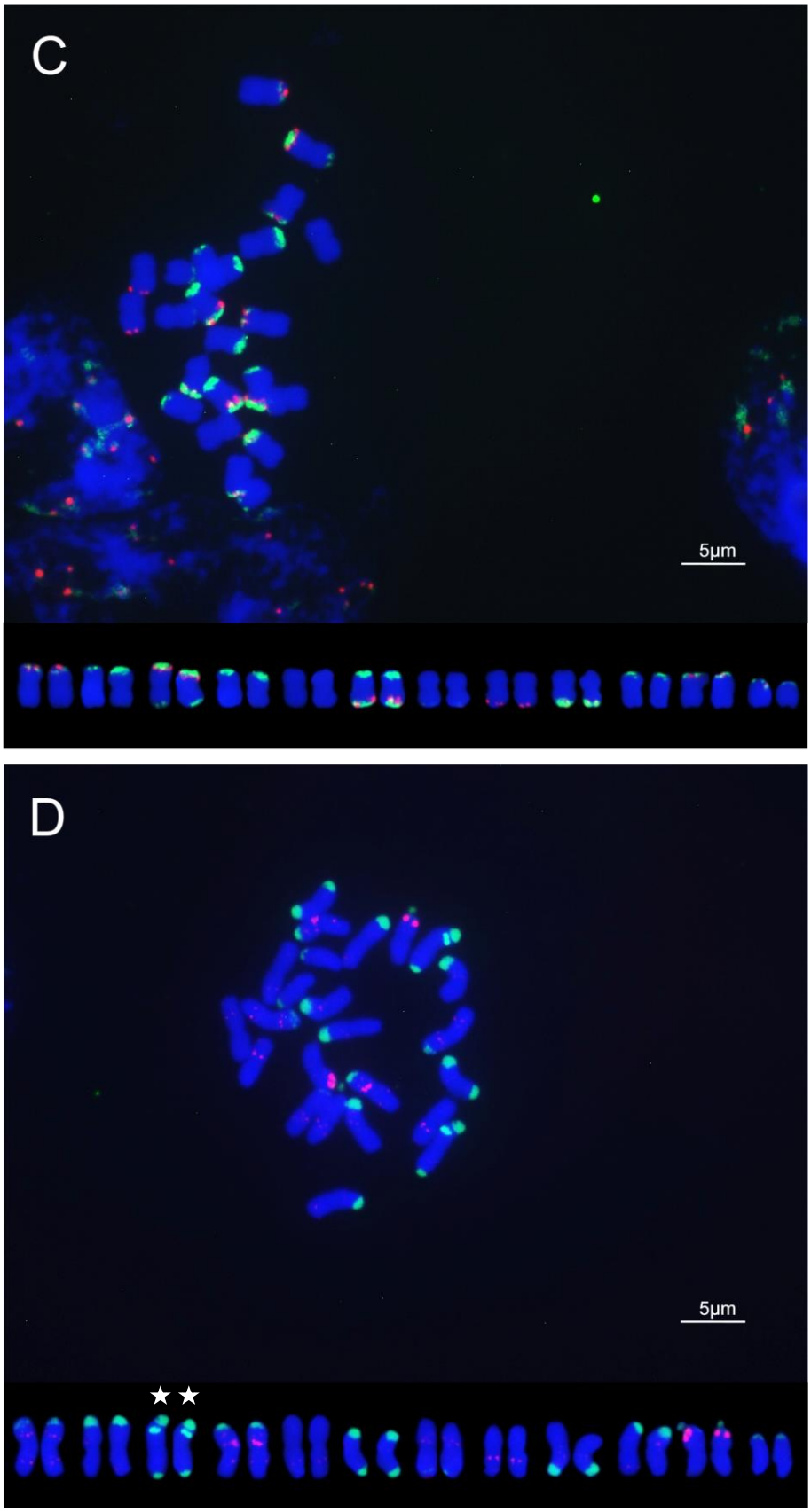

Figure 28 – Localization of 35S rDNA, and satellite DNAs in chromosomes of *C. pubescens* accession 56/1. Cut-out chromosome pairs are arranged by size and probe localization across Figures 27-31 to allow for comparative analysis among individuals. Probe combinations: (C) 35S rDNA (green) and CapSat4 (red), (D) 35S rDNA (green) and CapSat5 (red). White stars mark chromosomes in which additionally the 35S rDNA is colored in green.

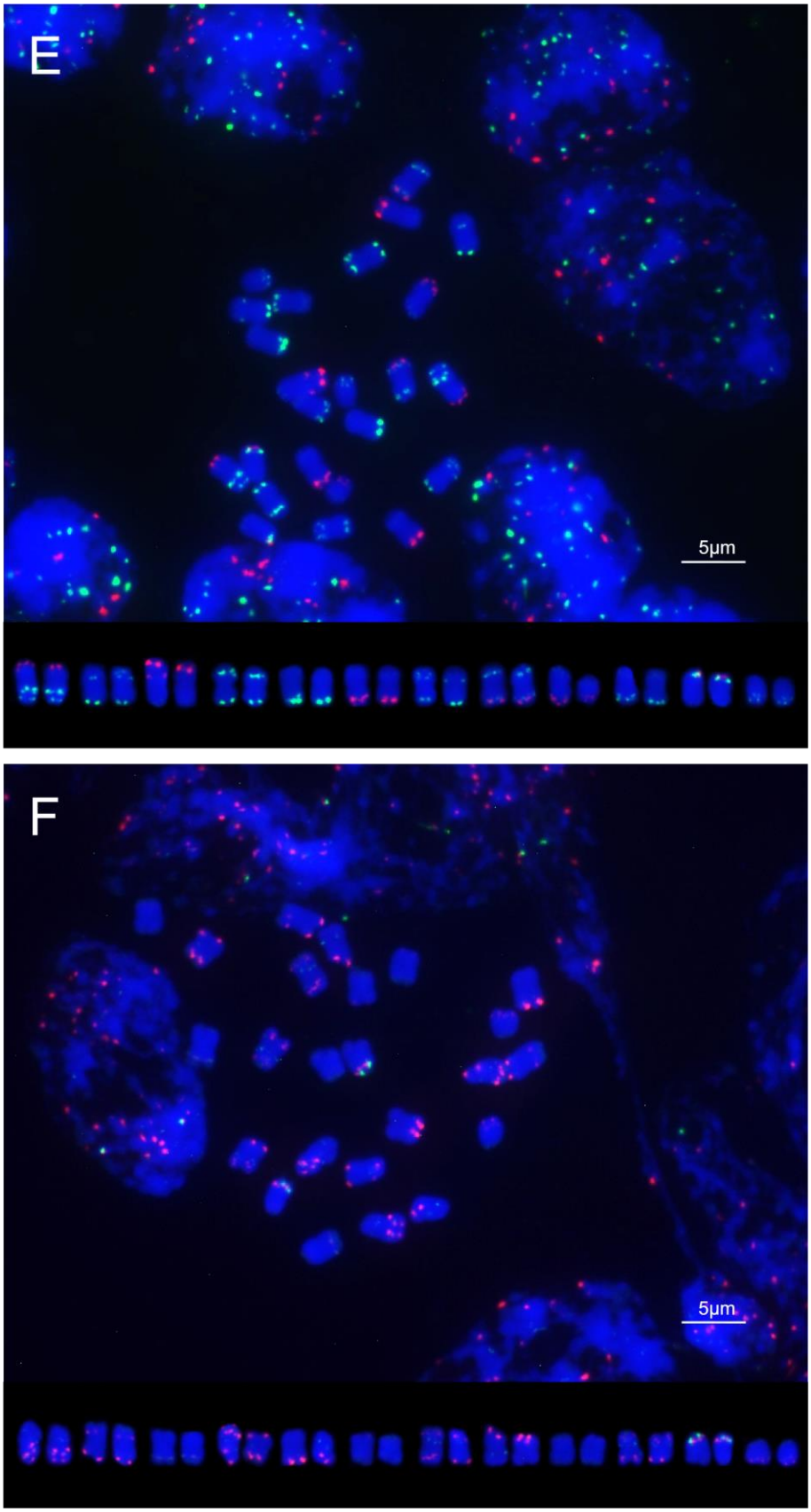

Figure 29 - Localization of satellite DNAs in chromosomes of *C. pubescens* accession 56/1. Cut-out chromosome pairs are arranged by size and probe localization across Figures 27-31 to allow for comparative analysis among individuals. Probe combinations: (E) CapSat1 (green) and CapSat4 (red), (F) CapSat5 (green) and CapSat1 (red).

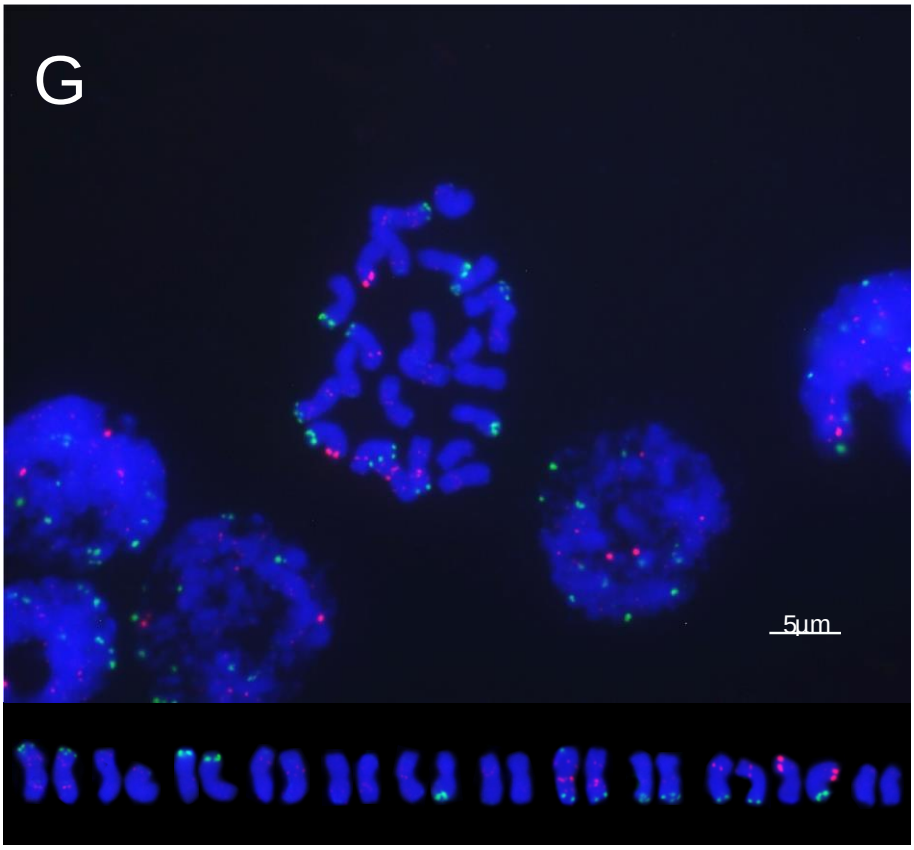

Figure 30 - Localization of satellite DNAs in chromosomes of *C. pubescens* accession 56/1. Cut-out chromosome pairs are arranged by size and probe localization across Figures 27-31 to allow for comparative analysis among individuals. Probe combinations: (G) CapSat4 (green) and CapSat5 (red).

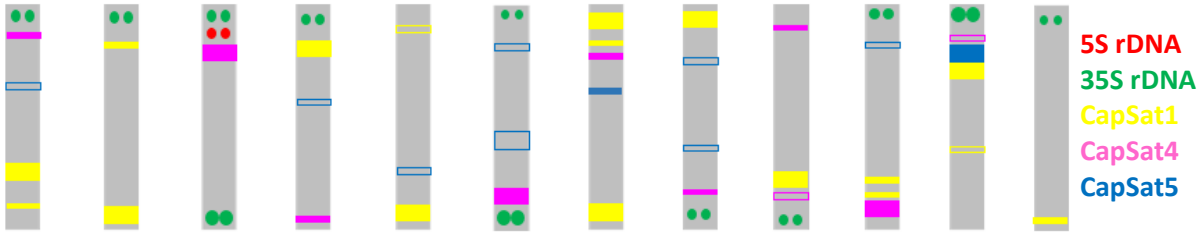

Figure 31 – Localization of all five tandemly repeated DNA probes in the haploid set of chromosomes (ideogram) of individual 1 of *C. pubescens* accession 56. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes in Figures 27-30. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 28-31. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

3.3. Localization of tandem repeats in *C. annuum* and *C. pubescens*

Five different tandem repeats were localized in the chromosomes of five individuals, representing two cultivated *Capsicum* species. In addition to the tandemly repeated coding and conserved rRNA gene loci, three novel satellites were identified and localized in the chromosomes. High variation in loci number, localization and relative size was detected both among individuals of the two species, and between the two species. Some of the loci were clearly on the border of detection and were classified as minor loci, as they were detected in some but not all chromosomal spreads of the same individual. The variation in rDNA and satellite DNA loci, however, was also high when only major loci, consistently detected in all chromosomal spreads, were considered.

Due to more extensive differences in the pattern of tandem repeat number and distribution in *Capsicum annuum* accession 61 individual 3 in comparison to the two other accessions of this species, this individual was not included in the following intra-and interspecific comparative analyzes.

3.3.1. *Capsicum annuum* (Figure 32, Tables 4, 6)

The *C. annuum* accessions 20141 and accession 50 both consistently and uniformly possessed one 5S rDNA locus in subterminal position on chromosome 5 and only one 35S rDNA locus was consistently found in the subterminal region of chromosome 11 (Figures 11, 16, 21, 32). However, an additional 35SrDNA locus of was detected in *C. annuum* accession 50 on chromosome 12.

Six out of nine major CapSat1 loci were shared by the two *C. annuum* individuals. Chromosomes 1, 3, 4, 8 and 11 of both individuals each had one locus of CapSat1 and two CapSat1 loci were found consistently on chromosome 2. *C. annuum* accession 20141/2 had two additional CapSat1 loci that the other accession did not have. One of these two CapSat1 loci was located on chromosome two and the other one was located on chromosome 5. *C. annuum* accession 50/2 had additional two unique CapSat1 loci located on chromosomes 6 and 7.

Both *C. annuum* accessions had five major CapSat4 loci, but only three of them were shared. Those three were consistently observed on chromosomes 3, 9 and 11. *C. annuum* accession 20141 had two additional unique CapSat4 loci on chromosomes 4 and 5 whilst *C. annuum* 50 had one unique locus on each of the chromosomes 1 and 2.

Four, out of maximum of five, major loci of CapSat5 were present in both *C. annuum* accessions. Chromosomes 2 and 11 both had one major locus, whilst chromosome 1 carried two major loci within the same chromosomal arm. In *C. annuum* accession 20141/2 an additional locus, not observed in the other accession, was found on chromosome 5.

3.3.2. *Capsicum pubescens* (Figure 33, Tables 5, 7)

The two analyzed *C. pubescens* individuals uniformly possessed one 5S rDNA locus in subterminal position on chromosome 3. Eleven loci of 35S rDNA were consistently detected in and shared by the two analyzed accessions (Figures 26, 31, 33). Chromosomes 1, 2, 4, 9, 10, 11 and 12 each carried one 35S rDNA locus. Two 35S rDNA loci were found consistently on chromosomes 3 and 6. Only one locus in chromosome 8 was not shared by the two individuals.

Thirteen out of a maximum of seventeen major CapSat1 loci were found in both analyzed *C. pubescens* individuals. One locus was found on chromosomes 4, 5, 8, 10, 11 and 12 and two loci were found on each of chromosomes 1, 2 and 11. Chromosome 7 carried three CapSat1 loci. *C. pubescens* accession 3858 had two additional unique major CapSat1 loci. One was found on chromosome 9 and the other on chromosome 10. In contrast, chromosomes 4 and 10 of *C. pubescens* accession 56 each carried one additional CapSat1 locus on the arm not carrying the shared CapSat1 locus. Furthermore, *C. pubescens* accession 56 had an additional major CapSat1 locus within the same arm as the already mentioned, shared CapSat1 loci on chromosomes 1 and 12.

Three out of a maximum of eight major loci of CapSat4 were detected in both *C. pubescens* individuals. Chromosomes 3, 6 and 8 each carried one CapSat4 locus in both accessions. Though both accessions had a CapSat4 locus on chromosome 9, in *C. pubescens* accession 56 it was located on the same chromosomal arm as the 35S rDNA locus, but in accession 3858 the CapSat4 locus was located on the other arm. Chromosomes 4, 7 and 10 of *C. pubescens* accession 3858, each had one major CapSat 4 locus that *C. pubescens* accession 56 did not have. In contrast, chromosome 11 of *C. pubescens* accession 56 possessed one non- shared major CapSat4 locus on both chromosomes 1 and 11.

C*. pubescens* accession 3858/2 and *C. pubescens* accession 56/1 had one CapSat5 locus on each of the chromosomes 7 and 11. *C. pubescens* accession 56 had four more major CapSat5 loci, which the other accession did not possess. Chromosome 1 possessed one major CapSat5 locus on each arm and the other two CapSat 5 loci were found on chromosomes 8 and 10.

Generally, *Capsicum pubescens* possessed a much higher amount of tandem repeat loci than *Capsicum annuum.* Both species had one locus of 5S rDNA, but the numbers of 35S rDNA loci were largely different in the two species. Whilst the *C. annuum* accessions showed a maximum of two 35S rDNA loci, the *C. pubescens* accessions showed eleven to twelve 35S rDNA loci. In both species, the most abundant atellite DNA was CapSat1. As many as thirteen CapSat1 loci were shared between the two *C. pubescens* accessions, whereas only seven shared CapSat1 loci were detected between the two *C. annuum*

accessions. Three shared CapSat4 loci were detected in both species. The number of shared CapSat5 loci was higher in *Capsicum annuum* accessions than in the *Capsicum pubescens* accessions.

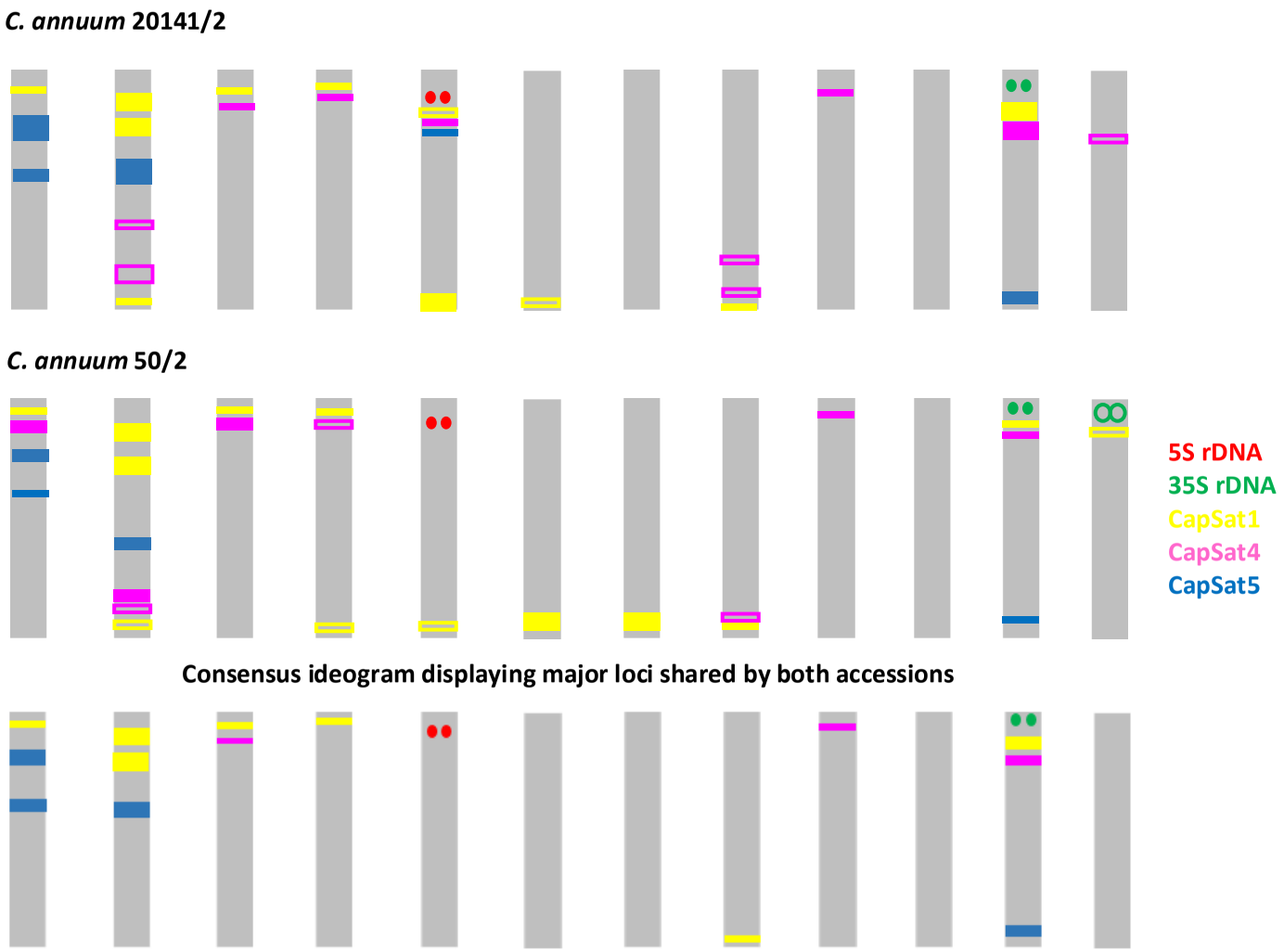

Figure 32 – Ideograms of *C. annuum* 20141/2, *C. annuum* 50/2 and ideogram displaying only major loci shared by both accessions. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 7-21. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

Table 4 - Number of major loci (bold) of 5S and 35S rDNAs and three satellite DNAs consistently found in individual chromosomes of both analyzed individuals of *Capsicum annuum*. Numbers is brackets refer to minimum and maximum number of all major loci of the given repeat detected in both individuals.

	Chr.1	Chr.2	Chr.3	Chr.4	Chr.5	Chr.6	Chr.7	Chr.8	Chr.9	Chr.10	Chr.11	Chr.12	Total number of major loci shared by both individuals
5S rDNA					**1** (1)								**1**
35S rDNA											**1** (1)		**1**
CapSat1	**1** (1)	**2** (2-3)	**1** (1)	**1** (1)	(0-1)	(0-1)	(0-1)	**1** (1)			**1** (1)		**7**
CapSat4	(0-1)	(0-1)	**1** (1)	(0-1)	(0-1)				**1** (1)		**1** (1)		**3**
CapSat5	**2** (2)	**1** (1)			(0-1)						**1** (1)		**4**

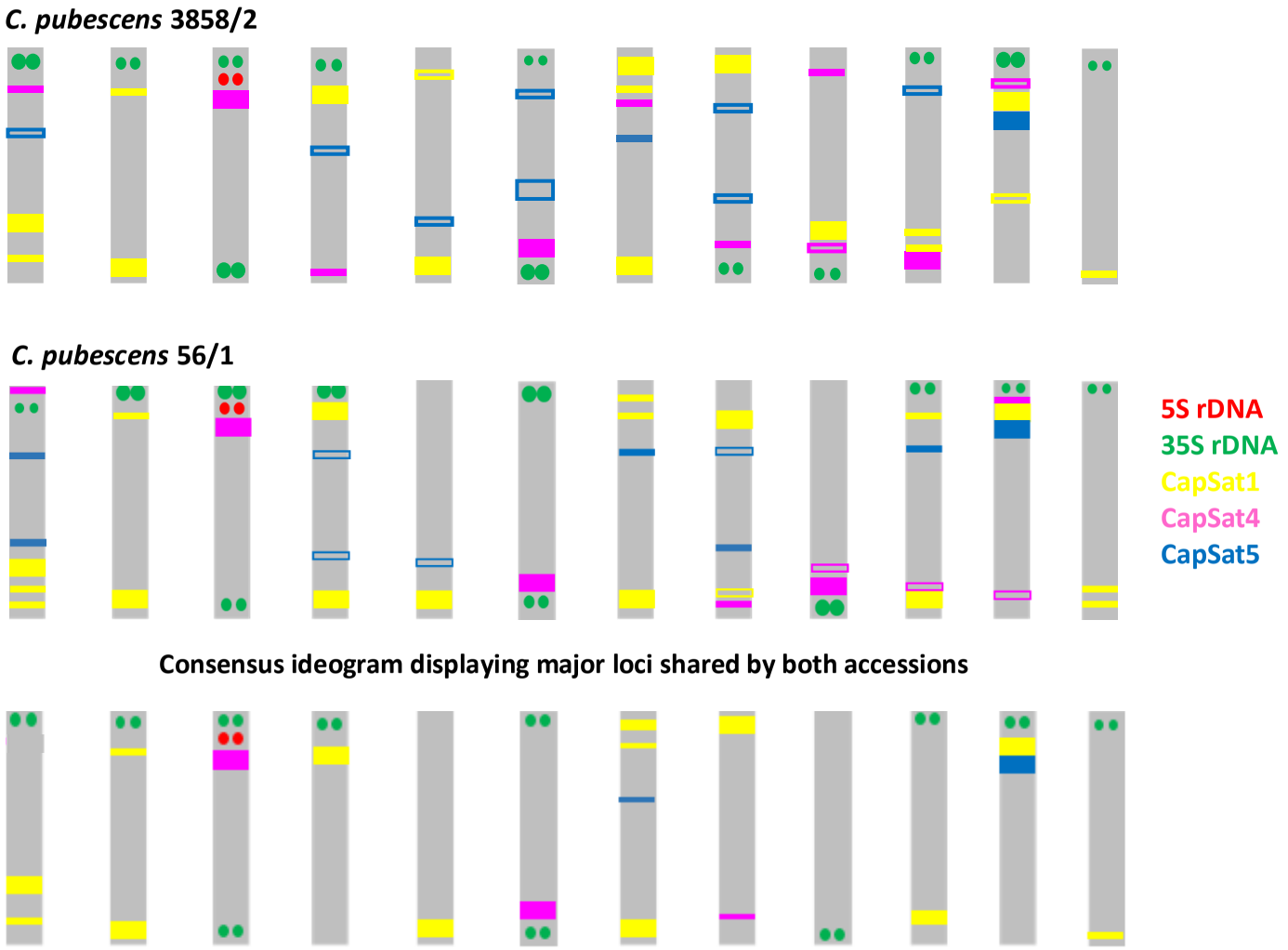

Figure 33 – Ideograms of *C. pubescens* 3858/2, *C. pubescens* 56/1 and ideogram displaying only major loci shared by both accessions. All chromosomes are drawn to the same size and centromeres are not indicated due to high levels of similarity of morphology of all chromosome pairs. The numbering of chromosomes corresponds to karyotypes. Probes mapped are: 5S rDNA (red circles), 35S rDNA (green circles), CapSat1 (yellow rectangles), CapSat4 (pink rectangles), CapSat5 (blue rectangles). Position and size of signals correspond to signals in karyotypes from Figures 22-31. Solid rectangles correspond to signals detected in all analyzed chromosomal spreads (major signals), empty rectangles correspond to signals detected only in some, but not all of the chromosomal spreads (minor signals).

Table 5 - Number of major loci (bold) of 5S and 35S rDNAs and three satellite DNAs consistently found in individual chromosomes of both analyzed individuals of *Capsicum pubescens*. Numbers is brackets refer to minimum and maximum number of all major loci of the given repeat detected in both individuals.

	Chr.1	Chr.2	Chr.3	Chr.4	Chr.5	Chr.6	Chr.7	Chr.8	Chr.9	Chr.10	Chr.11	Chr.12	Total number of major loci shared by both individuals
5S rDNA			**1** (1)										**1**
35S rDNA	**1** (1)	**1** (1)	**2** (2)	**1** (1)		**2** (2)		(0-1)	**1** (1)	**1** (1)	**1** (1)	**1** (1)	**11**
CapSat1	**2** (2-3)	**2** (2)		**1** (1-2)	**1** (1)		**3** (3)	**1** (1)	(0-1)	**1** (2)	**1** (1)	**1** (1-2)	**13**
CapSat4	(1)		**1** (1)	(0-1)		**1** (1)	(0-1)	**1** (1)	(1)	(0-1)	(0-1)		**3**
CapSat5	(0-2)						**1** (1)	(0-1)		(0-1)	**1** (1)		**2**

Table 6 – Overview of numbers of major + minor loci and the respective localization in chromosomes in *Capsicum annuum* individuals.

Species/Accession/Individual	Total number of loci per haploid genome (major + minor)	Number of major loci and their localization in individual chromosomes. Number in brackets indicates which chromosome carries the locus/loci.	Number of minor loci and their localization in individual chromosomes. Number in brackets indicates which chromosome carries the locus/loci.
		5S rDNA	
***C. annuum*/20141/2**	1 (major)	1(5)	
***C. annuum*/50/2**	1 (major)	1(5)	
***C. annuum*/61/3**	1 (major)	1(5)	
		35S rDNA	
***C. annuum*/20141/2**	1 (major)	1(11)	
***C. annuum*/50/2**	2 (major)	1(11), 1(12)	
***C. annuum*/61/3**	2 (major)	1(8), 1(11)	
		CapSat1	
***C. annuum*/20141/2**	11 (9 major + 2 minor)	1(1), 3(2), 1(3), 1(4), 1(5), 1(8), 1(11)	1(5), 1(6)
***C. annuum*/50/2**	13 (9 major + 4 minor)	1(1), 2(2), 1(3), 1(4), 1(6), 1(7), 1(8), 1(11)	1(2), 1(4), 1(5), 1(12)
***C. annuum*/61/3**	11 (10 major, 1 minor)	1(1), 3(2), 1(3), 1(4), 1(5), 1(6), 1(11), 1(12)	1(1)
		CapSat4	
***C. annuum*/20141/2**	10 (5 major + 5 minor)	1(3), 1(4), 1(5), 1(9), 1(11)	2(2), 2(8), 1(12)
***C. annuum*/50/2**	8 (5 major + 3 minor)	1(1), 1(2), 1(3), 1(9), 1(11)	1(2), 1(4), 1(8)
***C. annuum*/61/3**	13 (11 major, 2 minor)	2(1), 1(2), 1(3), 1(4), 1(5), 1(6), 1(8), 1(9), 2(11)	1(1), 1(10)
		CapSat5	
***C. annuum*/20141/2**	5 (5 major)	2(1), 1(2), 1(5), 1(11)	
***C. annuum*/50/2**	4 (major)	2(1), 1(2), 1(11)	
***C. annuum*/61/3**	7 (2 major, 5 minor)	2(10)	2(1), 1(2), 2(11)

Table 7 – Overview of numbers of major + minor loci and the respective localization in chromosomes in *Capsicum pubescens* individuals

Species/Accession/Individual	Total number of loci per haploid genome (major + minor)	Number of major loci and their localization in individual chromosomes. Number in brackets indicates which chromosome carries the locus/loci.	Number of minor loci and their localization in individual chromosomes. Number in brackets indicates which chromosome carries the locus/loci.
		5S rDNA	
***C. pubescens*/3858/2**	1 (major)	1(3)	
***C. pubescens*/56/1**	1 (major)	1(3)	
		35S rDNA	
***C. pubescens*/3858/2**	12 (major)	1(1), 1(2), 2(3), 1(4), 2(6), 1(8), 1(9), 1(10), 1(11), 1(12)	
***C. pubescens*/56/1**	11 (major)	1(1), 1(2), 2(3), 1(4), 2(6), 1(9), 1(10), 1(11), 1(12)	
		CapSat1	
***C. pubescens*/3858/2**	17 (15 major + 2 minor)	2(1), 2(2), 1(4), 1(5), 3(7), 1(8), 1(9), 2(10), 1(11), 1(12)	1(5), 1(11)
***C. pubescens*/56/1**	18 (17 major + 1 minor)	3(1), 2(2), 2(4), 1(5), 3(7), 1(8), 2(10), 1(11), 2(12)	1(8)
		CapSat4	
***C. pubescens*/3858/2**	10 (8 major + 2 minor)	1(1), 1(3), 1(4), 1(6), 1(7), 1(8), 1(9), 1(10)	1(9), 1(11)
***C. pubescens*/56/1**	9 (6 major + 3 minor)	1(1), 1(3), 1(6), 1(8), 1(9), 1(11)	1(9), 1(10), 1(11)
		CapSat5	
***C. pubescens*/3858/2**	10 (2 major + 8 minor)	1(7), 1(11)	1(1), 1(4), 1(5), 2(6), 2(8), 1(10)
***C. pubescens*/56/1**	10 (6 major + 4 minor)	2(1), 1(7), 1(8), 1(10), 1(11)	2(3), 1(5), 1(8)

Discussion

All eukaryotic genomes possess repetitive DNA. Two major classes of repeats, dispersed and tandemly repeated, make up a significant portion of the majority of higher plant genomes. Early hypothesis considered these repeats to be “junk DNA” or genomic parasites (Plohl et al. 2008). Recent developments and improvements of molecular techniques and analytical tools, however, continue to offer better insight into the biology of the repeats and allow for testing of the previous hypotheses. Thus, it is now commonly accepted and demonstrated in multiple systems that tandem repeats, due to their fast evolving nature, are not only involved in chromosomal and genomic rearrangements but are also useful tools to better understand genome evolution (Weiss-Schneeweiss et al. 2015; Garrido-Ramos 2017). Despite these new insights, still little is known about the patterns and dynamics of genomic abundance and chromosomal localization of tandem repeats, their role in the evolution of plant genomes and the functions of various tandem repeat families (Gemayel et al. 2010).

This study aimed to gain insight into the diversity, patterns and dynamics of tandem repeat variation in the two selected cultivated species of the genus *Capsicum*, *Capsicum annuum* and *Capsicum pubescens*. To this end, tandem repeats of 35S rDNA and 5S rDNA as well as three newly identified satellite DNAs were localized in the chromosomes of five *Capsicum* individuals using FISH. Three novel satellite DNAs: CapSat1, CapSat4 and CapSat5 were identified via analyses of Next Generation Sequencing data applying the bioinformatic pipeline RepeatExplorer (Novak et al. 2013).

All five tandem repeats were used as probes in different combinations, to allow for the most comprehensive characterization of individual chromosome pairs, of the two species analyzed. The localization of all five tandem repeats, relative to one another, allowed the construction of detailed ideograms for all five individuals and subsequently collectively for the construction of consensus karyotypes, for each of the two species. Some of the loci of the repeats mapped were consistently detected in all chromosomal complements/cells of each of the individuals and were referred to as major loci. Other loci were detected in some, but not all cells of a given individual and were thus referred to as minor loci. Minor loci were typically smaller and given, that FISH is only a semi-quantitative technique, their detection in some but not all cells likely resulted of the limitations of the method itself. Loci with fewer monomers in an array, especially if this number borders on a detection capability of the microscope concerning signal strength, were likely not consistently detected (Jang & Weiss-Schneeweiss 2015). The detection of the tandem repeats may also vary depending on the quality of the chromosomal preparations as well as the condensation degree of the chromosomes, that is, the accessibility of target

sequences for hybridization. All these factors likely influenced the numbers of observed minor satDNA loci.

High levels of variation in 35S rDNA loci contrast with conserved pattern of 5S rDNA locus distribution

All five analyzed *Capsicum* individuals, representing two species, possessed one subterminal 5S rDNA locus in one chromosome pair. These results are in accordance with previous reports on 5S rDNA loci numbers in those and 12 other *Capsicum* species belonging to several well defined evolutionary lineages (e.g.: Aguilera et al. 2016; Romero-da Cruz et al. 2017; Zhou et al. 2019; Scaldaferro & Moscone 2019). In the three *C. annuum* accessions analyzed in the current study, the 5S rDNA locus was found on chromosome 5, whilst in the two *C. pubescens* accessions it was found on chromosome 3. The only two previous studies, that analyzed the 5S rDNA localization in *C. pubescens,* both reported the 5S rDNA locus on chromosome 3 (Aguilera et al. 2016; Scaldaferro & Moscone 2019). In contrast, many more studies have analyzed the localization of the 5S rDNA locus in accessions belonging to the *C. annuum* clade. Whereas all the studies reported only one locus of this repeat, it was assigned to different chromosome pairs in different species e.g., on chromosome 3 (Aguilera et al. 2016), chromosome 4 (Romero-da Cruz et al. 2017) or chromosome 9 (Zhou et al. 2019). These differences in the localization of the 5S rDNA locus, especially in *C. annuum,* result from difficulties in identification of individual chromosome pairs in *Capsicum* as the majority of chromosomes of most *Capsicum* species are of very similar sizes and morphology. Thus, the order of the chromosomes in the karyotypes in this study was guided by the relative localization of the probes to each other, to allow for comparison within the species and as such, it does not directly correspond to the chromosome numbering in other studies. Nevertheless, one locus of the 5S rDNA has also been consistently found in this study, confirming it to be a conserved feature of all *Capsicum* genomes, in contrast to all other four tandem repeats analyzed here.

High levels of variation of both the number and localization of 35S rDNA loci have been reported in many species of *Capsicum* (Scaldaferro & Moscone 2019). The numbers of 35S rDNA loci varied in all analyzed *Capsicum* species between one and as many as 30 loci. Only one 35S rDNA locus was found in *C. annuum* var. *glabriusculum* (cytotype 1) and *C. rhomboideum,* whilst thirty 35S rDNA loci were found in *C. villosum* (Scaldaferro & Moscone 2019). In this study, one conserved and consistent major subterminal 35S rDNA locus was found on chromosome 11 in all three *C. annuum* accessions. However, two of the accessions (50/2 and 61/3) possessed an additional minor 35S rDNA locus localized either on

chromosome 12 in *C. annuum* 50/2, or on chromosome 8 in *C. annuum* 61/3. Several previous studies reported high levels of variation of 35S rDNA loci numbers among the analyzed accessions of *C. annuum*, ranging from a minimum of one locus to as many as eight loci (Romero-da Cruz et al. 2017; Zhou et al. 2019; Scaldaferro & Moscone 2019). However, out of the three analyzed accessions in the current study, only the accession of *C. annuum* 50/2 has previously been investigated and the same number of loci was reported (Romero-da Cruz et al. 2017).

High levels of variation of the number of 35S rDNA loci have also been reported previously for *C. pubescens* with loci numbers ranging from fourteen (Scaldaferro & Moscone, 2019) to 26 in the cultivar "Locoto rojo" (Grabiele et al. 2018). The two accessions analyzed in the current study have not been analyzed before, thus direct comparison is not possible. In both *C. pubescens* accessions analyzed here, eleven 35S rDNA loci were consistently found. One additional 35S rDNA locus was only observed in *C. pubescens* 3858/2. These results concerning the number and localization of 35S rDNA confirm higher level of intra-individual variation, since variation is present even within accessions of the same species in the genus *Capsicum.*

Higher intraspecific variation of satellite DNA patterns in *C. pubescens* than in *C. annuum*

Peppers are one of the most important cultivated vegetables in the world, but little is known about the composition of repetitive DNA in the genomes of the *Capsicum* species (Zhou et al. 2019). Despite the fact that three nuclear genome assemblies are available for *C. annuum* (Kim et al. 2014; Cheng et al. 2016) and plastid and mitochondrial genome sequences are available for few species (Jo et al. 2014; D'Agostino et al. 2018), only two studies so far have addressed the repetitive genome fraction of peppers. The evolution of intergenic-spacer sequences (IGS), localized between the 25S and 18S rRNA genes, was analyzed in five different cultivated *Capsicum* species (Jo et al. 2011). IGS typically consists of a central repeated region, rich in subrepeats of varying number. These subrepeats have been shown to give rise to satellite DNAs in several unrelated plant groups (e.g. genus *Phaseolus,* Almeida et al. 2012) also including some *Capsicum* species (Jo et al. 2011). This satellite repeat has been localized in some cultivated species (Jo et al. 2011). The only other satellite DNA characterized in peppers was "Ca167TR" composed of 167-bp monomers. It has been localized in subterminal position in two pairs of chromosomes in *C. annuum* (Zhou et al. 2019). No other tandem repeats have been identified in any of the nearly 40 species of peppers. Thus, the characterization and chromosomal localization of three

previously unknown tandem repeats in two pepper species reported in this study is novel and cannot be compared with other studies.

Satellite DNA distribution and localization in the three *Capsicum annuum* accessions reported in this study revealed, that two out of three accessions (20141/2 and 50/2) were more similar to one another than any of those to the third accession (61/3). Several hypotheses can be put forward to explain such patterns. Cultivated peppers are highly diverse with more than thousand varieties available and grown worldwide. These might differ in distribution of tandem repeats, as the varieties are often geographically isolated and under strong selective pressure and human-assisted selection. In addition to such existing variation, hybridization is also known to occur between varieties of *C. annuum* but also between *C. annuum* and other closely related cultivated species, especially *C. chinense* (Walter et al. 2019; Moreno-Peris et al. 2020; Scaldaferro & Moscone 2019). Thus, it is possible that either of these processes cause the deviating pattern of satellite DNAs distribution, observed in C. annuum individual 61/3. The satellite DNA with most differing pattern was CapSat5. *C. annuum* 61/3 had a total of five minor and two major CapSat5 loci, none of which was present in any of the two other accessions. Due to those differences, only *C. annuum* 20141/2 and *C. annuum* 50/2 were used for comparison of satellite DNA distribution and localization in this species. More extensive sampling and karyotyping of additional individuals and varieties of peppers as well as related cultivated species will allow for more and better understanding of the dynamics of this and other satellite DNAs in pepper genomes, both those that are cultivated and also in wild species.

The two *Capsicum annuum* accessions used in this study, *C. annuum* 20141/2 and *C. annuum* 50/2, showed largely similar distribution of major loci of all three novel satellite DNAs. The accessions shared seven CapSat1 loci and only differed by two major CapSat1 loci each. Both accessions shared three loci of CapSat4 and each had two loci that were unique. The two accessions shared all, but one of five detected CapSat loci.

The distribution and localization of the loci of three satellite DNAs in the two *Capsicum pubescens* individuals revealed high levels of variation between the two accessions. Both individuals shared thirteen CapSat1 loci, with only few loci being unique to one individual. In contrast, the majority of the loci of CapSat4 were unique to either of the individuals and only three loci were shared. Similarly, the majority of major CapSat5 loci in *Capsicum pubescens,* were unique and only two were shared.

Thus, *Capsicum pubescens* not only had more loci of 35S rDNA and more loci of two of the three satellite DNAs, but also the intraindividual variation in loci numbers and distribution was higher in this species when compared to *C. annuum*.

The differences in the numbers and/or localization of satellite DNA loci between the individuals and accessions within but also between the related species are also known to exist in other plant groups (genus *Brassicaceae,* Hloušková et al. 2019; Weiss-Schneeweiss et al. 2013). The repetitive DNAs, especially tandem repeats, are known to be much more dynamic in the genomes, in contrast to the more stable, coding part of the genome. This dynamic most often manifests itself in loci loss and gain, due to various molecular mechanisms often involving unequal or illegitimate recombination, (Garrido-Ramos 2017) inversions, translocations or extrachromosomal circular DNAs amplification (Sharma & Raina 2005; Lower et al. 2018).

C. annuum is the oldest domesticated and worldwide most cultivated pepper species and therefore under strong human selective pressure (Taitano et al. 2019) and prone to geographic isolation. The extant *Capsicum annuum* cultivars have been hypothesized to descend from just a few initial populations (Hulse-Kemp et al. 2016) and all global *C. annuum* accessions analyzed in a previous study showed high levels of admixture and limited genetic clustering (Taitano et al. 2019). *C. pubescens*, on the other hand, is considered to be the "wildest" among all cultivated pepper species and, in contrast to the globally cultivated *Capsicum annuum,* it is geographically limited and cultivated mostly locally in Central and South America. This implies that this species might have higher levels of genetic variation due to lower impact of human-assisted selection. It is also genetically isolated, not only from its closest relatives, but it does also not cross with any other of the cultivated species (Tripodi & Greco 2018). Therefore, *C. pubescens* might be under less human-assisted selection. This can have an impact on its genome dynamics, including the higher diversity, abundance and dynamics of the tandem repeats.

Localization of the rDNAs and three novel satellite DNAs allows for identification of at least seven of the twelve chromosome pairs in both *Capsicum* species

Satellite DNAs are useful cytogenetic markers that allow for tracing of the evolutionary rearrangements of the whole genomes, particularly on karyotypic level in various plant groups (Jang et al. 2016; Koo et al. 2016; Mlinarec et al. 2019). This study is the first to identify and apply the satellite DNAs for characterization of genome dynamics in peppers. Moreover, it characterizes and uses three novel

markers which, in addition to rDNAs, can be used for the identification of at least some chromosome pairs in the complements of the two cultivated pepper species.

Localization of 5S rDNA allows the identification of one chromosome pair in all analyzed individuals. In the three individuals, that were analyzed, mapping of 35S rDNA allowed consistent identification of one chromosome pair in *C. annuum* and two in *C. pubescens*. Mapping of 35S rDNA, in combination with the 5S rDNA, allowed discrimination between the two 35S-rDNA bearing chromosomal pairs in C. pubescens because chromosome 3 possessed two 35S rDNA loci as well as the 5S rDNA locus and chromosome 6 only had two 35S rDNA loci.

Further, five to six chromosome pairs in *C. annuum* (accessions 20141/2 and 50/2) could be discriminated using the combination of the two rDNAs, CapSat1, CapSat4 and CapSat5 probes. Chromosome 1 carried one locus of CapSat1 and two loci of CapSat5 on the same arm, chromosome 2 carried two loci of CapSat1 and one locus of CapSat5 on the same arm, chromosome 3 carried one locus of CapSat1 and CapSat4 on the same arm and chromosome 9 carried one CapSat4 locus on one arm. Chromosomes 4 and 8 could be distinguished from other pairs, because each of them possessed only one subterminal CapSat1 locus, but discrimination between these two was not possible.

In *Capsicum pubescens,* chromosome pairs 3 and 6 could be identified using 5S and 35S rDNA probes. Six other chromosomal pairs could further be identified using CapSat1, CapSat4 and CapSat 5 probes. Chromosome 1 had the 35S rDNA and the CapSat5 locus within the same arm and two CapSat1 loci within the other arm. Chromosome 2 not only possessed one 35S rDNA locus, but also one Sat1 locus in each of the arms. Chromosome 7 could be identified by a unique pattern of three CapSat1 loci, with one arm carrying two loci in close proximity to each other and the other arm carrying one subterminal CapSat1 locus. Chromosome 8 had one CapSat1 locus within one arm and a CapSat4 locus on the other arm. In both analyzed *C. pubsescens* accessions, chromosome 11 possessed one 35S locus, a CapSat5 locus and a CapSat1 locus within the same arm. The sixth chromosome, that could unequivocally be discriminated using rDNA and CapSat1 probes, was chromosome 12 with one 35S rDNA locus within one arm and one CapSat1 locus within the opposite arm.

This study has provided new insights into the dynamics and variation of rDNA and the three novel satellite DNAs (CapSat1, CapSat4 and Capsat5) in two cultivated *Capsicum* species, *Capsicum annuum* and *Capsicum pubescens*. Furthermore, it demonstrated that the number and localization of those tandem repeats have been highly variable between the accessions of each of the species, thus suggesting a high level of intra-individual variation. *In situ* hybridization, using a combination of the two rDNA types together with the three novel satellites, allowed for the unequivocal identification of seven to eight chromosome pairs in *Capsicum annuum* and eight chromosome pairs in *Capsicum pubescens.* Therefore, this study provides valuable markers for chromosome identification, which is not only very important for genetic and evolutionary analyses, but also useful for breeding programs.

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Abstract

Peppers are important worldwide grown spice crops. Although it was shown that genome of *Capsicum annuum* contain 60-83% of repeats, little is known about their composition and evolution. Cytogenetically, tandem repeats, which include satellite DNAs and rRNA genes are most interesting as they are often involved in genome rearrangements and provide valuable tools for investigating genome evolution.

This study aimed to characterize and determine the extent of variation of three novel satellite DNAs and two rDNA repeats in the genomes of two cultivated *Capsicum* species. Genome sizes of five individuals representing three accessions of *C. annuum* and two of *C. pubescens* were measured by flow cytometry. The RepeatExplorer pipeline was used to identify three novel satellite DNAs (CapSat1, CapSat4 and CapSat5) in NGS genome skimming data. These three satellite DNAs, together with two rDNAs (5S rDNA, 35S rDNA), were mapped in chromosomes of the five individuals using fluorescence *in situ* hybridization (FISH).

The comparative analyses of the karyotypes revealed high levels of variation of number and localization of 35S rDNA and satellite DNAs loci, but not of 5S rDNA. *C. pubescens* possessed more loci of nearly all analyzed tandem repeats and higher intraspecific loci variation than *C. annuum*. This might be the result of their different evolutionary history during their domestication. In both species, the most abundant satellite DNA was CapSat1. Furthermore, seven to eight chromosome pairs in *Capsicum annuum* and eight chromosome pairs in *Capsicum pubescens* could unequivocally be identified using the five tandem repeats.

Zusammenfassung

Chilis sind wichtige, weltweit angebaute Gewürzpflanzen. Obwohl gezeigt werden konnte, dass die Genome von *Capsicum annuum* aus 60-83% repetitiver DNA bestehen, ist wenig bekannt über ihre Genomzusammensetzung und -evolution. Zytogenetisch betrachtet sind „tandem repeats“, welche Satelliten DNA und rDNA Gene inkludieren, höchstinteressant, da sie an genomischen Umordnungen beteiligt sind und praktische Hilfsmittel zur Untersuchung der Genomevolution darstellen.

Das Ziel dieser Arbeit war die Charakterisierung und Untersuchung der Variation von drei neuen Satelliten DNAs und zwei rDNAs in den Genomen von zwei kultivierten *Capsicum* Arten. Genomgrößen von fünf Individuen, welche drei Sorten von *C. annuum* und zwei Sorten von *C. pubescens* repräsentieren, wurden anhand von Durchflusszytometrie gemessen. Mithilfe einer „RepeatExplorer“ Pipeline wurden drei neue Satelliten DNAs identifiziert (CapSat1, CapSat4 und CapSat5). Diese drei Satelliten DNAs wurden zusammen mit zwei rDNAs (5S rDNA, 35S rDNA) mithilfe von Fluoreszenz-*in-situ*-Hybridisierung auf den Chromosomen von den fünf ausgewählten Individuen abgebildet.

Durch vergleichende Karyotypen-Analyse konnten große Unterschiede in Bezug auf Anzahl und Lage der 35S rDNA- und Satelliten DNA Loci, jedoch nicht im Fall der 5S rDNA, festgestellt werden. *C. pubescens* zeigte nicht nur eine höhere Gesamtzahl der Loci von beinahe allen analysierten tandem repeats sondern auch höhere intraspezifische Variabilität der Loci als *C. annuum.* Dies könnte das Resultat ihrer unterschiedlichen Domestikationsgeschichte sein. Die in beiden Arten am häufigsten vorkommende Satelliten DNA, war CapSat1. Außerdem war es möglich, sieben bis acht Chromosomenpaare in der Art *Capsicum annuum* sowie acht Chromosomenpaare in der Art *Capsicum pubescens* eindeutig zu identifizieren.