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Evoluce genomů v tribu Camelineae (brukvovité)

Diplomová práce

Martin Polák

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Abstrakt

Tribus Camelineae patří do rostlinné čeledi brukvovitých (Brassicaceae). Přestože zahrnuje nejvýznamnější modelový rostlinný druh huseníček rolní (*Arabidopsis thaliana*) nebo důležitou plodinu lničku setou (*Camelina sativa*), zůstává většina zástupců tohoto tribu prakticky neprozkoumána. Pro cytogenetické analýzy bylo vybráno pět zástupců tribu Camelineae: *Chrysochamela elliptica* (2n = 16), *C. noeana* (2n = 16), *C. velutina* (2n = 16), *Neslia paniculata* (2n = 14) a *N. apiculata* (2n = 42). Struktura karyotypů těchto druhů byla rekonstruována pomocí FISH lokalizace základních cytogenetických markerů (35S a 5S rDNA) a především pomocí metody srovnávacího malování chromozomů (CCP). Zjištěné struktury karyotypů byly porovnány s referenčním ancestrálním karyotypem brukvovitých. Výsledky popisují tři odlišné postupy evoluce struktury karyotypů. Celý rod *Chrysochamela* nevykazuje žádnou změnu ve svých karyotypech oproti ancestrálnímu karyotypu. U *N. paniculata* byla identifikována jedna pericentromerická inverze následovanou fúzí chromozomů. Druhá identifikovaná pericentromerická inverze dala vzniknout telocentrickému chromozomu. U *N. apiculata* byly identifikovány tři homeologní kopie genomických bloků a mnohé genomové přestavby. Ze získaných dat i z dat již publikovaných vyplývá, že evoluce genomů v tribu Camelineae postupovala třemi z pěti směrů evoluce genomů dosud popsaných u brukvovitých: **i)** genomovou stabilitou, **ii)** přeskupením a redukcí chromosomálního počtu a **iii)** celogenomovou triplikací následovanou přestavbami genomu a návratem do diploidního stavu.

Abstract

Tribe Camelineae belongs to the plant family of crucifers (Brassicaceae). Although it includes the most important model plant organism thale cress (*Arabidopsis thaliana*) or an important crop false flax (*Camelina sativa*), the most of the tribe's species remain practically unexamined. Five species were selected from this tribe for cytogenetic analyses: *Chrysochamela elliptica* (2n = 16), *C. noeana* (2n = 16), *C. velutina* (2n = 16), *Neslia paniculata* (2n = 14) and *N. apiculata* (2n = 42). Karyotype structure in those species was reconstructed by using FISH to localise basic cytogenetic markers (35S and 5S rDNA) and especially by comparative chromosome painting (CCP). Observed karyotype structures were compared to the reference Ancestral Crucifer Karyotype. The results describe three different pathways of evolution in karyotype structures. The whole genus *Chrysochamela* has structurally unchanged karyotype in contrast to ancestral karyotype. For *N. paniculata* one pericentromeric inversion was detected triggering fusion of two chromosomes. The second identified pericentromeric inversion led one chromosome to become telocentric. For *N. apiculata* three homeologous copies of genomic blocks and many genomic rearrangements were detected. Given results in congruence with literature suggest the genome evolution in the tribe Camelineae taking three of five directions so far described for Brassicaceae: **i)** genome stasis, **ii)** reshuffling and descending dysploidy and **iii)** whole-genome triplication followed by subsequent diploidisation.

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Název práce: Evoluce genomů v tribu Camelineae (brukvovité)

Název práce anglicky: Genome evolution in the tribe Camelineae (Brassicaceae)

Oficiální zadání:
Tribus Camelineae obsahující rostlinný modelový rod Arabidopsis je z vědeckého hlediska nejdůležitější skupinou brukvovitých (Brassicaceae). Vedle několika modelových druhů tento tribus také zahrnuje významné plevelné druhy a sekvenovaný genom lničky seté (Camelina sativa; stále populárnější zdroj biopaliv). Navzdory své potenciální důležitosti, genomy několika druhů tribu Camelineae zůstávají neprozkoumány nebo jsou zcela neznámé. O vzniku a evoluci těchto přehlížených genomů existuje překvapivě málo informací. Cílem diplomové práce je (re)konstruovat srovnávací cytogenomické mapy a ancestrální genomy pro zástupce tribu Camelineae, identifikovat mechanismy a evoluční význam genomových přestaveb, a rekonstruovat fylogenetickou strukturu a postavení tribu Camelineae.

Jazyk závěrečné práce: angličtina

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student	**vedoucí práce**	**ředitel Ústavu experimentální biologie**

Poděkování

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Prohlášení

Prohlašuji, že jsem svoji diplomovou práci vypracoval/a samostatně s využitím informačních zdrojů, které jsou v práci citovány.

Brno 10. května 2018

Polák
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Martin Polák

CONTENT

ABBREVIATIONS

ACK Ancestral Crucifer Karyotype – all the chromosomes of hypothetical ancestor of Brassicaceae

AK ancestral chromosome from the ACK or a chromosomal probe designed to match the arrangement of genomic blocks in this chromosome

BAC bacterial artificial chromosome

BIO Biotin – combined with Texas Red (the red signal)

DIG Digoxigenin – combined with Alexa Fluor 488 (the green signal)

CCP comparative chromosomal painting

CP chromosomal painting

MCR major chromosomal rearrangement

NOR nucleolar organiser region

PCK Proto-Calepineae Karyotype

WGD whole-genome duplication

WGT whole-genome triplication

1. INTRODUCTION

„Comparative genomics is … an important field to study genome evolution, sequence collinearity and transfer of information from extensively studied model organisms to species of commercial interest“ (Sharma et al., 2014)

1.1. Brassicaceae

Much of the knowledge in the field of flowering plants comes mostly from various and deep studies of *Arabidopsis thaliana*. Obtaining its complete genome sequence affected every plant field of study and laid down the foundations for comparative plant studies on a molecular level. *Arabidopsis* is a member of the mustard family (Brassicaceae). Apart from this little weed, the family comprise about 3976 other species included in 341 genera and 52 tribes, as declared by the project BrassiBase (URL 1). Many of these species remain poorly explored. Thanks to the use of *A. thaliana* in various experiments as well as the agricultural importance of the family, Brassicaceae pose a group of high interest. According to NCBI database, 83 Brassicaceae species have their genomes completely sequenced so far or are in the process (URL 2) of which 17 belong to the tribe Camelineae (URL 3). The family's multiplicity and diversity of species together with its worldwide distribution, a content of popular model organisms and a large piece of knowledge obtained so far make it suitable for evolutionary studies.

1.1.1. Phylogeny of Brassicaceae

The era of molecular approaches brought new insights into the evolution and the phylogeny of Brassicaceae. Within the family, the tribe Aethionemeae is a sister taxon to the group containing all the other tribes (the group of core Brassicaceae) (Beilstein et al., 2006). Within the core Bressicaceae, Beilstein et al. (2006) established three major monophyletic lineages (LI, LII and LIII) for core Brassicaceae according to chloroplast gene *ndh*F sequence (Fig. 1A). These gained support in later studies finally resulting in the work of Huang et al. (2016) in which more than 100 orthologous nuclear markers in 55 genomic and transcriptome datasets were used to introduce robust phylogenetic tree resolving the early diversification defining the family's diversity (Fig. 1B). Clades *sensu* Huang et al. (2016) were introduced. Clade A for Lineage I, Clade B for Lineage II and Clade C and E for Lineage III and also poorly resolved Expanded lineage II (EII – Franzke et al., 2011) was revised. Guo et al. (2017) introduced so far the latest phylogenetic tree of Brassicaceae reconstructed with the use of plastomes of 51 species (Fig. 1C). Most notably, the position of Clade E (Lineage III) is revised to have a common ancestor with just Clades B + C group instead of all A + B + C Clades. That makes Clade A a sister group to Clades B + C + E. Guo et al. also place the origin of major lineages and clades between Oligocene and Miocene followed by rapid diversification. Though the family Brassicaceae is distributed worldwide, the highest diversity is observed in Irano-Turanian and Mediterranean regions (Appel and Al-Shehbaz, 2003). This suggests the origin of this family be located in Turkey and adjacent regions.

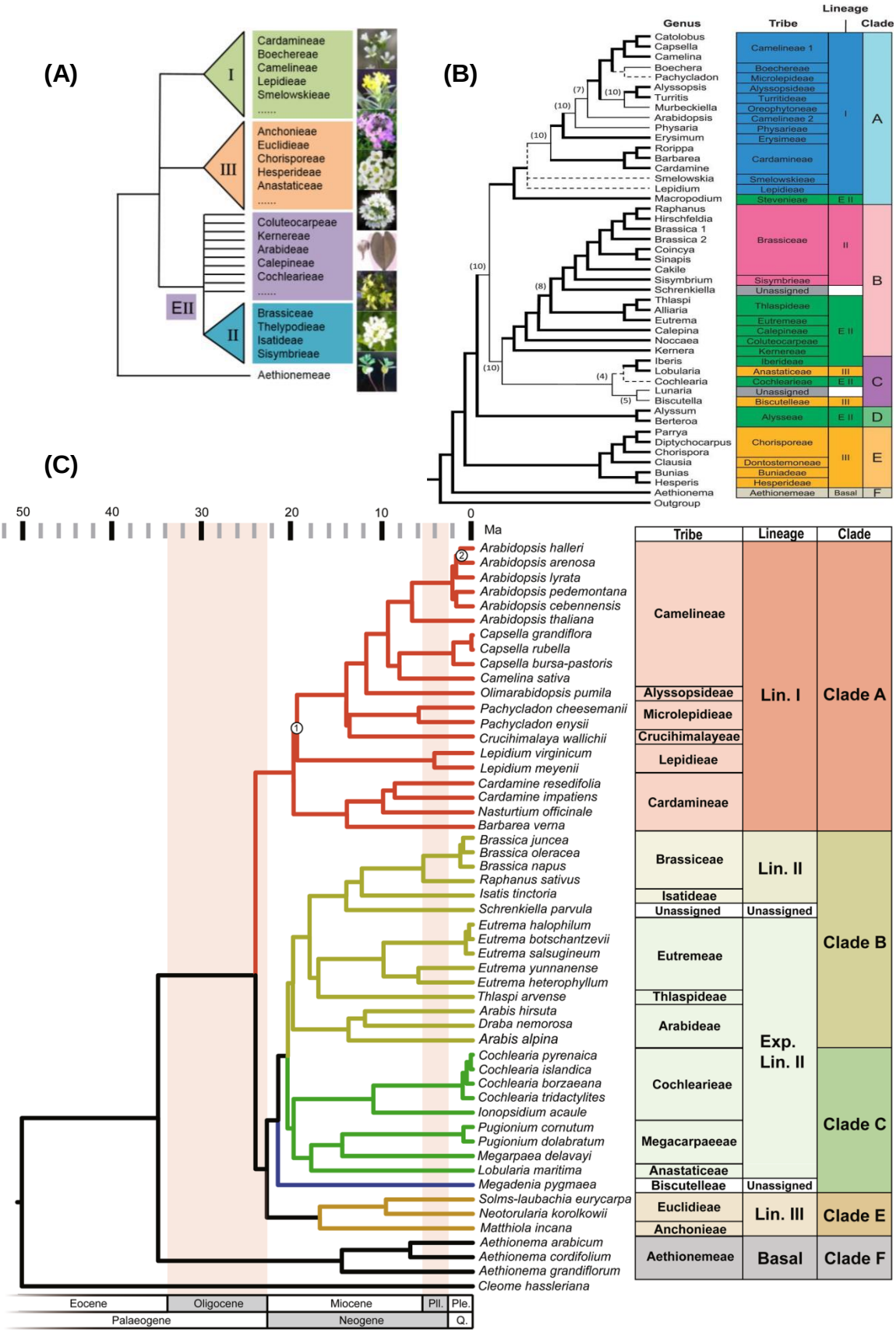

Fig. 1: Phylogenetic trees of the family *Brassicaceae*: **A)** according to Franzke et al. (2011) showing three lineages (I, II, III and unresolved EII), **B)** according to Huang et al. (2016) adding five clades (A – E) and **C)** newest phylogenetic tree according to Guo et al., (2017) which also provides time calibration (Ma = million years ago, Ple. = Pleistocene, Pli. = Pliocene, Q. = Quaternary). Tribe Aethionemeae is used in all trees as sister group to all core Brassicaceae.

1.1.2. Comparative chromosomal painting and its application in Brassicaceae

Pinkel et al. (1988) were the first to use the term „chromosome painting“ (CP). This method involves labelling whole chromosomes or their large segments with fluorescently dyed probes. The aim is not to observe particular gene or locus but to analyse chromosomal structure as a whole. The realm of the application typically includes a clinical diagnostics (e.g. Langer et al., 2004) and as such uses the same genomes as probes and template. But when probes derived from one species are hybridized on chromosomes of other species, an evolution of these two genomes can be compared. This method is called comparative chromosome painting (CCP).

The initial success in this field belongs to evolutionary studies of hominoids (Jauch et al., 1992). Replacing or being added to the previous combination of comparative cytogenomic banding techniques and gene mapping, CCP has become a valuable tool to help elucidate the evolution of animal genomes (Chowdhary and Raudsepp, 2001). Comparative painting techniques open possibilities for studying not only rearrangements but also chromosomes involved in aneuploidy, break points involved in reshuffling or identification of homologous regions across the species.

For the kingdom of plants, this method proved to be not suitable (Fuchs et al., 1996). Probes prepared with chromosomal flow-sorting or microdissection contained high proportions of complex dispersed repeats. They hybridized unspecifically creating dispersed signals. This was caused by high amount of repetitive sequences in the genomes of the examined species. Unfortunately, plant genomes are overflowing with repeats (up to 90% of a genome), especially tandem repetitive sequences (Mehrotra and Goyal, 2014). In contrast to mammalian genomes, plant genomes comprise a high diversity of repeats and at the same time interchromosomal distribution of these repeats is more homogeneous (Schubert et al., 2001).

The unspecific hybridisation can be prevented by using prehybridisation of unlabeled whole genome probes in excess (Lichter et al., 1988). This method is called chromosomal *in situ* suppression (CISS) hybridisation. However, this didn’t work for plants due to a multitude of dispersed repeats with high complexity (Fuchs et al., 1996) and their interchromosomal homogenisation (Schwarzacher et al., 1997). In addition, RFLP or cDNA clones of genetically defined regions used as probes have also failed to paint the corresponding chromosome segments with sufficient signal intensity (Fuchs et al., 1996).

Unique sequences are too short and too widely separated to provide sufficient signal intensity (Schubert et al., 2001). Thus, the probe signals are either dispersed across the chromosomes unspecifically or too weak to be properly identified. In conclusion, probes homologous to large portions of chromosomes cannot be used for plants since they include unspecific dispersed repeats. Different painting techniques have to be used in contrast to those applied to the analyses of animal or human genomes. These use genomic *in situ* hybridisation, probes made of repetitive sequences or fluorescence *in situ* hybridization in small genomes with a low amount of repetitive sequences.

Genomic *in situ* hybridization (GISH) was achieved in plants by Schwarzacher et al. (1989) but this technique enables only to distinguish parental genomes in interspecific hybrids. It allows observing alien sequences in genomes, translocations between parental chromosomes or unbalanced deletions and duplications. This is possible due to the usage of species-specific repetitions and blocking of shared repetitive sequences.

In another method, the chromosome-specific repeats are used. Only B chromosomes (Houben et al., 1996) and sex chromosome Y (Shibata et al., 1999) were painted in plants with these chromosome derived probes. This technique is based on different fluorescence intensity of labeled chromosomes due to a different amount of particular repetition. Multicolour chromosome-specific probes based on repetitions were also used (Kato et al., 2004).

Fluorescence *in situ* hybridization (FISH) using bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs) to prepare probes with locus-specific sequences have been also applied. It was successfully used for plants with small genomes and low content of repetitive sequences such as sorghum, cotton, tomato or potato (e.g. Woo et al., 1994; Hanson et al., 1995; Dong et al., 2000). Term BAC-FISH refers to the application of one or few BAC clones as a probe. On the other hand, chromosomal painting in crucifer cytogenetics uses BAC contigs made up from many clones covering large chromosomal regions or whole chromosomes.

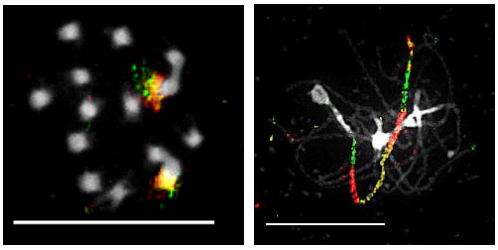

Fig. 2: The comparison of the chromosomal painting applied on mitotic chromosomes (left) and pachytene chromosomes (right). Mitotic chromosomes are too small and condensed to provide a suitable resolution.

The genome of crucifers is, in general, small and with a low amount of repetitive sequences organised in clusters (i.e. in centromere, pericentromeric regions and NORs) (Schmidt and

Bancroft, 2010, p. 19). Small mitotic chromosomes of *A. thaliana* dissuaded from cytogenetic analyses. However good resolution was obtained using much larger pachytene chromosomes (see Fig. 2) which have led to detailed cytogenetic descriptions (Fransz et al., 1998).

The genome of *Arabidopsis thaliana* comprises a very low amount of repetitions (approximately 15% – Leutwiler et al., 1984). The complete sequencing of its genome led to the assembly of chromosome-specific contigs composed of its BAC clones (Mozo et al., 1998). Lysak et al. (2002) used these BAC clones to successfully visualise chromosome 4 of *A. thaliana* with the method of CP during mitosis, meiosis and as well as interphase. In 2004 *A. thaliana* became the first plant species with completely painted karyotype (Pecinka et al., 2004). This enabled to introduce CP into genome studies of Brassicaceae. Since then, BAC clones of *A. thaliana* have been used in the most of the comparative studies analysing genomes of Brassicaceae.

1.1.3. Ancestral Crucifer Karyotype

Though the probes are routinely prepared from DNA of *A. thaliana*, its genome cannot be used as a reference point because of its complex reshuffling (Lysak et al., 2006). Five inversions, three fusions and two translocations are assumed as responsible for chromosome number reduction of *A. thaliana* to n = 5 (Fig. 3). The most common chromosome numbers of crucifers, however, are based on x = 8 (Warwick and Al-Shehbaz, 2006). This raised a question whether the ancestor of all Brassicaceae could have 8 chromosomes in haploid cells.

Hypothetical ancestral crucifer karyotype (ACK, n = 8) based on *A. lyrata* (Lysak et al., 2006) was established as a reference point and divided into 24 genomic blocks (Schranz et al., 2006). The concept of ACK was based on the comparative studies of *A. thaliana* with *A. lyrata* (Kuittinen et al., 2004), *A. thaliana* with *Capsella rubella* (Boivin et al., 2004), *A. thaliana* with *Brassica napus* (Parkin et al., 2005) and five other species (Lysak et al., 2006). Later were genomic blocks revisited and their number reduced from 24 to 22 (Fig. 3; Lysak et al., 2016). The reason for that was that the genomic blocks **K** and **L** are in fact separated only in *Arabidopsis* and blocks **M** and **N** only in *Brassica* species. That is why combined blocks **K+L** and **M+N** were introduced and are used in this study. Since its introduction, the concept of ACK has been used in numerous comparative studies of crucifer genomes.

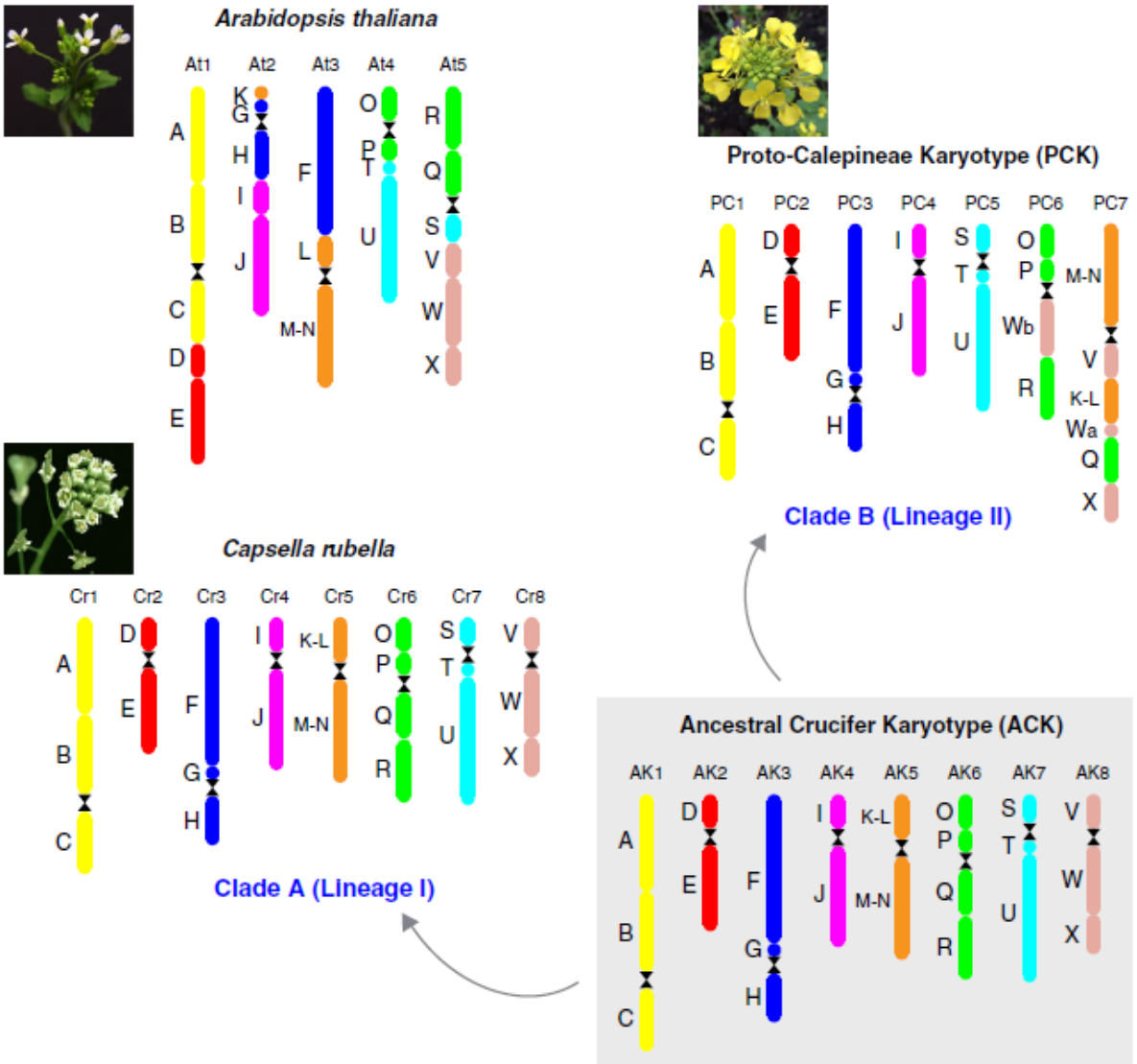

Fig. 3: Ancestral Crucifer Karyotype (ACK) and karyotypes of several crucifer species. ACK has 8 chromosomes (AK1, AK2, … AK8) consisting of genomic blocks named with letters (A, B, … X). *Arabidopsis thaliana* from the phylogenetic Clade A (Lineage I) has highly reshuffled genome folded into 5 chromosomes (At1, At2, … At5). On the other hand *Capsella rubella* of the same Clade has the same number and structure of chromosomes (Cr1, Cr2, …) as ACK. An important ancestral karyotype of n = 7 species of Clade B (Lineage II) is shown as derived from the ACK. Two chromosomes (PC6 and PC7) originated through inversions and translocations in AK5, AK6 and AK8. This figure was taken from Lysak et al. (2016) and adjusted.

1.1.4. Karyotype structure and evolution in the diploid Brassicaceae

ACK was probably the ancestral karyotype of all the Brassicaceae. When analysing karyotype evolution in six tribes which were all sharing x = 7 chromosome numbers a second ancestral karyotype was introduced (Mandakova and Lysak, 2008). Proto-Calepineae Karyotype (PCK) shares the same structure of 5 chromosomes with ACK but two of its chromosomes are derived from three chromosomes of ACK (Fig. 3) through chromosomal rearrangements (also supported later by Murat et al., 2015). This is an example of karyotype evolution through descending dysploidy, i.e. a reduction of chromosome number (the same as in the case of *A. thaliana*). Together with ACK, both ancestral karyotypes remain important reference points in comparative studies.

PCK is the ancestral karyotype for all species of Clade B (Lineage II) since all these share chromosome number reduced to 7. Descending dysploidy forming PCK probably occurred before the diversification of the whole clade (Mandakova and Lysak, 2008). Karyotypes of tribes Calepineae and Conringieae do not differ from PCK as described in Mandakova and Lysak, 2008. Chromosomal structure of tribe Coluteocarpeae secondary altered through inversions (Mandakova et al., 2015b). The PCK derived genome seems to be relatively stable. In general, the diversification of the tribe was not accompanied with any major repatterning so at least in this lineage, major chromosomal rearrangements (MCRs) did not have any important role during speciation. The only major rearrangement found in this clade was a whole arm translocation leading to the establishment of tPCK as an evolutionary younger variant of PCK. This was the ancestral karyotype of tribes Brassiceae (Cheng et al., 2013), Eutremeae (Wu et al., 2012), Isatideae and Sisymbrieae and in *Schrenkiella parvula* (Mandakova and Lysak, 2008).

For Clade A (Lineage I) ACK pose clearly as the direct ancestral genome (Lysak et al., 2006; Murat et al., 2015). None rearrangement in contrast to ACK occurred in the karyotype of n = 8 *Arabidopsis* species (Lysak et al., 2006) and *Capsella rubella* (Slotte et al., 2013). Cardamineae keep number of their chromosomes n = 8 but have those chromosomes reshuffled like *C. amara* or *C. rivularis* (Mandakova et al., 2013) or *C. hirsuta* (Hay et al., 2014), whereas *C. flexuosa* contains in its allopolyploid genome sequences of its diploid progenitors (Mandakova et al., 2014) and genome of *C. schulzii* is built even from three diploid progenitors (Mandakova et al., 2013). Descending dysploidy described as initial event for the karyotype evolution of Clade B also occurred in the evolution of Clade A in several occasions. Through descending dysploidy original n = 8 ACK turned into derived n = 7

chromosome number in two cases. One descending dysploidy reported for tribe Boechereae (Mandakova et al., 2015a) and another for tribe Descurainieae (Lysak et al., 2006). In Turritideae the number of chromosomes in karyotype dropped to 6 (Lysak et al., 2006).

Through the descending dysploidy ancestral karyotype of Clade E (CEK) got 7 chromosomes (Mandakova et al., 2017a). Since then the genome structure of species from the Clade E mostly didn't change except for translocations in Euclidieae. CEK was derived from older ancestral karyotype of 8 chromosomes which was a progenitor to tribe Arabideae (Willing et al., 2015; Mandakova et al., 2017a).

To conclude, at least four evolutionary events can make effect in chromosomal structures of diploid Brassicaceae according to Lysak (2016): **i)** genome stasis without major chromosomal reshuffling (n = 8) as in case of *Capsella rubella*, **ii)** genome stasis with a few structural rearrangements (n = 8) as observed in Cardamineae or **iii)** with more structural rearrangements (e.g. Arabideae – Willing et al., 2015) and **iv)** genome reshuffling accompanied with descending dysploidy (n = 7, 6 or 5) as described for *A. thaliana* (Lysak et al., 2006), PCK (Mandakova and Lysak, 2008), etc.

1.1.5. The whole-genome duplication or triplication and subsequent diploidisation

The previous chapter described the unveiled possibilities of karyotype evolution observed for diploid crucifer species. Besides these four evolutionary pathways, however, there is a fifth way involving whole-genome duplication (WGT) or triplication (WGT). Polyploidy used to be considered as a problematic factor rather retarding than promoting evolution by earlier authors (Mable, 2004) but in fact, it provides the potential for establishing new "playground" for evolution liberating single-copy sequences and genes from the necessity to not be altered.

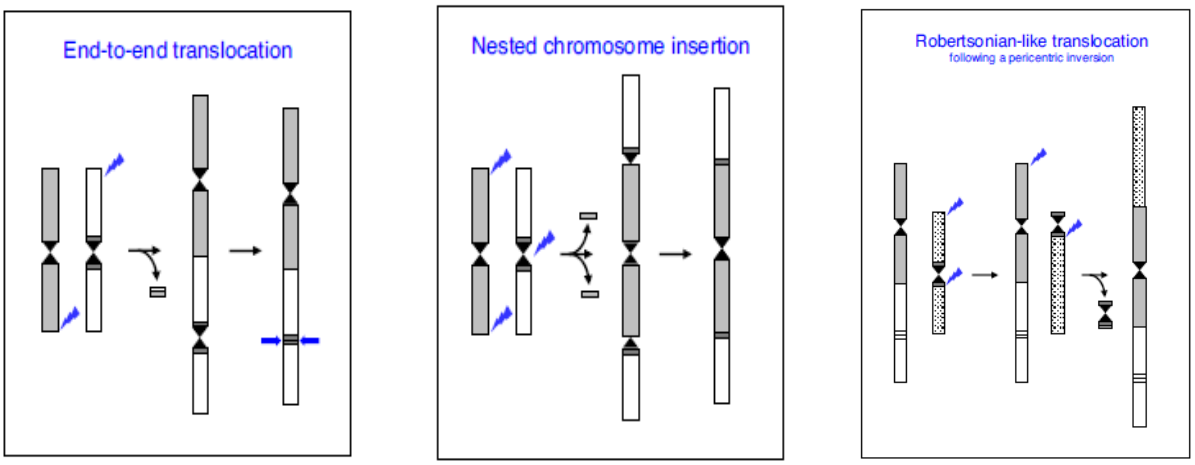

Fig. 4: Three possible principles involved in chromosome number reduction following polyploidisation event (adjusted from Mandakova and Lysak, 2018). The left scheme shows End-to-end translocation. The middle scheme shows nested chromosome insertion. The right one shows Robertsonian-like translocation following a pericentric inversion. Black arrows divide the processes to individual steps. Blue lightnings represent double strand breaks and blue arrows highlight the loss of functional centromere.

The changes take place in a period called post-polyploid diploidisation (PPD) (Mandakova and Lysak, 2018). Unfortunately, this complex process has remained largely overlooked topic until nowadays, although the whole-genome duplication and the post-polyploid diploidisation repeat throughout the history of species (Mandakova and Lysak, 2018). It includes chromosomal rearrangements shown in the Fig. 4. End to end translocations (EET) requires two double-strand breaks, each on the ends of different chromosomes. By these ends, chromosomes fuse together creating one dicentric chromosome. One of the centromeres has to be inactivated and/or removed to stabilise new chromosome. Another option requires three double-strand breaks to enable one chromosome to be inserted inside recipient chromosome, respectively inside or near its centromere (Luo et al., 2009). This is called nested chromosome insertion (NCI). A pericentric inversion can turn meta- or submetacentric chromosome into a telocentric (or acrocentric) chromosome or this kind of chromosome is already present. Such chromosomes can recombine with any other chromosome by mean of Robertsonian-like translocation (in general terminal chromosome translocations) resulting in one large stable chromosome (comprises mostly all four original chromosomal arms) and an unstable small one consisting predominantly of the telo/acrocentric's centromere. This minichromosome is discarded. Nevertheless, also non-

dysploid rearrangements accompany diploidisation such as inversions, duplication, deletions or reciprocal translocations (Mandakova and Lysak, 2018).

1.1.6. Karyotype structure and evolution in the polyploid Brassicaceae

Plant genomes, in fact, underwent many polyploidisation events in their history. The family Brassicaceae went through three polyploidizations prior its first radiation. At first, a triplication γ is shared in the common history of all dicotyledons (Jaillon et al., 2007). A paleotetraploidy event (ß) was detected in the history of the core Brassicales including Brassicaceae (Ming et al. 2008; Tang et al. 2008). The latest tetraploidisation event (α or At-α) occurred before the split of the Brassicaceae crown group from the tribe Aethionemeae (Blanc et al., 2003; Bowers et al., 2003). These events are ranked as paleopolyploidisations. After WGD, organisms can be broadly classified into neopolyploids, mesopolyploids and paleopolyploids according to time between WGD and the current rate of diploidisation (Mandakova et al., 2010; Mandakova and Lysak, 2018). Neopolyploids are the most recent polyploids. Their parental genomes remain mostly intact and distinguishable and parental species are still being extant. They are detectable with painting techniques. The reverse applies to paleopolyploids as their genomes are *quasi*-diploid with very reshuffled structure. Their parental subgenomes can be distinguished only with bioinformatic searches for orthologous and paralogous sequences. In genomes of mesopolyploids it is still possible to differ parental subgenomes with cytogenetics and phylogenetic approaches.

CCP was used mostly to uncover chromosomal rearrangements in mesopolyploids. Species of the tribe Microlepidieae, endemic to Australia and New Zealand, are an example of polyploidisation effects on karyotype evolution. They probably came across two hybridization events. Though they don't have high numbers of chromosomes, Australian Microlepideae passed through allopolyploid whole-genome duplication followed by the extensive reduction of the chromosome number (Mandakova et al., 2010b). Genus *Pachycladon* also underwent allopolyploidisation followed by diploidisation (Mandakova et al., 2010a). Origin of polyploid genomes of Microlepidieae was further analysed in Mandakova et al. (2017b). WGD was also observed in genomes of Cardamineae (Mandakova et al., 2013; 2014; 2016), Brassiceae (Lysak et al., 2005; Wang et al., 2011) or

Camelina sativa (Kagale et al., 2014). From the Clade C a tetraploid genome of *Biscutella laevigata* was analysed (Geiser et al., 2016).

1.2. The tribe Camelineae

Camelineae is a tribe of core Brassicaceae from lineage I, clade A (Fig. 1). It includes important model species like *A. thaliana* or economically important crop *Camelina sativa*. It is karyologically a very diverse group with chromosome number rising from 2n = 10 to 2n = 42 (source BrasiBase): The most common chromosome numbers of crucifers are based on x = 8 and this is valid to Camelineae as well (Warwick and Al-Shehbaz, 2006).

The tribe includes 7 genera: *Arabidopsis*, *Camelina*, *Capsella*, *Catolobus*, *Chrysochamela*, *Neslia* and *Pseudoarabidopsis* (Fig. 5, Tab. 1). Except for *A. thaliana* (n = 5) for all remaining *Arabidopsis* species, 16 or 32 (x = 8) chromosomes in diploid cells were reported. This is the same also for *Chrysochamela velutina* and genus *Capsella*. *A. arenosa*, *A. lyrata*, *A. kamchatica* and *A. suecica* are tetraploids (Bomblies and Madlung, 2014, Shimizu-Inatsugi et al., 2009). *C. bursa-pastoris* (2n = 32) is also tetraploid of probable origin through hybridization between *C. orientalis* and *C. grandiflora* (Douglas et al., 2015). Chromosome numbers 2n = 16 and 21 were reported for Catolobus and chromosome counts based on x = 7 for Neslia (14 and 42). The origin of polyploid *Neslia apiculata* has not yet been clarified. Camelina seems to be very variable genus with counts ranging from 2n = 12 up to 2n = 40 (i.e., 12, 16, 26, 32, 38, and 40) and Camelina sativa (2n = 40) is probably former hexaploid (Kagale et al., 2014). 50% of Camelineae species are neopolyploids (Hohmann et al., 2015).

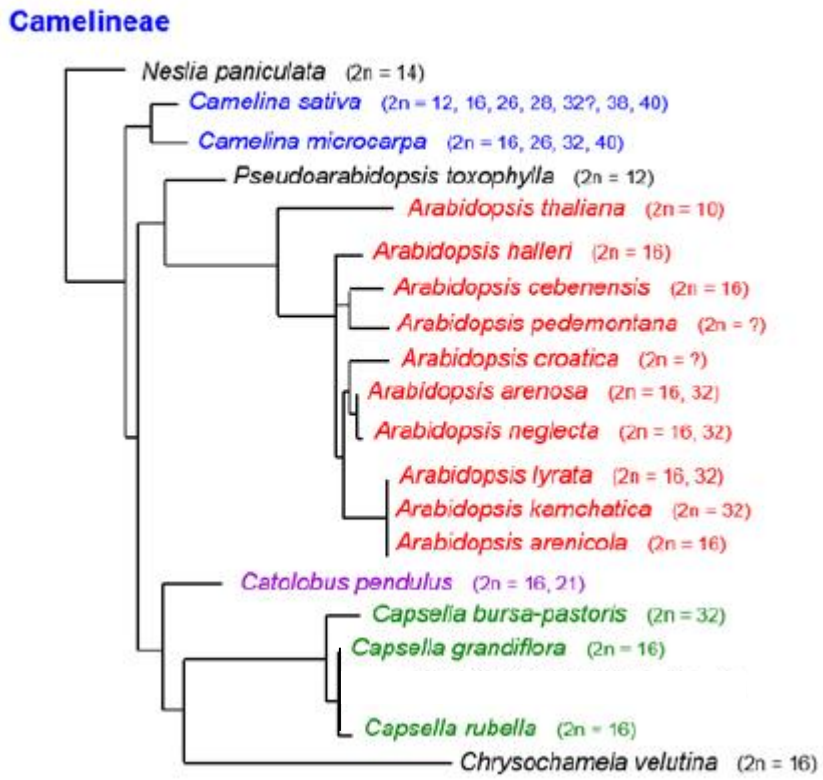

Fig. 5: The so far established phylogenetic tree of the tribe Camelineae. The colours highlight affiliation to the same genera and chromosome numbers taken from BrassiBase are included (adjusted by Mandakova, 2016 and further in this work according to newest phylogeny of Brassicaceae by Guo et al., 2017).

Tab. 1: Genera of the tribe Camelineae. Information is gathered from Brassibase (URL 1) and Mandakova 2016.

Genus	Number of species	Distribution	Number of chromosomes (2n)
Arabidopsis	10	Eurasia, N America	10, 16, 32
Camelina	8	SE Europe, E Maditerranean	12, 16, 26, 28, 32, 38, 40
Capsella	5	Eurasia (C. bursa-pastoris worldwide)	16, 32
Catolobus	1	Europe, Russia, Japan	16, 21
Chrysochamela	3	Turkey, Lebanon, Syria, Russia	16
Neslia	2	Eurasia, N Africa	14, 42
Pseudoarabidopsis	1	Russia, Kazakhstan, W China	12

2. AIM OF THE STUDY

This study examines the arrangements of the genomic blocks in genomes of selected species and compares them with supposed ancestral arrangements in order to fulfil these goals:

- to identify karyotype structures for selected species
- to provide explanations for the observed arrangements of the genomic blocks and to unveil the evolution of karyotypes in the tribe Camelineae

3. MATERIALS AND METHODS

3.1. List of chemicals and mixtures

4T buffer: 4× SSC, pH 7.0; 0.05% Tween-20

Blocking solution: 5% bovine serum albumin; 0.2% Tween-20 in 4× SSC

Carnoy's fixative I: 3:1 ethanol and glacial acetic acid

Citrate buffer: 40 ml of 100 mM citric acid; 60 ml of 100 mM trisodium citrate; adjust pH to 4.8 using 1 M HCl; dilute to 1× with distilled, deionized H_2O

Hybridisation buffer: 50% formamide; 10% dextran sulfate in 2× SSC

NT buffer 10×: 500 mM Tris·Cl, pH 7.5; 50 mM $MgCl_2$; 0.05% bovine serum albumin

Pectolytic enzyme mixture: 0.3% (weight/volume) pectolyase "Y-23" (Duchefa Biochemie, cat. no. P8004); 0.3% (w/v) cellulase "Onozuka R-10" (SERVA, cat. no. 16419.03); 0.3% (w/v) cytohelicase (Sigma, cat. no. C8274)

SSC 20×: 3 M sodium chloride; 300 mM trisodium citrate, pH 7.0

TNB buffer: 100 mM Tris·Cl, pH 7.5; 150 mM NaCl; 0.5% blocking reagent (Roche)

TNT buffer: 100 mM Tris·Cl, pH 7.5; 150 mM NaCl; 0.05% Tween-20

3.2. Plant material

The examined species (Tab. 2) covered two whole genera of the tribe Camelineae (*Chrysochamela* and *Neslia*): *Chrysochamela velutina* (2n = 16), *Chrysochamela elliptica* (2n unknown before this study), *Chrysochamela noeana* (2n unknown before this study), *Neslia paniculata* (2n = 14) and *Neslia apiculata* (2n = 42).

Tab. 2: Species investigated in the study. Information is gathered through a comparison of several databases (URL 1, 4 to 9).

Image[a)]	Name[b)]	Distribution[c)]	Number of chromosomes[b)]	Genome size (C-value)[b)]
	*Chrysochamela elliptica* Boissier, 1867	Turkey	?	?
	*Chrysochamela noeana* Boissier, 1867	Turkey	?	?
	*Chrysochamela velutina* Boissier, 1867	Turkey, Syria, Lebanon	16	0.35
	*Neslia paniculata* (L.) Desvaux, 1815	continental Europe, central Asia	14	0.2
	*Neslia apiculata* Fischer and Meyer, 1842	Mediterran, NW Africa, central Asia, Himalaya, Pakistan	42	?

a) URL 4,5,6,7,8

b) URL 1

c) URL 9, 10, 11, 12, 13

3.3. Chromosome preparation

Young inflorescences with closed flower buds were harvested into freshly made Carnoy's fixative I and stored overnight at 4°C. Then they were rinsed in distilled water (2× 5 min) and flower buds were selected (buds with fully developed yellow anthers containing pollen were removed from inflorescences so only those with white anthers remained, see Fig. 6). After washing flower buds in 1× citrate buffer (2× 5 min), they were transferred in 0.3% pectolytic enzyme mixture and incubated for approximately 3 hours or longer in a humid box placed in an incubator (37°C). To stop digestion, the flower buds were washed in citrate buffer.

Fig. 6: Harvested inflorescences after the fixation. Only the buds with not fully developed pollen grains (clearly white anthers) in the middle were used.

Individual flower buds were put on microscopic slides and disintegrated with a needle in a drop of citrate buffer. They were fixed with 20 µl of 60% acetic acid and heat of 50°C while stirring the suspension with preparation needle without touching the slide, then with 100 µl of Carnoy's fixative I. The dry preparations were staged with a phase contrast microscope. Suitable slides were postfixed before next steps in 4% formaldehyde in distilled water for 10 minutes. Only slides containing chromosomes in a stage of pachytene, diakinesis or undergoing mitosis were relevant for the study (see different stages of meiosis observed for *Chrysochamela velutina* in Appendix Fig. 44). The pachytene chromosomes were required for CCP experiments. The other two types of chromosomes were used to establish chromosome numbers and to localize rDNA sequences. Additionally, on the same slides as pachytene chromosomes, the mitotic chromosomes or those in the phase of diakinesis were needed to be present as well. These served as a control whether fluorescent probes really hybridize to single chromosomal bivalent.

The chosen chromosome preparations were digested in pepsin to remove cytoplasm (Fig. 7) as described here: At first, the slides were washed in 2× SSC (2× 5 min). 100 µl of RNase solution was applied, covered with a coverslip and incubated in a humid box at 37°C for 1 hour. The slides were washed in 2× SSC (2× 5 min) again. Subsequent incubation in the solution of pepsin in HCl (0.1 mg/ml) lasted up to 5 minutes but this was

adjusted according to the visually estimated amount of cytoplasm and according to previous experience. For *C. noeana*, mostly 1 or 2 minutes of digestion was sufficient, for other species it was longer (this was a significant deflection from established protocol). To observe the results of digestion or to continue with probe application, slides with properly removed cytoplasm were washed again in 2× SSC (2× 5 min), post-fixated in 4% formaldehyde in SSC for 10 minutes (if evaluation of the result of digestion was made) and dehydrated in ethanol series (70%, 80%, and 96% ethanol, 3 min each). The process was almost identical to the protocols of Mandakova and Lysak (2016a; 2016b).

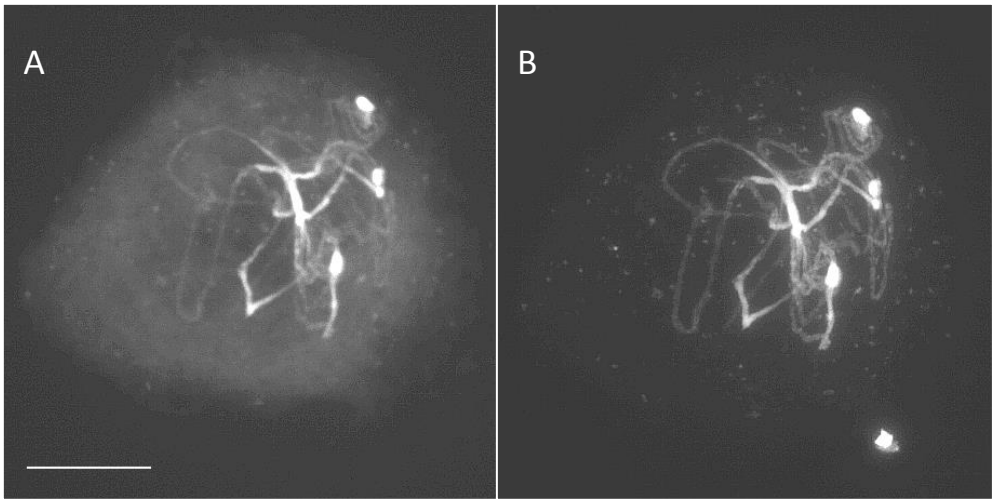

Fig. 7: Effect of pepsin treatment on chromosome visibility as described inMethods. **A)** Before the treatment cytoplasm immobilises DAPI creating unwanted background. **B)** Cytoplasm was removed after the pepsin treatment. The white scale bar in down-left corner stands for 10 μm.

3.4. Probes preparation

The BACs from IGF BAC library of Altmann (Mozo et al., 1998) were obtained from Arabidopsis Biological Resource Center (Columbus, Ohio) and were chosen to cover all genomic blocks of ACK (Tab. 2; Lysak et al., 2016).

AK	At	GB	Block borders Start		End	
			Gene	BAC clone (GenBank Accession)	Gene	BAC clone (GenBank Accession)
AK1	At1	A	At1g01010	T25K16 (AC007323)	At1g19840	F14P1 (AC024609)
	At1	B	At1g19850	F6F9 (AC007797)	At1g37130	F12K21 (AC023279)
	At1	C	At1g43020	F2J6 (AC009526)	At1g56190	T6H22 (AC009894)
AK2	At1	D	At1g64670	F1N19 (AC009519)	At1g56210	F14G9 (AC069159)
	At1	E	At1g64960	F13O11 (AC006193)	At1g80950	F23A5 (AC011713)
AK3	At3	F	At3g01015	T4P13 (AC008261)	At3g25520	MWL2 (AB025639)
	At2	G	At2g05170	F16J10 (AC007289)	At2g07690	T25N22 (AC005693)
	At2	H	At2g10940	T10F5 (AC007063)	At2g20900	F5H14 (AC006234)
AK4	At2	I	At2g20920	F7O24 (AC007142)	At2g31035	T19L18 (AC004747)
	At2	J	At2g31040	T9J22 (AC002505)	At2g48150	T8I13 (AC002337)
AK5	At2	K-L	At2g01060	F2I9 (AC005560)	At2g05160	F3C11 (AC007167)
	At3	K-L	At3g25540	T5M7(AP001313)	At3g32960	T4A2 (AP002066)
	At3	M-N	At3g42180	T10D17 (AL353865)	At3g63530	F16M2 (AL138648)
AK6	At4	O	At4g00026	F6N15 (AF069299)	At4g05450	T1J1 (AF128393)
	At4	P	At4g12620	T1P17 (AL049730)	At4g07390	T3H13 (AF128396)
	At5	Q	At5g30510	T8M17 (AF296835)	At5g23010	T20O7 (AB026660)
	At5	R	At5g23000	MRN17 (AB005243)	At5g01010	F7J8 (AL137189)
AK7	At5	S	At5g42110	MPK23 (AB020748)	At5g32470	F5H8 (AB025605)
	At4	T	At4g12700	T20K18 (AL049640)	At4g16240	F18A5 (AL035528)
	At4	U	At4g16250	T6K21 (AL021889)	At4g40100	T5J17 (AL035708)
AK8	At5	V	At5g47810	MGC1 (AB028612)	At5g42130	MJC20 (AB017067)
	At5	W	At5g47820	K16F13 (AB024025)	At5g60800	MUP24 (AB005246)
	At5	X	At5g60805	MSL3 (AB008269)	At5g67640	K9I9 (AB013390)

Tab. 2: Genomic blocks (GB) characteristics as described in (Lysak et al., 2016). Colour of background matches artificial colour coding for ACK chromosomes (see Introduction).

To visualise 35S rDNA BAC clone T10P10 was used and for 5S clone pCT 4.2 was used. The overall process of probe preparation is demonstrated in Fig. 8.

The phenol-chloroform DNA isolation was provided after overnight cultivation of bacterial libraries with the proper antibody (each BAC clone isolated from 50 ml of bacterial culture): A centrifugation (3,220 × g, 30 min, 4°C) separated bacteria from medium. For bacterial lysis three buffers were used (Buffer 1: 50 mM glucose, 10 mM EDTA (pH 8,0), 25 mM Tris-HCl (pH 8,0); Buffer 2: 0,2 M NaOH and 1% SDS in Milli-Q; Buffer 3: 5 M acetic acid, 3 M potassium acetate). The bacterial pellets were resuspended in 1 ml of the Buffer 1 and 2 ml of the freshly made Buffer 2 were added into each tube. The mixtures were shaken in hand and then kept on ice for 10 minutes. Then 1.5 ml of cold Buffer 3 was added. Again, the mixtures were shaken in hand and kept on ice for 10 minutes. The centrifugation took place again (3,220 × g, 30 min, 4°C). The supernatants were transferred to new tubes and the same amount of phenol-chloroform was added. The subsequent centrifugation (3,220 × g, 20 min, 4°C) separated proteins from DNA. The chloroform containing the DNA was transferred into new tubes and the same amount of isopropanol was added. Tubes were shaken in hands and incubated at -20°C for 30 minutes. This precipitated the DNA. After the centrifugation (3,220 × g, 20 min, 4°C), the supernatant was removed. The pellets of DNA were dried in a desiccator and 70% ethanol was added. After 10 minutes, the tubes

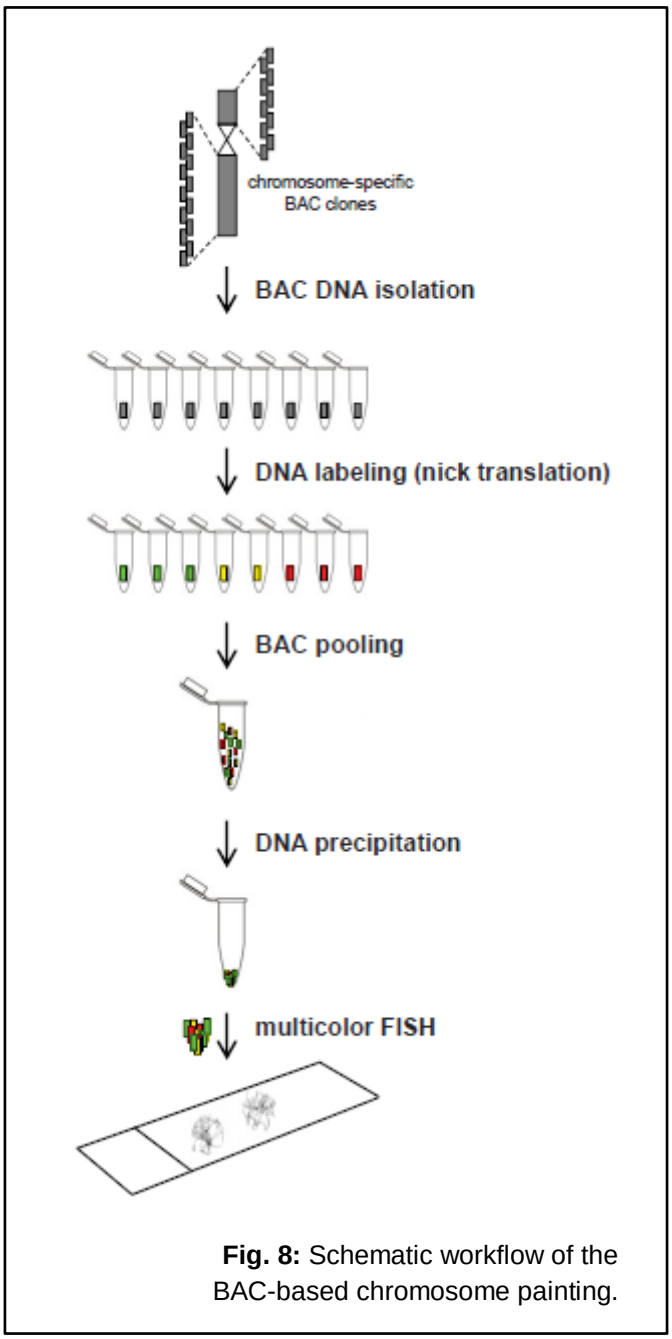

Fig. 8: Schematic workflow of the BAC-based chromosome painting.

were centrifuged (3,220 × g, 20 min, 4°C). The supernatant was discarded and the pellets of DNA were dried again in the desiccator. The dried pellets were resuspended in 100 µl RNase (1 mg/ml, pH 7.5) and incubated for 60 min at 37°C. Then stored in aliquots at -20°C.

From the BAC clones, contigs were arranged and differentially labeled according to genomic blocks (see Fig. 9) as described in next paragraphs. Each half of a genomic block was labeled with different colours to visualise an orientation of the block within a chromosome.

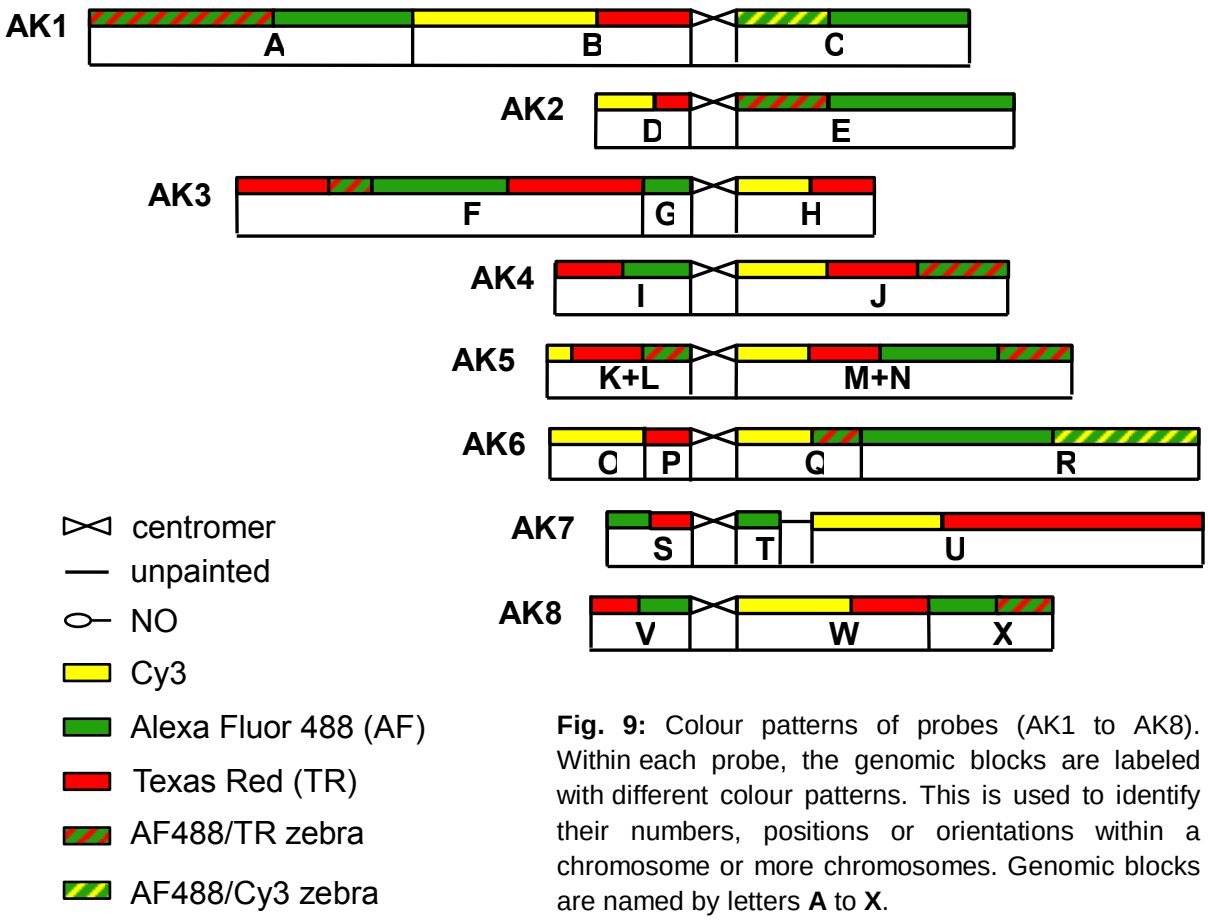

Fig. 9: Colour patterns of probes (AK1 to AK8). Within each probe, the genomic blocks are labeled with different colour patterns. This is used to identify their numbers, positions or orientations within a chromosome or more chromosomes. Genomic blocks are named by letters **A** to **X**.

All the BAC clones were labeled by nick translation (according to protocol of Mandakova and Lysak; 2016a) with biotin-dUTP, digoxigenin-dUTP, or Cy3-dUTP as follows: In a 0.5 ml microcentrifuge tube the following was combined: 1 µg of BAC DNA in 32 µl of distilled water, 5 µl of 10× NT buffer, 5 µl nucleotide mixture, 1 µl of 1 mM commercial x-dUTP, 5 µl of 0.1 M ß-mercaptoethanol, 1 µl DNase I, and 1 µl DNA polymerase I. The "x" in "x-dUTP" stands for biotin or digoxigenin. The total volume of the mixture was 50 µl. An incubation for 90 minutes was provided at 15°C, then the tubes were transferred on ice. A sample of 5 µl from each reaction mixture with 2 µl of loading dye was loaded on a 1% agarose gel with 2 µl of 100 bp ladder DNA marker. The optimal amount

of DNase I and the time of incubation were determined empirically for each probe. Extended incubation of additional 30 minutes was required if labeled segments, detected on the gel, were larger than 500 bp. If the smear of segments on the gel was between 200 and 500 bp (Fig. 10), the reaction was terminated with 1 µl of 0.5 M EDTA and heating for 10 min at 60°C. Complete probes can be stored in -20°C until use.

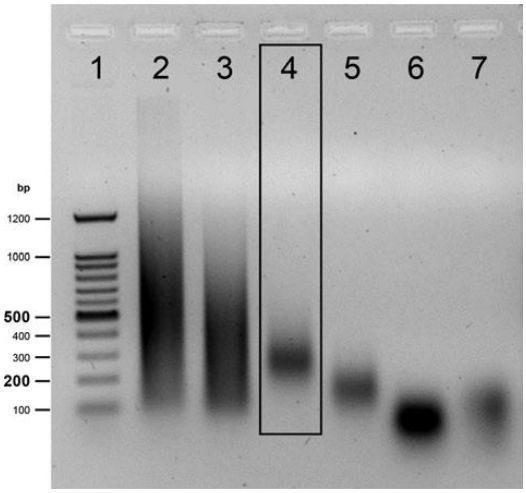

Fig. 10: Agarose gel analysis of probe segment sizes after nick translation. Lane 1: 100 bp DNA ladder. Lanes 2 and 3: too long DNA segments; >1 µg of BAC DNA and/or <1 µl of DNase I was added into the nick translation reaction. Lane 4: optimal probe segment size between 200 and 500 bp. Lanes 5 through 7: samples sheared to segments <200 bp; <1 µg of (degraded) BAC DNA and/or >1 µl of DNase I was added into the nick translation reaction.

From the labeled BAC clones probes were prepared: Each probe included BAC contigs covering one ancestral chromosome (Fig. 9). Selected BAC clones were therefore pooled into 5 µl mixtures (each mixture served as one probe). To reduce probe volume and to remove unincorporated nucleotides, DNA was precipitated with 0.1× 3 M sodium acetate and 2.5× ice-cold 96% ethanol. After vortexing, the probes were stored in -20°C for 30 minutes and then centrifuged (13,000 × g, 30 min, 4°C). The pellets were mixed with 500 µl of 70% ethanol and again centrifuged for 5 minutes. The supernatant was removed and pellets were dried in the desiccator and resuspended in 20 µl of hybridisation buffer at 37°C. Prepared probes were then stored at -20°C in dark or applied to chromosomes as described in the next chapter.

3.5. Comparative chromosomal painting

Finally, complete probes were applied to the slides. 20 µl of only one probe to one slide each time. The drop was covered with a coverslip and sealed around edges with a technical glue. This was done to prevent drying out or dilution of the probe with condensing water. The probes and chromosomes were denatured together on a hot plate at 80°C for precisely 2 minutes. Hybridisation was carried out by incubation in a moist chamber at 37°C for 48 hours.

The next steps included indirect immunofluorescence to attach fluorescent dyes to BAC contigs labeled with biotin and digoxigenin: After coverslip was removed, slides were washed in 2× SSC (2 min). Unbound and loosely bound probes were removed in 20% formamide stringent wash buffer (3× 5 min) and washed again in SSC. 2× 5 minutes washing in 4T buffer proceeded 30 minutes of incubation with 100 µl of avidin-Texas Red in TNB buffer (1:1000). 2× 5 minutes washing in TNT buffer proceeded 30 minutes of incubation with 100 µl of mixed goat biotinylated goat anti-avidin (1:200) and mouse anti-digoxigenin (1:250) in TNB buffer. Again 2× 5 minutes washing in TNT buffer proceeded 30 minutes of incubation with 100 µl of mixed avidin-Texas Red (1:1000) and goat anti-mouse-Alexa Fluor 488 (1:200) in TNB buffer. Then the slides were washed again in the same way, dehydrated in ethanol series (70%, 80%, and 96% ethanol, 3 min each time) and let to dry. All washing steps were carried out at 42°C.

3.6. Fluorescence microscopy and data analysis

The localisation of probe sequences on particular chromosomes was observed under a fluorescence microscope. 15 µl of DAPI with Vectashield was applied on each slide. ZEISS Axio Imager.Z2 microscope and MetaSystems CCD camera were used to take photos of selected nuclei. The photos were taken at a magnification of 1000.

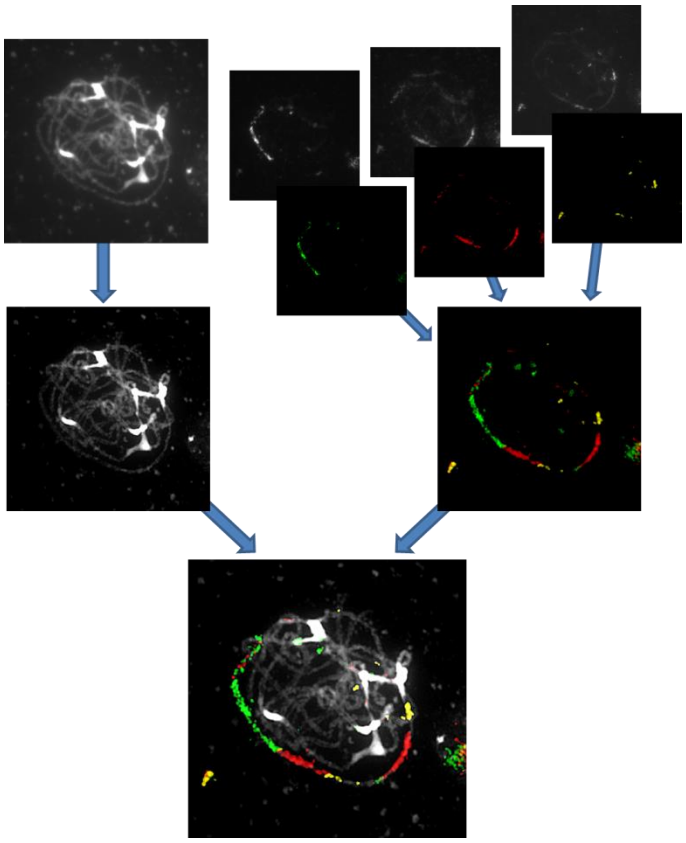

Fig. 11: Raw photo from microscope is sharpened in photoshop and its contrast is enhanced. All raw photos are in a scale of grey, that is why colours are arbitrary changed in corresponding photos (green, yellow and red; DAPI is left in grey scale). All four colours (DAPI photo and 3 colour signals) are then fused together. In the next step is the image cropped and occasionally turned around axis in ImageJ and scale bar is added. No other transformations are allowed to keep unchanged information from raw microscopic photo.

Raw photos from microscope were processed in programme Adobe Photoshop CS6 (64 Bit) according to steps visualised in Fig. 11. Programme ImageJ (Schneider et al., 2012) was used to further adjust photos and to straighten pachytene chromosomes or painted chromosomal segments.

Observed colour patterns in pachytene chromosomes were compared to genomic blocks arrangement in ACK (Fig. 9). If any differences from ACK appeared to be present in photos, these were compared with other meiotic nuclei of the species with the same probes. Irregular colour regions were then considered to appear as an effect of non-specific probe binding. However, regular differences in colour patterns need not to necessarily mean successful binding of probes to correct genomic blocks. NORs or pericentromeric regions tend to bind probes non-specifically due to the high amount of unspecific repetitive sequences. The length of observed blocks of colours need not to match with physical length of the genomic blocks due to narrowing, stretching or coiling of corresponding chromosomal regions. Also, it is often not easy to see proper order of the colours or recognise chromosomal ends. That is why more than one meiotic nucleus had to be observed for each pattern (see Appendix Fig. 45 to 56).

Beside pachytene chromosomes, those in the stage of diakinesis or undergoing mitosis were also observed on the same slides as pachytene chromosomes. They were less tangled than the long pachytene bivalents and they were hybridized with the same probes as pachytene chromosomes. This enabled to better distinguish whether the genomic blocks were present on the same chromosome or different chromosomes (see Appendix Fig. 45 to 56). Since homologous pairs of pachytene chromosomes form bivalents, all the genomic blocks seem to be present in one copy for genome but in fact there are two homologous genomic blocks in each genome.

In addition, rDNA as a traditional cytogenomic marker was localised in two steps. At first, only 35S and 5S complementary probes were hybridized to mitotic chromosomes to provide a basic estimate (relative location and the number of loci). In the second step chromosome structure resolved with CCP was utilized. The localization was specified further on particular chromosomes with rDNA probes used in combination with chromosome-specific BAC clones. This specific probe hybridized to one of the examined genomic blocks served as an orientation point marking particular chromosome.

When analysing mitoses, it can be confusing when two mitotic chromosomes are close to each other and the signal is between them. Also, homologous regions sometimes tend to be close to each other even in mitosis so sometimes signals merge into one bridge of colour between chromosomes.

4. RESULTS

4.1. Chromosome numbers

All three *Chrysochamela* species (*C. elliptica*, *C. noeana* and *C. velutina*) have 16 chromosomes within the mitotic chromosome complement (Fig. 12). *N. paniculata* has 14 chromosomes. For *N. apiculata* 42 chromosomes were detected within the mitotic chromosome complement.

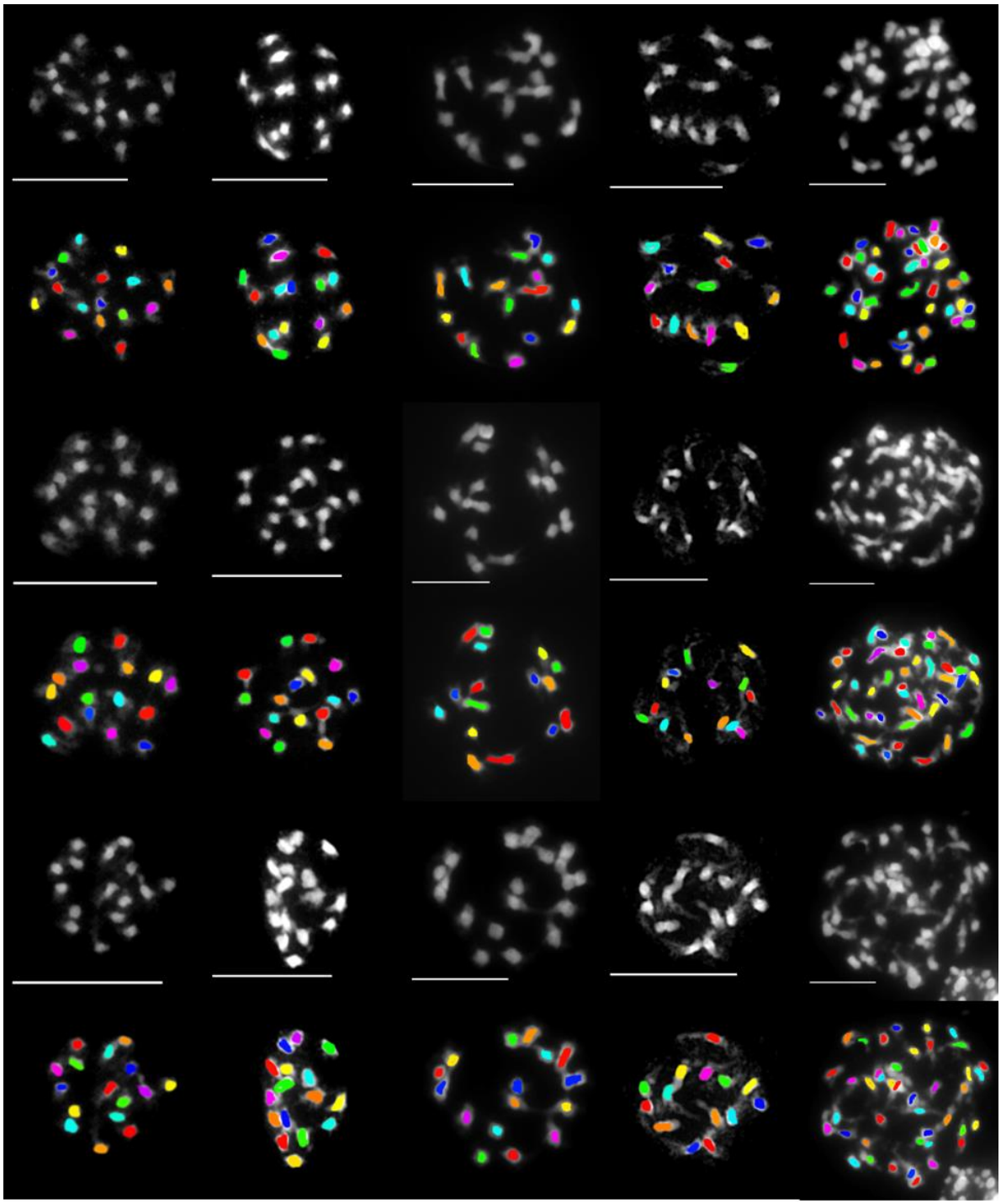

Fig. 12: Chromosome numbers determined according to observations of mitotic chromosomes. In each column three photos of mitotic chromosomes dyed with DAPI are presented for particular species. From the left: *C. elliptica* (2n = 16), *C. noeana* (2n = 16), *C. velutina* (2n = 16), *N. paniculata* (2n = 14), *N. apiculata* (2n = 42). For better orientation, the chromosomes were visualised with artificial random colours in computer (pictures under the DAPI photos) Scale bars = 10 µm.

4.2. Preliminary rDNA localization

To uncover the number and position of rDNA loci, probes corresponding to 35S and 5S rDNA were simultaneously hybridized on chromosomes of the investigated species (Fig. 13). In the genome of *C. elliptica* two 35S homologous pairs of loci are present. These are located on separate chromosomes in their terminal regions. Two 5S rDNA pairs of loci are observed on other chromosomes in internal regions. *C. noeana* has five terminal 35S pairs of loci. 5S rDNA can be found on two pairs of chromosomes in their internal regions. One pair of chromosomes comprises both 35S and 5S loci. The green signals on separate chromosomes of *C. velutina* indicate six 35S pairs of loci, however two of them share the same chromosomes with internal 5S pairs of loci. Other two 5S pairs of loci are located alone on other chromosomes again in internal regions. *N. paniculata* has three terminal 35S pairs of loci and three 5S pairs of loci in its genome. One pair of chromosomes contains both kinds of rDNA.

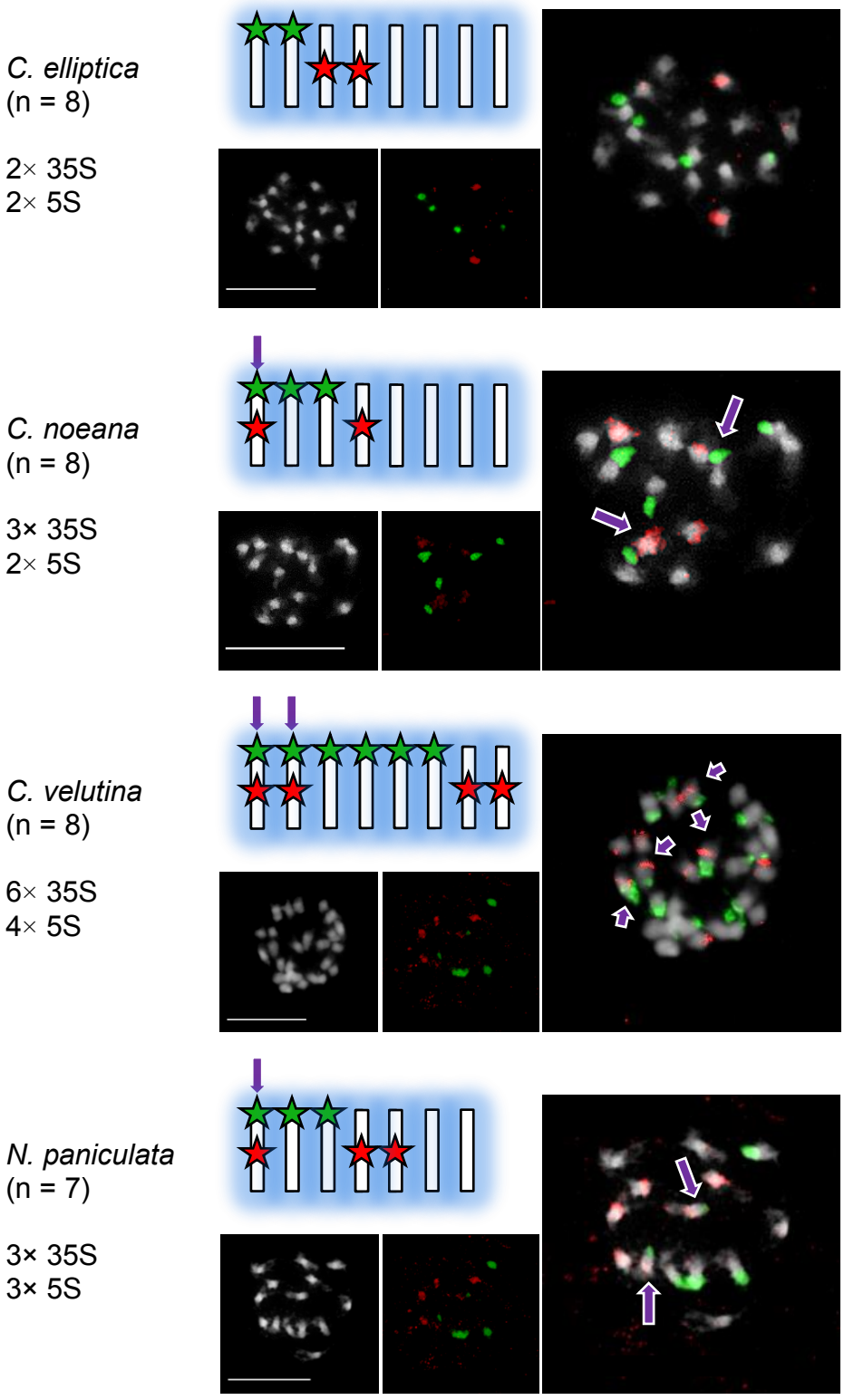

Fig. 13: Mitotic chromosomes of *C. elliptica*, *C. noeana*, *C. velutina* and *N. paniculata* painted with probes corresponding to 35S rDNA (green) and 5S rDNA (red). For each species one photo of mitotic chromosomes dyed with DAPI (left), one picture of combined probe signals (middle) and one picture of chromosomes with probe signals (right) are present. Each homologous pair of chromosomes is represented by white rectangle in the scheme above the photos. Green and red stars represent signals and their approximate location within chromosomes (terminal or internal). For each red or green star two homologous loci exist in genomes. Violet arrows highlight chromosomes containing both kinds of loci. Scale bars = 10 μm.

Locations and numbers of rDNA loci in the genome of *N. apiculata* are shown separately in larger pictures (Fig. 14) since it is more difficult to distinguish chromosomes or signals from each other. 12 pairs of 35S rDNA loci and 6 pairs of 5S rDNA loci were counted. All 35S are clearly terminal, however two pairs of 5S loci seem to be terminal as well in contrast to usual 5S location. Five pairs of chromosomes comprise both 35S and 5S rDNA loci.

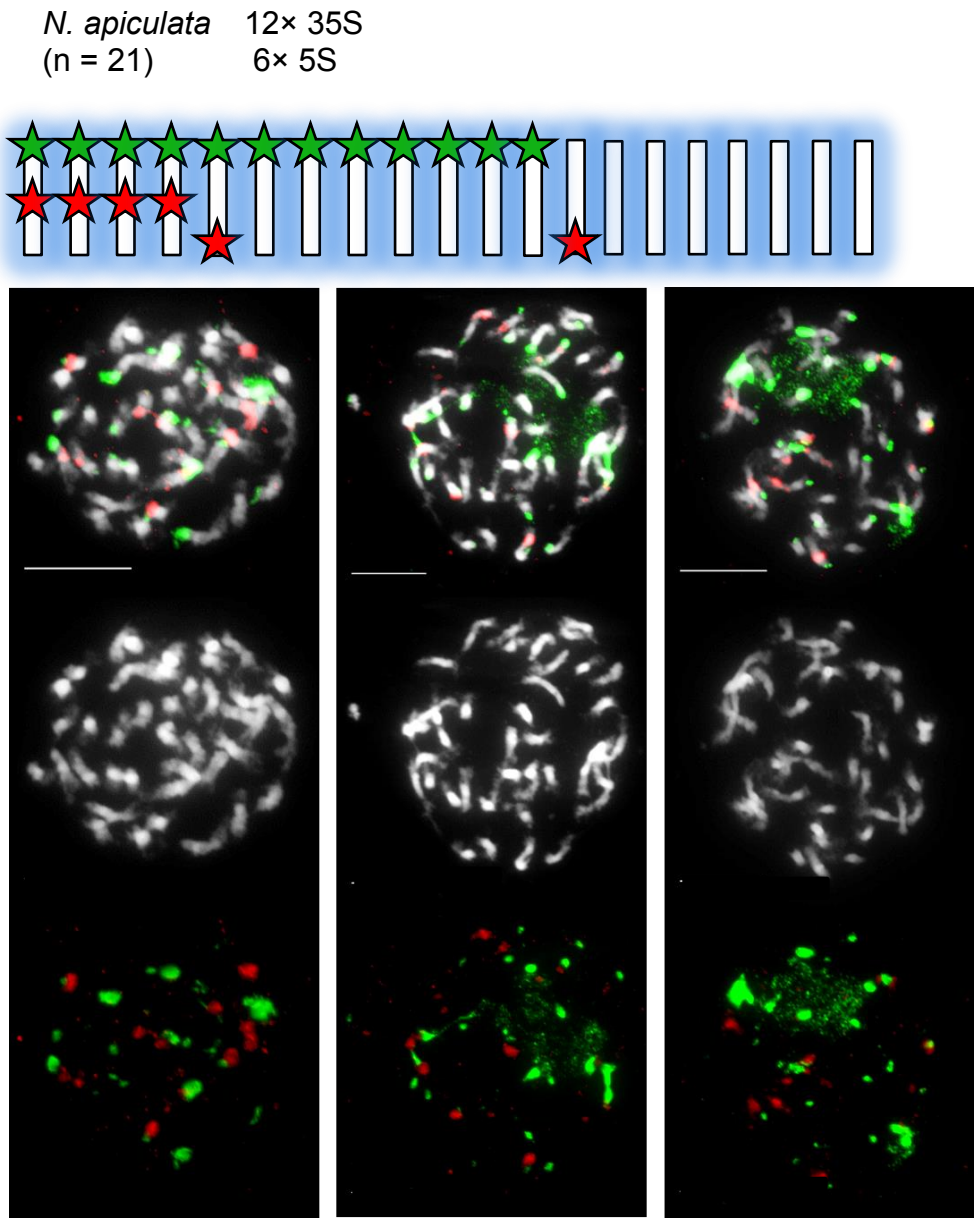

Fig. 14: rDNA locations and numbers in *N. apiculata*. 35S (green) and 5S (red) probes were hybridized on mitotic chromosomes (upper photos). The middle photos show chromosomes dyed only with DAPI. The pictures at the bottom show both rDNA signals combined. The white rectangles in the sheme above photos represent 21 homeologous pairs of chromosomes. Red and green stars represent signal locations on pairs of chromosomes (terminal or internal). For each red or green star two homologous loci exist in genomes. Scale bars = 10 μm.

4.3. Karyotypes and chromosome structures

This chapter introduces karyotypes for examined species as they were reconstructed according to the observed arrangements of genomic blocks on particular chromosomes. Pachytene bivalents of *C. noeana*, *C. elliptica*, *C. velutina*, *N. paniculata* and *N. apiculata* were painted with BAC probes in colour pattern representing corresponding genomic blocks (see Fig. 9 in chapter 3.4.). For each colour pattern of an AK chromosome applied (AK1 to 8) one picture of pachytene chromosomes is presented in Fig. 15 to 32. However, the colour pattern in the most cases cannot be properly observed from just one example. For other chosen examples for each chromosome see Appendix Fig. 45 to 56. The declared colour pattern was always observed in at least three images. It can be confusing, that a number of observed genomic blocks in a photo seems to be the half of a chromosome number (described in chapter 4.1.). This is because chromosomes in the stage of pachytene are present in the form of bivalents. Also, the karyotypes present only one chromosome for each homologous pair. Homologous chromosomes share the same arrangement of the genomic blocks.

4.3.1. Genus *Chrysochamela*

The arrangement of the genomic blocks is same in all three Chrysochamela species (Fig. 15 to 20). The probes in every case hybridized on entire chromosomal bivalents and the patterns of genomic blocks were always identical to ancestral chromosomes from the ACK (see Fig. 9 in chapter 3.4.). Chromosome 4 of *C. noeana* was unfortunately not resolved in appropriate quality with CCP. Chromosome 8 of *C. elliptica* was not painted since not enough slides containing good pachytene chromosomes was prepared. For this, photos of AK4 painted chromosomes of *C. noeana* and AK8 painted chromosomes of *C. elliptica* are not included. However, photos of chromosome 4 from *C. noeana* with the highest quality of probe signals can be seen in Appendix Fig. 46. Surprisingly, for the long arm of chromosome 3 of *C. elliptica* additional red signal in the pericentromeric region was observed in all photos. Nevertheless, this is considered to be the result of unspecific hybridization. According to given results karyotypes for *C. elliptica*, *C. noeana* and *C. velutina* were reconstructed (Fig. 21).

C. elliptica

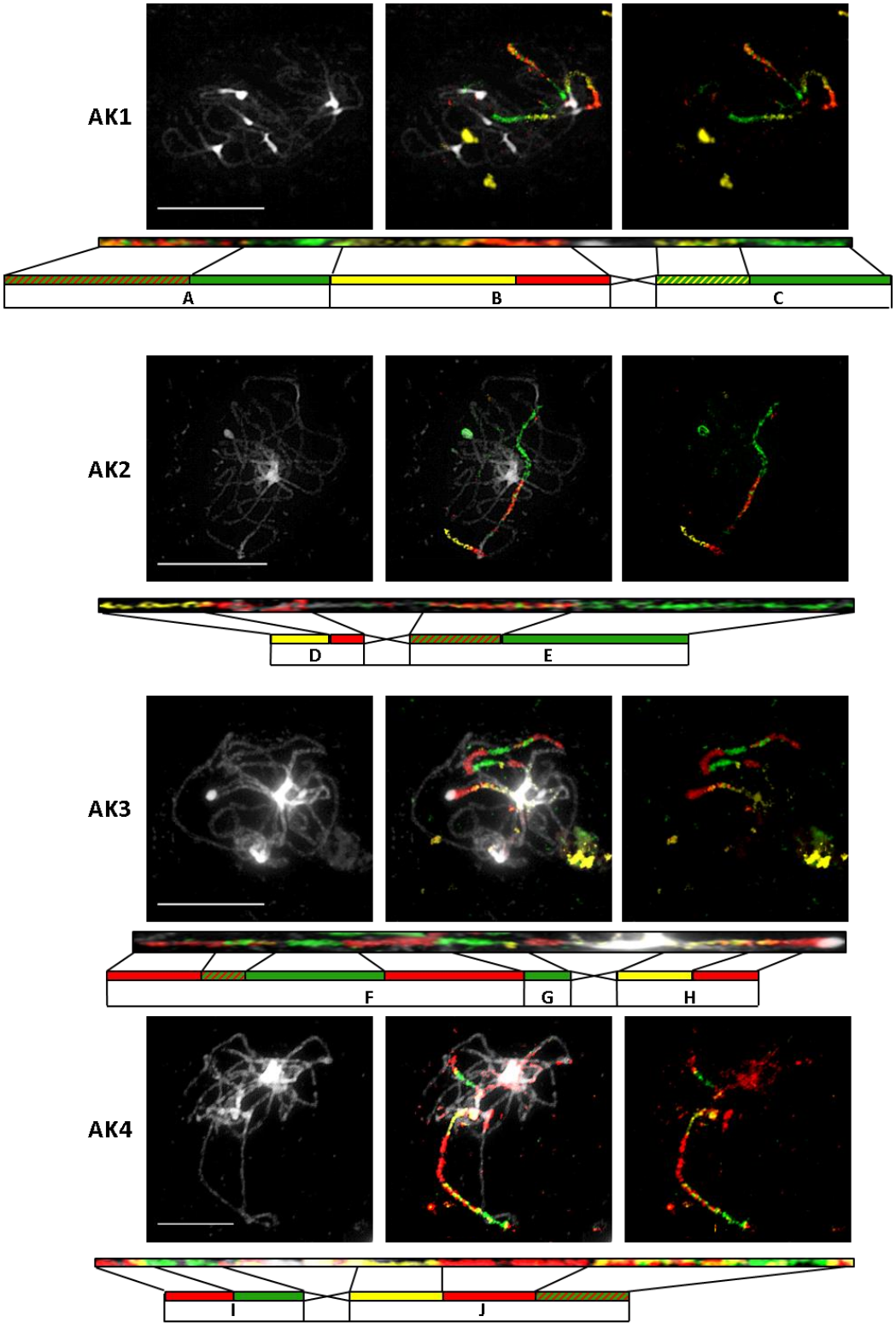

Fig. 15: Each line of photos represents chromosomes of *Chrysochamela elliptica* painted with a colour pattern of one chromosome from ACK (1 to 4). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 μm.

C. elliptica

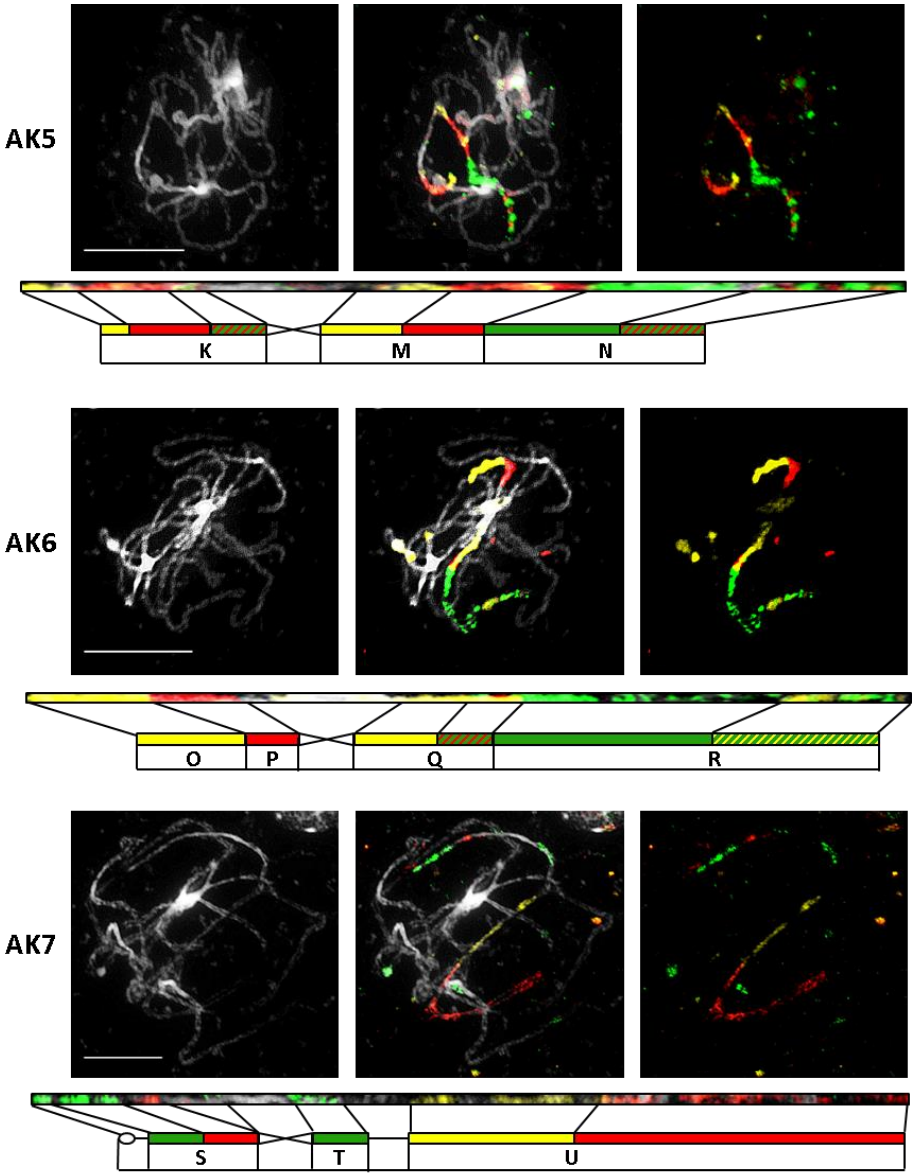

Fig. 16: Each line of photos represents chromosomes of *Chrysochamela elliptica* painted with a colour pattern of one chromosome from ACK (5 to 7). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 μm.

C. noeana

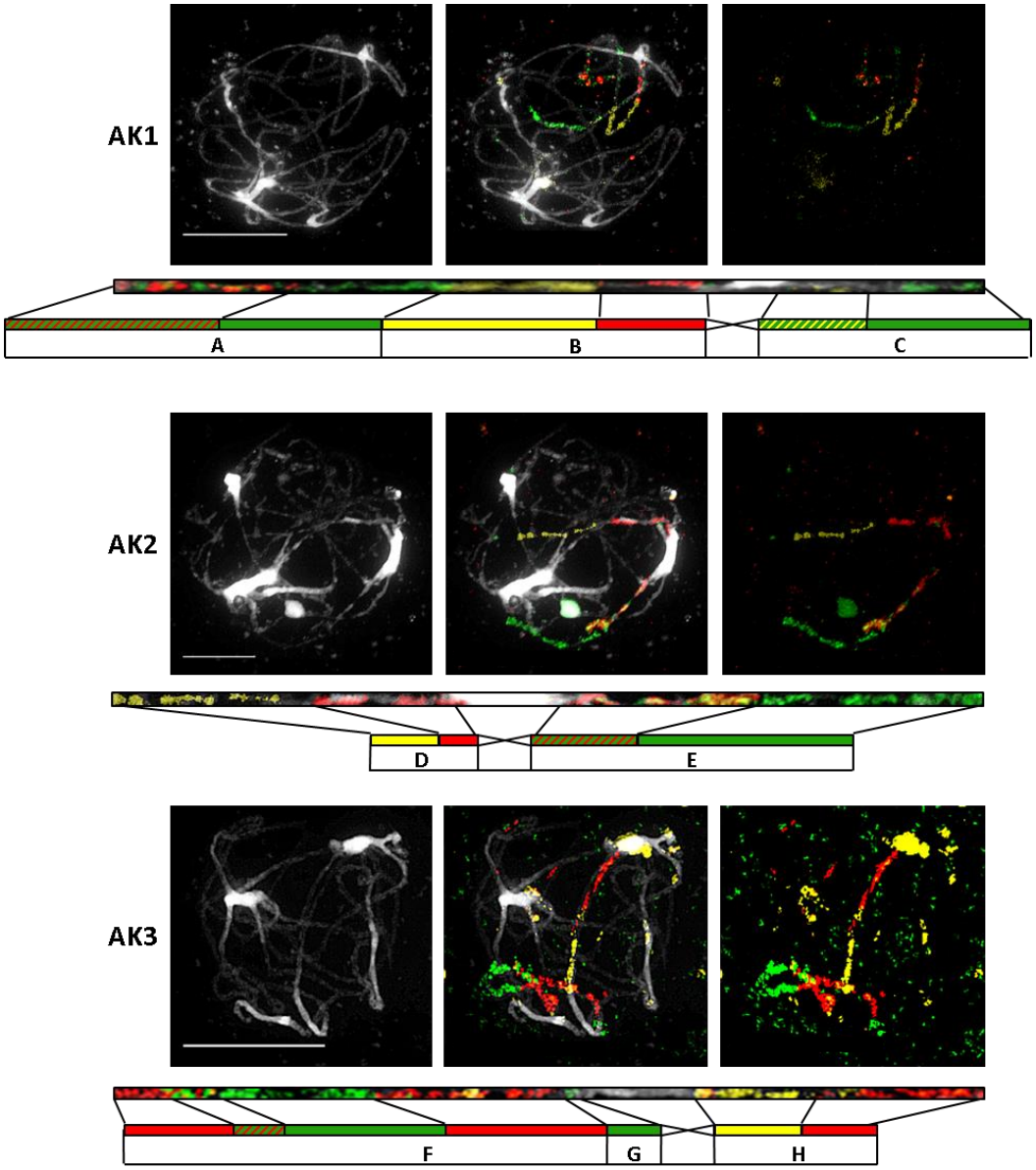

Fig. 17: Each line of photos represents chromosomes of *Chrysochamela noeana* painted with a colour pattern of one chromosome from ACK (1 to 4). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 µm.

C. noeana

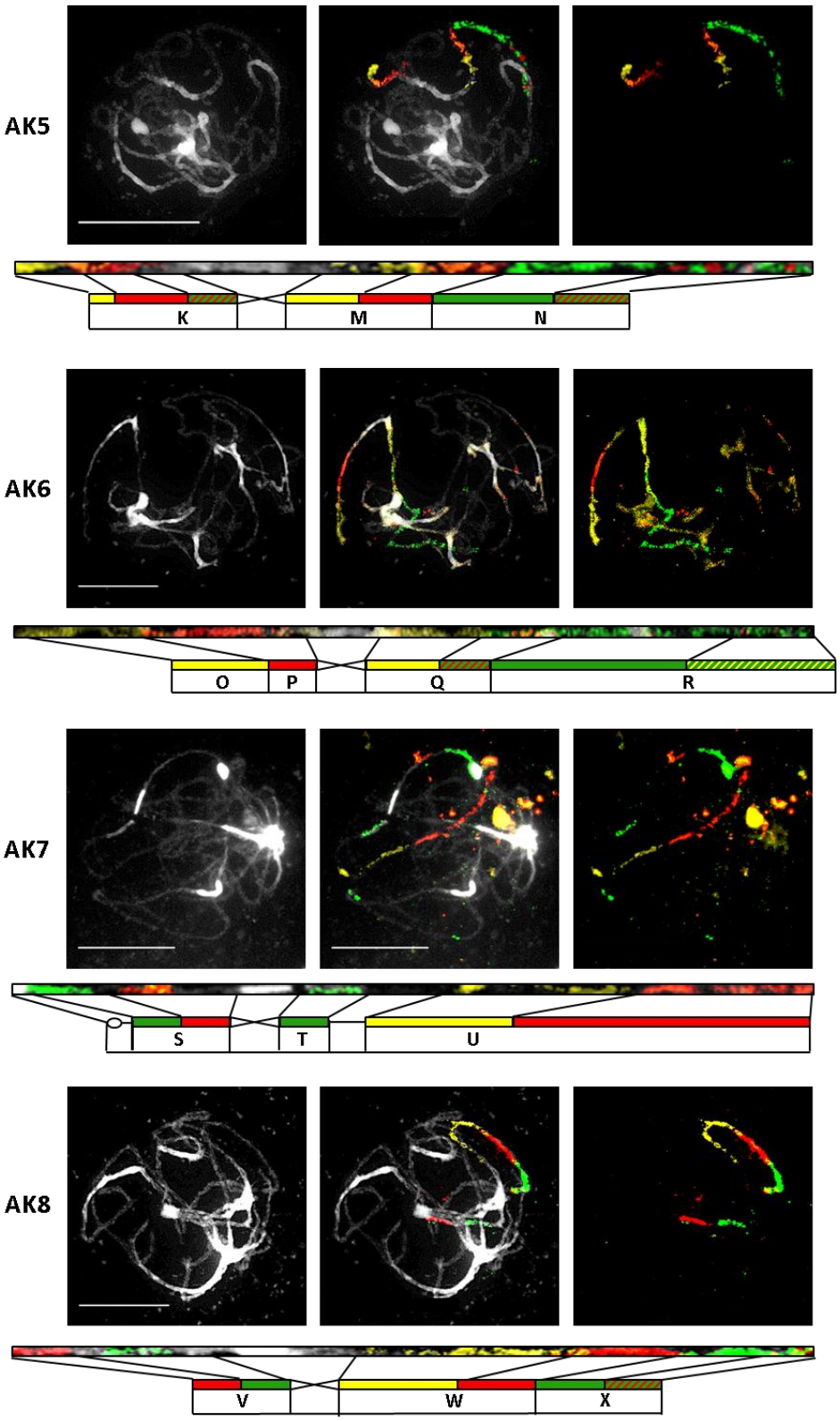

Fig. 18: Each line of photos represents chromosomes of *Chrysochamela noeana* painted with a colour pattern of one chromosome from ACK (5 to 8). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 μm.

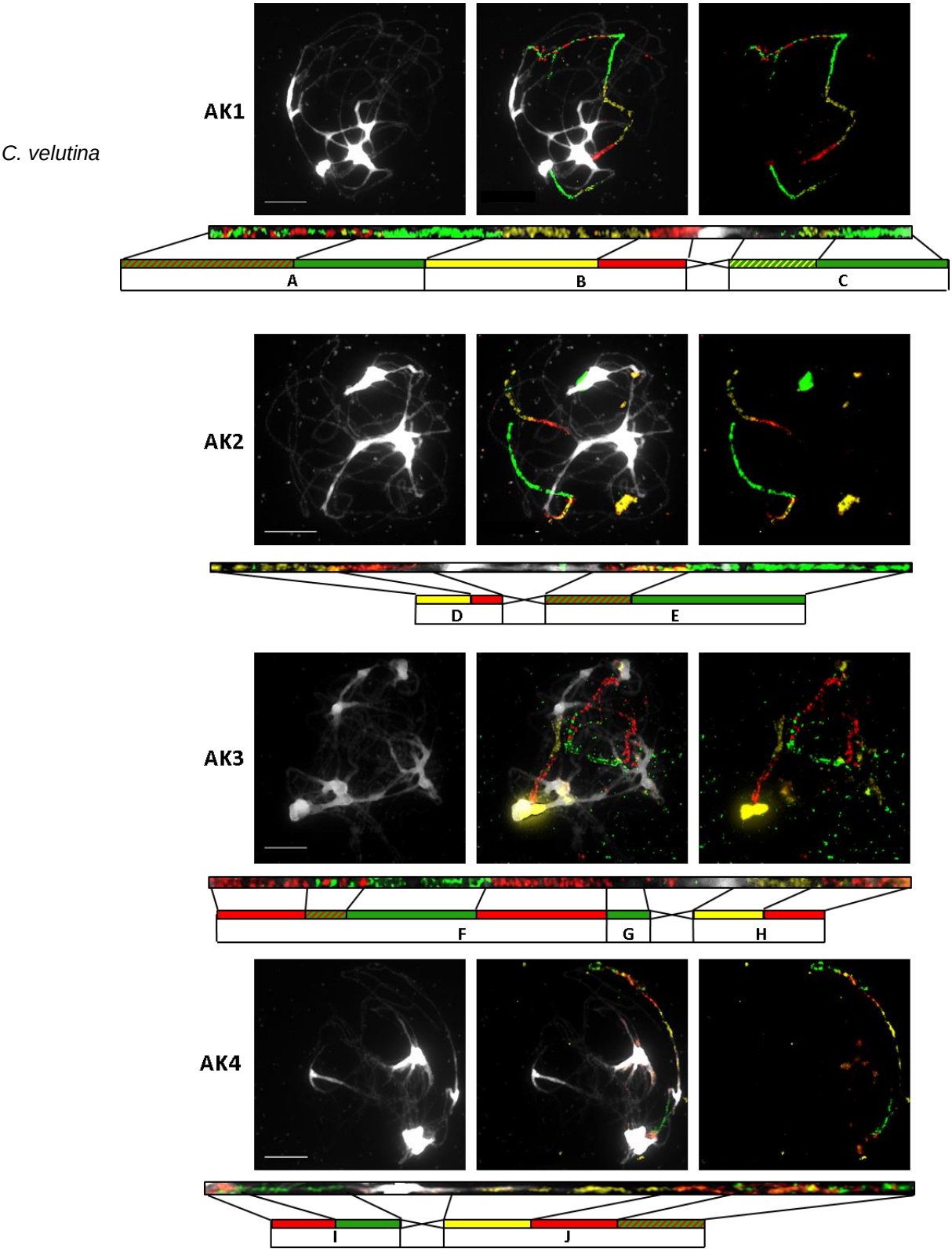

Fig. 19: Each line of photos represents chromosomes of *Chrysochamela velutina* painted with a colour pattern of one chromosome from ACK (1 to 4). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 μm.

C. velutina

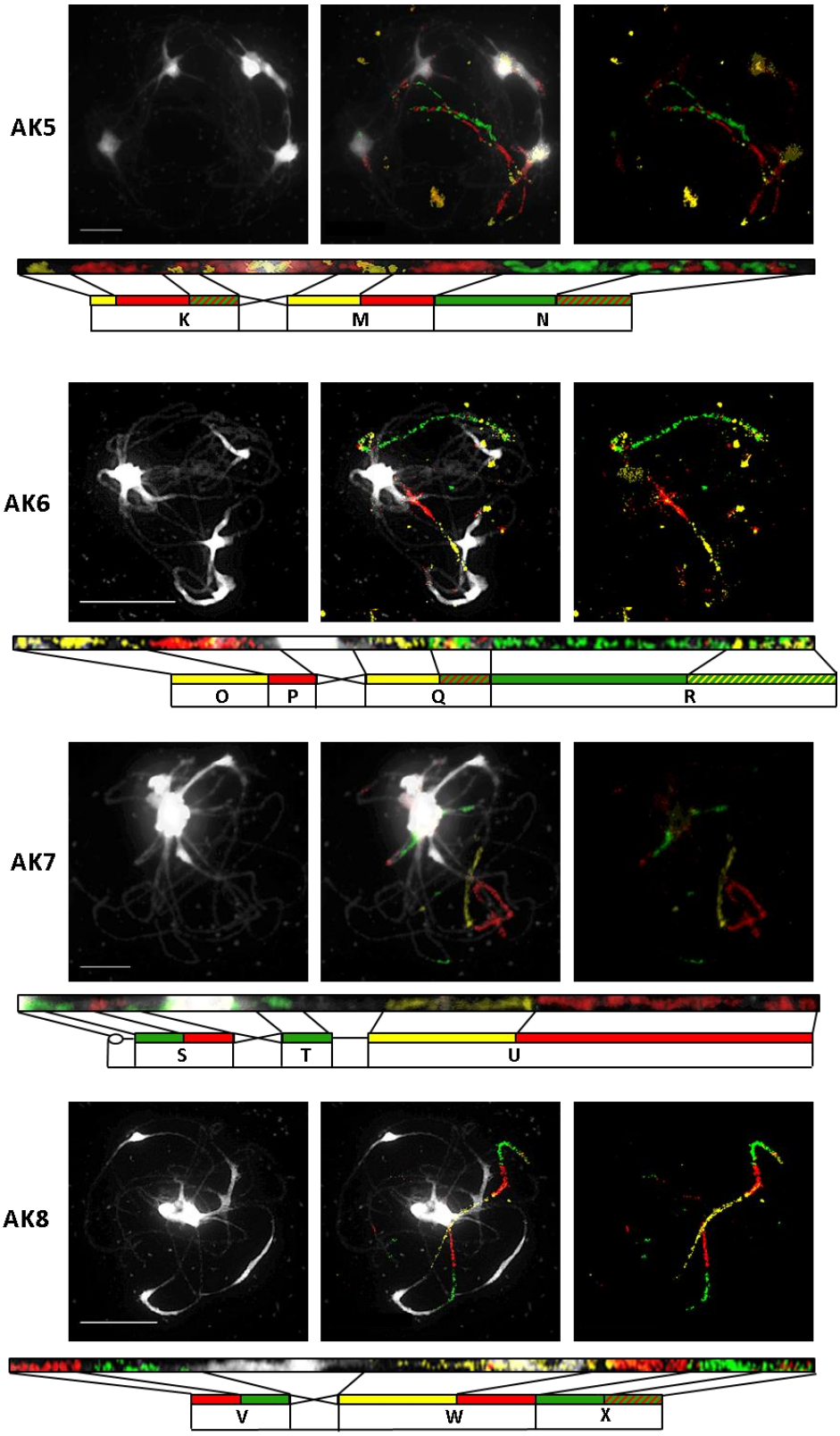

Fig. 20: Each line of photos represents chromosomes of *Chrysochamela velutina* painted with a colour pattern of one chromosome from ACK (5 to 8). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. In each line, the painted chromosome from the middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. Scale bars = 10 μm.

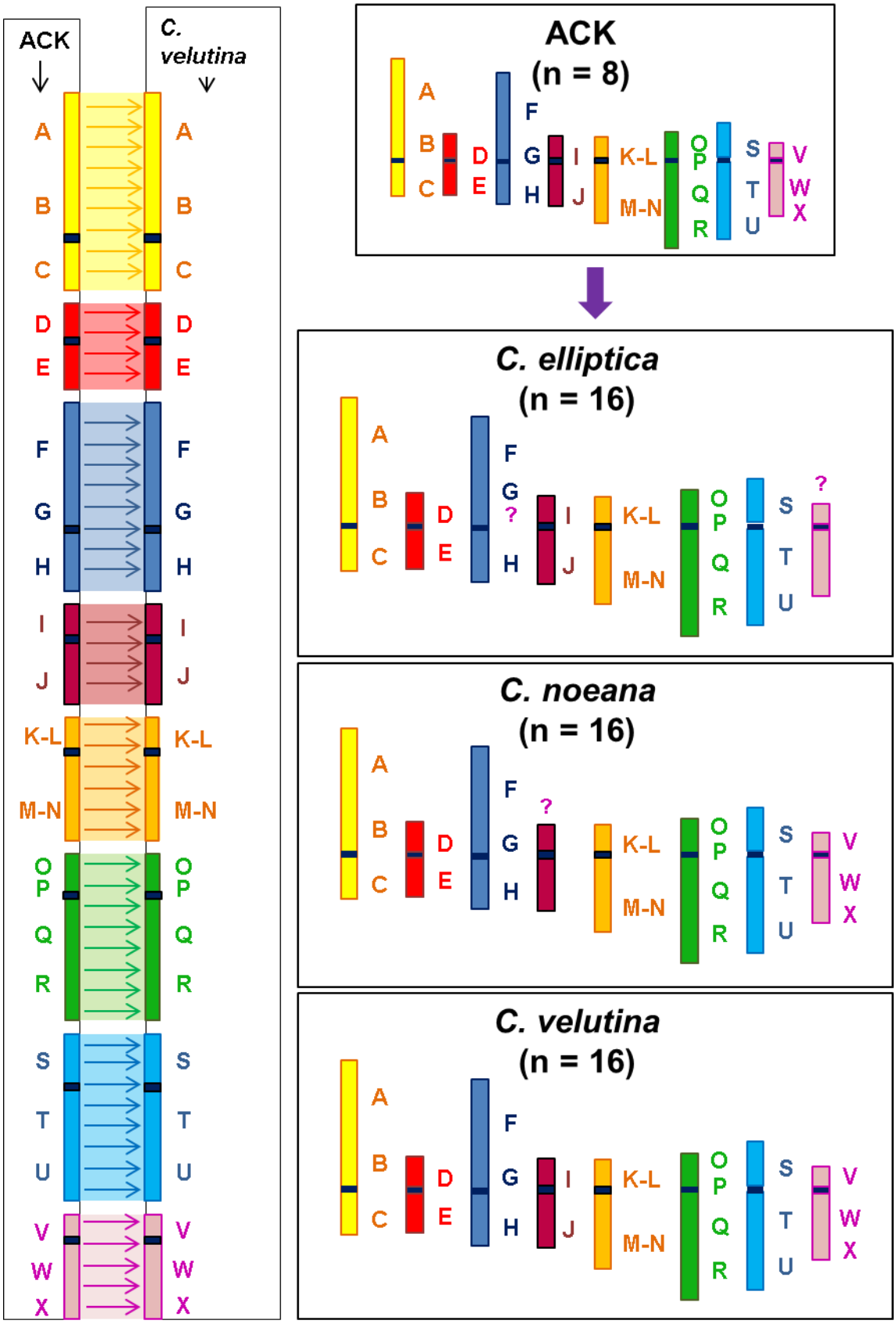

Fig. 21: Karyotypes of *Chrysochamela* species reconstructed according to observations of chromosomes after CCP (on the right) and assumed evolution of chromosome structures in *C. velutina* (on the left). The violet question marks represent unresolved chromosomes 8 in *C. elliptica* and chromosomes 4 in *C. noeana* and uncertain structure of chromosome 3 in *C. elliptica.*

4.3.2. *Neslia paniculata*

In the case of *N. paniculata* (Fig. 22 and 23) the comparative painting of chromosomes revealed different organisation of the genome in contrast to genus *Chrysochamela*. Chromosomes 1, 2, 3, 6 and 7 of *N. paniculata* were identified as fully painted with particular probes and without any rearrangements of genomic blocks. These chromosomes are then identical to ancestral chromosomes of ACK (see Fig. 9 in chapter 3.4.).

Two pericentromeric inversions were observed. One inverted region is the genomic block **V** together with centromere on chromosome 8. This chromosome was identified as telocentric. The second inversion is observable on chromosome 4 where genomic block **I** together with centromere is reversed.

Moreover, the centromere of chromosome 5 was not located in the same position as on ancestral chromosome 5 in ACK and it divided painted chromosomal region from an unpainted chromosomal region. Also, centromere of chromosome 4 divided painted and unpainted regions. These observations suggest that both regions painted with AK4 and AK5 probes are parts of a larger chromosome. Since all other chromosomes beside 4 and 5 were painted across their whole size and since *N. paniculata* has 7 chromosomal bivalents, the only possible explanation is that these two regions are parts of the same chromosome. Fig. 24 demonstrates probable chromosomal rearrangements for *N. paniculata*. Suggested karyotype of *N. paniculata* with AK4/AK5 chromosome is shown in Fig. 25.

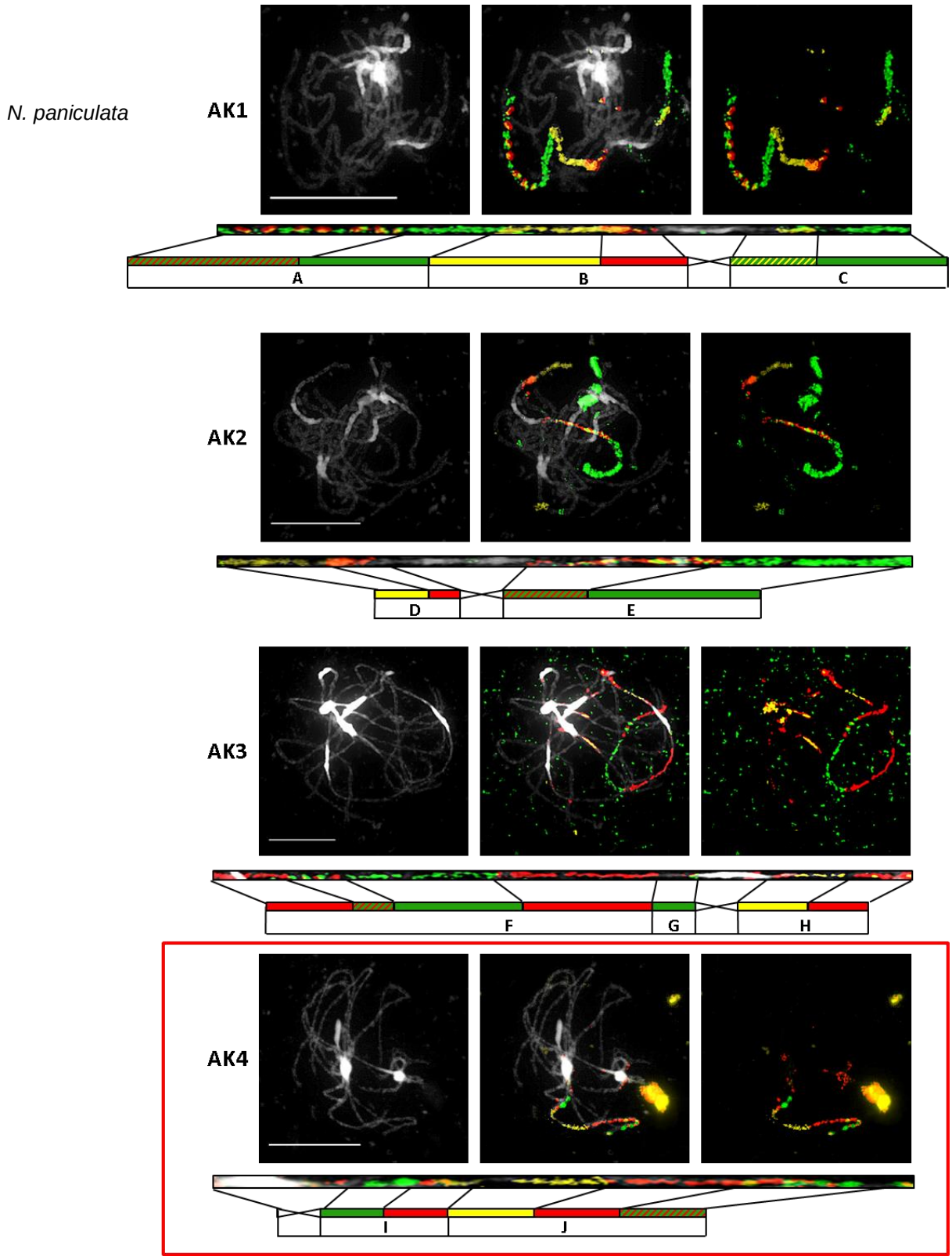

Fig. 22: Each line of photos represents chromosomes of *Neslia paniculata* painted with colour pattern of one chromosome from ACK (1 to 4). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. The painted chromosome from middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. The red frame highlights chromosomes which differ from the ACK colour pattern (see Fig. 24). Scale bars = 10 μm.

N. paniculata

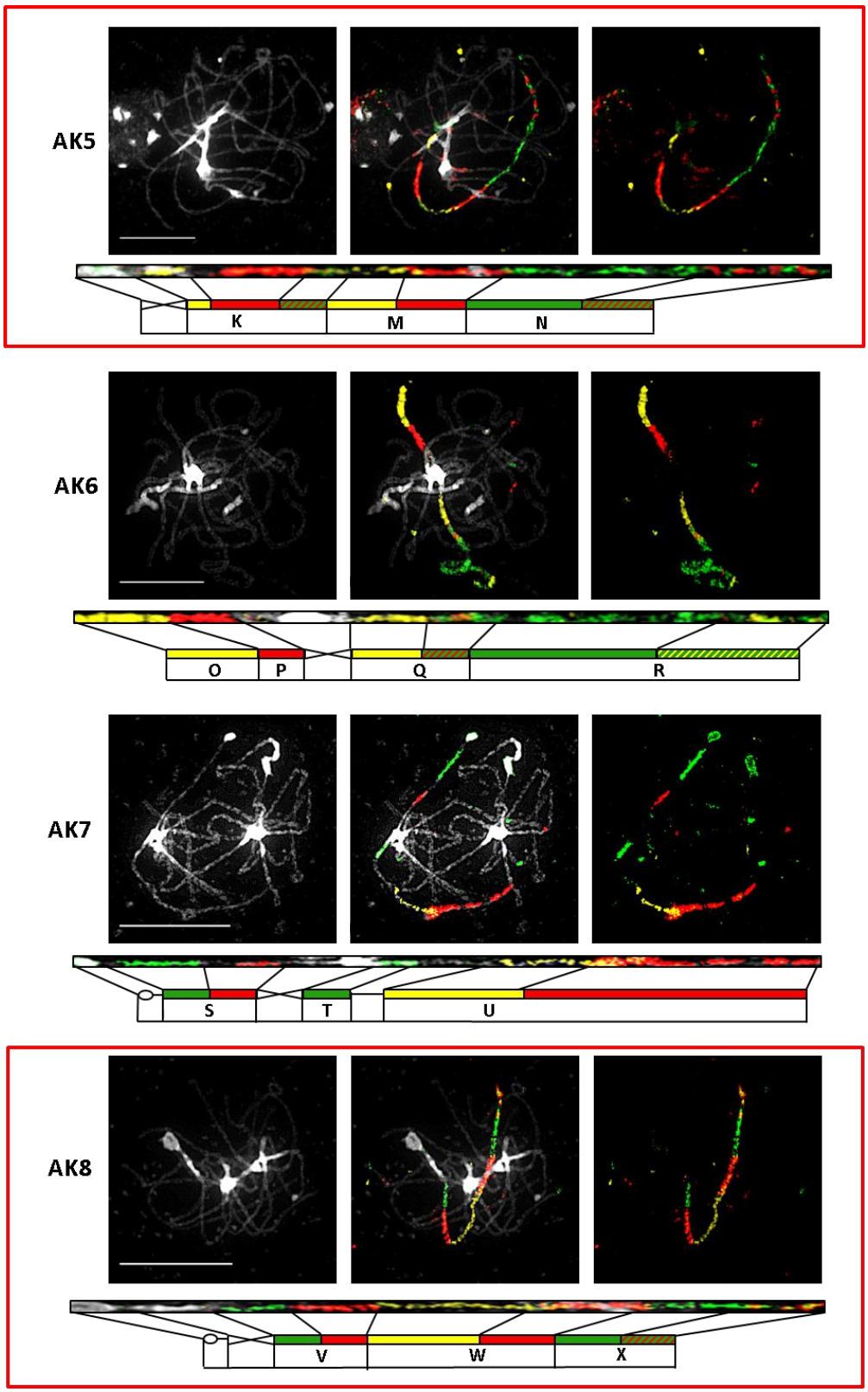

Fig. 23: Each line of photos represents chromosomes of *Neslia paniculata* painted with colour pattern of one chromosome from ACK (5 to 8). The left image of each line is an adjusted photo of species' chromosomes dyed with DAPI. The right image combines all probe signals enhanced with immunodetection. The middle image combines the previous two together. The painted chromosome from middle image is shown separately below photos straightened with ImageJ software. Lower scheme presents genomic blocks (see also Fig. 9) and their relation to the painted chromosome. The red frame highlights chromosomes which differ from the ACK colour pattern (see Fig. 24). Scale bars = 10 μm.

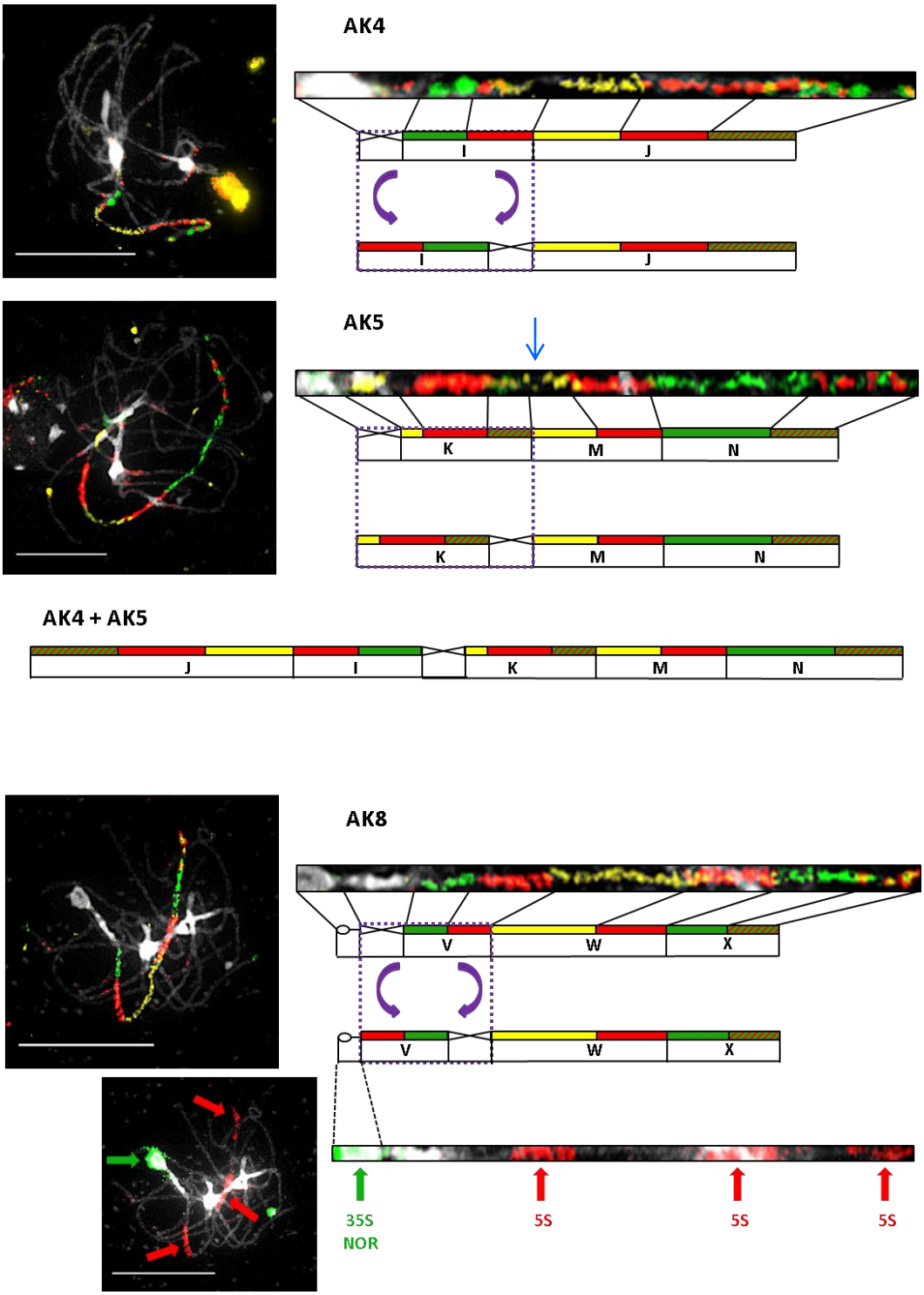

Fig. 24: Chromosomal rearrangements in genome of *Neslia paniculata* (images highlighted with red frames in Fig. 22 and 23). Chromosome related to AK4 has reversed colour pattern of the genomic block **I** + reversed position of the centromere suggesting pericentromeric inversion. The inverted region is highlighted with violet dots and put in contrast with pattern from ACK (lower sheme). AK5 related chromosome shows loss of the centromere in its former location (a violet frame). The blue arrow shows observable gap in colour pattern suggesting possible presence of a centromeric remnant. Similar feature as on chromosome 4 (AK4) is observed on chromosome related to AK8 (genomic block **V** and centromere). In addition, chromosome 8 has a NOR attached to the centromere. This chromosome is therefore telocentric. On the other hand, former chromosomes 4 and 5 (AK4 and AK5) have been fused into one chromosome. Scale bars = 10 μm.

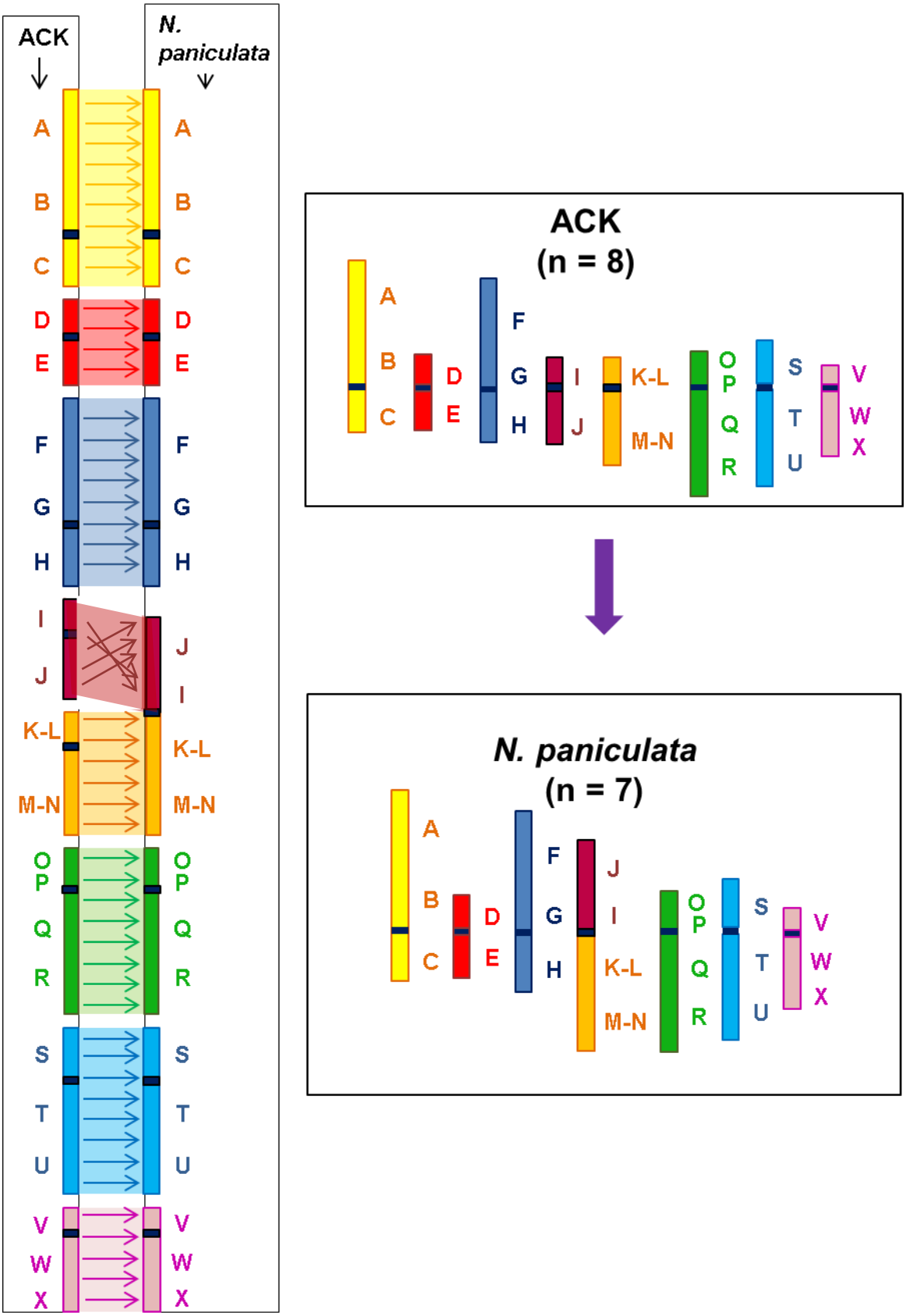

Fig. 25: Karyotype of *N. paniculata* built up according to observations of chromosomes after CCP (on the right) and assumed evolution of chromosome structures (on the left).

4.3.3. *Neslia apiculata*

Results for *N. apiculata* are presented in larger figures in contrast to the rest of the species. This is so due to the high complexity of the rearrangements. Hybridized probes do not form a compact line but instead paint many segmented regions in the large genome of *N. apiculata* with many chromosomes. The application of AK4 probe to *N. apiculata* didn't provide any conclusive results and for this reason it is not presented here. However, chosen photos of the best quality of the AK4 hybridisation can be found in Appendix Fig. 52.

Painted genomic regions of *N. apiculata* in Fig. 26–32 are described as painted segments. The term "painted segments" is used in this work for observed painted distinguishable chromosomal regions. Each segment is supposed to occupy its own chromosome which is proved with hybridization of AK probes on mitotic chromosomes (Appendix Fig. 49 to 56). Some segments cover whole chromosomes, others include only painted regions inside of an unpainted rest of a chromosome.

The AK1 probe painting covered whole three chromosomal bivalents. Two of them keep the pattern of genomic blocks same as in the ACK. The colour pattern of the other one includes two additional colour signals in the block **A** suggesting an inversion. The AK2 probe painted regions on five separate chromosomes. All of them seem to be just parts of larger chromosomes. At least two of them cover whole arms of chromosomes but it is impossible to determine whether short or long arms. The rest seems to be mixed with some other unpainted genomic blocks around pericentromeric regions. On the other hand, the AK3 probe painted mostly regions close to the centromeres. The same statement can be made about regions on at least four chromosomes painted with the AK5 probe. The rest of the segments painted with the AK5 probe on the other hand mostly occupy terminal regions of the chromosomes. Difficult patterns were obtained after application of the AK6 probe. Especially the short arm of ancestral chromosome 6 is rearranged complexly. Regions painted with AK7 seem to cover one whole chromosome, two-times whole chromosomal arms and once a large part of another arm of another chromosome (maybe the whole arm). AK8 probe exposed one large region painted with the same pattern as in ACK but having unpainted region attached. Other segments are incorporated inside different chromosomes where they have similar positions as in ancestral chromosome 8. An inversion is observable in one of these segments. Structural rearrangements in the genome of *N. apiculata* are so intricate that they make impossible proper chromosome identification without deeper analyses. Thus no karyotype

could be established for *N. apiculata*. However, assumed evolution of these rearrangements is presented in chapter 4.3.4.

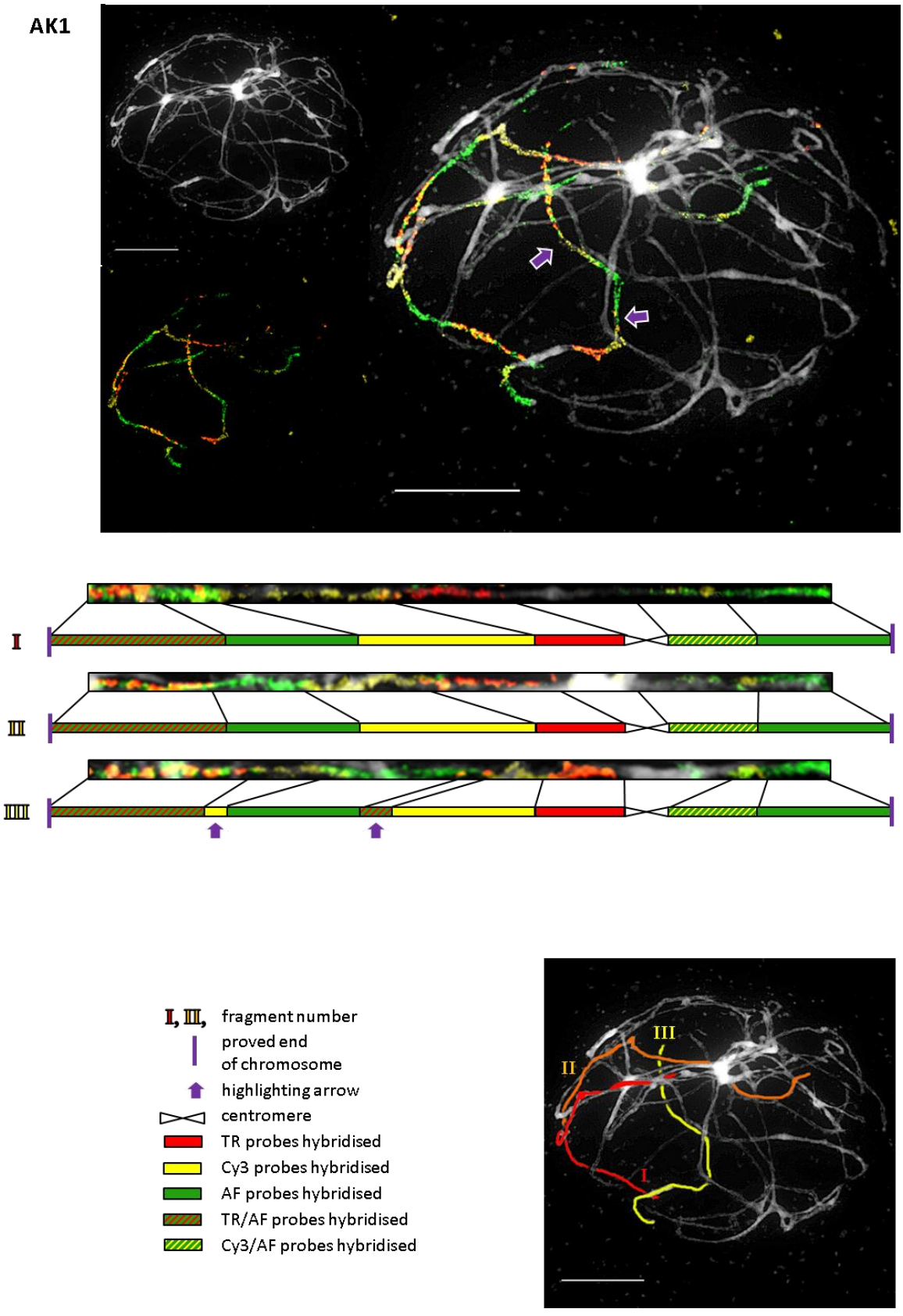

Fig. 26: Pachytene chromosomes of *N. apiculata* painted with AK1 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 49. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photo). Each segment was given a number (**I** to **III**) and its own colour for better recognition (lower image). Scale bars = 10 μm.

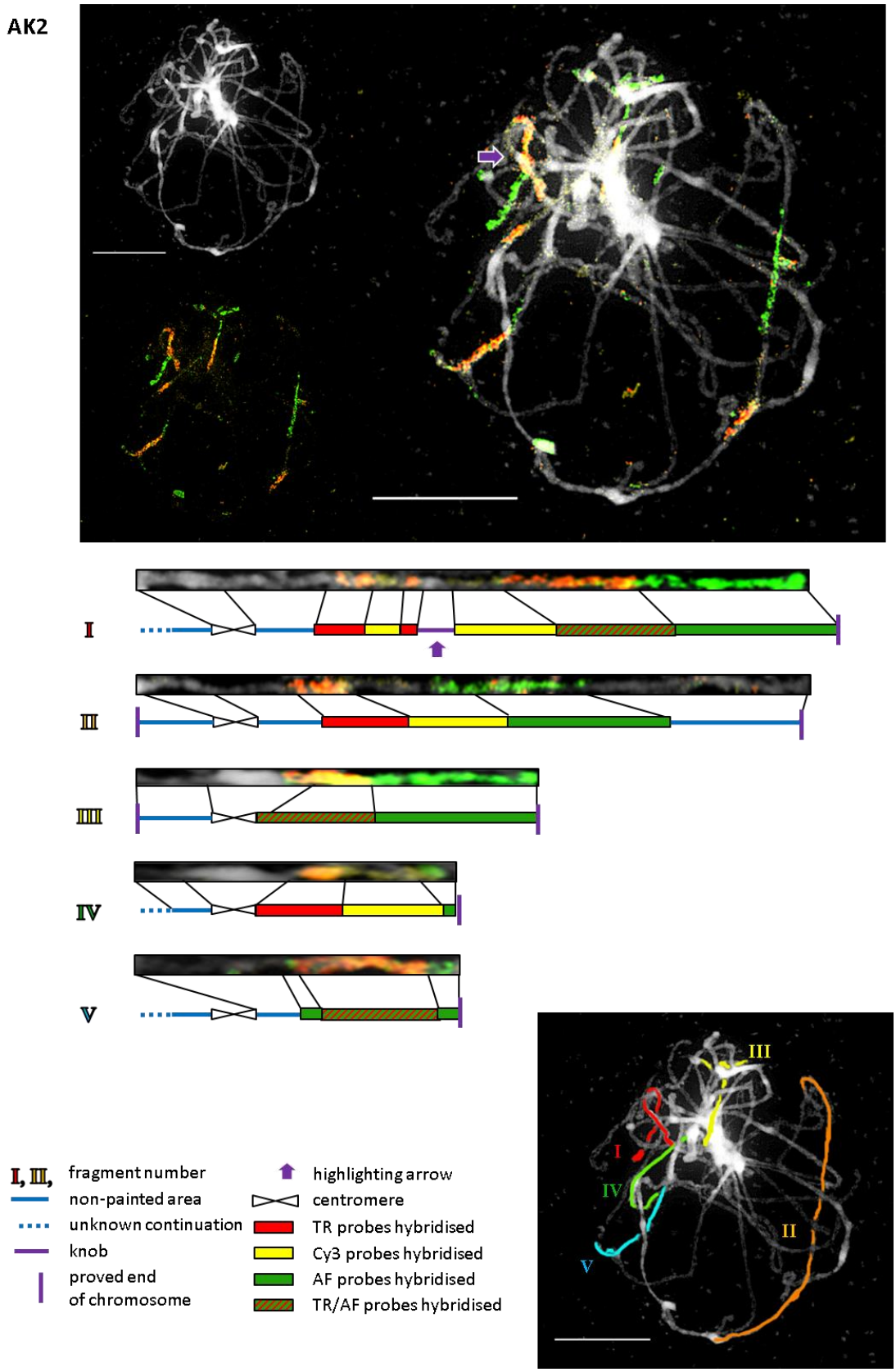

Fig. 27: Pachytene chromosomes of *N. apiculata* painted with AK2 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 50. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photo). Each segment was given a number (**I** to **V**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

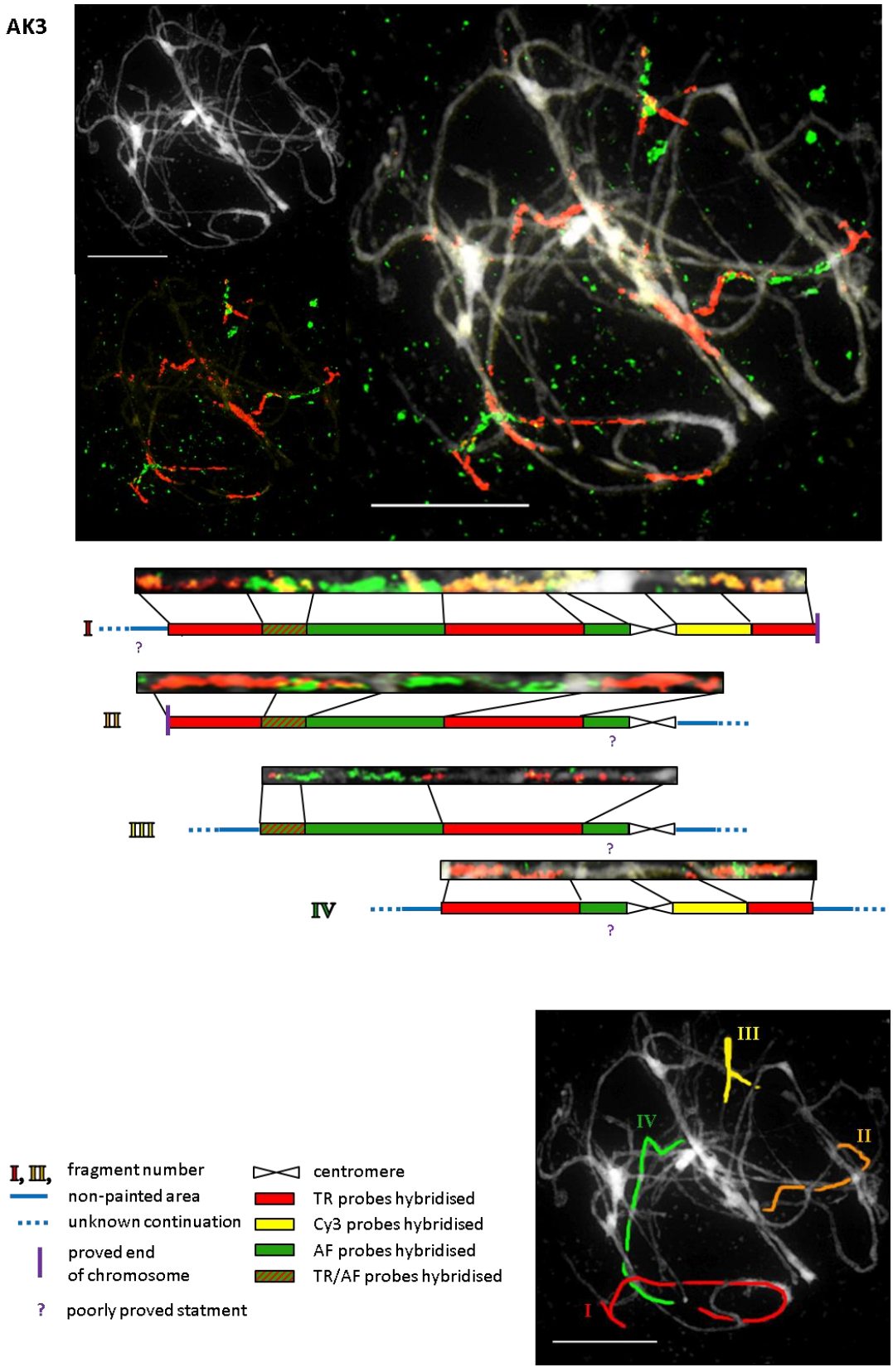

Fig. 28: Pachytene chromosomes of *N. apiculata* painted with AK3 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 51. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photo). Each segment was given a number (**I** to **IV**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

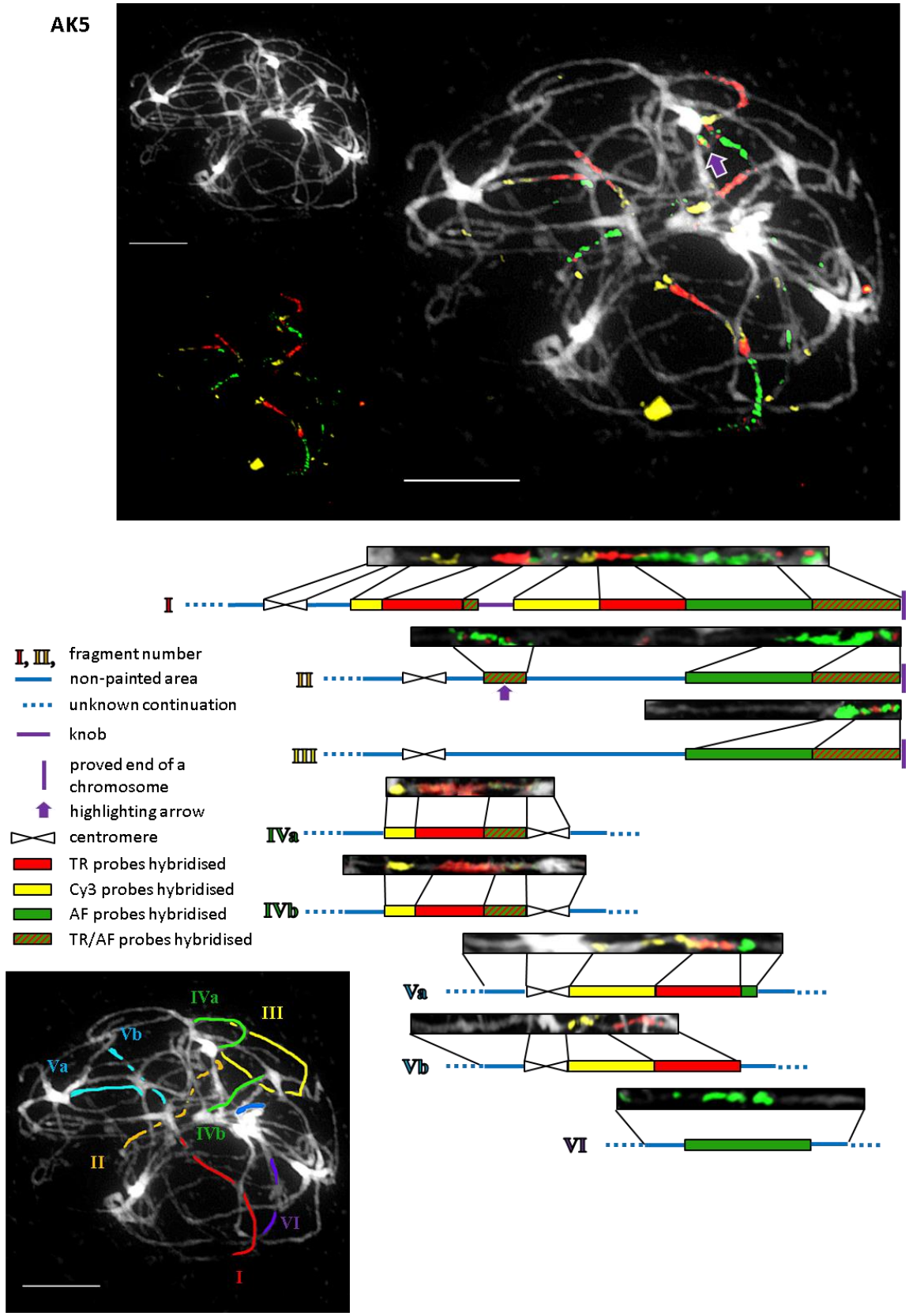

Fig. 29: Pachytene chromosomes of *N. apiculata* painted with AK5 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 53. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photo). Each segment was given a number (**I** to **VI**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

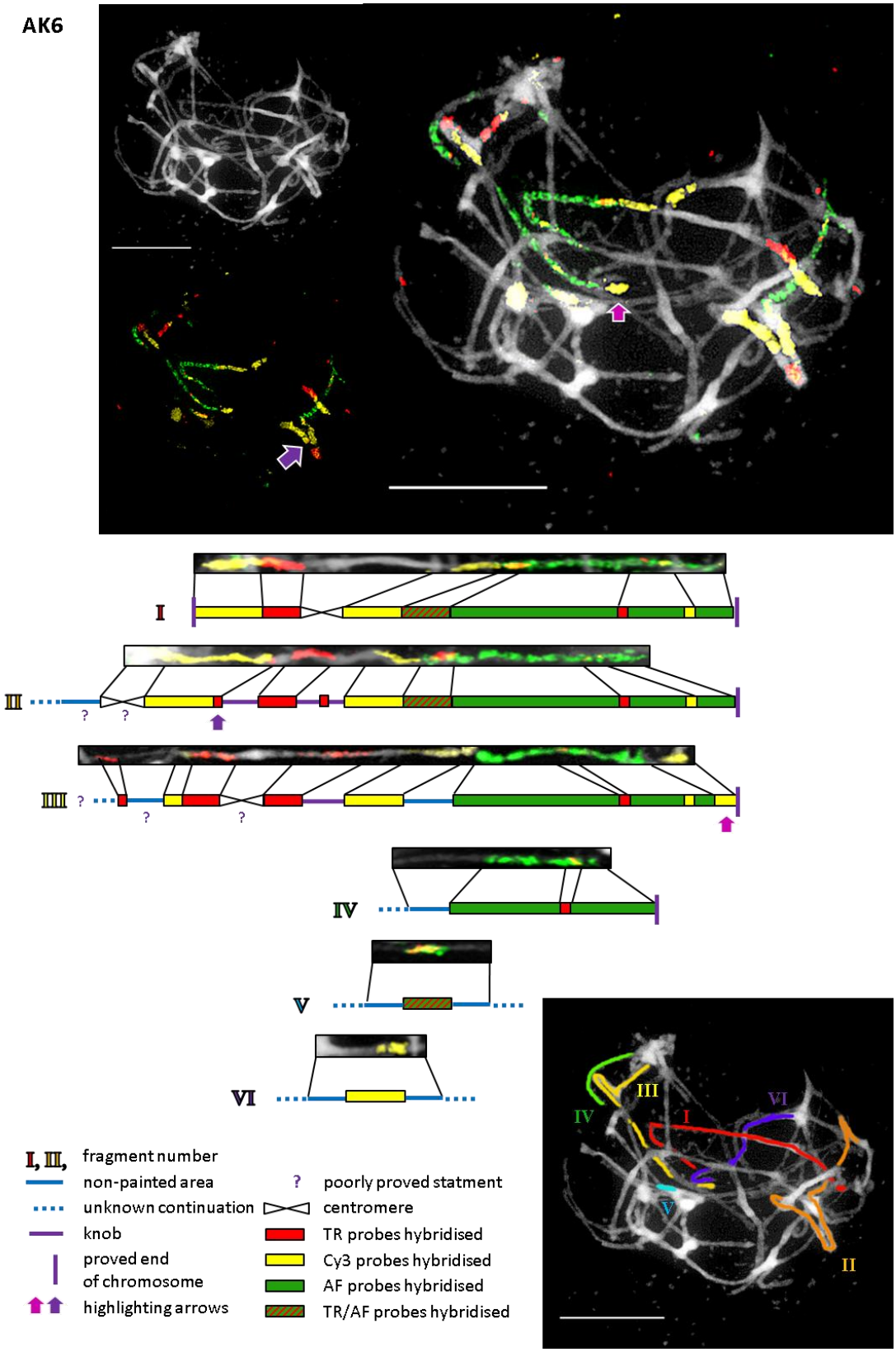

Fig. 30: Pachytene chromosomes of *N. apiculata* painted with AK6 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 54. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photo). Each segment was given a number (**I** to **VI**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

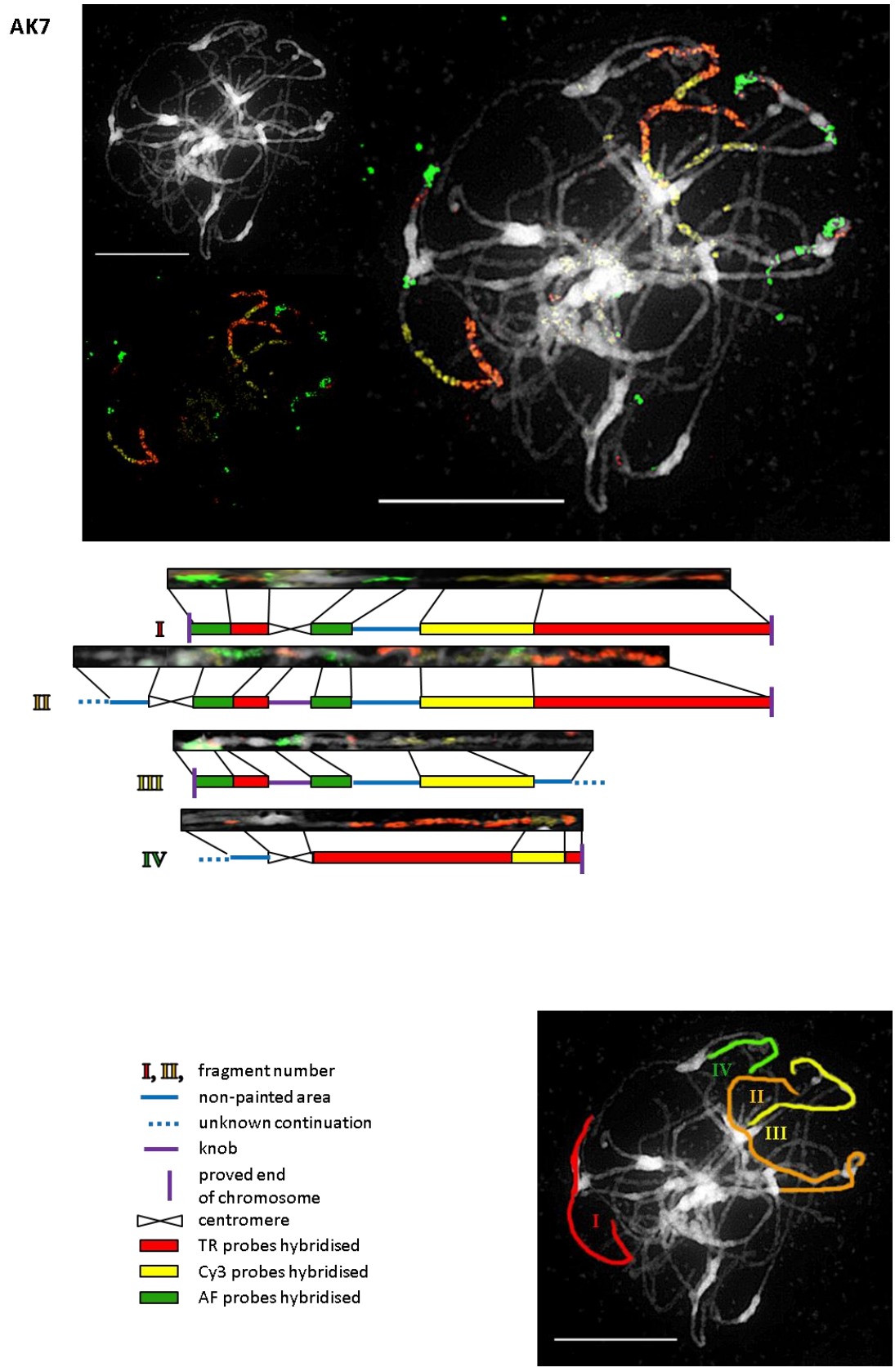

Fig. 31: Pachytene chromosomes of *N. apiculata* painted with AK7 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 55. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photos). Each segment was given a number (**I** to **IV**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

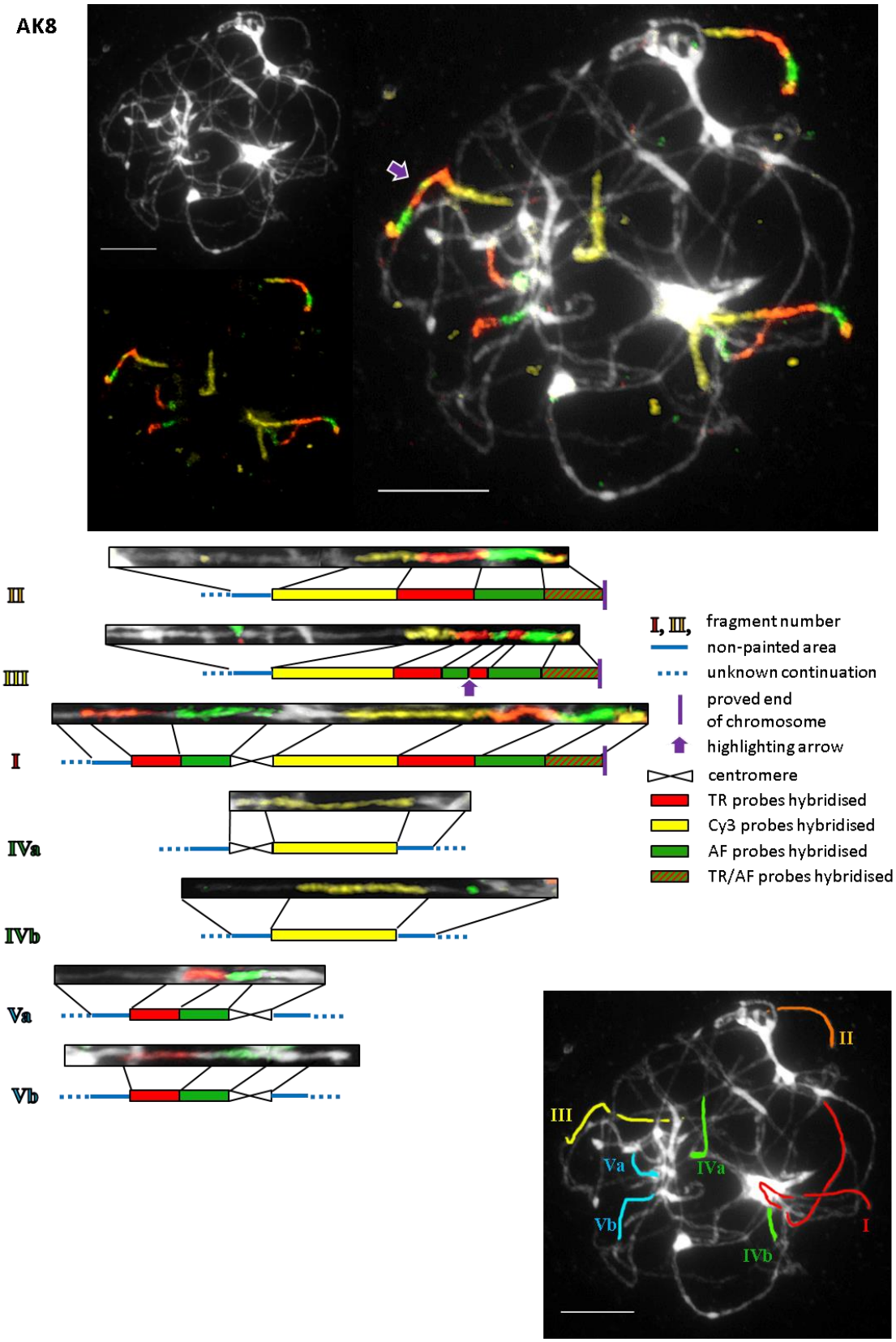

Fig. 32: Pachytene chromosomes of *N. apiculata* painted with AK8 probes. Upper images include a DAPI photo, an image of all probe signals and an image of previous two combined. For more examples see Appendix Fig. 56. The schemes below present observed colour patterns (the lengths of the schemes correspond with neither genomic blocks' lengths nor with the lengths in the photos). Each segment was given a number (**I** to **Vb**) and its own colour for better recognition (lower image). Scale bars = 10 µm.

4.3.4. Assumed rearrangements in the genome of *Neslia apiculata*

Photos of pachytene chromosomes of *N. apiculata* hybridized with probe corresponding to AK1 revealed three homeologous chromosome regions (artificially named segments **I, II, III**). Each of them covers a whole chromosome and colour patterns are in two cases identical to AK1 colour pattern. In the third segment, a slightly different pattern was observed. This can be clarified with an inversion as demonstrated in Fig. 33. In conclusion, former ancestral chromosome pair AK1 came across whole genome triplication resulting in three copies of its bivalents. Besides, an inversion occurred in one of resulting chromosomal pairs.

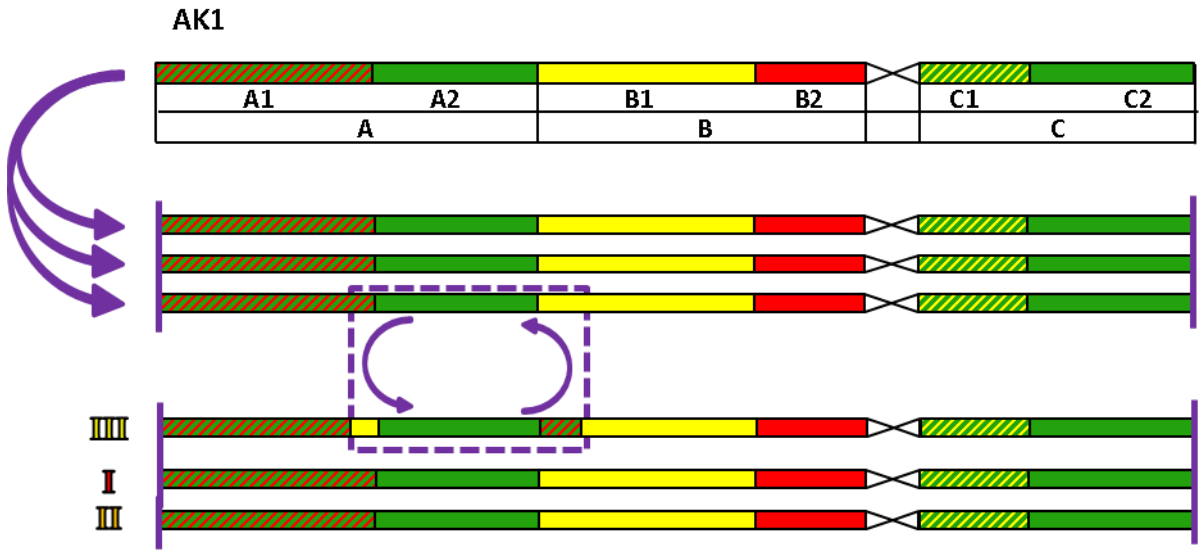

Fig. 33: Suggested scenario of origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK1 probe. Each line represents one chromosomal bivalent. For explanatory caption see Fig. 26. Violet arrows demonstrate evolutionary events (triplication, inversion) and violet frame highlights a region of a change.

AK2 probe painting photos (Fig. 27) indicate again that ancestral genome underwent a triplication of former AK2 chromosomal pair creating three pairs for each genomic block. However genomic blocks became mostly separated and integrated inside different chromosomes. Thus, as a result, five painted segments (**I** to **V**) can be observed. With the number **I** was named the largest of segments resembling original AK2 chromosome at the highest rate. The scenario of the creation of this segment can involve an inversion of **D** block followed by the second inversion inside this block (see Fig. 34). It seems that within the breakage point a centromere was once integrated but was knocked out when the whole segment fused with another chromosome with its own centromere. The second and the third pair of former hexaploid chromosomes didn't last as compact as the first one and each got split in two segments. One of them made segments **II** and **V** and the division of other one led to segments **III** and **IV**. The segment **III** is probably unchanged **E** block which fused with some other chromosome. Likewise, segment **IV** is genomic block **D** fused with a different chromosome. The significant green signal can be observed on its end. This can be either a NOR because these regions tend to be painted unspecifically, or a little region of **E** block. The segment **V** is again genomic block **E** but the only green region (**E2**) probably moved to join genomic block **D** in segment **II**.

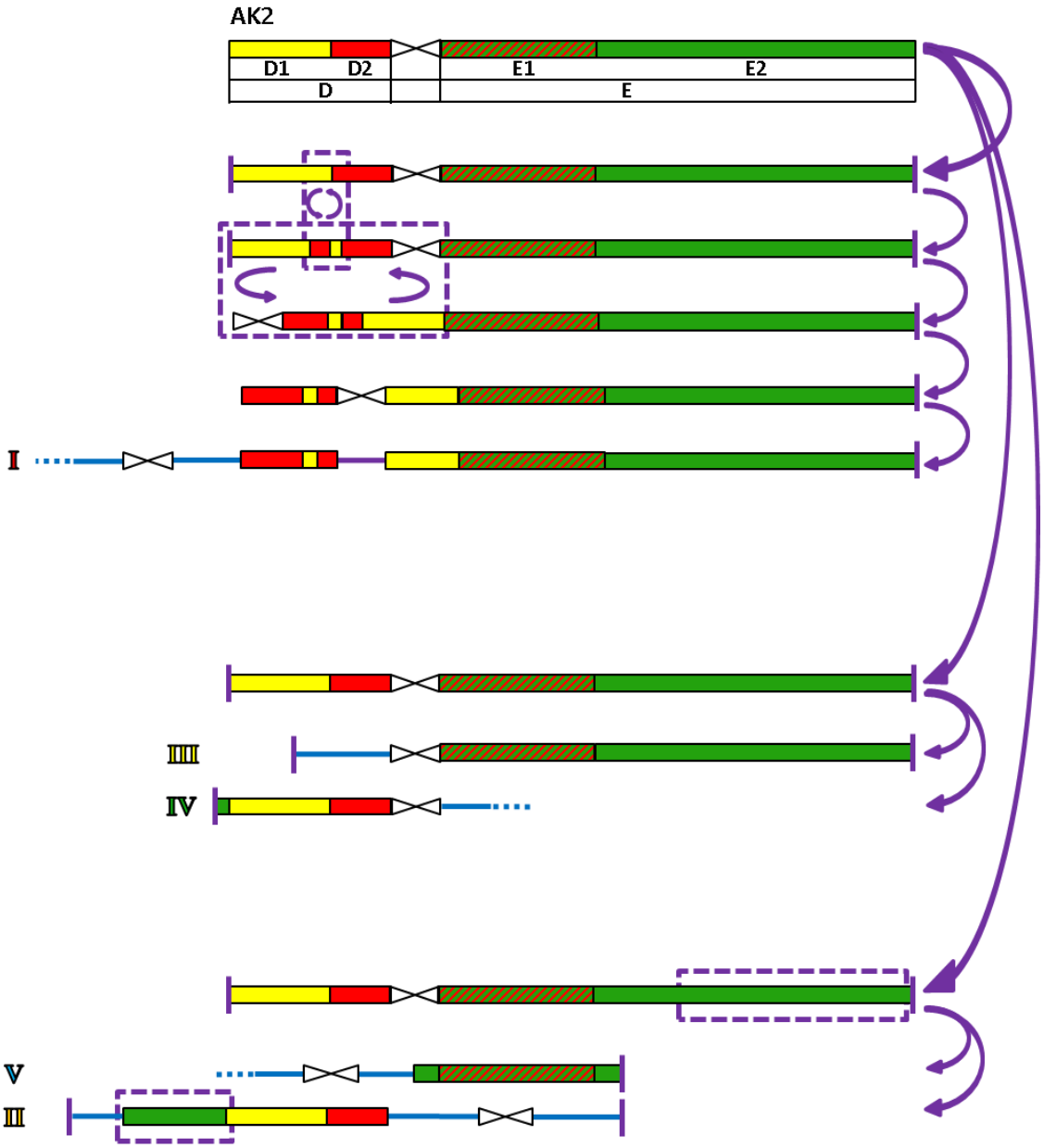

Fig. 34: Suggested scenario of origin for observed colour pattern and signal distribution in the genome of *N. apiculate* painted with AK2 probe. Each line represents one chromosomal bivalent or its painted segment. For explanatory caption see Fig. 27. Violet arrows demonstrate evolutionary events (triplication, inversion, separation of blocks) and violet frames highlight regions of a change.

For the case of AK3 probe painting, it seems that again whole-genome triplication created segments **I**, **II** and **III** and segment **IV** consists of the missing parts (Fig. 35). However, one **H** block is missing and probably disappeared from the genome in process of descending dysploidy.

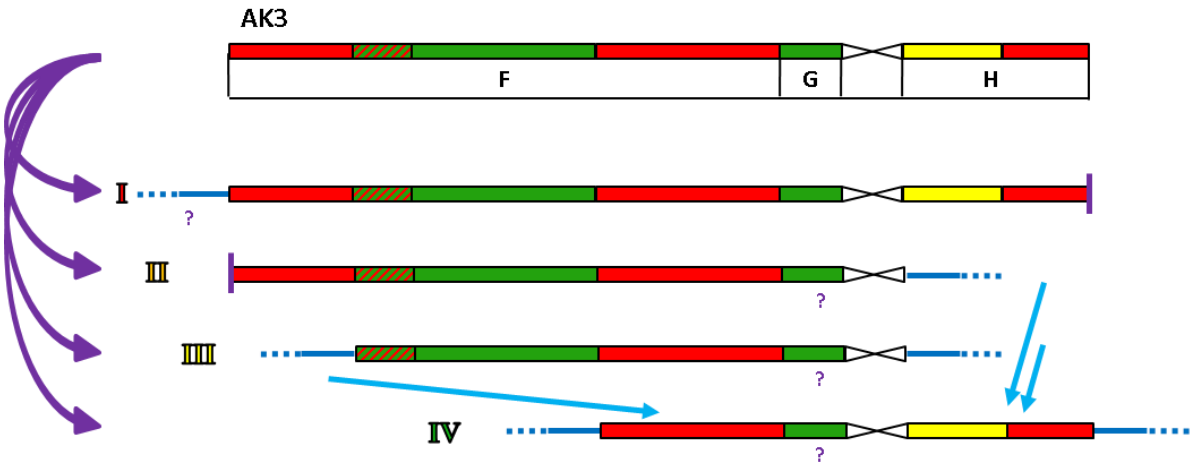

Fig. 35: Suggested scenario of origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK3 probe. Each line represents one chromosomal bivalent or its painted segment. For explanatory caption see Fig. 28. Violet and blue arrows demonstrate evolutionary events (segment **IV** could be created either through the violet or the blue way).

Unfortunately, AK4 probe painting was not successful. It failed to provide distinguishable fluorescent signals for all the genomic blocks. This probably happened because of a poor quality of the cytogenetic preparation.

AK5 painting again suggests a triplication from former ancestral chromosome 5 (Fig. 29). However, none of the segments covers a whole individual chromosome and so each colour segment is a part of some unpainted chromosome. In one case, the colour pattern of the ancestral chromosome was maintained unchanged in colour segment number **I**. This segment probably covers the whole region of the ancestral chromosome which fused with its short arm with some other unpainted chromosome. This unpainted chromosome has been keeping its centromere, thus original centromere of chromosome 5 was knocked-out and knob remained on its place. Other two pairs of chromosome underwent segmentation. Consequently, six other colour segments are observed in photos. Segments **IVa** and **IVb** are separate **K** blocks. Segments **Va** and **Vb** are **M** blocks and **II** and **III** are **N** blocks. **Va** segment also contains a little green signal attached to **M** block, which is probably a small part of **N1**. Surprising is a presence of colour segment **VI** which seems to contain material from genomic block **N1**. The second additional painted region is a red-green zebra in segment **II** (highlighted with a violet arrow in Fig. 36). It can be formed with genomic blocks **K3** or **N2** and it is located on the same chromosome as **N** block in segment **II**. Those two additional regions can be the result of a translocation, partial sequence multiplication and insertion of the amplicon into a new region or non-specific hybridisation due to similar, probably repetitive, regions.

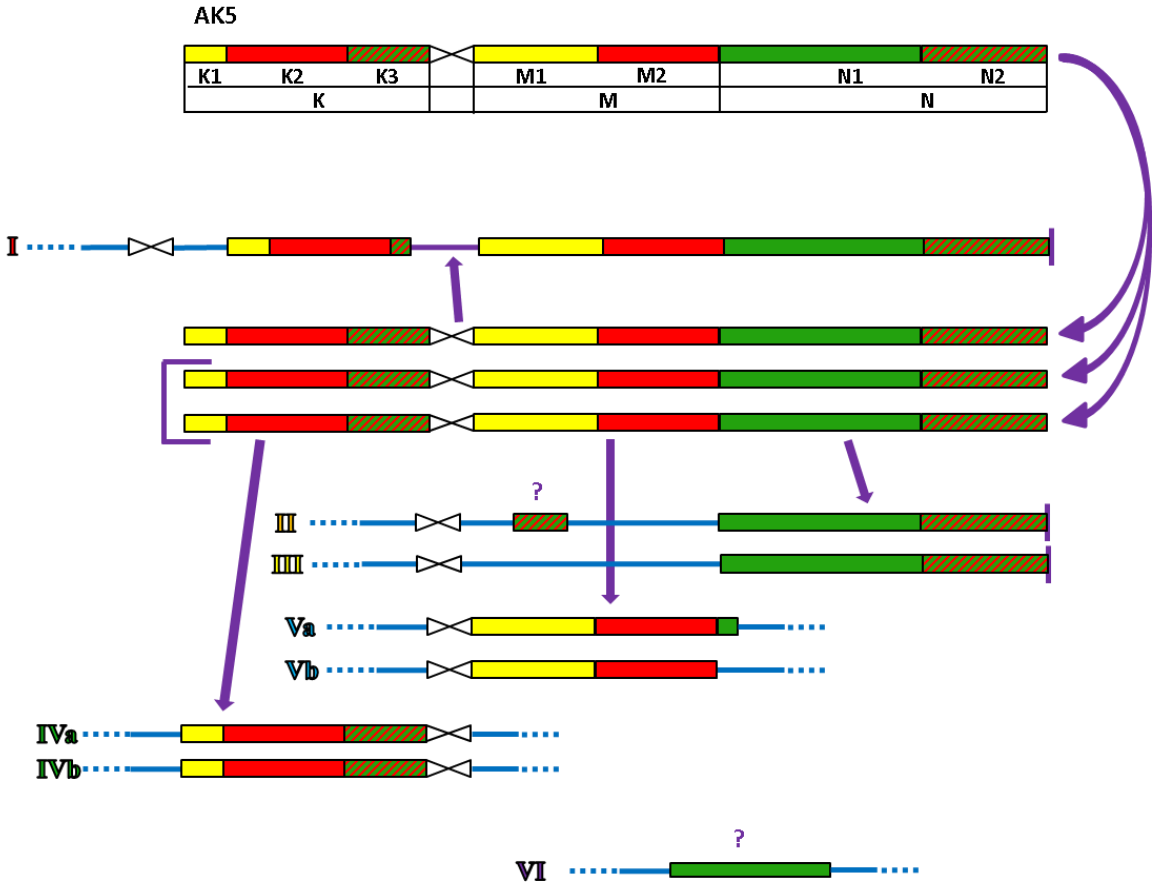

Fig. 36: Suggested scenario of origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK5 probe. Each line represents one chromosomal bivalent or its painted segment. For explanatory caption see Fig. 29. Violet arrows demonstrate evolutionary events (triplication, separation of blocks) and violet clamp demonstrates similar evolution in two chromosomes. Violet question marks here express a debatable origin of painted regions.

Segment **I** in the genome painted with AK6 probe (Fig. 30) covers the whole chromosome with the unchanged colour pattern of AK6 except for block **R** (Fig. 37). The yellow-green zebra of the block **R2** seems to be either missing or changed into little red and yellow signals inside a solid green region. Interestingly these two dots appear regularly in all uncluttered photos and the red signal is definitely not an outcome of red-yellow spectrum coverage. The same pattern applies to the second segment (**II**) but with a few differences. The original centromere was replaced with other one integrated in the block **P**. This might have happened even twice for at least three red signals separated with unpainted regions can be observed on clear photos (see Appendix Fig. 54). Lately, this centromere was also knocked out when the former chromosome 6 was fused through its short arm with another chromosome containing its own centromere. Another explanation for this pattern can be a fusion with the short arm of the third copy of the chromosome 6 and late reduction of the

redundant blocks. Unfortunately, the continuity of the chromosome with a centromere and unpainted region is badly proved and what seems to be a centromere can also be a NOR. The segment **III** is similar to previous two and again resembles the original chromosome 6. A noticeable yellow signal was observed on the distant end of **R** block. As in the upper case, former centromere was replaced by new one integrated inside the block **P**, suggesting these two segments evolved from the common ancestral segment (represented with green arrows in Fig. 37). That also explains why several blocks seem to be present in four instead of three copies. However, due to a lack of enough persuasive evidence, it is difficult to determine whether the unpainted region is really the centromere or a knob. Equally, an existence of continuation of the unpainted chromosome after block **O** is neither proved nor disproved. In some photos, little red signal was observed in the relatively regular close distance from the end of the painted segment. This colour pattern can be also a remnant of the third copy instead of the pattern in the segment **II**. Moreover, **Q2** was switched with the unpainted region and probably moved during crossing over to different chromosome creating segment **V** (the blue arrow in Fig. 37). Nevertheless, this is not the only possible explanation of the segments origin. Triplication can also explain segment **V** as well as segments **IV** and **VI** as remnants of the third copy of the chromosome 6. These contain sequences hybridized with probes for blocks **R** in case of segment **IV** and **O** or **Q1** in case of segment **VI**.

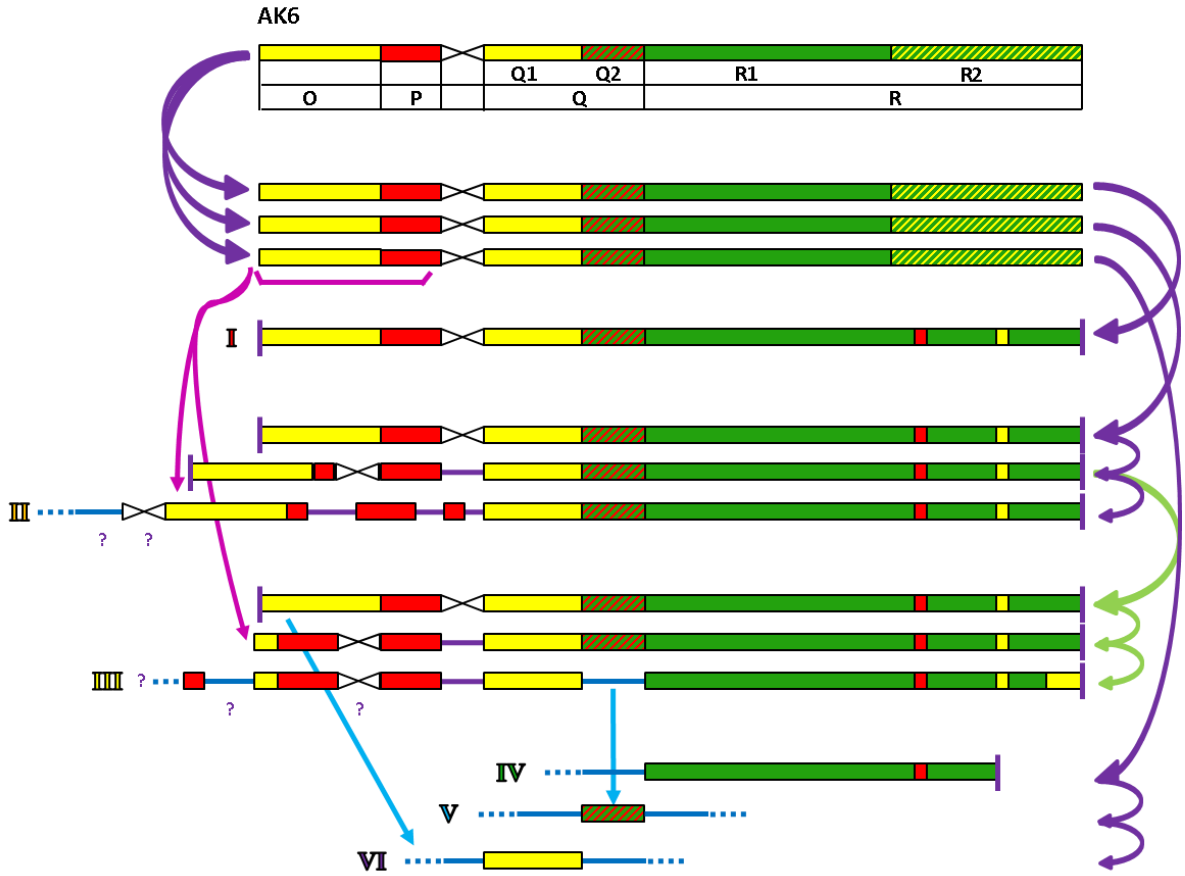

Fig. 37: Suggested scenario of the origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK6 probe. Each line represents one chromosomal bivalent or its painted segment. The arrows demonstrate evolutionary events (multiplication, separation of blocks). The light green arrows follow a possible evolution of the fourth copy of several genomic blocks. The blue arrow stands for a different possible origin of segment **V** via translocation and for possible alternative for segment **VI** explanation which can enlighten observed significantly short yellow signal in segment **III**. For the rest of the explanatory caption see Fig. 30.

Also, a former ancestral chromosome 7 underwent the triplication (Fig. 38). One homologous diploid pair of chromosomes remained unchanged as seen in segment **I**. Coloured segment **II** shows the second copy of the chromosome 7 to be attached to its short arm to another unpainted chromosome. The distant end of the block **S** seems to be directly followed by a foreign centromere. Therefore, the original centromere of chromosome 7 was, in this case, knocked out. Also, in the segment **III** was the former centromere knocked out, but this time is a new centromere suspected to lie somewhere on unpainted chromosome attached to the block **U1** of the long arm of chromosome 7. **U2** probably moved to another chromosome creating the segment **IV**. Before that, an inversion inside block **U** occurred explaining a yellow region inside the red block.

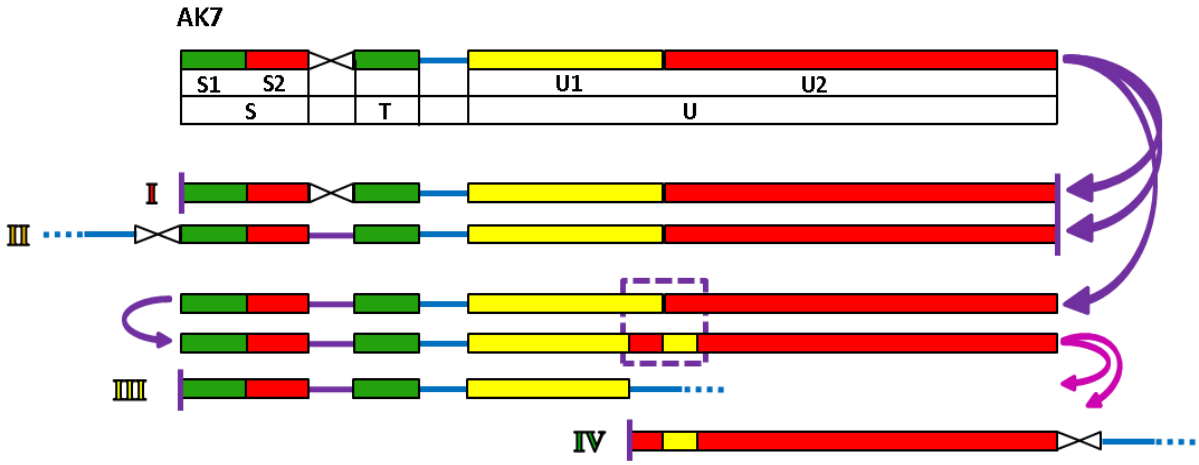

Fig. 38: Suggested scenario of the origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK7 probes. Each line represents one chromosomal bivalent or its painted segment. Violet arrows demonstrate evolutionary events (triplication, inversion) and purple arrows demonstrate separation of former blocks. Violet frame highlights the region of inversion. For further explanatory caption see Fig. 31.

Painted with AK8 probes (Fig. 32), one of the chromosomes was observed being not changed from the ancestral one except for a fusion with unpainted regions on the end of the short arm (**I**). Segments **II** and **III** include a colour pattern of the whole long arm of ancestral chromosome 8 joined with unpainted chromosomes. Furthermore, the colour pattern in segment **III** underwent an inversion in the border region of blocks **W** and **X**. The short chromosomal arms related to those two segments were observed on different chromosomes forming segments **Va** and **Vb**. Again, segments **IVa** and **IVb** do not fit in the simple triplication scenario, thus their origin is deemed to be either from duplication followed with translocation, reduction of other former copies of chromosome 6 or unspecific hybridisation due to similar sequences.

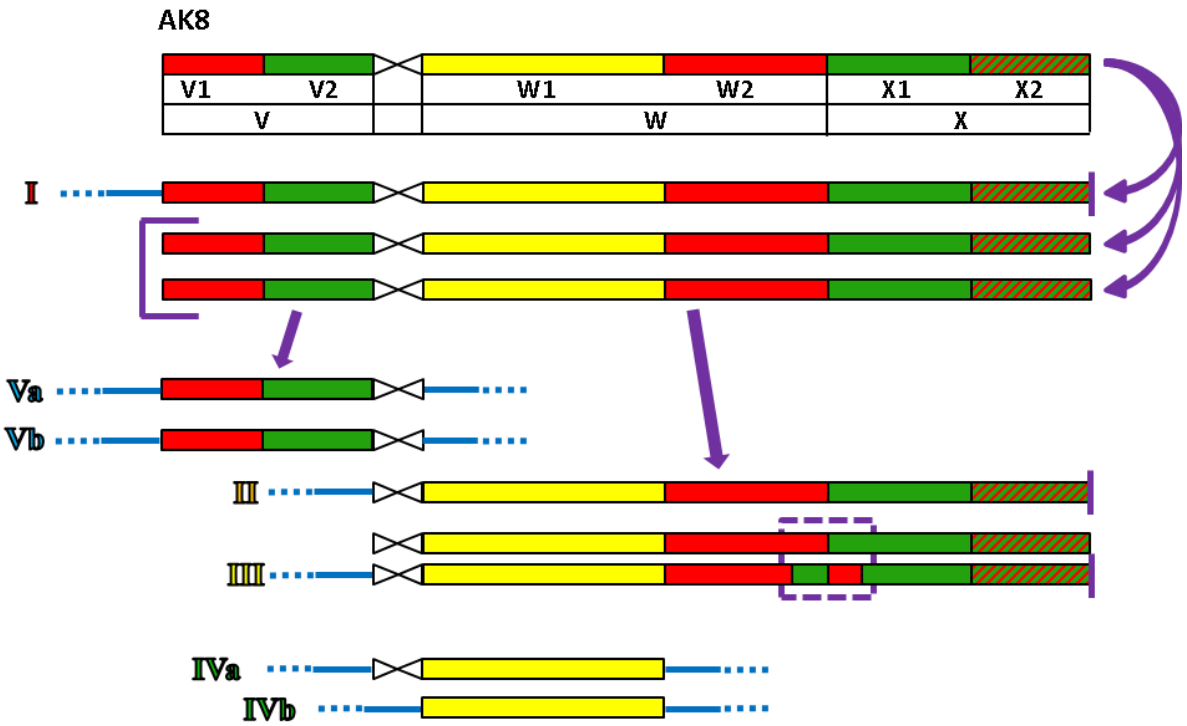

Fig. 39: Suggested scenario of the origin for observed colour pattern and signal distribution in the genome of *N. apiculata* painted with AK8. Each line represents one chromosomal bivalent or its painted segment. Violet arrows show possible evolutionary events (triplication, segmentation). Violet frame highlights the region of inversion. Violet clamps demonstrate shared evolution for both copies. For further explanatory caption see Fig. 32.

4.4. Localization of rDNA on particular chromosomes

With the knowledge of the arrangement of genomic blocks in the investigated species rDNA loci could be localized more specifically on particular chromosomes (Fig. 42). Chromosome-specific BAC clones were used to design probes according to results from chapter 4.3. On each slide chromosome-specific probe, 35S rDNA probe and 5S rDNA probe were simultaneously hybridized on mitotic chromosomes. Probes were labeled with different colours. A combination of rDNA and chromosome-specific probe signal occurring on the same chromosome marks the location of particular rDNA on a particular

chromosome (Fig. 40 and 41). This could not be applied on *N. apiculata* since no map of genomic blocks could be established from obtained results.

In conclusion: i) 35S rDNA is always present in terminal regions, ii) 35S rDNA sequences are present in more copies than 5S rDNA in all studied species, iii) no evolutionary pattern can be deduced from observed rDNA locations since they seem to be randomly distributed on different chromosomes and independent on the positions of genomic blocks. Only 35S rDNA locations in chromosome 7 are shared in all species (curiously, the 35S region forming a NOR is visible also in photos of pachytene chromosomes in previous chapters).

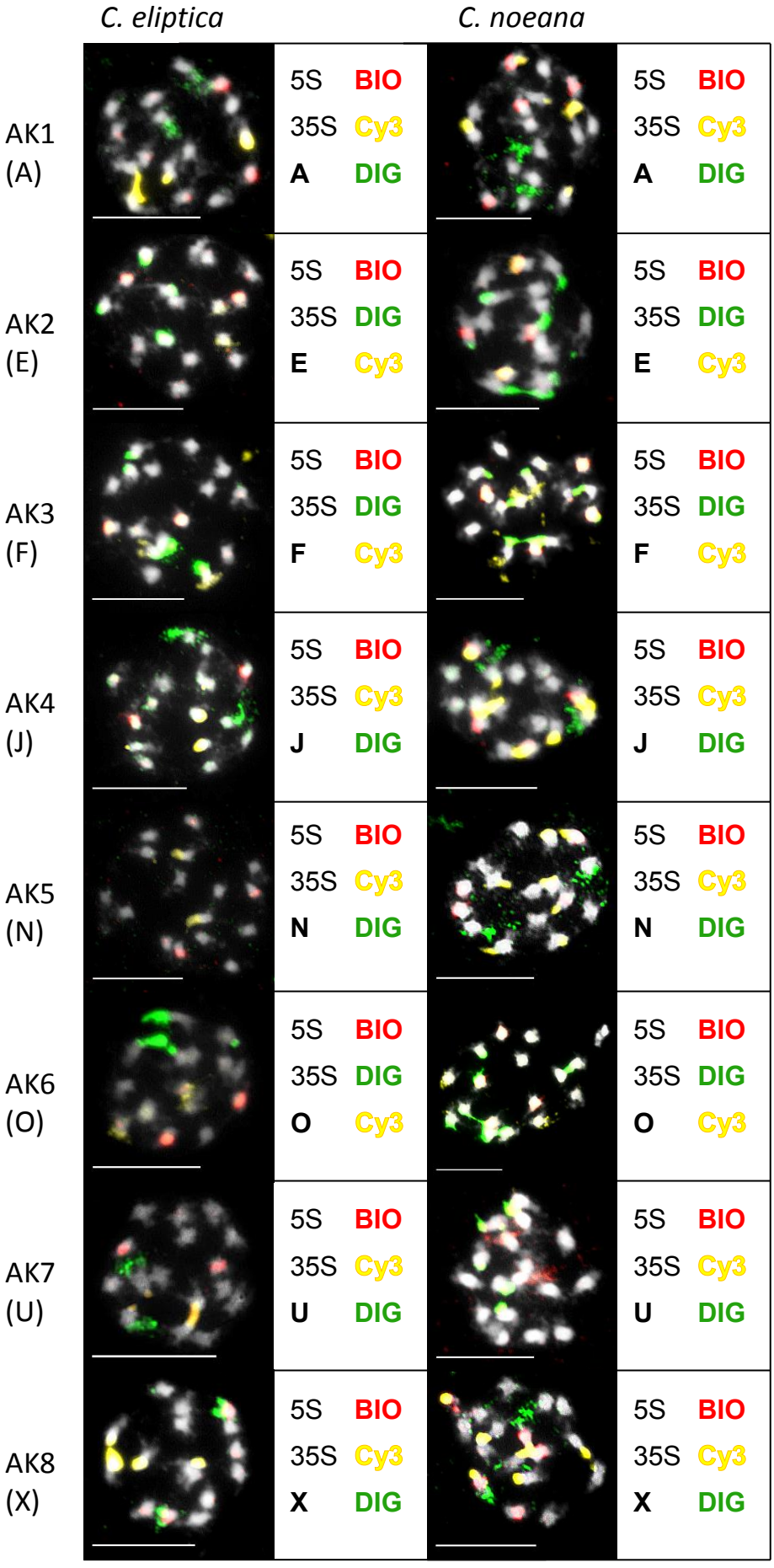

Fig. 40: rDNA localization in *C. elliptica* and *C. noeana*. Each photo includes 3 probe signals of unique colours (a genomic block (A to X), 35S rDNA and 5S rDNA). Each probe can be labeled differently in different photos either with biotin (BIO), digoxigenin (DIG) or directly with Cy3. The colours correspond with particular signal colour provided with a fluorochrome (Alexa Fluor 488 – green; Texas Red – red; Cy3 – yellow). Scale bars = 10 μm.

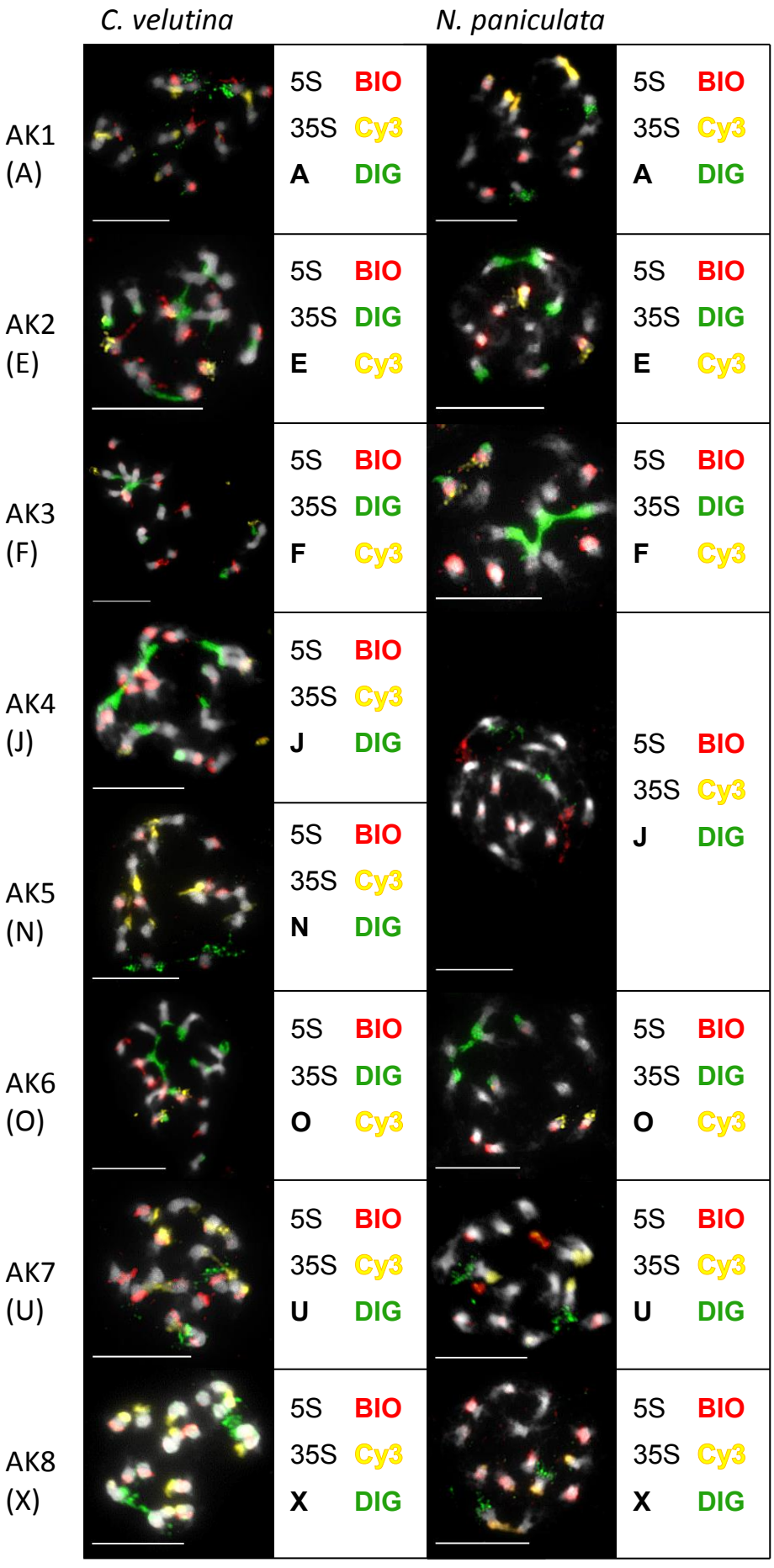

Fig. 41: rDNA localization in *C. velutina* and *N. paniculata*. Each photo includes 3 probe signals of unique colours (a genomic block (A to X), 35S rDNA and 5S rDNA). Each probe can be labeled differently in different photos either with biotin (BIO), digoxigenin (DIG) or directly with Cy3. The colours correspond with particular signal colour provided with a fluorochrome (Alexa Fluor 488 – green; Texas Red – red; Cy3 – yellow). Scale bars = 10 µm.

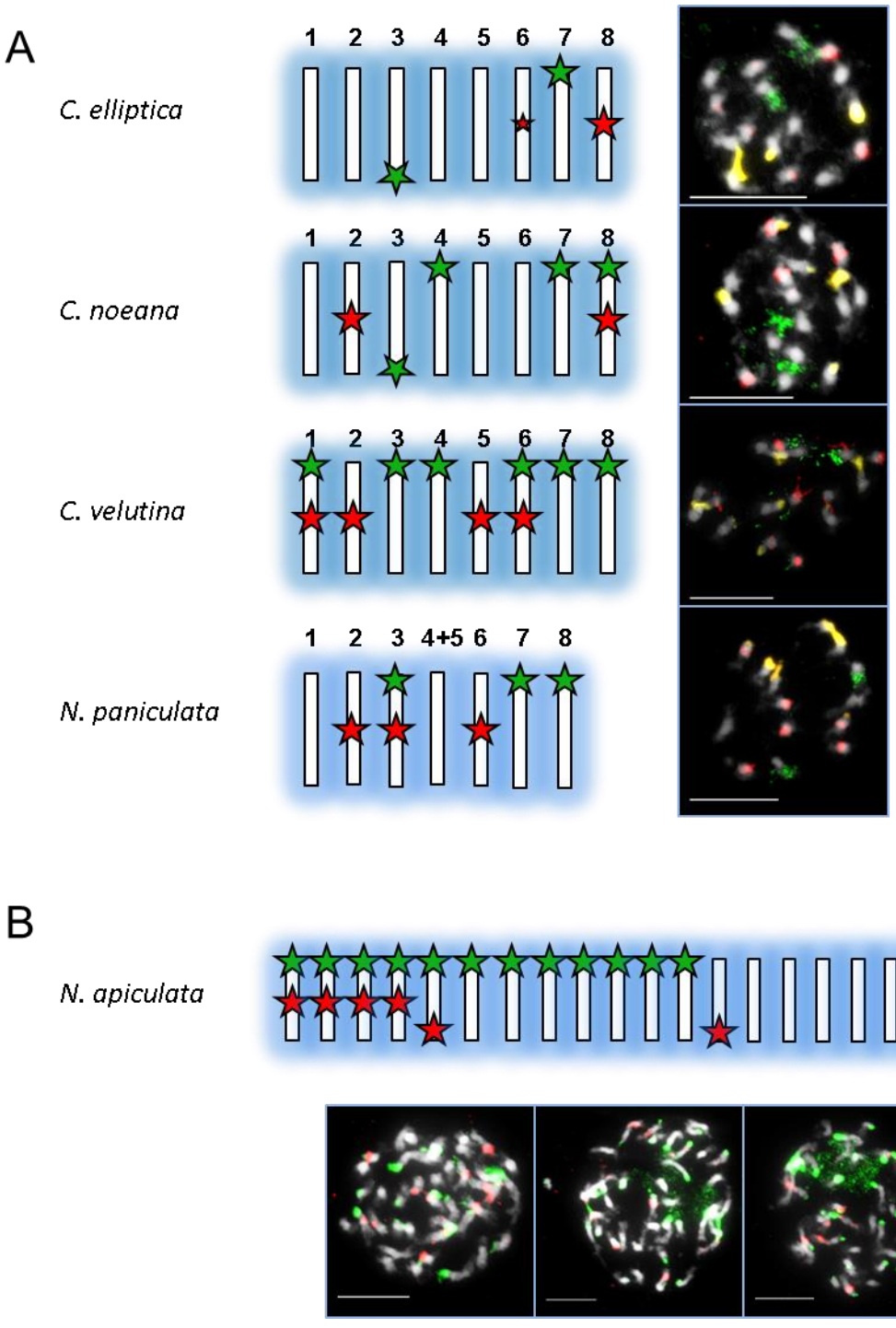

Fig. 42: Mitotic chromosomes of investigated species painted with probes homologous to 35S rDNA (green) and 5S rDNA (red). The scheme shows homologous or homeologous pairs of chromosomes represented by white rectangles. One rectangle stands for one pair of chromosomes. Green and red stars represent probe signals and their approximate location within a chromosome (either the internal or terminal region). The size of the objects has no meaning. However smaller red star on chromosome 6 of *C. elliptica* demonstrates very weak probe signal. **A)** Diploid species with rDNA localised on specific chromosomes. **B)** rDNA locations in *N. apiculate* as described in chapter 4.2. Scale bars = 10 μm.

5. DISCUSSION

5.1. rDNA locations in the examined genomes

The number and location of ribosomal RNA genes were investigated to provide additional information to the analysis of genomic blocks arrangements to better elucidate the evolution of genomes. However, the given results do not match rDNA locations and numbers obtained for *C. velutina* and *N. paniculata* by Ali et al. (2005). Investigated species had always more copies of 35S rDNA than 5S rDNA which fits the results of previous studies (Garcia et al., 2017). In angiosperms, 35S rDNA sequences occur preferentially in terminal regions (Roa and Guerra, 2012) which corresponds to the results preseted here. In contrast to 35S rDNA, 5S rDNA was observed mostly in internal regions. According to work of Roa and Guerra (2015), 5S rDNA is mostly distributed in proximal regions and in angiosperms tend to be present as only one pair of loci in karyotype. Garcia et al. (2017) also accent a migration of rDNA sequences independent on other chromosome rearrangements. The same phenomenon was observed in my work and thus rDNA locations seem to have no value for the analysis of genome evolution in the tribe Camelineae.

5.2. Genome evolution in the tribe Camelineae

For all the three *Chrysochamela* species the genome stasis was observed. They have 8 pairs of homologous chromosomes (2n = 16) as proved in photos of mitotic chromosomes and most of them were proved to be identical to the original chromosomes of the ancestral karyotype. The only exceptions are the chromosome 4 of *C. noeana* and the chromosome 8 of *C. elliptica* (also possibly the chromosome 3). The chromosome 4 of *C. noena* was not resolved in appropriate quality with CCP so its pattern of genomic blocks remain unclear. This was caused probably by a damage of chromosomes obtained during pepsin treatment. However, As all the other chromosomes of *C. noeana* do not differ from ACK, only internal changes within chromosome 4 would be possible and the equivalent chromosomes of *C. elliptica* and *C. velutina* don't have any changed patterns. The pattern of genomic blocks in the chromosome 3 of *C. elliptica* was obtained, however in all the photos additional painted region was observed. Since this region is next to the centromere it is probably a result

of unspecific probe hybridization caused by the high amount of unspecific repetitive sequences in pericentromeric regions. For equivalent chromosomes of *C. noeana* and *C. velutina* no difference from the AK3 was observed. Photos of chromosomes painted with AK8 probe were not obtained because not enough cytogenetic slides was prepared from *C. elliptica*. It was very difficult to find any suitable pachytene chromosomes for this species. Again, equivalent chromosomes of *C. noeana* and *C. velutina* have the arrangement of genomic blocks constant. Since no translocation was detected in the rest of species' chromosomes, the chromosome 8 of *C. elliptica* can possibly only have internal rearrangements. The karyotypes of *C. noeana* and *C. elliptica* are almost complete and it seems they have not differentiated from the structure of the ACK during the evolution. Also, the karyotype of *C. velutina* has remained without any doubt in genome stasis during the evolution.

The genome stasis was also detected in other species from the tribe Camelineae namely *Arabidopsis lyrata* (Lysak et al., 2006) and *Capsella rubella* (Slotte et al., 2013). So far, the genome stasis was observed only in the tribe Camelineae in contrast to the other tribes of Brassicaceae.

The genus *Neslia* exhibits two different evolutionary scenarios for the two of its species – the descending dysploidy through genome reshuffling and the whole-genome duplication followed by diploidisation (which is also descending dysploidy accompanied with chromosomal rearrangements but this term will not be used in this context to prevent confusion). *Neslia paniculata* remained diploid and almost unchanged except for the pericentromeric inversion on chromosome 8 and chromosome 4 which led to the fusion of chromosomes 4 and 5 and subsequent loss of centromere on former chromosome 5 (this supports the previous study of Lysak et al., 2006). That explains why *Neslia paniculata* has only 7 pairs of homologous chromosomes (2n = 14) in contrast to the common x = 8 in Camelineae.

Pericentromeric inversion on chromosome 8 can possibly be a player in future chromosomal fusions just as chromosome 4 has been. After all, the same events occurred in the evolution of *A. thaliana* and led to the reduction of its chromosome number to n = 5. It seems however that only these two species alone in the tribe Camelineae underwent descending dysploidy through genome reshuffling (not *sensu* descending dysploidy after WGD or WGT). On the other hand, in context of the whole family Brassicaceae the desceding dysploidy through chromosome fusions is quite common. In the Clade A it was reported for tribes Boechereae (Mandakova et al., 2015a) and Descurainieae (Lysak et al., 2006), both

reducing their chromosome number to 7, and in Turritideae reducing it even to 6 pairs of homologous chromosomes (Lysak et al., 2006). Five chromosomes of *A. thaliana* remain the lowest point of descending dysploidy so far. Through the descending dysploidy ancestral karyotype of Clade E (CEK) got 7 chromosomes (Mandakova et al., 2017a). And of course most notably all n = 7 tribes from Clade B share the common ancestor with 7 chromosomes in PCK (Mandakova and Lysak, 2008).

Surprisingly, *Neslia paniculata* is considered to be basal species in the context of the Camelineae and genus *Chrysochamela* lies in more advanced branches of the phylogenetic tree (Huang et al., 2016). In condition of the robust phylogenetic tree this indicates at least two things: I) the genome reshuffles in other genera of Camelineae appeared independently and II) the evolution of the sequence level and the whole genome level goes separately and can have even opposite tendencies (*Chrysochamela velutina* has one of the most derived molecular markers in Camelineae but is identical to the ancestor of all crucifers on the genomic level).

Neslia apiculata has 42 chromosomes. This number could possibly be achieved through the triplication of former 14 chromosomes. Three copies of the genomic blocks revealed by CCP support this. Except for AK1-like chromosomes, all the chromosomes of *N. apiculata* are reshuffled in contrast to the chromosomes from the ACK. This indicates that *N. apiculata* is a mesopolyploid in the process of diploidisation. The hypothetical chromosomal rearrangements in *N. apiculata* are described in Fig. 33 to 39.

In the tribe Camelineae, also *Camelina sativa* underwent the whole-genome triplication (Kagale et al., 2014). Mesopolyploids in Brassicaceae include Microlepidieae (Mandakova et al., 2010a; Mandakova et al., 2010b; Mandakova et al., 2017b), Brassiceae (Lysak et al., 2005; Wang et al., 2011) and some Cardamineae (Mandakova et al., 2013; (Mandakova et al., 2014; Mandakova et al., 2016). Geiser et al. (2016) analysed a tetraploid genome of *Biscutella laevigata* from Clade C. Heliophileae underwent the whole-genome triplication too (Mandakova et al., 2012).

What are the pathways of the genome evolution in the tribe Camelineae then? In conclusion, three of five so far encountered evolutionary scenarios were observed in the tribe Camelineae. Either no genomic rearrangement happens and the chromosome number stays conserved in genomic stasis as was the case of *C. elliptica*, *C. noeana*, *C. velutina* or *A. lyrata* and *C. rubella*. Or the genomic blocks of chromosomes rearrange and cause chromosome number reduction as for *N. paniculata* or for *A. thaliana* was observed. The last principle of chromosome evolution in the tribe Camelineae is the whole-genome triplication

which results later in complex structural rearrangements and chromosome number reduction as polyploids return to a diploid state. This can be found in the genomes of *N. apiculata* or *C. sativa*. These evolutionary pathways are demonstrated in the context of the whole family Brassicaceae in Fig. 43 with genera *Chrysochamela* and *Neslia* newly incorporated. The other two possibilities in evolution (reshuffling without descending dysploidy in low or high scale) have not been observed for Camelineae. Neither in previous studies nor in this work.

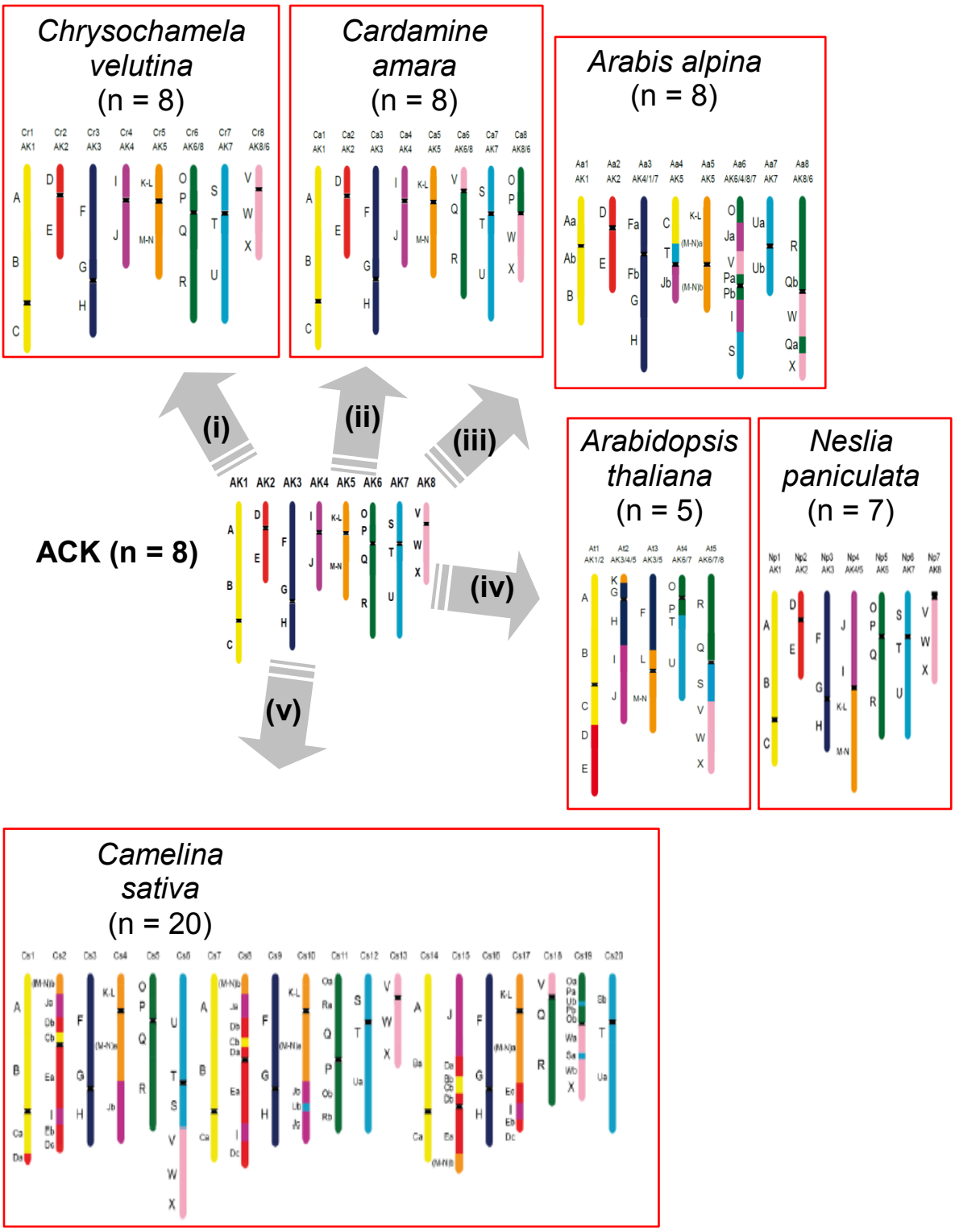

Fig. 43: All the so far observed evolutionary pathways for karyotypes in Brassicaceae as they evolve from ancestral crucifer karyotype (ACK, n = 8) (adjusted original from poster of Mandakova, 2016). **i)** genome stasis without major chromosomal reshuffling (n = 8) represented by *Chrysochamela velutina*, **ii)** genome stasis with a few structural rearrangements (n = 8) represented by *Cardamine amara*. **iii)** genome reshuffling without chromosome number change (n = 8) represented by *Arabis alpina*. **iv)** genome reshuffling accompanied with descending dysploidy (n = 7, 6 or 5) represented by *A. thaliana* and *Neslia paniculata*. **v)** whole-genome duplication or triplication followed by diploidisation represented by *Camelina sativa*.

6. SUMMARY

This diploma thesis widens the knowledge of the chromosomal evolution in the family Brassicaceae. The target group of the focus was one of the family's tribes – Camelineae. To examine evolution of the genomes in this tribe comparative chromosomal painting (CCP) was applied on five chosen species: *Chrysochamela elliptica* (2n = 16), *C. noeana* (2n = 16), *C. velutina* (2n = 16), *Neslia paniculata* (2n = 14) and *N. apiculata* (2n = 42).

CCP implementation was feasible due to the BAC library of the complete genome of A. thaliana, which is included in Camelineae, and due to the suitability of Brassicaceae. These plants actually comprise a relatively low amount of repetitive sequences in the context of the plant kingdom.

From young anthers of selected species, cytogenetic slides containing pachytene or mitotic chromosomes or chromosomes in the phase of diakinesis were prepared. Probes built up from BAC contigs were applied on proper chromosomes labelling particular genomic blocks or rDNA sequences. 35S and 5S sequences were observed on mitotic chromosomes but the results turned to be not valuable for evolution analysis in the tribe Camelineae. On pachytene chromosomes probes homologous to particular genomic blocks were applied. These were arranged according to reference ancestral crucifer karyotype (ACK).

Obtained results showed the conservative genomic structure in genus *Chrysochamela* since all chromosomes of observed species have an identical arrangement of genomic blocks as ACK do. Almost the same result was obtained for *N. paniculata*, however two pericentromeric inversions were detected in contrast to ACK and two former ancestral chromosomes were fused into a single chromosome. *N. apiculata* as polyploid appeared to have too many rearrangements in its genome to provide a good view into the chromosomal structure of the blocks. At least it provided basic information available for future studies.

Obtained results demonstrate the outcomes of the three of five known evolutionary pathways documented for the family Brassicaceae. The first is genome stasis when no genomic rearrangement occurs and the number of chromosomes is constant. The second is a descending dysploidy as a result of genome reshuffling. The last is whole-genome triplication followed by complex genomic rearrangements and subsequent gradual loss of the chromosomes. Thus, at least these three evolutionary pathways certainly shaped the genome evolution in the tribe Camelineae.

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Appendix

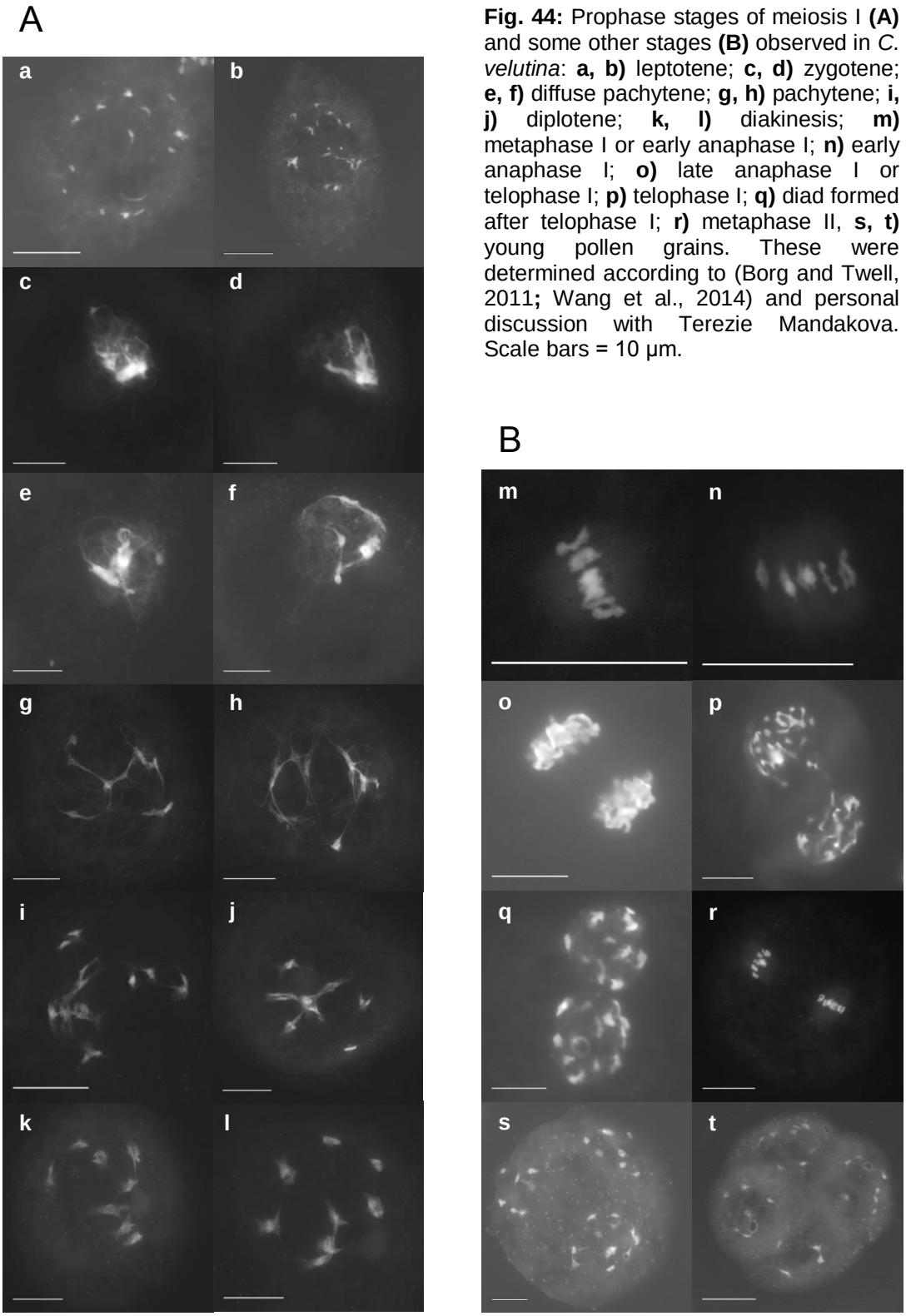


Fig. 44: Prophase stages of meiosis I **(A)** and some other stages **(B)** observed in *C. velutina*: **a, b)** leptotene; **c, d)** zygotene; **e, f)** diffuse pachytene; **g, h)** pachytene; **i, j)** diplotene; **k, l)** diakinesis; **m)** metaphase I or early anaphase I; **n)** early anaphase I; **o)** late anaphase I or telophase I; **p)** telophase I; **q)** diad formed after telophase I; **r)** metaphase II, **s, t)** young pollen grains. These were determined according to (Borg and Twell, 2011**;** Wang et al., 2014) and personal discussion with Terezie Mandakova. Scale bars = 10 µm.

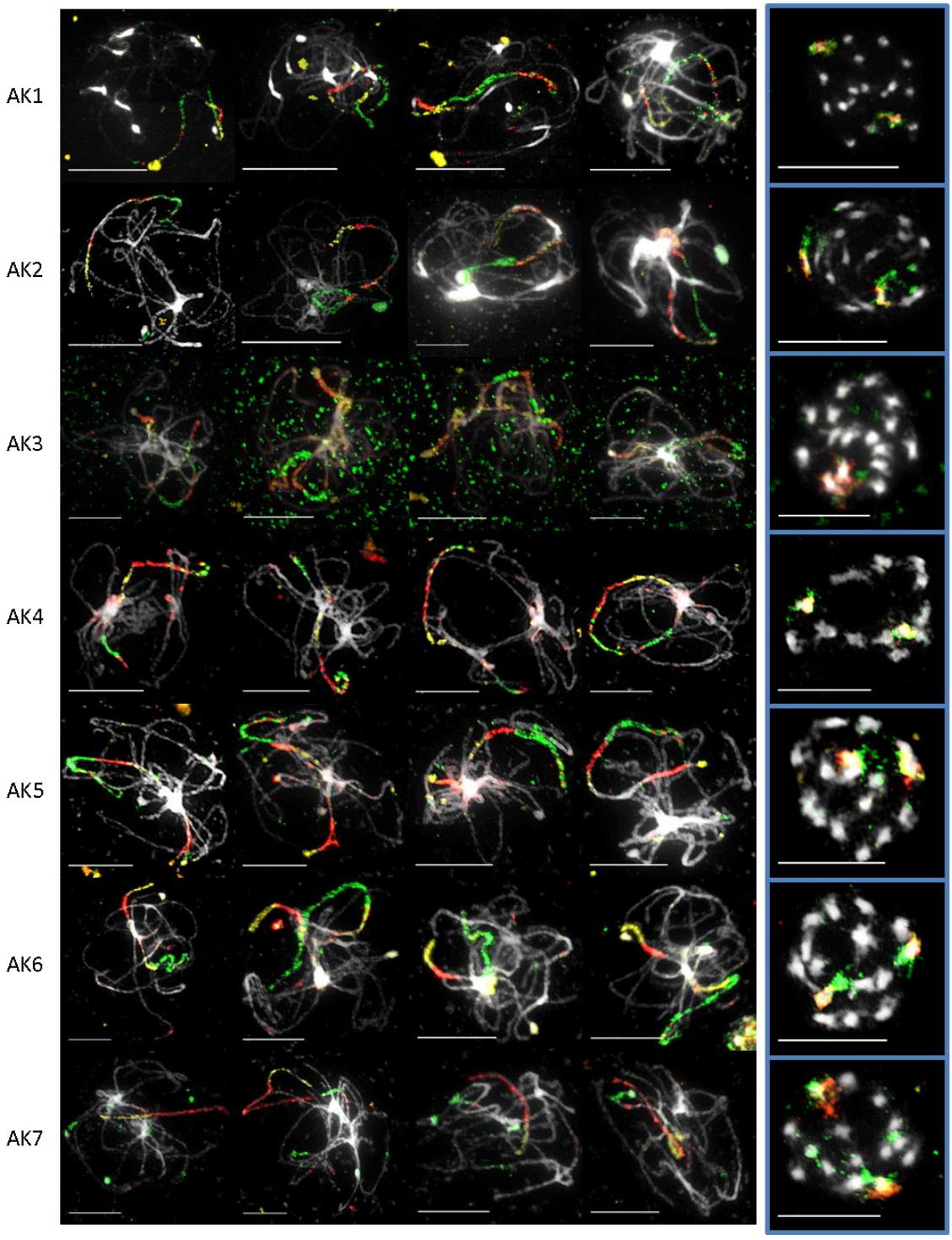


Fig. 45: Other examples of painted chromosomes for *Chrysochamela elliptica* proving declared coulour pattern. Each line presents application of one probe matching colour pattern given to one ACK chromosome (AK1 to AK7). Photos in blue frames show mitotic chromosomes or diakineses painted with given probes (AK1 to AK7). Scale bars = 10 μm.

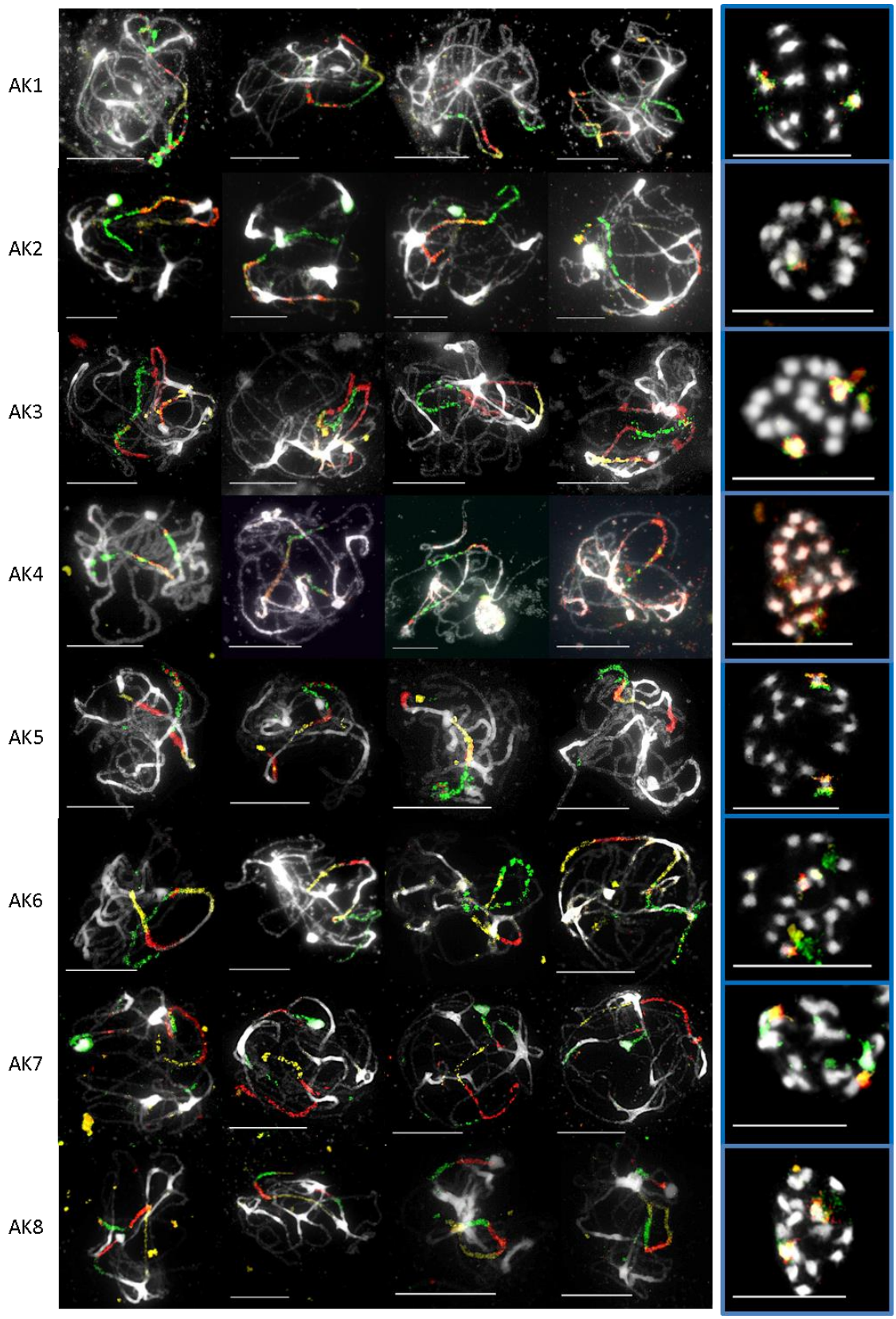


Fig. 46: Other examples of painted chromosomes for *Chrysochamela noeana* proving declared coulour pattern. Each line presents application of one probe matching colour pattern given to one ACK chromosome (AK1 to AK8). Photos in blue frames show mitotic chromosomes or diakineses painted with given probes (AK1 to AK8). Scale bars = 10 µm.

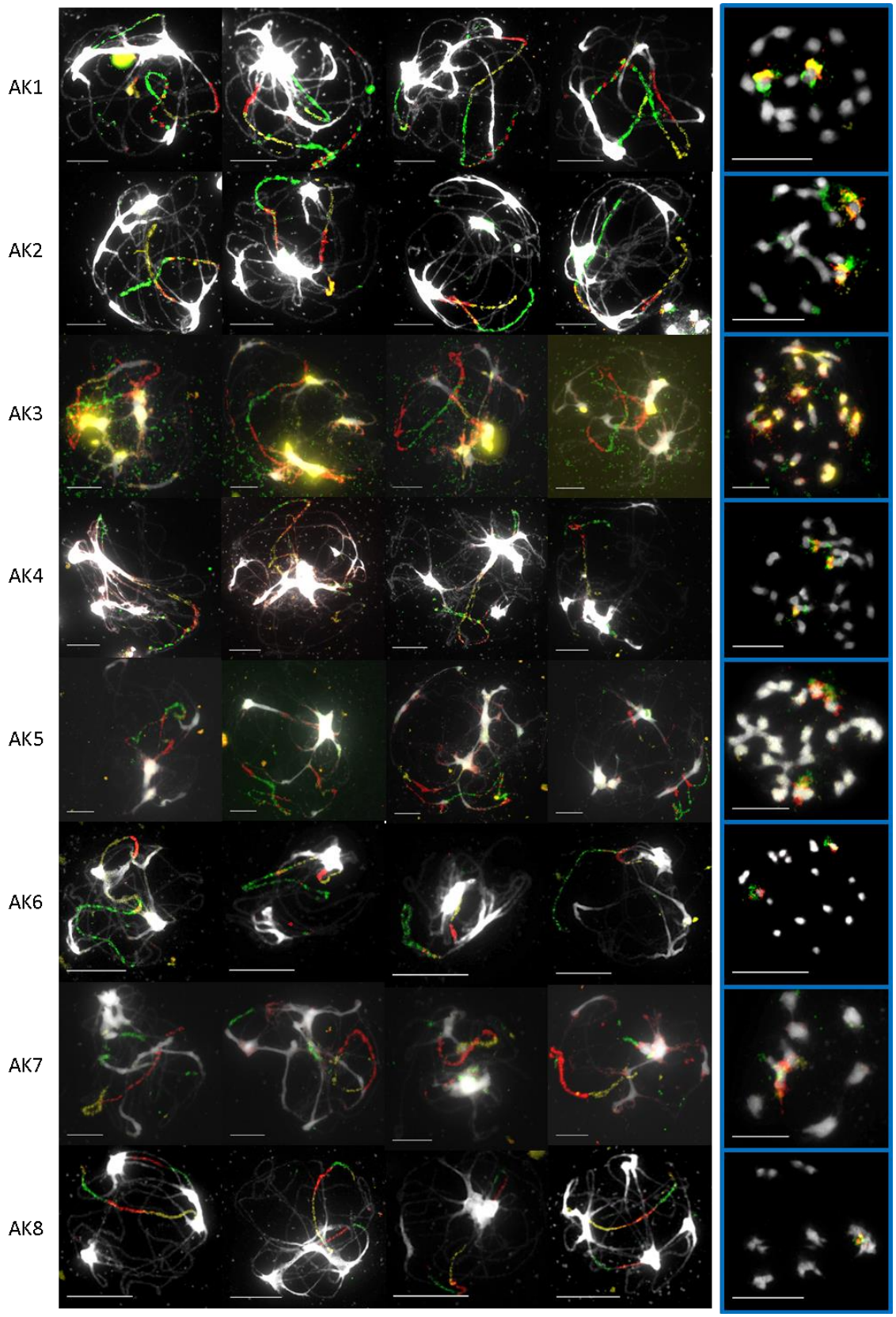


Fig. 47: Other examples of painted chromosomes for *Chrysochamela velutina* proving declared coulour pattern. Each line presents application of one probe matching colour pattern given to one ACK chromosome (AK1 to AK8). Photos in blue frames show mitotic chromosomes or diakineses painted with given probes (AK1 to AK8). Scale bars = 10 μm.

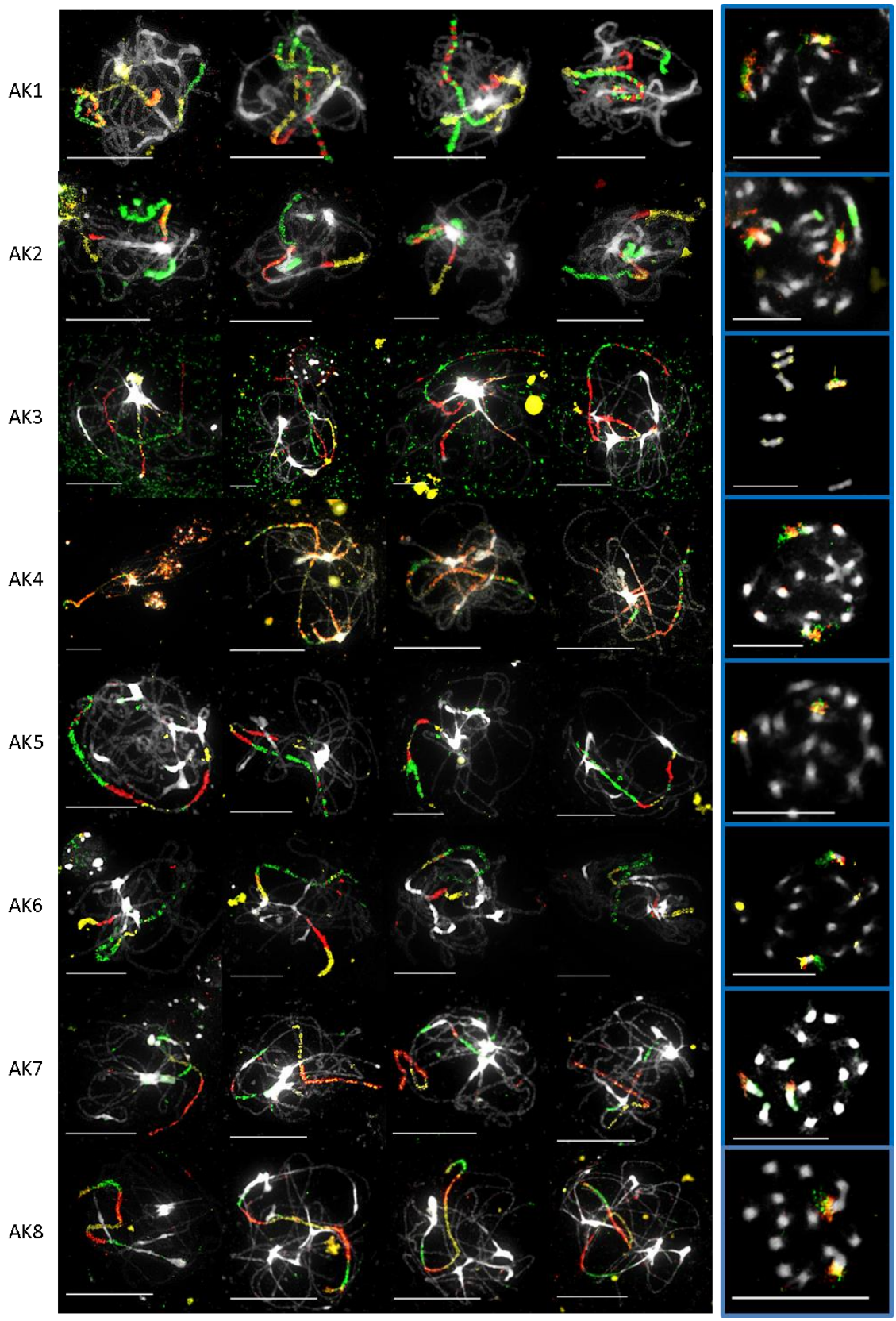


Fig. 48: Other examples of painted chromosomes for *Neslia paniculata* proving declared coulour pattern. Each line presents application of one probe matching colour pattern given to one ACK chromosome (AK1 to AK8). Photos in blue frames show mitotic chromosomes or diakineses painted with given probes (AK1 to AK8). Scale bars = 10 μm.

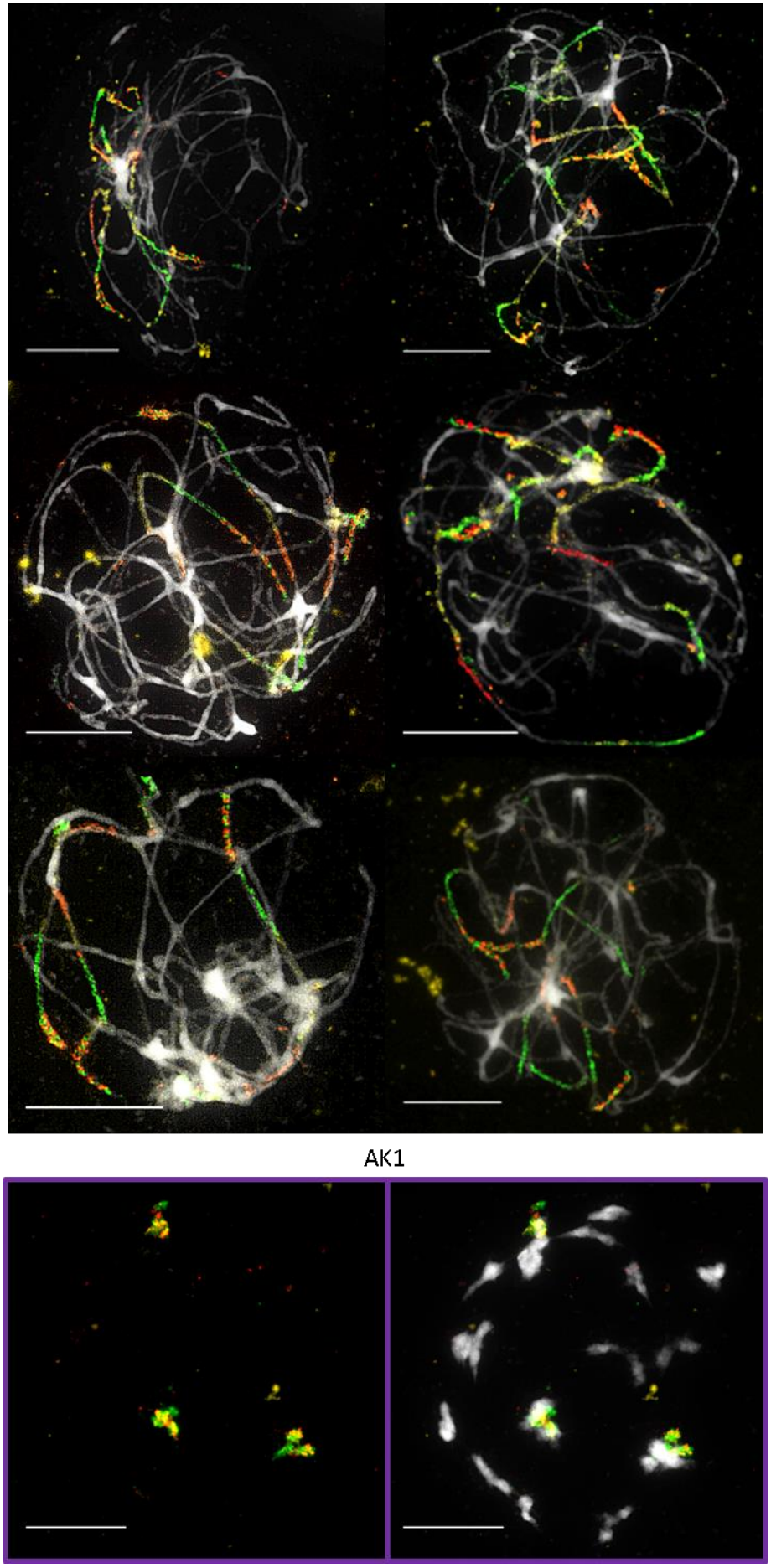


Fig. 49:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK1 probes (upper images). In the photos with a violet frame, chromosomes in diakinesis (DAPI + AK1 on the left, only AK1 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts). Scale bars = 10 µm.

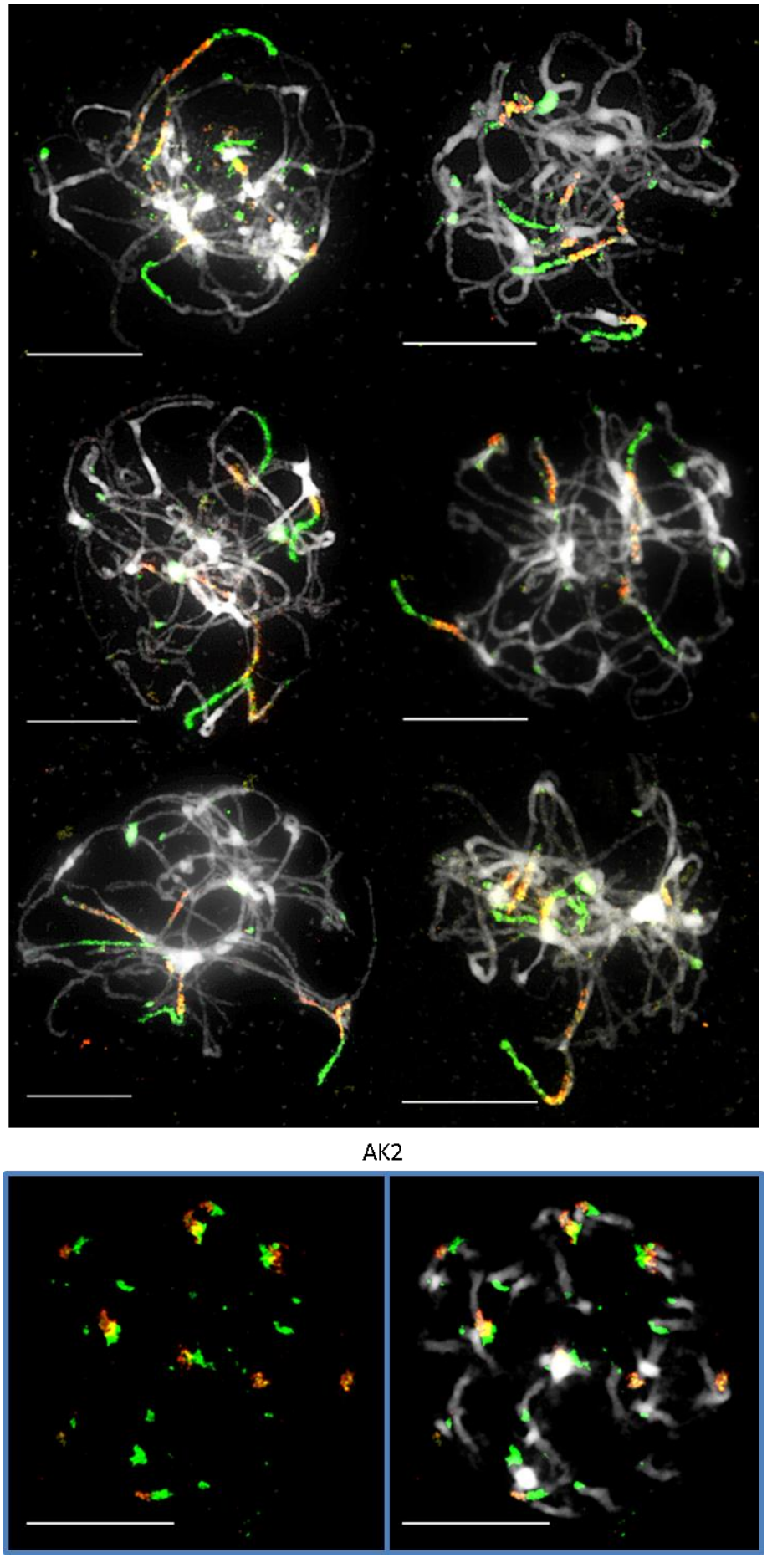


Fig. 50:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK2 probes (upper images). In the photos with a blue frame, mitotic chromosomes (DAPI + AK2 on the left, only AK2 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts). Scale bars = 10 µm.

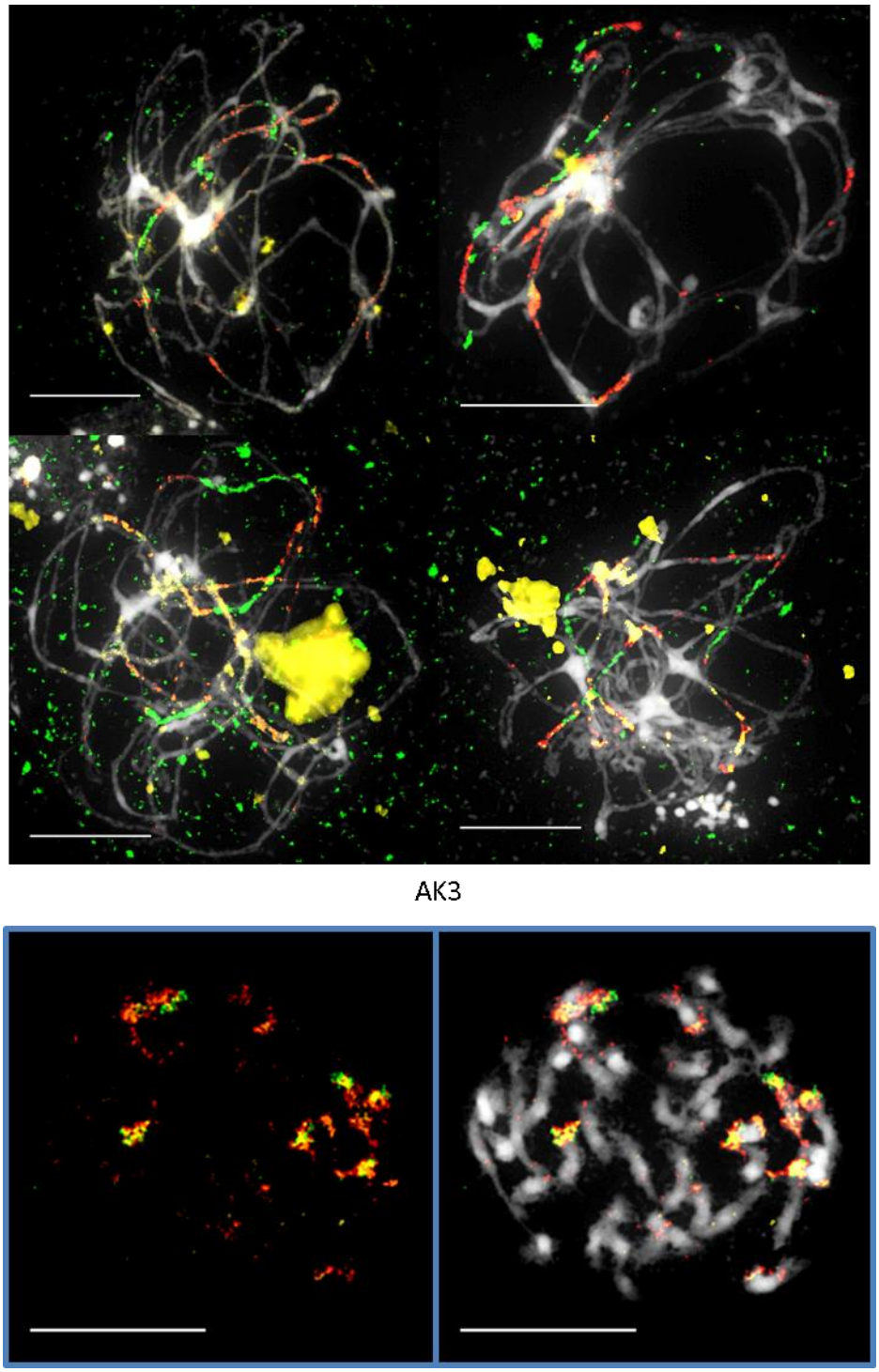


Fig. 51:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK3 probes (upper images).
In the photos with a blue frame, mitotic chromosomes (DAPI + AK3 on the left, only AK3 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts).
Scale bars = 10 µm.

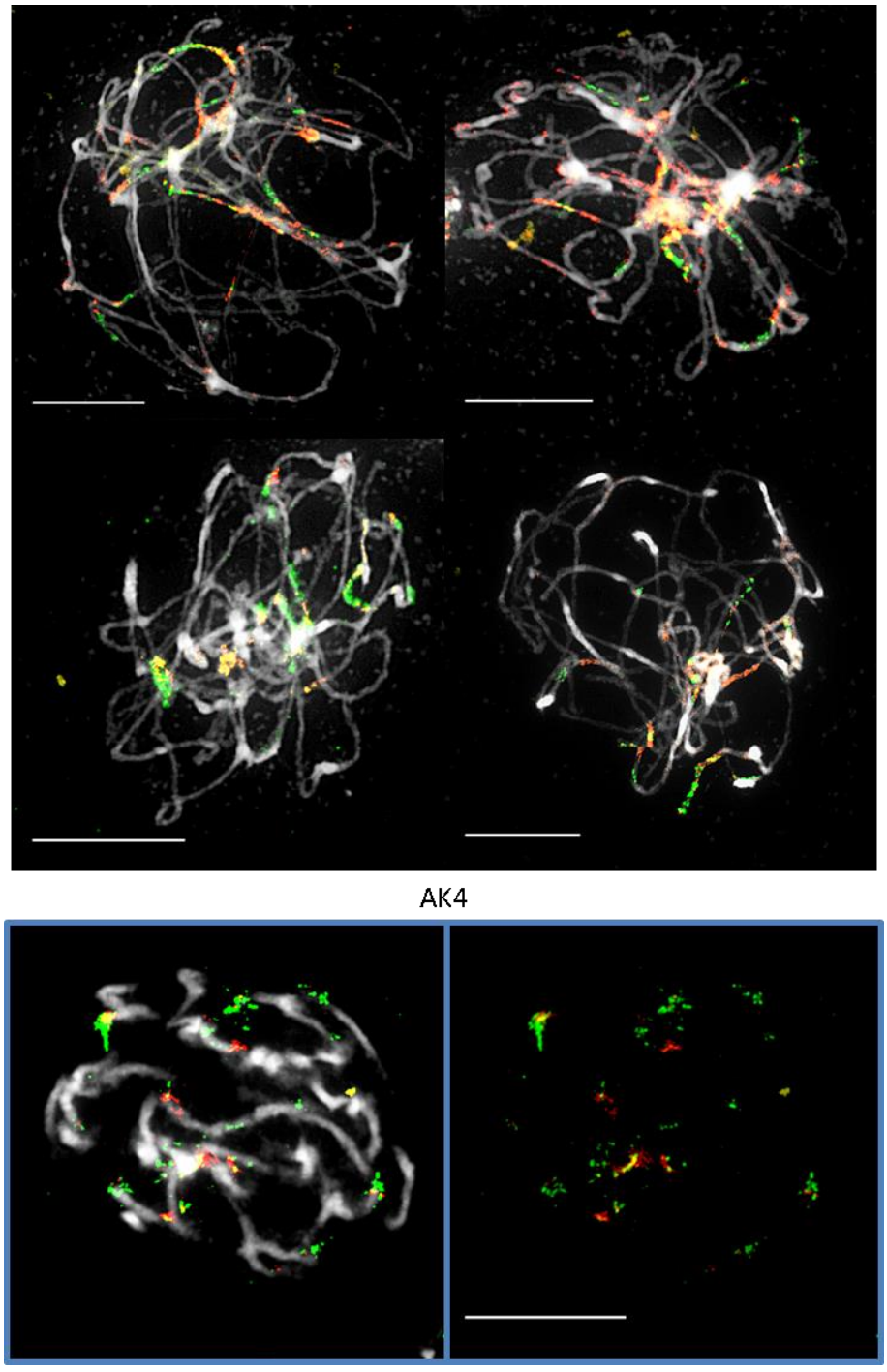
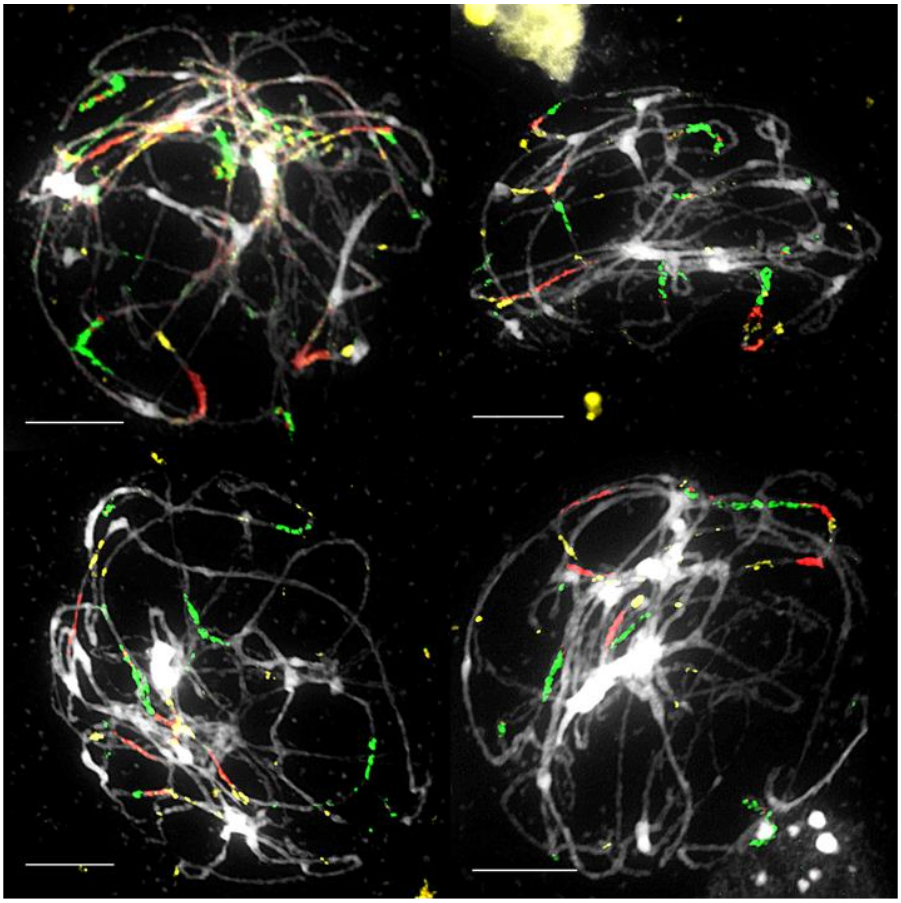


Fig. 52:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK4 probes (upper images).
In the photos with a blue frame, mitotic chromosomes (DAPI + AK4 on the left, only AK4 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts).
Scale bars = 10 µm.



AK5

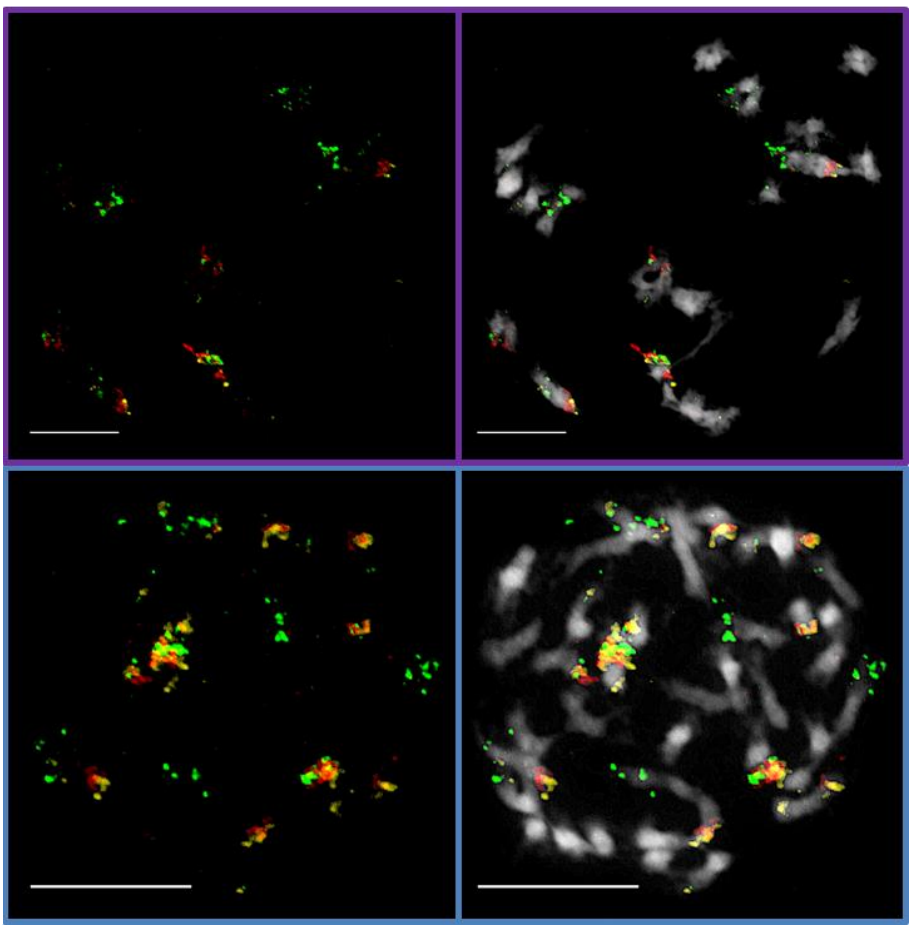
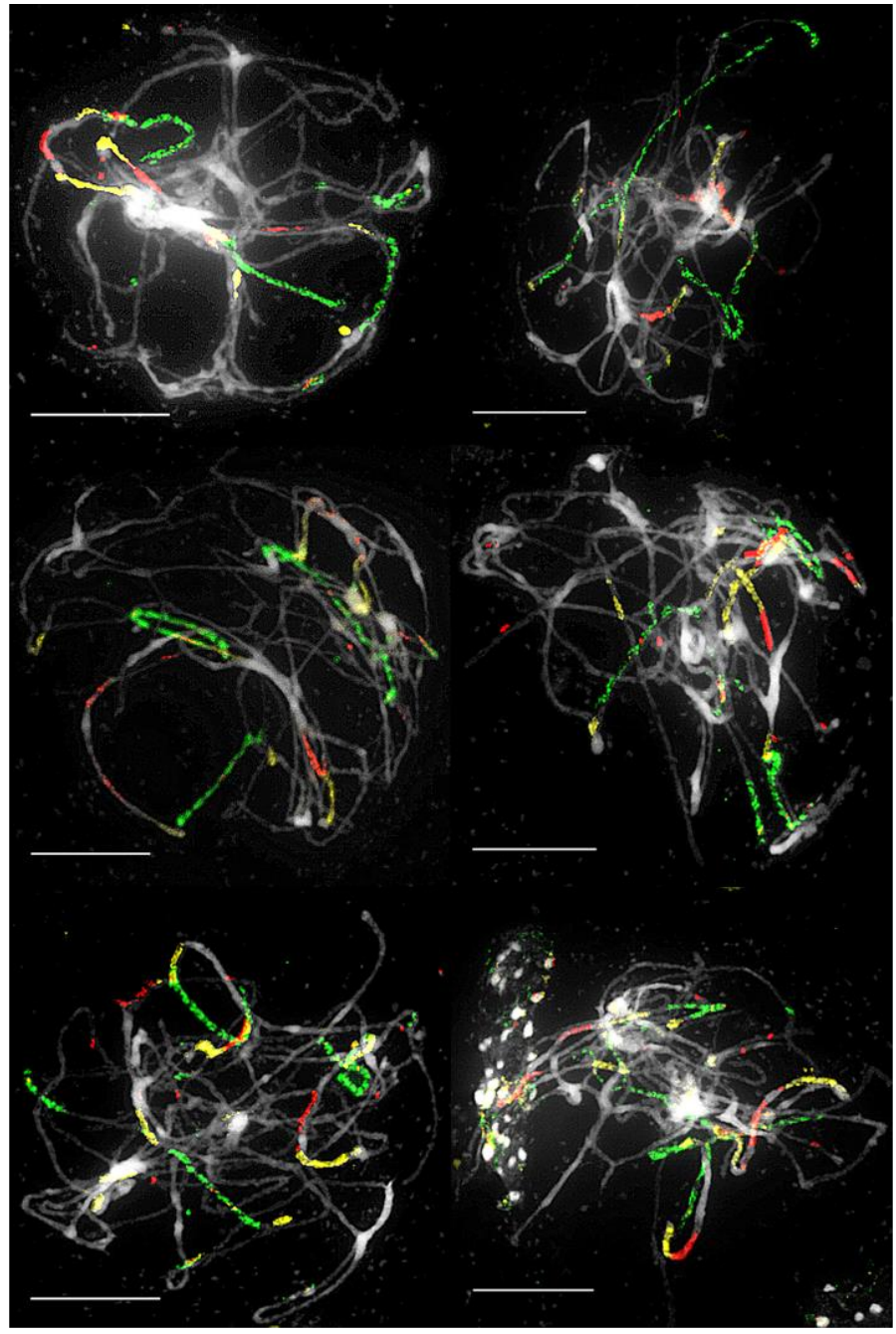


Fig. 53:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK5 probes (upper images). The mitotic chromosomes are shown and in the photos with a blue frame and photos with a violet frame show a diakinesis (DAPI + AK5 on the right, only AK5 on the left). These prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts). Scale bars = 10 µm.



AK6

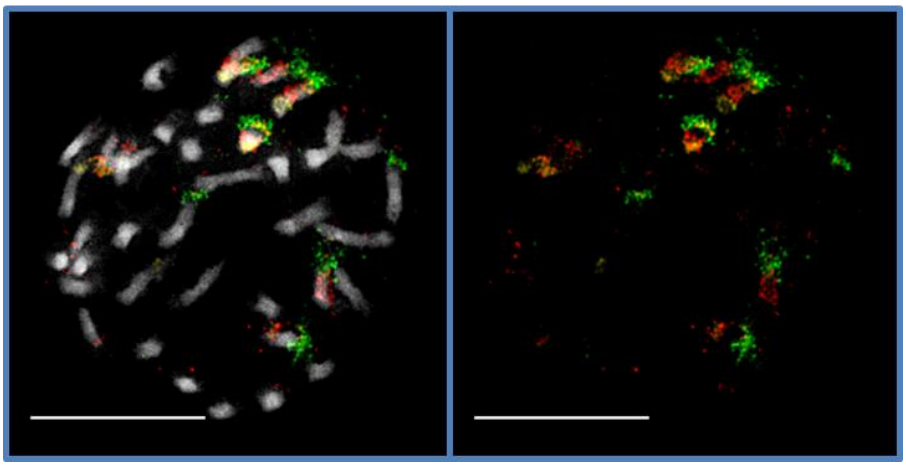


Fig. 54:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK6 probes (upper images).
In the photos with a blue frame, mitotic chromosomes (DAPI + AK6 on the left, only AK6 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts).
Scale bars = 10 µm.

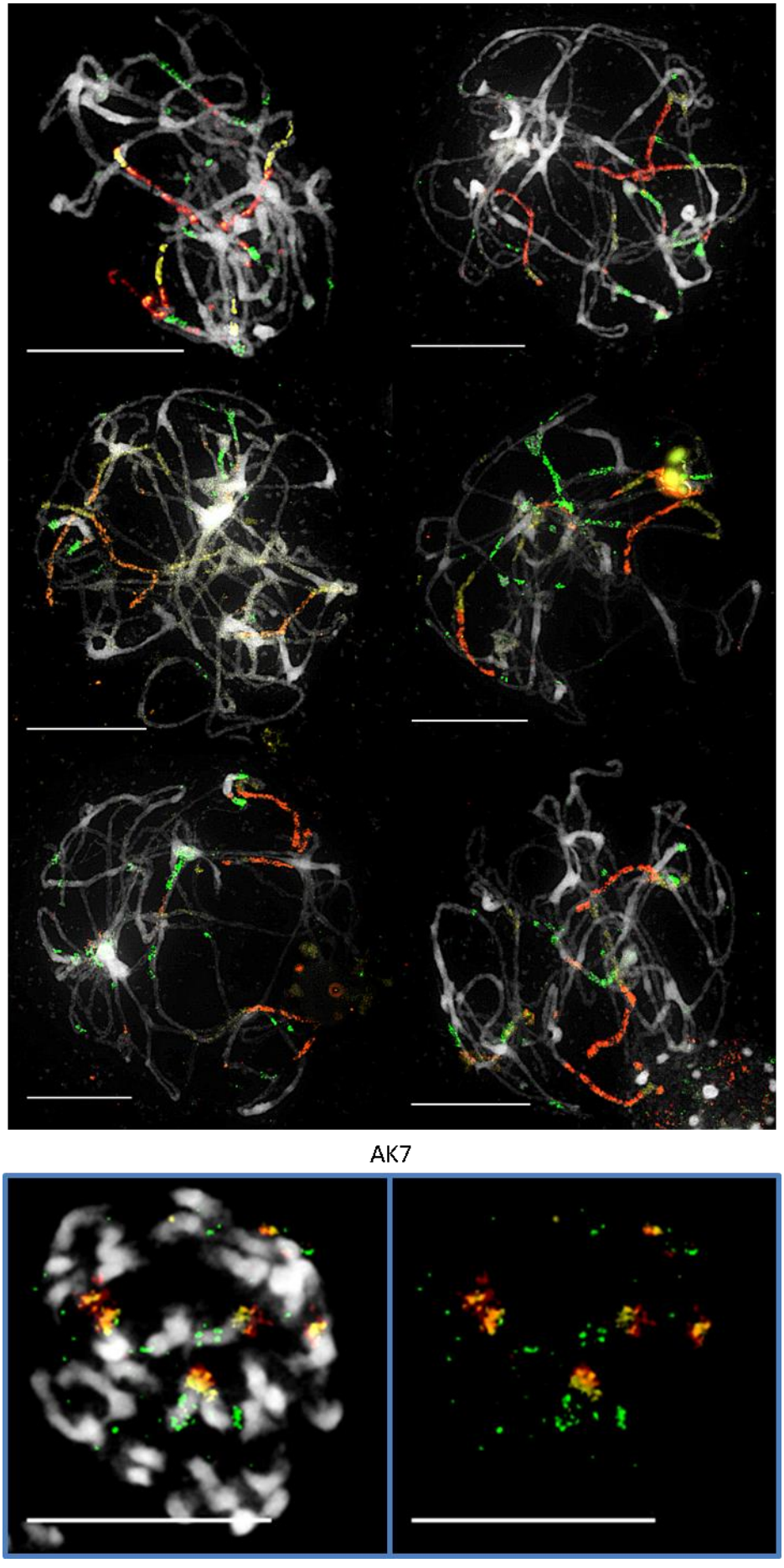


Fig. 55:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK7 probes (upper images).
In the photos with a blue frame, mitotic chromosomes (DAPI + AK7 on the left, only AK7 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts).
Scale bars = 10 µm.

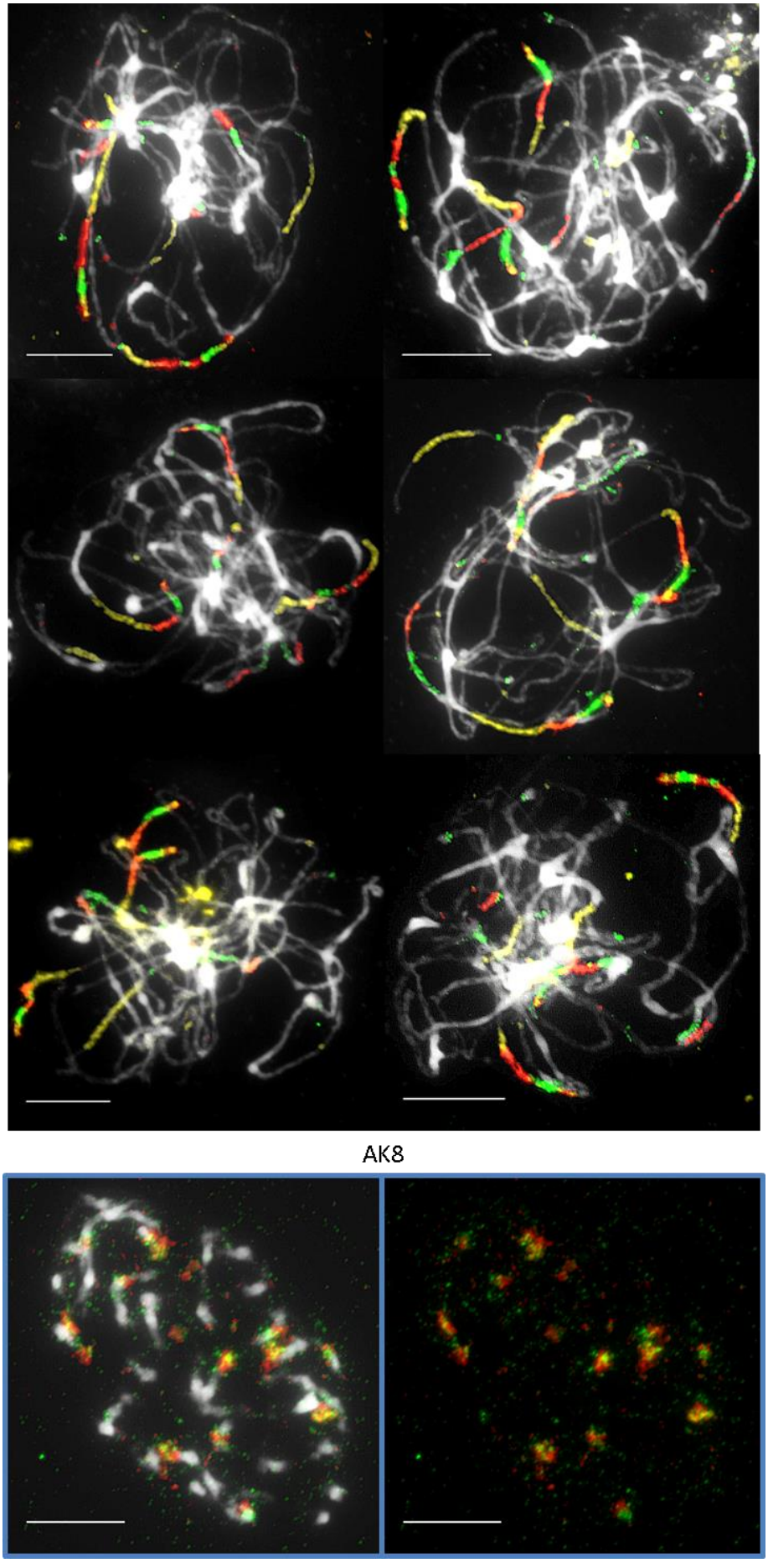


Fig. 56:
Other examples of pachytene chromosomes for *N. apiculata* painted with AK8 probes (upper images). In the photos with a blue frame, mitotic chromosomes (DAPI + AK8 on the left, only AK8 on the right) prove the distribution of the hybridization sites (for each genomic block two regions of the same colour are present, little colour dots can be random artefacts). Scale bars = 10 µm.