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Physical localisation of *in silico* identified tandem repeats in diploid *Ae. umbellulata* and *Ae. comosa* and their tetraploid hybrids by FISH

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3) - Fyzické mapovaní tandemových repetic na U a M genomických chromosomů diploidních a tetraploidních druhů Aegilops metodami FISH a GISH
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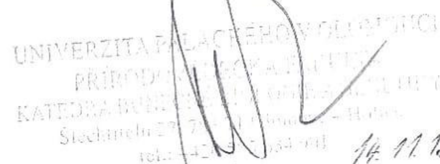

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SUMMARY

This thesis focuses on the genus *Aegilops*, its genetic proximity to wheat and the evolution of wheat and *Aegilops* genomes. Furthermore, it briefly discusses methods of crossing wheat with alien species, alien chromosome identification and probe labelling. Lastly, it describes the process of *in silico* tandem repeat identification, which was the basis for the experimental part.

The experimental part discusses use of newly identified tandem repeats as probes on two diploid species *Ae. umbellulata* and *Ae. comosa*, as well as on an allotetraploid hybrid *Ae. biuncialis* which shares their genome types. On *Ae. biuncialis*, genomic *in situ* hybridisation was also performed to identify the genomic origin of each chromosome. The same experiment has been performed on a *Ae. biuncialis*/*Triticum* amphiploid, however without any publishable results, therefore it is not included in the *Results* section.

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SOUHRN

Tato diplomová práce se zaměřuje na rod *Aegilops*, jeho genetickou příbuznost pšenici a evoluci genomů pšenice a *Aegilops*. Dále stručně popisuje metody křížení pšenice s cizím druhem, metody identifikace nepůvodních chromosomů a značení sond. Na konec popisuje princip identifikace tandemových repetic *in silico*, což sloužilo jako základ pro praktickou část.

Praktická část se zabývá použitím nově identifikovaných randemových repetic jako prób u dvou diploidních druhů *Ae. umbellulata* a *Ae. comosa*, a také allotetraploidního hybrida *Ae. biuncialis*, jež nese stejné typy genomů. Na *Ae. biuncialis* bylá take provedena genomová *in situ* hybridizace na identifikaci genomového původu každého chromosomu. Ten samý experiment byl proveden na ampfiploidním hybridu pšenice a *Ae. biuncialis*, nicméně bez jakýhkoliv publikovatelných výsledků, a není tedy zahrnut v sekci *Výsledky*.

I declare that I wrote this diploma thesis by myself under supervision of Ms.C. István Molnár, Ph.D., using the sources provided at the end of the thesis.

Date: ………………….   Signature: ………………

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LIST OF ABBREVIATIONS

AFLP – amplified fragment length polymorphism

CCD camera – Charge-coupled device camera

CY3 – cyanine 3

DAPI – 4′,6-diamidino-2-phenylindole

DNase – deoxyribonuclease

EDTA – Ethylenediaminetetraacetic acid

FISH – fluorescent *in-situ* hybridisation

FITC – fluorescein isothiocyanate

GISH – genomic *in-situ* hybridisation

SSC – Saline sodium citrate

TAE – Tris-acetate-EDTA

Tris – trisaminomethane

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Tab. 1: Species of the genus Aegilops and their genomes (van Slageren 1994).

Tab. 2. Primer sequences and anealing temperatures (Ta) used for the PCR reactions to amplify the in silico identified tandem repeats from the genomic DNA of Ae. umbellulata.

1 INTRODUCTION

Aegilops (goatgrass), a genus of monocot plants, are closely related to wheat and some *Aegilops* species played sygnificant role in the evolution of tetra- and hexaploid wheat genome. Thanks to its close genetic proximity the species of *Aegilops* are also considered as important gene source for wheat improvement. However, the chromosome mediated gene transfer to wheat would be greatly facilitated by developing molecular tools suitable for the identification of the *Aegilops* chromosomes.

The most popular approach used to identify *Aegilops* chromosomes is the fluorescent in situ hybridisation of DNA repeat probes. Many previously identified repeats, like Afa family and pSc119.2 exhibit signals in telomeric regions making the identification of interstitial chromosome segments difficult. Therefore finding new repeat probes located in intersticial parts of the chromosomes would greatly improve the chromosome identification.

Genome analysis of *Aegilops* can be simplified by flow sorting. The flow sorted chromosomes can be sequenced and the sequence data can be used for many genomic applications, like gene cloning, repeat analysis or marker design. In a previous work, the U- or M-genome chromosomes of diploid *Ae. umbellulata* and *Ae. comosa*, respectively, were flow sorted and sequenced. The *in silico* analysis of chromosomal sequences identified several putative tandem repeats but their distribution along the chromosomes haven't been investigated.

The aim of this thesis was to investigate the ability to use these *in silico* identified tandem repeats as FISH probes. Using fluorescent *in situ* hybridisation, the chromosomal distribution of these repeats was investigated on diploid *Ae. umbellulata* and *Ae. comosa* and on their natural allotetraploid hybrid *Ae. biuncialis*. In order to assign the hybridisation pattern of the new tandem repeats to the chromosomes of *Aegilops* we applied sequential FISH with the new repeats and with standard repeat probes with known karyotype. In case of *Ae. Biuncialis,* I also applied genomic *in situ* hybridisation as a third step hybridisation experiment to discriminate the U and M genomes.

2 MAIN GOALS

- Learn the practical steps of fluorescent and genomic *in situ* hybridisation on plant chromosomes.
- Prepare new FISH probes from *in silico* identified tandem repeats
- Physical mapping of the tandem repeats on U and M mitotic chromosomes of diploid and allotetraploid species using FISH and GISH
- Test the usability of the new probes for *Aegilops* chromosome identification in wheat/*Aegilops* hybrids

3 LITERARY OVERVIEW

3.1 The genus *Aegilops*

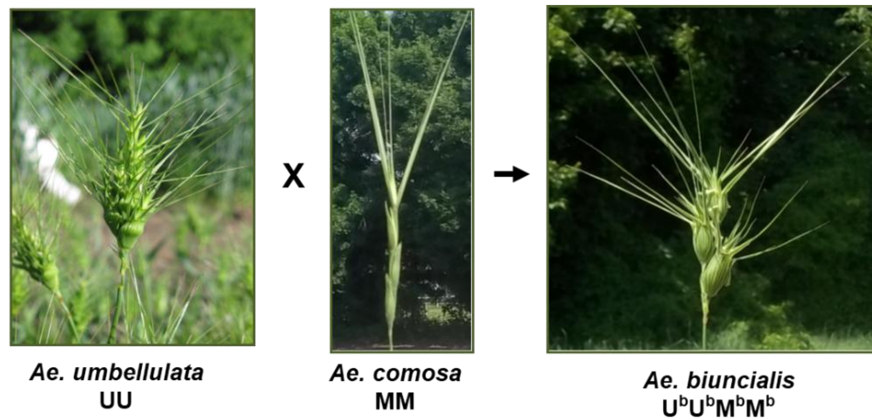
Aegilops (goatgrass) is a genus of annual plants belonging to the tribe *Triticeae* from the grass family *Poaceae*. It is the closest related genus to *Triticum* (wheat) and because of that, *Aegilops* species are widely used as a gene source for wheat improvement. *Aegilops* species grow mainly around the Mediterranean Sea and in Central and Western Asia (van Slageren, 1994). To North America, *Aegilops* has been introduced artificially, with *Ae. cylindrica* being the most widespread.

3.1.1 Taxonomy and genome of the *Aegilops* genus

All of the taxonomy and genetics in this chapter has been described by van Slageren in 1994. The genus *Aegilops* contains five sections: *Aegilops*, *Cylindropyrum*, *Vertebrata*, *Comopyrum* and *Sitopsis*. The first three contain species both diploid and polyploid, while the last two contain only diploid species. There are 11 diploid, 10 allotetraploid and 2 allohexaploid species (Table 1). The base chromosome number is 7.

Based on chromosome pairing during meiosis of inter-specific and inter-generic hybrids, different types of genomes were identified within the *Aegilops* genus: C, D, M, N, S, T and U (Kihara, 1954; Feldman et al., 1979; Kimber and Tsunewaki, 1988). Multiple species can have the same genome type. The polyploid species have got different combinations of two (in case of allotetraploid species) or three (in case of allohexaploid species) of these genome types. The three *Aegilops* species examined in this thesis were *Ae. umbellulata* Zhuk. (2n=2x=14, UU) and *Ae. comosa* Sm. in Sibth. et Sm. (2n=2x=14, MM) and *Aegilops biuncialis* Vis. (2n=4x=28; $U^bU^bM^bM^b$).

Fig. 1: Spikes of Ae. umbellulata, Ae. comosa and their allopolyploid hybrid, Ae. biuncialis. Source: Pictures taken by I. Molnár in a field nursery in Martonvásár, Hungary.



Tab. 1: Species of the genus Aegilops and their genomes (van Slageren 1994).

Species	**Genomic constitution**
Aegilops bicornis (Forssk.) Jaub. & Spach	S^bS^b
Aegilops biuncialis Vis.	UUMM
Aegilops caudata L.	CC
Aegilops columnaris Zhuk.	UUMM
Aegilops comosa Sm. in Sibth. & Sm.	MM
Aegilops crassa Boiss.	DDMM
Aegilops cylindrica Host.	DDCC
Aegilops geniculata Roth.	UUMM
Aegilops juvenalis (Theil.) Eig	DDMMUU
Aegilops kotschyi Boiss.	UUSS
Aegilops longissima Schweinf. & Musch.	SS
Amblyopyrum muticum (Boiss.) Eig	TT
Aegilops neglecta Req. ex Bertol	UUMM
Aegilops neglecta var. recta	UUMMNN
Aegilops peregrina (Hack. in J.Fraser)	UUSS
Aegilops searsii Feldman & Kislev ex Hammer	SS
Aegilops sharonensis Eig	SS
Aegilops speltoides Tausch	SS
Aegilops tauschii Coss.	DD
Aegilops triuncialis L.	UUCC
Aegilops umbellulata Zhuk.	UU
Aegilops uniaristata Vis.	NN
Aegilops vavilovii (Zhuk.) Chennav.	DDMMSS
Aegilops ventricosa Tausch	DDNN

3.1.2 Evolution of the *Aegilops/Triticum* genus

The studies of van Slageren (1994) have shown that based on different morphological markers, *Triticum* and *Aegilops* are two different genera. Studies of Sakamura (1918), Sax and Sax (1924) and Kihara (1924) have discovered that wheat species show different levels of ploidy. There are diploid species, called einkorn wheats; allotetraploid species, called emmer wheats; and allohexaploid species, called bread wheats. The base chromosome number is 7. These studies also led to the discovery of different genome types, namely A, B, C, D, G, M, N, S, T and U. 7 of these 10 genome types are also present in *Aegilops* species, as described in the section above. According to the recent classification based on different morphological markers van Slageren (1994) devided *Triticum* and *Aegilops* into two different genera.

Huang et al. (2002) attribute the origin of the A genome to wild diploid *Triticum urartu* (AA) and the B genome to *Aegilops speltoides* (SS). However, *Ae. speltoides* is nowadays believed to have the S genome and also having been the origin of the S genome, so the origin of the B genome is still a subject of debate. To shed some more light into this problem, Killian et al. (2007) did a series of AFLP analyses on different loci of the S-genome *Aegilops* species, comparing these loci to those found in *Triticum aestivum* lines (AABB and AAGG tetraploid lines). B-genome specific markers helped trace the origin of the B genome to the S-genome chromosomes of *Ae. speltoides*. The origin of the G genome was discovered in a similar way as the B genome and it likewise showed a strong similarity to the S genome of *Ae. speltoides*. It can be concluded that the S genome is evolutionary younger than the B and G genomes. C genome was discovered to be very closely related to the A genome and as such, being derived from it (Wang et al., 2011). D genome is believed to originate from *Ae. tauschii* (DD) (Cox, 1998). The M and N genomes seem to be closely related to the D genome (Wang et al. 1997). Tanaka (1985) believes, that the species *Ae. comosa* (MM) and *Ae. uniaristata* (NN) had either a common ancestor or that one derived from the other. The T genome has its origin in *Ae. mutica* (Hyunh et al., 2019). The progenitor of the U genome was discovered to be *Ae. umbellulata* (UU) (Badaeva et al., 2004).

Based on morphological traits that Eig (1929) defined as primitive, *Ae. mutica*, *Ae. speltoides*, *T. monococcum* and *T. urartu* are believed to be the ancestral species in the diploid group of the *Aegilops* genus and *Ae. umbellulata* and *Ae. uniaristata* to be the youngest.

Additionally, Senyaninova-Korchagina (1932) and Chennaveeraiah (1960) discovered that certain diploid species, namely those with A, D, S and T genomes, have got only metacentric or submetacentric chromosomes, while species with genomes C, M, N and U have got only subtelocentric chromosomes. Stebbins (1950) proposes, that chromosomal arm-length asymmetry means a species with this trait is evolutionary younger than a species with chromosomes that exhibit arm-length symmetry.

Spontaneous chromosome doubling, and therefore allopolyploidisation, was discovered to be one of the mechanisms of speciation in the *Triticeae* group (von Tschermak and Bleier, 1926). Allopolyploidisation is an instant step that takes effect from one generation to another and as such, the offspring is genetically very distant from its two parents. Doubling of the genetic dose causes problems and has to be overcome. Many allopolyploid hybrids undergo a series of epigenetic changes, and deletion of low-copy sequences. These sequences are eliminated from one of the two genomes in case of tetraploids and from two of the three genomes in hexaploids (Ozkan et al., 2001). The eliminations are undertaken only in the first generation and the changes are inherited by all successive generations. These changes are done in order to reduce the genome size and to prevent pairing between homologous chromosomes of closely related genomes (Morris and Sears, 1967).

3.1.3 Wheat gene pools

In breeding point of view, Friebe (1996) has divided wild relatives into primary, secondary and tertiary gene pools of hexaploid wheat (2n=6x=42, AABBDD) based on their genomic constitution. The primary gene pool consists of species with genomes homologous to *T. aestivum*. *Ae. tauschii* (DD) and *T. urartu* (A^uA^u), belongs to this pool along with other species containing A, D or B genomes such as *T. aestivum ssp. spelta* (BBAADD) or *T. turgidum ssp. durum* (BBAA). Crossing of primary gene pool species is easily done.

The secondary gene pool consists of species with at least one, but not all, genomes homologous to *T. aestivum,* such as *T. timopheevii* (GGA^tA^t), *T. zhukovsky* ($GGA^tA^tA^mA^m$). Intercrossing of these species is possible, but recombination occurs only between the chromosomes of homologous genomes. Many of the *Aegilops* species with S genomes belong to the secondary gene pool (*Ae. speltoides*, *Ae. longissima*, *Ae. sharonensis* etc.).

Within the tertiary gene pool, the species of *Secale*, *Hordeum*, *Agropyron*, *Thynopyrum* and *Aegilops* (like *Ae. umbellulata*, *Ae. comosa* and *Ae. biuncialis*) are genetically most distant from wheat and their chromosomes are not homologous to those of wheat. Crossing of these species with *T. aestivum* is only possible after employing special cytogenetic techniques. Genetic distance has got one advantage however, which is higher probability of new gene introduction. Rye (*Secale cereale*) belongs to the tertiary gene pool and is one of the most widely used species in wheat improvement (Kaur, 2018).

3.1.4 Importance of *Aegilops* species in cereal breeding

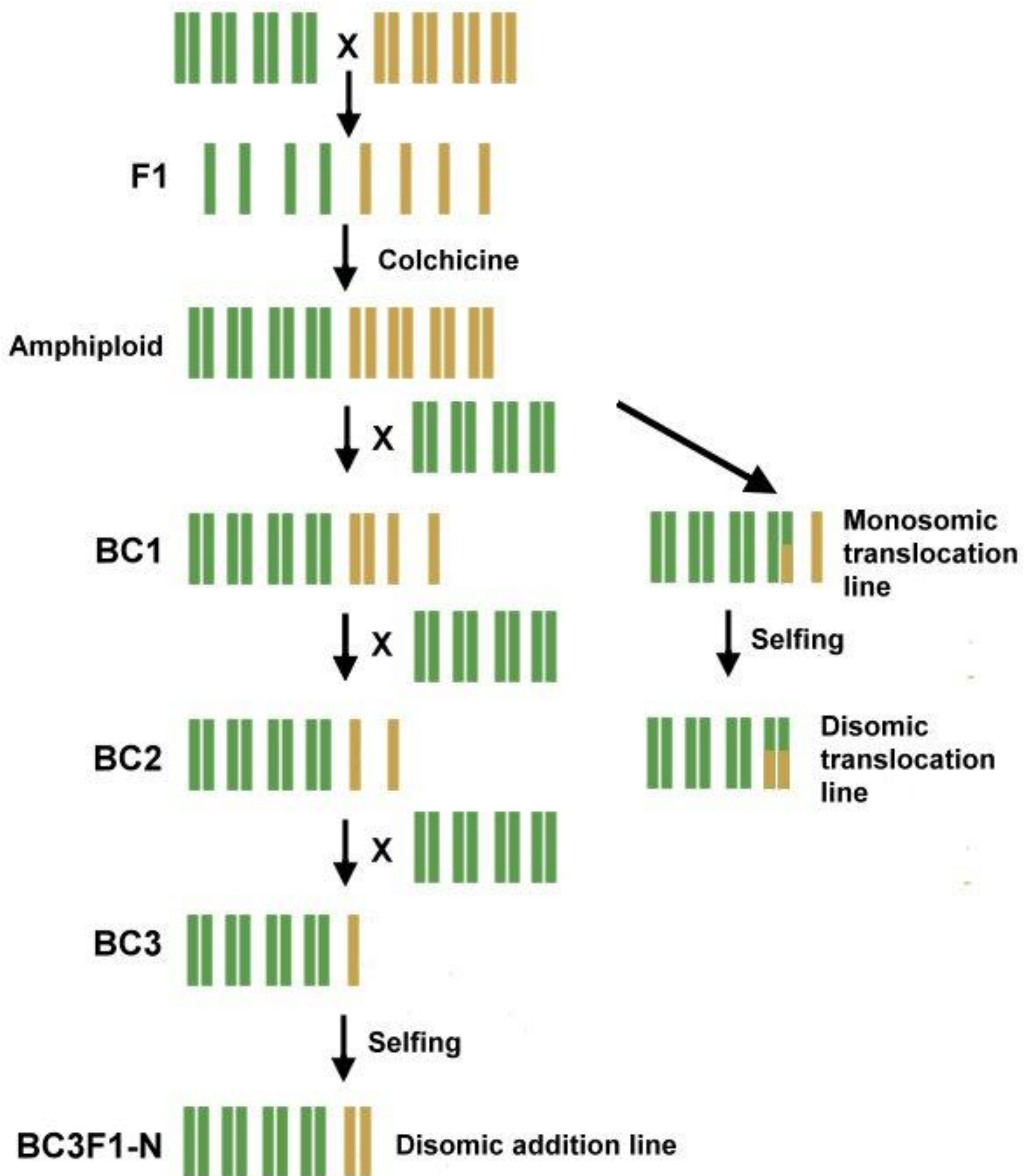
Feeding the world in 2050 and ensuring global food security will require high-yielding, stress tolerant wheat cultivars with improved nutritional quality. Efforst to breed such cultivars are compromised by narrow genetic diversity of the current wheat cultivars. On the other hand, *Aegilops* species contain a considerable variability in biotic and abiotic stress tolerance and nutritional quality and can be used to improve wheat by sexual crossing (Schneider et al. 2008). The production of wheat- *Aegilops* chromosome addition and translocation lines by interspecific hybridisation is a suitable tool to utilize genetic diversity of *Aegilops*.

Allotetraploid *Ae. biuncialis* Vis. with $U^bU^bM^bM^b$ genome (2n=4x=28) has a large ecological adaptation ability and, together with its diploid ancestors *Ae. comosa* Sm. in Sibth. & Sm. (2n=2x=14, MM genome) and *Ae. umbellulata* Zhuk. (2n=2x=14, UU genome), they are attractive sources of agronomically important genes. To date, several biotic stress resistance genes (*Lr9, Lr57, Sr34, Yr8, Yr40, Pm29*) have been transferred from the *Aegilops* species to wheat. Among them, *Lr9* is one the most effective genes providing resistance to leaf rust (Sears 1956). Several accessions of *Ae. biuncialis*, have been considered as gene sources to improve drought tolerance of wheat (Molnár et al. 2004; Dulai et al. 2014), while others are good sources to increase the grain dietary fibre- (β-glucan and arabinoxylans) and micronutrient (K, Zn, Fe, and Mn) content (Farkas et al. 2014; Rakszegi et al. 2017).

3.2 Main steps of chromosome mediated gene transfer in wheat

3.2.1 Crossing of wheat with alien species

Fig. 2: A diagram showing the process of crossing wheat (green) with Aegilops (yellow) and resulting lines. BC = Backcross generation



The first step in the chromosome mediated gene transfer from wild relatives into wheat is the production of F_1 hybrids. The ability to produce F1 seeds depends on the parental wheat genotypes. It is reported that crossability is a quantitative trait and controlled by several genes (Molnár-Láng), two dominant genes *Kr1* (5BL) and *Kr2* (5AL) are the major genes. According to Lein's classification (Lein, 1943) wheat genotypes with *Kr1Kr2* exhibit >10% crossability with rye, *Kr1kr2* showed between 10 and 25% crossability, *kr1Kr2* between 25 and 50% and plants with the *kr1kr2* genotype more than 50% crossability. East Asian wheat genotypes like Chinese Spring, generally have higher crossability rate with rye as they has kr1kr1kr2kr2 genotype. However, these genotypes usually showed bad agronomic performance. The transfer of recessive crossability alleles from Chinese Spring into a winter wheat genotype Mv9 resulted in a wheat line Mv9kr1 with good agronomic traits (Molnár-Láng et al. 1996) that was used in several introgression breeding programmes (Molnár-Láng 2015).

The F_1 hybrids can be subsequently backcrossed with the bread wheat parent, in order to reduce the number of alien chromosomes and produce wheat-alien addition, substitution and translocation lines. This method was used by Gill and Raupp (1987) to successfully introduce genes for resistance to Hessian fly and rust into the wheat genome.

3.2.2 Genome doubling

F1 hybrids are usually sterile due to their haploid genome composition. One option to restore the fertility is the chromosome doubling using colchicine and the production of wheat-alien amphiploids. The wheat-alien amphiploids contain the parental genomes in two copynumber thus they are partially fertile.

Colchicine, first discovered in the 1930s (Marzougui et al., 2011), is an inhibitor of microtubule formation, which prevents chromosome segregation during meiosis, which then causes polyploidisation. Eng (2018) has discovered that higher concentrations have to be used because colchicine exhibits lower affinity to plant tubulin.

Colchicine is however highly toxic, therefore using a lower concentration but prolonging the exposure usually yields the best results (Sajjad, 2013). This treatment is used on pre-germinated seeds. If used on older plants, there is a chance of producing cytochimeras (Manzoor, 2019). The mechanism of colchicine genome doubling was further examined by Zhou in 2017 and he discovered, that colchicine also operates on transcription level, inhibiting the expression of genes for spindle formation and attachment and fragmoplast formation.

3.2.3 Backcrossing

The wheat as an allohexaploid species tolerate well the presence of extra chromosomes. Because the amphiploids have only partial fertility and several undesired traits, it is needed to reduce the alien chromosome number by recurrent backcrossing with the parental wheat genotype until only one alien homologous chromosome pair will be present. The importance of these sets of disomic addition lines is that the fertility is much higher and the effect of alien chromosomes on wheat can be studied.

By the use of addition lines, several traits, like disease resistance, quality traits, abiotic stress tolerance etc. can be assigned to alien chromosomes. The addition lines with desirable agronomic traits can be used for the production of wheat-alien translocations. The selection of disomic addition lines recure at least three backcrossing and selfing.

Over the past decades, wheat-*Ae. umbellulata* disomic 1U, 2U, 5U, 6U, 7U and monosomic 3U 4U additions and wheat- *Ae. comosa* 2M-7M disomic addition lines have been developed (Kimber 1967, Friebe et al. 1995, Liu et al. 2019). The potential of *Ae. biuncialis* remains underutilized in wheat improvement and out of the 14 possible wheat-*Ae. biuncialis* addition lines, only seven have been produced (Schneider et al. 2005). The investigations revealed that the chromosomes $1U^b$, $3U^b$ and $3M^b$ of *Ae. biuncialis* have positive effect on wheat drought tolerance, chromosome $7M^b$ improves edible fiber content and chromosomes $3M^b$ and $7M^b$ contain loci for increased grain micronutrient (Fe, Mn, Zn) content (Farkas et al. 2014). However, it would be desirable to develop further wheat-*Aegilops* disomic additionlines (together with substitution and translocation lines).

3.2.4 Production of wheat-alien translocations

The main goal of the interspecific hybridisation is to transfer only the alien genomic region responsible for the useful agronomic traits without any negative effect. In order to reach this theoretical goal, a further reduction of the alien chromosomes is needed via the production of wheat-alien translocation lines. Wheat-alien translocations can be formed spontaneously during the backcrossing steps or it is possible to actively stimulate chromosome rearrangements.

It is desired if the loss of wheat chromatin (genes) can be functionally compensated by the alien chromosome segment. This compensating wheat-alien translocations can be obtained through the induced homoeologous chromosome pairing during the meiosis resulting in homoeologous recombinations. In wheat only the homologous chromosomes can pair during the meiosis due to the strict genetic control where the major locus is the *Ph1* on the long arm of chromosome 5B (Sears 1976). However in the absence of *Ph1* homoeologous chromosomes can also pair as it was observed in ph1b mutant lines carrying a 70Mb deletion on the Ph1 locus (Sears 1977).

The crossing this Chinese Spring *ph1b* mutant lines with wheat-alien addition lines may results in compensating homoeologus translocations as it was reported for: rye (Lukaszewski 2000), barley (Islam et al. 1992) and *Aegilops* (Zhang et al. 2015).

If the wheat and alien chromosomes doesn't pair even in the absence of *Ph1*, other approaches can be used to induce random chromosome rearrangements such as ionizing irradiation (most frequently ^{60}Co γ-ray) (Molnár et al. 2009), chemical treatment (Friebe 1994) or tissue cultre (Molnár-Láng et al. 2000).

3.3 Methods of alien chromosome identification

The efficient selection of wheat-alien addition, substitution and translocation lines requires the detection and identification of alien chromatine in the wheat genetic background in every backcrossed and selfed generation. Several approaches can be used to identify alien chromosomes such as morphological characters, molecular markers and cytogenetic methods.

As the present thesis focuses to the improvement of molecular cytogenetic methods to identify the *Aegilops* chromosomes, only the main cytogenetic methods have been discussed in the following pages.

3.3.1 Chromosome banding

Some of the older methods for chromosome identification include chromosome banding. There are multiple banding types, namely G-banding, Q-banding, C- banding and R-banding. G-banding uses Giemsa stain (Giemsa, 1904) to dye condensed gene-poor regions (AT-rich) with a darker stain than gene-rich less condensed regions (GC-rich). R-banding works similarly to G-banding, but the colour profile is reversed. Q-banding works in the same way as G-banding, but uses quinacrine instead of Geimsa stain and a fluorescent microscope is necessary to observe the results. C-banding uses Barium Hydroxide and is used mainly to observe constitutive heterochromatin.

Each chromosome banding method provides a characteristic pattern of bands on each chromosome. These bands can be used to identify individual chromosomes within the karyotype and also individual segments on the chromosomes, including aberrations. Banding methods are highly reproducible and consistent.

3.3.2 Fluorescent *in situ* hybridisation

A modern way of chromosome identification in plants is *in situ* hybridisation (ISH), developed 40 years ago (Gall and Pardue 1969) have been extensively used to study the structure, function, organisation, and evolution of the genome. *In situ* hybridisation allows the location of a DNA sequence to be determined by hybridisation of a labelled DNA probe to a DNA target in a microscope preparation – chromosomes, nuclei or DNA fibres (Schwarzacher and Heslop-Harrison 2000).

Fluorescence in situ hybridisation (FISH) uses fluorochromes for signal detection (Langer-Safer et al. 1982). The advantage of FISH is different DNA probes can be labelled with different haptens and simultaneously detected using different fluorochromes (multicolour FISH). Moreover, fluorescence signals can be captured by fluorescence microscope equipped with a CCD camera and analysed with digital imaging systems, allowing more precise mapping.

Fluorescent *in situ* hybridisation (FISH) with repetitive DNA probes results in chromosome specific hybridisation pattern allowing the identification of the individual chromosomes in the species of *Triticeae* (Mukai et al. 1993a, Rayburn and Gill 1985, 1986). Moreover, in the multicolour procedure, several differentially labelled probes can be used simultaneously allowing the more accurate identification of the chromosomes.

Genomic *in situ* hybridisation (GISH), another type of the ISH technique (Schwarzacher et al. 1989) use total genomic DNA from one of the parental species as a probe and unlabelled total DNA of the other parent apply as a block. Alternatively, total DNA from both parents can be differentially labelled and used as probes, each one detected with a different fluorochrome (Sepsi et al. 2008). GISH enables the discrimination of parental genomes in allopolyploid species such as in bread wheat (Mukai et al. 1993b), and it has also been used extensively to detect alien chromatin in a wheat background as well as for detection of irradiation induced intergenomic chromosome rearrangements (Mukai et al. 1993b, Molnár et al. 2009).

In order to facilitate the introgression of *Ae. biuncialis* chromosomes into the hexaploid wheat genome, the fluorescence *in situ* hybridisation (FISH) pattern of *Ae. biuncialis* chromosomes was compared with the diploid progenitors *Ae. umbellulata* and *Ae. comosa*, using repetitive DNA probes (pSc119.2, pAs1/Afa family, pTa71, $(GAA)_n$ and $(ACG)_n$) and multicolour GISH (Molnár et al. 2011ab).

However, new probes providing alternative hybridisation patterns would be needed for the precise identification of small *Aegilops* chromosome segments in the wheat genetic background.

3.4 Probe labelling

In order to provide an observable signal under a fluorescent microscope, the probe has to be labelled. The labelling can be indirect or direct.

In case of indirect labelling, the probe is first labelled with a non-fluorescent molecule, to which a fluorescent ligand is attached in a subsequent detection reaction. A commonly used indirect label is biotin, whose high affinity to avidin conjugated with a fluorophore can be easily exploited (Langer, 1981).

Other widely used labels are digoxygenin and FITC. FITC has got a high affinity for its antibody but can also be directly conjugated with a nucleotide for direct labelling methods. Direct labelling methods utilise nucleotides conjugated to fluorophores. An advantage of direct labelling methods over the indirect is that no detection reaction, since the probes fluoresce by themselves.

Nick translation is best used for total genomic DNA. Two enzymes are at work during nick translation: DNase I and DNA polymerase I. The DNase makes single-strand breaks (nicks) in the target DNA. The polymerase then attaches itself to the DNA at the nick site and excises nucleotides in the 5'-3' direction and adds fluorescently labelled nucleotides according to the template strand.

For shorter fragments, random primer labelling is used. In this method, Klenow fragment is used to amplify a single-stranded DNA molecule, using a mixture of random commercially available oligonucleotides (usually hexanucleotides). The hexanucleotides bind to the DNA at random positions and serve as primers in the 3'-5' direction. All possible sequence combinations should be theoretically covered by the hexanucleotides. The new DNA strand is then synthesised by the Klenow fragment using a mixture of labelled and non-labelled nucleotides.

PCR labelling can be used to label specific fragments of DNA using primers designed to anneal to specific sequences.

The procedure follows a standard PCR reaction, with the same steps and reagents (denaturation, annealing, elongation). Usually, the reaction is twofold, with the first using only non-labelled nucleotide and labelled in the second reaction. That way, there is more template to label.

3.5 *In silico* tandem repeat identification

Flow cytometric chromosome sorting represents an effective approach for analyzing molecular organisation of chromosomes in cereals including the wild relatives of wheat and for development molecular tools to support alien gene transfer in wheat improvement programmes (Doležel et al. 2012).

The most up-to-date chromosome sorting method is based on bivariate flow karyotyping of DAPI stained chromosome suspensions labelled with GAA-FITC oligonucleotide probes by FISH in suspension (FISHIS) (Giorgi et al. 2013).

The U and M genomes of *Aegilops* has characteristic GAA clusters allowing flow-sorting the whole chromosome complement from *Ae. umbellulata* and *Ae. comosa* at 72-99% purity (Molnár et al. 2016). The flow sorted chromosomes were shot gun sequenced by Illumina and the short reads were assembled resulting in a mean assembly coverage of x0.45 and x0.66 per chromosome in *Ae. umbellulata* and *Ae. comosa*, respectively (personal communication).

Hřibová has randomly selected 500 000 read data sets for each chromosome and reconstructed several putative repetitive DNA elements. The repeats were identified by RepeatMasker using data of RepeatExplorer database containing protein-coding domains of retroelements (Novák et al., 2010). Further searches were done by BLAST using GenBank database nucleotide data and database of repeats specific to 5M chromosome of *Ae. geniculata* (Tiwari et al., 2015). It was these specific repeats that were used in the experimental part of this thesis.

4 MATERIALS AND METHODS

4.1 Plant material

The accessions *Aegilops umbelullata* AE740/03, *Ae. comosa* MvGB1039, *Ae. biuncialis* MvGB382 and *Aegilops biuncialis*/*Triticum* amphiploid Mv9kr1 are maintained in the Cereal Genebank of the Agricultural Institute, ATK (Martonvásár, Hungary) and their seeds were provided for the present study.

4.2 Chemicals

Agarose (Sigma-Aldrich, USA)
Acetic acid (Lach-ner, Czech Republic)
DAPI-vectashield (Vector Chemicals, USA)
Dextran sulphate (Vector Chemicals, USA)
EDTA (Lach-ner, Czech Republic)
Ethanol (Lach-ner, Czech Republic)
Formaldehyde (Sigma-Aldrich, USA)
Formamide (Sigma-Aldrich, USA)
Methanol (Sigma-Aldrich, USA)
Potassium chloride (Sigma-Aldrich, USA)
Tris (Roche Applied Science, Germany)

4.3 Enzymes, buffers a molecular biology components

Taq DNA polymerase (New England Biolabs, USA)
dNTPs (Sigma-Aldrich, USA)
biotin-dNTPs (Roche Applied Science, Germany)
digoxygenin-dNTPs (Roche Applied Science, Germany)
PCR buffer 5× (New England Biolabs, USA)
TAE buffer (Thermo Fisher Scientific, USA)
Anti-dig FITC (Roche Applied Science, Germany)
Cellulase Onozuka R-10 (Duchefa Biochemie, Netherlands)
KCl buffer: 75 mmol/l KCl, 7,5 mmol/l EDTA pH 4,0 (Thermo Fisher Scientific, USA)
Pectolyase Y-23 (Duchefa Biochemie, Netherlands)
Salmon sperm DNA: 10 mg/ml (Roche Applied Science, Germany)

SSC buffer (Thermo Fisher Scientific, USA)

Streptavidin-CY3 (Roche Applied Science, Germany)

TE buffer pH 7,6 (Thermo Fisher Scientific, USA)

4.4 Laboratory equipments

Waterbath WNB10 (Memmert, Germany)

Heating and drying table 12801 (Medax, Germany)

Accublock digital dry bath D1200 (Labnet International, USA)

Slidemoat slide hybridizer 240000 (Boekel Scientific, USA)

Primo Star educational microscope (Carl Zeiss, Germany)

Microscope Axio Imager Z2 (Carl Zeiss, Germany)

X-Cite XCT10A fluorescent illumination (EXFO, Canada)

Eplax 232 power supply (Carl Zeiss, Germany)

Peltier thermal cycler PTC-200 (Bio-Rad Laboratories, USA)

Hoefer PS-200HC power supply (Fisher Scientific, USA)

InGenius LHR gel documentation system (Syngene, UK)

4.5 Solutions

4 % cellulose + 1 % pectolyase: 0,2 g of cellulose Onozuka R-10 and 0,05 g of pectolyase Y-23 in total volume of 5 ml of 1× KCl buffer. Stored in 20 µl aliquots in -20 °C.

PCR mix for new repeats: 4 µl of 5× PCR buffer, 2 µl of 10 mmol/l non-labelled dNTPs, 0,2 µl of 100 µmol/l forward and reverse primers each, 10 ng of template DNA, 0,4 µl of Taq DNA polymerase, dH_2O to reach total volume of 20 µl.

PCR mix standard repeats: 10 µl of 5× PCR buffer, 1 µl of 10 mmol/l non-labelled dNTPs, 0,5 µl of 25 mmol/l forward and reverse primers each, 20 ng of template DNA, 1 µl of Taq DNA polymerase, dH_2O to reach total volume of 50 µl.

5× NICK translation mix: 50 % glycerol, DNA polymerase I and DNase I (part of a commercial kit: Roche Applied Science, Germany)

NICK-labelling mix: 4 µl of 25 mmol/l biotin-dNTPs or digoxygenin-dNTPs, 1 µg of template DNA (in dH_2O, total volume 12 µl) and 4 µl of 5× NICK translation mix

Hybridisation solution: 15 µl of 100 % formamide, 3 µl of 20× SSC, 0,6 µl of salmon sperm DNA, 0,3 µl of a corresponding probe and 11,5 µl of dextran sulphate. Total volume of the solution for one reaction was 30 µl.

Detection solution: 59,2 µl of blocking reagent, 0,4 µl of anti-dig FITC and 0,4 µl of streptavidin CY3. Total volume for one reaction was 60 µl.

Blocking reagent (Roche Applied Science, Germany)

Immersion oil (Carl Zeiss, Germany)

4.6 Procedures

4.6.1 Probe amplification and labelling

The probe preparation consisted of twofold PCR amplification with subsequent labelling by NICK translation.

4.6.1.1 Probes for newly identified tandem repeats

The newly identified tandem repeats were amplified in two consequtive PCR using the total genomic DNA of *Ae. umbellulata* as template for the first amplification reaction. The primer sequences and anealing temperatures of each tandem repeats are summarised in Table 2.

Tab. 2. Primer sequences and anealing temperatures (Ta) used for the PCR reactions to amplify the in silico identified tandem repeats from the genomic DNA of Ae. umbellulata.

Primer name	5‘-3‘ sequence	Ta (°C)
AUMBCL174C22 F	TGGAGGGCAAAGGAAAATAA	56
AUMBCL174C22 R	GCTTATCAATTGTTCGCTCCA	56
5MCL199C6 F	TTGAATAGCCGCGAGACC	58
5MCL199C6 R	CCTCTTTTCCCCTTTTGTCC	58
AUMBCL107 F	CCGTTTTGTACACGAAGTGC	58
AUMBCL107 R	AAGGTTGAAAGTTGGCATGG	58
AUMBCL179C37 F	GAGGCAGAATCCGCAATAAC	58
AUMBCL179C37 R	CGGAGCGCATTTACGTGT	58
6UCL273 F	TCAGTGAAACGTGACCGAAG	58
6UCL273 R	AAACTCGCAGTTTTGGTTGG	58
6UCL298 F	TCATCAAATGTGGCCTACCA	56
6UCL298 R	GCAACATTGTTTGCCATCAC	56
1UBICL18C52 F	AGGGCACCCTTTTAATTTGG	58
1UBICL18C52 R	CAGATGCTCCGTTTACGTTG	58
1UBICL20C353 F	GCACCCAAGGACACAGATTT	58
1UBICL20C353 R	AGCATGATGGTTTCGGTAGG	58
5MCL36C7 F	AGCGGATGCATTATTCTTGG	58
5MCL36C7 R	TGAGCCATCTTGCACAACTC	58
5MCL70C149 F	CCGTTTTGTACACGAAGTGC	58
5MCL70C149 R	GGTGGAAAGTTGGCATGGTA	58
5MCL147C59 F	ATTCCTGGCATCGGTCAATA	58
5MCL147C59 R	ACGGACTCCGAATGACAAAG	58
5MCL125C35 F	TCGATGAAATGTGTGGCAAT	58

5MCL125C35 R	GAGGCCGTATGTTGATCGTT	58
5MCL191C11 F	TCTACACGGAGGGGATCTTG	60
5MCL191C11 R	ACCCTGTAGCGTTTGACCAC	60
1UBICL94C50 F	ACCGTTATGGTTAGGCGTTG	60
1UBICL94C50 R	ACACCCCTCACAAACTGGAG	60
AUMBCL217C21 F	CCTCTGAGTGGGGGTGTATG	62
AUMBCL217C21 R	GATCTAGACTGGCCCCACAG	62
AUMBCL269 F	CGCTCAATTTTCAACGGAAT	58
AUMBCL269 R	GATGGCATCCTTTGACTCGT	58
2ULCL318 F	TGGTTAATCCGGCAAACATT	58
2ULCL318 R	AGCACTTTATCGATGGCAGTC	58
5UCL101 F	CAAAACTGGAGCAGTGACGA	58
5UCL101 R	GCTTCGCAGTTTTCTTGGAC	58
5UCL201 F	TGTTCGTGACCATCAACGAT	58
5UCL201 R	CTTCCTGCATTTGTCCAGGT	58
5UCL233 F	ACGTTGGCAGAACGTAGCTT	58
5UCL233 R	AGCGGATTGAGCAGAACACT	58

The PCR mix was prepared according to the Solutions section above and the tandem repeats were amplified using a thermal reaction profile: 94°C (5 min); 30 cycles of 94°C (50 sec), Ta °C (50 sec), 72°C (50 sec); hold at 72°C (5 min).

4.6.1.2 Control gel electrophoresis

PCR products of the tandem repeats were separated on 2% agarose gel along with 4 µl O'RangeRuler™ 100 bp DNA size marker (Fermentas, Vilnius, Lithuania). Two µl PCR reaction sample was mixed with a loading dye in the ratio 1:2, (total volume: 6 µl) and used for the fragment analysis with 120V for 1 hour. The electrophoretic patterns were documented and analysed using GeneGenius gel documentation system (Syngene, Cambridge, UK).

4.6.1.3 Standard probes: Afa family and pSc119.2

The sequences of pSc119.2 and Afa family repeats were amplified in two PCR reactions. In the first amplification the PCR mix, prepared as described in the Solutions section, contained 20 ng total genomic DNA of hexaploid wheat genotype Chinese Spring as a template for Afa repeats or 650 ng of pSc119.2 plasmid DNA for pSc119 repeat. By the use of specific primers (Afa: AS-A and AS-B; pSc119.2: M13 F+R) the Afa repeats were amplified using a thermal reaction profile: 94°C (30 sec); 39 cycles of 94°C (15 sec), 55 °C (15 sec), 72°C (50 sec); 72°C (5 min), hold at 4 oC, while the reaction profile for the pSc119.2 was slightly modified: 93°C (5 min); 33 cycles of 94°C (30 sec), 54 °C (30 sec), 72°C (50 sec); 72°C (5 min) hold at 4 °C.

For the second amplification, only two changes were made to the PCR reaction mix. First, the total volume was 25 µl, so all the reagent volumes were halved; and second, PCR product from the first amplification was used as the template DNA. The amount of used DNA was ~ 400 ng.

4.6.1.4 Indirect probe labelling by NICK translation

The amplified DNA of Afa repeats was labelled with Biotin-16-dUTP, while pSc119.2 was labelled with Digoxygenin-11-dUTP using nick translation (Biotin- or Dig-Nick Translation Mix, Roche). In case of 45S rDNA no previous amplification was done and the pTa71 plasmid DNA was labelled with biotin or digoxygenin by nick translation as described above and the biotin and digoxygenin labelled probes were used in 50-50% for the in situ hybridisation experiments to provide a yellow signal under a fluorescent microscope. The NICK translation mix, detailed in the Solutions section, was incubated at 15°C for 90 minutes using a PCR thermocycler. The reaction was then stopped by adding 1 µl of 0,5 mol/L EDTA ph 8,0 and keeping at 65 °C for 10 minutes.

4.6.2 Root fixation

Sinchronisation of cell devision in root tip meristems and its accumulation in mitotic metaphase has been carried out as described by Steuernagel et al. (2017). Breafly, the seeds of the *Aegilops* genotypes were germinated on a moist filter paper until optimal root length (3 cm) is reached. In order to synchronize the cell division the seedlings were transfered into 2 mM hydroxyurea in 0.1x Hoagland's nutrient solution for 18 hours in dark at 25 ^{o}C. Then the seedlings were incubated in 0.1x Hoagland's solution in the dark at 25 ^{o}C for 5.5 hours. In order to accumulate cells in metaphase, the seedlings were transferred into 2.5 µM Amiprophos methyl solution for 2 hours in dark at 25 ^{o}C. Then the seedlings were transferred into ice water at 5 ^{o}C for overnight.

Roots were cut off the germinated *Aegilops* seeds and transferred into a 1,5 ml Eppendorf microtube onto ice. Here, they were treated for 10 minutes in ice cold acetic acid. After the treatment, the roots were washed three times in 70 % ethanol. The roots can be kept in 70 % ethanol in -20 °C for several months.

4.6.3 Microscopy slide preparation via drop method

All steps were undertaken on ice, unless specified otherwise. Five fixed roots were transferred into a 1,5 ml Eppendorf tube and washed three times in water for 10 minutes followed by a washing step in 1× KCl buffer for 5 minutes. The root tips were then cut off with a scalpel and placed into a tube containing 20 µl of 4 % cellulose + 1 % pectolyase solution and incubated in 37 °C for 56 minutes in a water bath. After the incubation, the reaction was stopped by adding an aliquot of TE buffer (just enough to fill the tube) and keeping the root tips on ice for 5 minutes followed by three times washing in 100 % ethanol. Then 32 µl of freshly prepared ice-cold 9:1 acetic acid - methanol mixture was added to the root tips, which was gently crushed and 7 µl suspension was dropped onto a glass microscopy slides which incubated in a humid Styrofoam box at room temperature. The slides were kept to dry over night and stored at +5 ^{o}C until using.

4.6.4 FISH and GISH

4.6.4.1 Slide washing and hybridisation; FISH with newly identified repeats

Dry slides with chromosome spreads were treated with 2× SSC for 5 minutes then transferred into 45 % acetic acid for 10 minutes and washed in 2× SSC for 10 minutes. Subsequent washing steps included two times 5 minutes in 2× SSC, 10 minutes in 4 % formaldehyde (dissolved in 2× SSC), three times 4 minutes in 2× SSC and finally a series of 70 %, 90 % and 100 % ethanol 2 minutes of each, respectively.

The hybridisation solution detailed in the ‘Solutions‘ section with a tandem repeat probe was incubated for 10-12 minutes at 90 °C and then put on ice for 5 minutes. The hybridisation solution (30 µl) was pipetted onto the chromosomal spreads, covered with coverslips and denatured at 80 °C for 2.5 minutes in humid environment and allowed to hybridize over night at 37 °C.

4.6.4.2 Post-treatment and microscopy

After the hybridisation, the coverslips were removed under 2× SSC and the slides were washed in 2× SSC for 5 minutes at room temperature, 20 minutes in 2× SSC at 50 °C and 5 mintues in 2× SSC at room temperature again. Finally, the slides were dehidrated using the ethanol series as described in the previous paragraph and air dried in dark. Dry slides were mounted with 17 µl Vectashield + DAPI and covered with coverslips. The FISH signals were

detected using a fluorescent microscope ZEISS and documented with a CCD camera and analysed with ISIS software. Pictures of at least 20 good cells were made.

4.6.4.3 Reprobing; FISH with standard probes

After the first FISH, the immersion oil was removed from the coverslips, which were then also removed the same way, as in the first hybridisation steps. Three 30-minute washing steps in 4× SSC were performed, followed by two 5-minute washes in 2× SSC. The slides were then dehydrated using an ethanol series 70 %, 90 % and 100% for 5, 5 and 10 minutes, respectively, and airdried. The denaturation and hybridisation steps of the slides in the presence of the hybridisation solution (30 µl) containing the probes pSc119.2-digoxygenin, Afa-biotin, 45S-rhodamin and 45S-digoxygenin (0,1µl of each) were the same as described in the first FISH experiment.

4.6.4.4 Post-treatment and microscopy

The post-treatments have been done as described in the first experiment with minor modifications due to the standard repeats were labelled indirectly. After the post hybridisation washing steps, the slides were rinsed in 4×SSC, and incubated at 37 °C for 21 min at the presence of 60 µl detection solution in a humid chamber followed by a 5-minute washing in 4× SSC in the dark. The FISH signals of the probes pSc119.2, Afa-family and 45S rDNA was documented on the same cells as examined in the first experiments using a fluorescent microscope as described before.

4.6.4.5 Reprobing; GISH and final post-treatment

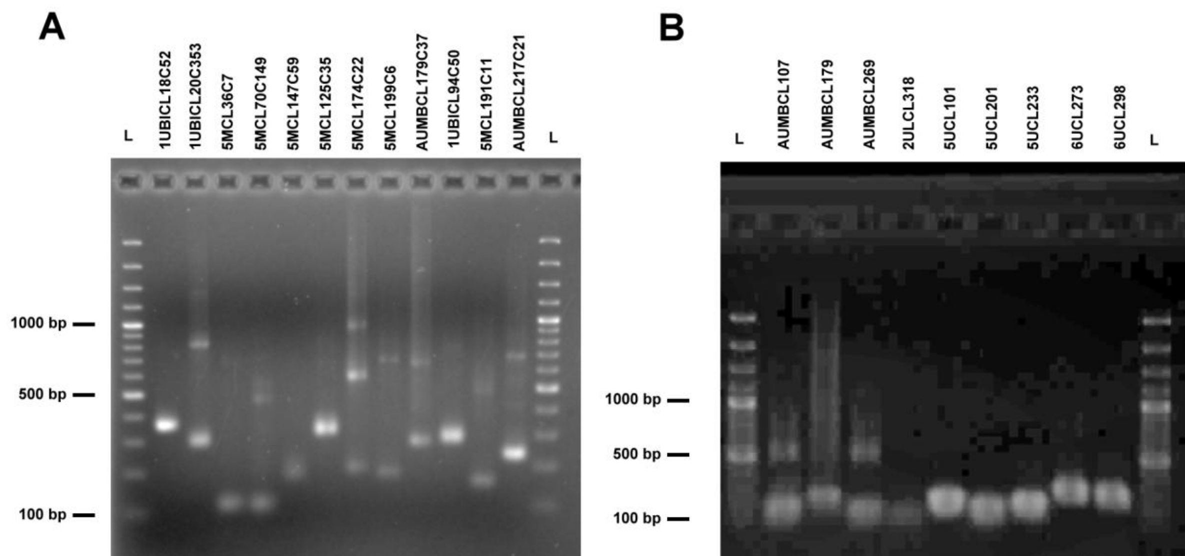
After the second FISH, the slides were treated the same way as in the first reprobing steps. The same hybridisation solution (30 µl) contained 0.3 µl of biotin-labelled U genomic probe and 0.3 µlof digoxygenin-labelled M genomic probe. The denaturation conditions were the same as during the last two FISH reactions but the hybridisation was carried out at 42 °C. The post-treatment steps were identical to those of the second FISH (including the antibody detection steps.). The GISH signal of the same cells investigated previously by the use of new tandem repeats (1. experiment) and standard repeat probes (2. experiment) was documented under a fluorescent microscope.

5 RESULTS

5.1 Probe preparation

The sequence analysis of chromosomes flow sorted from *Ae. umbellulata* and *Ae. biuncialis* identified putative tandem repeats and designed specific primer pairs. As a first step of the MSc. research, we tested the ability of these primer pairs to amplify specific DNA fragments from the U or M genomes of *Ae. umbellulata* and *Ae. comosa*. After the fragment analysis with agarose electrophoresis we detected specific PCR amplicons (most of them in the 100-500 bp interval) with all of the tested primer pairs (Fig. 3.). Because of the repeats showed the same electrophoretic pattern irrespective of the used template DNA, we used Ae. umbellulata template in the further experiments.

Fig. 3: PCR products of the tandem repeats amplified from Ae. umbellulata accession AE740/03 separated by 2% agarose gelelectrophoresis.



5.2 FISH on diploid *Ae. umbelullata* and *Ae. comosa*

In the next step, we labelled the PCR amplicons of the tandem repeats and used as probes for fluorescence in situ hybridisation on diploid genome progenitors of U and M genomes, Ae. umbellulata and Ae. comosa, respectively. We applied consequtive FISH with the new repeat probes and standard probes Afa family, pSc119.2 and 45S rDNA which has a known karyotype on the U and M chromosomes (Molnár et al. 2016) thereby the hybridisation

signals of the new repeats could be assigned to the chromosomes. Out of the twenty tandem repeats, six exhibited well defined hybridisation signals on the U or M genome chromosomes. Specific hybridisation signals were detected with the repeats AUMBCL174C22, 5MCL199C6, AUMBCL107, AUMBCL179C37, 6UCL273 and 6UCL298 on *Ae. umbellulata* (Fig. 4-7) and *Ae. comosa* (Fig. 8-11) excluding 6UCL273 which didn't provide any signal on the M-genome chromosomes.

Fig. 4: FISH on mitotic metaphase chromosomes of Ae. umbellulata. The chromosomes were first hybridised with a probe for tandem repeat AUMBCL174C22 (A) or 5MCL199C6 (C) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B and D). Bar=10 µm

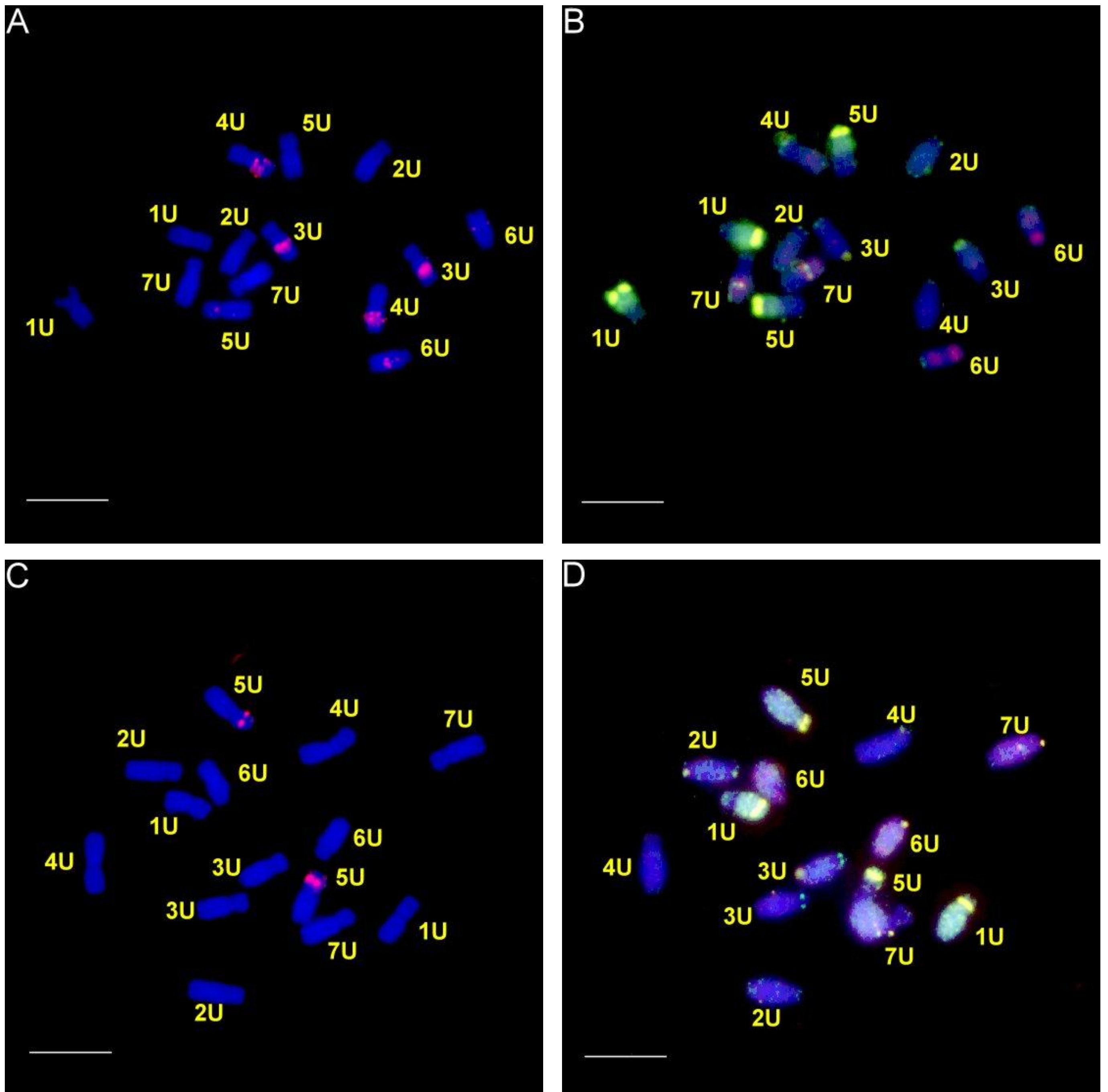


Fig. 5: FISH on mitotic metaphase chromosomes of Ae. umbellulata. The chromosomes were first hybridised with a probe for tandem repeat AUMBCL107 (A) or AUMBCL179C37 (C) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B and D). Bar=10 µm

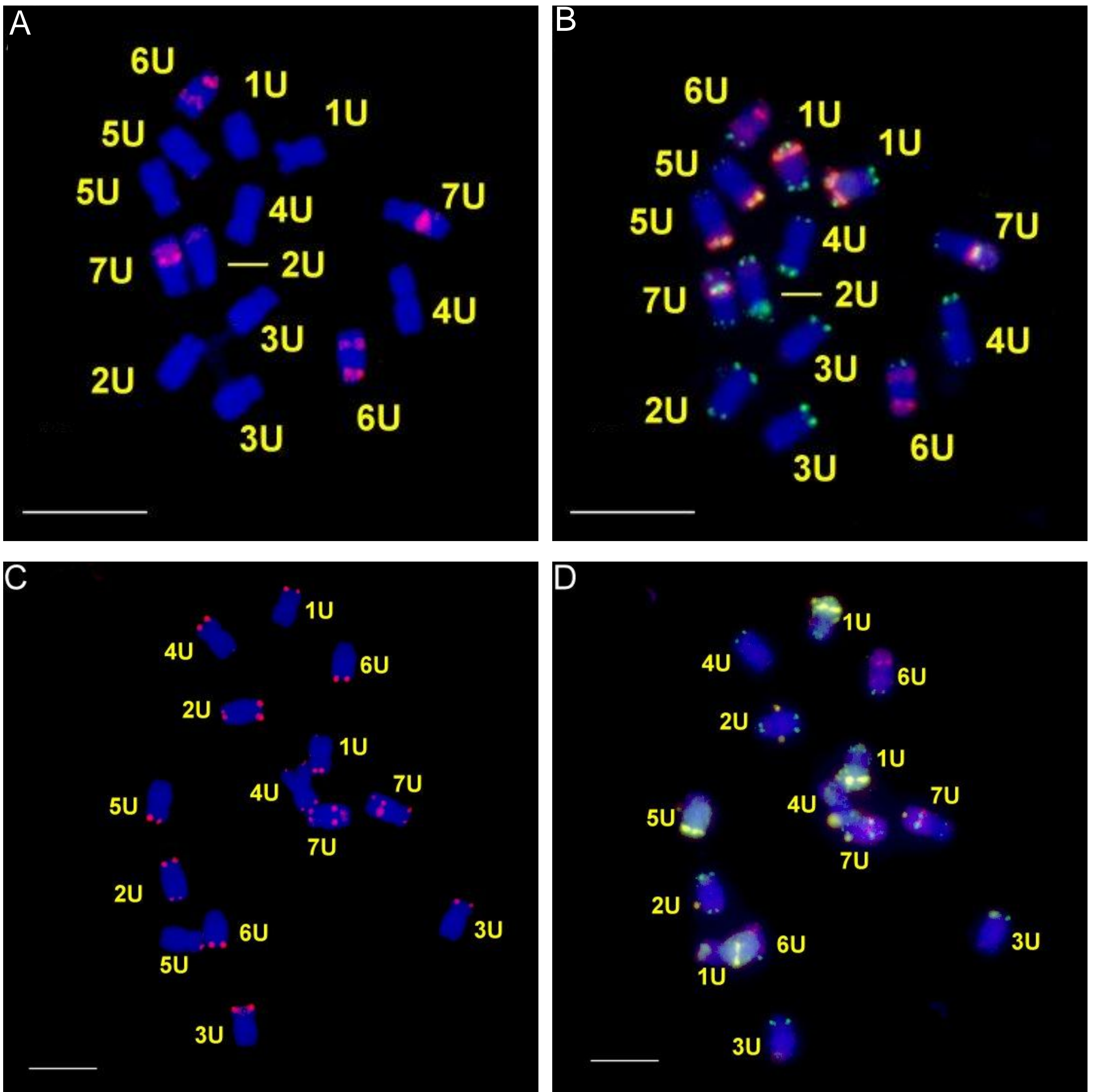


Fig. 6: FISH on mitotic metaphase chromosomes of Ae. umbellulata. The chromosomes were first hybridised with a probe for tandem repeat 6UCL273 (A) or 6UCL298 (C) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B and D). Bar=10 µm

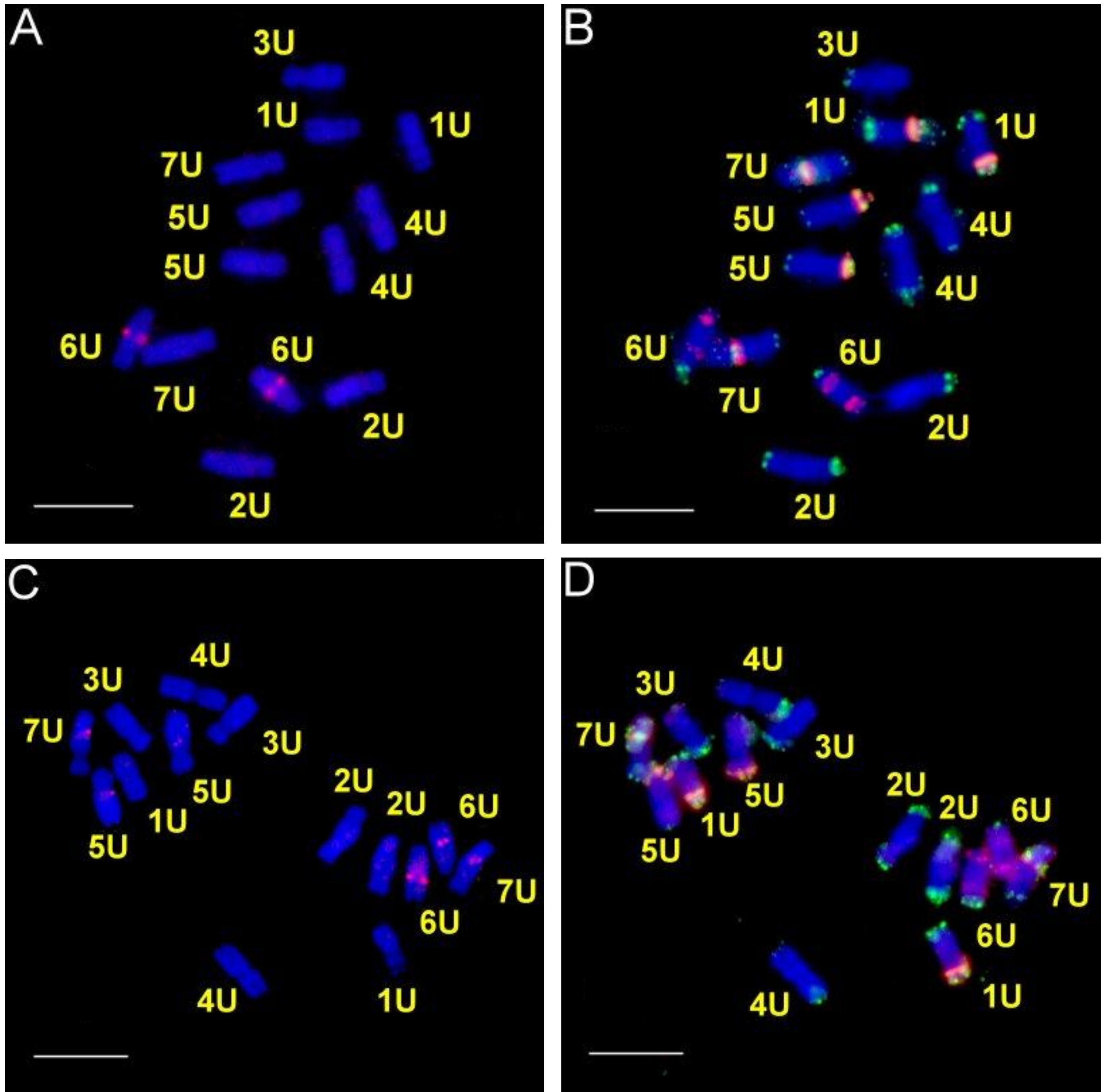


Fig. 7: Karyotype of standard repeat probes Afa family (red), pSc119.2 (green) and 45S rDNA (yellow) on mitotic metaphase chromosomes of diploid Ae. umbellulata (A) and of the newly identified repeats on Ae. umbellulata (B).

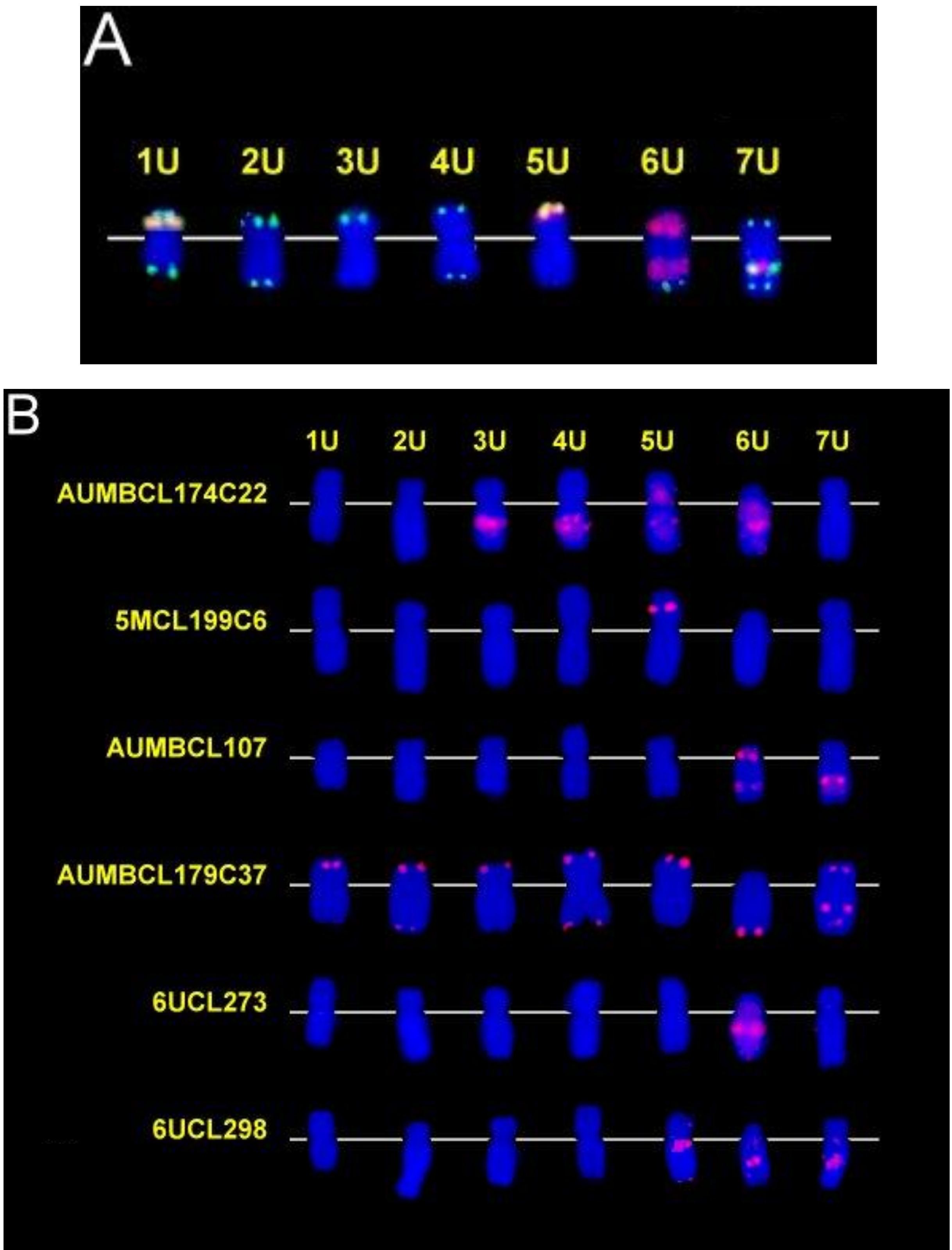


Fig. 8: FISH on mitotic metaphase chromosomes of Ae. comosa. The chromosomes were first hybridised with a probe for tandem repeat AUMBCL174C22 (A) or 5MCL199C6 (C) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B and D). Bar=10 µm

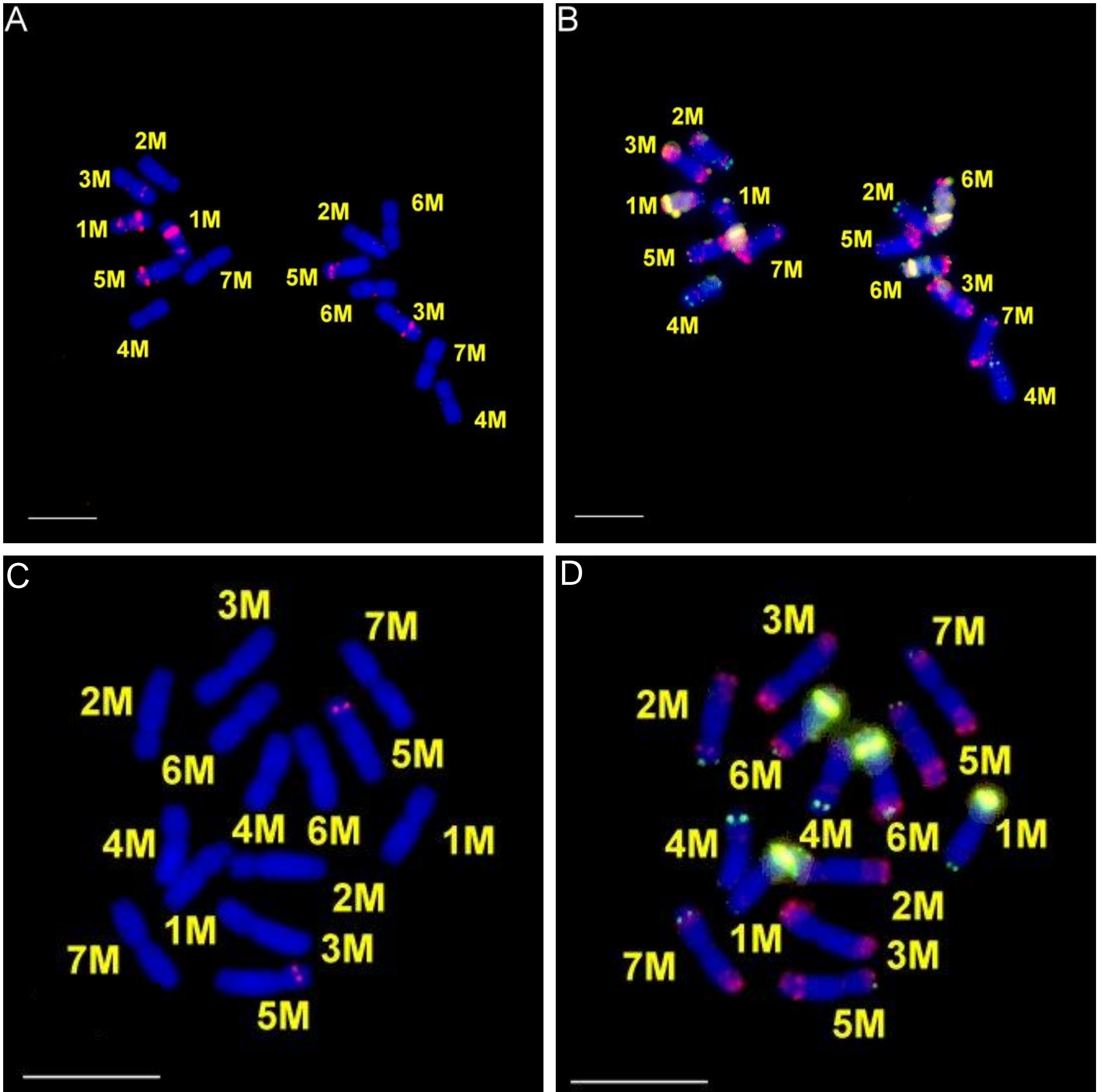


Fig. 9: FISH on mitotic metaphase chromosomes of Ae. comosa. The chromosomes were first hybridised with a probe for tandem repeat AUMBCL107 (A) or AUMBCL179C37 (C) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B and D). Bar=10 µm

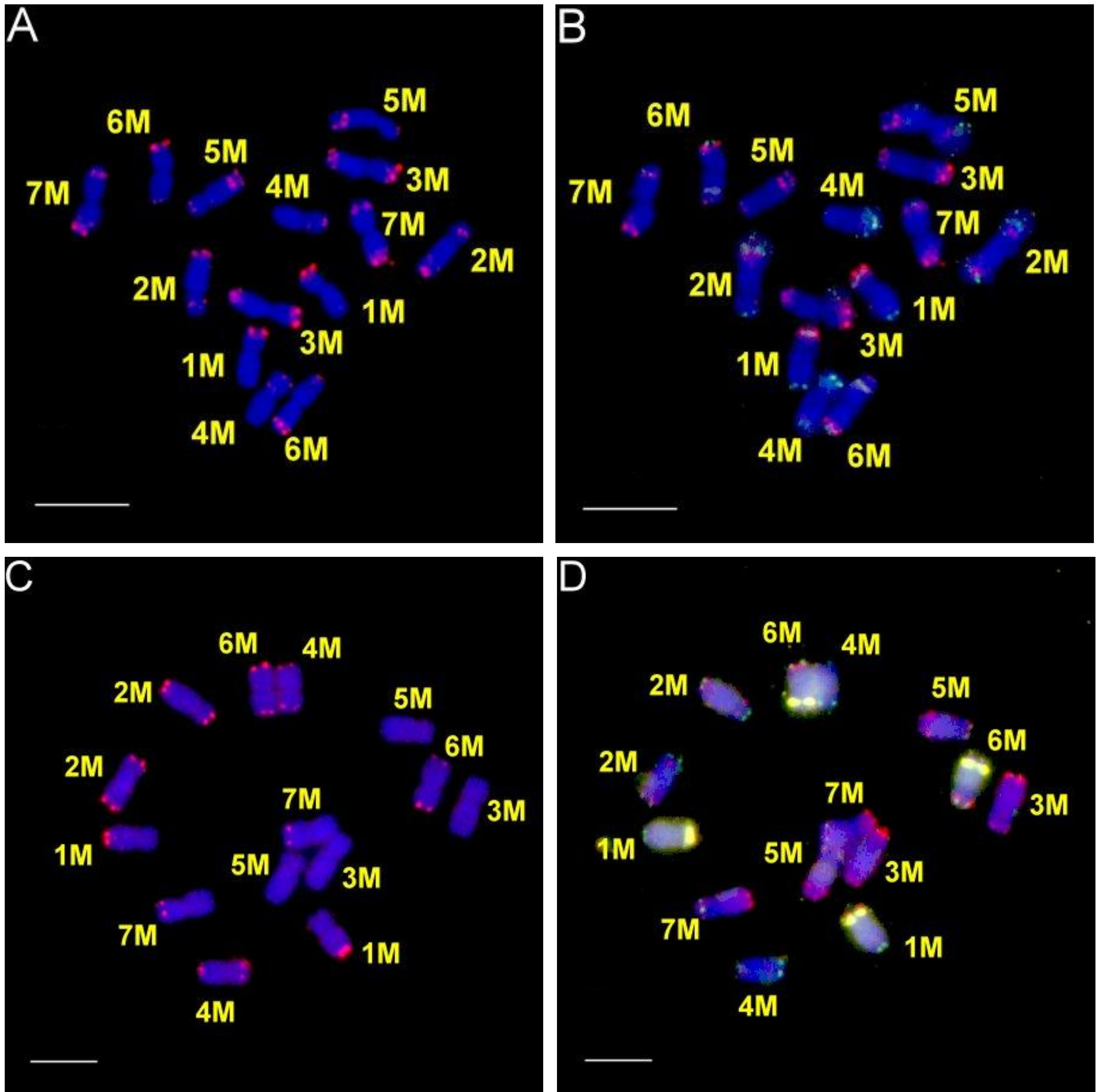


Fig. 10: FISH on mitotic metaphase chromosomes of Ae. comosa. The chromosomes were first hybridised with a probe for tandem repeat 6UCL298 (A) and subsequently reprobed with Afa, pSc119.2 and 45S probes (B). Bar=10 µm

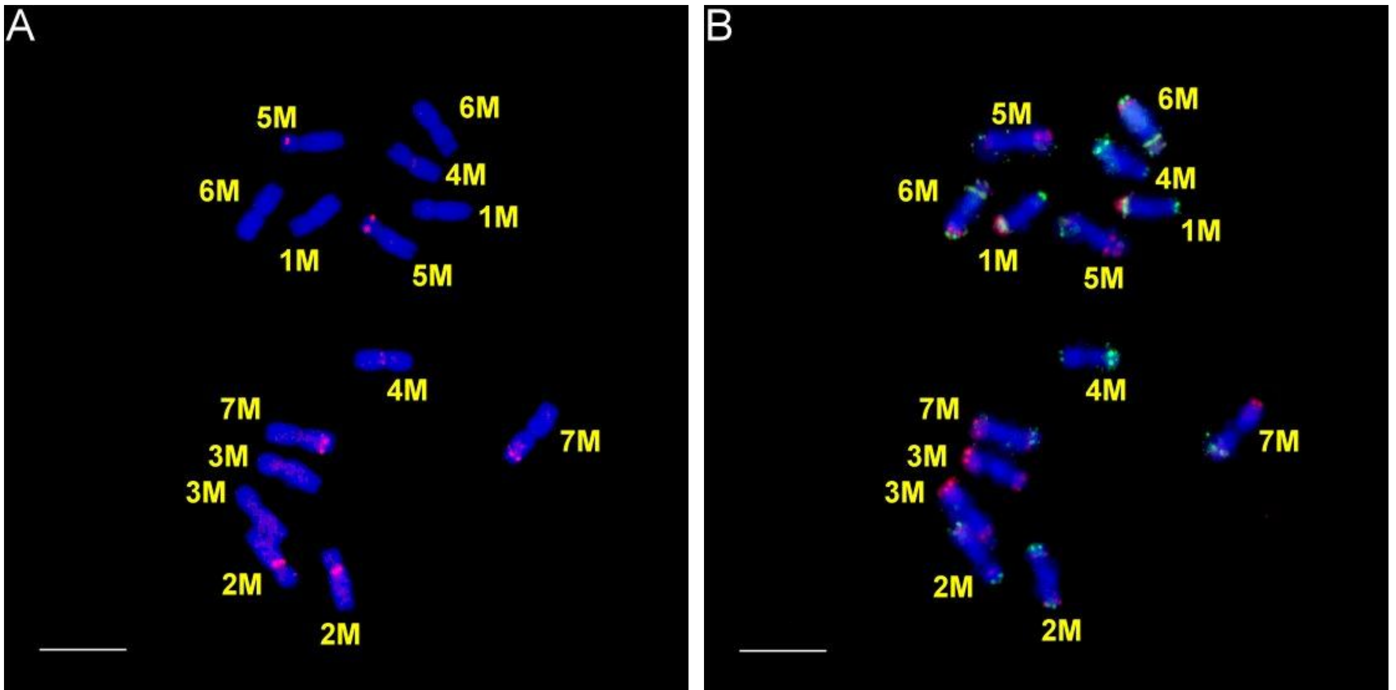
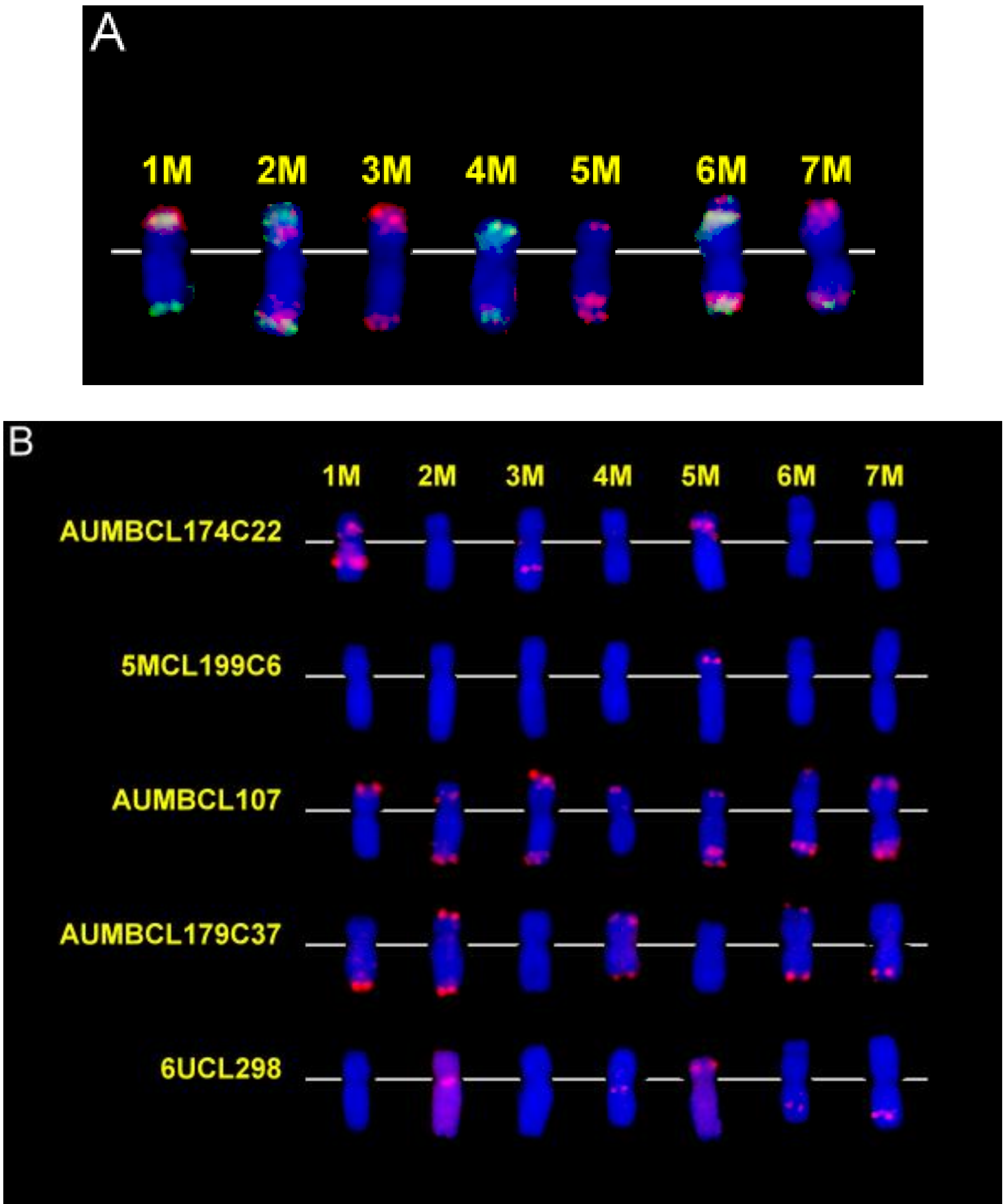


Fig. 11: Karyotype of standard repeat probes Afa (red), pSc119.2 (green) and 45S rDNA (yellow) on mitotic metaphase chromosomes of diploid Ae. comosa (A) and of the newly identified repeats on Ae. comosa (B).



5.3 FISH on allotetraploid *Ae. biuncialis*

I also wanted to know whether the new tandem repeats give similar hybridisation pattern on the U- and M-genome chromosomes of allotetraploid Ae. biuncialis as it was observed on the diploid progenitors. In order to determine the chromosomal location of the repeat clusters we allied a three step consequtive in situ hybridisation experiment. In the first hybridisation, similar to *Ae. umbellulata* and *Ae. comosa*, the repeats AUMBCL174C22, 5MCL199C6, AUMBCL107, AUMBCL179C37, 6UCL273 and 6UCL298 were hybridized to the mitotic metaphase cells of Ae. biuncialis. After documentation of the results, the chromosomes of the same cells were identified by FISH with the use of standard repeat probes Afa, pSc119.2 and 45S using the previously described karyotype (Molnár et al. 2010). Finally, the same cells were rehybridized with U- and M-genomic probes (GISH) in order to discriminate the U and M genomes of *Ae. biuncialis*. On *Ae. biuncialis*, all of the newly identified probes except probe 6UCL273 gave clear and repeatable FISH signals.

Fig. 12: FISH on mitotic metaphase chromosomes of Ae. biuncialis. The chromosomes were first hybridised with probes for tandem repeat AUMBCL174C22 (A) or 5MCL199C6 (D), subsequently reprobed with Afa (red), pSc119.2 (green) and 45S rDNA (yellow) probes (B, E) and then with total genomic DNA of Ae. umbellulata and Ae. comosa as probes for U (red) and M genomes (green), respectively (C, F). Bar=10 µm

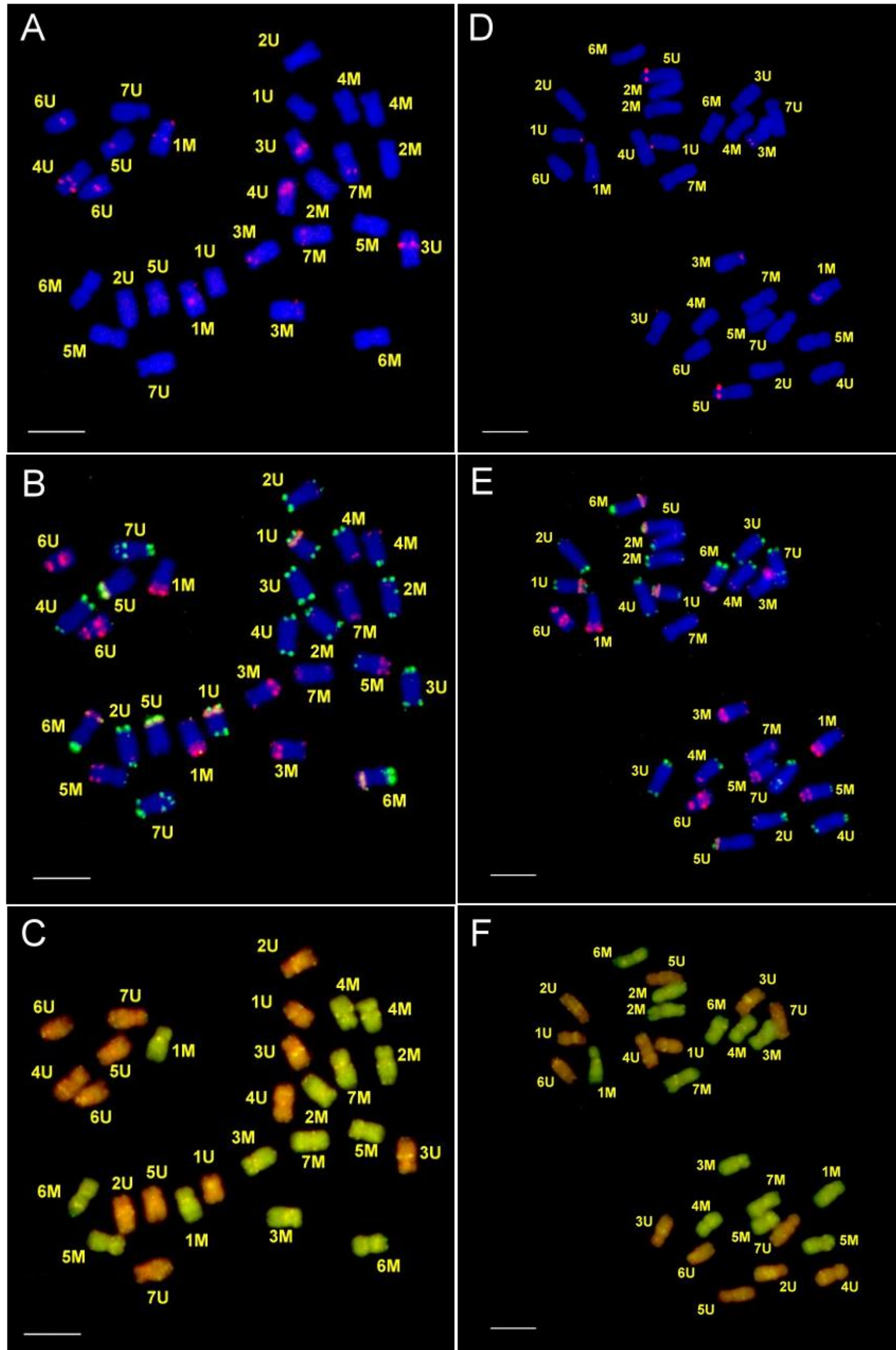


Fig. 13: FISH on mitotic metaphase chromosomes of Ae. biuncialis. The chromosomes were first hybridised with probes for tandem repeat AUMBCL107 (A) or AUMBCL179C37 (D), subsequently reprobed with Afa (red), pSc119.2 (green) and 45S rDNA (yellow) probes (B, E) and then with total genomic DNA of Ae. umbellulata and Ae. comosa as probes for U (red) and M genomes (green), respectively (C, F). Bar=10 µm

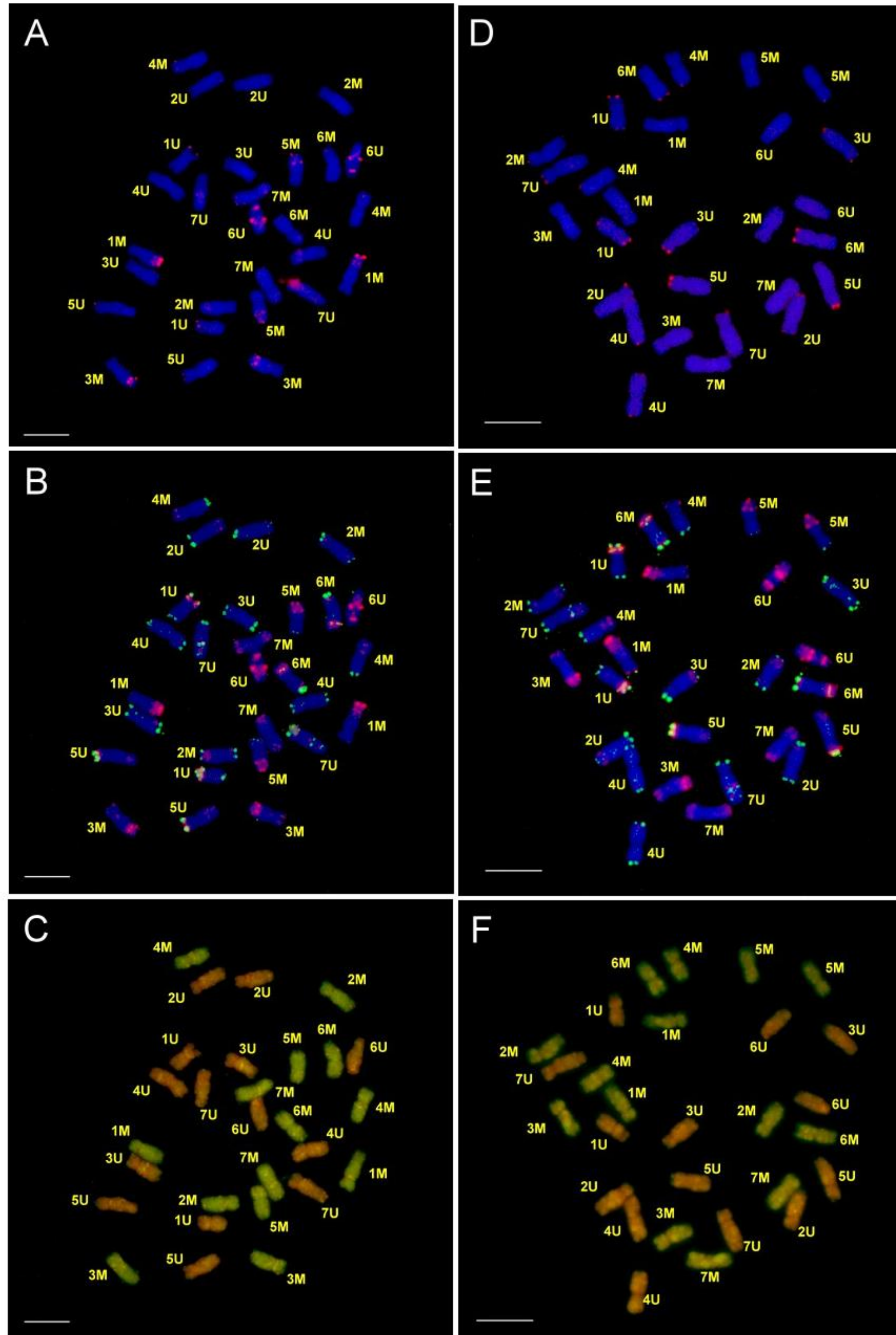


Fig. 14: FISH on somatic metaphase chromosomes of Ae. biuncialis. The chromosomes were first hybridised with a probe for tandem repeat 6UCL298 (A) and subsequently reprobed with Afa, pSc119.2 and 45s probes (B) and then with total Ae. umbellulata and Ae. comosa DNA as genomic probes for U and M genomes (C). Bar=10 µm

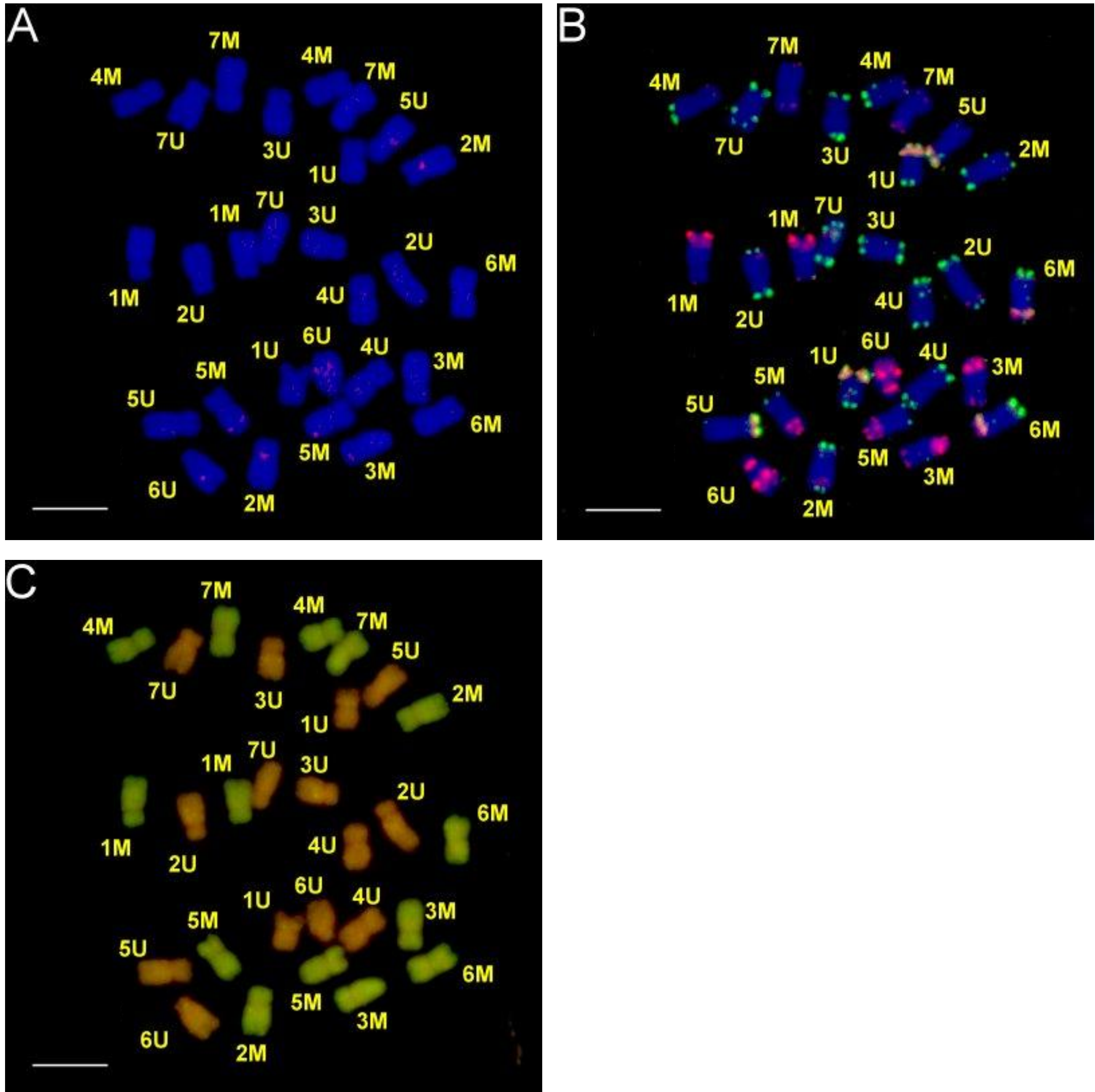
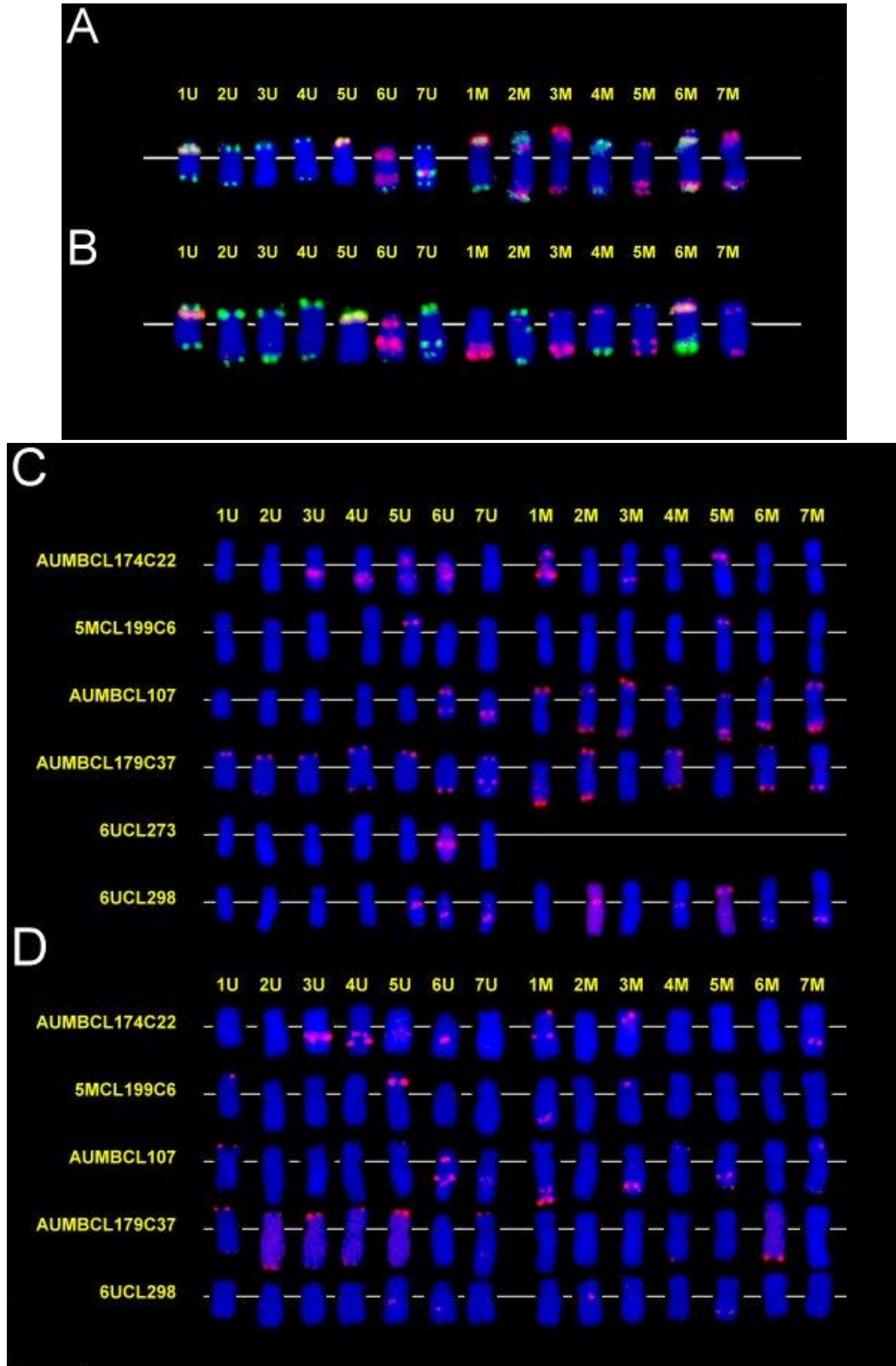


Fig. 15: Karyotype of standard DNA repeat probes Afa-family (red), pSc119.2 (green) and 45S rDNA (yellow) on mitotic metaphase chromosomes of diploid Ae. umbellulata (1U-7U) and Ae. comosa (1M-7M) (A) and their distribution on U and M genomes of allotetraploid Ae. biuncialis (B), and karyotype of the newly identified tandem repeats on U and M genomes of Ae. umbellulata and Ae. comosa (C) and their distribution on the chromosomes of allotetraploid Ae. biuncialis (D).



5.4 Distribution of studied tandem repeats in examined *Aegilops* species

5.4.1 AUMBCL174C22

In *Ae. umbellulata* this repeat showed a strong interstitial signal on the long arm (L) of chromosome 3U, two characteristic interstitial bands on the chromosome arm 4UL and on the 6UL. One clearly visible band was detected on the centromere and two weak signals on the long arm of 5U. In *Ae. comosa,* the repeat showed a strong interstitial signals on both arms of chromosome 1M and on the chromosome arm 5MS. A clearly visible interstitial band was also observed on 3ML.

In *Ae. biuncialis*, strong interstitial signals on the chromosome arms 3UL, 4UL and 6UL were detected similar to *Ae. umbellulata*. However, differently from the diploid progenitor, no hybridisation signals were detected on 5U and on the centromere of 6U. In M genome, the bands detected on 1M were similar as those in Ae. comosa, while the signal on 3M appeared on the short arm, it disappeared on 5M and a new locus appeared on the long arm of 7M.

5.4.2 5MCL199C6

This repeat was specific for the group five chromosomes in *Ae. umbellulata* and *Ae. comosa* as clear interstitial signals were detected on the short arm of chromosomes 5U and 5M.

In *Ae. biuncialis*, the signal on 5U was also visible and a new weak band appeared in the satellite of 1U. Moreover, weak subtelomeric signals appeared in the chromosome arms 1ML and 3MS, while the signal detected on 5M in the diploid progenitor disappeared in the tetraploid *Aegilops*.

5.4.3 AUMBCL107

The chromosomal distribution of this repeat showed strong homology with the probe Afa family in the three *Aegilops* species. In *Ae. umbellulata*, this repeat was present on the short and long arm of 6U, and on the long arm of 7U. In *Ae. comosa*, the repeat was present in telomeric regions on the short arms of 1M, 3M, 4M, 5M and 6M, telomeric regions on the long arms of 2M, 3M, 5M and 7M. Additonally, in subtelomeric region on the short arm of 7M and subtelomeric regions on long arms of 3M, 5M and 6M. One locus was also present on the short arm of 2M.

In *Ae. biuncialis*, the loci on 6U and 7U were also visible and a new signal appeared in the telomeric region of 1US. In M genome, the loci detected on 2M and 6M in *Ae. comosa* disappeared in *Ae. biuncialis*, as well as the loci detected previously on the short arms, except the chromosomes 4M and 7M. However, two strong hybridisation bands appeared in telomeric and subtelomeric regions of 1ML.

5.4.4 AUMBCL179C37

The hybridisation pattern of this repeat was the same as those of pSc119.2 in all of the investigated Aegilops species. In *Ae. umbellulata*, this repeat was present in telomeric regions on the short arms of 1U, 2U, 3U, 4U and 5U and 7U and long arms of 2U, 4U and 6U. The repeat also showed a diagnostic bands on interstitial and subtelomeric parts of 7UL. In *Ae. comosa*, the repeat was present in telomeric regions on the short arms of 2M, 4M and 6M, and telomeric regions of the long arms of 1M, 2M, 4M, 6M and 7M.

In *Ae. biuncialis*, the loci on short arms of U genome chromosomes maintained their presence but a new locus appeared in the telomeric region of the long arm of 3U and in the middle of the long arm of 5U. In the M genome chromosomes, all of the loci except those on the long arms of 4M and 6M disappeared.

5.4.5 6UCL273

This repeat was present only in a single locus on the long arm of 6U of *Ae. umbellulata*.

5.4.6 6UCL298

In *Ae. umbellulata*, this repeat showed visible signals on the pericentromeric part of 5UL and interstitial parts of 6UL and 7UL. We also detected strong 6UCL298 signals on the centromere of 2M and on the telomere of 5MS in *Ae. comosa*. Moreover, a well visible subtelomeric signal was located on 7ML and weak signals on the pericentromeric parrt of 4ML and subtelomeric part of 6ML.

In *Ae. biuncialis*, only weak pericentromeric and interstitial signals were detected on 5UL and 6UL, respectively, and on the centromere of 2M and subtelomeric part of 5ML.

6 DISCUSSION

The aim of this thesis was to use fluorescent *in situ* hybridisation to study the presence of different tandem repeats within the genome of diploid *Aegilops umbellulata*, *Aegilops comosa*, allotetraploid *Aegilops biuncialis* and a *Ae. biuncialiss/Triticum* amphiploid and their distribution on the individual chromosomes. Out of the twenty putative tandem repeats investigated, six formed cytogenetically visible clasters on the U- and M-genome chromosomes that could be detected by fluorescence microscopy. By the use of Afa family, pSc119.2 and 45S rDNA probes we were able to identify all the chromosomes of *Ae. umbellulata*, *Ae. comosa* and *Ae. biuncialis* according to their previously described karyotype (Badaeva et al. 2004; Molnár et al. 2016). We found that karyotypes of the repeats AUMBCL179C37 and AUMBCL107 were highly similar to those of pSc119.2 and Afa family, respectively, indicating that the sequence data were enough representative and the bioinformatic approach used for the repeat analysis (Koo et al. 2016) was suitable to identify these previously mapped repeats.

The karyotypic analysis showed that significant parts of the hybridisation signals were detected in the interstitial regions of the *Aegilops* chromosomes especially in case of the repeat AUMBCL174C22. The diagnostic bands of this repeat on the chromosomes of 3U, 4U and 1M make it possible to discriminate 2U (no signal) from 3U (one signal) and 4U (two signal) in diploid and tetraploid Aegilops species, and also the chromosome 1M from 3M in *Ae. biuncialis*, which chromosomes were hard to discriminate with the previously used pSc119.2 and Afa probes. This repeat probe together with 5MCL199C6, 6UCL273 and 6UCL298 means additional possibilities for the identification of *Aegilops* chromosome segments if they are involved in wheat-*Aegilops* translocation lines (Molnár et al. 2009).

At first, the experiments were not successful; the probes had either very strong background (many red dots all around the chromosomes) or provided too strong signal that prevented distinguishing the signals on two adjacent chromatids. Several different hybridisation conditions were tried, namely different hybridisation times and trying out humid and dry conditions. Different dilution of the standard probes had to be tested as well. After finding the optimal hybridisation conditions and optimal probe dilution, the experiments worked on *Ae. umbellulata*, *Ae. comosa*, and *Ae. biuncialis*. On the *Triticum*/*Ae. biuncialis* amphiploid, no probes provided any signal.

In some cases the new tandem repeat probes provided different signals on the diploid species than on their allotetraploid hybrid. This can be explained by evolutionary changes in the U and M genomes related to the allopolyploidisation process. It has been reported that polyploidisation induce rapid genomic changes such as the elimination of non-coding, low-copy DNA sequences from homoeologous chromosomes and genomes, the complete elimination or partial changes of copy number of high-copy DNA sequences, the elimination of rRNA genes and chromosomal repatterning (Wendel, 2000; Feldman and Levy, 2005). Probably the frequent decreasing in the number of chromosomal clusters of the tandem repeats in *Ae. biuncialis* relative to the diploid progenitors could also be explained by allopoliploidisation-related processes. In some cases, however, new loci appeared on *Ae. biuncialis*, which could be also explained by evolutionary changes.

7 CONCLUSION

Probes for 6 different tandem repeats that have been previously identified *in silico* on *Aegilops* U and M genomes have been found to be suitable for use as FISH probes to identify the chromosomes of diploid *Aegilops umbellulata*, *Aegilops comosa* and of allotetraploid *Aegilops biuncialis* and probably in *Triticum/Ae. biuncialis* amphiploid hybrid. 5 of these probes provided FISH signals on both diploid species as well as the allotetraploid. The hybridisation pattern of the probes AUMBCL179C37 and AUMBCL107 were identical with those of pSc119.2 and Afa family, while the remaining four repeats provided new karyotypes and diagnostic bands. These repeats represents further oportunity for the identification of *Aegilops* chromosomes and to facilitate gene transfer from these wild relatives to wheat. Further optimisation of hybridisation proces needs to use the new tandem repeat probes for the identification of *Aegilops* chromosomes in *Triticum*/*Ae. biuncialis* hybrid progenies. In the future, this experiment should be performed on additional accessions of *Ae. umbellulata*, *Ae. comosa* and *Ae. biuncialis* as well, to examine genetic diversity within the species.

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