Final report

Tertiary gene pool of hexaploid wheat including many wild species - such as *Thinopyrum* - can serve as valuable genetic resources for wheat improvement. Most of these species can be crossed naturally with bread wheat and numerous transfers have been made already. This makes it possible producing pre-breeding materials carrying useful resistance genes from the wild species. However, to date, despite heavy use as crossing partner in wheat breeding programs, no high-resolution karyotype existed. Understanding the organization of the genomes in the *Thinopyrum* genus and their phylogenetic relationships with other related genotypes (with hexaploid wheat) will greatly facilitate the utilization of them for transferring agronomically useful genes into bread wheat.

The main objective of our research project was to develop detailed FISH-based karyotype of several perennial diploid *Thinopyrum* species and physically map unique DNA sequences in order to characterize these genomes.

During the first year of our research project, seeds from each diploid *Thinopyrum* genotypes carrying JJ, PP, StSt and NN genomes with different geographical origin were collected. Martonvásár Cereal Genebank maintains several *Thinopyrum* accessions but also additional genotypes were collected from different gene banks from all over the world. In the frame of the present project, a perennial nursery was set up in Martonvásár. The collected accessions were successfully germinated and viable plants maintained in our greenhouse, phytotron chambers and in a perennial nursery in Martonvásár.
During the first year period 610 seeds were germinated. Producing only 73 roots shows all the difficulties of this project. Germinating and maintaining perennial species is more complicated and time consuming than non-perennials. We were able to produce squash preparations, which were necessary to the further in situ hybridization experiments.

During the second and third year of our research project, we carried out detailed FISH-based chromosome analysis of three diploid wheatgrass species:*Agropyroncristatum* (L.)Beauv, *Thinopyrum bessarabicum* (Savul&Rayss) A. Löve, *Pseudoroegneria spicata* (Pursh) A. Löve, the supposed ancestors of hexaploid *Thinopyrum intermedium* (Host) Barkworth & D.R.Dewey, using DNA repeats and comparative genome analysis based on COS markers.
Multicolor fluorescence in situ hybridization (mcFISH) was performed using repetitive DNA probes: Afa-family, pTa71, and the rye subtelomeric

heterochromatic sequence pSc119.2, on each genotypes. The DNA probe pTa71 contains an insert of the clone pTA71 recloned in the EcoRI site of the vector pUC19. The 9.05 kbp fragment is a part of an rDNA repetitive unit consisting of one copy each of 18S rDNA, 5.8S rDNA, 25S rDNA, and an intergenic spacer from hexaploid wheat cv. Chinese Spring. The clone pSc119 contains a 120 bp repeat derived from an EcoRI relic DNA of rye cv. King II inserted into the plasmid pBR322. Afa-family is a subfamily clone of the pAs1 repetitive sequence originally cloned by Rayburn and Gill (1986) from *Aegilops squarrosa* (*Aegilops tauschii*). Each DNA probes were labelled individually with different fluorochromes and FISH experiments were carried out using all labelled probes. For 3-color FISH, the pSc119.2 and Afa-family DNA sequence were amplified and labelled by PCR with biotin-11-dUTP (Roche) and digoxigenin-11-dUTP (Roche) by Nick Translation. The clone pTa71 was labelled combinatorial with 50% biotin-11-dUTP and 50% digoxigenin-16-dUTP. Digoxigenin and biotin were detected using anti-digoxigenin-Rhodamine Fab fragments (Roche) and Alexa Fluor 488 Streptavidin (Life Technologies). In situ hybridization was carried out on mitotic chromosomes of each Thinopyrum species according to Linc et al. (1999), with minor modifications. Using 3-color fluorescence in situ hybridization with repetitive DNA clones, different species show specific chromosome pattern. Based on the chromosome polymorphism, all investigated species was studied via different accessions. FISH-based karyotypes were generated using the chromosome polymorphism data as well.

All seven P genome chromosomes of both *A. cristatum* (PP) accessions carried specific pSc119.2 and Afa signals, so they could all be distinguished from each other. However, this species had the least characteristic chromosomes based on their FISH patterns, and many chromosome polymorphisms affecting all seven chromosome pairs were detected between the two accessions. Two of the seven chromosome pairs possessed NOR regions. The 5P chromosome had a clearly distinguishable satellite in both accessions, while the satellite on 1P was much narrower and was only visible in one analysed accession. The Afa signals on 5P appeared mainly in terminal and subterminal positions in both accessions. Instead of showing a pTa71 signal on 1PS, one accession had a characteristic pSc119.2 site at the chromosome tip. Chromosome 2P was found to be (sub)metacentric and carried Afa-family sites on both arm, mainly terminally (2PS) and subterminally (2PL). There was a characteristic Afa signal on 2PS in the pericentromere region. 3P, a large metacentric chromosome, had mainly Afa-family signal on both arms in subterminal and terminal positions. There

was a faint pSc119.2 signal at the tip of 3PS, but only in one accession. The submetacentric chromosome 4P carried Afa signals in both accessions, in subterminal-terminal positions. A strong pSc119.2 signal was located at the tip of 4PS in one accession. The submetacentric chromosome 6P possessed Afa-family repeats on both arms, in terminal-subterminal positions. The 7P submetacentric chromosome showed strong FISH-pattern polymorphism between the two chromosomes of the accessions analyzed. In one accession, 7PS carried a faint Afa-family signal at the pericentromere and a pSc119.2 signal at the tip of the chromosome arm.

All *Thinopyrum bessarabicum* (JJ) chromosomes could be distinguished from each other according to their FISH signals (three different accessions analysed). The 1J chromosome had a strong terminal pSc119.2 and a subterminal Afa-family site on its long arm, and both telomeres showed faint Afa-family signal. The 2J chromosome showed strong pSc119.2 signal on the short arm and a faint telomeric Afa-family site on both arms. Distinct pSc119.2 signals were visible on both arms of chromosome 3J in the terminal position and a strong subterminal Afa-family locus on the long arm. The 4J chromosome is a satellite chromosome with a strong terminal Afa-family signal on its long-, and a faint telomeric pSc119.2 signal on the short arm. The other satellite chromosome, 5J, showed a strong terminal pSc119.2 position on both arms. Both satellite chromosomes showed a distinct, strong pTa71 site subterminally on the short arms. A terminal pSc119.2 signal was detected on both arms of the 6J chromosome and a distinct subterminal Afa-family locus on the short arm. The 7J chromosome showed strong telomeric pSc119.2 signal on both arms. The 4J and 5J carry 45S rDNA locus. There was no difference between the three populations' karyotypes.
Pseudoroegneria spicata (StSt) (two accessions analysed) 1S submetacentric chromosome carried a telomeric Afa-family sign on both arms and a telomeric pSc119.2 signal on the short arm. Interestingly, only one of the sister chromatids carried specific pSc119.2 sign on the long arm, subterminally. The 2S chromosome had a secondary constriction but it failed to show a pTa71 sign. However, it carried an Afa-family sign on both arms, subterminally. The 3S chromosome showed faint Afa-family signal in the subterminal position on its short arm and in the terminal position on the long arm. The 4S submetacentric chromosome had a faint Afa-family signal on both arms, terminally. The 5S chromosome is a satellite chromosome, with the most complex FISH pattern. It had a strong pSc119.2 signal terminally, a strong Afa-family signal subterminally and a pTa71 sign on the secondary constriction site on the short arms. Distinct pSc119.2 signals

were visible on the long arm in the pericentromeric position. A subterminal Afa-family site was detected on both arms of the 6S chromosome. The 7S chromosome showed a strong pSc119.2 and a faint Afa-family sign in the terminal position on both arms.

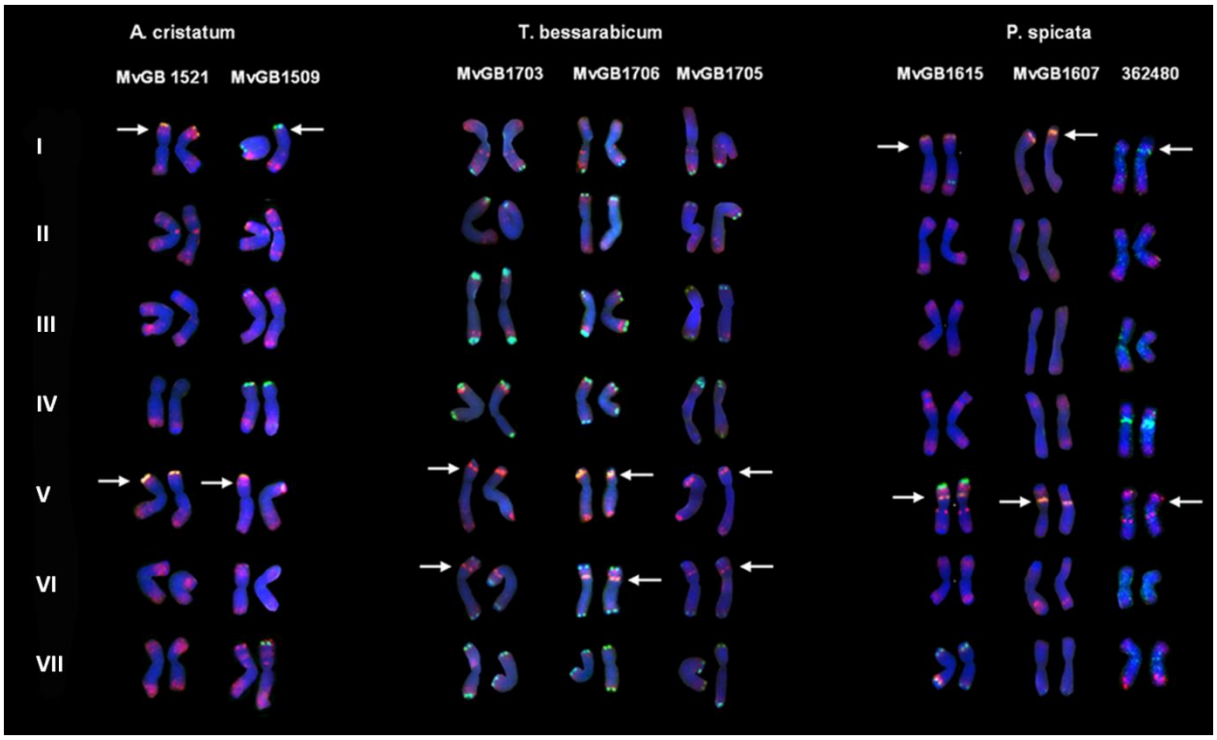

Fig 1. FISH-based chromosome karyotpe of three diploid *Thinopyrum* species (analysing different geographical origin' accessions) using DNA repetitive probes: red- Afa family, green- pSc119.2, yellow- pTa71.

Fluorescence *in situ* hybridization (FISH) with repetitive DNA probes proved suitable for the identification of individual chromosomes in the diploid JJ, StSt and PP genomes. Trinucleotide repeats were tested for suitability of species and chromosome specific DNA markers using FISH. Of the seven microsatellite markers tested only the $(GAA)_n$trinucleotide sequence was appropriate for use as a single chromosome marker for the *P. spicata* 1St chromosome.

Based on COS marker analysis, the phylogenetic relationship between diploid wheatgrasses and the hexaploid bread wheat genomes was established. These findings confirmed that the J and E genomes are in neighbouring clusters. COS markers specific for wheat homoeologous groups 1-7 were used for PCR analysis of the total genomic DNA of the perennial species in order to provide tools for detecting the genomes E, J, St and P in the wheat genetic background and to establish their genetic

similarity relative to wheat. Among the 117 COS markers studied, 102 showed PCR products in the wheat control genotype or in at least one of the 6 wheatgrass lines, while 15 markers amplified no product. The 102 markers (wheat chromosome group 1: 12, group 2: 11, group 3: 18, group 4: 10, group 5: 12, group 6: 19, group 7: 20) resulted in 396 bands of various sizes.

Out of the 119 loci (85 markers) detected in the E- genome of diploid *T. elongatum* 54 (43 markers) showed significant length polymorphism (≥5bp) relative to the control wheat genotype. In the case of *T. bessarabicum*, where 113 loci (81 markers) were located on the J- genome, 49 loci (44 markers) were polymorphic.

In the case of *P. spicata* and *A. cristatum* two accessions of each species were investigated and only amplicons exhibiting similar size within the species but size polymorphism relative to wheat were considered to be suitable for pre-breeding purposes. In *P. spicata* two genotypes (MvGB1607 and MvGB1615), 123 and 140 loci, respectively, were assigned to the St genome, of which 62 loci (50 markers) showed significant size polymorphism. Finally, 42 loci (36 markers) were polymorphic among the 126 and 134 located on *A. cristatum* two genotypes. Genome-specific loci of the COS markers with significant (≥5bp) length polymorphism between control wheat genotype and the perennial grasses were considered to be suitable for the marker-assisted selection of new wheat-alien introgression lines in pre-breeding programs.

For phylogenetic analysis, the PCR amplicons were scored as present (1) or absent (0) for each marker and were used as character states. Amplicons of identical size were considered to be the same. Only bands shared between at least two genotypes were considered in the calculation of Jaccard's similarity coefficient. A total of 206 loci of 97 COS markers were scored in a global matrix of which 188 (91%) were polymorphic between the species. The genetic similarities ranged from 0.26 to 0.74. Differences in the degree of similarity between species were confirmed by statistical analysis. The dendrogram generated from the analysis of similarity separated most of the populations and all the species, which split into three groups. The first group included the E-genome species *T. elongatum,* the J-genome species *T. bessarabicum* and *T. aestivum*. Both genotypes of the P-genome species *A. cristatum* fell in the second group, while the two genotypes of the St-genome species *P. spicata* formed a third, distantly separated clade.

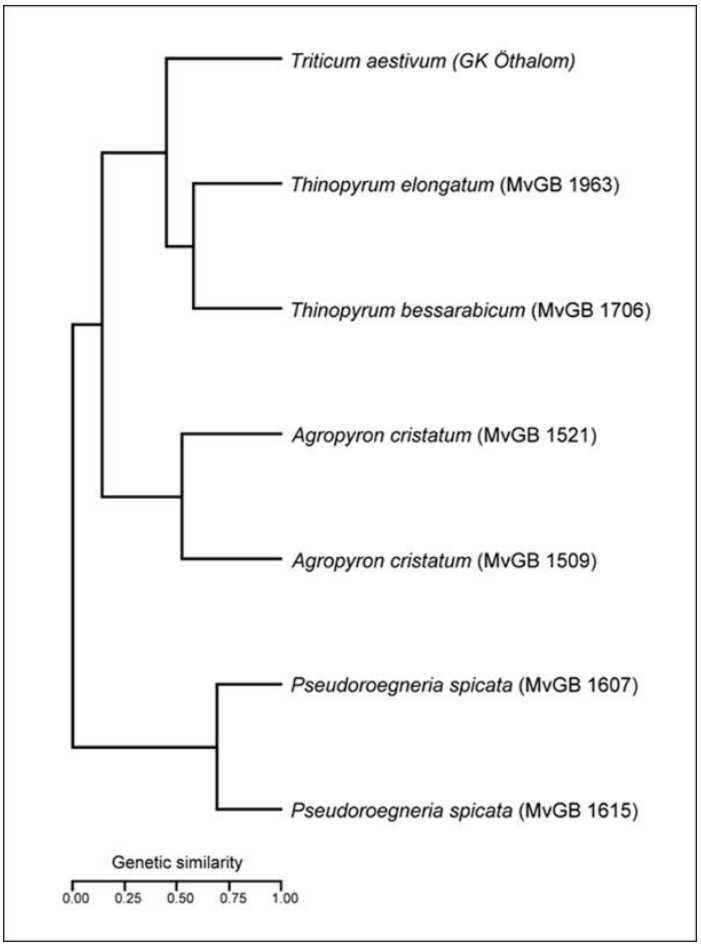

Fig 2. Dendrogram illustrating genetic similarities between the genera *Thinopyrum*, *Agropyron*, *Pseudoroegneria* and hexaploid wheat.

It can be summarized that in the frame of this research project we were able to characterize 3 diploid *Thinopyrum* species, which belong to the tertiary gene pool of bread wheat. By using molecular cytogenetic (FISH) and molecular (COS marker analysis) genetic techniques, we successfully set up high resolution FISH-based karyotypes for different genotypes of 3 diploid *Thinopyrum*. Based on COS marker analysis we selected species- and chromosome specific DNA sequences, which - together with FISH markers - could be useful for efficient screening of pre-breeding materials produced with *Tinopyrum* species.

Unfortunately two more scientific paper is under evaluation, which contains data from this project and have the NKFI identification number.