PhD THESES

Studies on nuclear and chloroplast ribosomal DNA sequences of hexaploid bread wheat and its relatives

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Introduction

Wheat certainly is the most important crop of the European civilization. Bread, made of wheat flour, has been our fundamental daily food for ages. Nowadays *winter wheat* types of *bread wheat* are primarily grown worldwide but there are regions where *durum* and other species and cultivars play also important role. For instance *spelt wheat* is cultivated more and more widely as it became popular in healthy nutrition. Further species, such as *einkorn* and *emmer* wheats were staple crops formerly and what is more their cultivation began earlier than that of bread wheat.

Considering its importance no wonder that wheat has always been in the centre of botanical research, since recognition and change of its characters and utilization of advantageous varieties offer particular economic benefits.

Bread wheat is an allohexaploid plant species, meaning it has three genomes i.e. three complete sets of chromosomes in somatic cells. As it was discovered, researchers raised up the question: which diploid species were the ancestors of wheat, what of the several relative species took part in the development of bread wheat? Answer can be important in terms of breeding and gene bank saving.

Three genomes of hexaploid bread whet are designated A, B and D. More and more proofs on origin of A and D genomes have been recently found. Although bread wheat is grown on more acreage than any other cereal crop so it is in the foreground of the scientific research, origin of B genome is still a matter of debate. Aims of our work were i) getting new results on evolution and relationships of hexaploid bread wheat by molecular methods, ii) confirm or deny earlier results on origin of genomes A and D, iii) clarify or light the origin of genome B by new results and last but not least iiii) examine the effect of wheat complex genomic structure on ribosomal DNA sequences.

Main goals of the work

Analyses of wheat ribosomal DNA (rDNA) regions have revealed new and interesting results and also offer further opportunities in studies on wheat origin and evolution. Nuclear ribosomal ITS region is especially suitable for examination of species boundaries even between close relatives. Consequently it is employable in studies on evolution and genomics of wheat and its relatives.

Main goals of my work are summarized below:

1. Studies on main lines of origin and evolution of hexaploid bread wheat by PCR-based characterization of ribosomal DNA regions

2. Broadening, confirming or denying the recent knowledge on origin of A and D genomes by the abovementioned methods

3. Lighting or (if possible) clarifying the origin of genome B by new results

4. Examination of the effect of wheat complex genomic structure on ribosomal DNA sequences

Materials and methods

Wheat species and cultivars used in the experiments:

Triticum aestivum Mv15 and Galahad cultivars, *Triticum urartu* Mv110, *Triticum turgidum* ssp. *dicoccum* Mv300, *Triticum monococcum* Mv515, *Aegilops tauschii* Mv363, *Aegilops speltoides* Mv621, *Aegilops squarrosa* Mv605. All samples are originated from the Agricultural Research Institute of the Hungarian Academy of Sciences. Identification codes correspond with those of the gene bank of the above institute. Since DNA extraction was carried out from mature plant leaves and vernalized seedlings, plant growing was corresponding with these methods, respectively.

DNA extraction, PCR, sequencing:

Samples were thoroughly ground with liquid nitrogen and quartz sand in mortars then resuspended in 2% CTAB lysis buffer. Proteins were excluded by precipitation with chloroform. DNA was precipitated with two volumes of absolute ethanol and washed twice. Pellet was resuspended in Tris buffer (0,1 M, pH=8) or ultrapure water.

For RCR Perkin Elmer GeneAmp PCR System 2400 and Techne TC-312 instruments were executed. PCR was carried out in 50 µl reaction volumes with 2 mM final Mg^{2+}concentration. Result of the PCR was checked by agarose gel electrophoresis carried out in Gibco Horizon 11-14 gel running system. DNA was visualized with ethidium bromide.

PCR products were purified by Montage-PCR centrifugal devices (Millipore). For cycle sequencing Abi Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems) was used. Capillary electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Special PCR and cloning:

For identification of the three genomes of bread wheat a special PCR method was designed based on PCR used for successful amplification from Glomalean spore DNA that hardly amplifiable because of highly methylated DNA. According to the experiences higher denaturing temperatures increase the success in PCR. The reaction was carried out in two steps, a first with high denaturation temperatures with raised amount of polymerase enzyme, and a second with usual parameters serving for amplification of PCR products formed in the first step. *Pfu* polimerase was used in the PCR and then post-amplification adenilation was executed for the TA-cloning.

Products of this special PCR were cloned into JM109 bacterial cells by T4 DN ligase and pGEM plasmid vector (pGEM-T Easy Vector System II, Promega). Transformed cells were spread on ampicillin LB-agar and white colonies were directly screened by PCR for the presence of inserts of the expected size. After the PCR, clone samples were sequenced and further analyzed.

Sequence handling, primers, phylogenetic analysis:

BlastN 2.2.2 and Fasta33_t softwares were used for screening the international databases (GenBank, EMBL) for sequence similarities. Correct alignment of sequences was carried out by the ClustalW program. Fourteen primers were used for PCR and sequencing during the work and ten of them were designed by me.

Phylogenetic analyses were carried out by the programs of MEGA 3.1 package. Tree Explorer software of MEGA package was used for building, editing and visualizing of phylogenetic trees. For tree reconstruction neighbor-joining and maximum parsimony analyses were used with default parameters of MEGA programs. One thousand replicates were used in bootstrap tests with both of tree-building methods.

Results and Discussion

Results of direct nrITS sequencing:

In the first phase of my work I determined the nrITS sequences of bread wheat and some of its main relatives. Our *T. aestivum* cv. Mv15 sample remarkably differentiated from all other materials, even from its parent species *T. turgidum* ssp. *dicoccum* and *Ae. tauschii*. This separation definitely required further analysis of ITS of Mv15 wheat. Mv15 is one of wheat cultivars of Agricultural Research Institute at Martonvásár that bear 1RS translocation chromosome element. This element can contain ribosomal RNA arrays, so it could affect our results. Attaching the rye nrITS to the data confirms this: Mv15 and rye ITS sequences gets close to each other on the phylogenetic tree in a position far from all others. Thus we proved that rye chromosome element has an unambiguous strong effect on direct nrITS sequence of Mv15 wheat.

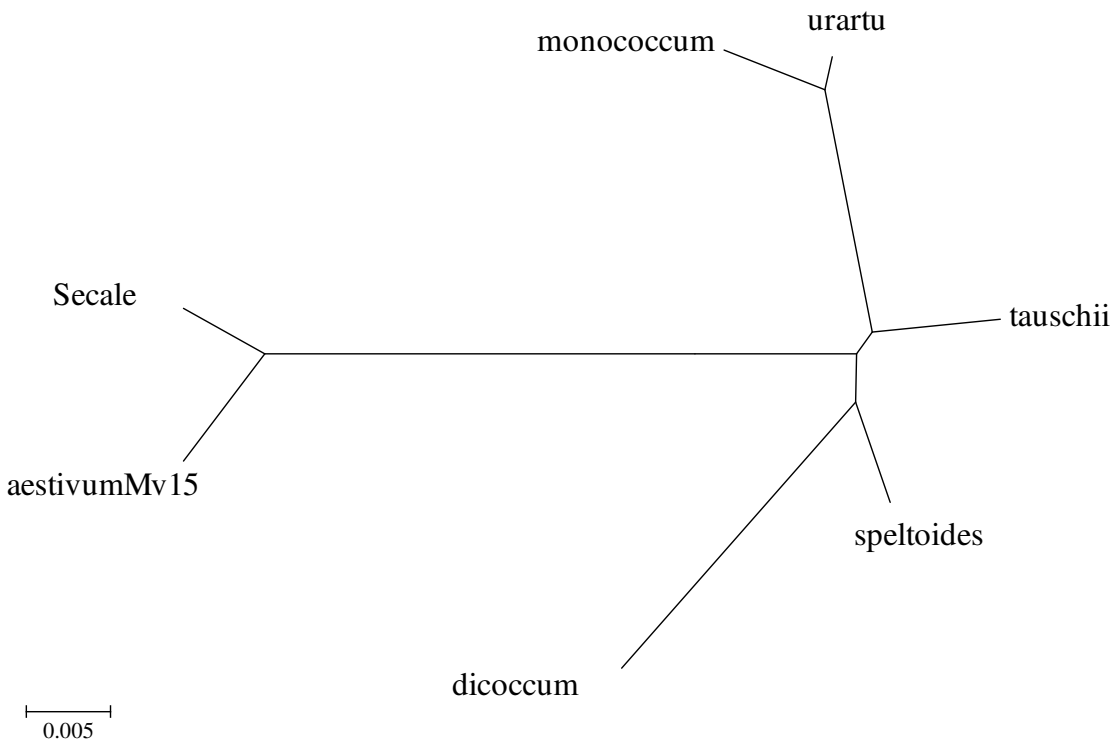


Figure 1. Neighbor-joining unrooted tree based on Kimura-2-parameter distance matrix.

Rye rDNA effect on Mv15 nrITS sequences adverted our consideration for the chloroplast genome independent from nuclear translocations. However the studied plastid rDNA markers did not prove to be variable enough. So we tried to get new results by analysis

of nrITS with special methods. We attempted to get inner information from bread wheat on rye and A, B and D subgenome ITS sequences.

Results of nrITS sequences carried out by special PCR and cloning:

High denaturation two-step PCR and cloning resulted in 67 clone ITS sequences from two samples, Mv15 and Galahad cultivars of bread wheat. 20 clones originated from Mv15 (M01-M20), and 47 from Galahad (G01-G47). Remarkable differences were found among particular clones. The clones could be rated into four types (Table 1).

	all	*aestivum* type	D genome type	A genome type	rye type
ITS clone sequences	67	47	8	2	10
from					
cv. Mv15	20	7	1	2	10
cv. Galahad	47	40	7	0	0

Table 1. ITS clone sequences (pcs) per types and wheat cultivars

Among clones of Mv15 there was not any identical with the original direct nrITS sequence. Rye type clones mostly were identical to the rye ITS or very similar to that (1 or 2 nucleotide difference). A and D genome type clones also have been found among Mv15 clones. Clones of cv. Galahad fitted into two groups, *aestivum* and D genome types. Among *aestivum* type clones could be found some sequences identical to the original Galahad ITS, while one of the D genome type clones was identical to an Mv15 D genome type clone sequence.

Considering all clones, the sequences of a type mostly differed from each other in a few bases but identical ones also could be found even between the two cultivars. An instance of the latter is a group of identical *aestivum* type sequences from Mv15 and Galahad wheat cultivars.

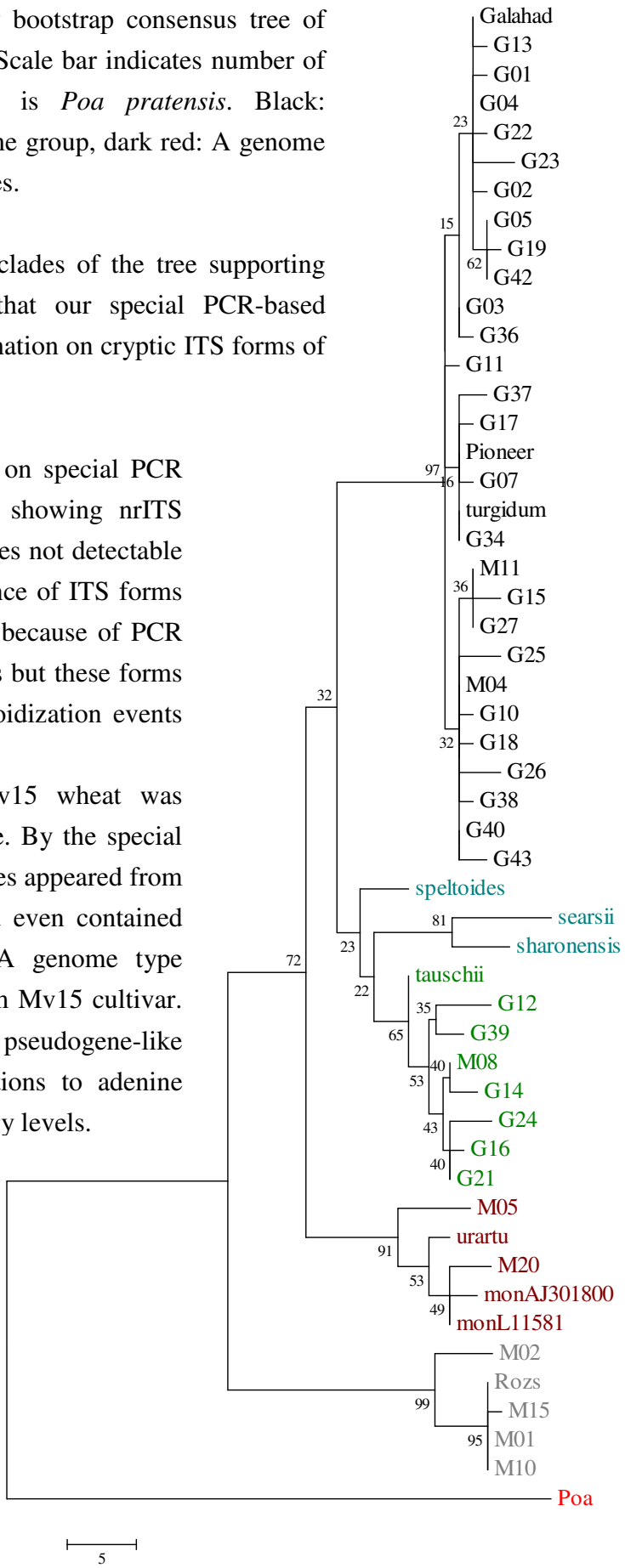
Figure 2. Maximum parsimony bootstrap consensus tree of clone and reference sequences. Scale bar indicates number of nucleotide changes. Outgroup is *Poa pratensis*. Black: *aestivum* group, green: D genome group, dark red: A genome group, dark cyan: Sitopsis species.

Clone sequences suit well the clades of the tree supporting their grouping and showing that our special PCR-based method is suitable for get information on cryptic ITS forms of bread wheat genomes.

By the invented method based on special PCR and cloning we succeeded in showing nrITS copies from bread wheat genomes not detectable by conventional PCR. Appearance of ITS forms similar to parental types is not because of PCR errors or other accidental effects but these forms are traces of former allopolyploidization events or more recent hybridizations.

Direct nrITS sequence of Mv15 wheat was unambiguously of hybrid nature. By the special method D genome type sequences appeared from both of the wheat samples and even contained identical clones. In addition A genome type sequences were found only from Mv15 cultivar. Sequences did not have pseudogene-like characteristics i.e. many mutations to adenine and thymine or higher free energy levels.

Because of the low error rate of the method, clone sequences can show the real nrITS copy rates. It seems that several minor nrDNA arrays coexist in bread wheat subgenomes that differ from each other and reflect on traces of former hybridization events.



Most important results of my work are summarized below:

- Application opportunities of plastid and nuclear rDNA regions for wheat origin and evolution studies were examined during my work. According to my results cp16S rRNA gene and spacer region between cp23S and 5S genes are hardly suitable for such purposes. On the other hand nuclear ribosomal ITS region is a good choice for that and also suitable for founding sequences bearing traces of former hybridizations
- Without using the described method these sequences (see above) mostly remain hidden
- Direct nrITS sequence of bread wheat could be of hybrid nature (cv. Mv15)
- Direct nrITS sequence of Galahad wheat (and other wheat cultivars) is almost identical to ITS of *T. turgidum*. It is probably a hybrid of ancient sequences homogenized by concerted evolution, and so it is not suitable for studying wheat evolution history
- Origin of genome D, as the youngest subgenome of wheat, was supported by the most proofs. My results support the accepted hypothesis that D genome donor was *Aegilops tauschii*. Concerning genome A, my results neither deny nor confirm exclusive donor role of *T. urartu*, however raise the issue that nrITS sequence of the former donor was not identical to either of recent einkorn wheat species (*T. urartu* or *T. monococcum*) but refer to a common ancestor ancient einkorn ITS sequence
- Sequence of unambiguously B genome origin could not be detected from the studied samples. It may caused by concerted evolution that could homogenize original parental ITS forms in the time elapsed from hybridization of A and B genomes into a new sequence type of hybrid nature
- Direct sequencing results gained from wheat and other allopolyploids should be handled with care. The method described in my work should be used for avoiding mistakes as it allows to reveal traces of former hybridization events in wheat genome through disclosing minor nrITS copies that mostly remain hidden by conventional PCR. Therefore direct information could be gained from certain elements of complex genomic structure of wheat

Publications

Papers in referred scientific journals:

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