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Detection of genome-specific ribosomal DNA sequences from bread wheat by a modified PCR-based method

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Summary

Although the nuclear ribosomal ITS region is the marker most frequently used for the molecular analysis of plant origin, little use has been made of this region to determine the origin of common wheat. The present work demonstrates that the nrITS region is suitable for research on the origin or evolution of wheat, not via direct PCR and sequencing, but by means of a novel PCR technique. This PCR analysis involved a combination of high denaturing temperature and high-fidelity *Pfu* polymerase, followed by product cloning and the sequencing. In this way sequences were revealed that remained undetected using the conventional technique and that bore traces of earlier hybridisations, allowing conclusions to be drawn on the original ITS sequences of the units involved in the hybridisation. It was demonstrated that the direct nrITS sequence of common wheat may be hybrid in nature, and that the results obtained by means of direct sequencing must be treated with caution in wheat and other allopolyploid organisms. With the help of the method described here, it should be possible to avoid such errors.

Introduction

Over the last ten years numerous molecular markers have been tested and numerous techniques applied in the hope of clarifying the origin, evolution and relationships of various wheat species. These include the fluorescence *in situ* hybridisation (FISH) method (BELYAYEV et al., 2001; ZHANG et al., 2004) and sequence analysis (LELLEY et al., 2000; LIU et al., 2003; CALDWELL et al., 2004). In wheats, as in the case of other plant groups, the 18S-26S *NOR* region, a type of nuclear ribosomal DNA (nrDNA), is one of the most frequently analysed loci (DVORAK et al., 1998; PARÁDI et al., 2003; SALLARES and BROWN, 1999, 2004).

Nevertheless, the Internal Transcribed Spacer of the 18S-26S rDNA region (nrITS), used most frequently for phylogenetic purposes (ALVAREZ and WENDEL, 2003), is surprisingly under-represented in the relevant literature on wheat research (HSIAO et al., 1995; WANG et al., 2000; BAUM et al., 2001; ZHANG et al., 2002).

Several hundreds or thousands of copies of the 18S-26S nrDNA arrays containing the ITS region are present in the haploid genome at one or more chromosome loci. Due to the homogenising effect of concerted evolution, the nrDNA arrays do not generally vary within the genome (BALDWIN et al., 1995; ALVAREZ and WENDEL, 2003), though there are also exceptions (BUCKLER et al., 1997; MAYOL and ROSSELLO, 2001). If homogenisation is not complete, the nrDNA arrays may form diverse orthologous/paralogous and functional/pseudogene series, scattered over loci with a smaller or larger number of copies. In polyploids epigenetic changes serve to silence the superfluous gene copies. One of the most important of these is methylation, which also plays a significant role in enhancing the mutation rate (WENDEL, 2000; LIU and WENDEL, 2003).

Unmethylated functional sequences and methylated pseudogenes have been reported to behave differently during molecular analysis using the PCR method (BUCKLER et al., 1997; RAUSCHER et al., 2004; RAZAFIMANDIMBISON et al., 2004). The results suggest, however, that the strongly denaturing PCR conditions (20% DMSO or a higher denaturing temperature) promote the amplification of both methylated and unmethylated copies. Other results also indicate that DNA in a strongly methylated state gives very poor results under normal PCR conditions, but can be amplified well if the denaturing temperature is adjusted to a higher value.

In the present work a two-step PCR profile including a higher denaturing temperature was developed in order to obtain genome-specific nrITS copies from hexaploid bread wheat. By comparing the sequences of the copies it was hoped to obtain a clear picture of the relationship between bread wheat and its probable ancestors. In the course of the work it was demonstrated that nrITS or sequences belonging to other multigene families could be reliably identified using special PCR techniques and cloning.

Materials and methods

Plant material. The analyses were carried out on two varieties of *T. aestivum*: Mv 15, cultivated in Hungary in recent decades, and Galahad, cultivated in France. Mv 15 contains the 1B.1R wheat/rye translocation chromosome (KOSZEGI et al., 2000). Both varieties were provided by the Agricultural Research Institute of the Hungarian Academy of Sciences (ARI HAS), Martonvásár.

DNA extraction and PCR. DNA was extracted from 3-week-old seedlings grown in the phytotron of ARI HAS (5°C, 3 weeks, G 30 chamber, Conviron, Canada) according to DOYLE and DOYLE (1987) and 30 ng genomic DNA was used for the special two-step PCR reaction. The first step involved 10 min/99°C preliminary denaturation, followed by 10 three-step cycles of 1 min/99°C, 30 s/51°C, 1 min/72°C, and 2 min/72°C final synthesis. The second step consisted of normal PCR for 25 cycles, i.e. 2 min/94°C preliminary denaturation, followed by 25 three-step cycles of 30 s/94°C, 30 s/51°C and 45 s/72°C, during which the synthesis time was increased by 1 s per cycle. The ITS1A (5'-GACGTCGCGAGAAGTCCA) forward (GULYÁS et al., 2005) and ITS4 (5'-TCCTCCGCTTATTGATATGC) reverse (WHITE et al., 1990) primers were used for the PCR. For the most reliable results, the proofreading *Pfu* polymerase (Zenon Bio Ltd., Szeged, Hungary) was used and the 3' end adenine nucleotide required for TA cloning was synthesised into the PCR products in a separate reaction (3 min/94°C, 30 min/72°C). Montage-PCR centrifugal filters (Millipore, Billerica, MA, USA) were used to purify the products.

Cloning and sequencing. The purified PCR products were ligated into pGEM-T Easy Vector plasmids (pGEM-T Easy Vector System II, Promega, Madison, WI, USA) using T4 DNA-ligase. The closed plasmids were transformed into *E. coli* JM109 bacterium cells with a heat-shock procedure and transformants containing inserted DNA fragments were screened by the blue/white method after an overnight incubation on LB/ampicillin/IPTG/X-Gal plates. After plasmid

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isolation (Wizard Plus Miniprep DNA purification system, Promega) the presence of inserts of the expected size was tested by direct PCR using plant-specific ITS1P (5'-CCGTACCATTTAGAGGAAGGAG) forward (GULYÁS et al., 2005) and ITS4 reverse primers.
A BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used to sequence the purified PCR products. The sequencing primers were forward ITS1 (WHITE et al., 1990) and reverse ITS4. Sequencing based on capillary electrophoresis using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions.

Sequence analysis. The BlastN 2.2.2. program (ALTSCHUL et al., 1997) was used to search for published data similar to the monitored sequences in the international database (GenBank-EMBL). The exact alignment of the sequences was executed using the ClustalW algorithm (THOMPSON et al., 1994) of the MEGA 3.1 program package (KUMAR et al., 2004). Programs from the MEGA 3.1 program package (KUMAR et al., 2004) were also used for the phylogenetic analysis, while the Tree Explorer application from this program package was employed to display and edit the dendrograms. The "Neighbor-Joining" and "Maximum Parsimony" applications were used in the analyses, with the default parameters. The reliability of the phylogenetic analyses was tested with the bootstrap method, using 1000 replications in each case. The sequences reported here were submitted to EMBL/GenBank/DDBJ database under the accession numbers FM998862-FM998928.

Results

In order to clarify the origin of the genome donors of hexaploid bread wheat, the nrITS sequences of two wheat varieties (Mv 15, Galahad) were analysed using a PCR-based method. After two-step PCR with a high denaturing temperature, followed by cloning and plasmid isolation, a total of 67 ITS clone sequences were produced from the two samples, 20 from Mv 15 (code-named M01 to M20) and 47 from Galahad (code-named G01 to G47). A comparison of these sequences revealed substantial differences between the clones, which could be separated into four basic groups. The largest group contained clone sequences most closely resembling the ITS sequence of the *T. aestivum* cultivar Pioneer (Z11761) used as a reference (CHATTERTON et al., 1992). The second group had sequences similar to the nrITS region of rye. None of the Galahad clones were included in this group, which consisted of half the clones of Mv 15, which contains a rye chromosome. The clones in group three exhibited the greatest similarity to the ITS sequences of the D genome donor, *Ae. tauschii*, though there was also some resemblance to other *Aegilops* species. Group four included only two ITS clones, which bore the greatest resemblance to diploid species containing the A genome (*T. monococcum, T. urartu*).
None of the Mv 15 clones were found to be identical to the AJ301799 ITS sequence previously determined by direct sequencing from the Mv 15 variety. The *aestivum*-type clones of Mv 15 belonged to two extremely similar sequence types. Within the groups there was no difference between the clones, while the two groups differed from each other at a single position and from the ITS of the reference wheat, Pioneer, at 4 and 3 positions, respectively.
Ten clones of the rye type were identified, eight of which were either identical to rye ITS or differed from it for only 1 or 2 bases, while the remaining two clones exhibited differences at 10 and 19 positions. One of the latter was clearly a chimeric type of ITS, since the ITS-1 section was similar to that of einkorn wheats, while the ITS-2 section was identical to the corresponding sequence of rye.
In addition to the above, one clone of the D genome type and two of the A genome type were isolated from Mv 15, i.e. it proved possible to detect paralogous sequences similar to all the parental ITS types.
Clones of the *aestivum* and D genome type were isolated from the Galahad variety. None of the *aestivum*-type clones were identical to the ITS of Pioneer, but six were the same as the ITS sequence (AM040486) determined from Galahad directly, i.e. without cloning. The *aestivum*-type clones of Galahad were not as uniform as those of Mv 15, where only two sequence types were found. Based on sequence alignment, the majority of the clones differed from both the Pioneer and Galahad reference ITS, but at various positions. The D genome clones also exhibited a relatively high level of variability, differing from the reference ITS sequence of *Ae. tauschii* (AJ301802) at 3-11 bases.
It was characteristic of all the clones that the sequences generally differed from each other within each type at one or more bases, though in both the *aestivum* and D genome groups there were paralogues that were found in both Mv 15 and Galahad.
On the phylogenetic trees illustrating the relationships between the sequences (Figs. 1 and 2) bootstrap values of over 10% are displayed near the given branches. The reference sequences used in the analysis were as follows: *Ae. speltoides* (AJ301804), *Ae. searsii* (AF149194), *Ae. sharonensis* (AF149195), *Ae. tauschii* (AJ301802), *T. urartu* (AJ301803), *T. monococcum* (AJ301800), *T. monococcum* (L11581), *S. cereale* (AF303400), *T. aestivum* cv. Pioneer (Z11761), *T. aestivum* cv. Galahad (AM040486) and *T. turgidum* ssp. *dicoccum* (AJ301801).
The most distinct clade in the ingroup was the rye group. This corresponds with the fact that the *Aegilops* and *Triticum* genera are more closely related to each other than to the *Secale* genus, though all three are members of the Triticeae tribe. Sequences in the rye group hardly differed from each other, with the exception of the clones M02 and M09, the special traits of which were mentioned above. Among the wheat-type sequences, the A genome group was the most distinct, though it was naturally close to the group of *aestivum*-type sequences that made up most of the tree. The group was depicted as an independent clade on the basis of the high bootstrap support (88-97%).
The *aestivum* group was the most uniform: the clade had bootstrap values of 97-99% and the distances between any two components were extremely small. The *aestivum* clade was closer to the *Aegilops*-type sequences than to the A genome group. In both cases the D genome group was monophyletic and mapped as a uniform group, unlike the *Sitopsis* species, which were not on the same branch of the Neighbor-Joining tree, but were combined in a common clade with the D genome group in the Maximum Parsimony analysis. The uncertain mapping of the *Ae. speltoides* ITS sequence is reminiscent

Tab. 1: Number and distribution of ITS clone sequences for each type and variety

	Total	*aestivum* type	D genome type	A genome type	Rye type
ITS clone sequences	67	47	8	2	10
From variety Mv 15	20	7	1	2	10
From variety Galahad	47	40	7	0	0

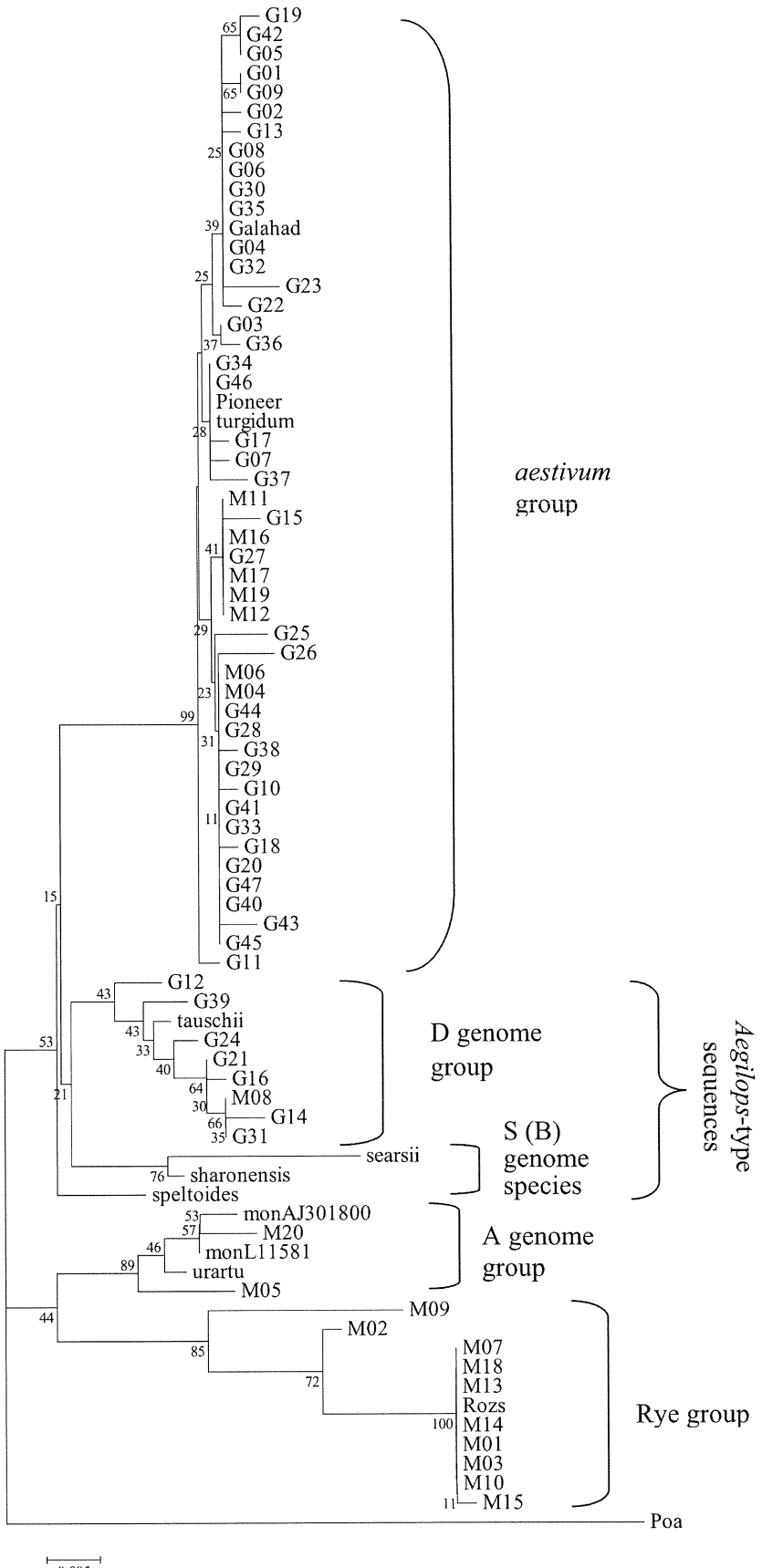

Fig. 1: "Neighbour-Joining" consensus tree based on all the clone sequences and references. The tree was prepared with the MEGA 3.1 program package. Bootstrap values (% of 1000 replications) are given for selected nodes. The outgroup was *Poa pratensis*. Scale bar indicates nucleotide changes per position.

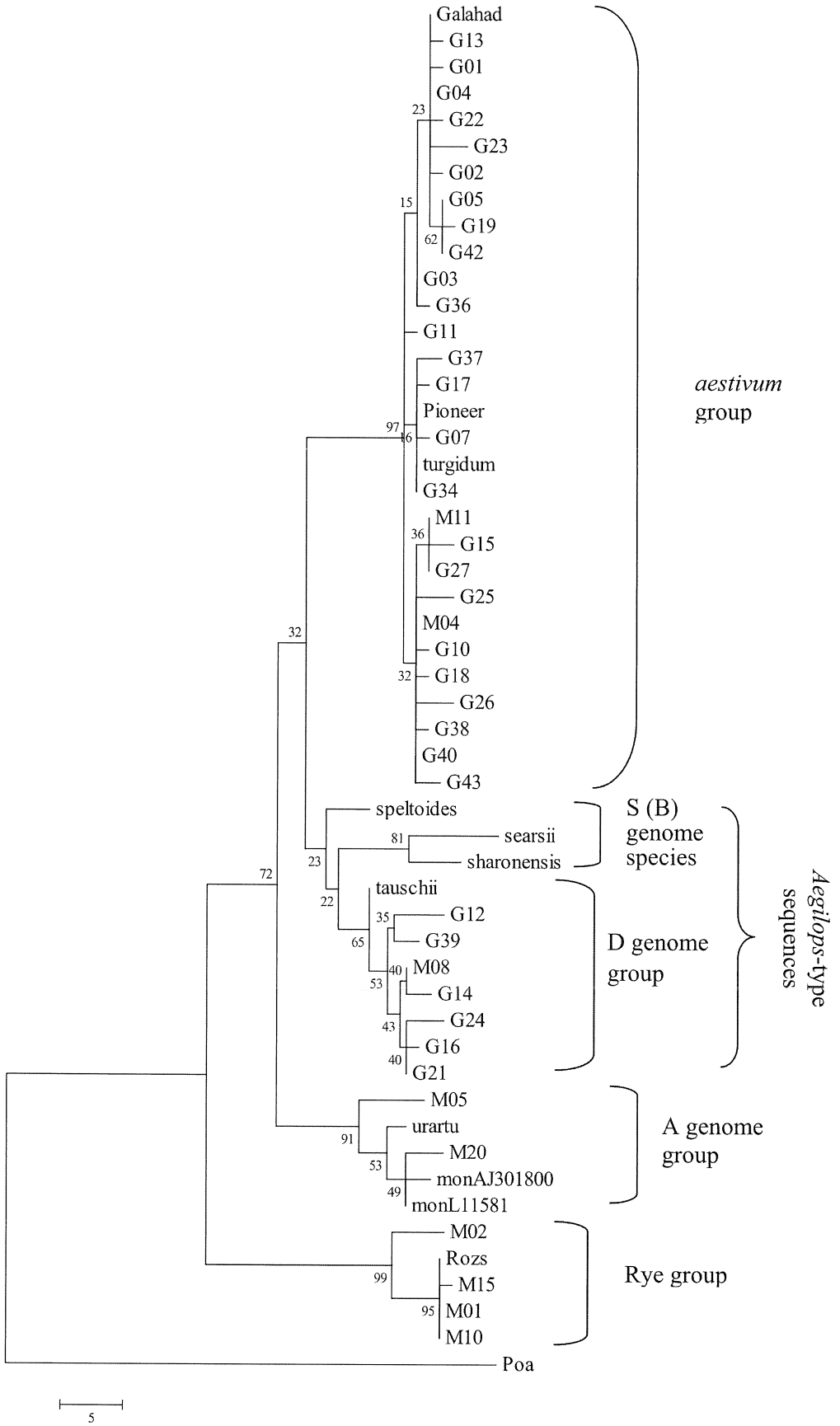

Fig. 2: "Maximum Parsimony" consensus tree based on 118 equally parsimonious trees with the consideration of 52 sequences. Repetitive and chimeric sequences were excluded from the analysis. The tree was prepared with the MEGA 3.1 program package. Bootstrap values (% of 1000 replications) are given for selected nodes. The outgroup was *Poa pratensis*. Scale bar indicates nucleotide changes.

of the results published by ZHANG et al. (2002), who found that this species did not conform phylogenetically with the *Sitopsis* group, while their results suggested that it was most closely related to the B and G genomes of the tetraploids.

It is important to note that the clone sequences fit extremely well to the clades depicted on the phylogenetic trees, which confirms their classification in the given groups and demonstrates that the special method applied in the present work provides a good picture of the ITS forms "latent" in the common wheat genomes. This could serve as a good basis for wheat origin research, while also shedding light on important methodological problems.

Discussion

With the use of a special PCR/cloning method it proved possible to detect the presence of nrITS copies in the common wheat genomes that could not be identified with the normal PCR technique. The appearance of forms similar to the ITS types of the genome donors is not the result of chance or of PCR errors, but reflects earlier hybridisation events. The direct ITS sequence of Mv 15 was clearly hybrid in nature: none of the clones was identical to it, but a clear distinction could be made between sequences of the *aestivum* and rye types, traces of which were also perceptible in the direct sequence. The situation was different for the Galahad variety: six of the 40 *aestivum*-type clone sequences were identical to the direct sequence, but the presence of 34 diverse clones was a clear indication that the ITS copies of the rDNA arrays were not identical here either. This is also indicated by the D genome-type sequences, no trace of which could be seen in the direct ITS sequence, probably due to the level of methylation or to the consequent poorer denaturising tendency.

During the amplification of the sequences two main questions arose from the methodological point of view: **(1)** what sequences are formed during direct PCR and are there positions of artefacts and (2) what sequences remain hidden.

(1) In the variety Mv 15 the sequence formed using the traditional PCR technique (AJ301799) bears a resemblance to both the *aestivum* and rye types of ITS sequences. This obviously means that several original templates were involved in the formation of the final sequence, on the one hand the *aestivum*-type ITS series and on the other hand the ITS copies of the rye chromosome component. If several templates are present together in the initial material, the characteristic result of PCR is a "mixed" sequence, but in the present case the final result is a sensible sequence, indicating that one of the sequence types becomes dominant by the end of the reaction, though this also has hybrid characters.

It was demonstrated by WAGNER et al. (1994) that when a primer pair has several binding sites on the genome (e.g. in the case of gene families), the products may not appear in their real proportions at the end of the reaction. The authors suggested two reasons for this: PCR selection, which is always selectively more efficient for one of the sequences, and PCR drift, when chance decides which of the sequences will become dominant by the end of the reaction. As a combination of strongly denaturing PCR and cloning did not lead to the appearance of a form identical to the direct sequence among the products, it is highly probable that the dominant ITS was not a copy of an ITS already present in the genetic material, but a hybrid ITS that had evolved during early cycles of PCR. In the course of the reaction copies are made simultaneously from a number of very similar templates. There is a considerable likelihood that these will become renatured, forming hybrid double strands, leading to mixed hybrid amplicons due to the poor error-correcting ability of *Taq* polymerase. As the difference between the possible ITS forms is relatively small and the binding sites of the primers are almost certainly the same, it seems very likely that chance plays the greatest role in the development of hybrid sequences.

(2) Through the joint application of strongly denaturing PCR and cloning it proved possible to detect ITS copies which are present in the gene pool but do not produce detectable copies during PCR under conventional conditions. This is almost certainly the result of the methylated state associated with the allopolyploid nature of wheat. Allopolyploid plants possess several possible alleles of the same gene, but in the majority of cases only the alleles of one of the genomes are functional, while the others are repressed, primarily due to methylation (WENDEL, 2000). For this reason, many sequences in wheat are in the methylated state and these behave differently from the functional loci in the course of PCR. Several authors have reported on the PCR behaviour of functional and non-functional ITS sequences (BUCKLER et al., 1997; RAUSCHER et al., 2004; RAZAFIMANDIMBISON et al., 2004), but their results are somewhat contradictory and do not address the direct role of methylation. Experience shows, however, that methylated DNA, especially that which is strongly methylated, is much more difficult to amplify in the course of PCR, though the use of strongly denaturing steps (DMSO, higher temperature) may lead to greater success.

In the case of the wheat variety Galahad, the whole of the D genome group consists of clone sequences that leave no trace during direct sequencing. It is highly probable that these sequences are not amplified by conventional techniques due to their methylated state, suggesting that the rDNA arrays of the D subgenome are silenced by methylation.

For the wheat variety Mv 15 four types of ITS group were obtained after clone analysis. Of these, the *aestivum* type was the most common, with a considerable number of rye type and only a small number of D genome- and A genome-type sequences. As in the Galahad variety, the D and A genome-type sequences could only be detected using special methods involving strongly denaturing PCR combined with cloning, suggesting that they are probably present in the genome in the methylated state. The clone sequences of the *aestivum* type present a more uniform picture than in Galahad and exhibit little deviation from the reference sequences. As they can be traced in hybrid-type direct ITS sequences, it is unlikely that they are methylated. The same is true of the rye-type copies, which make up half the ITS clones of Mv 15, i.e. they can be excellently amplified both with conventional methods and under strongly denaturing conditions. This suggests that they are not strongly methylated, though this is contradicted by the results of HOUCHINS et al. (1997), who assumed that rDNA of rye origin was not expressed in hybrid and addition lines of wheat because it was methylated at many sites, including key positions. It is known, however, that strongly methylated DNA compromises the success of PCR, as confirmed by the ITS copies of the A and D genome type, which can only be detected in the case of strongly denaturing PCR. One reason for this contradiction could be that the extent of methylation and the copy number have a combined effect on the ease of DNA amplification during PCR, while another reason could be that the pattern of methylation (methylated key positions) is more important than the extent of methylation in the course of gene expression.

There was a surprising level of variability between the clone sequences themselves and compared to the references. As the point mutation error rate of the method is negligible, this variability level no doubt reflects the real situation (Tab. 2).

Even diploid plant species may contain many kinds of ITS paralogues (BALDWIN et al., 1995), so their presence in a polyploid species of hybrid origin is not surprising. What is surprising is the variability of the sequences and the large number of paralogue forms. This suggests that in common wheat, which is a young polyploid species, there may be numerous nrDNA sequences of various sizes which either preserve the traces of earlier hybridisations or which are proof of genetic or epigenetic changes of more recent origin.

Tab. 2: Number of different paralogue sequences for each type. In the case of the aestivum and D-genome types, the two varieties contained clones with identical sequences

	No. of different paralogues for each type			
	Rye type	*aestivum* type	D genome type	A genome type
Mv 15	5	2	1	2
Galahad	-	26	7	-
Total	5	27	7	2

The majority of the rye-type sequences are identical with the ITS of the *Secale* species, but a few clones differ at one or two bases, making it clear that even a short period may be sufficient for the formation of diverse paralogues.

Sequences of the D-genome type were isolated from both varieties, seven from Galahad and one from Mv 15, the latter was identical to one of the D-type clones of Galahad. The clones differed at 3-11 bases from the *Ae. tauschii* ITS used as reference, and assuming that the ITS sequence of *Ae. tauschii* was not greatly different 8000 years ago from what it is today, it can be said that these changes have been fixed in these D genome-type sequences during the intervening period. This also suggests that in the allopolyploid subgenomes the nrITS regions may change much more rapidly than in the diploid parental species.

Two ITS clones of the A-genome type were isolated, both from the variety Mv 15. It is currently thought that the A genome of common wheat originates from *T. urartu* (DVORAK et al., 1993; SALLARES and BROWN, 1999). When the two clones were compared with the ITS sequences of various diploid species carrying the A genome, it was found that the ITS-1 section of clone M20 was completely identical to the sequences of *T. monococcum*, while it differed from that of *T. urartu* at two bases (Tab. 3). It is unlikely that this is proof of *T. monococcum* as the donor of the A genome. It is much more likely to indicate partial hybridisation with a variant of *T. monococcum* in the recent past, perhaps with a cultivated line of einkorn. It can be concluded from these results that at the time of the first hybridisation the ITS sequences of the two modern einkorns did not differ substantially from each other, and that the slight difference between them has developed since then.

Information was obtained on both the A and the D genome, but ITS sequences that were unambiguously of the B genome type could not be detected from either variety. At the same time, the most frequently detected sequence was the *aestivum* type, which exhibits no outstanding resemblance to the ITS region of any related diploid species, but is almost identical to that of the tetraploid *T. turgidum* ssp. *dicoccum* (cultivated emmer, AJ301801). This appears to be an instance of the homogenising effect of concerted evolution, previously observed, for example, by KOVARIK et al. (2004) in allotetraploid tobacco samples, where the number and location of rDNA loci proved to be additive when deduced from those of the diploid ancestors, but not on the basis of restriction patterns: the rDNA of one of the parents was almost or completely over-written by hybrid-type sequences. As one of the wild polyploid samples had retained the parental rDNA patterns to a greater extent, it was concluded that cultivation and repeated origin may accelerate the homogenisation of the rDNA. The present results suggest that the more recent the given hybridisation, the more likely it is that the parental ITS will be retained in the hybrid, polyploid genome. On the other hand, it is possible that the two varieties examined were the result of reticulate derivation, since one important difference between them is that the A genome was retrieved from Mv 15 but not from Galahad, while they had common *aestivum* and D-genome ITS forms, but no B-type sequences could be isolated from either of them.

It can be concluded from the results that after the hybridisation of the genomes there are first changes in the ITS sequences, and only later do they begin to be deleted. This is confirmed by the fact that the ancient A- and B-genome types have almost completely disappeared, the younger D-type has changed and has a reduced number of copies, while the still younger rye type can be found in Mv 15 in large numbers of mostly unchanged copies. It appears that after the hybridisation of the A and B genomes there was sufficient time for concerted evolution to homogenise the parental nrITS forms in the direction of a new, hybrid-type sequence. This hybrid form could be represented by the *aestivum* type which was found in large numbers among the clones, while the original parental copies have disappeared or are present in very small numbers – which may differ in varieties or variants derived following different pathways. This process had probably been largely completed by the time the stabilised tetraploid species underwent a second hybridisation step. As the introduction of the D genome took place only around 8000 years ago, the parental copies can still be traced, though they are now present in reduced number and with various changes in the sequences, suggesting that these sequences are no longer part of the functional nrDNA.

Scientists in the past seem to have largely ignored the fact that, due to the allohexaploid genomic structure of wheat, PCR can be expected to produce several kinds of possibly hybrid products. It is true that the homoeologous loci of two of the three sub-genomes are generally suppressed, but possible differences in the paralogous sequences may leave their mark on the PCR products generated. To date more than a million nucleotide sequences originating from common wheat have been stored in the public international database (GenBank, http://www.ncbi.nlm.nih.gov), the majority of which, particularly the older data, were obtained from a combination of conventional PCR and sequencing. This means that they may contain errors caused by the involvement of diverse paralogous sequences, resulting in artificial products, or hybrid sequences. The same assumption can be made for the nucleotide sequences of all allopolyploid organisms, particularly as regards the rDNA locus, but basically for all DNA elements that are present in more than one copy in the given genome, irrespective of the origin of the copies.

The method discussed in the paper is therefore able on the one hand to reveal nrITS forms that cannot be displayed independently by conventional PCR, thus providing information on the evolutionary

Tab. 3: Deviations (bp) between the ITS-1, 5.8S rRNA and ITS-2 regions of the A genome-type clone sequences (M05 and M20) and the diploid A genome species used as reference

	T. urartu (AJ301803)				*T. monococcum* (L11581)				*T. monococcum* (AJ301800)			
	ITS-1	5.8S	ITS-2	Entire ITS	ITS-1	5.8S	ITS-2	Entire ITS	ITS-1	5.8S	ITS-2	Entire ITS
M05	9	1	2	12	9	1	2	12	9	1	5	15
M20	2	0	2	4	0	0	2	2	0	0	5	5

history of wheat, on the other hand, it draws attention to a major methodological problem: in the case of samples which are polyploid or contain several homoeologous genetic elements, a combination of strongly denaturing PCR conditions, proofreading DNA-polymerase, and cloning should be applied to ensure the discovery of correct sequences.

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