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Isolation, characterization and application of a species-specific repeated sequence from *Haynaldia villosa*

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Abstract A species-specific repeated sequence, pHvNAU62, was cloned from *Haynaldia villosa*, a wheat relative of great importance. It strongly hybridized to *H. villosa*, but not to wheat. In situ hybridization localized this sequence to six of seven *H. villosa* chromosome pairs in telomeric or sub-telomeric regions. Southern hybridization to wheat–*H. villosa* addition lines showed that chromosomes 1V through 6V gave strong signals in ladders while chromosome 7V escaped detection. In addition to *H. villosa*, several Triticeae species were identified for a high abundance of the pHvNAU62 repeated sequence, among which *Thinopyrum bassarabicum* and *Leymus racemosus* produced the strongest signals. Sequence analysis indicated that the cloned fragment was 292 bp long, being AT rich (61%), and showed 67% homology of pSc7235, a rye repeated sequence. Isochizomer analysis suggested that the present repeated sequence was heavily methylated at the cytosine of the CpG dimer in the genome of *H. villosa*. It was also demonstrated that pHvNAU62 is useful in tagging the introduced 6VS chromosome arm, which confers a resistance gene to wheat powdery mildew, in the segregating generations.

Key words *Haynaldia villosa* · Repeated sequence In situ hybridization · Gene transfer

Introduction

Repeated nucleotide sequences occupy a large proportion of plant genomes and for genomes larger than 2 pg over 75% of the total DNA is in a repeated form (Flavell 1986). The rapid amplification and divergence of these sequences, whether tandem or dispersed, have made a major contribution to the evolution of plant genomes (Flavell 1986), bult also lead to some difficulties in plant genome analysis (Vilageliu and Tyler-Smith 1992). Isolation and characterization of repetitive DNA sequences not only enable us to better understand plant genome organization and to develop appropriate strategies for the analysis and manipulation of plant genomes, but also provide an opportunity to study plant phylogeny at the molecular level and to devise molecular markers to tag the alien chromatin introduced into cultivated plants (see review by Lapitan 1992). For the latter case, species-specific repeated DNA sequences have been shown to be especially useful. Species-specific repeated sequences have been isolated from many genera of the tribe Triticeae, such as *Elymus* (Tsujimoto and Gill 1991), *Haynaldia* (De Pace et al. 1992), *Secale* (Bedbrook et al. 1980; Appels et al. 1981, 1986; McIntyre et al. 1990; Guidet et al. 1991), *Thinopyrum* (Zhang and Dvorak 1990) and *Triticum* (Rayburn and Gill 1986; Talbert and Clack 1991; Anamthawat-Jonsson and Heslop-Harrison 1993; McNeil et al. 1994).

Haynaldia villosa (L) Schur [Syn: *Dasypyrum villosum* (L) Candargy 2n = 14, VV], is an annual relative of wheat, native to the Mediterranean and Caucasian areas. With multiple disease resistance, drought tolerance, and high protein content, *H. villosa* has been thought to be a potential source of useful genes for wheat improvement (Qualset et al. 1981). Moreover, much progress has been made in transferring genes from *H. villosa* into wheat through the development of addition (Hyde 1953; Blanco et al. 1987; Liu et al. 1988), substitution (Liu et al. 1988; A. K. M. R. Islam and K.W. Shepherd unpublished) and translocation lines (Qi et al. 1993). Although morphological (Hyde 1953; Liu et al. 1988; E. R. Sears, unpublished), isozyme (Montebrove et al. 1987; De Pace et al. 1988; Liu et al. 1993), and seed storage protein (Montebrove et al. 1987; Blanco et al. 1991) markers, as well as chromosome banding (Chen and Liu 1986; Fribe et al. 1987), have all been employed to identify the introduced *H. villosa* chromosomes in wheat, these techniques have proven to be

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either difficult or unsatisfactory, especially in respect of the detection of small introduced segments. Therefore, the development of *H. villosa*-specific DNA (repeated or single-copy) markers provides an attractive alternative. In the present paper, we report on the cloning and characterization of *H. villosa*-specific repeated sequences and their application in monitoring the *Pm21* gene.

Material and methods

Plant materials

The accessions or cultivars of Triticeae species and the genetic stocks used, together with their genome formulae and sources, are listed in Table 1. The wheat/*H. villosa* addition and substitution lines are designated Add and Sub, respectively.

Cloning and screening of repeated sequences

Genomic DNA of *H. villosa* and plasmid DNA of pUC19 were digested with *Sau*3AI and *Bam*HI, respectively. After extraction with phenol-chloroform, precipitation with ethanol, and resuspension in double-distilled water, they were ligated with T4 ligase. The strain JM103 of *E. coli* was transformed and distributed on LB/ampicillin/X-gal/IPTG plates. The white colonies were selected as recombinants and transferred to LB/ampicillin plates.

The recombinant plasmids were prepared by the alkaline-lysis method (Sambrook et al. 1989). After denaturation by boiling at 100 °C and chilling in ice, about 50 ng of individual recombinants were spotted onto a Hybond N^+ membrane (Amersham) and then fixed by baking at 80 °C for 30 min. The dot blots were hybridized with ^{32}P-labelled genomic DNA of *Triticum aestivum* cv CS and *H. villosa* by nick translation, respectively. The conditions for hybridization and subsequent washing followed the description of Sharp et al. (1988). The recombinants which hybridized with genomic DNA of *H. villosa*, but not to that of CS, were recognized as putative clones containing *H. villosa*-specific repeated nucleotide sequences.

Table 1 Plant materials

Material	Accession	Genome(n)	Source
Species of genetic stocks			
Triticum aestivum	Chinese Spring (CS)	ABD	E.R. Sears, University of Missouri, Columbia
	Yangmai 5	ABD	D.J. Liu, Nanjing Agricultural University
T. bicorne	As138	S^b	C. Yen, Sichuan Agricultual University
T. caudatum	As54	C	C. Yen
T. comosum		M	C. Yen
T. longissimum	As960	S^l	C. Yen
T. searsii	As915	S^s	C. Yen
T. sharonense	As913	S^{sh}	C. Yen
T. speltoides	As905	S	C. Yen
T. tauschii	As74	D	C. Yen
T. timopheevii	As274	AG	C. Yen
T. tripsacoides	As142	T	C. Yen
T. turgidum	1320	AB	D. J. Liu
T. umbellulatum	As2	U	C. Yen
T. uniaristatum	As136	N	C. Yen
Agropyron cristatum	Y419	P	C. Yen
Cristesion bogdanii	Y2655	H	C. Yen
C. chilense	P2-283324	H	C. Yen
Elymus ciliaria		SY	D. J. Liu
Haynaldia villosa	PG008	V	D. J. Liu
Heteranthelium piliferum	As177	Q	C. Yen
Hordeum vulgare	Zaosu3	I	D. J. Liu
Leymus racemosus		JN*	D. J. Liu
Psathyrostachys juncea	Y2578	N*	C. Yen
P. strigosa	MA-69-1-42	NN*	C. Yen
Pseudoroegneria libanotica	PI-228392	S*	C. Yen
Secale cereale	Jinzhou	R	D. J. Liu
S. vavilovii	As163	R	C. Yen
Thinopyrum bassarabicum		J	C. Yen
Thinopyrum elongatum		E	P. D. Chen, Nanjing Agricultural University
Addition lines of *H. villosa* chromosome in common wheats			
Add2V-7V		ABD	D. J. Liu
Addition lines of *H. villosa* chromosome in CS			
Add1Ha, 2Ha, 4Ha-7Ha		ABD	P. J. Gustafson, University of Missouri, Columbia
Substitution line of 6V/6A in common wheat			
Sub6V		ABD	D. J. Liu
Translocation lines of 6AL-6VS in Yangmai 5			
T1-11		ABD	D. J. Liu

* N and S genomes are not relevant to those of *Triticum*

Southern-blot hybridization

Genomic DNA was extracted from leaves of all genotypes using the protocol described by Gill et al. (1991). After restriction, the genomic DNAs were subjected to electrophoresis in an 0.8% agarose gel containing 0.5 ng/l of ethidium bromide, and then blotted to a Hybond N^+ membrane by the alkaline-transfer protocol recommended by the manufacturer. Southern hybridization and post-hybridization membrane washing were conducted using the procedure of Sharp et al. (1988). The membranes were exposed to X-ray film for a period of overnight to 2 days.

DNA sequence analysis

Sequencing was carried out using a *Taq* Dye Primer Cycle Sequencing kit (ABI) and analysed on automated DNA sequencer ABI 373A. The resultant sequence was compared with those in GenBank.

In situ hybridization

An insert of clone pHvNAU62 (hereafter pHv62) was localized on metaphase chromosomes of *H. villosa* by in situ hybridization. The insert was labelled with biotin-11-dUTP by nick-translation (Enzo-biochemicals) following the manufacturer's instruction. In situ hybridization and detection followed the protocol of Mukai et al. (1991).

Results

Isolation of V genome-specific repeated DNA sequences

More than 500 white colonies were screened by dot-blot hybridization. The result showed that 108 colonies hybridized to the genomic DNA of *H. villosa*, but not to that of *T. aestivum* cv CS; of these 38 gave strong signals. Ten of the 38 selected clones were used as probes for Southern analysis. pHv62 was chosen for characterization because of its high specificity to *H. villosa*.

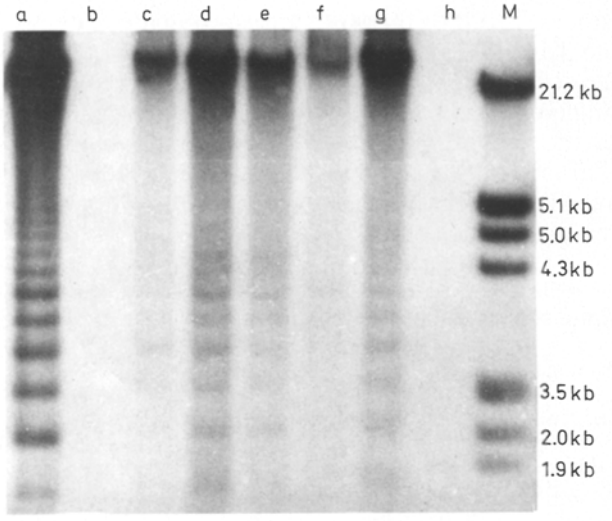

Fig. 1 Hybridization of pHv62 with Southern blots of *Eco*RI digests of *H. villosa* (*a*), CS, (*b*), Add1Ha (*c*) Add2Ha (*d*), Add4Ha (*e*), Add5Ha (*f*), Add6Ha (*g*), and Add7Ha(*h*). The amount of DNA loaded per lane was 4 µg from *H. villosa* and 10 µg from the rest. *M* λDNA digested with *Hin*dIII and *Eco*RI

Characterization of pHv62

Distribution within the V genome: Southern hybridization indicated the abundance of this repeated sequence in the genome of *H. villosa* and its scarcity in wheat. It hybridized into ladders in the Southern blots of *H. villosa* and Add1Ha, Add2Ha, Add4Ha, Add5Ha and Add6Ha, but not at all in those of CS and Add7Ha (Fig. 1). The hybridization pattern of another set of addition lines was similar to Fig. 1. Signals were absent in the blots of parental wheat cultivars and Add7V. Add5V showed a faint signal at the top (Fig. 2). Combining the results of the two sets of addition lines on the

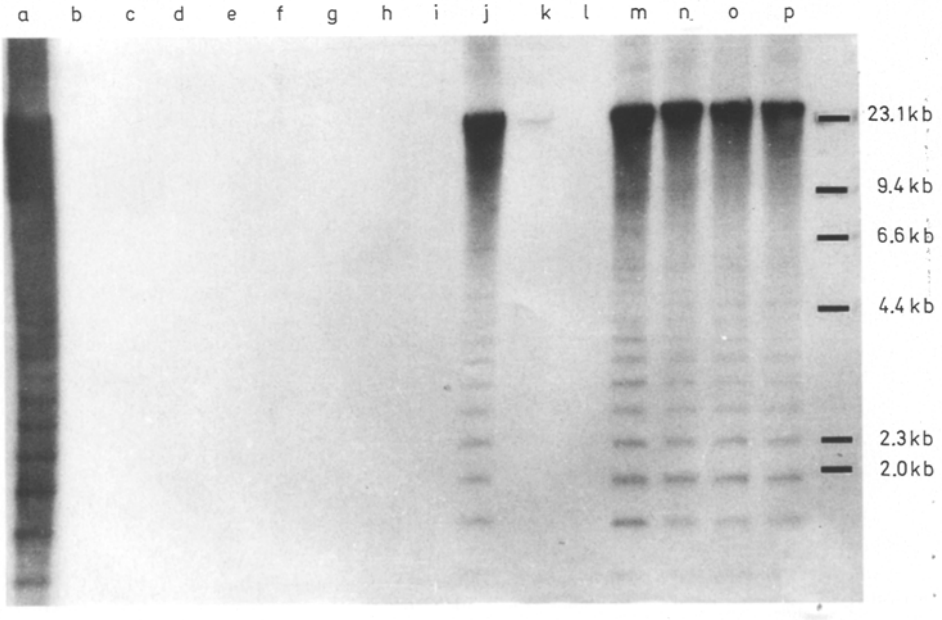

Fig. 2 Hybridization of pHv62 with Southern blots of *Eco*RV digests of *H. villosa* (*a*), *durum* (*b*), and parental wheat cultivars of addition lines (*c*) through (*i*), Add6V (*j*), Add5V (*k*), Add7V (*l*), Add3V (*m*), Add 4V (*n*) and (*o*), and Add2V (*p*). The amount of DNA loaded per lane was 4 µg from *H. villosa* and 10 µg from the rest. *M* λDNA digested with *Hin*dIII

basis of chromosome homology, we can infer that six of the seven chromosome pairs of *H. villosa* contain the pHv62 repeated sequence, i.e., 1V, 2V, 3V, 4V, 5V and 6V.

Distribution within the Triticeae: to understand the phylogenetic relationship of *H. villosa* with other species of the Triticeae, and to explore the possibility of using pHv62 as a molecular marker for other genomes besides the V genome, the DNAs from 29 species (representing 25 genomes) were digested with *Eco*RI and hybridized with pHv62. All species of *Tritricum* gave unanimously negative results (Fig. 3). It is evident that the *Triticum* species tested contain too few copies of the pHv62 sequence to be detected by hybridization. The remaining 14 species can be divided into four groups according to the intensity of the detected signal (Fig. 4). The first group, including *Heteranthelium piliferum* (genome Q), *Hordeum vulgare* (I), *Psathyrostachys juncea* (N), *P. strigosa* (NN) and *Thinopyrum elongatum* (E), showed no signal; the second group species, *Cristesion chilense* (H), *Pseudoroegneria libanotica* (S) and *Elymus ciliaris* (SY), produced signals solely on the top of the lanes; the third group consisting of *Agropyrum cristatum* (P), *Cristesion bogdanii* (H), *Secale cereale* (R) and *S. vavilovii* (R), yielded signals of medium intensity; the last group species, *Thinopyrum bassarabicum* (J) and *Leymus racemosus* (JN), gave as strong a signal as the host species *H. villosa*. Moreover, the ladder signal pattern in the species of groups 3 and 4 is the same as that of *H. villosa*. Therefore, pHv62 can be used to detect the chromosomes of *Th. bassarabicum* and *Ley. racemosus*.

Sequence organization: the cloned fragment in pHv62 was 292 bp long, 61% of which was composed of AT nucleotide pairs (Fig. 5). Polypurine-polypyrimidine stretches and duplications (direct and inverted) of CAAAA were also present in this sequence. Sequence searching in GenBank showed that the insert of pHv62 shares considerable homology (67%) to pSc7235, a rye sub-telomeric repeated sequence (Appels et al. 1981). This agrees with the result of Southern hybridization (Fig. 4).

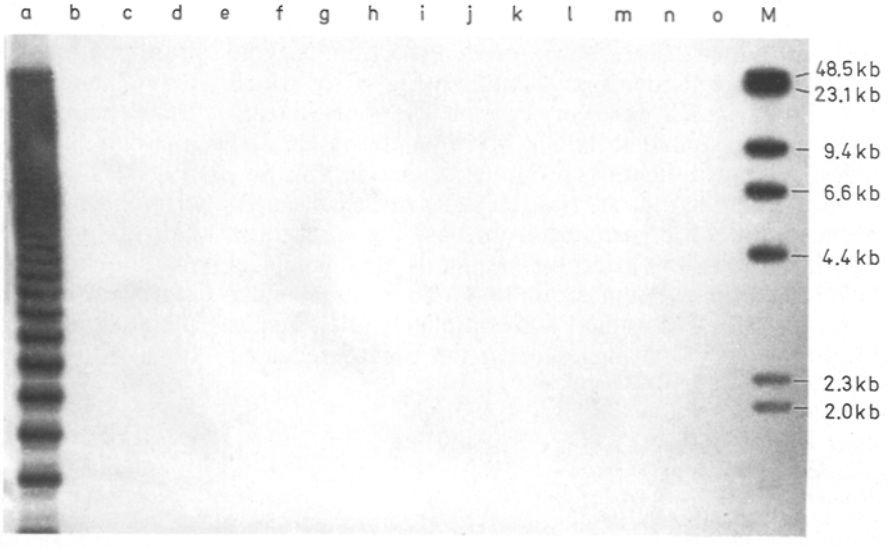

Fig. 3 Hybridization of pHv62 with Southern blots of *Eco*RI digests of *H. villosa* (*a*), *Triticum turgidum* (*b*), *T. timopheevii*, (*c*), *T. aestivum* (*d*), *T. caudatum* (*e*), *T. bicorne* (*f*) *T. longissimum* (*g*), *T. searsii* (*h*), *T. sharonense* (*i*), *T. speltoides* (*j*), *T. tauschii* (*k*), *T. umbellulatum* (*l*), *T. uniaristatum* (*m*), *T. comosum* (*n*), and *T. tripsacoides* (*o*). The amount of DNA loaded per lane was 3 μg from diploids, 6 μg from tetraploids and 9 μg from hexaploids. *M* λDNA digested with *Hin*dIII

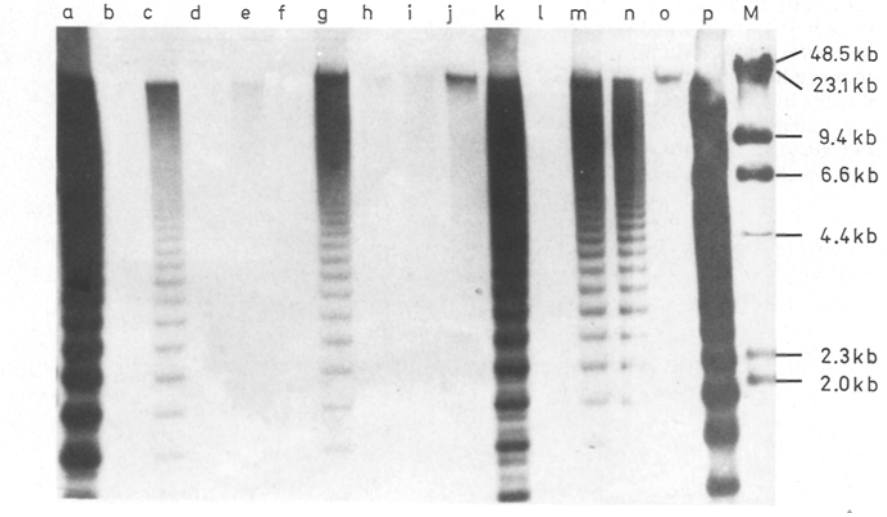

Fig. 4 Hybridization of pHv62 with Southern blots of *Eco*RI digests of *H. villosa* (*a*), CS (*b*), *Agropyron cristatum* (*c*), *Heteranthelium piliferum* (*d*), *Cristesion chilence* (*e*), *Hordeum vulgare* (*f*), *Cristestion bogdanii* (*g*), *Psathyrostachys juncea* (*h*), *Psa. strigosa* (*i*), *Pseudoroegneria libanotica* (*j*), *Thinopyrum bassarabicum* (*k*), *Th. elongatum* (*l*), *Secale cereale* (*m*), *S. vavilovii* (*n*), *Elymus ciliaris* (*o*), and *Leymus racemosus* (*p*). The amount of DNA loaded per lane was 3 μg from diploids, 6 μg from tetraploids and 9 μg from hexaploids. *M* λDNA digested with *Hin*dIII

GATCGAAGGATTGAAAAAAGGAACAATTTCGCACTTACAGCTCAAAAATA

TATGGGACAACTCATCCGTATGGTCTGTAGAAATATGCACCTTGACCATG

AATTGGGTGAACATGGTGTTAGGCTCACATTAGGGAAGAATCGGTGAACA

AAGAAAAGACAAAATTCACCGTATAGAGGGTATTGAATGAGACTATCAAC

GTGACTCATAACGAGCTCCGTTTGAGTAATTTTTGTAGGTGTTGGAAAGG

TATCGAGGAATTGAGATGATAGGAATTCTAATAGTCATGATC

Fig. 5 The nucleotide sequence of the cloned *Sau*3AI fragment in pHv62. The shaded parts are polypurine-polypyrimidine stretches. *Arrows* indicate CAAAA

The genomic DNA of *H. villosa* was cleaved with 12 restriction endonucleases (including two pairs of isochizomers, i.e., *Mbo*I/*Sau*3AI and *Hpa*II/*Msp*I). After separation in 1.2% agarose gel, digests of *Hpa*II and *Pst*I were concentrated near the sample well (data not shown), indicating the high occurrence of m5CpG and m5CpNpG since these two enzymes are sensitive to these two sites, respectively. Probing with the pHv62 insert, Southern blots of six-base restriction enzyme digests gave a ladder signal pattern (Fig. 6), of which *Eco*RI and *Eco*RV were very typical. The shortest fragment was estimated to be 386 bp in length. A *Hin*dIII digest produced the most prominent signal in this fragment, suggesting that the *Hin*dIII site is more conserved because the ladder resulted from base-pair mutation. Thy hybridization pattern of *Msp*I was also in typical ladders, quite different from that of its isochizomer *Hpa*II, in which the signal was completely anchored at the top of gel. This suggests that the pHv62 repeated sequence in *H. villosa* was heavily methylated at the cytosine of the CpG dimer. *Mbo*I and its isochizomer *Sau*3AI produced a similar hybridization pattern with the signals concentrated at positions 94 bp, 292 bp and 386 bp. This provides evidence that little methylation had occurred in the adenine.

In situ hybridization: metaphase chromosomes of *H. villosa* and the *T. turgidum-H. villosa* amphiploid were hybridized with clone pHv62. Six of the seven chromosome pairs of *H. villosa* gave signals at 24 sites, most of which were localized in telomeric or sub-telomeric regions. As expected, all wheat chromosomes were devoid of detection in the amphiploid. This confirmed the results of Southern hybridization and proved that the pHv62 repeated sequence is tandemly organized in the V genome.

Application of pHv62 in monitoring gene *Pm21*

Gene *Pm21*, a wheat powdery mildew resistance gene conferred by the chromosome arm 6VS of *H. villosa*, has been transferred to wheat in the form of a 6AL-6VS translocation (Qi et al. 1993). To explore possible use of pHv62 in tagging such resistance, Southern blots of the translocation lines were probed. Eleven translocation lines gave signals similar to those of Add6V and Sub6V (Fig. 7). Dot-blot hybridization of 30 F_2 plants from a cross of the translocation line and a susceptible wheat cultivar produced 20 positives and ten negatives (data not shown), which was in complete agreement with the segregation of the resistance gene. This may be attributed to the fact that 6VS hardly ever pairs and recombines with its wheat homoeologue 6AS (Qi et al. 1993).

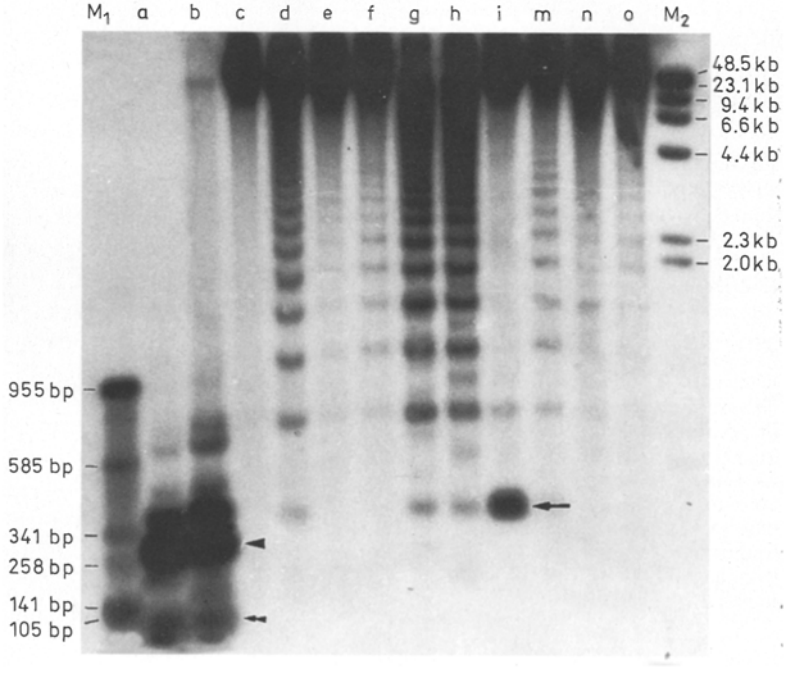

Fig. 6 Hybridization of pHv62 with Southern blots of *H. villosa* genomic DNA (4 µg) cleaved with *Mbo*I (*a*), *Sau*3AI (*b*), *Hpa*II (*c*), *Msp*I (*d*), *Bam*HI (*e*), *Bgl*II (*f*), *Eco*RI (*g*), *Eco*RV (*h*), *Hin*dIII (*i*), *Pst*I (*j*), *Pvu*II (*k*), and *Xho*I (*l*). *M1* pUC19 plasmid digested with *Sau*3AI; *M2* uncleaved λDNA and *Hin*dIII-digested λDNA. *Arrow*, *arrow head* and *double arrow head* indicate the fragments of 386 bp, 292 bp and 94 bp, respectively

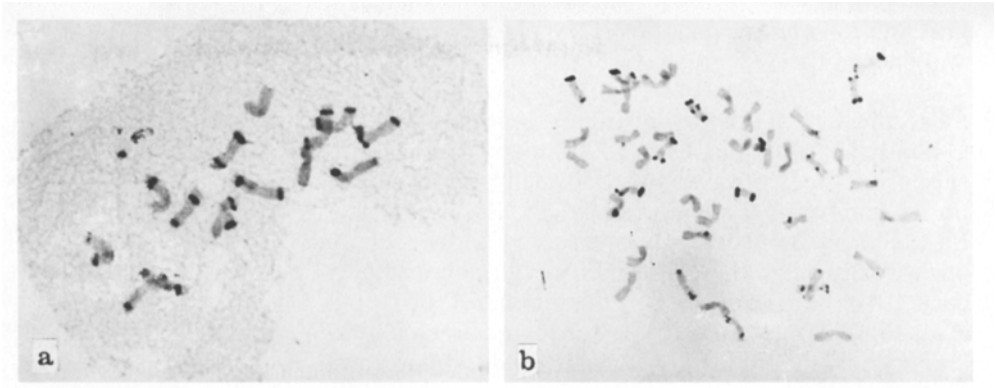

Fig. 7 In situ hybridization of pHv62 with chromosomes of *H. villosa* (**a**), and amphiploid *Triticum turgidum* × *H. villosa* (**b**)

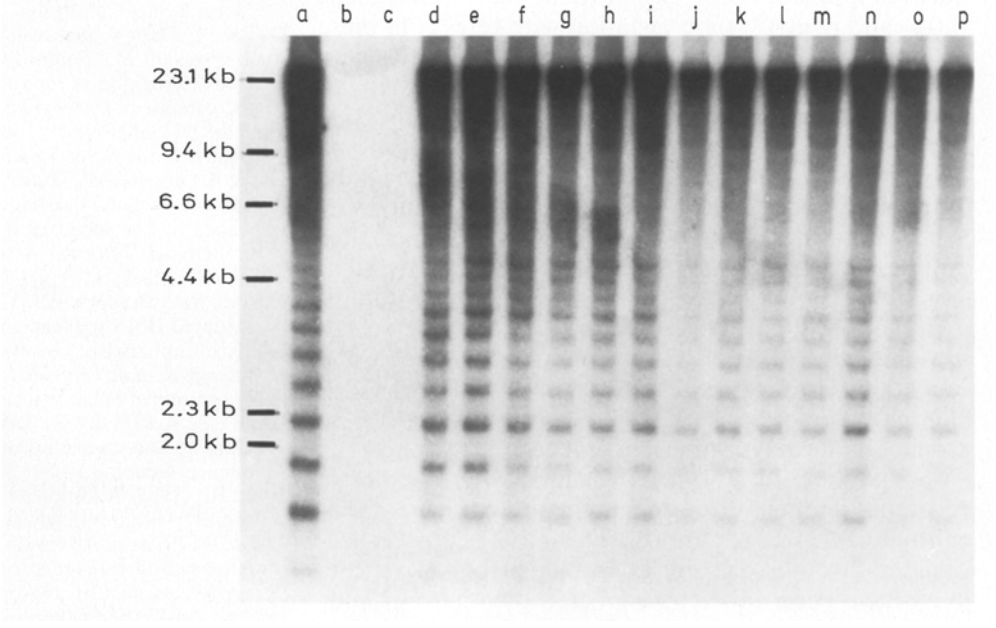

Fig. 8 Hybridization of pHv62 with Southern blots of *Eco*RI digests of *H. villosa* (**a**), CS (**b**), wheat YM5 (**c**), Add6V (**d**), Sub6V (**e**), and 6AL-6VS translocation lines (**f**) *through* (**p**). The amount of DNA loaded per lane was 4 µg from *H. villosa* and 10 µg from the rest

Discussion

Comparison of pHv62 with other repeated DNA sequences

Many species-specific repeated DNA sequences have been isolated from species of the Triticeae. In the context of tandem repetitive sequences, the R350 family has been characterized in most detail (Bedbrook et al. 1980; Appels et al. 1981; Jones and Flavell 1982). PHv62 and pAeSBK52, an S-genome-specific repeated sequence from *Triticum speltoides* (Anamthawat-Jonsson and Heslop-Harrison 1993), have 67% and 65% homology to pSc7235 (a member of the R350 family), respectively. As pHv62 failed to hybridize with the S-genome species of *Triticum* in Southern blots, it clearly lacks homology to pAeSKB52. This might be explained by the homology of the two sequences to two different regions of pSc7235 or else to variation in different directions. Nevertheless, they do share some other features; for example, they both occupy a sub-telomeric location, and are characterised by a lack of hybridization signals in probing blots of wheat. This may also indicate their possible evolutionary relationship. The considerable size variation and wider distribution in the Triticeae imply that the pHv62 repeated sequence has been the subject of a more ancient amplification, and hence a longer exposure for base-pair mutation after amplification, as compared with pAeSKB52. Another noteworthy characteristic of these three repeated sequences is a duplication of CAAAA, a putative motif responsible for a breakage-reunion mechanism (Appels et al. 1986). The conservation of the CAAAA duplication might confirm its important role in the amplification of these sequences.

In *H. villosa*, another specific repeated sequence, p380, was extracted by De Pace et al. (1992). pHv62 is similar to p380 in hybridization pattern and monomeric unit length, but lacks homology to it. This is inferred from their hybridization with blots of rye, i.e., p380 did not produce a hybridization signal in a dot blot of rye (De Pace et al. 1992) whereas pHv62 did (Fig. 4). Therefore, pHv62 is a new repeated sequence specific to *H. villosa*.

Distribution and localization of the pHv62 repeated sequence in the V genome

Since the first discovery of highly repeated DNA in C-bands (Pardue and Gall 1970), many studies have supported the hypothesis that heterochromatin is composed mainly of tandemly repeated DNA sequences. In the tribe Triticeae, some repeated sequences are anchored around the centromeres (Gerlach and Peacock 1980), some are close to the telomeres (Bedbrook et al. 1980; Appels et al. 1981; Jones and Flavell 1982; Tsujimoto and Gill 1991; Anamthawat-Jonsson and Heslop-Harrison 1993), and others are interstitial (Tsujimoto and Gill 1991). However, few repeated sequences coincide with all the heterochromatin sites. Intra- and inter-genome variation with respect to these sites has also been observed. pCbTag4. 14, a repeated sequence specific to the H genome, occurred in all seven chromosomes of the H genome of *Cristesion bogdanii*, but in only six chromosomes of the H^t genome of *Elymus trachycaulus*, the exception being $5H^t$ (Tsujimoto and Gill 1991). The authors attributed this variation to the loss of the pCbTag4.14 sequence after polyploidization. In the present study, Southern and in situ hybridization have demonstrated that the amplification of pHv62 also occurred in six of the seven chromosome pairs of *H. villosa*; chromosome 7V being the exception. However, unlike the case of CbTag4.14 *H. villosa* is a diploid. Several possible explanations for the absence of the pHv62 repeated sequence in chromosome 7V are possible. First, 7V may be unusual in respect of its resistance to integration, and/or the subsequent amplification by unequal recombination, of this sequence. Second, the chromosome block occupied by pHv62 repeats must have been either translocated to other chromosomes or deleted in evolution. Third, base-pair mutation of this repeated sequence in 7V might have occurred so frequently that little homology has remained. A clarification of these possibilities will be a subject for further study.

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