Mol Gen Genet (2000) 263: 741–751
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ORIGINAL PAPER

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Two new families of tandem repeats isolated from genus *Vicia* using genomic self-priming PCR

Received: 15 December 1999 / Accepted: 8 March 2000

Abstract A modified genomic self-priming technique was used for rapid isolation of tandem repeats from several *Vicia* species. Based on homologies of their nucleotide sequences the newly isolated clones were assigned to two repeat families named VicTR-A and VicTR-B. Both families are rich in AT (74%) and are organized as long blocks of tandemly repeated units. The VicTR-A repeats are characterized by a monomer size of 69 bp, whereas the VicTR-B repeat monomer is about 38 bp long, and the two families do not share significant sequence homology. VicTR sequences show different degrees of amplification (up to 10^6–10^7 copies/haploid genome) in individual *Vicia* species and are not amplified in other legumes. The abundances of these repeats do not correlate with genome sizes but are similar in species that belong to the same taxonomic section within the genus *Vicia*. Primed in situ (PRINS) labeling of metaphase chromosomes of *V. pannonica* revealed that VicTR-A sequences are located predominantly in the telomeric regions of the short arms of all chromosomes. In contrast, labeling of VicTR-B repeats in *V. sativa* resulted in mainly intercalary bands of various intensities and only weak telomeric signals.

Key words Tandem repeats · Satellite DNA · Genomic self-priming PCR · *Vicia* · Primed in situ DNA labeling (PRINS)

Communicated by R. G. Herrmann

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Introduction

Repetitive DNA sequences arranged in tandem arrays are ubiquitous in higher plant genomes. Based on the length of the repeat monomer they are usually classified as microsatellites (1–5 bp), minisatellites (up to 40 bp) or satellites (varying lengths, the most frequent are 140–180 and 300–360 bp). The satellites are also characterized by high copy numbers and arrangement in long arrays spanning up to 100 Mb (Charlesworth et al. 1994; Schmidt and Heslop-Harrison 1998). Apart from a few exceptional cases (Vogt 1992), no function or coding capacity has been found to be associated with the majority of the tandem repeats. In this respect it is striking that individual families of tandem repeats can comprise up to 20% of the nuclear genomes of some plant species (Ingham et al. 1993), corresponding to 10^6–10^7 copies per haploid genome (Kato et al. 1984; Ingham et al. 1993; Irifune et al. 1995).

A typical feature of tandem repeats is their preferential amplification in a single species or a limited number of related species (Crowhurst and Gardner 1991; Schmidt et al. 1991; Unfried et al. 1991; King et al. 1995; Maggini et al. 1995; Schmidt and Kudla 1996; Fahleson et al. 1997; Miller et al. 1998; Nouzová et al. 1999). This has led to their utilization as genome-specific molecular markers for the characterization of interspecific somatic hybrids (Schweizer et al. 1988; Pehu et al. 1990; Forsberg et al. 1994; Stadler et al. 1995; Calderini et al. 1997; Fahleson et al. 1997) and in taxonomic and evolutionary studies (De Kochko et al. 1991; Wu and Wu 1992; Ingham et al. 1993; Vershinin et al. 1994; King et al. 1995; Helm and Hemleben 1997; Alix et al. 1998; Nagaki et al. 1998). When used as a probe for in situ hybridization, some tandem repeats produce labeling patterns that allow the discrimination of chromosomes within a karyotype. This can be used for karyotyping and other cytogenetic studies (Fuchs et al. 1994; Brandes et al. 1995; Busch et al. 1996; Cuadrado and Jouve 1997; Kamstra et al. 1997), and for

the preparation of purified fractions of individual chromosome types using flow sorting (Macas et al. 1995; Pich et al. 1995).

In spite of the growing interest in satellite DNA in recent years, relatively few data are available that throw any light on the possible role(s) of these sequences in genome evolution and function. Therefore, isolation of new repeats from various plant taxa is required, especially for studies of mechanisms of satellite DNA amplification and to allow an assessment of the possible contribution of such amplification to the remarkable size differences between plant genomes (Bennett and Leitch 1995, 1997; Bennett 1998). The isolation of new tandem repeats can be achieved by several techniques, based on specific properties of these sequences (the arrangement of multiple copies of repeat monomers in a head-to-tail orientation, differences in AT/GC content, conserved length of subrepeats). These methods include centrifugation in density gradients (Deumling 1981; Peacock et al. 1981), isolation of prominent bands from restriction enzyme-digested genomic DNAs fractionated by gel electrophoresis (Kato et al. 1984; Martinez-Zapater et al. 1986; Schweizer et al. 1988; Ingham et al. 1993; Nakajima et al. 1996; Nouzova et al. 1999), the cloning of high-molecular-weight "relic" DNA remaining after enzymatic digestion (Bedbrook et al. 1980; Metzlaff et al. 1986; Belostotsky and Ananiev 1990; Maggini et al. 1991; Salina et al. 1998), and screening of genomic DNA libraries (Metzlaff et al. 1986; Schweizer et al. 1988; Simoens et al. 1988; Dong et al. 1998). Recently, a new technique termed genomic self-priming PCR (GSP-PCR) has been described (Buntjer and Lenstra 1998) that is based on interactions of tandem repeat units in a modified PCR setup. These authors have shown that in a mixture of sheared and high-molecular-weight genomic DNA subjected to PCR without primers, the tandem repeats anneal together and are repeatedly extended. This leads to their amplification in the form of very long concatemers that can be visualized after gel electrophoresis as a smear of high-molecular-weight DNA fragments. Digestion of these amplification products with a restriction endonuclease that cuts within an amplified repeat sequence facilitates its cloning.

In this study we have applied GSP-PCR to thirteen legume species belonging to the genus *Vicia*. This genus is an interesting model for the study of plant genome evolution since it contains species that differ by about 7.5-fold in the size of their nuclear genomes (Bennett and Leitch 1995, 1997). One possible reason for these differences is variation in the proportions of satellite DNAs; however, only three types of tandem repeat (*Fok*I, TIII15 and IGS-related elements) have been identified up to now, all in the genome of the field bean (*V. faba* L.) (Kato et al. 1984; Maggini et al. 1991; Nouzová et al. 1999). We have refined the GSP-PCR technique by using restriction enzyme-digested instead of randomly sheared DNA; this modification led to improved sensitivity and to the isolation of two new families of tandem repeats. Sequence composition, genomic organization, abundance, and distribution among related legume species was studied for both of these repeat families.

Materials and methods

Plant material and genomic DNA isolation

Seeds of field bean (*V. faba* ssp. *faba* var. *equina* Pers., $2n = 12$) cv. 'Inovec' were obtained from Dr. M. Vavák (Horná Streda, Slovakia). Seeds of the other species were from the legume gene bank at Agritec, Sumperk, Czech Republic [*V. narbonensis* L., *Phaseolus vulgaris* L., *Pisum sativum* L., *Pisum elatius* (M.B.) Stev., *Glycine max* (L.) Merr., *V. sativa* L., *Lupinus angustifolius* L., *Cicer arietinum* L.] or from the IPK, Gatersleben, Germany [*V. melanops* Sibth. and Smith, *V. lathyroides* L., *V. lutea* L., *V. michauxii* Sprengel, *V. pannonica* Crantz, *V. grandiflora* Scop., *V. peregrina* L., *V. hybrida* L., *V. villosa* Roth, *V. sepium* L., *Vigna unguiculata* (L.) Walp.]. Total genomic DNA was extracted from leaves as described by Dellaporta et al. (1983). All DNA concentration measurements were done using the PicoGreen dye (Molecular Probes) according to manufacturer's recommendations.

Genomic self-priming PCR

Genomic DNA (35 ng/μl) was incubated at 100 °C for 15 min in order to break high-molecular-weight molecules into smaller fragments (Buntjer and Lenstra 1998). Alternatively, the DNA was digested with one of the following restriction endonucleases: *Alu*I, *Mbo*I, *Rsa*I, *Taq*I, or *Tru*9I, and adjusted to a concentration of 30 ng/μl. Two microliters of heat- or restriction enzyme-treated DNA were added, together with 35 ng of intact genomic DNA, to a PCR mix (total volume 30 μl) consisting of 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.2 U of *Taq* DNA polymerase (Promega). The reaction was carried out in a Techne Cyclogene cycler and involved 35 cycles of 95 °C for 30 s, 55 °C for 2 min, and 72 °C for 2 min. Cycling was preceded by an initial denaturation step (95 °C, 2 min), and followed by a final extension step (72 °C, 10 min). The reaction products were then analyzed by agarose gel electrophoresis (the products of tandem repeat self-priming formed smears of very low mobility due to their high molecular weight). The amplification products were digested with an appropriate restriction nuclease, and the resulting bands were visualized on 10% native polyacrylamide gels stained with a 1:10,000 dilution of SybrGreen (Molecular Probes) by scanning using a Storm scanner (Molecular Dynamics).

Cloning and analysis of amplified sequences

The amplification products digested with *Tru*9I were separated from longer (uncleavable) background fragments present in the reaction mix using molecular weight cut-off column (Microcon-100, Millipore). The fragments that passed through the column were filled-in using Klenow enzyme, dephosphorylated, and cloned, using the Zero Blunt TOPO kit, into the pCR4Blunt-TOPO plasmid vector (Invitrogen). Resulting clones were sequenced by the dideoxy chain termination method (Sanger et al. 1977). Nucleotide sequences were searched for homology to sequences in the GenBank database (release 114) using BLASTN or BLASTX 2.0.2 (Altschul et al. 1997) or FASTA 3.2 (Pearson and Lipman 1988). Comparisons between the sequences, as well as various sequence analyses (searches for direct and inverted repeats, secondary structure analysis), were performed using PC/Gene 6.60 (IntelliGenetics) and Dot-plot 3.0 (created by Ramin Nakisa, Oxford University). Dinucleotide frequencies were determined

according to Burge et al. (1992), using a computer program written in Turbo Pascal.

Dot-blot and Southern hybridizations

Dot-blot and Southern blot hybridizations were performed on Hybond-N+ membranes (Amersham). To estimate the copy numbers of newly isolated repeats in the genomes of individual species, serial dilutions of genomic DNAs corresponding to 50–10^5 haploid genomes were dot-blotted, together with 10^7–5×10^9 copies of clones of the respective repetitive sequences (haploid genome sizes of individual species were taken from the Angiosperm DNA C-Values Database Release 2.0, http://www.rbgkew.org.uk/cval/database1.html). The clones used as standards were N7 and P5 (both containing two subrepeats of the VicTR-A sequence) and S1 (containing five VicTR-B subrepeats). The same clones were used as hybridization probes. Following hybridization, signal densities of the individual dots were compared and dilutions of genomic and standard (plasmid) DNAs giving the same signals were identified. On the basis of these values the copy numbers of individual probes in genomic DNAs were estimated. Calculations of the percentage of a genome occupied by individual sequences were based on length and estimated copy numbers of individual probes, assuming that 1 pg of genomic DNA equals 9.65×10^8 bp (Bennett and Smith 1976).

For Southern hybridizations, 1.5 μg of digested genomic DNA was fractionated on 1.5% or 2.0% agarose gels and blotted onto membranes by capillary transfer. The inserts selected as hybridization probes were amplified from the plasmids by PCR using T3 and T7 primers, and digested with *Eco*RI to remove surrounding polylinker sequences. The fragments were gel-purified, labeled with alkaline phosphatase and hybridized using the AlkPhos Direct Kit (Amersham) according to manufacturer's recommendations (hybridization and washing temperatures were 55 °C). Signal detection was done by blot incubation with chemiluminescent substrate (CDP Star, Amersham) followed by exposure of the membranes to X-ray film for periods ranging from 3 min to several hours at room temperature.

Primed in situ DNA labeling (PRINS)

Squash preparations of *V. sativa* and *V. pannonica* mitotic chromosomes were prepared from synchronized root tip meristems as described by Leitch et al. (1994). Cell cycle synchronization and accumulation of cells in metaphase was done according to Doležel et al. (1992) with the following modifications: hydroxyurea treatment was carried out for 18 h at 1 mM concentration for *V. sativa* and at 0.75 mM for *V. pannonica*, the time in the medium without hydroxyurea was 2 h, and metaphase accumulation was achieved using 15 μM oryzalin for 4 h.

The PRINS reaction was performed using Frame-Seal chambers and a PTC-200 thermal cycler equipped with a Twin Tower block (MJ Research). The reaction mix (65 μl) contained 1× PCR buffer, 4 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP, 0.017 mM dTTP, 0.008 mM fluorescein-dUTP, 0.2 μM primers, and 5.2 U of *Taq* polymerase (Promega). The primers were derived from the consensus sequences of VicTR-A (5′-TACATAA-AAGTCAYGAAGTT-3′ and 5′-TASTATAACAYAAGAYAA-TC-3′) and VicTR-B (5′-ATATAAGTCTTCARAAAAT-3′ and 5′-GAAGACTTATATTCACTT-3′). The temperature profile consisted of a denaturation step (94 °C, 3 min), primer annealing (40 or 45 °C, 5 min), and primer extension (72 °C, 30 min). The heating rate between the primer annealing and extension steps was 0.1 °C/s. The reaction was stopped by rinsing the slides in 0.5 M TRIS-HCl, 0.05 M EDTA (pH 8) at room temperature. The chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole, 2 μg/ml) and observed using a Nikon Eclipse-600 epifluorescence microscope equipped with a CCD camera. DAPI and fluorescein signals were collected separately using UV-2A and B-2A filter sets and further processed using LUCIA software (Laboratory Imaging).

Results

Isolation of new tandem repeats using refined GSP-PCR

GSP-PCR was tested on 13 *Vicia* species selected because they differ widely in genome size (ranging from 2.3 pg/1C to 13.3 pg/1C) and on the basis of their assignment to various taxonomic sections within the genus. When the the reaction was carried out using mixtures of heat-degraded and intact genomic DNAs as described by Buntjer and Lenstra (1998) clear amplification products were obtained only from *V. faba* DNA (Fig. 1A). Restriction digestion of the amplified DNA using *Taq*I resulted in a ladder of fragments (monomer size 59 bp) showing a size distribution corresponding to that of the previously described FokI elements (Kato et al. 1984) (not shown). Subjecting the reaction mixtures to two additional reamplifications failed to yield any products in any of the samples except for *V. faba*.

We therefore attempted to increase the sensitivity of the technique by replacing the randomly fragmented DNA with DNA that had been digested with various restriction endonucleases. In theory, if a tandem repeat contains a recognition site for a given endonuclease then digestion with that enzyme should produce many short

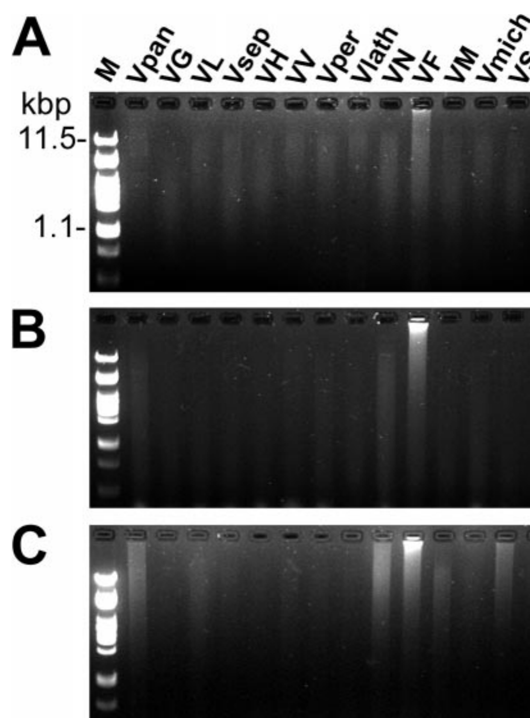

Fig. 1A–C Products of genomic self-priming PCR, generated using heat-fragmented or restriction enzyme-digested genomic DNA. The reactions were performed using high-molecular-weight DNAs from individual species, mixed with the same DNAs degraded either by boiling (**A**) or by digestion with *Taq*I (**B**) and *Tru*9I (**C**). The products were resolved on an ethidium bromide-stained 1.5% agarose gel. The abbreviations for the species names correspond to those used in Table 1. M, λ DNA digested with *Pst*I

fragments that will be in molar excess over other fragments, and therefore could serve as more efficient primers. We tested five endonucleases selected because their recognition sites are expected to occur frequently and cleavage is insensitive to DNA methylation. The reactions with the samples digested with *Alu*I, *Mbo*I, and *Rsa*I did not yield any products, except for a very weak smear in *V. faba*. Using genomic DNAs digested with *Taq*I resulted in generation of clearly visible amplification products in *V. faba*, which were obviously stronger than those generated in the reaction with randomly fragmented DNA (Fig. 1B). As expected, digestion of these products gave a pattern typical for the FokI repeats (Fig. 2, lane 1). GSP-PCR using DNA digested with *Tru*9I produced smears in the samples of *V. pannonica*, *V. narbonensis*, *V. faba* and *V. sativa* (Fig. 1C). Digestion of these amplification products with *Tru*9I generated distinct bands in the samples from *V. pannonica*, *V. narbonensis*, and *V. sativa*, whereas no bands were detectable in the *V. faba* sample (Fig. 2, lanes 2–5). The most prominent bands obtained in *V. pannonica* and *V. narbonensis* ranged from about 50 to 200 bp, with some of the bands being common to both species. Fragments released by the digestion of the *V. sativa* sample appeared in a ladder pattern with the band spacing of about 40 bp and with the most prominent band at about 120 bp (Fig. 2). These short fragments were cloned in bulk from all three samples, and clones with insert lengths corresponding to the bands visible on the gel were selected for sequencing.

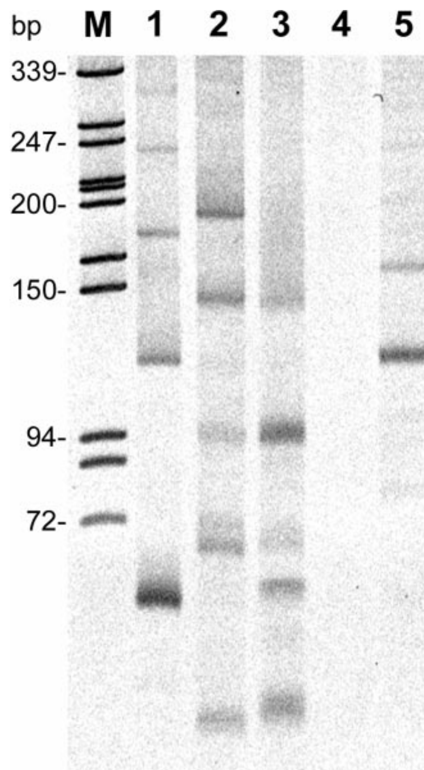

Fig. 2 Bands produced by restriction enzyme digestion of GSP-PCR products. Lane 1, products of GSP-PCR with *Taq*I-digested *V. faba* DNA; lanes 2–5, products of GSP-PCR with *Tru*9I-digested DNAs from *V. pannonica* (lane 2), *V. narbonensis* (lane 3), *V. faba* (lane 4), and *V. sativa* (lane 5). Following the reaction, the products were purified and digested with *Taq*I (lane 1) or *Tru*9I (lanes 2–5). Samples were fractionated by electrophoresis on 10% polyacrylamide and visualized by staining with SybrGreen

Sequence analysis

A total of sixteen clones were sequenced (five or six each from three species – *V. narbonensis*, *V. pannonica* and *V. sativa*; Fig. 3) and their sequences were subjected to computer analysis. FASTA and BLAST searches of nucleotide sequence databases did not find significant homologies between the newly isolated repeats and other known sequences. Based on their mutual homologies, the clones were divided into two groups, named VicTR (Vicia Tandem Repeat) A and B, respectively. Group A comprised all eleven clones isolated from *V. narbonensis* and *V. pannonica*, whereas all five clones obtained from *V. sativa* belonged to group B.

The clones belonging to the VicTR-A group were 48–186 bp long and their alignment revealed their tandem sequence organization. Based on this alignment a consensus sequence of 69 bp was reconstructed (Fig. 3A). The sequence is AT-rich (74% AT) and does not contain any obvious subrepeats except for a duplicated 7-bp motif (AAATTTG), one copy of which is highly conserved in all clones. As depicted on Fig. 3A, the *Tru*9I restriction site may occur at any of several positions within the repeat monomers, and thus the observed clone lengths did not correspond to simple multiples of the consensus sequence. Pairwise homology scores for individual clones containing at least one full repeat ranged from 37 to 97% and the degree of homology of individual clones to the consensus sequence ranged from 59 to 88% (average 76%).

The five VicTR-B clones were 118–232 bp long and contained three to six tandemly arranged repeat units (Fig. 3B). Sequence comparisons with the sequences obtained in the course of other experiments performed in our laboratory (M. Nouzová et al., manuscript in preparation) revealed sequence homology between these clones and one isolated from a *V. sativa* genomic library. This clone (GS38) contained eight repeated units and was included in a multiple sequence alignment with the sequences obtained by GSP-PCR. The consensus sequence derived from this analysis was 38 bp long (74% AT) and, similarly to VicTR-A, the *Tru*9I restriction site could occur at any of several positions (Fig. 3B). Pairwise homology scores of individual repeats were 58–97% and the degree of homology to the consensus ranged from 76 to 97% (average 88%). The homology values for the subrepeats from one clone were not significantly higher than those for repeats from independent clones; this was also true for comparisons between the genomic clone GS38 and the clones isolated by the GSP-PCR (data not shown). Interestingly, the same 7-bp motif as

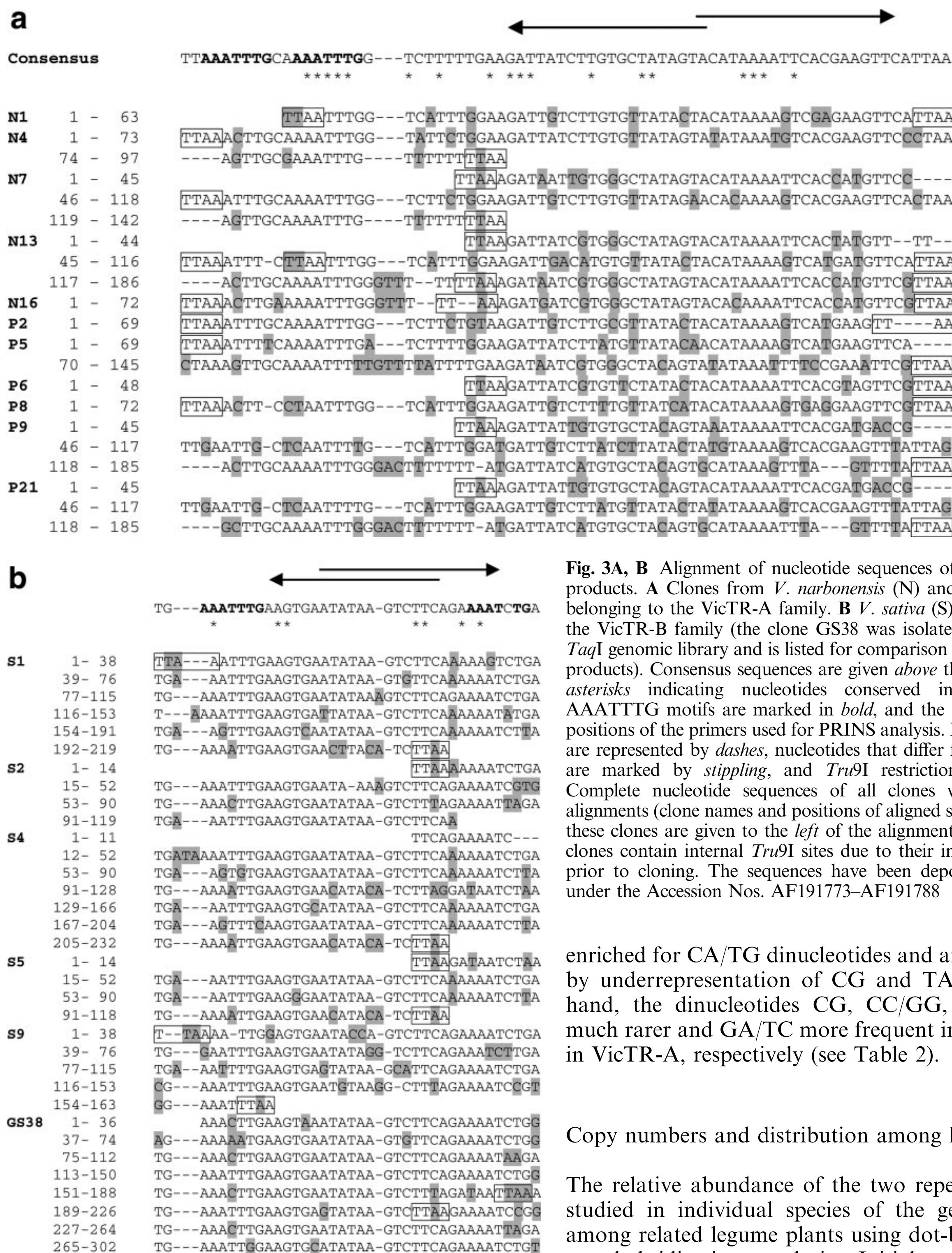

Fig. 3A, B Alignment of nucleotide sequences of cloned GSP-PCR products. **A** Clones from *V. narbonensis* (N) and *V. pannonica* (P), belonging to the VicTR-A family. **B** *V. sativa* (S) clones assigned to the VicTR-B family (the clone GS38 was isolated from a *V. sativa* *Taq*I genomic library and is listed for comparison with the GSP-PCR products). Consensus sequences are given *above* the alignments, with *asterisks* indicating nucleotides conserved in all clones; the AAATTTG motifs are marked in *bold*, and the *arrows* indicate the positions of the primers used for PRINS analysis. Missing nucleotides are represented by *dashes*, nucleotides that differ from the consensus are marked by *stippling*, and *Tru*9I restriction sites are *boxed*. Complete nucleotide sequences of all clones were used for the alignments (clone names and positions of aligned subsequences within these clones are given to the *left* of the alignments). Note that some clones contain internal *Tru*9I sites due to their incomplete digestion prior to cloning. The sequences have been deposited in GenBank under the Accession Nos. AF191773–AF191788

in VicTR-A sequences is present in VicTR-B, once as the sequence AAATTTG and once with a 1-bp substitution (AAATC̲TG) (Fig. 3B). Although they have similar AT/GC ratios and share the aforementioned 7-bp motif, we could not detect any other similarities between the sequences of the A and B families. However, analysis of dinucleotide frequencies showed that both families are enriched for CA/TG dinucleotides and are characterized by underrepresentation of CG and TA. On the other hand, the dinucleotides CG, CC/GG, and GC were much rarer and GA/TC more frequent in VicTR-B than in VicTR-A, respectively (see Table 2).

Copy numbers and distribution among legume species

The relative abundance of the two repeat families was studied in individual species of the genus *Vicia* and among related legume plants using dot-blot and Southern hybridization analysis. Initial experiments had shown that none of the repeats was detectable in the genomes of *C. arietinum* (chickpea), *G. max* (soybean), *L. angustifolius* (narrow-leaved blue lupine), *P. vulgaris* (kidney bean) or *V. unguiculata* (cowpea), and that they were present in about several hundred or less copies in *P. sativum* (garden pea) and *P. elatius*, and at very variable copy numbers among the *Vicia* species (data not shown). Copy numbers per haploid genome were then

determined more precisely for the *Vicia* species; they ranged from undetectable amounts up to 10^5–10^6 for VicTR-A and up to 10^6–10^7 for VicTR-B, respectively (Table 1). The degree of repeat amplification was not correlated with genome size. It was, however, similar in species that belong to the same systematic sections. For example, all four species from the section Hypechusa contained highly amplified VicTR-A and moderate or low copy numbers of the VicTR-B repeats. In contrast, in the two species from the section Vicia only the VicTR-B family is highly amplified, and neither of the two families is amplified in the species from the section Peregrinae (Table 1).

The proportion of the repeats in individual genomes can be estimated from the length of the probes, their copy numbers and the genome sizes of the species under study. These calculations (using the mean values for copy numbers given in Table 1) show that VicTR-B sequences comprise very large portions of the genome in some species (about 25% in *V. sativa*, 17% in *V. grandiflora*, 2.4% in *V. sepium*, and 1.5% in *V. narbonensis*). VicTR-A repeats, on the other hand, represent only up to about 1% of the genome in the species in which they are most abundant (*V. hybrida*, *V. lutea*, *V. melanops*, *V. pannonica* and *V. narbonensis*).

Genomic organization

The distributions of VicTR-A and B repeats in the genomes of individual *Vicia* species were studied by restriction digestion and Southern hybridization. The VicTR-A-specific probe revealed only limited number of bands when *Tru*9I-digested genomic DNAs were used (Fig. 4A). In the extreme case of *V. narbonensis* only two very strongly hybridizing bands were detected, corresponding to the monomer and dimer of the consensus sequence (about 70 and 140 bp long). Patterns were

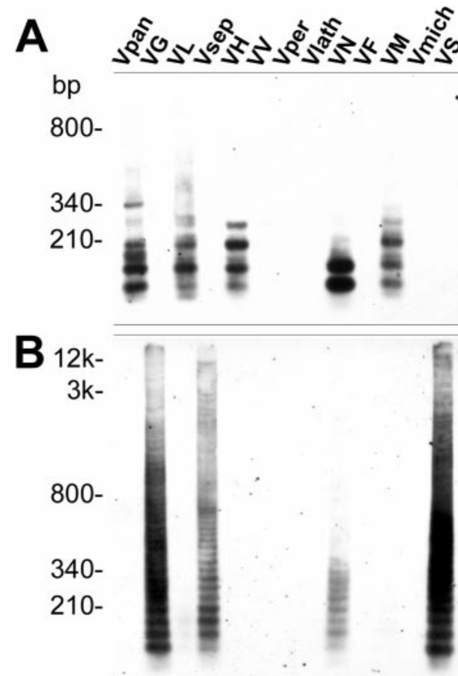

Fig. 4A, B Molecular organization and abundance of newly isolated repeats in the genomes of individual *Vicia* species. A Southern blot of *Tru*9I-digested genomic DNAs resolved on a 2% agarose gel was hybridized with labeled clone N7 (VicTR-A repeat family, **A**), and with clone S1 (VicTR-B family, **B**)

Table 1 Distribution of VicTR-A and B repeats within *Vicia* species

Section[a]	Species	Code	2*n*[b]	1C (pg)[b]	Abundance[c]		
					N7	P5	S1
Cracca	*V. villosa*	VV	14	2.3	+++	++	–
Hypechusa	*V. hybrida*	VH	12	6.8	++++	+++	–
	V. lutea	VL	14	7.4	++++	+++	++
	V. melanops	VM	10	10.0	++++	++++	++
	V. pannonica	Vpan	12	6.8	++++	++++	(+)
Narbonensis	*V. narbonensis*	VN	14	7.3	++++	++++	++++
Vicia	*V. grandiflora*	VG	14	3.4	+	(+)	+++++
	V. sativa	VS	12	2.3	–	–	+++++
Attosa	*V. sepium*	Vsep	14	4.7	–	–	++++
Wiggersia	*V. lathyroides*	Vlath	12	2.6	–	–	++
Faba	*V. faba*	VF	12	13.3	–	–	+
Peregrinae	*V. michauxii*	Vmich	14	8.3	–	–	–
	V. peregrina	Vper	14	9.5	(+)	–	–

[a] The species are sorted according to taxonomic sections recognized in the *Vicia* genus (Kupicha 1976; Maxted 1995)

[b] Chromosome numbers (2*n*), haploid DNA content (1C), and abundance estimates for three hybridization probes are given

[c] Abundance is indicated in copy numbers of hybridized fragments per haploid genome [+++++, 10^6–5×10^6; ++++, 10^5–10^6; +++, 10^4–10^5; ++, 10^3–10^4; +, 100–1000; (+), ~ 100; –, < 100]. The probes N7 and P5 represent homologous clones of the VicTR-A repeats isolated from *V. narbonensis* and *V. pannonica*, respectively; the probe S1 belongs to the VicTR-B repeat family isolated from *V. sativa* (see Fig. 3 for a detailed description of the clones). Note that N7 and P5 contain two and S1 contains five monomer units

more complicated in other species, but in all cases hybridization was mostly restricted to fragments less than 340 bp in size. Analysis using other enzymes showed the higher-order organization of the VicTR-A repeats, which was reflected in band patterns spaced by 140 bp (Fig. 5A). These patterns were conserved in all species in which VicTR-A sequences were abundant and differed only in intensities of the individual bands.

In contrast to the VicTR-A probe, the probe specific for VicTR-B detected prominent ladder patterns in the genomic DNAs digested with *Tru*9I (Fig. 4B). The ladders with spacing corresponding to the repeat monomer ranged up to multimers several kilobases in size. However, no higher-order patterns were detected in *V. sativa*, *V. sepium* or *V. narbonensis* when other restriction enzymes were used. The only exception was seen with DNA from *V. grandiflora*, which showed ladders of bands spaced by about 180 bp on digestion with three out of the six enzymes tested (Fig. 5B).

In order to investigate the long-range genomic organization of the newly isolated repeats, we determined their location on mitotic chromosomes using primed in situ DNA labeling (PRINS). *V. pannonica* and *V. sativa* were selected for this study since they contain abundant VicTR-A and VicTR-B repeats, respectively. The PRINS reaction using VicTR-A specific primers on *V. pannonica* chromosomes resulted in strong signals in telomeric and/or subtelomeric regions of the short arms of all chromosomes. Additional weaker signals were detectable at telomeres of the long arms in the majority of the chromosomes and as intercalary bands within the long arms of two chromosome pairs (Fig. 6A).

The use of VicTR-B-specific primers produced bands of various intensities on all *V. sativa* chromosomes. Based on these band patterns all six chromosome types could be distinguished (Fig. 6B). Most bands were located in intercalary regions of the long arms of acrocentric chromosomes and they were absent or of very low intensity on a pair of metacentric chromosomes. All chromosomes showed additional signals in the telomeric regions of their short arms.

Discussion

Genomic self-priming PCR was first applied to genomic DNA of selected mammalian and bird species. Besides amplification of several known repeats, this technique led to identification of a novel satellite specific for ostrich (Buntjer and Lenstra 1998). The data presented in this paper demonstrate the feasibility of GSP-PCR for rapid isolation of tandem repeats in plants. The method performed as described in the original paper (Buntjer and Lenstra 1998) generated amplification products only in one (*V. faba*) of thirteen *Vicia* species subjected to analysis. Based on the presence of *Taq*I restriction sites and the monomer size of 59 bp these products were identified as the already known FokI elements (Kato et al. 1984). FokI elements are among the most abundant plant repeats described so far (2.5×10^7 copies per haploid genome) and were found to be present in high copy numbers only in *V. faba* (Kato et al. 1984; Maggini et al. 1995). The FokI repeats are readily amplified by GSP-PCR, regardless of the DNA fragmentation method. Amplification products similar to those generated from heat-fragmented DNA (Fig. 1A) were present even in the *V. faba* DNA samples digested with endonucleases that do not cut in the majority of FokI genomic sequences (*Alu*I, *Mbo*I, *Rsa*I, and *Tru*9I). Analysis of GSP-PCR products from *Rsa*I-digested DNA confirmed the presence of FokI repeats (data not shown), and the amplification of these repeats in the *Tru*9I-treated sample is therefore the most likely explanation for the absence of bands after product digestion with this enzyme (Fig. 2, lane 4).

Replacement of randomly sheared DNA by restriction endonuclease-digested DNA improved the sensitivity of the technique and resulted in amplification of

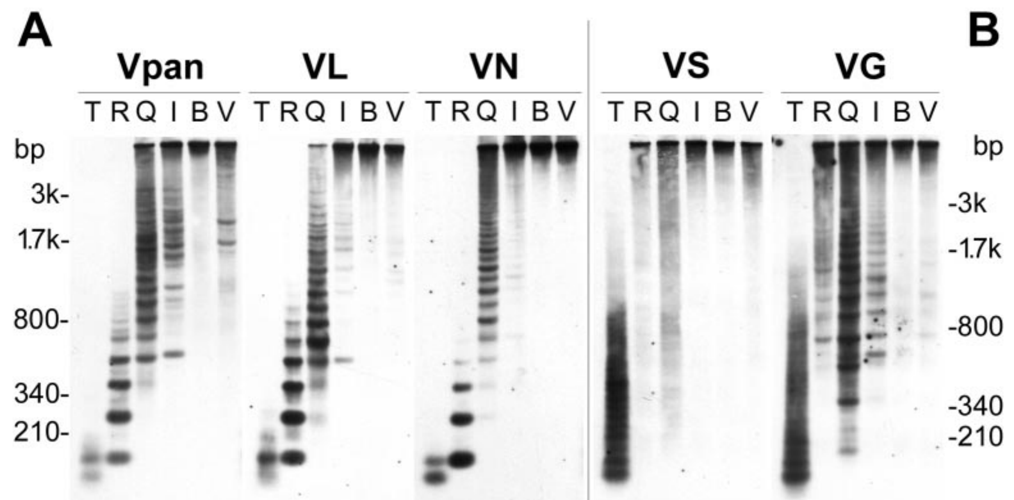

Fig. 5A, B Genomic organization of VicTR-A and B repeats. Southern blots of genomic DNAs digested with various restriction enzymes and resolved on a 1.5% agarose gel were probed with the labeled clones N7 (**A**) and S1 (**B**). Enzymes used for DNA digestion were: T, *Tru*9I; R, *Rsa*I; Q, *Taq*I; I, *Eco*RI; B, *Bam*HI; V, *Eco*RV. Abbreviations of the species names are given *above* the blots

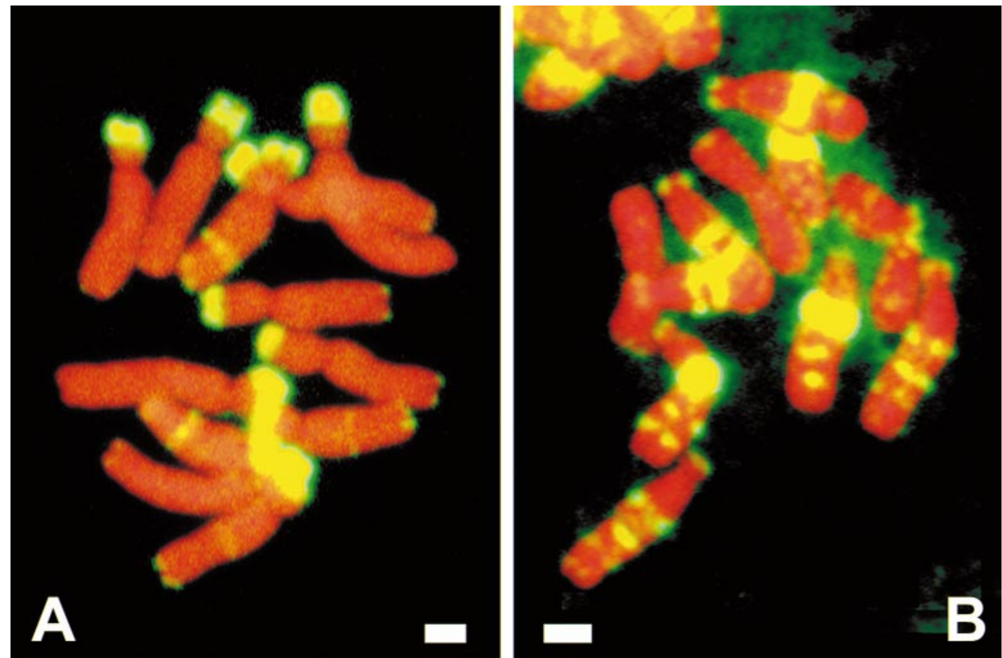

Fig. 6A, B PRINS labeling of metaphase chromosomes. The repeats were localized on chromosomes by direct incorporation of fluorescein-labeled nucleotides in the PRINS reaction, using primers specific for the respective repeat families. **A** *V. pannonica* chromosomes ($2n = 12$) labeled using VicTR-A specific primers. **B** *V. sativa* chromosomes ($2n = 12$) after the reaction using VicTR-B specific primers. Fluorescein-labeled sites appear yellow-green, the chromosomes are counterstained with DAPI (displayed in red for better contrast with the fluorescein signal). Bar = 1 μm

satellite DNAs from three additional species. However, it is obvious from the estimated abundance of the two newly isolated repeats that successful amplification by GSP-PCR does not depend solely on the contribution made by a given satellite to the genome of a given species. Thus, VicTR-A repeats were amplified only from *V. pannonica* and *V. narbonensis*, and no products were obtained from the samples of three species that contain similar proportions (about 1%) of VicTR-A sequences in their genomes (*V. hybrida*, *V. lutea*, *V. melanops*). VicTR-B repeats were amplified only from *V. sativa* DNA, where they comprise about 25% of the genome, but not from *V. grandiflora* in which they are also very abundant (17%). Furthermore, in *V. narbonensis*, where the VicTR-A and -B families are of similar abundance (1% and 1.5%, respectively), only the former was found among amplified and cloned sequences. A similar failure to amplify certain satellite DNAs was also reported by Buntjer and Lenstra (1998) and probably represents a feature typical for GSP-PCR. This difference might theoretically be explained by differences in the degree of heterogeneity of satellite DNAs in individual species and by the interactions of tandem repeats with other genomic sequences during the reaction. However, more experimental data is needed to prove this hypothesis. Therefore, while easy and straightforward cloning of tandem repeats represents the main advantage of the GSP-PCR technique, the absence of reaction products cannot be taken as evidence for the absence of abundant satellite(s) in a genome under study.

The VicTR sequences have one of the highest A + T contents (74%) found among plant tandem repeats. A similar proportion of A + T has been reported only for *Dra*I repeats in *V. unguiculata* (Galasso et al. 1995). VicTR-B sequences also belong to the tandem repeats with the shortest monomers, together with 34-bp repeats of *Scilla siberica*. In contrast to VicTR-B, these repeats contain only 22% A + T (Deumling 1981).

Although both VicTR repeat families are AT rich and have some common sequence features, it is not clear whether they originated from the same ancestral sequence. Apart from sharing the same 7-bp subrepeats, no other significant homologies were found. Moreover, the two families diverge significantly in the frequencies of several dinucleotide motifs and in the length of their monomers (Table 2). On the other hand, the VicTR-A

Table 2 Dinucleotide frequencies in VicTR repeat families

Family[a]	Input[a]	Dinucleotide									
		AA/TT	AC/GT	AG/CT	AT	CA/TG	CC/GG	CG	GA/TC	GC	TA
VicTR-A	C	1.22	0.90	0.90	0.85	1.35	0.43	0.85	1.05	1.70	0.64
	A	1.19	1.06	0.83	0.85	1.31	0.98	0.67	0.93	1.03	0.71
VicTR-B	C	1.07	0.54	1.36	0.97	1.36	0	0	2.17	0	0.39
	A	1.14	0.77	1.24	0.86	1.24	0.40	0.25	1.73	0.20	0.52

[a] The frequencies of all possible dinucleotides were calculated for consensus sequences (rows C) and for the sum of all complete monomer units present in the sequenced clones (rows A). The method of Burge et al. (1992), which takes account of both DNA strands and is therefore independent of sequence orientation was used for the calculations. The values diverging from 1 indicate over- (> 1) or under-representation (< 1) of the given dinucleotide

monomers (69 bp) could have originated as dimers of the shorter VicTR-B repeats (38 bp) provided some other rearrangements including deletions/insertions were involved in their evolution. There are indications that different mechanisms of homogenization may act on tandem repeats localized in different chromosomal regions (Vershinin et al. 1995). The VicTR-A and B repeat families differ in their chromosomal locations, the former being present mostly in telomeric regions whereas the latter form mainly intercalary bands on metaphase chromosomes (Fig. 6). It is interesting that the 7-bp motif present in both VicTR families (AAATTTG) was found to be conserved also in the *Olea europaea Sau*3AI tandem repeat family (Katsiotis et al. 1998). Moreover, the *Sau*3AI and VicTR repeats also share a subsequence, CAAAA, that is supposed to be involved in a breakage-reunion mechanism of repeated sequences (Katsiotis et al. 1998 and references therein). There is evidence that oligo A/T tracts are major centers of DNA curvature in tobacco GRS and NTRS tandem repeats (Gazdová et al. 1995; Matyášek et al. 1997). The presence of such motifs in presumably unrelated repeat families suggests their significance either for molecular mechanisms of tandem repeat amplification and maintenance in a genome, or for the determination of specific chromatin properties of loci containing satellite DNA.

Our results indicate that, during evolution, the VicTR families were amplified to high copy numbers only in the genomes of certain *Vicia* species. The copy numbers of VicTR-A and B repeats reach 10^6 and 10^7 per haploid genome, approaching the highest values reported for plants (FokI in *V. faba*: 2.5×10^7/1C, Kato et al. 1984; PG140 in *Pennisetum glaucum*: 3.5×10^6/1C, Ingham et al. 1993; pAfi100 in *Allium fistulosum*: 2.8×10^6/1C, Irifune et al. 1995). However, the abundance of the repeats does not correlate with genome size (Table 1). Thus, the different copy numbers of VicTR repeats alone do not account for genome size variations among the species investigated in this study.

In contrast to FokI and TIII15 sequences, which have been amplified only in *V. faba* genome (Maggini et al. 1995; Nouzová et al. 1999), the VicTR families are abundant in several species of the genus *Vicia*. Genus-specific tandem repeats are common in plants, and most of them are present in variable numbers in individual species within a genus (Wu and Wu 1992; King et al. 1995; Helm and Hemleben 1997). The degree of satellite amplification has been used as an additional molecular evidence for the evolutionary relationships among species in the genera *Cucurbita* (King et al. 1995), *Cucumis* (Helm and Hemleben 1997) and *Beta* (Kubis et al. 1997). The data obtained for the VicTR repeats are consistent with the taxonomy of the genus *Vicia* and it is likely that their quantification in genomes of a broader range of species would help to assess phylogenetic divergences of individual sections in this genus.

The banding patterns produced by labeling of VicTR repeats could provide a tool for karyotyping and evolutionary studies of *Vicia* chromosomes. Like the A001-I and D32-13 probes in *Alstroemeria* (Kamstra et al. 1997) and FokI elements in *V. faba* (Fuchs et al. 1994; Macas et al. 1995; Pich et al. 1995), the VicTR-B repeats produce patterns that can be used for identification of individual *V. sativa* chromosomes. If they are conserved among related species, these patterns would facilitate identification of homologous chromosomes among species containing abundant VicTR-B sequences. Such comparisons would be interesting, especially for species that differ in chromosome numbers, like *V. sativa* ($2n = 12$) and *V. grandiflora* ($2n = 14$).

Acknowledgements We thank Dr. M. Vavák (Horná Streda, Slovakia), Dr. U. Pich (IPK, Gatersleben, Germany), and Dr. M. Griga (AGRITEC, Sumperk, Czech Republic) for the seeds of *Vicia* and other legume species; Ms. H. Štěpančíková and Ms. O. Šonková for excellent technical assistance, and Ms. S. Rafelski for assistance in preparation of the manuscript. This work was supported by grant No. 521/96/K117 from the Grant Agency of the Czech Republic.

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