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The Structure, Amount and Chromosomal Localisation of Defined Repeated DNA Sequences in Species of the Genus *Secale*

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Abstract. The structure, copy number and chromosomal location of arrays of four families of highly repeated sequences have been investigated in representative species of the genus *Secale*. The four unrelated families, previously characterised in *Secale cereale*, have repeating units of 480, 610, 630 and 120 base pairs respectively. The following general conclusions can be drawn in addition to detailed knowledge of the sequence content of heterochromatin in each accession studied: (1) Every species is unique in its complement or chromosomal distribution or both of the four highly repeated sequence families. *S. montanum* and *S. cereale* accessions studied here show the same complement of repeated sequences, but they differ substantially in the amounts they contain of the 610 and 630 base pair (bp) families, and in the distribution over the chromosomes of the 480 bp family. The structure of the repeating unit is also different in many members of the 480 bp family in *S. montanum*. – (2) The substantial differences between species in the amounts of the most highly repeated DNA sequences exist in the absence of any such conspicuous differences in most other repeated sequences which were detected as fluorescent bands after restriction enzyme digestion and gel electrophoresis. – (3) Each of the different highly repeated families can exist independently of the other families, though all the families have telomeric sites. Also, in the outbreeding species, heteromorphisms are frequent, and are particularly conspicuous in hybridisation detecting the 480 bp sequence family. – (4) The association of the highly repeated sequences with heterochromatin, discussed in the accompanying paper is generally true for other species in the genus, and the lower amounts of heterochromatin in other *Secale* species compared to *S. cereale* are associated with lower amounts of specific families of highly repeated DNA sequences. – (5) Analysis of highly repeated sequence families is likely to provide an easy method of identification of new accessions of *Secale*.

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Introduction

To understand more about the molecular basis of chromosome structure, evolution and variation it is often useful to carry out comparisons between chromosomes from related species which have been characterised by cytogeneticists. The genus *Secale* is composed of such species. *Secale* chromosomes are very suitable for cytogenetical analysis and can be characterised in detail at pachytene (Lima-de-Faria, 1952), or in mitosis. There are several stocks carrying different B chromosomes and autotetraploids have been produced (Jain, 1960). Addition lines have been constructed, placing each rye (*S. cereale*) chromosome pair separately in a wheat genetic background (Riley and Chapman, 1958). Many studies have been made of the numbers of multivalents in meiosis of F_1 hybrids between different taxa. This approach has provided data for theories about the evolution of the genus, and particularly about the evolution of *S. cereale* from other species (Khush and Stebbins, 1961; Khush, 1962, 1963a, b; Stutz, 1972; Kranz, 1976; Singh and Röbbelen, 1977). However the number of distinct species in the genus is still contentious.

Following the discovery that rye telomeric heterochromatin could be visualised by C-banding (Sarma and Natarajan, 1973), the C-banding technique has been extended to other *Secale* species (Singh and Röbbelen, 1975), and C-value differences between the different species have been analysed (Bennett et al., 1977).

Appels et al. (1978) were the first to report the presence of highly repeated DNA sequences in *S. cereale* telomeric heterochromatin. The structures of four families of highly repeated sequences found in *S. cereale* heterochromatin were characterised through molecular cloning by Bedbrook et al. (1980a) and the structure of a fifth type of repeat was described more recently (Bedbrook et al., 1980b).

The molecular cloning of members of these families of *S. cereale* repeats provided probes for detecting homologous repeats in other genomes. Other species of the *Secale* genus have therefore now been examined for the presence, structure and chromosomal locations of these families. The studies were undertaken (1) to see if there was any substantial correlation between changes in the complement and/or chromosomal location of highly repeated DNA sequences and presumed speciation events; (2) to understand better the evolution of defined repeated sequences in related but different genetic backgrounds (species); (3) to test throughout the genus the correlation between the presence of tandem arrays of defined repeated sequences and a "*heterochromatic*" chromatin structure, and (4) to investigate the value of repeated sequences for sorting out phylogenetic relationships between species or assigning new accessions to defined species.

Five of the six fundamental *Secale* species described by Stutz (1972) have been studied, accepting the view that *S. anatolicum* is a *S. montanum* type. Another, and as yet poorly characterised, species, *S. iranicum* (Kobylyanskii 1975) was also included to see if analysis of its repeated sequence DNA provided interesting taxonomic clues.

Materials and Methods

Plant Genotypes

DNA for restriction enzyme analysis was extracted from accessions of five of the six major species defined by Stutz (1972). These were *S. cereale* (Petkus),
S. montanum (Guss ssp montanum PBI R99, Leningrad WiR 9598),
S. vavilovii (Grossh from Dr. A. Kranz),
S. silvestre (Host (syn *S. fragile*) PBI R52), and
S. africanum (Staff PBI R102, Univ. Manitoba 2D-127).
A sixth was also included, *S. iranicum* (described by Kobylyanskii, V.D., 1975; PBI R105, Leningrad WIR 10431) which is as yet poorly characterised taxonomically.

In situ hybridisation was carried out to chromosomes of several different accessions of *S. cereale, S. montanum* (including *S. anatolicum*) and *S. africanum*, in addition to the ones from which DNA was extracted. They were taken from the collection of rye accessions maintained at the Plant Breeding Institute, Cambridge:
S. africanum PBI R107, Karoo desert,
S. cereale L. ssp. ancestrale Zhisk, PBI R27,
S. cereale L. ssp. dighoricum (Vav) Rosher, PBI R40,
S. montanum PBI R15, Pania, Hungary 1-S-14-1,
S. anatolicum Boiss PBI R98, Leningrad WIR 10086,
S. montanum spp. Kuprijanovii Grossh PBI R100, Leningrad WIR 10053,
S. montanum PBI R101,
S. vavilovii PBI R30,
S. vavilovii PBI R11.

These *Secale* taxa will all cross pollinate. Precautions were taken to maintain purity (e.g. bagging together ears of different plants of the same accession), but the possibility that some cross fertilisation had given rise to some of the polymorphisms observed cannot be completely excluded.

DNA Extraction

Plants were grown to about 10 cm high in pots. Green leaves were harvested and frozen in liquid nitrogen prior to storage at −80° C. The tissue was ground to a powder in a coffee grinder and added to (5 ml/gm plant tissue) 0.1 M NaCl 50 mM EDTA pH 8.5 at 4° C (Graham 1978). The slurry was rapidly made up to 2% SARKOSYL, 1 M $NaClO_4$ and 0.1% diethyl pyrocarbonate. 0.5 vol of redistilled phenol and 0.5 vol of $CHCl_3$/isoamyl-alcohol were added, and the homogenate stirred at 4° C for 1 h. The aqueous phase was recovered by centrifugation at 8000 rpm for 15 min and re-extracted with $CHCl_3$/isoamyl alcohol and then recentrifuged. The DNA was recovered from the second supernatant by ethanol precipitation, and redissolved in 4–10 ml (depending on the quantity of DNA) of 50 mM Tris/10 mM EDTA, pH 8.0, containing 1 mg/ml pronase which had been autodigested for 2 h before use. After shaking for 2 to 5 h (or until the DNA had completely dissolved) at 37° C in a siliconised beaker, the solution was brought to 8.4 ml (or a multiple "n" thereof), "n" × 8 g of CsCl and "n" × 0.5 ml of a 10 mg/ml Ethidium bromide solution were added and gently mixed. The DNA was banded by centriguation at 40 K rpm for 48 h and recovered. The DNA was then recentrifuged in another caesium chloride-ethidium bromide gradient. Ethidium bromide was extracted from the recovered DNA band shaking repeatedly with amyl alcohol saturated with 10 mM Tris pH 8.0. The DNA solution was then dialysed against 10 mM Tris/0.25 mM EDTA, pH 8.0, and stored at 4° C.

Restriction Enzyme Digestion of DNA, Electrophoresis, Transfer to Paper and cRNA/DNA or DNA/DNA Hybridisation

Eco RI, Hind III, Taq I and Hae III were purified in this laboratory. DNA digested by restriction endonucleases was fractionated by electrophoresis on either agarose gels as described previously (Bedbrook et al. 1980a) or on 6% acrylamide gels (Maniatis et al., 1975a). DNA

from agarose gels was transferred to millipore nitrocellulose sheets for hybridisation analysis as described by Southern (1975) or to diazotised cellulose paper as described by Wahl et al. (1979). Lower molecular weight fragments were fractionated before transfer on composite 0.75% agarose/6% acrylamide gels and transferred to diazotized cellulose paper as described by Alwine et al. (1977). ^{32}P cRNA was prepared on chimaeric plasmid templates using *E. coli* RNA polymerase as described in Bedrook et al. (1980a). Plasmids were labelled by nick translation as described by Maniatis et al. (1975b). All hybridisations were carried out in 50% formamide 5 × SSC 1% glycine, 0.1% SDS 0.1 M sodium phosphate buffer pH 6.3, 1 × Denhardts solution (1966) and 100 μg/ml *E. coli* rRNA or denatured salmon sperm DNA. ^{32}P cRNA was hybridised to the nitrocellulose filters, but for hybridisations to DBM paper, nick translated DNA was used. These latter hybridisations were done in the presence of 10% dextran sulphate which substantially improved the signal to noise ratios and shortened the autoradiographic exposure times (Wahl et al., 1979).

In situ Hybridisation and Karyotype Analysis

In situ hybridisation using ^{3}H-labelled cRNAs made on chimaeric plasmid templates was carried out as described in Bedbrook et al. (1980a) as amended in Jones and Flavell (1982).

Recombinant DNA Plasmids Containing S. cereale Repeated DNA Sequences

Hybridisation probes were made from the following recombinant DNA plasmids whose isolation and detailed characterisation is described in Bedbrook et al. (1980a): (a) pSC 210 which contains pACYC 184 and a 480 bp *S. cereale* repeat from a family which constitutes about 6% of the *S. cereale* genome; (b) pSC 34 which contains pBR 322 and a 610 bp repeat from a family which constitutes about 3% of the *S. cereale* genome; (c) pSC 119 which contains pBR 322 and a 120 bp sequence from a family which constitutes about 2% of the *S. cereale* genome; (d) pSC 33 which contains pBR 322 and a 630 bp repeat which hybridises to sequences occupying about 0.5% of the *S. cereale* genome.

A fifth plasmid pSC 310 contains pACYC 184 and a 2200 bp repeat from *S. cereale*. This 2200 bp contains a few copies of the 120 bp sequence homologous to that in pSC 119, and other, unrelated sequences (Bedbrook et al., 1980b).

Results

I. Restriction Enyzme, Gel and "Blot" Hybridisation Analysis

A. Comparison of Repeated Sequences in *Secale* Species by Digestion of DNA with Restriction Enzymes

DNAs isolated from *S. vavilovii, S. iranicum, S. africanum, S. montanum, S. cereale* and *S. silvestre* were first compared by restriction enzyme analysis. When the DNAs were restricted with Eco RI or Hind III, and fractionated on agarose gels (results not shown), all showed very similar fragment distributions although minor variations could be discerned. The fragment size distributions of *S. cereale* DNA isolated from nuclei of embryos and from green leaves were indistinguishable, indicating that chloroplast and mitochondrial DNA constitute only a small proportion of total leaf DNA.

Densitometer traces of photographic negatives of the Hind III digests were used to compare the amount of each DNA spared by the enzyme and consequently left at the limiting mobility of the gel (so-called relic DNA – Bedbrook et al., 1980a). This DNA in *S. cereale* is highly enriched for

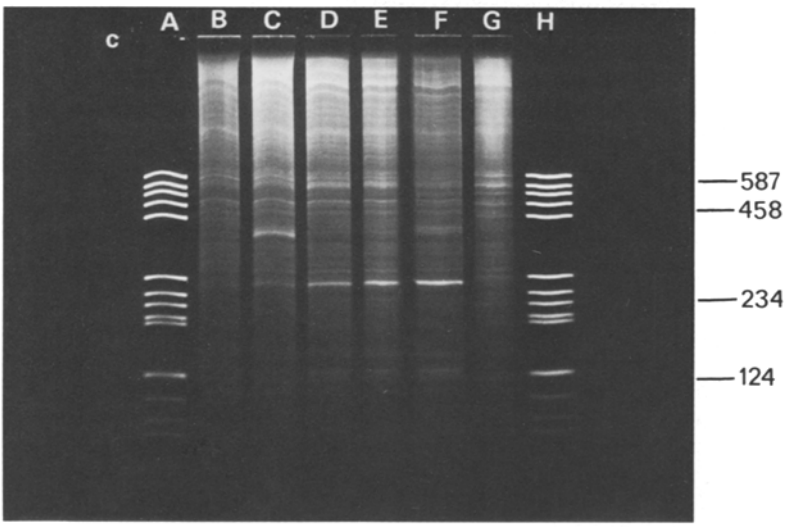


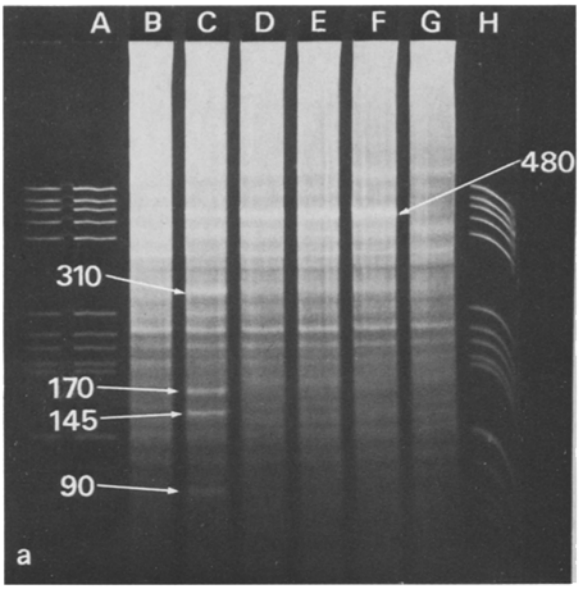
Fig. 1. Electrophoretic fractionation of *Secale* DNAs digested with Hae III. In the tracks are 2 μg of DNA from *S. africanum* (B), *S. montanum* (C), *s. vavilovii* (D), *S. iranicum* (E), *S. cereale* (F). *S. silvestre* (G) and 1 μg of Hae III digests of pBR 322 DNA for marker fragments (A and H). These marker fragments are respectively 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80 etc. bp (Sutcliffe, 1978), as shown on the figure

the four families of repeated sequence featured in this paper (Bedbrook et al., 1980a). The proportion of each DNA which appeared as bands was also estimated. The percentage of relic DNA was about 2.5% for all the species except *S. montanum* (4%) and *S. cereale* (5%). This variation between species in the proportion of DNA appearing as relic was considerably greater than the variation in the proportion of DNA in bands.

Digestion of the different *Secale* DNAs with the restriction enzymes Hae III (Fig. 1.) and Taq (Fig. 2.) which recognise four base sequences, followed by fractionation in 6% acrylamide gels reveals some obvious differences between the species, though extensive similarities in the repeated sequence spectrum are still dominant.

In the Hae III digests ((Fig. 1) all species possessed bands which comigrate with the 115 and 120 bp fragments of *S. cereale* (track F; see the legend for sizes of marker fragments) and *S. silvestre* (track G and see also Bedbrook et al. 1980a). The strong band in *S. cereale* DNA digests at 240 bp and two other bands at 127 and 138 bp have counterparts in *S. iranicum* (track D) and *S. vavilovii* (track E) and a weak counterpart in *S. montanum* (track C). In contrast, the strongest band in *S. montanum* DNA digests is at about 380 bp which has only a weak counterpart in *S. cereale*. Otherwise, all the digests look very similar. The bands in the *S. silvestre* track which comigrate with the marker fragments are due to the inclusion of a small amount of pBR322 marker DNA digested with Hae III to assess (for the purpose of fragment size analysis) the effects of differing salt concentrations on fragment migration.

In the Taq I digests (Fig. 2a) *S. iranicum* (track E), *S. vavilovii* (track



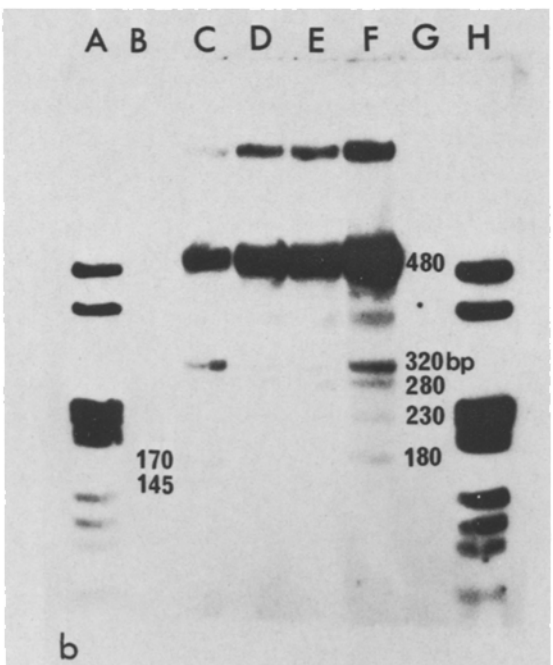


Fig. 2a and b. Electrophoretic separation and hybridisation of pSC 210 to *Secale* DNAs digested with Taq I. The DNAs fractionated in the different tracks are pBR 322 (digested with Hae III) (A and H), *S. silvestre* (B), *S. montanum* (C), *S. vavilovii* (D), *S. iranicum* (E), *S. cereale* (F), and *S. africanum* (G). In (**a**), 1 μg DNA was digested with Taq 1 and fractionated on the gel, while in (**b**) 0.8 μg DNA was digested with Taq I, fractionated on a composite gel and transferred to DBM paper prior to hybridisation with nicktranslated pSC 210 DNA. This probe is not homologous to all fragments of pBR 322 digested with Hae III (tracks A and H) because the Taq I fragment of the 480 bp repeat is cloned in the vector plasmid pACYC 184. The 587, 540 and 458 bp and 267 bp fragments of pBR 322/Hae III digests do not hybridise with pACYC 184. The fact that in the composite gel the major site of hybridisation migrates more slowly than the (largest) 504 bp marker band, whereas it migrates faster in the acrylamide gel illustrates the problem of different relative fragment mobilities in composite agarose/polyacrylamide and pure polyacrylamide gels

D) and *S. cereale* (track F) DNAs showed very strong bands at 480 bp, while this band was weak in *S. montanum* (track C), and very weak or absent in *S. africanum* (track G) and *S. silvestre* (track B) DNAs. *S. montanum* but none of the other DNA digests showed strong bands of 310, 170, 145 and 90 bp.

B. Hybridisation of Cloned *S. cereale* Repeats to Restriction Enzyme Digests of *Secale* DNAs

DNA of each of the six *Secale* species was digested with Hae III or Taq I, fractionated in composite agarose/polyacrylamide gels, transferred to diazobenzyloxymethyl paper and hybridised with ^{32}P cRNA to pSC 34 or nick translated ^{32}P DNA of pSC 33, pSC 119, or pSC 210. The hybridisation of repeated sequence probes to fragments of the DNA of each species was then detected by autoradiography.

(i) Hybridisation with pSC 210 (480 bp Repeat Family). Nick translated pSC 210 DNA was hybridised to Hae III digests (Fig. 3a) or Taq I digests (Fig. 2b) of DNA from the various *Secale* species. The Hae III digests were not all complete and slightly different quantities of each DNA were fractionated on this gel (Fig. 3a). However, the following conclusions can be drawn. Firstly, *S. iranicum* (track E), *S. cereale* (track F), *S. vavilovii* (track D) and *S. montanum* (track C) possess substantial amounts of the 480 bp family, whereas it cannot be detected in *S. africanum* (track G) and *S. silvestre* (track B), even on a much longer exposure of the autoradiograph (see Bedbrook et al., 1980a). Secondly, the distribution of Hae III sites in the 480 bp repeating unit is identical in *S. iranicum* (E), *S. vavilovii* (D) and *S. cereale* (F) DNAs, but is different in *S. montanum* (C) DNA. A smaller proportion of the copies of this sequence in *S. montanum* possess the Hae III site which leads to fragments of 240 bp and 127 or 138 bp in *S. cereale* DNA. Instead the strongest hybridisation is to fragments of about 380 bp, which is approximately the sum of the (240+127) or (240+138) bp fragments in *S. cereale* (F).

However, some copies of the *S. montanum* 480 bp repeat do appear to contain this Hae III site, because hybridisation does occur to bands of about 240 bp and 130 bp. The reduction in the proportion of members of this family which contain this Hae III site is further corroborated by the very weak hybridisation to 600 bp and 720 bp fragments compared with the hybridisation to fragments of this size in *S. iranicum* and *S. vavilovii* (tracks E and D repectively). These sizes correspond approximately to (480+130) bp and (480+240) bp. This weak hybridisation is in contrast to the hybridisation at 860 bp (480+380) bp which is as strong in the *S. montanum* track (C) as in the *S. vavilovii* or *S. iranicum* tracks. Taken together with the fluorescence data (Fig. 1), this evidence strongly suggests seqeunce divergence of the 480 bp family between *S. montanum*, and the other three species *S. cereale, S. vavilovii* and *S. iranicum*.

Hybridisation to DBM papers carrying DNA after Taq I digestion is shown in Fig. 2b. Two faint hybridisation bands (170 bp and 145 bp) are unique to *S. montanum* (track C), and these correspond with two of the

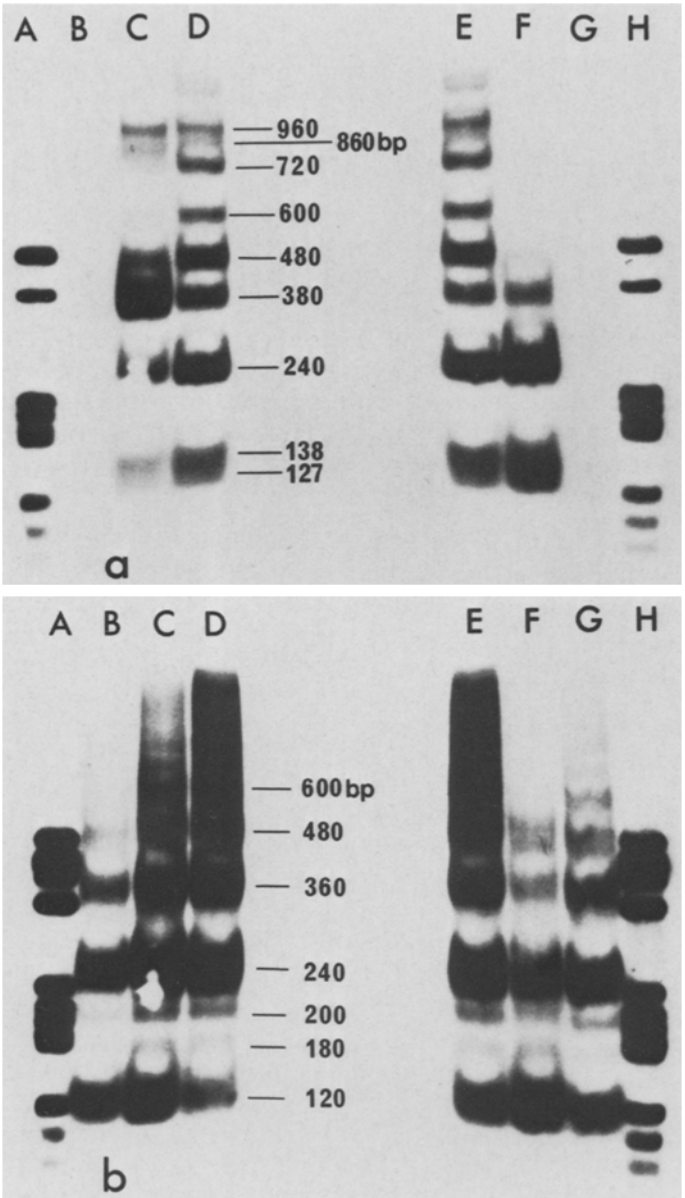


Fig. 3a–b. Hybridisation of pSC 210 and pSC 119 to *Secale* DNAs digested with Hae III. DNAs from *S. silvestre* (track B, 4.2 μg), *S. montanum* (track C, 6.6 μg), *S. vavilovii* (track D, 7.6 μg), *S. iranicum* (track E, 8.0 μg), *S. cereale* (track F, 6.8 μg) and *S. africanum* (track G, 5.6 μg) were digested with enough Hae III to digest completely about 6 μg of DNA and fractionated on composite agarose/polyacrylamide gels prior to transfer to DBM paper and hybridisation with nick-translated probe in the presence of 10% Dextran- Sulphate. For **a** 1.5×10^6 cpm of pSC 210 probe ($\simeq 5 \times 10^6$ cpm/μg) were hybridised for 6 h in 10 ml of hybridisation buffer, and for **b** 2×10^6 cpm of pSC 119 probe ($\simeq 10^7$ cpm/μg) were hybridised for 6 h in 8 ml of hybridisation buffer. Papers were washed through three changes of 2 × SSC/0.1% SDS for 5 min at room temperature, and then for three changes (20 min each) of 2 × SSC/0.1% SDS, 65° C, in 1.5 litres in a shaking water bath. Autoradiography was for 20 h in **a** and 40 h in **b**. The fragment sizes shown in bp were determined from the size markers on the gels stained with ethidium bromide (Fig. 1)

brightly fluorescing bands which are also essentially unique to *S. montanum* digests (Fig 2a). Two other bands on the autoradiograph, at 230 bp and 180 bp, appear to be unique to the *S. cereale* track. However, tracks C, D, E, and F all show some hybridisation to fragments of 280 and 320 bp. This hybridisation is very weak for *S. vavilovii* (track D) and *S. iranicum* (track E) DNAs and quite strong for *S. cereale* (track F) DNA. 280 bp and 320 bp added to 230 bp and 170 bp respectively give about 500 bp i.e. approximately the size of the repeating unit of the family. One might expect then that weak hybridisation to 280 bp and 320 bp fragments would be accompanied by weak hybridisation to 230 bp and 180 bp fragments. In the *S. vavilovii* and *S. iranicum* tracks such hybridisation would probably be below the limit of detection. It is therefore premature to assume that the 230 bp and 180 bp fragments are *S. cereale* specific.

The transfer of DNA from the left lower part of the gel (Fig. 2b) was probably poorer, and this would explain the low hybridisation to the lower molecular weight *S. montanum* fragments, and perhaps explains the absence of hybridisation to a 90 bp fragment visualised by fluorescence in *S. montanum* DNA digests (Fig. 2a). The appearance of the Taq I site in the *S. montanum* 480 family, which does not appear in the 480 bp families of the other three species, provides further evidence for sequence divergence in this family during evolution.

Estimates of the ratios of the copy numbers of the 480 bp repeat in the genomes of *S. vavilovii, S. iranicum* and *S. montanum* to that in *S. cereale* were made by scanning autoradiographs in a densitometer. In the Hae III blot (a shorter autoradiographic exposure of Fig. 3a was used) the ratios were 1:0.8:0.8:0.7 (*S. cereale: S. vavilovii: S. iranicum: S. montanum*) with these figures expressed as optical density per μg of loaded DNA. In hybridisations to blots of two different Taq I digests fractionated on agarose or composite agarose/polyacrylamide gels (Fig. 2b) the ratios were 1:0.7:0.5:0.2 and 1:0.3:0.3:0.3 respectively. This variation between autoradiographs is not unexpected given the difficulties inherent in making the measurements (see legend Table 1). However, it seems reasonable to conclude that the number of 480 bp sequences is highest in the *S. cereale* accession and lowest in the *S. montanum* accession studied.

(ii) Hybridisation with pSC 119 (120 bp Repeat Family). The same Hae III digests of *Secale* DNA which had been transferred to DBM paper and probed with nick translated pSC 210 were also probed with nick translated pSC 119 (after melting off the pSC 210 hybrids in 0.4 M NaOH at 37° C for 30 min). All species (Fig. 3b) contain the 120 bp family. Even faint bands migrating between the monomer and the dimer are held in common. These may be due to rare additional Hae III sites in the unit repeat, although this seems unlikely, because fragments shorter than the monomer were not detected, or they may be due to fragments of the 120 bp repeat unit organised in different conformations with other sequences (Flavell et al., 1980; Bedbrook et al., 1980b).

Scanning the negative of Figure 1 suggests that the 120 bp sequence is present in all species in roughly equal amounts. Parallel in situ hybridisations

Table 1. Approximate copy number of repeats and proportion of the genome in each family in different *Secale* species

Repeated sequence family	*S. cereale*	*S. vavilovii*	*S. iranicum*	*S. montanum*	*S. africanum*	*S. silvestre*
480 bp	10^6 copies 6.1% of genome	$3–8 \times 10^5$ 1.8–4.9%	$3–8 \times 10^5$ 1.8–4.9%	$2–7 \times 10^5$ 1.2–4.3%	Blod	Blod
610 bp	3.5×10^5 2.7%	Blod	Blod	$\simeq 6.5 \times 10^4$ 0.5%	5×10^3 0.04%	Blod
120 bp	1.5×10^6 2.4%	$1–2 \times 10^6$ 1.6–3.2%	1.2×10^6 1.6–3.2%	1.2×10^6 1.6–3.2%	$\simeq 1.5 \times 10^6$ $\simeq 2.4\%$	$\simeq 1.5 \times 10^6$ $\simeq 2.4\%$
630 bp	7.5×10^4 0.6%	$1–5 \times 10^3$ 0.01–0.04%	$1–5 \times 10^3$ 0.01–0.04%	2×10^4 0.16%	Blod	Blod

Blod signifies "*below limit of detection*"

These copy number estimates are derived from those reported for *S. cereale* in Bedbrook et al. (1980a). The *S. cereale* values were determined by renaturation kinetics or measuring the proportion of the total DNA present in specific restriction fragments (Bedbrook et al., 1980a) or both. To derive the values in the table the optical densities of autoradiographs or photographic negatives made from the autoradiographs shown in this paper were scanned, the areas under the curves estimated using a Keichert-Jung Vidorplan and the values for the *Secale* species compared with those for *S. cereale*. The biggest error in this procedure is probably the assignment of the base line to the scans. Estimation of the area itself is probably accurate within 2–5%. Other errors could enter through non-linear response of autoradiographic emulsion or of negative film. However, these ought to affect the different tracks equally in the autoradiographs chosen, and not invalidate comparisons. A more severe problem in the comparisons of tracks in Figures 3 and 4 is that low molecular weight fragments (e.g. 120 bp) might diffuse more rapidly out of the gel prior to transfer and hence lead to an underestimate of the copy number in those genomes whose DNA was completely digested. The minimum copy number which could have been detected in these in situ and "*blot*" hybridisations is probably about 1000 copies per haploid genome of the 610, 480 or 630 bp repeats

(see later) with the same amount of ^{3}H pSC 119 cRNA led to similar grain counts after similar exposure times in all species. However, a densitometer scan of an autoradiograph of the pSC 119 blot hybridisation shown in Figure 3b (but exposed for a shorter time to reduce non-linear responses due to saturation of the emulsion) shows twice as much hybridisation to *S. vavilovii, S. iranicum* and *S. montanum* DNAs as the *S. cereale, S. silvestre* and *S. africanum* DNAs. This might be due to greater losses of smaller fragments from the gel after periodic acid cleavage of the DATD polyacrylamide. It seems reasonable to conclude that the copy number of this sequence does show some but not substantial variation between species.

(iii) Hybridisation with pSC 34 (610 bp Repeat Family). Nick translated pSC 34 showed strong hybridisation to *S. cereale* (Fig. 4a, track F) and weaker hybridisation to *S. montanum* (track C). After long autoradiographic exposure and a higher loading weak hybridisation was also observed to *S. africanum* DNA (track B, Fig. 4b). The sequence was below the limit

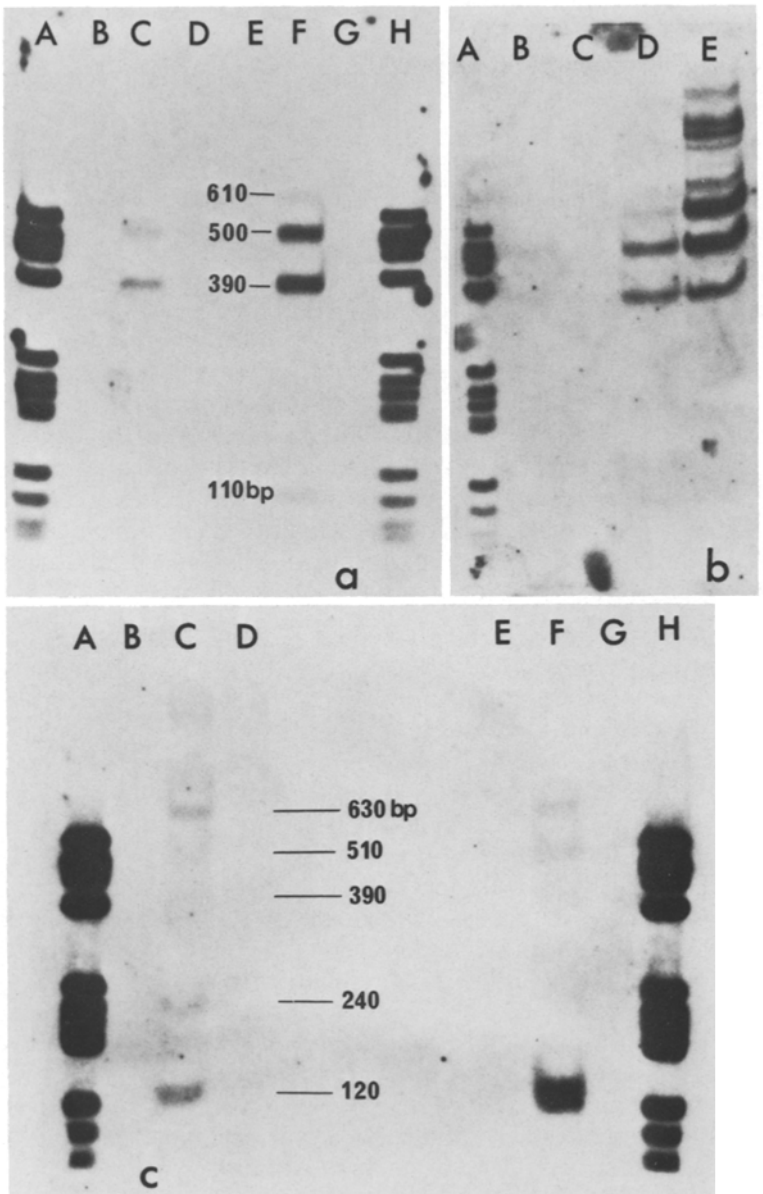


Fig. 4a–c. Hybridisation of pSC 34 and pSC 33 to *Secale* DNAs digested with Hae III. **a** 5 µg of DNA from *S. silvestre* (Track B), *S. montanum* (Track C), *S. vavilovii* (Track D), *S. iranicum* (Track E), *S. cereale* (Track F) and *S. africanum* (Track G) were digested with Hae III and fractionated on a composite agarose/polyacrylamide gel and transferred to DBM paper. Tracks A and H carried 1 µg of pBR 322 DNA digested with Hae III. The paper was hybridised overnight with 8×10^5 cpm of ^{32}P cRNA prepared from pSC 34. Autoradiographic exposure time 40 h. **b** 10^6 cpm of ^{32}P cRNA prepared from pSC 34 were hybridised to 0.5 µg pBR 322 (A), 10 µg *S. africanum* DNA (B), no DNA (C), 5 µg *S. montanum* DNA (D), 5 µg *S. cereale* DNA (partial digest) (E) digested with Hae III fractionated and transferred to DBM paper as in **a**. Autoradiographic exposure time was 1 week. **c** pSC 119 DNA was melted off the DBM paper used for Figure 3a, b and the filter rehybridised with 2×10^6 cpm pSC 33 DNA

of detection in the other three species. The conformation of the sequence is identical in the three species in which it appears. By scanning the negatives of the autoradiographs it was possible to estimate that if there are 3.5×10^5 copies per haploid genome in *S. cereale* DNA (Bedbrook et al., 1980a), then there were approximately 6.5×10^4 copies in *S. montanum* DNA and 5×10^3 in *S. africanum* DNA (Table 1).

(iv) Hybridisation with pSC 33 (630 bp Repeat Family). This sequence was readily detectable in *S. cereale* (Fig. 4c, track F) and *S. montanum* (track C) and also in much lower copy number in *S. vavilovii* (track D) and *S. iranicum* (track E) DNAs. In *S. montanum* the conformation of the sequence is the same as in *S. cereale,* but Alu I digests and hybridisations were not performed to detect the 356 bp variant of this family (see Bedbrook et al. 1980a). These experiments did not permit the detection of this sequence in *S. silvestre* DNA. If *S. cereale* DNA possesses 7.5×10^4 copies of this sequence per haploid genome (Bedbrook et al., 1980a), then *S. montanum* possesses approximately 2×10^4 copies. The quantitative results of these investigations are summarised in Table 1.

(v) Hybridisation with pSC 310 (2,200 bp Repeat Family). Bedbrook et al. (1980b) have described a complex *S. cereale* repeat which has a few copies of a 120 bp sequence interspersed with unrelated sequences in a repeating unit of 2,200 bp. The distribution of this repeat in the six species was investigated using both the clone of the 120 bp sequence (pSC 119) and a Pst/Eco RI fragment of the 2,200 bp repeat as probes to Southern blots of Eco RI and Hind III digests of DNA (results not shown). The results for *S. cereale* and *S. silvestre* have already been published by Bedbrook et al. (1980b). All the DNAs had almost identical electrophoretic banding patterns except *S. silvestre* DNA. This is also shown by the results in Fig. 5 where five *Secale* DNAs were hybridised to the complete 2,200 bp repeat (pSC 310) after restriction with Hind III. Careful scrutiny of the autoradiograph shows that the *Secale* species *cereale* (c), *vavilovii* (v) and *iranicum* (i) have identical hybridisation patterns and *S. montanum* (m) differs only in not possessing a band of 1.95 kb. *S. silvestre* (s) however, shows five detectable differences from the other *Secale* species. It lacks hybridisation bands of 0.66, 1.06, 1.95 and 5.10 kbp and has an additional band of 2.25 kb. These results suggest that *S. silvestre* is more distantly related to other *Secale* species than the other species are to each other.

II. In situ Hybridisation Analysis Using pSC 210, pSC 119, pSC 33 and pSC 34 as Probes

The results are presented as ordered karyotypes except where few chromosomes are labelled. The characteristics of the *S. cereale* karyotype are defined in the accompanying paper (Jones and Flavell, 1982), but two accessions defined by karyotype analysis of F_1 hybrids (Stutz, 1972) to be *S. cereale* types are included here. These are *S. cereale* ssp *dighoricum* and *S. cereale* ssp *ancestrale* (see Khush, 1963a). All the karyotypes were constructed by

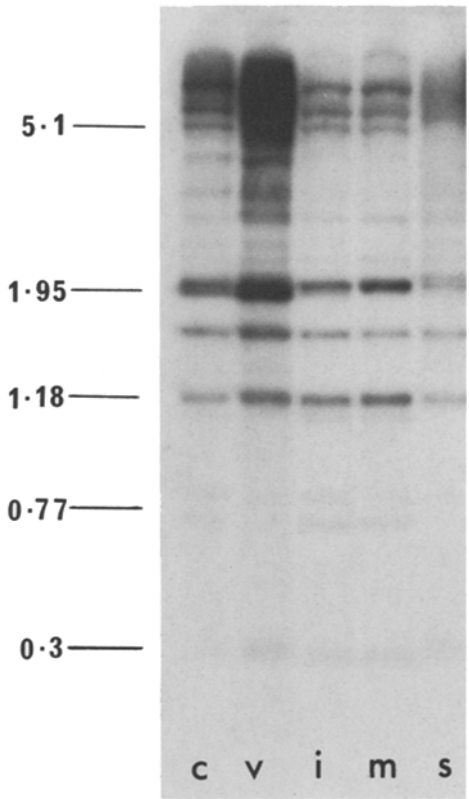


Fig. 5. Hybridisation of pSC 310 to *Secale* DNAs digested with Hind III. Approximately 5 µg of DNA was loaded on each track after digestion with Hind III. The DNAs are lettered as follows: **c** *S. cereale;* **v** *S. vavilovii;* **i** *S. iranicum;* **m** *S. montanum*; **s** *S. silvestre*. Hybridisation was with approximately 1×10^6 cpm of ^{32}P labelled pSC 310 and autoradiographic exposure time was approximately three days

placing on the extreme left the NOR chromosome, which was nearly always distinguishable, and subsequently ordering the pairs by decreasing arm ratio. Consequently the most metacentric pair of chromosomes is on the extreme right. It is most important to note that this means that comparisons between chromosomes aligned vertically is not valid.

A. *S. cereale* ssp *dighoricum* (R40) and *S. cereale* ssp *ancestrale* (R27)

These two species appear to be correctly classified as *S. cereale* types (see Jones and Flavell 1982) both on the basis of the strength of their hybridisation with the 480 bp repeat, the number of chromosomes which show hybridisation, and presence of heterobrachial chromosomes with interstitial sites for the 480 bp repeat on their arms (Fig. 6). There appear to be heterozygosities for the 480 bp sequence. The level of hybridisation with the 610 bp sequence is slightly less than one might expect from a *S. cereale* type, but the 630 bp hybridisation is typical, and much stronger than that of a *S. montanum* (see later). Hybridisation with the 120 bp repeat resembles in general that of all other *Secale* species.

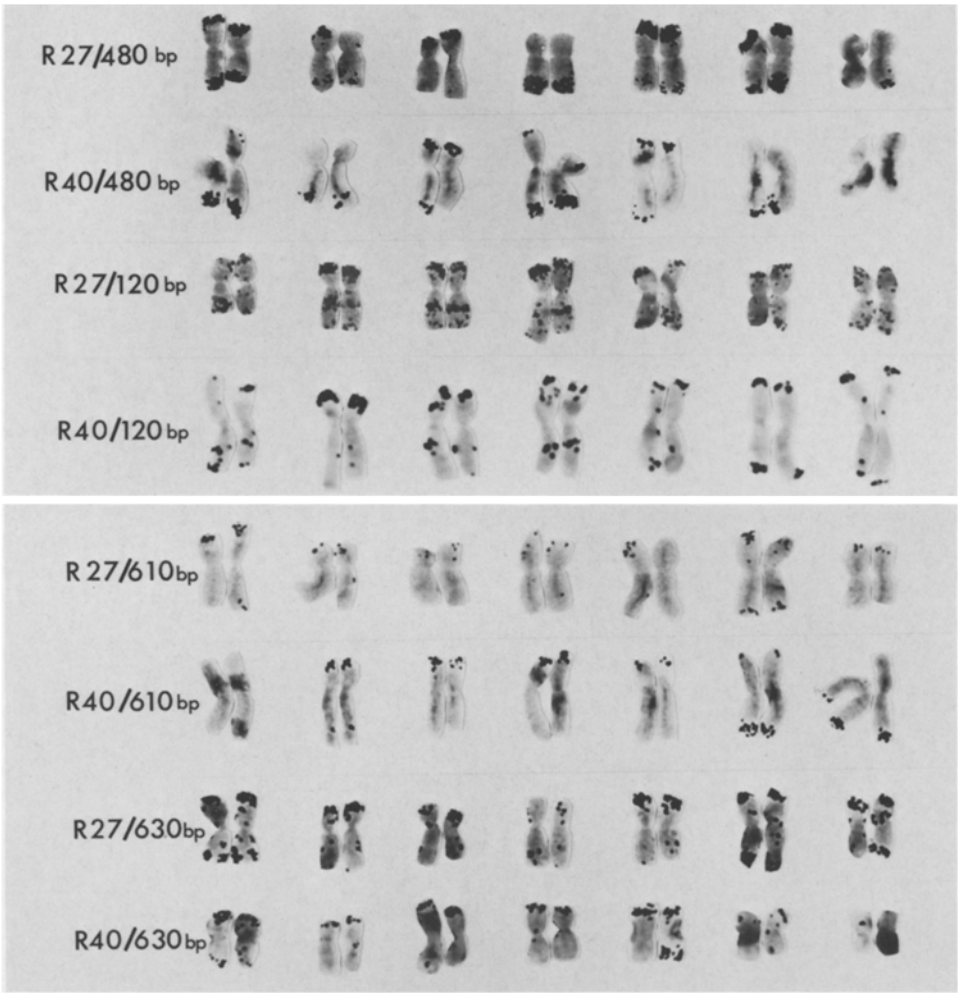


Fig. 6. In situ hybridisations of ^{3}H cRNA to R27 (*S. ancestrale*) and R40 (*S. dighoricum*). 480 bp repeat (pSC 210): 65,000 cpm slide, 21 days exposure, 120 bp repeat (pSC 119): 65,000 cpm/slide, 30 days exposure. 610 bp repeat (pSC 34): 60,000 cpm/slide, 15 days exposure. 630 bp repeat (pSC 33): 200,000 cpm per slide, 30 days exposure

B. *S. vavilovii* and *S. iranicum*

Two PBI accessions of *S. vavilovii* (PBI R11, PBI R30) were hybridised with pSC 210 to detect the 480 bp family. It was clear that they both resembled *S. cereale* in possessing interstitial long arm sites of hybridisation with the 480 bp sequence (Fig. 8a). This is consistent with the contention of Jain (1960) that some *S. cereale* types had been wrongly classified as *S. vavilovii*, and also with the findings of Khush (1963b).

It was for this reason that seed of another *S. vavilovii* accession was

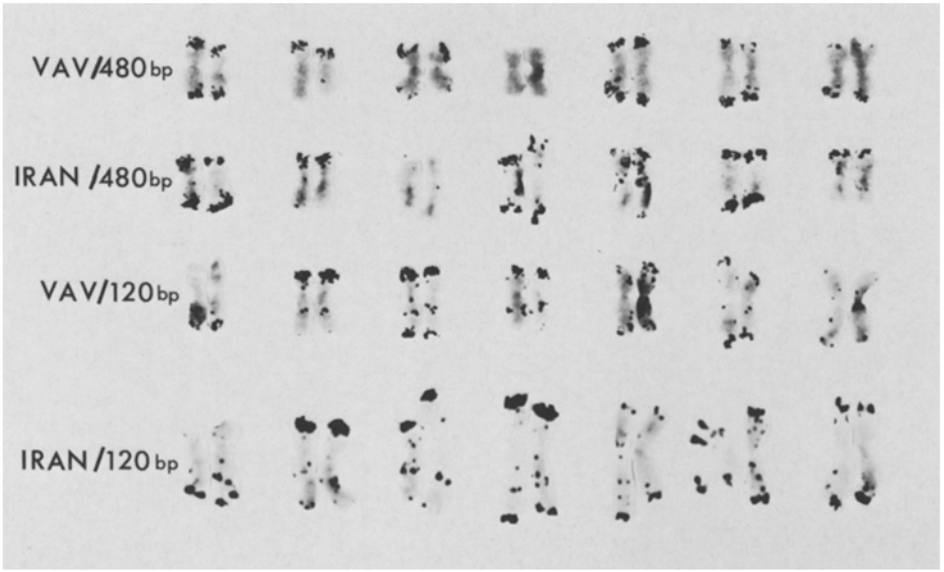


Fig. 7. In situ hybridisations of ^{3}H cRNA to *S. vavilovii* and *S. iranicum*. With 480 bp repeat (65,000 cpm/slide) autoradiographic exposure time was 21 days. With 120 bp repeat (65,000 cpm per slide) autoradiographic exposure time was 30 days

sought from Dr. A. Kranz. As shown by blot hybridisations, the highly repeated sequence complements of this accession of *S. vavilovii* appear to be essentially identical to *S. iranicum*. In situ hybridisation corroborates this similarity (Fig. 7). With the 480 bp repeat, both *S. iranicum* and *S. vavilovii* show labelling on six pairs of chromosomes, and most importantly, they show no interstitial sites, even on relatively uncontracted chromosomes (Fig. 8b). Hybridisation with pSC 119 to reveal the 120 bp repeat family showed labelling patterns typical for *Secale* (Fig. 7). The 610 bp and 630 bp families could not be detected by in situ hybridisation with pSC 34 and pSC 33 in several separate experiments.

C. *S. montanum*

The chromosomes of five different *S. montanum* accessions were hybridised in situ. Only one of these, R15, has been extensively checked by analysis of meiosis in F_1 hybrids with *S. cereale* (T.E. Miller, personal communication). The other populations could to varying extents have acquired some *S. cereale* chromosomes by outbreeding.

(i) Hybridisations Using pSC 210 to Detect the 480 bp Family. Some very disjunct distributions of this family around the chromosomes are apparent (Fig. 8c, d, e). *S. anatolicum* (R98) is the most conspicuous example of this having only one pair of heavily labelled chromosomes (Fig. 8e). Some individuals of the *S. anatolicum* stock (data not shown) were heterozygous

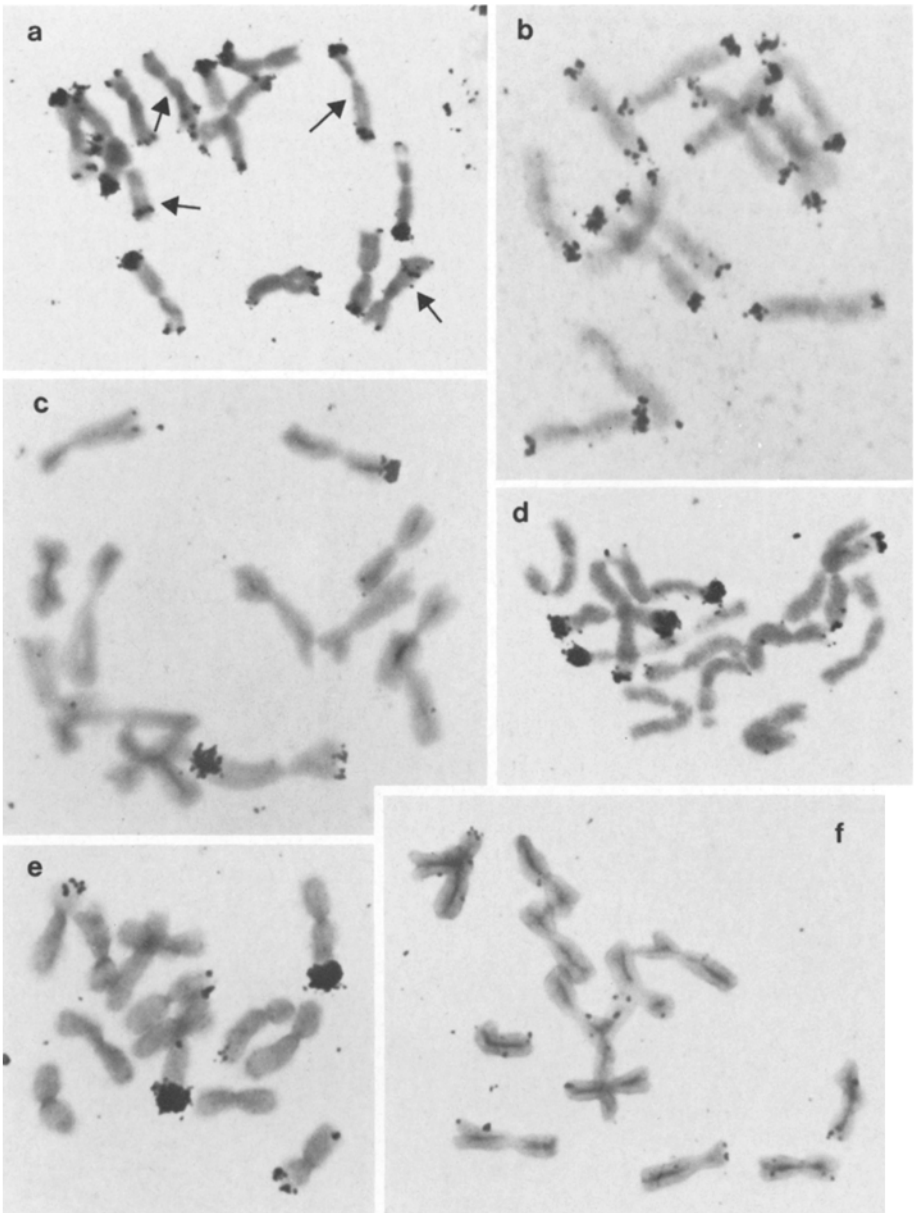


Fig. 8a–f. In situ hybridisation of pSC 210 (480 bp repeat family) to accessions of *S. vavilovii* and *S. montanum* types and of pSC 33 (630 bp repeat family to *S. kuprijanovii*). **a** *S. vavilovii* (PBI R11) hybridised with pSC 210 (480 bp repeat family) 65,000 cpm/slide with an autoradiographic exposure time of 14 days. Chromosomes with interstitial labelling typical of chromosomes 5R and 6R of *S. cereale* are arrowed. **b** *S. vavilovii* (from Kranz) hybridised with pSC 210 (480 bp repeat family) 65,000 cpm/slide with autoradiographic exposure time of 14 days. Note that no interstitial labelling is obvious. Compare with **a**. **c** *S. anatolicum* (R98) hybridised with pSC 210 (480 bp repeat family) 65,000 cpm/slide with an autoradiographic exposure time of 15 days. Note strong hybridisation to two dissimilar chromosomes. Compare with **e**. **d** *S. montanum* (R15) hybridised with pSC 210 (480 bp repeat family). 65,000 cpm/slide with an autoradiographic exposure time of 31 days. At least one heterozygosity is present. **e** *S. anatolicum* (R98) hybridised with pSC 210 (480 bp repeat family). 65,000 cpm/slide with an autoradiographic exposure time of 30 days. The two heavily labelled chromosomes appear homologous. Compare with **c**. **f** *S. kuprijanovii* (R100) hybridised with pSC 33 (630 bp repeat family). 200,000 cpm/slide with an autoradiographic exposure time of 60 days

for the chromosome carrying large amounts of the 480 bp family, with only one out of fourteen chromosomes displaying extremely heavy hybridisation. Sometimes (Fig. 8c) the two chromosomes showing label differ in arm ratio; this could mean the chromosomes involved are non-homologous, or that they are homologous with blocks of the sequence at opposite telomeres, as described for heterochromatin in a bivalent observed at meiosis in this accession by Bennett et al. (1977). However no observations have been made by us on meiosis in any accessions, so the identity of some of these heterozygosities cannot be defined precisely. *S. montanum* (R15) shows a clear heterozygosity, with only one out of the complement showing label at both ends (Fig. 8d), and *S. kuprijanovii* (R100) (Fig. 9) showed a strong heterozygosity for the NOR chromosome satellited arm. The heterozygous labelling pattern on this chromosome was present in all four individuals of this accession which were examined. R99 and R101 (both *S. montanum* accessions) on the other hand show some features of *S. cereale*, with long arm interstitial sites (Fig. 9). The *S. montanum* DNA which was extracted and assayed by filter hybridisation came from R99, whose in situ karyotype paradoxically most resembles *S. cereale*.

(ii) Hybridisations to Detect the 610, 630 and 120 bp Families. With the probe to detect the 610 bp family all accessions were only lightly labelled compared with *S. cereale* (Fig. 9). R15 was hybridised with four times more counts per slide and exposed for five weeks instead of thirty days, and so the stronger hybridisation should not be taken as evidence of a quantitative difference between the accessions. A heterozygosity is apparent in R15, for a chromosome labelled on both telomeres. The level of in situ hybridisation is consistent with the estimate of a five-fold lower copy number of the 610 bp sequences in DNA from R99 compared with *S. cereale* DNA obtained by blot hybridisations (Table 1).

The 630 bp family was hard to detect by in situ hybridisation in *S. montanum* accessions; all except R101 show very low hybridisation (see Fig. 10). R101 looks almost comparable to a *S. cereale* level of hybridisation, though this accession looks comparable with the other *S. montanum* types after hybridisation with the 610 bp repeats.

All species showed a typical *S. cereale* pattern (Jones and Flavell 1982) of hybridisation with pSC 119 which detects the 120 bp repeat family (Fig. 10).

D. *S. africanum* and *S. silvestre*

S. africanum was first shown to possess the 610 bp repeat family by in situ hybridisation (Fig. 11a). A second accession (R107) shoes an identical in situ hybridisation karyotype (data not shown). The sequence can be detected only on the short arm of one pair of chromosomes, and its presence is corroborated by blot hybridisations (Fig. 4b). The 630 bp and 480 bp repeat families cannot be detected in this genotype.

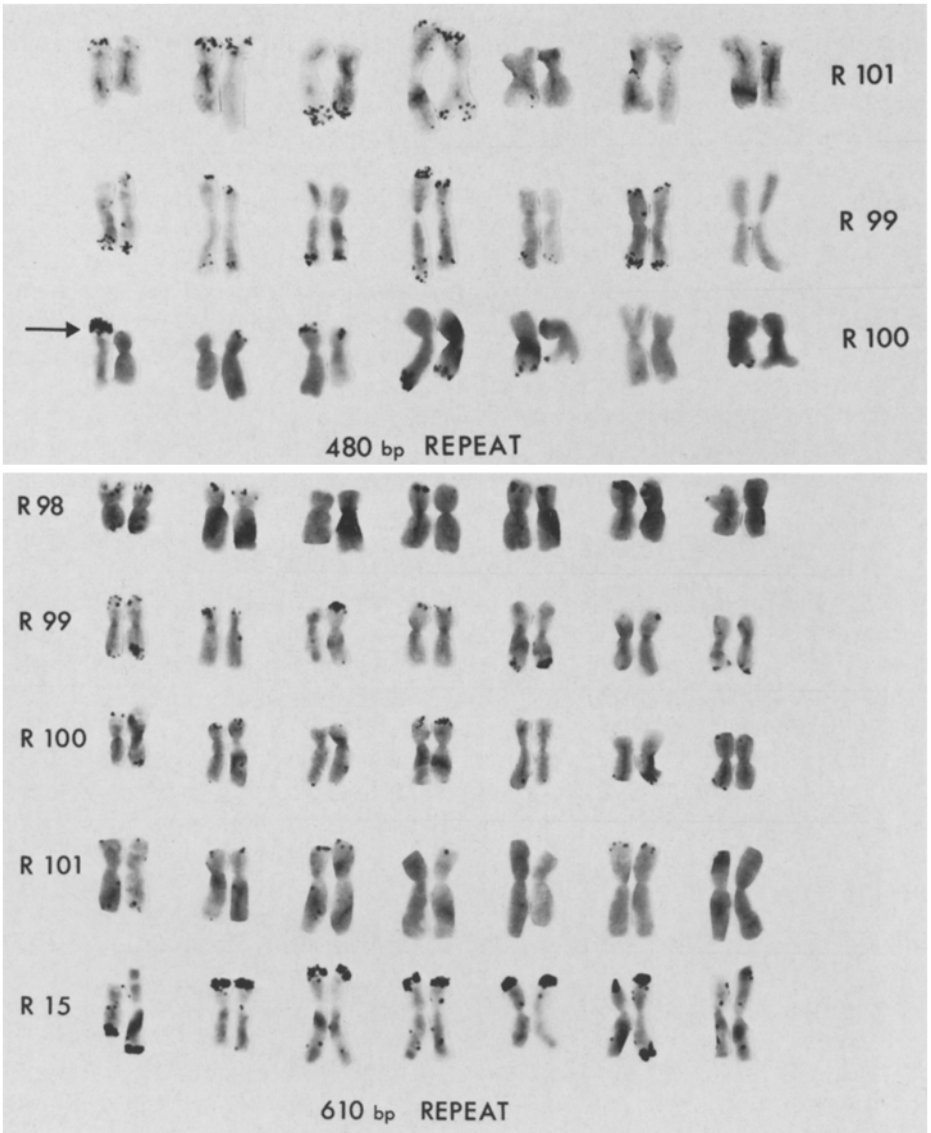


Fig. 9. In situ hybridisation to *S. montanum* types. *S. anatolicum* (R98), *S. montanum* (R99), *S. montanum* (*Kuprijanovii*, R100), *S. montanum* (R101) and *S. montanum* (R15) were hybridised with 480 bp repeat (pSC 210) and/or 610 bp repeat (pSC 34). With 610 bp repeat 60,000 cpm/slide autoradiographic exposure time was 21 days except with R15 when 150,000 cpm/slide were added and exposure time was 35 days. With the 480 bp repeat, 65,000 cpm/slide, the exposure time was 11 days

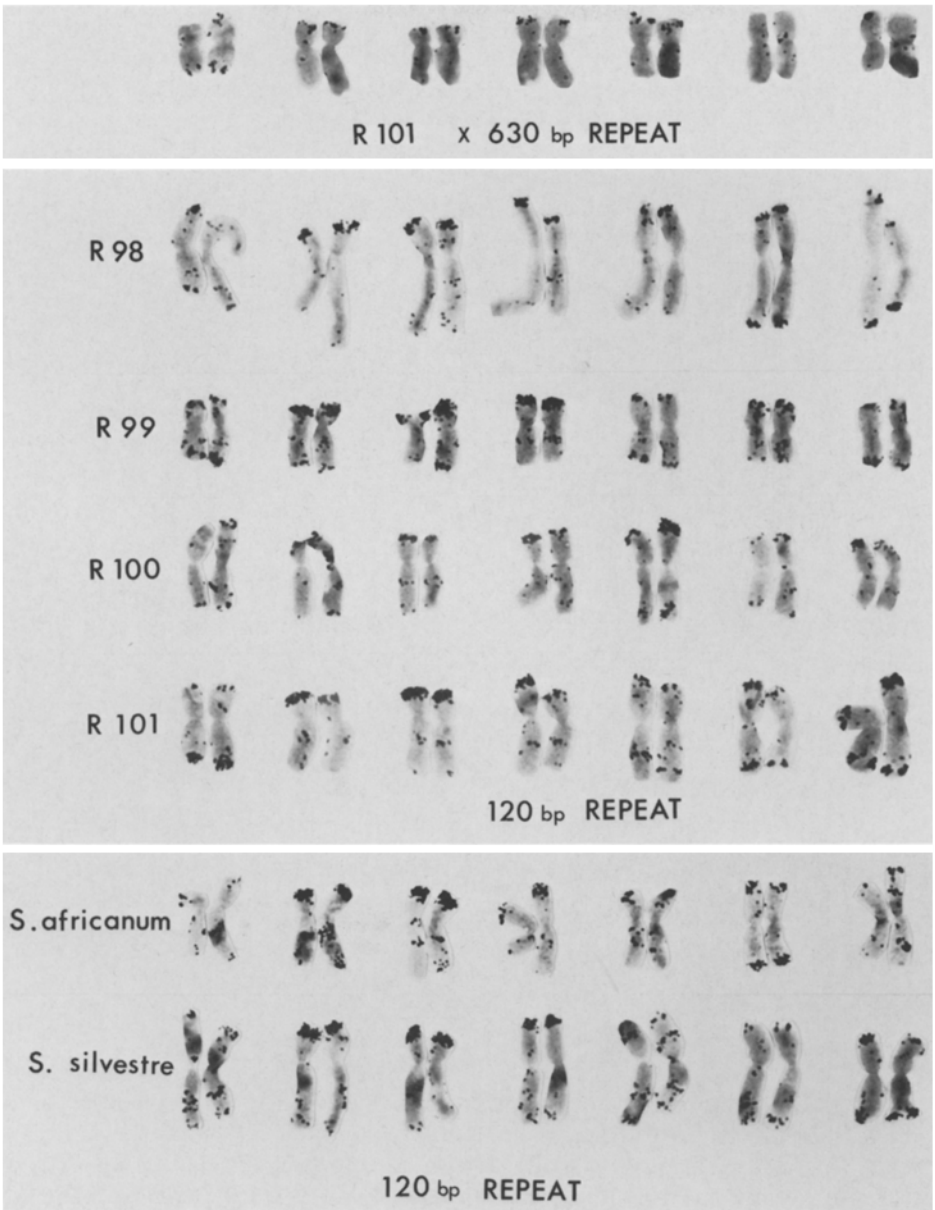


Fig. 10. In situ hybridisation of 120 bp repeat and 630 bp repeat to *S. montanum* types and the 120 bp repeat to *S. silvestre* and *S. africanum*. *S. montanum* (R101) was hybridised with the 630 bp repeat (200,000 cpm/slide) with an autoradiographic exposure time of 60 days. *S. montanum* (R101), *S. montanum* (*Kuprijanovii*) (R100), *S. anatolicum* (R98), *S. montanum* (R99), *S. silvestre* and *S. africanum* were hybridised with 120 bp repeat using 65,000 cpm/slide and an autoradiographic exposure time of 30 days

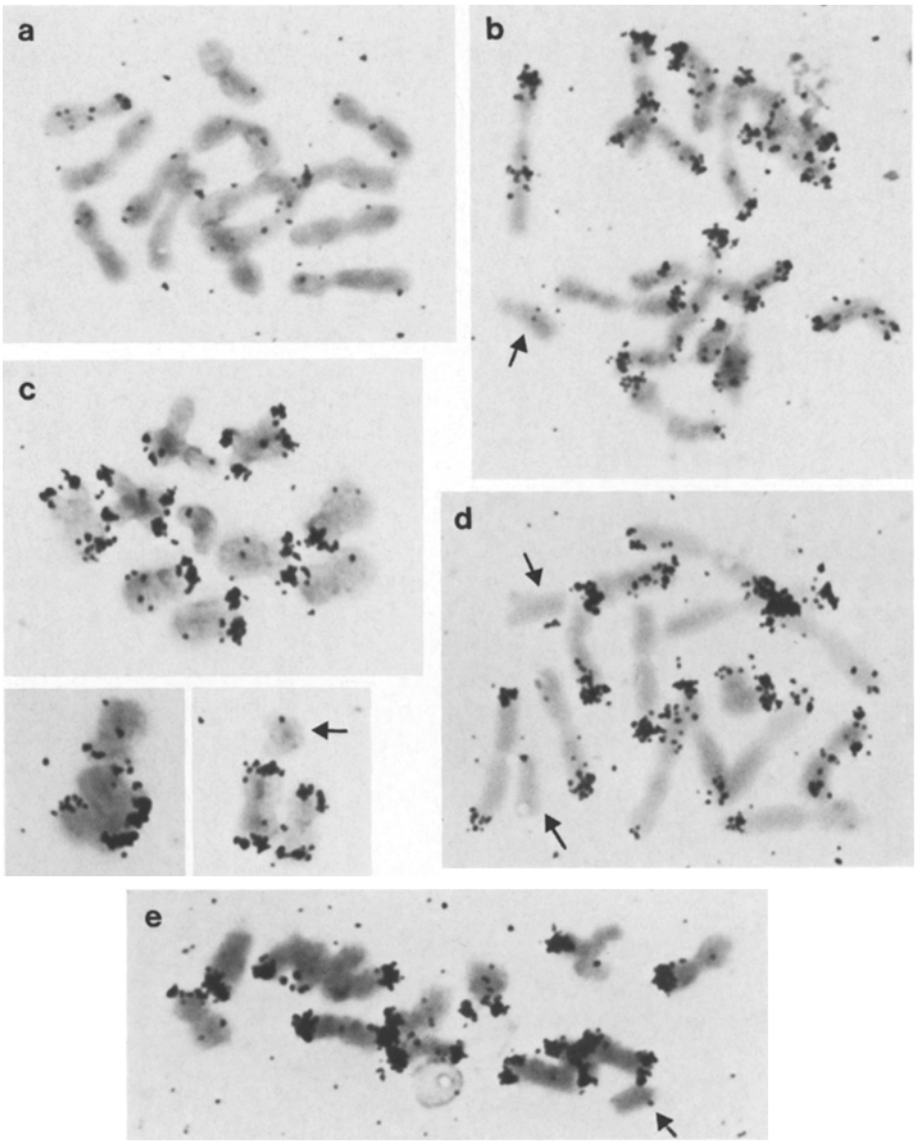


Fig. 11a–e. Hybridisation of *S. africanum* with pSC 34 (610 bp repeat family) and B chromosomes with members of 480, 120, 630 and 610 bp families. **a** *S. africanum* (R102) hybridised with H cRNA prepared from pSC 34 (610 bp repeat family). 150,000 cpm/slide with an autoradiographic exposure time of 21 days. **b** Hybridisation of pSC 119 (120 bp repeat family) to rye containing a B chromosome (*arrowed*). 100,000 cpm/slide with an autoradiographic exposure time of 21 days. **c** Hybridisation of pSC 33 (630 bp repeat family) to rye containing B chromosomes (*arrowed*). 200,000 cpm/slide, with an autoradiographic exposure time of 30 days. **d** Hybridisation of pSC 210 (480 bp repeat family) to rye containing a B chromosome (*arrowed*). 65,000 cpm/slide, with an autoradiographic exposure time of 21 days. **e** Hybridisation of pSC 34 (610 bp repeat family) to rye containing a B chromosome (*arrowed*). 150,000 cpm/slide with an autoradiographic exposure time of 10 days

Both *S. africanum* and *S. silvestre* show the typical *S. cereale* in situ hybridisation pattern for pSC 119 which detects the 120 bp repeat family (Fig. 10). No hybridisation of pSC 210, pSC 33 or pSC 34 could be detected to *S. silvestre* chromosomes.

E. B Chromosomes

The plants carrying B chromosomes assayed for the presence of the four repeated sequence families was an experimental rye population obtained from Aberystwyth, into which had been crossed B chromosomes from a stock initially classified as *S. vavilovii*. The hybridisation results are shown in Figure 11 b→e. None of the four probe sequences showed significant hybridisation to B chromosomes. This is curious in view of the presence of telomeric heterochromatin on some B chromsomes (Gill and Kimber, 1974). Another B chromosome stock, in which B chromosomes from a Transbaikal *S. cereale* accession had been crossed into Chinese Spring, was shown to lack sites of hybridisation for the probes to the 480 bp, 610 bp and 120 bp sequences families (data not shown). This is consistent with the observation of Appels et al. (1978) that in situ hybridisation with cRNA prepared from Cot 10^{-2} DNA of *Secale* failed to reveal substantial sites of hybridisation to B chromosome stocks.

When DNA extracted from rye plants with eight B chromsomes was digested with Hae III and fractionated on a polyacrylamide gel no new bands were detectable but there was a slight reduction in the concentration of DNA in the 240 bp band derived from the 480 bp repeats (data not shown). These plants would be expected to have nearly 50% of their DNA in B chromosomes (Jones and Rees, 1968).

Discussion

These studies on the repeated sequence DNA families in *Secale* contribute to our understanding of the evolution of repeated sequences and the *Secale* genus in numerous ways. The principal conclusions, included in the abstract are developed below.

The Complement and Chromosomal Distribution of Very Highly Repeated Sequence Families is Unique in Each Species

The evidence for each species having a unique complement of very highly repeated sequences in summarised in Table 1. The results where particular families were not detected cannot be taken to mean that the sequences are entirely absent from the species. The quantitative limits of detection of a sequence in each kind of experiment are difficult to assess accurately. However, on the basis of kinetic analyses reported in Bedbrook et al.

(1980a), and the results in this paper, the copy number of the 480 bp sequence must be at least 1000-fold greater in *S. cereale, S. montanum, S. iranicum* and *S. vavilovii* than it is in *S. africanum* and *S. silvestre*. Similarly, because no hybridisation of the 610 bp sequences to *S. vavilovii* or *S. iranicum* could be detected in experiments where approximately 5000 copies were detectable in *S. africanum* DNA, there is probably at least a 350-fold difference in the copy number of this sequence per haploid genome between *S. cereale* and *S. vavilovii* or *S. iranicum*.

Where specific sequences are detected in two or more species the copy numbers are often different. For example, there are considerably more copies of the 610 bp sequences in *S. cereale* than in *S. montanum* and *S. africanum* and of the 630 base pairs sequences in *S. cereale* than in *S. montanum, S. vavilovii* and *S. iranicum*. Summing the estimates in Table 2 of the proportions of total DNA occupied by each of the four families, shows that *S. cereale* contains considerably more DNA in these repeated sequence families than the other *Secale* species. This is consistent with the estimates of the proportions of DNA not digested with Hind III because this "relic" fraction is highly enriched for these particular families (Bedbrook et al., 1980a).

In addition to copy number variation, there is variation between species in the chromosomal distribution of common sequences. This is particularly marked for the 480 bp sequences (compare Fig 8c–e). Arrays of 480 base pair sequences are present at distal interstitial sites in *S. cereale* (Fig. 8a) but no interstitial sites have been observed in *S. vavilovii, S. iranicum* or *S. montanum*. In *S. cereale* and *S. vavilovii* the 480 bp sequences are somewhat evenly distributed over specific sites on most of the chromosomes, but the quantitative distribution of 480 bp sequences in the *S. anatolicum* and other *S. montanum* accessions is very different. From estimations of grain counts at different exposure times in the in situ hybridisations the heaviest labelled telomeres in *S. anatolicum* might have up to twice as many copies of the 480 bp sequences as most telomeres of *S. cereale*. However, the quantitative chromosomal distribution of repeated sequences cannot be solely interpreted on a species basis because we have observed considerable variation between individuals within accessions, between accessions and between varieties (see Figs. 8 and 9; Jones and Flavell, 1982).

The 480 base pair family of sequences was detected in high copy number in all the species except *S. africanum* and *S. silvestre*. However, a fraction of the repeats in *S. montanum* had a slightly different structure, possessing an additional Hae III restriction site. This suggests that a variant has been reamplified in *S. montanum* and emphasises the point that extensive sequence data may be necessary to assess the extent of homology between hybridising sequences in different species or even between hybridising sequences within a species.

The unique complements of very highly repeated sequences in each of the species suggests that arrays of each of the 480, 610, 630 and 120 base pair sequences can exist independently of each other in *Secale* genomes, although again the conclusions are dependent upon the lower limits of detec-

tion in our experiments. Arrays of the 610 bp repeats appear to exist in the absence of 630 bp repeats in *S. africanum* and in the absence of the 480 bp repeats at the telomeres of some *S. montanum* chromosomes. The 610 bp family is detected only at telomeres where 120 bp repeats are also found but this may be fortuitous because 120 bp sequences are present at most telomeres and 610 bp sequences seem confined to telomeric sites.

The apparent independence of these sequences in consistent with the idea that they exist in separate blocks, even when they are all present together in *S. cereale* (see Jones and Flavell, 1982). These blocks may constitute the telomeric pachytene "*knobs*" described by Lima-de-Faria (1952) which never number more than four at any one telomere.

The Variation in the 480, 610 and 630 Base Pair Families Between Secale Species is in Contrast to Most Other Repeated Sequence Families

The 480, 610 and 630 base pair repeats were originally selected for study because they were likely candidates to be the molecular basis of the quantitative differences between the *S. cereale* and *S. silvestre* genomes (Bedbrook et al., 1980a). The interspecies variation in these families can be compared with that of a large fraction of the other families from comparisons of the Eco RI, Hind III (not shown) and Hae III restriction enzyme digest patterns shown in Figure 1. The ethidium bromide stained bands are due to repeated sequences. The patterns of bands from the different *Secale* species are almost indistinguishable with the exception of those belonging to the 480 base pair family. Proof that similarly migrating bands constitute essentially identical repeats requires extensive characterisation, as has been carried out for the 480, 610, 120 and 630 base pair families and also the 2.2 kb bands found in Eco RI digests of *Secale* DNAs. However, it is reasonable to conclude that the very similar banding patterns in the different *Secale* species implies that most families of repeated sequences are quantitatively and qualitatively similar within the genus. Previous studies (Bedbrook et al., 1980a) have shown that essentially all the repeats in *S. silvestre* are present in *S. cereale* and that most, if not all, the repeats in *S. cereale* not present in *S. silvestre* are members of the 480, 610 and 630 base pair families. The 480, 610 and 630 base pair family differences account for only about 50% of the total DNA differences between *S. cereale* and *S. silvestre*. Most of the remaining DNA difference (6% of the genome) is probably due to quantitative differences in repeat families which are found in both species.

Highly Repeated Sequences, Heterochromatic C Bands and B Chromosomes

The strong correlation of the location of arrays of highly repeated DNA sequences with the position of major C bands in *S. cereale*, discussed in the accompanying paper (Jones and Flavell, 1982) appears to hold true

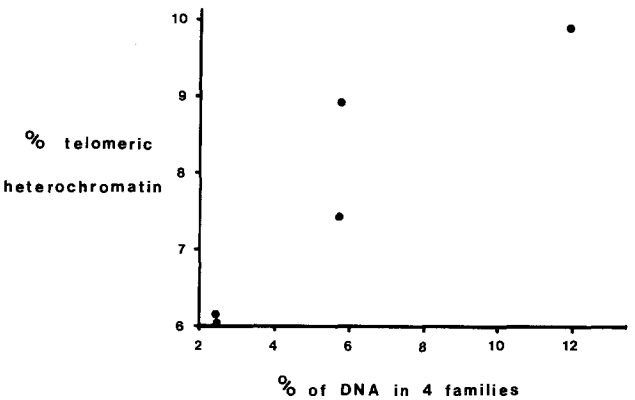


Fig. 12. Relationship between the percentage of telomeric heterochromatin and the four highly repeated DNA families. The percentages of the highly repeated DNA are taken from the mean values in Table 1. The percentages of the length of metaphase chromosomes occupied by telomeric heterochromatin are taken from Bennett et al. (1977)

in the rest of the genus. The major blocks of heterochromatin throughout the genus are telomeric (Bennett et al., 1977) as are the blocks of highly repeated sequences.

In Figure 12 the percentage of the total length of metaphase chromosomes occupied by heterochromatin in accessions of *Secale* species measured by Bennett et al. (1977) is plotted against the mean value of the percentage of total DNA occupied by the four families in the accessions studied here (data taken from Table 1). There is a clear relationship between the two parameters, confirming that these sequences are the major components of the variation in *Secale* heterochromatin (Bedbrook et al., 1980a).

Bennett et al. (1977) reported major differences in the amounts of heterochromatin at different telomeres in *S. anatolicum* and *S. kuprijanovii*, all of which correlate with the disjunct distribution of 480 bp repeats at the telomeres (Fig. 8c–e) and contrast with the more uniform distribution of heterochromatin and amounts of highly repeated DNA in *S. cereale* (Jones and Flavell, 1982). A particularly interesting example of the distribution of 480 bp repeats and heterochromatin occurs in some plants of *S. anatolicum*, accession R98, which possess a pair of chromosomes heteromorphic for large C bands at both telomeres (described in Bennett et al., 1977). These chromosomes possess the largest blocks of heterochromatin in the karyotype and are almost certainly the pair of chromosomes in Figure 8c which have different arm ratios and large blocks of 480 bp repeats at one telomere. If so, then the large blocks of 480 bp repeats are probably responsible for the major band of heterochromatin on these chromosomes.

Small interstitial C bands in *Secale* species have been reported (Sigh and Röbbelen, 1975; Bennett et al., 1977) at sites similar to those detected by in situ hybridisation using 120 bp repeats as a probe. Only the commonly observed C band at the nucleolus organiser was not detected by in situ hybridisation. However, some of the interstitial sites of blocks of 120 bp repeats have not been reported as C bands.

The absence of large blocks of sequences of the four families tested on the long arm telomeres of two types of B chromosome indicates another example of heterochromatin, in addition to the centromeric bands and the NOR bands, which presumably consists of repeats other than those tested. Singh and Röbbelen (1975) showed that the B chromosomes for the Transbaikal rye have a minor interstitial C band too. The position of this corresponds to the site of a few silver grains after in situ hybridisation with pSC 119 (results not shown).

In summary, at least one of these highly repeated sequence families is usually but not always present in *Secale* heterochromatin. It is obviously tempting to suggest that tandem arrays of these sequences specify the contracted state of heterochromatin.

The Highly Repeated Sequences and Secale Phylogeny

The evolutionary tree of the *Secale* species as concluded from morphological chromosome pairing and hybrid fertility assays is shown in Figure 13. It seems well-established that the progenitors of *S. silvestre* (an annual inbreeder) and *S. montanum* (a perennial outbreeder) separated first and that more recently *S. africanum* (a perennial inbreeder) evolved separately from *S. montanum* in North Africa. *S. vavilovii* (an annual inbreeder) and *S. cereale* (an annual outbreeder) also evolved from *S. montanum* relatively recently (Khush and Stebbins, 1961; Khush, 1962, 1963a, b; Stutz, 1972; Kranz, 1976; Singh and Röbbelen, 1977). The early separation and greater distinctness of *S. silvestre* is supported by this species having a different complement of sequences homologous to pSC 310 (Fig. 5). The *Secale* species all differ from one another by one or two translocations and so it is thought that chromosome translocation has been an important process in speciation. It is not possible to superimpose on this evolutionary scheme a bifurcating series of sequence amplifications to give the repeated sequence distributions in Table 1. For example, *S. vavilovii* and *S. africanum* have both evolved relatively recently from *S. montanum* but *S. vavilovii* lacks the 610 bp repeat family present in *S. montanum* and *S. africanum* does not. On the other hand *S. africanum* lacks the 480 bp family present in

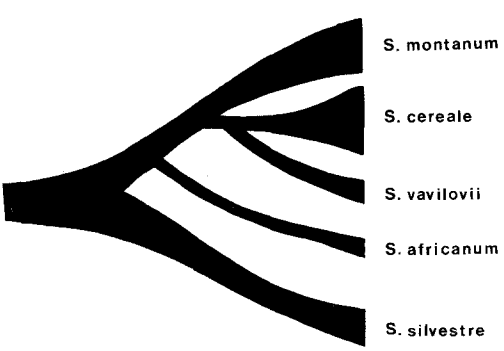


Fig. 13 A schematic representation of *Secale* evolution

S. montanum and *S. vavilovii*. These anomalies can be explained by three possibilities: (1) separate amplification of the same family in diverged species, (2) deletion of a family from an evolving species, and (3) introgression of a family of repeats from one species to another.

The amplification of the same or a closely related sequence in diverged species to explain a disjunct distribution of similar or related repeats in related species or genera has been discussed by Fry and Salser (1977). The probability of amplification of the same complex, single or few copy sequence in different species is very low unless the sequence is predisposed to being amplified. The stable appearance of such repeats in different species would, of course, be helped if there was strong selection for them. In the absence of knowledge about the predisposition of the 480, 610 and 630 bp families to be amplified or about possible functions (see later), more speculation is not warranted. However, it should be noted that reamplification or turnover of these repeats occurs (Flavell et al., 1977) as supported by the finding that a variant repeat of the 480 bp family forms a large fraction of the family in *S. montanum* (Fig. 2).

Major quantitative changes in tandem arrays of repeats, which are interpreted as amplifications or deletions, can result from unequal crossing over or intrastrand recombinations. Within the few plants studied here we have observed many examples of heteromorphisms and deletions of sequences. The deletions of the 480 and 610 bp sequences of *S. cereale* chromosomes in wheat lines are particularly striking (Jones and Flavell, 1982). Thus the loss of a family of repeats from a species is not an improbable occurrence.

The third possibility to explain the distributions of families of repeats in the *Secale* genus, that of introgression, is also possible because in spite of the translocations between, for example, *S. cereale* and *S. montanum*, some cross hybridisation does occur in the wild and a hybrid population has been detected (Zohary, 1971).

The inability to make a common bifurcating lineage of species and sequences means, of course, that these highly repeated sequences cannot be used to shed more light on *Secale* phylogeny. It is probable that species relationships can, however, be established better by considering *together* many families or banding patterns after restriction endonuclease digestion (e.g. Fig. 5). This is to be the subject of a separate paper (Flavell et al., in preparation).

The genus *Secale* contains species which are closely related. Classification of accessions to distinct species is sometimes difficult. The finding that defined species appear to have unique complements of the most highly repeated DNA families means that these repeats should help in the classification of new accessions. This can be illustrated by the study of the two *S. vavilovii* accessions in the PBI collection. Their classification was suspect before analysis of their repeated sequences. Only one of them was self-fertile, for example. On the first assessment of their repeated sequence complement, they both looked clear *S. cereale* types, and so the *S. vavilovii* seed collected from Hamadan, Iran, by Professor H. Kuckuck's 1958 expedition was obtained. This seed proved to be significantly different from *S. cereale*. Jain

(1960) and Khush (1963b) have both reported that many supposed *S. vavilovii* accessions are in fact *S. cereale* types. The cloned highly repeated sequences are clearly useful in characterising *S. vavilovii* accessions.

Two other taxonomic conclusions can be drawn from the repeated sequence assays described in this paper: (1) *S. iranicum* is very closely related to *S. vavilovii*, and (2) *S. ancestrale* and *S. dighoricum* are very similar to *S. cereale* as also was concluded on other grounds (Zohary, 1971).

A number of intraspecies heteromorphisms and other kinds of variants have also been described in this paper. These illustrate the value of in situ hybridisation using defined repeated sequence DNA probes to detect genetic variants (Hutchinson et al. 1981).

Evolution of the Repeated Sequence Families in the Genus

The absence of the 480, 610 and 630 bp families in *S. silvestre* suggests that these sequences may have been first amplified in *Secale* evolution after *S. silvestre* diverged from the ancestor of the other *Secale* species. This conclusion is further supported for the 610 bp family by the absence from *S. silvestre* of the 610 bp unit repeat when assayed under conditions which detect unique sequences (Bedbrook et al., 1981). More precise conclusions on the evolutionary intervals when the 480, 610 and 630 bp families were amplified cannot be made from this species distribution for reasons described earlier (section d). The 120 bp family, present in all the *Secale* species, is also in *Triticum*, *Elymus*, *Agropyron*, *Hystrix* and *Haynaldia* (unpublished results) which implies this family is the oldest of the four studied here. It is also the most heterogeneous, with respect to its constituent sequences (Bedbrook et al., 1980a, b) and as shown here, arrays of this sequence are present in up to eight interstitial sites (Figs. 6, 7, 10, 11). The most homogeneous family, the 610 bp, was detected in no interstitial sites (Figs. 6, 9, 11) while the 480 and 630 bp families are found in one to three interstitial sites (Figs. 6–8, 10, 11). This suggests that the primary sites of accumulation of arrays of these families in *Secale* are the telomeres but that during evolution blocks evolve at interstitial sites. The sequences could be moved to interstitial sites by chromosome fusion and breakage, translocation, inversion or by excision and reintegration. Because arrays of each family are present on many telomeres, the repeats must be capable of dispersion to other telomeres in the complement. This could occur by recombination between telomeric DNA during DNA replication (Holmquist and Dancis, 1979), or during the "*leptotene bouquet*" stage of meiosis (Thomas and Kaltsikes, 1976), by chromosome fusion and breakage, or sequence excision and reintegration. The transfer of sequences between nonhomologous chromosomes due to specific chromosome association in the nucleus is discussed in the accompanying paper (Jones and Flavell, 1982).

It is particularly interesting that the four families all accumulate at the telomeres. It is probable that this is the position in *Secale* species where these sequences (or the heterochromatin they specify) can be tolerated or

are functional. The idea that the positions of the sequences in different chromosomes is a consequence of how the chromosomes interact physically is discussed in the accompanying paper (Jones and Flavell, 1982).

One conspicuous feature of the highly repeated sequences in *Secale* is the heteromorphism within the karyotypes of outbreeding species. The heteromorphism is presumably the consequence of unequal crossing over, deletions or amplifications, events which happen frequently to arrays of repeated sequences. If any cell activity is regulated by repeated sequences in blocks of heterochromatin because of their position, copy number or sequence (Flavell 1981) then heteromorphism may affect the phenotype of individuals in the population. An influence on certain cell activities by sequences which rapidly undergo quantitative changes could be both a source and a regulator of variation in natural populations.

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