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Chromosomal Variation in *Crocus vernus* Hill (Iridaceae) Investigated by *in situ* Hybridization of rDNA and a Tandemly Repeated Sequence

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The physical localization of three tandemly-organized repetitive DNA sequences was investigated by *in situ* hybridization to metaphase chromosomes of 11 *Crocus vernus* accessions. The sequences included were the 18S–25S rDNA, the 5S rDNA and a tandemly-repeated sequence cloned from *C. vernus* (clone pCvKB8). Ten $2n = 8$ karyotypes from accessions ranging across the Alps and the Pyrenees could be interpreted as variations of a standard karyotype. Polymorphisms were found involving size of the satellite chromosomes, extra 5S rDNA sites, and extensive differences in size and number of pCvKB8 loci. The $2n = 16$ type did not correspond to any possible tetraploid derived from the $2n = 8$ types.

Key words: Evolution, phylogeny, *Crocus vernus* Hill (Iridaceae), *in situ* hybridization, chromosomal polymorphism, karyotype evolution, repetitive DNA.

INTRODUCTION

Crocus vernus Hill is a morphologically and karyologically variable species, distributed throughout most southern mountainous regions of Europe. Two subspecies are recognized: *C. vernus* Hill ssp. *albiflorus* (Schultes) Asch. & Graeb., a small white, purple-striped or purple type growing mostly at high altitude in the Alps and Pyrenees, and in Sicily ($2n = 8$); and *C. vernus* ssp. *vernus*, a somewhat larger and more richly coloured type of the Carpaters, the former Yugoslavia and central Italy ($2n = 8$, 10, 12, 16, 18, 20, 22). Although many different names have been used for these plants (and some still are used), the overlapping variation in morphology and lack of correlation between chromosome number and morphology, makes a reliable division into two or more species impossible (Mathew, 1982). Widely different karyotypes have been found among plants with the same chromosome number, and the relationships between karyotypes are not easily inferred from the morphology of the chromosomes (Brighton, 1976).

Since the widespread adoption of *in situ* hybridization, repetitive DNA sequences have become important markers for identifying chromosomes and following their evolution and alteration during evolution over long time-scales associated with speciation, and shorter periods associated with plant breeding. Repetitive DNA sequences constitute a large percentage of the genome of most investigated plant species (see Flavell, 1986; Kubis *et al.*, 1998; Schmidt and Heslop-Harrison, 1998). Their organization ranges from dispersed elements (more or less evenly distributed throughout the genome) to tandem repeats in discrete clusters at a number of loci. Within the genome, tandemly-repeated sequences might show chromosome-specific distribution patterns that can be used as a tool for recognition of single chromosomes, either within a single species (Busch *et al.*, 1995; Cuadrado *et al.*, 1995), or between species (de Bustos *et al.*, 1996). Often subtelomeric or centromeric sequences are tandemly-organized repeats (Galan *et al.*, 1991; Maluszynska and Heslop-Harrison, 1991; Busch *et al.*, 1995; Chung-Mong *et al.*, 1997). As suggested by the taxonomic and phylogenetic relationship of species, many genes and the genetic order of genes may be conserved between species, with genes showing allelic types of difference (Jacobsen and Orgaard, 1996), and collinearity or conserved synteny (Moore *et al.*, 1995). In contrast, repetitive sequences may vary extensively in their nature, copy number and chromosomal distribution even within a species (Cuadrado *et al.*, 1995), and hence their study is of value in plant classification and phylogenetic analyses.

The study of the nature and distribution of repetitive DNA sequences thus complements and adds to studies of chromosome numbers and morphology, C-banding, meiotic analyses and plant morphology which have already been published for *Crocus*. Most previous studies using molecular cytogenetic methods have been carried out with economically-important crop species and their wild relatives. It is now important to: (1) investigate chromosomal evolution in a wider range of species because of the value of such analyses in species studied already; (2) study a broader range of wild species; and (3) make comparative studies to reveal common features of genome organization and evolution (Heslop-Harrison, 1996).

We aimed to investigate the physical organization of some major tandemly-organized repetitive DNA sequences using *in situ* hybridization along the chromosomes of different *C. vernus* accessions. Here we present an investigation of

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three clones of tandemly-organized repetitive DNA: the ribosomal rDNA genes of 18S–5·8S–25S and the 5S rDNA, and a clone of highly-repeated, tandemly-organized DNA of no known function, isolated from *C. vernus*. We aimed to use the sequences as chromosome markers to identify homologous chromosomes or chromosome segments in the different cytotypes. The results are used to examine the variation and possible relationships between ten different karyotypes with $2n = 8$; for comparison a karyotype with $2n = 16$ was also included. Although *in situ* hybridization has been performed on wild populations (Linde-Laursen *et al*., 1992; Garrido *et al*., 1994) to our knowledge this is the most comprehensive study to date of chromosomal variation and evolution in a wild species performed by *in situ* hybridization.

MATERIALS AND METHODS

Plant material

Accession numbers and sources are shown in Table 1. All accessions are under cultivation at the Section of Botany, The Royal Agricultural University (KVL), Copenhagen.

Chromosome preparation and in situ *hybridization*

Three clones were used as probes in these experiments. Clone pTa794 contains a 410 bp *Bam*HI fragment of the 5S rDNA, with the 120 bp coding sequence for the 5S rRNA and the intergenic spacers isolated from common wheat, *Triticum aestivum* L. (Gerlach and Dyer, 1980). Clone pTa71 is a 9 kb *Eco*RI fragment from *T. aestivum* (Gerlach and Bedbrook, 1979), containing the coding sequences for the 18S, 5·8S, and 25S ribosomal RNA genes and the intergenic spacer sequences (called 45S rDNA henceforth). Clone pCvKB8 is a 177 bp tandemly-organized repetitive sequence isolated from *C. vernus*. For *in situ* hybridization, probes were labelled with biotin-11-dUTP or digoxigenin-11-dUTP by PCR or nick translation. Pre-treatments of roots aimed to give extended chromosomes since strong condensation reduces the resolution of *in situ* hybridization. Root-tip digestion followed standard methods (Orgaard *et al*., 1995; Schwarzacher and Heslop-Harrison, 2000), with a digestion time of 40–50 min. Some roots were digested with addition of 0·5 % pectolyase. The major problem encountered was cytoplasm overlaying chromosomes; treatment with proteinase K or pepsin partly removed this layer. *In situ* hybridization used 40 ng labelled DNA per slide in 40 µl; preparations with denatured probe were denatured at 70°C for 8 min, and allowed to hybridize overnight at 37°C. The most stringent wash following hybridization was at 42°C in 0·1 × SSC and 20 % formamide, allowing only target sequences of more than 85 % homology to remain hybridized. Streptavidin Cy3 and anti-digoxigenin-FITC were used as fluorochromes with DAPI (4′,6-diamidino-2-phenylindole) as counterstain. Photographs were made on 400ASA Fuji SuperHG colour print film with a Leitz epiflorescence microscope at 1000 times magnification, negatives scanned to Kodak PhotoCD, and printed from Adobe Photoshop after optimization using only functions which affect the whole image equally.

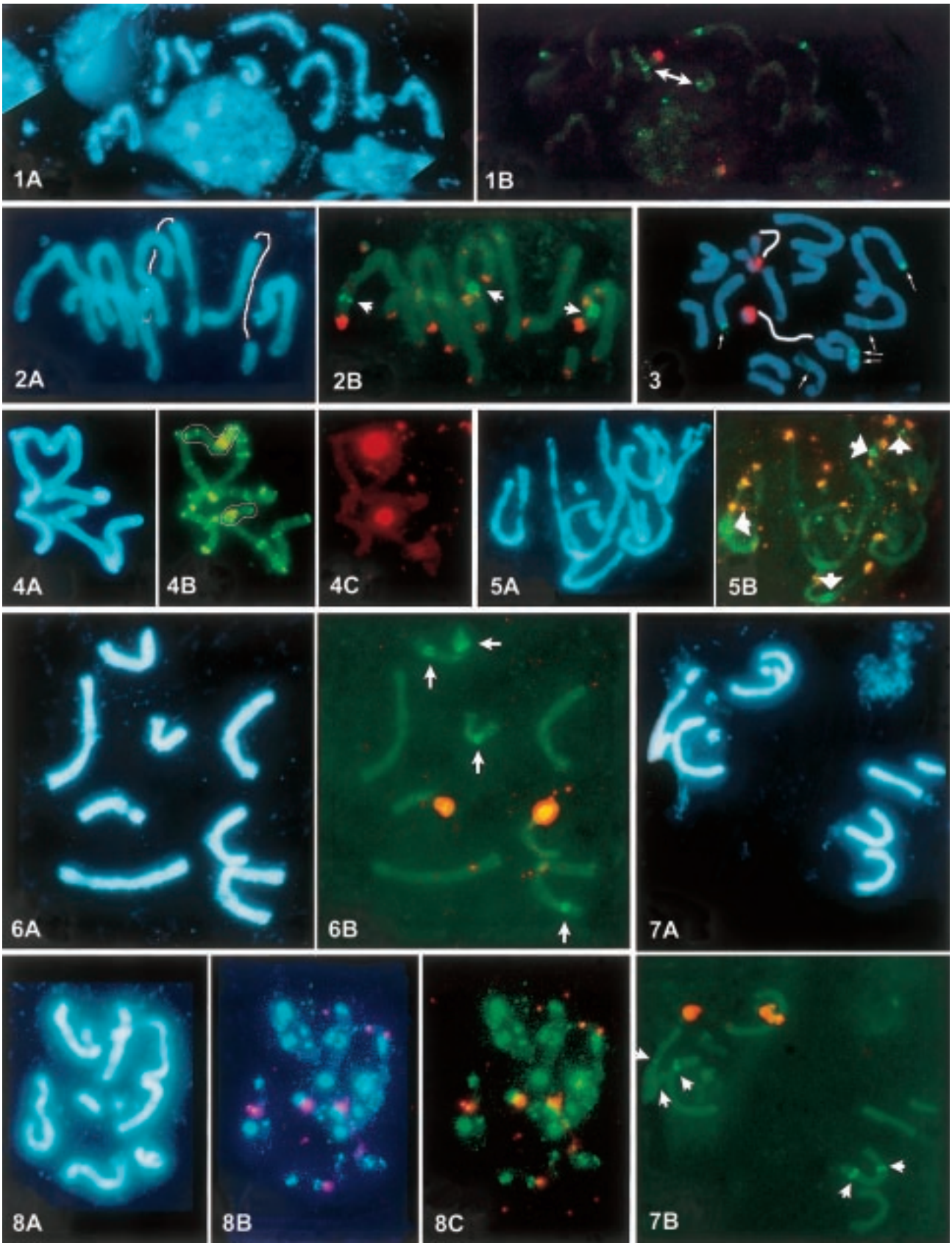
RESULTS

The micrographs (Figs 1–8) and idiograms (Figs 9–11) illustrate metaphase chromosomes following *in situ* hybridization of three repetitive DNA sequences to a tetraploid ($2n = 16$) and different diploid ($2n = 8$) accessions of *Crocus vernus*. Prometaphases with extended chromosomes were measured to make conclusions about morphology and to draw idiograms, although more condensed metaphases are normally illustrated for clarity. 45S and 5S rDNA and pCvKB8 were localized at discrete sites on the four pairs of chromosomes in diploid *C. vernus* accessions. PCvKB8 also showed a dispersed hybridization along the chromosome arms, presumably because of the presence of homologous sequence fragments or other related sequences. There were substantial polymorphisms between accessions in number and locations of 5S rDNA and pCvKB8 sites.

For some karyotypes, differences in the relative condensation of various chromosomal segments made precise localization of signals difficult, a well-known problem in karyotype analysis (Schrader *et al*., 1997). Satellites were often separated from any chromosome and, even in more condensed metaphases, were not near their chromosome of origin as determined by strands of DNA labelled with the 45S rDNA probe connecting the satellite and the rest of the chromosome arm. Such separation is a frequent source of

TABLE 1. *List of plant accessions used in this study*

KVL Acc. number	2*n*	Locality	*In situ* Figure number	Subspecies
95-2	8	Austria: Near Musau. Alt. 800 m	1	ssp. *albiflorus*
95-37	8	Italy: Between Anzi and Potenza. Alt. 1200 m	2	ssp. *vernus*
95-50	8	Italy: 10 km north of Montella. Alt. 1150 m		ssp. *vernus*
95-60	16	Italy: Top of Mt. Pisano, North of Pisa. Alt. 900 m	3	ssp. *vernus*
95-82	8	Italy: Near Cuneo, 4 km from the French border. Alt. 1850 m		ssp. *albiflorus*
95-106	8	Austria: South of Deutschlandsberg, near Soboth. Alt. 900 m	6	ssp. *vernus*
95-114	8	Austria: near Italian border, Passo monte Croci. Alt. 1250 m		ssp. *albiflorus*
95-213	8	France: Massif Central.	4 and 5	ssp. *albiflorus*
96-12	8	Spain: Catalonia, Superespot skilift, Espot. Alt. 1850 m		ssp. *albiflorus*
C-354	8	Cultivated material of "heuffelianus"-type	7	ssp. *vernus*
96-13	8	Spain: Catalonia, near Puerto de la Bondigea. Alt. 1900 m	8	ssp. *albiflorus*



FIGS 1–8. For legend see page 320.

error in published karyotypes, and has probably led to false reports of variable numbers of B-chromosomes. Figure 9 shows idiograms of ten different accessions of *C. venus* (see Table 1). For each accession, the idiogram represents an average from measurements of a number of metaphases and prometaphases, and does not necessarily correspond exactly with the illustrated metaphase; centromere constrictions were not always visible after *in situ* hybridization. The convention of numbering the chromosomes from the largest to the smallest, placing the satellite-chromosome last, was followed. Some pCvKB8 sites were close to, or collocalized with the centromere, and these were placed with less certainty. From the analysis, a consensus karyotype (Fig. 10) was defined with deviations from it shown by each accession (Table 2). The satellite chromosome was present in a longer and shorter form (Fig. 10). Karyotypes of accessions C-354 and 95-106 showed the greatest differences. Individual karyotypes are described under the second author's website (www.jic.bbsrc.ac.uk/staff/pat-heslop-harrison).

TABLE 2. *Schematic representation of karyotypes*

Accession number	Chromosome pair number 1	2	3	4
95-2	con; con	con; con	con[1]; con	con-a; con-a
95-37	8; 8	8; 8	8,8; 8,8	con-a; con-a
95-50	con; con	con; con	5,8; 5,8	con-b; con-b
95-82	con; con	8,8; con	8,8; 8,8	con-a; con-a
95-106	con; con	−5; con	5; con*	con-b; con-a
95-114	con; con	8; con	con; con	con-a; con-a
95-213	8,8; con	8; 8,8	8,8; 8,8	con-a,8; con-b
96-12	con; con	con; con	5; 5	con-a; con-a
96-13	45; 45	8,8; 8,8	con; con	con-a,8; con-b
C-354	con; con	−5*; con	5; 5	con-b; con-a

Each chromosome type is described with the homologues separated by ';'. con, The consensus chromosome type (see Fig. 10) with variants indicated. 95-106 had further rearrangements, probably due to translocation, so chromosomes could not be paired easily.
con-a, Chromosome type 4a, con-b, chromosome type 4b.
*, A chromosome with a deletion relative to the consensus.
[1], 5S rDNA at an unusual position.
5, Additional 5S rDNA sites.
8, Altered pCvKB8 sites (repeated where more than one site).
45, Additional 45S rDNA sites.
−, Sites missing.

The consensus karyotype for C. vernus *2n = 8 (Fig. 10)*

Chromosome pair no. 1: Submetacentric chromosome without rDNA sites, and with large pCvKB8 sites near both telomeres and an intercalary site on the long arm near the centromere.

Chromosome pair no. 2: Metacentric to submetacentric chromosome with one intercalary 5S-rDNA site, near the telomere of the short arm. Large pCvKB8 sites near both telomeres and a variable intercalary site near the centromere apparently on the short arm.

Chromosome pair no. 3: Metacentric chromosome with one 5S rDNA site on one arm near the centromere. Large pCvKB8 sites near both telomeres and a variable intercalary site near the centromere on the other arm.

Chromosome pair no. 4: Two forms of chromosome 4 were observed in the accessions: 4a) a larger submetacentric chromosome, and 4b) a more metacentric chromosome with shorter long arm. The short arm carried a secondary constriction, with 45S rDNA signal lying on the satellite proximal to the secondary constriction, and a smaller site distal. Large subtelomeric pCvKB8 sites were present on the satellite and on the long arm. No intercalary sites of pCvKB8 were visible.

Weaker and highly variable intercalary pCvKB8 sites were present on all chromosomes. The chromosomes in the $2n = 16$ accession no. 95-60. could not be described in terms of the consensus. One pair of large chromosomes showed intercalary 5S rDNA sites, while one pair had terminal 5S rDNA sites. One short chromosome showed two 5S rDNA sites, while one short chromosome had one intercalary 5S rDNA site. Eight chromosomes showed no rDNA sites. Two satellites belonged to one of the shortest chromosome pairs. The uniqueness of the karyotype of accession 95-60 might indicate that this 4*x C. vernus* accession is best regarded as a hybrid between two species, with overlapping plant morphology.

DISCUSSION

A consensus karyotype could be derived from ten accessions of *C. vernus*, 2*x*, from the Alps and Pyrenees, representative of a large part of its geographical range, but individual karyotypes were characterized by high polymorphism in the distribution of repetitive DNA. Morphological differences between chromosomes of the satellited pair have been reported previously (Brighton, 1976; Rafinski and Passakas, 1976). With the benefit of markers to identify each chromosome pair, we show here that the level of polymorphism on the three pairs of chromosomes without satellites is essentially as high, with respect to the size and morphology of chromosomes, the number of

FIGS 1–8. Metaphase chromosomes of *Crocus vernus* following *in situ* hybridization with 45S and 5S rDNA probes and the tandemly-repeated sequence pCvKB8. Blue signals show DAPI counterstaining of chromosomal DNA. Red signals are biotin-labelled DNA detected by Cy3 fluorescence. Green signals are digoxigenin-labelled DNA detected by FITC fluorescence. All figures × 1600. Fig. 1. Metaphase from acc. 95-2. A, DAPI counterstaining. B, Probes: 45S rDNA (red) and 5S rDNA (green). The polymorphism of 5S rDNA position on chromosome 3 is visible (arrow, left shows intercalary site, right shows sub-terminal site). Fig. 2. Metaphase from acc. 95-37 (one chromosome 3 is missing). A, DAPI counterstaining. B, Probes: pCvKB8 (red) and 5S rDNA (green, three sites arrowed). Fig. 3. Metaphase from acc. 95-60 probed with 45S rDNA (red) and 5S rDNA (green, arrows). Lines connect satellites to their corresponding chromosomes. Fig. 4. A metaphase from acc. 95-213. A, DAPI counterstaining. B, Probes: pCvKB8 (green) and 45S rDNA (yellow; the two satellited chromosomes are circled to show the polymorphisms). C, Probe 45S rDNA (red). Fig. 5. A prometaphase from acc. 95-213. A, DAPI counterstaining. B, Probes pCvKB8 (red) and 5S rDNA (green; four sites arrowed). Fig. 6. Metaphase from acc. 95-106. A, DAPI counterstaining. B, Probes: 45S rDNA (red) and 5S rDNA (green; four sites arrowed on three chromosomes). Fig. 7. Metaphase from acc. C-354. A, DAPI counterstaining. B, Probes: 45S rDNA (red) and 5S rDNA (green; five sites arrowed). Fig. 8. Metaphase from acc. 96-13. A, DAPI counterstaining. B and C, Probes: 45S rDNA (red) and pCvKB8 (green).

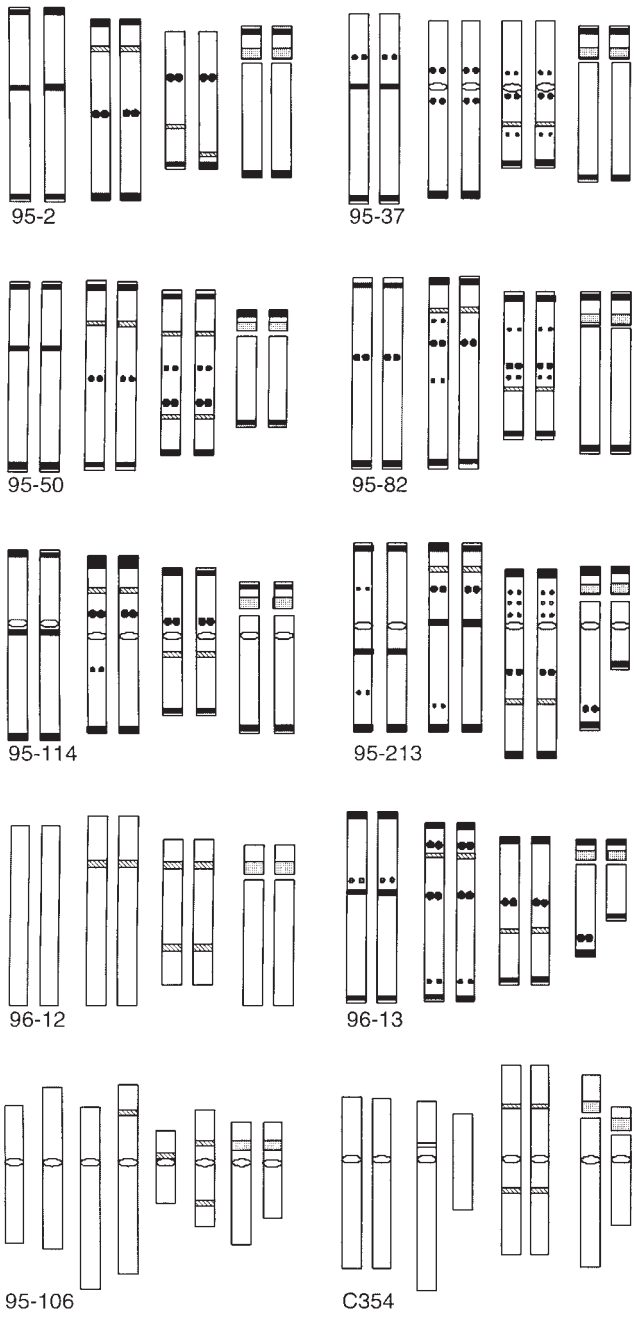


FIG. 9. Idiograms of the karyotypes of the $2n = 8$ accessions. The distribution of pCvKB8 is not shown in the accessions where it was not investigated in detail.

5S rDNA sites, and the number and size of a major repetitive DNA sequence, pCvKB8. Similar polymorphisms in number of 5S rDNA sites were found in both Alpine and Pyrenean populations. It is possible that the polymorphism is ancient and has been retained, or is carried through the range by gene flow, but it is perhaps more likely that the polymorphism has originated multiple times.

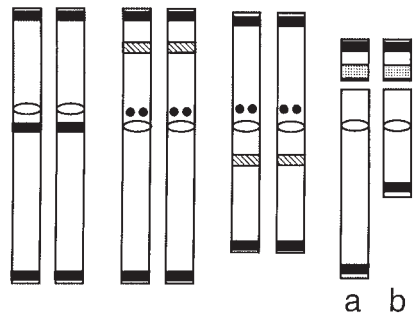


FIG. 10. Idiogram of consensus karyotype from the $2n = 8$ accessions shown in Fig. 9. Symbols as in Fig. 9. For the satellited chromosome 4, two variants were found with similar frequency.

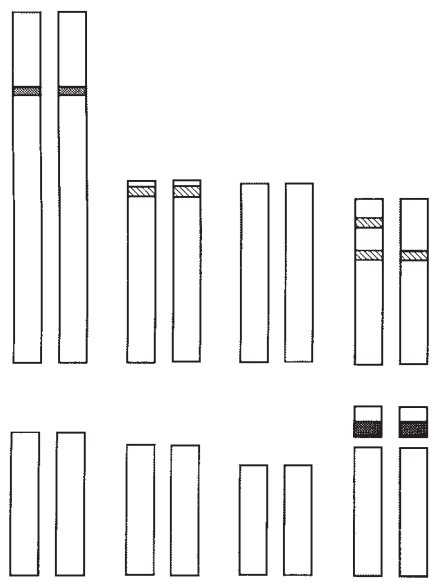


FIG. 11. Idiogram showing 5S and 45S rDNA sites in the $2n = 16$ accession 95-60. Symbols as in Fig. 9.

The intercalary sites of pCvKB8 showed high levels of variation between the investigated karyotypes, although the terminal and centromeric sites were more constant, as well as generally larger. Like other tandemly-repeated DNA sequences, pCvKB8 may have a structural role in stabilizing chromosomes and chromosome segregation. pCvKB8 is unusual in having both centromeric and subtelomeric locations (Schmidt and Heslop-Harrison, 1998).

Though several chromosome types were found in more than one karyotype, we believe that we have sampled only part of the polymorphism in this species. Clearly *C. vernus* tolerates great plasticity in its chromosomal constitution. In this light, an estimation of the gene flow between populations would be valuable, as would controlled hybridization between karyotypes, both with the same and with different chromosome number. Karyotype variability and molecular cytogenetics of natural populations, using multiple accessions from the geographical range, have rarely been studied in plants. Some *Hordeum* species show variation in rDNA, although much less than that found here in chromosome morphology (and, although not examined here, chromosome number) (de Bustos *et al.*, 1996; Taketa *et al.*, 1999).

Brassicaceae species have been studied in separate rDNA studies (Maluszynska and Heslop-Harrison, 1993; Snowdon *et al*., 1997). Some examples of intensive rDNA site variation in natural populations have been reported in the Lilliales: *Allium* species (Jamilena *et al*., 1990; Garrido *et al*., 1994) and in *Scilla autumnalis* (Parker *et al*., 1991).

C. vernus is a good model for the investigation of chromosome evolution in wild species: the diploid number based on only four pairs of large chromosomes limits problems of chromosome identification. The variation in chromosome number and wide geographical range make it likely that multiple evolutionary events have taken place. In future studies, questions about the origin of the chromosomal polymorphism should be addressed. Much could be learned about the evolution of the species and, in general, chromosomal variation in a wild, perennial species. This study stresses the variation on diploid ($2n = 8$) karotypes; future investigations can address the polymorphisms of tetraploid ($2n = 16$) karyotypes, and the identification of diploid ancestors of tetraploids.

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