Theor Appl Genet (1995) 90:477–486


S. N. Raina · Y. Ogihara

# Ribosomal DNA repeat unit polymorphism in 49 *Vicia* species

Received: 2 May 1994 / Accepted: 10 June 1994

**Abstract** DNA restriction endonuclease fragment analysis was used to obtain new information on the genomic organization of *Vicia* ribosomal DNA (rDNA), more particularly among *V. faba* and its close relatives and the taxa within three (Narbonensis, Villosa, Sativa) species' complexes. Total genomic DNA of 90 accessions representing 49 *Vicia* species was restricted with 11 enzymes, and the restriction fragments were probed with three ribosomal clones. Twenty-eight repeat unit length classes were identified. The number of length classes (1–2) per accession did not correspond to the number of nucleolar organizing regions (NORs). The number of rRNA genes was independent of the 2C nuclear DNA amount present in the taxon. Each of the 90 accessions had 2 (rarely 1)–4 *Dra*I sites. Those taxa with the same number of *Dra*I sites generally could be distinguished from each other by different configurations. Probing of the DNA samples digested with tetranucleotide recognition restriction endonucleases emphasized differences between divergent spacer regions and enabled relative homologies between the coding regions to be established. Overall, rDNA restriction site variation among the species showed a good correlation with taxonomic classification. The rDNA analysis indicated evolutionary relatedness of the various taxa within the Narbonensis species complex. rDNA diversity within two other species complexes (Villosa, Sativa), on the other hand, was more extensive than expected. With few exceptions, data on the two complexes give evidence of taxon-specific divergences not seen with other approaches. The restriction site variability and repeat length heterogeneity in the rDNA repeat exhibited startling differences between *V. faba* and its close wild relatives included in the Narbonensis species complex. This analysis provides new evidence that none of the species within the complex can be considered to be putative allies of broad bean.

**Key words** *Vicia* species · Ribosomal DNA · RFLP Diversity · Phylogenetic relationships

Communicated by J. Mackey

S. N. Raina[1] (✉) · Y. Ogihara
Kihara Institute for Biological Research, Yokohama City University, Nakamura-cho 2-120-3, Yokohama 232, Japan

*Present address:*
[1] Laboratory of Cellular and Molecular Cytogenetics, Department of Botany, University of Delhi, Delhi 110007, India

## Introduction

The genus *Vicia* L. (Leguminosae, Vicieae) includes approximately 166 species (Allkin et al. 1986) widely distributed throughout the temperate zones of both hemispheres and extratropical South America (Hanelt and Mettin 1989; van de Ven et al. 1993). The most striking species diversity is to be found in the Mediterranean region and the Caucasus (Hanelt and Mettin 1989). Some 40 species, mainly of Eurasian origin, are widely cultivated for food, green manure, fodder and cover crop purposes (Harlan 1956); the most important of these, broad bean (*V. faba*), common vetch (*V. sativa*) and hairy vetch (*V. villosa*), are conspicuous features of the agricultural system (Duke 1981).

Fifty percent of the species are diploids with a basic number of x = 7; 21% and 5% are diploids with x = 6 and x = 5, respectively. Only 8 species are polyploids (2n = 4x = 24, 2n = 4x = 28), about 7% of the total. In addition, there are several species that have more than one basic number, and a few species inlcude both diploid and tetraploid cytotypes (Hanelt and Mettin 1989; unpublished data). Apart from the numerical chromosome variation, there is large-scale variation in chromosome size, 2C nuclear DNA content (3.85–27.07 pg), repetitive and non-repetitive DNA sequences and chromosomal repatterning in non-nucleolar and nucleolar chromosomes (Raina and Rees 1983; Raina and Narayan 1984; Narayan et al. 1985; Raina 1990;

Raina and Bisht 1988; Raina et al. 1989; Chooi 1971; unpublished data).

The taxonomic history of the genus is extensive and contentious, there being 20 major (re) classifications of the genus since Linnaeus (Maxted 1993). The present difficulties over the correct identification, and therefore nomenclature, of the taxa within the genus *Vicia* arise partly because it is difficult to assess inter-species relationships more precisely by conventional taxonomic, cytological and hybridization studies. The elucidation of taxonomic relationships among *Vicia* species using restriction fragment length polymorphism (RFLP) analysis (Raina and Ogihara 1994; van de Ven et al. 1990, 1993) is still at its early stages in comparison to other crops.

Restriction fragment analysis of nuclear ribosomal RNA genes (rDNA), although not with the same impact as chloroplast DNA, has proven to be of tremendous utility in phylogenetic reconstruction (Doyle et al. 1984; Schaal and Learn 1988; Lapitan et al. 1987; Gill and Appels 1988; Appels and Dvorák 1982a, b; Hamby and Zimmer 1988; Sytsma and Schaal 1985; Terauchi et al. 1992). The tandem arrays of rDNA repeat units, generally located in the nucleolar organizing regions (NORs) of chromosomes, combine highly conserved gene regions, encoding ribsomal RNA (18S, 5.8S, 25S), with more variable intergenic spacer regions (IGS, see Long and Dawid 1980; Appels and Honeycutt 1986). Because ribosomal gene sequences are subject to relatively rapid rates of concerted evolution (Arnheim et al. 1980), they produce DNA fragmentation patterns that are highly homogeneous within individuals and among closely related populations or species, yet exhibit characteristic heterogeneity between groups. In comparison, single-copy gene markers (Johns et al. 1983) often tend to exhibit as much within-group as between-group variation in plants. Ribosomal DNA polymorphisms can, therefore, constitute useful genetic markers. In addition to its value in phylogenetic reconstruction, the biparentally inherited nuclear rDNA variation also provides valuable genetic markers for the analysis of genomic relationships among cultivated species and its wild relatives (Zimmer et al. 1988; Springer et al. 1989; Saghai-Maroof et al. 1984; Breiman et al. 1987; Doyle and Beachy 1985; Appels and Dvorák 1982a, b; Santoni and Bervillé 1992).

With the exception of *V. faba* (Kato et al. 1985; Rogers et al. 1986; Rogers and Bendich 1987a; Yakura et al. 1984; Yakura and Tanifuji 1981, 1983), very little is known about the structure and variability of rDNA repeats in the genus *Vicia* (see Rogers and Bendich 1987b). Thus, in the study presented here we have examined 90 accessions of 49 *Vicia* species to determine the extent and nature of intra- and interspecific variation in the evolution of this gene family. Specifically, we will emphasize that rDNA has proven to be powerful tool for (1) determining the genetic variation between *V. faba* and its close wild relatives and (2) providing novel insights into the evolution of three species complexes.

## Materials and methods

### Plant materials

A list of the species investigated is given in Table 1. Most of the accessions selected within the species were from different geographical regions. The seed samples were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria and Vicieae Project Genebank, Department of Biology, The University, Southampton, UK.

### DNA isolation

Total cellular DNA was isolated from seedling leaves by following a modification of the CTAB method of Murray and Thompson (1980): about 1–2 g of fresh leaf tissue was homogenized in a warm (60 °C) mortar and pestle containing 2X CTABb at 60 °C.

### Restriction endonuclease digests and Southern blotting

Total DNAs were digested with 11 enzymes (*Xba*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, BamHI, *Taq*I, *Mbo*I, *Hin*fI, *Alu*I, *Msp*I) according to the manfacturer's instructions and fractionated by 0.85% or 1.5% agarose gel electrophoresis (AGE) in TAE buffer (Maniatis et al. 1982). After AGE, DNA was transferred to nylon membranes (Hybond $N^+$, Amersham Co. Ltd.) by means of the alkaline method (Reed and Mann 1985).

### DNA probing

The ribosomal gene-specific probes used in this study were pTa 71 (Gerlach and Bedrook 1979), and Ver 18–6 and Ver 6–5 (Yakura et al. 1984). pTa 71 contained an 8.95-kpb *Eco*RI fragment of a full-length nuclear rDNA repeat unit of wheat; Ver 18–6 and Ver 6–5 (rDNA repeat units of *V. faba*) consisted of 3.7-kbp *Eco*RI and 5.7-kbp *Eco*RI + *Bam*HI fragments containing spacer regions flanked by small coding regions of 25S and 18S rRNA, respectively. To separate inserts from the vectors, plasmid DNAs were digested with appropriate enzymes (*Eco*RI, *Eco*RI + *Bam*HI) and run on 1.5% agarose gels. The gel-purified DNA probes were chemically labelled with digoxigenin-dUTP and detected enzymatically according to the manufacturer's (Boehringer Mannheim) protocol. The molecular weights of the hybridizing fragments were determined with reference to digested lambda phage DNA samples run on the same gel. A few anomalous bands wherever observed were not considered in the data analysis.

## Results

There are two basic parameters for which an understanding is essential in an uncharacterized system like *Vicia*, the repeat sizes of the rDNA unit and the locations of the regions of variability within the unit. Initially, therefore, the rDNA repeats in taxonomically 3 divergent species (*V. narbonesis*, *V. ervilia*, *V. sativa* ssp. *sativa*) were characterized with 6 enzymes (*Xba*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI) to check the validity of these enzymes for RFLP analysis (Appels and Honeycutt 1986). The results enabled us to develop a strategy for ordering restriction enzyme analysis in 90 *Vicia* accessions (data not shown). After *Hin*dIII digestion, wheat rDNA hybridized only with repeats of high molecular weight (over 30 kb), indicating thereby that unlike pea (Ellis et al. 1984) there was no *Hin*dIII restriciton site in the rDNA repeat units of any of these 3 species. *Xba*I and *Dra*I were chosen for

**Table 1** Ribosomal DNA repeat unit length variants in *Vicia* species

| Section (subgenus) | Species | Complex | Number of accessions | 2n | DNA amount ($\times 10^{-12}$) | rDNA repeat unit length (kb) | Number of satellited chromosoms[a] |
|---|---|---|---|---|---|---|---|
| Faba (Vicia) | *V. faba* | | 1 | 12 | 27.07 | 11.1, 9.5 | 1 |
| | *V. narbonensis* | Narbonensis | 4 | 14 | 16.11 | 9.0 | 1 |
| | | | 1 | | | 10.2 | |
| | *V. narbonensis* ssp. *salmonea* | Narbonensis | 1 | 14 | 16.11 | 9.0 | 1 |
| | *V. narbonensis* ssp. *jordanica* | Narbonensis | 1 | 14 | | 9.0 | 1 |
| | *V. serratifolia* | Narbonensis | 2 | 14 | 15.63 | 9.0 | 1 |
| | *V. johannis* | Narbonensis | 1 | 14 | 14.14 | 9.0 | 1 |
| | *V. galilaea* | Narbonensis | 1 | 14 | | 8.6 | 1 |
| | *V. hyaeniscyamus* | Narbonensis | 1 | 14 | | 9.0 | 1 |
| | *V. kalakhensis* | Narbonensis | 1 | 14 | | 9.0 | 1 |
| | *V. bithynica* | | 1 | 14 | 9.98 | 7.3[b] | 1 |
| | | | | | | 6.9[b] | |
| Hypechusa (Vicia) | | | | | | | |
| | *V. assyriaca* | | 1 | | | 8.2 | |
| | *V. esdraelonensis* | | 1 | 14 | | 8.9[b] | 1 |
| | *V. galeata* | | 1 | 14 | | 8.4 | 1 |
| | *V. hybrida* | | 1 | 12 | 16.46 | 8.4 | 2 |
| | *V. lutea* | | 1 | 14 | 18.03 | 9.3 | 1 |
| | *V. melanops* | | 1 | 10 | 20.02 | 8.5 | 2 |
| | *V. pannonica* | | 1 | 10 | 13.52 | 11.7 | 2 |
| | *V. sericocarpa* | | 1 | 12 | 18.40 | 6.9 | 2 |
| Peregrinae (Vicia) | | | | | | | |
| | *V. michauxii* | | 1 | 14 | 20.68 | 8.4 | 2 |
| | *V. peregrina* | | 1 | 14 | 19.15 | 7.7 | 2 |
| Vicia (Vicia) | *V. sativa* ssp. *sativa* | Sativa | 7 | 12 | 4.50 | 8.8[b] | 1 |
| | | | | | | 8.0[b] | 1 |
| | *V. cordata* | Sativa | 3 | 10 | 3.95 | 9.1 | 1 |
| | | | 2 | | | 9.6 | |
| | *V. amphicarpa* | Sativa | 2 | 14 | | 8.4[b] | 1 |
| | | | 2 | | | 10.2 | |
| | | | 1 | | | 8.5[b], 9.0[b] | |
| | *V. incisa* | Sativa | 2 | 14 | | 7.9 | 1 |
| | *V. macrocarpa* | Sativa | 1 | 12 | 5.40 | 8.6[b] | 1 |
| | | | 1 | | | 8.2[b] | |
| | | | 3 | | | 8.4[b] | |
| | *V. nigra* | Sativa | 3 | 12 | | 8.0[b] | 1 |
| | *V. angustifolia* | Sativa | 1 | 12 | 5.00 | 8.1[b] | 1 |
| | *V. grandiflora* | | 1 | 14 | 9.05 | 7.2 | 1 |
| | *V. lathyroides* | | 1 | 12 | 6.47 | 8.8 | 1 |
| Cracca (Vicilla) | *V. villosa* | Villosa | 1 | 14 | 4.67 | 6.3 | 1 |
| | *V. villosa* ssp. *villosa* | Villosa | 1 | 14 | | 10.5 | 1 |
| | *V. varia* | Villosa | 1 | 14 | | 9.4 | 1 |
| | | | 2 | | | 6.3 | |
| | *V. eriocarpa* | Villosa | 6 | 14 | 4.50 | 6.3 | 1 |
| | | | 1 | | | 9.1 | |
| | *V. dasycarpa* | Villosa | 2 | 14 | 4.42 | 6.3 | |
| | | | 6 | | | 8.9 | |
| | *V. benghalensis* | | 1 | 14 | 6.95 | 8.6 | 1 |
| | *V. monantha* | | 1 | 14 | 3.85 | 7.8 | 1 |
| | *V. disperma* | | 1 | 14 | 5.18 | 8.4 | 1 |
| | *V. vicioides* | | 1 | 14 | 8.64 | 10.3[b] | 2 |
| | *V. tenuifolia* | | 1 | 24 | 15.99 | 7.9 | 2 |
| | *V. hirsuta* | | 1 | 14 | 8.95 | 7.1 | 1 |
| | *V. ludoviciana* | | 1 | 14 | 8.48 | 7.7 | 1 |
| Cassubicae (Vicilla) | *V. orobus* | | 1 | 12 | 10.60 | 7.8 | 1 |
| Americanae (Vicilla) | *V. americana* | | 1 | 14 | | 8.4[b] | 1 |
| | | | | | | 7.9[b] | |
| Ervilia (Vicilla) | *V. ervilia* | | 1 | 14 | 8.41 | 8.8 | 1 |

**Table 1** (Continued)

| Section (subgenus) | Species | Complex | Number of accessions | 2n | DNA amount ($\times 10^{-12}$) | rDNA repeat unit length (kb) | Number of satellited chromosoms[a] |
|---|---|---|---|---|---|---|---|
| Australes (Vicilla) | *V. epetiolaris* | | 1 | | | 8.9 | |
| Variegatae (Vicilla) | *V. megalotropis* | | 1 | | | 10.9 | |
| Not known | | | | | | | |
| | *V. dalimatica* | | 1 | 12 | 8.23 | 10.9 | |
| | *V. microphylla* | | 1 | | | 8.8 | |
| | *V. atropurpurea* | | 1 | 14 | 7.09 | 8.5 | |
| Total | 49 | | 90 | | | | |

[a] Number of satellited chromosomes for haploid genome
[b] Refers to total length of the two fragments

rDNA analysis in the *Vicia* accessions. Similarly, 5 (*Mbo*I, *Hin*fI, *Alu*I, *Msp*I, *Taq*I) four base-pair cutting restriction enzymes were initially used to assess the level of polymorphism detectable in the above-mentioned 3 species. Among these, 2 (*Taq*I, *Mbo*I) were found to be the most suitable for further analysis.

## Survey of variation in rDNA unit length

A representative survey of the fragment patterns produced by *Xba*I and pTA 71 probe combination is shown in Fig. 1 to illustrate several salient features of *Vicia* rDNAs. Each of the 66 accessions of the *Vicia* species produced a single DNA band, thus showing the existence of a single recognition site in their rDNA units. The length of the ribosomal repeat varies considerably within the genus (Table 1), with several species having repeats in the range of 8.2 to 9.0 kb. A few taxa were found to have rather short repeats (Table 1). This is not surprising in the light of finding that the minimum IGS length in a few *Vicia* species may be of the order of 1.1–1.9 kb (see Rogers and Bendich 1987b). One common pattern in a few accessions of *V. villosa, V. varia, V. eriocarpa* and *V. dasycarpa* (Fig. 1e) (all belonging to the Villosa complex) was a very hazy fragment of either 10.8 or 11.8 kb of variable intensity and a prominent fragment of 6.3 kb. We have no explanation for this. The hazy band could be an artifact, or it could have arisen through the methylation of some *Xba*I site preventing cleavage (Gruenbaun et al. 1981). The *Xba*I site, other than the predominantly visible repeat fragment (6.3 kb) encompassing the conservative region as well, presumably located in IGS region, could not be detected by the heterologous spacer probe. This type of phenomenon was observed to occur in the same and a few more taxa in the *Dra*I and pTa 71 probe combination as well (Figs. 2, 3). Two and 3 *Xba*I sites were also detected in a few accessions. Barring *V. faba*, the 2 restriction fragments, wherever present, added up to a total repeat length of about 8–9 kb. In the case of taxa with three restriction fragments the estimates gave values equivalent to 2 repeat units. The 2-kb fragment in taxa with 2–3 *Xba*I sites was relative to 1–2 bands of very low intensity, sometimes hardly visible on the membrane. The reason for this inconsistency in intensity is not known. It is likely that the fragment in question originates from the spacer region, with little affinity and/or more base-pair mismatching with the heterologous wheat rDNA, and thus the probe in the latter case is partially lost from the membrane during washing. The accessions of only 4 out of 11 species investigated for intraspecific variation were identical in their repeat unit lengths.

## Localization of repeat length heterogeneity

### *DraI restriction sites*

Digestion with *Dra*I gave 2- (rarely 1) 4 fragments with detectable hybridization to the wheat rDNA probe, indicating the existence of internal *Xba*I sites. Twenty-one species produced a 1.7 kb-*Dra*I digestion fragment that hydribized to the probe. At a position corresponding to a size of 2.0–2.8 kb, 3.6–4.1 kb and 4.7–5.7 kb, several different bands could be seen in 31, 16 and 13 species, respectively. Figures 2 and 3 also illustrate that the samples were polymorphic for additional restriction sites as well. *V. assyriaca, V. esdraelonesis, V. galeata, V. hybrida, V. lutea* and *V. melanops* were more closely related to each other than to *V. pannonica* and *V. sericocarpa* in the section Hypechusa; each of the former consistently showed a 1.7-kb fragment and a 2.0- to 2.5-kb fragment besides the 5.2- to 5.7-kb fragment present in *V. assyriaca, V. galeata, V. hybrida* and *V. lutea.* Similar results were obtained in the species listed in sections Faba, Peregrinae, Vicia and Cracca. Several species within section Vicia and Cracca were characterized by the presence of novel 4.7- and 3.4-kb fragments, respectively. Seven species/subspecies within the Narbonensis complex in section Faba similarly produced novel 3.7- and 2.6-kb fragments. The

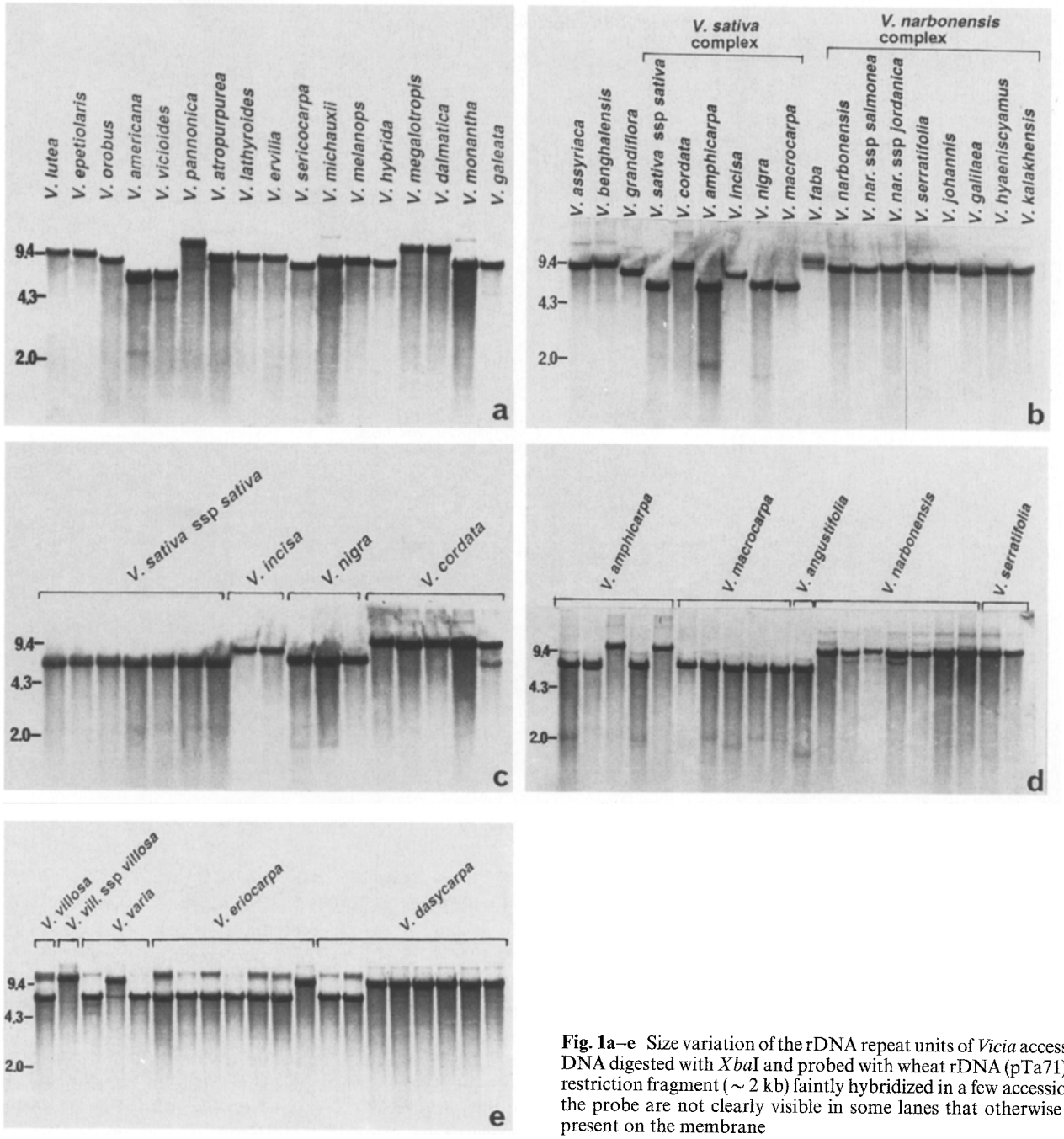


**Fig. 1a–e** Size variation of the rDNA repeat units of *Vicia* accessions. DNA digested with *Xba*I and probed with wheat rDNA (pTa71). The restriction fragment (~ 2 kb) faintly hybridized in a few accessions to the probe are not clearly visible in some lanes that otherwise were present on the membrane

probe-enzyme combination detected polymorphism between the accessions within 7 species as well (Fig. 3).

*Taq*I, *Mbo*I restriction sites

The spacer as well as rRNA coding regions show numerous sites for four-cutter restriction enzymes (Appels and Dvorák 1982a; Yakura et al. 1984). DNA samples for analysis were, therefore, digested with *Taq*I and *Mbo*I and probed with Ver 6–5. The fragment patterns produced by these enzymes clearly illustrate the considerable heterogeneity present in the samples (Fig. 4). The coding regions of the rDNA are present in the smaller fragment bands, 0.8 and 0.7 kb in *Taq*I and 0.9 or 0.6 and 0.7 kb in *Mbo*I, while the length variation (0.5–2.8 kb) is distinguishable in other sets of bands that presumably contain the IGS region. The additional fragments which could have arisen by the enzyme digestion were not detected because either the fragments derived from the IGS region are too small to be assayed with the procedure used here or the regions are too divergent to hybridize with the probe (Gill and Appels 1988). The fragments which have, nevertheless, hybridized emphasize, as in the case of Triticeae (Gill and Appels 1988; Appels and Dvorák 1982a), differences between the divergent spacer sequences of the *Vicia* species.

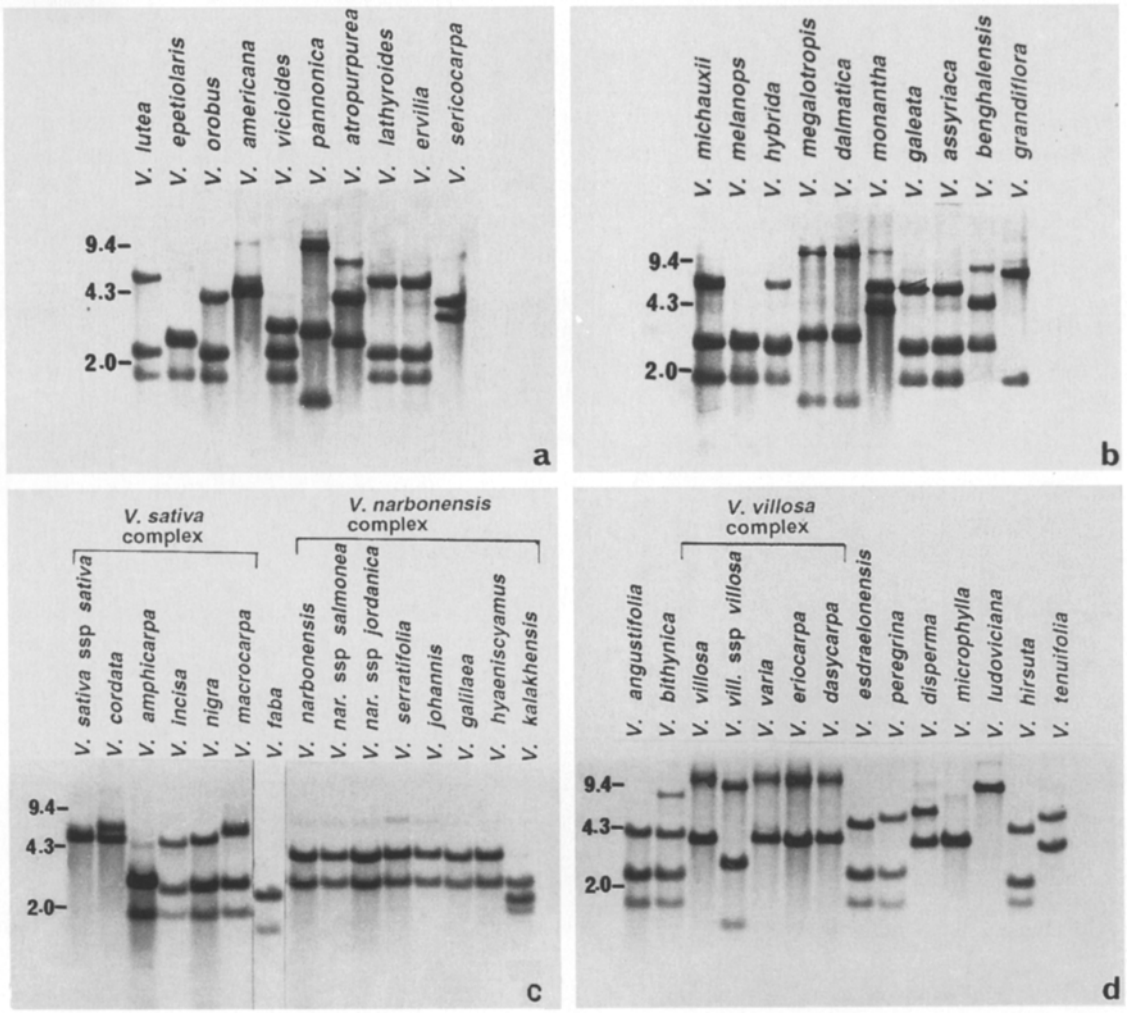


**Fig. 2a–d** Variation in *Dra*I restriction fragments of rDNA that hybridize to wheat rDNA among 49 *Vicia* species

## rDNA restriction site and length variation detected within three species complexes

Out of the 8 species/subspecies within the Narbonensis complex, 6 were identical and possessed repeat units of 9.0 kb only (Table 1, Fig. 1). Five restriction endonuclease (*Dra*I, *Taq*I, *Mbo*I, *Hinf* I, *Alu*I) digests probed with pTa 71, Ver 18-6 and Ver 6-5 yielded essentially identical fragmentation patterns in all but 2 of the species examined (*V. kalakhensis, V. serratifolia*) (Figs. 3, 4). In strong contrast, considerable variations in repeat unit length and also restriction site polymorphism occurring within rDNA arrays of taxa belonging to the Sativa and Villosa complexes were quite evident for various enzyme-probe combinations tested (Table 1, Figs. 1–4).

## rDNA restriction site and length variation detected among *V. faba* and its close wild relatives

The most striking feature to emerge from the present study is the extreme divergence between *V. faba* and its close allies (included in the Narbonensis complex), both in length of the ribosomal repeat as well as in the relative positions of the restriction sites within the repeat unit (Table 1, Figs. 1, 2, 4). Even one probe-enzyme combination was sufficient enough to distinguish between them. Compared to the 2 units of 11.1- and 9.4-kb in *V. faba*, the taxa within the complex possessed 1 repeat unit of either 10.2- or 9.0- or 8.6-kb alone. In other probe-enzyme combinations as well, the percentage of common fragments was estimated to be barely 20%.

## rDNA gene copy number and genome size

On the basis of band intensity in the above-mentioned enzyme-probe combinations, it was amply clear that the intensity differences, if any, were not correlated with the large-scale sevenfold (3.85–27.07 pg) nuclear DNA variation in the genus. These observations are a very indirect measure of the number of rDNA genes present, and the interpretation is tentative. Previous reports on *Vicia* also indicate that the genomic proportion of rDNA decreases with increasing amounts of DNA (Maher and Fox 1973).

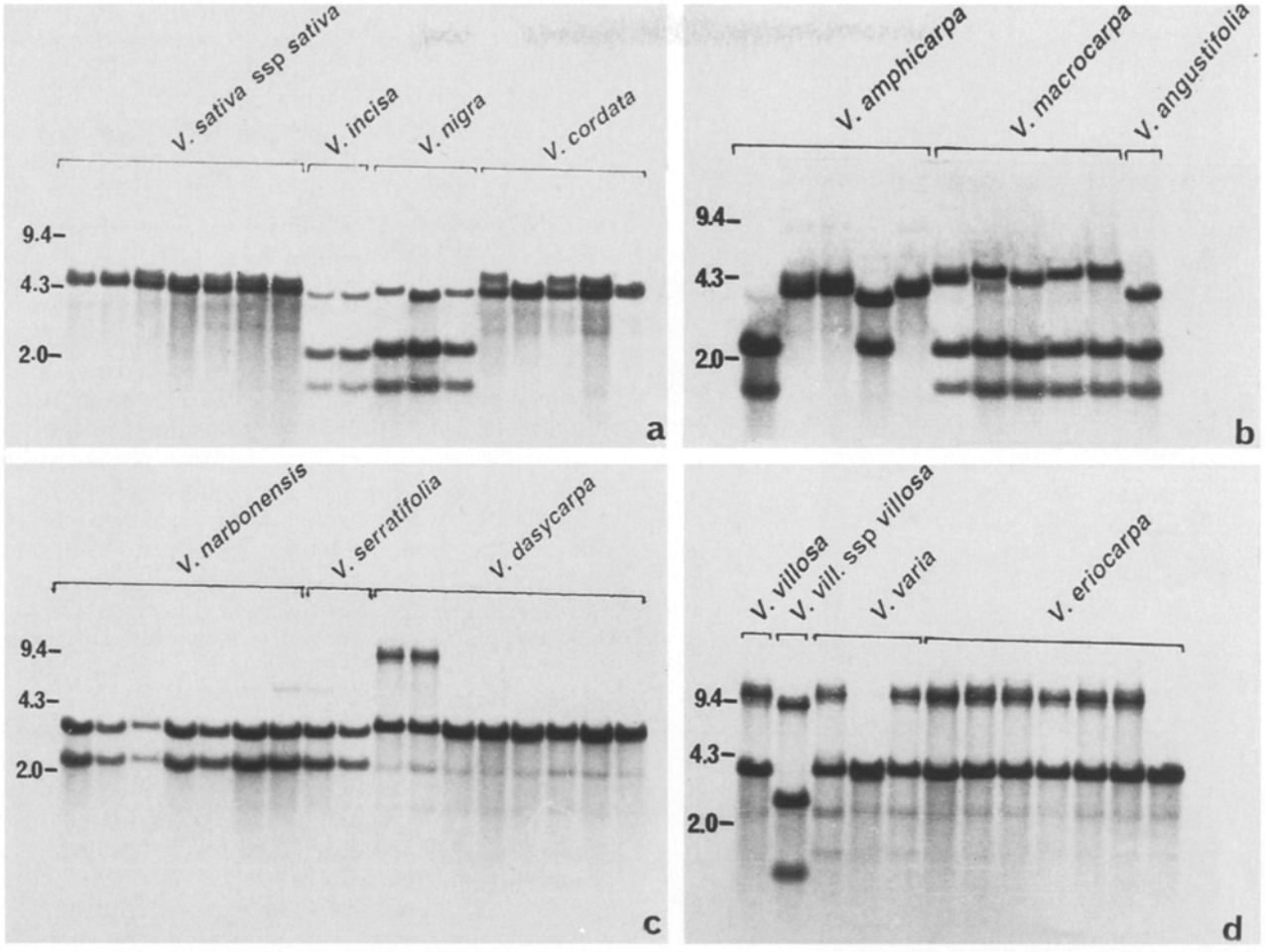


**Fig. 3a–d** Variation in *DraI* restriction fragments of rDNA that hybridize to wheat rDNA among 57 accessions belonging to 14 *Vicia* species

Number of satellited chromosomes

The chromosome complements ($2n = 2x = 10, 12, 14$) of *Vicia* species contain one or two pairs of nucleolar chromosomes (Table 1). Unlike *Hordeum* species (Molnar et al. 1989) the number of rDNA repeat lengths in the *Vicia* species do not correspond to the number of satellited chromosomes present within the complement.

## Discussion

To our knowledge the present study represents the first report of the utilization of variation in rDNA repeat units to provide novel evolutionary insights and generate biosystematically useful data on *Vicia*, more especially among *V. faba* and its wild relatives and within three species complexes. Variation among single accessions of 38 *Vicia* species/subspecies has been studied. Fifty-two accessions representing 11 species have been examined in order to determine intraspecific variation. The taxa stuided belong to 10 of the 22 sections recognized by Kupicha (1976). Of the 8 taxa recognized in each of the Narbonensis and Sativa complexes (Maxted et al. 1991; Maxted 1993; Hollings and Stace 1974; Hanelt and Mettin 1966), we have investigated 7 in each of both groups. In addition, we have characterized the rDNA of all 5 taxa within the Villosa complex (Rota-Michelozzi 1986; Plitmann 1967).

Two types of polymorphisms were observed. The first was in rDNA unit length. Among 45 species, only one repeat length was detected; together these represent 25 length classes. More than one repeat unit length occurred in 5 species, in 3 of which the combination was unique. In *Vicia* species, however, unlike *Hordeum* species (Molnar et al. 1989), there was no correlation between number of repeat unit length classes detected and the number of pairs of satellited chromosomes. The single class of repeat unit lengths in species with more than one pair of NORs suggests that the same class is maintained at different NORs and that they have evolved in conjunction with each other. Variation in repeat unit length was also detected at the intraspecific level. As in the case of *Aegilops* species (Kim et al. 1992), these intra- and interspecific variations were independent of geographical origin (data not shown) as there was no uniformity in the repeat unit length among accessions collected from the same region. The correlation between the repeat unit

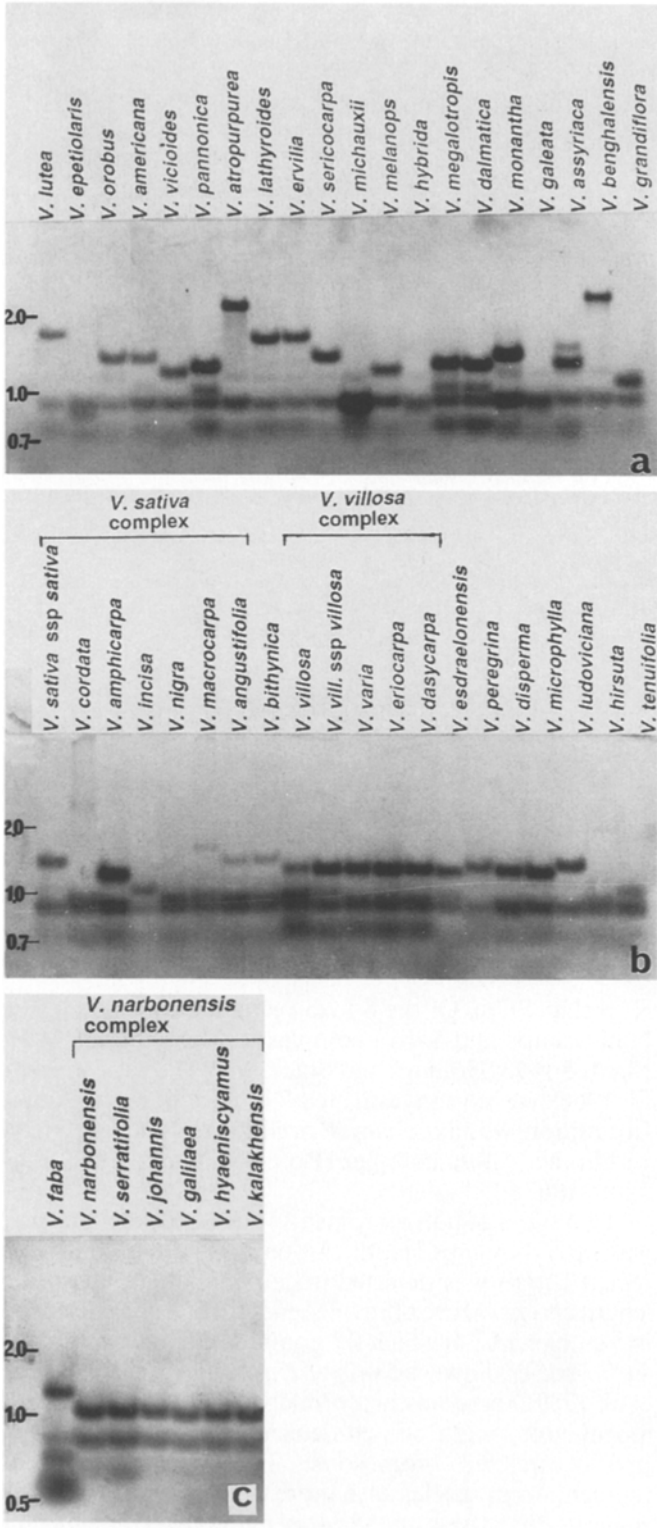


**Fig. 4a–c** *Taq*I restriction fragments of rDNA of *Vicia* species and probed with the spacer region of *V. faba* rDNA (Ver 6–5)

lengths and taxonomic sections was not tenable as a similar range of rDNA repeat unit lengths occurred in taxonomic sections of the genus *Vicia* and certain species were themselves polymorphic. The recent study of rDNA polymorphism in *Hordeum* (Molnar et al. 1989; Molnar and Fedak 1989) produced results very similar to ours in *Vicia*.

The second type of polymorphism that was obseved in rDNA repeat units was in the number and relative position of cleavage sites for six- and four-base-pair cutting enzymes. In particular, *Dra*I and *Taq*I enzyme analysis of the rDNA in *Vicia* species identified several distinct sites that can serve as characteristic markers (Figs. 2–4), and the former generally distinguished major sections of the genus. Restriction site variation, therefore, is more discriminating than length variation for characterization of *Vicia* species. DNA-DNA hybridization of mostly spacer-sequence probes to *Taq*I digests of DNA samples was found to provide a qualitative measure of relationships between the large-spacer *Taq*I fragments present in different *Vicia* species. The present results, therefore, demonstrate the potential of restriction site variation for further studying the dynamics of ribosomal gene evolution in *Vicia* species. A rigorous phylogenetic analysis of the nuclear genome-based evolutionary relationships within the genus would need more characters to be observed.

The taxonomy of the Sativa, Villosa and Narbonensis species complexes in *Vicia* is inconsistent. The groups have been considered to consist of from 1 to 14, 1 to 6 and 3 to 7 separate species, respectively (Baker 1970; Hollings and Stace 1974; Roti-Michelozzi 1986; Plitmann 1967; Maxted et al. 1991; Maxted 1993). The high frequency of bivalents (40–90%) and 1–2 quadrivalents in the hybrids between the different taxa within the Sativa and Narbonesis complexes nevertheless indicates that in general there is considerable chromosomal homoeology or homology between the analyzed genomes, with taxa differing by translocations alone (see Hanelt and Mettin 1989).

The present study on rDNA reflects unambiguously the close relatedness among genomes of the Narbonensis complex, both in the length of rDNA repeat units as well as restriction sites within the unit. Most of the taxa are indistinguishable on the basis of rDNA profiles. This situation is in marked contrast to the profiles observed in the Sativa and Villosa complexes. The presence of detectable quantities of repeats of either different lengths or restriction profiles, or both, from the closely related genomes of individual taxa within the Sativa and Villosa complexes is of interest not only from the point of view of being phylogenetically informative, but also since several distinct lengths and/or sites can serve as characteristic markers for the morophologically not-too-distinct germ plasms. In particular, we have identified polymorphisms that are diagnostic for all the 7 taxa within the Sativa complex. Several papers have descibed a very close, often indistinguishable resemblance between *V. sativa* ssp. *sativa, V. incisa* and *V. nigra* on the one hand and *V. villosa, V. villosa* ssp. *villosa* and *V. varia* on the other, and yet they show distinctive profiles. The present results are, therefore, best understood if it is assumed that the genomes of these taxa are sufficiently well-differentiated to be given special status. The variability within the taxa differs from taxon to taxon. The accessions of *V. serratifolia, V. sativa*

ssp. *sativa, V. incisa* are similar within each taxon, while those of *V. nigra, V. cordata, V. amphicarpa, V. macrocarpa, V. varia, V. eriocarpa, V. narbonensis* and *V. dasycarpa* are more variable. Barring *V. amphicarpa* and *V. narbonensis*, much of the variability in one taxon is, however, overlapping with accessions of other taxa. This illustrates the remarks made by several authors (Hollings and Stace 1974; Plitmann 1967; Hanelt and Mettin 1989, Roti-Michelozzi 1986) that the exact delimitation of each taxa is often difficult to set due to a lack of discontinuity for both morphological and karyotypic features. Spontaneous although rare interspecific hybridization produces offspring that initially are hardly distinguishable phenotically.

In this study, we also examined the structure of rDNA repeats in *V. faba* and its near relatives included in the Narbonensis complex. This complex is considered to stand morphologically closest to the wild relatives of *V. faba* (Zohary and Hopf 1973; Cubero 1984; Birch et al. 1985; Schäfer 1973). According to Zohary and Hopf (1973) and Schäfer (1973) this species complex share a common ancestry with *V. faba*. As mentioned before, the taxa within the Narbonensis complex were observed to have fairly similar rDNA monomer sizes and restriction maps, befitting their close common ancestry. A comparison of *V. faba* rDNA, however, with those from the other 8 species/subspecies in the complex reveals startling differences in the length of the ribosomal repeats and restriction sites. The variable number of sub-repeat units of 325 bp within the IGS region is considered to account for the length variation in *V. faba* (Yakura et al. 1984; Rogers et al. 1986). As in the case of *Hordeum vulgare, Sorghum bicolor, Zea mays*, wheats and their close relatives (Gerlach and Bedrook 1979; Saghai-Maroof et al. 1984; Breiman et al. 1987; Springer et al. 1989; Zimmer et al. 1988; Appels and Honeycutt 1986), there is the possibility of having repeat length variability in its presumed putative allies as well. Such was not the case in the limited population that we investigated. Further, in comparison to 2 repeat unit lengths in *V. faba*, the complex taxa contained only 1 repeat unit. More important, the restriction site polymorphism observed among the complex taxa in the coding and IGS regions was quite different to the one observed in *V. faba*. This is in sharp contrast to the situation found in *Glycine max, Sorghum bicolor, Zea mays, Hordeum vulgare*, wheat and their close relatives wherein the restriction site profiles between the cultivated and wild progenitors were either identical or the variation could be interpreted phylogenetically (Springer et al. 1989; Doyle and Beachy 1985; Zimmer et al. 1988; Molnar et al. 1989; Appels and Honeycutt 1986). There is a case, therefore, for maintaining that none of the wild species in the complex could be considered to be the immediate wild progenitor of *V. faba*. Other studies have also shown that *V. faba* is genetically distinct in its nuclear (Raina 1990; Raina and Rees 1983; Ladizinsky 1975; Perrino et al. 1989; Cubero 1981; van de Ven et al. 1993) and chloroplast genomes (Raina and Ogihara 1994) from the presumed allies. The present study adds yet another extra complication to the picture of Faba bean evolution.

**Acknowledgements** Grateful thanks are due to the Japanese Society for the Promotion of Science and Indian National Science Academy for financial assistance to SNR, and to the International Centre for Agricultural Research in the Dry Areas (ICARDA) and Department of Biology, The University, Southampton for supplying seed samples. We would like to thank Dr. K. Yakura (Kanagwa University, Japan) for providing ribosomal gene probes. Drs. T. Sasakuma and H. Tsujimoto are thanked for helpful suggestions and one of the authors (SNR) acknowledges the generous help and encouragement provided by H. Katayama, K. Hasegawa and S. Yanagawa. This work was supported, in part, by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government. This is contribution No. 156 from Kihara Institute for Biological Reserach, Yokohama City University.

## References

Allkin R, Goyder DJ, Bisby FA, White RJ (1986) Names and synonyms of species and subspecies in the Vicieae: Issue 3, Vicieae Database Project, Publication No. 7

Appels R, Dvorák J (1982a) The wheat ribosomal DNA spacer region: Its structure and variation in populations and among species. Theor Appl Genet 63:337–348

Appels R, Dvorák J (1982b) Relative rates of divergence of spacer and gene sequences within the rDNA region of species in the *Triticeae*: implications for the maintenance of homogeneity of a repeated gene family. Theor Appl Genet 63:361–371

Appels R, Honeycutt RL (1986) rDNA: Evolution over a billion years. In: Dutta SK (ed) DNA systematics, vol 2. CRC Press, Boca Raton, Fla., pp 81–135

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, Zimmer E (1980) Molecular evidence for genetic exchanges among ribosomal genes on non-homologous chromosomes in man and apes. Proc Natl Acad Sci USA 77:7323–7327

Baker HG (1970) Genetic resources in plants: their exploration and conservation. In: Frankel OH, Bennett E (eds) Taxonomy and the biological species concept in cultivated plants. Blackwell Scientific Publ, Oxford, pp 49–68

Birch ANE, Tithecott MT, Bisby FA (1985) *Vicia johannis* and wild relatives of Faba bean: a taxonomic study. Econ Bot 39:177–190

Breiman A, Rotem-Abbarbanell D, Karp A, Shaskin H (1987) Heritable somaclonal variation in wild barely (*Hordeum spontaneum*). Theor Appl Genet 74:104–112

Chooi WY (1971) Variatioin in nuclear DNA content in the genus *Vicia*. Genetics 68:195–211

Cubero JI (1981) Interspecific hybridization in *Vicia*. In: Hawton G, Webb C (eds) Faba bean improvement. Martinus Nijhoff, The Netherlands, pp 91–108

Cubero JI (1984) Taxonomy, distribution and evolution of the Faba bean and its wild relatives. In: Witcombe JR, Erskine W (eds) Genetic resources and their exploitation - chickpeas, faba beans and lentils. Martinus Nijhoff/Dr. W Junk Publ, The Netherlands, pp 131–143

Doyle JJ, Beachy RN (1985) Ribosomal gene variation in soybean (*Glycine*) and its relatives. Theor Appl Genet 70:369–376

Doyle JJ, Beachy RN, Lewis WH (1984) Evolution of rDNA in *Claytonia* polyploid complexes. In: Grant WF (ed) Plant biosystematics. Academic Press, Ontario, Canada, pp 321–341

Duke JA (1981) Handbook of legumes of world economic importance. Plenum Press, New York

Ellis THN, Davies DR, Castleton JA, Bredford ID (1984) The organization and genetics of rDNA length variants in peas. Chromosoma 91:74–81

Gerlach WL, Bedrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Gill BS, Appels R (1988) Relationships between *Nor*-loci from different Triticeae species. Plant Syst Evol 160:77–89

Gruenbaun Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860–862

Hamby RK, Zimmer EA (1988) Ribosomal RNA sequences for inferring phylogeny within the grass family (Poaceae). Plant Syst Evol 160:29–37

Hanelt P, Mettin D (1966) Cytosystematische Untersuchungen in der Artengruppe um *Vicia sativa* L. II. Kulturpflanzen 14:137–161

Hanelt P, Mettin D (1989) Biosystematics of the genus *Vicia* L. (Leguminosae). Annu Rev Ecol Syst 20:199–223

Harlan JR (1956) Theory and dynamics of grassland agriculture. D Van Nostrand, Princeton

Hollings E, Stace CA (1974) Karyotype variation and evolution in the *Vicia sativa* aggregate. New Phytol 73:195–208

Johns MA, Strommer JN, Freeling M (1983) Exceptionally high levels of restriction site Polymorphism in DNA near the maize *Adh*I gene. Genetics 105:733–743

Kato A, Yakura K, Tanifuji S (1985) Repeated DNA sequences found in the large spacer of *Vicia faba* rDNA. Biochem Biophys Acta 825:411–415

Kim WK, Innes RL, Kerber ER (1992) Ribosomal DNA repeat unit polymorphism in six *Aegilops* species. Genome 35:510–515

Kupicha FK (1976) The infrageneric structure of *Vicia*. Notes R Bot Gard Edinburgh 34:287–326

Ladizinsky G (1975) Seed protein electrophoresis of the wild and cultivated species of section Faba of *Vicia*. Euphytica 24:785–788

Lapitan NLV, Gill BS, Sears RG (1987) Genomic and phylogenetic relationships among rye and perennial species in the Triticeae. Crop Sci 27:682–687

Long EO, Dawid IB (1980) Repeated genes in eukaryotes. Annu Rev Biochem 49:727–764

Maher EP, Fox DP (1973) Multiplicity of ribosomal RNA genes in *Vicia* species with different nuclear DNA contents. Nature 245:170–172

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Maxted N (1993) A phenetic investigation of *Vicia* L. subgenus Vicia (Leguminosae, Vicieae). Bot J Linn Soc 111:155–182

Maxted N, Khattab AMA, Bisby FA (1991) The newly discovered relatives of *Vicia faba* L. do little to resolve the enigma of its origin. Bot Chron 10:435–465

Molnar SJ, Fedak G (1989) Polymorphism in ribosomal DNA repeat units of 12 *Hordeum* species. Genome 32:1124–1127

Molnar SJ, Gupta PK, Fedak G, Wheatcroft R (1989) Ribosomal DNA repeat unit polymorphism in 25 *Hordeum* species. Theor Appl Genet 78:387–392

Murray HG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Narayan RKJ, Ramachandran C, Raina SN (1985) The distribution of satellite DNA in the chromosome complements of *Vicia* species (Leguminosae). Genetica 66:115–121

Perrino P, Maruca G, Linsalata V, Bianco VV, Lester RN, Lattanzio V (1989) Flavonoid taxonomic analysis of *Vicia* species of section Faba. Can J Bot 67:3529–3533

Plitmnn U (1967) Biosystematical study in the annual species of *Vicia* of the Middle East. Hebrew University, Jerusalem

Raina SN (1990) Genome organisation and evolution in the genus *Vicia*. In: Kawano S (ed) Biological approaches and evolutionary trends in plants. Academic Press, London, pp 183–201

Raina SN, Bisht MS (1988) DNA amounts and chromatin compactness in *Vicia*. Genetica 77:65–77

Rains SN, Narayan RKJ (1984) Changes in DNA composition in the evolution of *Vicia* species. Theor Appl Genet 68:187–192

Raina SN, Ogihara Y (1994) Chloroplast DNA diversity in *Vicia faba* and its close wild relatives: implications for reassessment. Theor Appl Genet 88:261–266

Raina SN, Rees H (1983) DNA variation between and within chromosome complements of *Vicia* species. Heredity 51:335–346

Raina SN, Yamamoto K, Murakami M (1989) Intraspecific hybridization and its bearing on chormosome evolution in *Vicia narbonensis* (Fabaceae). Plant Syst Evol 167:201–217

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 3:7207–7221

Rogers SO, Bendich AJ (1987a) Heritability and variability in ribosomal RNA genes of *Vicia faba*. Genetics 117:285–295

Rogers SO, Bendich AJ (1987b) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol 9:509–520

Rogers SO, Honda S, Bendich AJ (1986) Variation in the ribosomal RNA genes among individuals of *Vicia faba*. Plant Mol Biol 6:339–345

Roti-Michelozzi G (1986) Biosystematic studies on the *Vicia villosa* complex in Europe. Candollea 41:399–411

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Santoni S, Bervillé A (1992) Characterization of the nuclear ribosomal DNA units and phylogeny of *Beta* L. wild forms and cultivated beets. Theor Appl Genet 83:533–542

Schaal BA, Learn GH Jr (1988) Ribosomal DNA variation within and among plant populations. Ann Mo Bot Gard 75:1207–1216

Schäfer HI (1973) Zür Taxonomie der *Vicia narbonensis* Gruppe. Kulturpflanzen 21:211–273

Springer PS, Zimmer EA, Bennetzen JL (1989) Genomic organization of the ribosomal DNA of *Sorghum* and its close relatives. Theor Appl Genet 77:844–850

Sytsma KJ, Schaal BA (1985) Phylogenetics of the *Lisianthius skinners* (Gentianaceae) species complex in Panama utilizing DNA restriction fragment analysis. Evolution 39:594–608

Terauchi R, Chikaleke VA, Thottappilly G, Hahn SK (1992) Origin and phylogeny of Guinea yams as revealed by RFLP analysis of chloroplast DNA and nuclear ribosomal DNA. Theor Appl Genet 83:743–751

van de Ven WTG, Duncan N, Ramsay G, Phillips M, Powell W, Waugh R (1993) Taxonomic relationships between *V. faba* and its relatives based on nuclear and mitochondrial RFLPs and PCR analysis. Theor Appl Genet 86:71–80

van de Ven WTG, Powell W, Ramsay G, Waugh R (1990) Restriction fragment length polymorphisms as genetic markers in *Vicia*. Heredity 65:329–342

Yakura K, Tanifuji S (1981) The organization of rRNA genes in *Vicia faba*. Plant Cell Physiol 22:1105–1111

Yakura K, Tanifujii S (1983) Molecular cloning and restriction analysis of *Eco*RI fragments of *Vicia faba* rDNA. Plant Cell Physiol 24:1327–1330

Yakura K, Kato A, Tanifuji S (1984) Length heterogeneity in the large spacer of *Vicia faba* rDNA is due to the differing number of 325-bp repetitive sequence element. Mol Gen Genet 193:400–405

Zimmer EA, Jupe ER, Walbot V (1988) Ribosomal gene structure, variation and inheritance in maize and its ancestors. Genetics 120:1125–1136

Zohary D, Hopf M (1973) Domestication of pulses in the old world. Science 182:887–894