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Physical mapping of 18S-5.8S-26S and 5S ribosomal RNA gene families in three important vetches (*Vicia* species) and their allied taxa constituting three species complexes

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Abstract The most-important vetch species, *Vicia narbonensis* (narbon vetch, section *Faba*), *Vicia villosa* (hairy vetch, section *Cracca*) and *Vicia sativa* (common vetch, section *Vicia*) and their close relatives (often difficult to circumscribe into distinct taxa) constitute respectively, Narbonensis, Villosa and Sativa species complexes in the genus *Vicia.* The distribution of the 18S-5.8S-26S (18S-26S) and 5S ribosomal RNA (rRNA) gene families on the chromosomes of 19 (2n=2*x*=10,12,14) of the 24 species and subspecies belonging to the three species complexes, and *Vicia bithynica* (2n=12, section *Faba*) and *Vicia hybrida* (2n=12, section *Hypechusa*) was studied by fluorescence in situ hybridization (FISH) with pTa 71 (18S-26S rDNA) and pTa 794 (5S rDNA) DNA clones. Computer – aided chromosome analysis was performed on the basis of chromosome length, the arm-length ratio and the position of the hybridization signals. The positions of the four (2+2) signals of the two rRNA gene families were similar between each of the three, as well as two subspecies of *V. narbonensis* and *Vicia johannis*, respectively. Two major 18S-26S rDNA loci were found in the nucleolus organiser regions (NORs) of each of the species except *V. hybrida*, where it was present in two out of four SAT chromosomes. In addition to major NORs, two minor loci have been physically mapped at the centromeric regions of chromosomes of group 1 in *Vicia amphicarpa*, *Vicia macrocarpa* and *V. sativa*, and two NORs of group 5 in *V. hybrida,* and on the long arms of group 4 in *V. bithynica*. Two or four 5S rDNA loci, observed in the short arms of groups 2–4 and 5, and 18S-26S rDNA loci were located in different chromosomes of all the species within the Narbonensis and Villosa species complexes, and *Vicia angustifolia* of the Sativa species complex. In the remaining six species of the Sativa species complex, and *V. bithynica* and *V. hybrida*, the two or four 5S rDNA sites were present in chromosomes which harbor 18S-26S rRNA genes. The tandemly repeated 5S rDNA sites, located at the proximal part of the long arm of groups 3–5, were diagnostic for *V. angustifolia*, *Vicia cordata*, *Vicia incisa*, *V. macrocarpa*, *Vicia nigra* and *V. sativa* of the Sativa species complex. In *V. amphicarpa* of the same complex, the tandem repeats were located at the distal part of the long arms of group 3. Variability in the number, size and location of two ribosomal DNA probes could generally distinguish species within the Narbonensis and Sativa species complex, *V. bithynica* and *V. hybrida.* With respect to the four species of the Villosa species complex the karyotypes could not be identified individually on the basis of the distribution of two ribosomal gene families in three out of seven pairs of chromosomes.

Keywords Vetch species complexes · 5S rRNA · 18S-5.8S-26S rRNA · In situ hybridization · Evolution

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Introduction

The genus *Vicia* L. (Leguminosae: Vicieae) comprises approximately 170 species (Allkin et al. 1986) which are widely distributed throughout the temperate zones of both northern and southern hemispheres. Kupicha (1976) recognized 22 sections in the two subgenera, *Vicia* and *Vicilla*. Section *Faba* (subgenus *Vicia*), *Cracca* (subgenus *Vicilla*) and *Vicia* (subgenus *Vicia*) is each comprised of two distinct units. One is of a clearly distinct species, and the other contains a group of closely related taxa re-

ferred to as the Narbonensis species complex (*Vicia narbonensis* and allied taxa, section *Faba*), the *Villosa* species complex (*Vicia villosa* and allied taxa, section *Cracca*) and the *Sativa* species complex (*Vicia sativa* and allied taxa, section *Vicia*) (Kupicha 1976; Roti-Michelozzi 1986). The three species, *V. narbonensis* (narbon vetch), *V. villosa* (hairy vetch) and *V. sativa* (field or common vetch), are widely cultivated as a green manure cover, as well as grain and straw crops for animal feed, and a soil binder throughout the North Temperate Zone of the New and Old Worlds (Duke 1981; McKee and Schoth 1949; Bennett and Maxted 1997). The wild or semi-cultivated taxa, such as *Vicia eristalioides*, *Vicia galilaea*, *Vicia hyaeniscyamus*, *Vicia johannis*, *Vicia kalakhensis* and *Vicia serratifolia*; *Vicia dasycarpa*, *Vicia eriocarpa* and *Vicia varia*; *Vicia amphicarpa*, *Vicia angustifolia*, *Vicia cordata*, *Vicia devia*, *Vicia incisa*, *Vicia macrocarpa*, *Vicia nigra*, *Vicia pilosa* and *Vicia segetalis,* related to *V. narbonensis*, *V. villosa* and *V. sativa* respectively, are of interest to agronomists and plant breeders as crop plants in their own right and as possible sources of germplasm for cultivated *Vicia faba* (the Narbonensis species complex is considered to be putative allies of faba bean), *V. narbonensis, V. villosa* and *V. sativa* breeding (Donnelly and Clark 1962; Zohary and Hopf 1973; Ladizinsky and Temkin 1978; Birch et al. 1985; Roti-Michelozzi 1986; Roupakias 1986; Maxted et al. 1991; Bennett and Maxted 1997). The associated taxa, as above, are, however, poorly known, and there are differences of opinions in the classifications used for them in various taxonomic treatments. The Narbonensis, Villosa and Sativa species complexes have been variously assigned to from 3–7, 1–6 and 1–14 separate species, respectively (Ball 1968; Hollings and Stace 1974; Roti-Michelozzi 1986; Maxted et al. 1991; Maxted 1993). As a consequence, materials that might be of use to breeders are named in different ways, or sometimes misidentified, and breeders are uncertain in using the taxonomy to help decide with which plants to experiment. The present difficulties over the correct identification and nomenclature of plants within these three complexes arise partly because it is difficult to circumscribe each taxon not only on morphological features but also on karyotypic, cytochemical, biochemical, molecular and crossability evidences (Hollings and Stace 1974, 1978; Ladizinsky 1975a, 1978, 1981; Ladizinsky and Temkin 1978; Perrino and Pignone 1981; Ladizinsky and Waines 1982; Raina and Rees 1983; Roti-Michelozzi 1986; Raina and Bisht 1988; Hanelt and Mettin 1989; Maxted et al. 1991; Raina and Ogihara 1995; Jaaska 1997; Bisht et al. 1998; Potokina et al. 1999; Shiran and Raina 2001). In the Sativa species complex, the situation is further complicated by the occurrence of intraspecific karyotypic polymorphism (Hollings and Stace 1974; Zohary and Plitmann 1979; Hanelt and Mettin 1989).

The alternative approach is to use recently developed molecular cytogenetic techniques, such as fluorescent in situ hybridization, which has been used to localize repeated DNA sequences both in interphase nuclei, or onto condensed chromosomes during mitosis or meiosis, in plant species. For example, the determination of the number of sites and the physical mapping of tandem repeated genes encoding the 18S-5.8S-26S ribosomal RNA (18S-26S rDNA) and 5S ribosomal RNA (5S rDNA) has allowed the identification and discrimination of different chromosomes within and between chromosome complements besides providing valuable information on genome organisation, chromosome evolution and cytotaxonomy (Mukai et al. 1990; Ricroch et al. 1992; Leitch and Heslop-Harrison 1993; Maluszynska and Heslop-Harrison 1993; Castilho and Heslop-Harrison 1994; Fukui et al. 1994a, b; Badaeva et al. 1996; Lubaretz et al. 1996; Shi et al. 1996; Xu and Earle 1996; Brown and Carlson 1997; Raina and Mukai 1999).

In the present work, we aimed to produce physical maps of the location of the 18S-26S rRNA genes and their chromosomal positions with respect to 5S rRNA genes on the chromosomes of various taxa within the Narbonensis, Villosa and Sativa species complexes. Hybridization patterns of these two RNA gene families will be useful for identifying chromosomes within complements of none too distinct chromosome pairs, and will also enable comparisons of chromosomal organisation to be made between related taxa within the three complexes.

Materials and methods

The seed samples of the species listed in Table 1 were obtained from the International Centre for Agricultural Research in Dry Areas (ICARDA), Aleppo, Syria. The voucher specimens are available in the Department of Botany, University of Delhi, Delhi, India. Three accessions of each species/subspecies were studied by in situ hybridization. In the Sativa species complex we have chosen the accessions that exhibited chromosome morphology similar to the majority of accessions investigated previously within each taxon.

The fluorescent in situ hybridization (FISH) procedure described by Raina et al. (1998) and Raina and Mukai (1999) was used to map the ribosomal RNA genes. Two DNA probes, pTa 71 and pTa 794, were employed. Probe pTa 71 contains a 8.95-kb *Eco*RI repeat unit of the 18S-5.8S-26S rDNA genes and the intergenic spacer (IGS) isolated from *Triticum aestivum* and re-cloned in the pUC 19 plasmid (Gerlach and Bedrook 1979). Probe pTa 794 is a 410 – bp *Bam*HI fragment of 5S rDNA isolated from *T. aestivum* and inserted in the pBR 322 plasmid (Gerlach and Dyer 1980). Clone pTa 794 contains the 120-bp 5S rRNA gene separated by a 290 – bp spacer.

For karyotypic analysis, DAPI-stained chromosomes were captured by a cooled CCD camera and analysed on a computer with the image-analysis software IPLAB SPECTURM H (signal analytics). Chromosomes were randomly numbered and total length and lengths of the short arm (p) and long arm (q) were measured for each chromosome. Using NIH image software, the homologous chromosome pairs were identified based on chromosome arm ratio (q/p), chromosome length and FISH sites of 5S rDNA and 18S-26S rDNA. In the karyogram constructed, chromosome pairs were ordered from longest to shortest, based on the relative length of each pair. At least three well-spread chromosome preparations of each accession were analysed to validate the karyogram constructed for each accession.

Table 1 Number of rDNA loci detected in *Vicia* species

Section (subgenus)	Species	2n	Number of SAT chromosomes	rDNA	
				18S-5.8S-26S	5S
Faba (*Vicia*)	Narbonensis species complex				
	1 *V. galilaea* Plitm. & Zoh.	14	2	2	2
	2 *V. hyaeniscyamus* Mout.	14	2	2	2
	3a *V. johannis* Tamamsch. ssp *johannis*	14	2	2	2
	b *V. johannis* ssp *procumbens* Schäf.	14	2	2	2
	4a *V. narbonensis* L. ssp *jordanica* Schäf.	14	2	2	2
	b *V. narbonensis* ssp *narbonensis*	14	2	2	2
	c *V. narbonensis* ssp *salmonea* (Mout.) Schäf.	14	2	2	2
	5 *V. serratifolia* Jacq.	14	2	2	4
	5				
	1 *V. bithynica* L.	12	2	2[c]+2[a]	2[a]
Cracca (*Vicilla*)	Villosa species complex				
	1 *V. dasycarpa* Tenore	14	2	2	4
	2 *V. eriocarpa* (Hausskn.) Hal.	14	2	2	4
	3 *V. varia* (Host) Corb.	14	2	2	4
	4 *V. villosa* Roth.	14	2	2	4
	4				
Vicia (*Vicia*)	Sativa species complex				
	1 *V. amphicarpa* (L.) Batt.	12	2	2[c]+2[a]	2[a, b]+2[a, b]
	2 *V. angustifolia* Grufb.	12	2	2	2[b]+2[b]
	3 *V. cordata* (Wulf ex. Hop.) Aschers. & Graebn.	10	2	2[a]	2[a, b]+2[a, b]
	4 *V. incisa* (M. Bieb.) Arcang.	12	2	2[a]	2[a, b]+2[a, b]
	5 *V. macrocarpa* (Moris) Arcang.	12	2	2[c]+2[a]	2[a, b]+2[a, b]
	6 *V. nigra* (L.) Ehrh.	10	2	2[a]	2[a, b]+2[a, b]
	7 *V. sativa* L.	12	2	2[c]+2[a]	2[a, b]+2[a, b]
	7				
Hypechusa (*Vicia*)	1 *V. hybrida* L.	12	4	2+2[a, c]	2[a]

[a] 18S-5.8S-26S and 5S in the same chromosome; [b] Tandemly located; [c] Inactive site

Results and discussion

In situ hybridization with fluorescein isothiocyanate (FITC)-labeled 18S-26S rDNA and digoxigenin-labeled 5S rDNA sequence repeats produced distinct chromosome – specific signal patterns after detection with anti-digoxigenin rhodamine (Fig. 1). Our data report the first application of FISH to *Vicia* species. The FISH patterns showed no variation within species or subspecies.

The chromosome complements

The species within the three species complexes had a very similar chromosome morphology. Their karyotype consisted of a gradually decreasing chromosome size. Barring *Vicia hybrida*, each karyotype carried one nucleolar organiser chromosome pair (SAT chromosome). The majority of the chromosomes within the Narbonensis and Villosa species complexes were submedian (>1:1–<3:1) whereas in the Sativa species complex all but a few chromosomes were subtelocentric (=>3:1–<0:1). The karyotypes of *Vicia bithynica* and *V. hybrida* consisted of all but one and two subtelocentric chromosomes, respectively.

In situ hybridization with 18S-5.8S-26S and 5S ribosomal DNA probes

The narbonensis species complex

Double target in situ hybridization distinguished two pairs of chromosomes by revealing two sites of both 5S and 18S-26S rRNA gene loci in seven out of eight species and subspecies (Figs. 1a–d and f–h). *V. serratifolia* was an exception by having four sites of 5S rRNA gene loci (Fig. 1e). Two large-sized signals for 18S-26S rDNA were associated with two nucleolus organizer regions (NOR) of the SAT chromosomes. Each of the species had a 5S RNA gene locus located in the mid region of the short arm of the chromosome. The distribution of the 18S-26S and 5S rDNA loci among the three *V. narbonensis* subspecies, and two *V. johannis* subspecies, were similar. The present finding of the number and location of 5S rDNA loci in *V. narbonensis* corresponds to the results of radioactive in situ patterns observed by Knälmann and Burger (1986). Perrino and Pignone (1981), Ladizinsky (1975b) and Yamamoto et al. (1982), investigating *V. galilaea*, *V. hyaeniscyamus*, *V. johannis*, *V. narbonensis* and *V. serratifolia* karyotypes and (or) using the banding technique, found very little differences

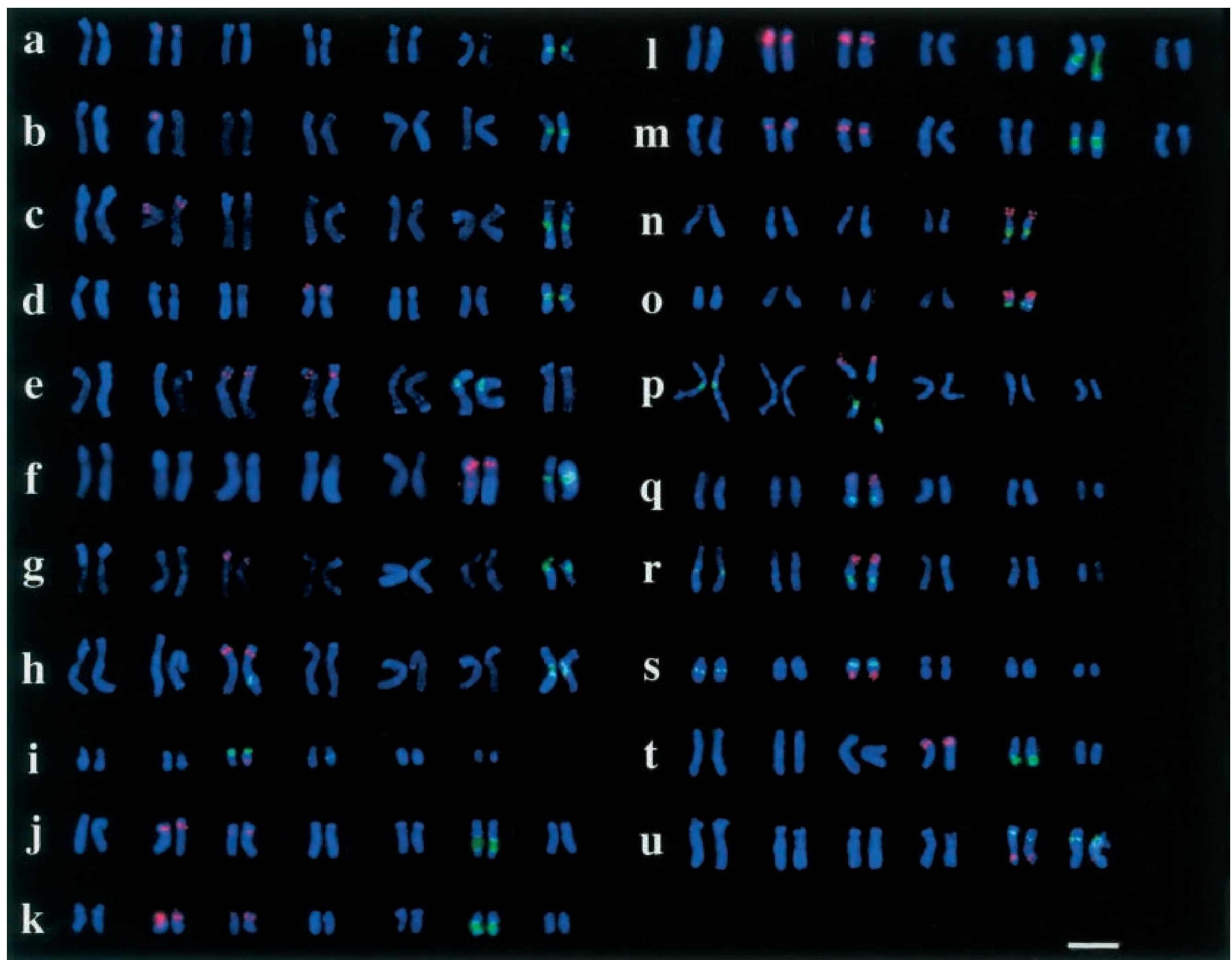

Fig. 1 Karyograms of *Vicia* species after fluorescence in situ hybridization with the 18S-5.8S-26S (*green fluorescence*) and 5S rDNA (red fluorescence) probes. Chromosomes were counterstained with DAPI. a *V. narbonensis* ssp. *jordanica*; b *V. narbonensis* ssp *narbonensis*; c *V. narbonensis* ssp *salmonea*; d *V. galilaea*; e *V. serratifolia*; f *V. hyaeniscyamus*; g *V. johannis* ssp *johannis*; h *V. johannis* ssp *procumbens*; i *V. bithynica*; j *V. villosa*; k *V. varia*; l *V. eriocarpa*; m *V. dasycarpa*; n *V. nigra*; o *V. cordata*; p *V. sativa*; q *V. incisa*; r *V. macrocarpa*; s *V. amphicarpa*; t *V. angustifolia*; u *V. hybrida.* The bar represents 10 μm

between the species. The variability in the number and distribution of 5S rRNA gene loci on chromosomes of the different groups clearly distinguished the five species within the complex (Figs. 1a–h).

Some authors believe that *V. serratifolia* is not sufficiently distinct from *V. narbonensis* to warrant specific status (Plitmann 1967; Ball 1968; Kupicha 1976; Chrtkova-Zertova 1979). However, Schäfer (1973) and Raina and Ogihara (1994), based on morphological and restriction fragment length polymorphism (RFLP) of chloroplast DNA evidence, respectively, suggested that *V. serratifolia* should be given the rank of a distinct species. The labelling patterns of the two ribosomal DNA probes also support this view. Similarly, Plitmann (1967), Schäfer (1973), Ladizinsky (1975b) and Birch et al. (1985) doubted the distinctness of *V. galilaea, V. hyaeniscyamus* and *V. johannis,* while Maxted et al. (1991) and Raina and Ogihara (1994) are of the view that at least two of them should be given species status. FISH with the 5S rRNA gene-specific probe yielded signals on different chromosomes in the three species (Figs. 1d and f–h), and, on the face of it, there is good reason to believe that the above species should be considered as distinct species.

V. bithynica is linked with the Narbonensis species complex (Maxted 1993). This species is clearly distinguished from the Narbonensis complex taxa by the presence of two large and two minute-sized signals for the 18S-26S rDNA loci, and two 5S rDNA loci characteristically located on the long arm of the SAT chromosomes (Fig. 1i). These unique FISH patterns for the two ribosomal DNA probes agrees with the results of Maxted et al. (1991) and Yamamoto et al. (1982) who proposed separate taxon clusters for the Narbonensis species complex and *V. bithynica*. *V. bithynica* is distinguished from the Narbonensis species complex by the presence of a truncate calyx mouth, a peduncle which is often longer than the flower and few, symmetric, elliptic leaflets.

Based on these characters, Maxted (1993) later placed it in the mono-specific section Bithynicae.

The Villosa species complex

The identity of three of the chromosome pairs carrying pTa 71 and pTa 794 signals was determined by in situ hybridization to the *V. dasycarpa*, *V. eriocarpa*, *V. varia*, and *V. villosa* chromosomes (Figs. 1j–m). FISH with the 18S-26S rRNA gene-specific probe yielded strong signals at the NOR of the two SAT chromosomes in all the the four species. The 5S rDNA gene-specific probe labelled four chromosomes in the short arm closely proximal to the centromere in each species. No difference in the number, location and size of hybridization sites for the two ribosomal DNA probes was detected in karyotypes of the four species.

According to Ball (1968), Plitmann (1970) and Zangheri (1976) the complex was considered to represent one species but with five distinctive subspecies. Hess et al. (1970), Gunn (1971), Yamamoto (1973), and Kuta (1980), on the other hand, consider that these subspecies merit specific status. From the similar positions of the six signals for the two ribosomal RNA gene families it seems reasonable to relegate these species to a rank, perhaps of subspecies, within the Villosa species complex.

The sativa species complex

Four hybridization sites for the 18S-26S ribosomal rDNA loci were observed on the *V. amphicarpa, V. macrocarpa* and *V. sativa* chromosomes. In one pair the location of strong signals corresponded to the nucleolar constrictions of the SAT chromosomes, indicating that these regions are actively transcribed. In the other, the minute-sized signals are located near the centromere of the first pair of submedian chromosomes. Each chromosome of the former pair carried two equal-sized and tandemly located 5S rDNA sites in the three species (Figs. 1p, r, and s). The physical location of 5S sites at the proximal and distal part of the long arm could clearly distinguish *V. macrocarpa* and *V. sativa* from the *V. amphicarpa* genome, respectively. The remaining four species, *V. angustifolia*, *V. cordata*, *V. incisa* and *V. nigra* were characterized by the presence of only two hybridization sites for 18S-26S rDNA loci at the NORs of two SAT chromosomes, and two equal sized tandemly repeated loci for 5S rDNA on each of the two chromosomes. In *V. cordata*, *V. incisa* and *V. nigra* these loci occur on chromosomes that harbor 18S-26S rRNA genes (Figs. 1n, o and q). In *V. angustifolia*, 18S-26S and 5S rDNA loci are located on two different pairs of chromosomes (Fig. 1t).

The data presented reveal great discrete interspecific genomic diversity of at least three out of the seven species within the complex. FISH did not show any polymorphism in the two ribosomal gene sites between *V. cordata* and *V. nigra.* Similarly, there were no differences between *V. macrocarpa* and *V. sativa.* However, these groups of two species each exhibited clearly distinct labelling patterns from other species within the Sativa complex. These results have a significant meaning, as several authors believe that it is not possible to classify the Sativa species complex into a small finite number of taxa which are clearly circumscribed. Following the thorough studies so far carried out, Mettin and Hanelt (1964) recognized six species, *V. amphicarpa*, *V. angustifolia*, *V. cordata*, *V. incisa*, *V. macrocarpa* and *V. sativa*. They later (Hanelt and Mettin 1966) added a seventh species, *V. pilosa*. Subsequent studies, however, revealed that morphological differences between 6–8 main entities that botanists recognize in this complex are often blurred. Thus a clear-cut species delimitation is practically impossible (Zohary and Plitmann 1979). According to Ball (1968), Davis and Plitmann (1970) and Maxted (1993) the complex was considered to represent one species but with five, six and six subspecies, respectively. *V. angustifolia* and *V. pilosa* were included by Ball (1968) in *V. sativa* ssp. *nigra*. Hollings and Stace (1974) and Potokina (1997), on the other hand, consider that the taxa within the complex merit specific status, and they recognized eight and seven species, respectively.

Based on results of the present study, the Sativa complex can be subdivided into five subgroups constituted by *V. cordata* and *V. nigra*; *V. macrocarpa* and *V. sativa*; *V. incisa*; *V. amphicarpa*; and *V. angustifolia.*

FISH analysis distinguished the section Hypechusa with one species, *V. hybrida*, from all other species (Fig. 1u) investigated. Four NOR-bearing chromosomes were specifically marked by using the 18S-26S gene as a probe. One of these chromosome pairs with minor NOR loci contained 5S rDNA loci in the distal region of the long arm. The taxonomic history shows that species of the section Hypechusa tend to cluster together rather than forming links with other sectional groups (Maxted 1993). The present results are in agreement with this conclusion.

The detection of one or two 18S-26S rDNA loci in the haploid complements of 19 out of 21 taxa in the present study corroborated well with the results of RFLP analysis (Raina and Ogihara 1995). All the taxa within the Narbonensis and Villosa species complexes, for example, had one repeat-unit length class and one hybridization site for 18S-26S rDNA loci, while *V. amphicarpa*, *V. bithynica* and *V. sativa* had two repeat-unit length classes and two hybridization sites. *V. angustifolia* and *V. hybrida* were exceptions in exhibiting two hybridization sites but only one repeat-unit class. It may be reasonable to assume that the ribosomal gene family was located on the same chromosome, and following genome divergence some of the major NORs were transferred to other chromosomes as a result of translocation (Badaeva et al. 1996).

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