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Nuclear DNA contents, rDNAs, and karyotype evolution in *Vicia* subgenus *Vicia*: II. Section *Peregrinae*

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Summary. Nuclear DNA contents, automated karyotype analyses, and sequences of rDNA spacers have been determined for the species of *Vicia* belonging to sect. *Peregrinae*, as well as for *V. mollis*. The phylogenetic data generated from the comparison of rDNA sequences and karyomorphological results would both indicate that *Vicia mollis* is a sister group to sect. *Peregrinae.* The relationships among the species belonging to the *Peregrinae* section and species enclosed in sections *Faba*, *Narbonensis*, and *Bithynicae* have been also investigated: a clade including *V. mollis* and sect. *Peregrinae* is a sister group to a clade including *V. bithynica* and sect. *Narbonensis.* With our choice of outgroup, *Vicia faba* (including subsp. *paucijuga*) is external to the above mentioned inclusive group.

Keywords: *Vicia* species; Karyotype; Nuclear DNA content; DNA sequence; Phylogeny.

Introduction

The genus *Vicia* L. includes approximately 190 species (ILDIS 1999) subdivided in two subgenera, *Vicia* and *Vicilla* (Schur) Rouy, and widely distributed throughout the temperate zone of both hemispheres (Kupicha 1976). The best know species of the genus is the faba bean, or broad bean, *Vicia faba* L., an important fodder and vegetable crop throughout North Africa and West Asia.

The relationship between *V. faba* and its putative allies of the subgenus *Vicia* has always been controversial, and in a historical review of more than 20 taxonomic studies since Linneus, no consensus was seen on the position of this species. Various studies suggested that the closest relatives of broad bean were to be found in sect. *Faba* sensu Kupicka (including *V. bithynica*, *V. faba*, and a group of species referred to as the Narbonensis complex); in particular, *V. narbonensis* or its closely related species gave strong evidence of being closely related to *V. faba* (Ball 1968, Kupicha 1976). More recently, Maxted (1993) subdivided the subgenus *Vicia* into nine sections and substituted the sect. *Faba* sensu Kupicka with three distinct sections: *Bithynicae* and *Faba*, monospecific, and *Narbonensis* containing the species before referred to as Narbonensis complex, namely, *V. narbonensis* L., *V. serratifolia* Jacq., *V. galilaea* Plitm. et Zoh., *V. hyaeniscyamus* Mout., *V. johannis* Tamasch., and the two newly discovered species *V. kalakhensis* Khatt., Maxt. et Bisby (Khattab et al. 1988) and *V. eristalioides* Maxt. (Maxted 1988).

The relationships within and among sections *Bithynicae*, *Narbonensis*, and *Faba* have been investigated by morphological, karyological, biochemical and molecular approaches (Venora et al. 2000 and references therein), and several of these studies confirmed the classification of Maxted (1993), emphasizing the remoteness of *V. faba* from *V. narbonensis* and from the other species of the group, in some cases also suggesting a closer relationship with the species belonging to other sections, including sect. *Peregrinae* (Van de Ven et al. 1993, Jaaska 1997, Fennell et al. 1998, Potokina et al. 1999, Leht and Jaaska 2002).

To improve the understanding of the relationships within subgenus *Vicia*, our team started the analysis of its nine sections by karyological and molecular methods; results for

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sections *Bithynicae*, *Faba*, and *Narbonensis* have already been published (Venora et al. 2000).

In the present paper we determine nuclear DNA contents, automated karyotype analysis, sequences of the internal transcribed spacers (ITS1 and ITS2), 5.8S rDNA and partial sequences of external transcribed spacer regions (ETS) of rDNA of the species belonging to sect. *Peregrinae*, namely, *V. michauxii* Sprengel (including the subsp. *stenophylla* Boiss), *V. aintabensis* Boiss., *V. peregrina* L., and *V. mollis* Boiss. The position of the last species, *V. mollis* Boiss, is not clear, since Plitman (1967) and Maxted (1994) did not include it in the section *Peregrinae*, whereas Kupicha (1976) did.

Results are compared with similar findings obtained in our previous studies (Venora et al. 2000), with the goal of understanding the relationships in sect. *Peregrinae* and of clarifying the position of *V. faba* in comparison to the species belonging to sections *Peregrinae*, *Bithynicae*, and *Narbonensis*.

Material and methods

Plant materials

The names, source, and accession numbers of the selected species are listed in Table 1.

Cytophotometric analysis

For cytophotometric analysis, 1 cm long roots of *Vicia* species were fixed in ethanol–acetic acid (3 : 1, v/v) and squashed under a cover slip in a drop of 45% acetic acid, after pectinase (Sigma, St. Louis, Mo., U.S.A.) treatment. All slides were then simultaneously hydrolysed either in 1 N HCl at 60 °C for 7 min or in 5 N HCl at room temperature for 30 min, then stained with Schiff's reagent and washed, according to Kotseruba et al. (2000). Feulgen DNA absorptions in individual cell nuclei in the same postsynthetic condition (G_2, 4C) were measured at a wavelength of 550 nm with a Leitz MPV3 integrating microdensitometer (Leica, Wetzlar, Federal Republic of Germany). All squashes were stained concurrently with *V. faba* preparations, used as an internal standard; nuclei in the same postsynthetic stage, from roots at the same developmental stage (1 cm long), were measured and relative Feulgen DNA units were converted into picograms of DNA by assuming for *V. faba* a 4C DNA content of 53.12 pg (Ceccarelli et al. 1995).

Karyomorphometry

Slides were prepared according to Venora et al. (1991). 1 cm long roots of *Vicia* species, from the same lots as those used for nuclear DNA determinations, were treated with 0.05% colchicine for 4 h, then fixed, squashed, and stained with Schiff's reagent. For each species at least five metaphases for each of five seedlings were analysed.

Microscopic investigations are carried out with a Zeiss Axioplan 2 microscope (Carl Zeiss Jena GmbH, Jena, Federal Republic of Germany), connected to a KS400 Zeiss image analysis system, with the dedicated karyotyping software Ikaros 3.4 (Metasystem Gmbh, Altlussheim, Federal Republic of Germany). The automated image analysis system ensures a high degree of accuracy since measurements are taken directly in the first image of the chromosomes (Venora et al. 1991): this method therefore

Table 1. Accession, source, chromosome number and mean nuclear DNA amount in *Vicia* species

Species	Accession nr.	Source [a]	Chromosome nr. (2n)	DNA amount 4C (pg)
V. michauxii Sprengel	831	IPK	14	31.86 ± 0.74
V. michauxii subsp. *stenophylla* Boiss	TR3/93	IPK	14	29.37 ± 1.01
V. peregrina L.	12096	USDA	14	36.22 ± 1.70
V. aintabensis Boiss.	17514	USDA	14	30.61 ± 0.67
V. mollis Boiss.	62649	IPK	10	31.31 ± 1.42
V. narbonensis L.	105786	IGV	14	29.10 ± 0.3 [b]
V. eristalioides Maxt.	877321	B	14	38.58 ± 0.5 [b]
V. galilaea Plitm. et Zoh.	112018	IGV	14	26.09 ± 0.2 [b]
V. hyaeniscyamus Mout.	112008	IGV	14	31.24 ± 0.1 [b]
V. johannis Tamash.	112019	IGV	14	25.08 ± 0.3 [b]
V. kalakhensis Khatt. & al.	867095	B	14	42.22 ± 0.4 [b]
V. serratifolia Jacq.	NAR 121/77	IPK	14	39.59 ± 0.4 [b]
V. bithynica L.	Vic 303/79	IPK	14	18.03 ± 0.2 [b]
V. faba L.	113064	IGV	12	53.12 [c]
V. faba subsp. *paucijuga* Murat.	113021	IGV	12	56.32 ± 1.1
V. sativa L.	253426	UDSA	12	9.00 [d]

[a] IGV, Istituto Genetica Vegetale, CNR, Bari, Italy; B, Genebank, University of Southampton, U.K.; IPK, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Federal Republic of Germany; USDA, United States Department of Agriculture, Pullman, U.S.A.
[b] Venora et al. 2000
[c] Ceccarelli et al. 1995
[d] Rees et al. 1966

allows a precise determination of the karyomorphological indices (TF%, Rec, and SYi) considered directly related to karyotype evolution.

Total lengths of short arms, long arms, and satellites were measured with the computer system. All data for each plate were then computed with the dedicated software Karyo 95 (Dipartimento di Botanica, Università di Catania, Italy) to improve accuracy in matching chromosome pairs on the basis of the data of each chromosome (Pavone et al. 1995).

The classification of Stebbins (1971), the TF% index (Huziwara 1962), and the Rec and SYi indices (Greilhuber and Speta 1976) were computed for each species. The classification of Stebbins (1971) is based on the relative frequency of chromosomes with a long-arm ratio greater than 2 and on the ratio between the length of the longest and that of the shortest chromosome in the complement; the TF% index is expressed by the ratio between the sum of the lengths of the short arms of individual chromosomes and total length of the complement; the Rec index expresses the average of the ratios between the length of each chromosome and that of the longest one; the SYi value indicates the ratio between the average length of the short arms and average length of the long arms.

Chromosome pairs were grouped by cluster analysis (Scott and Knott 1974); nomenclature by Levan et al. (1964) was followed, excluding the satellite length in computing arm ratios.

Table 2. Chromosome morphometric data of *Vicia* species

Species and chromosome nr.[a]	Length (μm) of:[b,c]		Long/short arm ratio[c]	Species and chromosome nr.[a]	Length (μm) of:[b,c]		Long/short arm ratio[c]
	Chromosome	Satellite			Chromosome	Satellite	
V. michauxii				*V. narbonensis*[d]			
1*	7.19 ± 0.60 a	2.73 ± 0.26	3.90 d	1	4.47 ± 0.24 a		1.79 d
2	5.28 ± 0.45 b		5.86 c	2	4.20 ± 0.12 b		1.62 e
3*	5.12 ± 0.42 c	0.89 ± 0.12	1.10 e	3	4.02 ± 0.09 c		1.93 c
4	4.83 ± 0.37 d		5.27 d	4	3.85 ± 0.29 d		1.50 f
5	4.73 ± 0.38 e		10.00 a	5	3.76 ± 0.10 e		1.81 d
6	4.45 ± 0.41 f		6.42 c	6	3.56 ± 0.27 f		1.99 b
7	4.36 ± 0.25 f		8.91 b	7*	3.29 ± 0.17 g	1.02 ± 0.01	2.07 a
V. michauxii subsp. *stenophylla*				*V. bithynica*[d]			
1	8.49 ± 1.06 a		1.38 c	1	3.87 ± 0.35 a		4.86 a
2	6.32 ± 0.62 b		6.56 a	2	3.75 ± 0.28 a		4.43 a
3	5.86 ± 0.58 c		5.10 b	3*	3.62 ± 0.45 a	0.86 ± 0.17	4.11 b
4	5.64 ± 0.61 c		6.29 b	4	3.44 ± 0.22 b		4.55 a
5	5.43 ± 0.40 c		6.71 a	5	3.41 ± 0.27 b		3.61 c
6*	5.30 ± 0.36 c	1.36 ± 0.13	2.07 c	6	3.11 ± 0.30 c		3.57 c
7	4.87 ± 0.45 c		5.26 b	7	2.87 ± 0.15 d		4.52 a
V. peregrina				*V. faba*[d]			
1*	8.45 ± 0.27 a	3.08 ± 0.12	4.84 d	1*	17.60 ± 1.95 a	3.26 ± 0.30	1.83 e
2	6.27 ± 0.16 b		6.13 c	2	8.91 ± 1.01 b		5.36 d
3*	6.00 ± 0.19 c	0.69 ± 0.05	5.82 c	3	8.44 ± 0.79 c		5.75 d
4	5.74 ± 0.29 d		1.06 e	4	8.21 ± 0.76 d		8.33 b
5	5.74 ± 0.23 d		10.48 b	5	7.58 ± 0.45 d		9.46 a
6	5.57 ± 0.39 d		5.26 d	6	7.38 ± 0.53 d		6.60 c
7	4.52 ± 0.31 e		11.91 a				
V. aintabensis				*V. sativa*			
1*	8.12 ± 0.32 a	2.96 ± 0.26	4.67 d	1	3.27 ± 0.13 a		4.84 a
2	5.87 ± 0.40 b		5.67 d	2*	3.06 ± 0.20 b	1.03 ± 0.13	2.38 d
3*	5.71 ± 0.32 c	0.80 ± 0.06	1.08 e	3	2.90 ± 0.18 c		3.68 b
4	5.56 ± 0.29 d		6.51 c	4	2.78 ± 0.28 d		1.46 e
5	5.46 ± 0.15 d		8.58 b	5	2.57 ± 0.15 d		3.51 c
6	5.35 ± 0.26 d		5.77 d	6	1.56 ± 0.08 e		2.71 d
7	4.50 ± 0.24 e		9.98 a				
V. mollis							
1*	13.00 ± 0.09 a	2.47 ± 0.06	1.51 b				
2	7.59 ± 0.10 b		3.54 a				
3	6.40 ± 0.07 c		3.16 a				
4	6.11 ± 0.15 c		3.66 a				
5	5.02 ± 1.00 d		3.44 a				

[a] Asterisk indicates satellite chromosome

[b] Values are means with standard errors

[c] Values followed by the same letter are not significantly different, according to the cluster analysis of Scott and Knott (1974), P = 0.05

[d] Venora et al. 2000

DNA sequencing

Nuclear DNAs were extracted and purified from secondary roots produced after decapitation of the primary one, as described in Maggini et al. (1978), for the species listed in Table 1.

The entire region including ITS1, 5.8S, and ITS2 rDNA was amplified by standard polymerase chain reaction (PCR). Amplifications were carried out using the following parameters: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min, and finally 72 °C for 5 min. The primers, designed by using the conserved coding region of the 18S and 26S ribosomal genes, were 18Sdir, 5′-CGTAACAAGGTTTC CGTAGG-3′, and 26Scom, 5′-AGCGGGTAGTCCCGCCTGA-3′.

Partial sequences of the ETS regions (precisely the portion adjacent to the 5′ end of 18S rDNA) were obtained by amplification experiments using the following primers: 5′-GTGTTTGCTGGCTCCATGCT-3′ and 5′-AGAT TAAGCCATGCATGTGTCAGT-3′. These oligonucleotides correspond to the sequence located from bp 2883 to bp 2902 in the intergenic spacer of *V. faba* (3490 bp long [Kato et al. 1990]) and to a well conserved region present at the 5′ end of the 18S rDNA in many angiosperms, respectively. Amplifications were carried out using the following parameters: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 52 °C for 2 min, 72 °C for 2 min, and finally 72 °C for 5 min. All the PCR products were sequenced directly for both strands by using the automated sequencer ABI Prism 310 (Applied Biosystems, Foster City, Calif., U.S.A.)

ITS and ETS sequences were aligned separately by the Clustal 1.83 software with default values (Thompson et al. 1994), run as a daughter process of Bioedit (Hall 1999), which was also used for sequence editing and manipulation. Aligned sequences were then visually inspected to correct the very few gap distributions devoid of biological meaning. The complete alignment used for all further analyses is available upon request to the senior author. ETS sequences were then individually subjected to a dot plot comparison (20 bp window) to detect repeats by a strategy similar to that in Linder et al. (2000). A preliminary phylogenetic analysis was undertaken on the repeats to discriminate between paralogous and orthologous sequences (Sanderson and Doyle 1992, Linder et al. 2000). The combined ITS and ETS sequence matrix, after removal of the ETS repeats (except the first one), underwent a combined parsimony analysis.

All the manipulations of the matrices, as well as the cladistic analyses, were carried out by the cladistic software environment Winclada (Nixon 1999), running Nona (Goloboff 1999) as a daughter process. Settings included the following: a maximum storage space of 100000 trees (hold 100000); a tree storage space per iteration of 100 (hold/100); one hundred iterations of an algorithm which randomizes the addition order of taxa, creates Wagner tree, and swaps its branches by tree bisection-reconnection (mult*100); further branch swapping on all the found trees (max). *Vicia sativa* was employed as an outgroup. The resulting cladograms were investigated with Winclada, which was also used for 1000 bootstrap replicas (Felsenstein 1985); Nona was used for the evaluation of Bremer support (Bremer 1994).

Results

Cytophotometry

The chromosome numbers and the nuclear DNA contents of the species investigated in this paper are listed in Table 1.

For the determination of nuclear DNA content, we tested two different methods of hydrolysis (Kotseruba et al. 2000), and since the two methods gave the same values of nuclear DNA content, we used the cold hydrolysis method. The DNA content in sect. *Peregrinae* (picograms per 4C nucleus) ranged from 29.37 pg in *V. michauxii* subsp. *stenophylla* to 36.22 pg in *V. peregrina*.

Karyomorphometry

All karyological data from the analyzed species are reported in Table 2; the Rec, SYi, and TF% indices, as well as the classification after Stebbins (1971), are summarised in Table 3.

The species of sect. *Peregrinae* presented a complement 2n = 14 and two couples of satellite chromosomes, except *V. michauxii* subsp. *stenophylla*; *V. mollis* had a chromosome complement of 2n = 10 and only one satellite couple (Fig. 1).

The chromosome typology is summarised in the karyotype formulas (Table 3). All the species of sect. *Peregrinae* showed the formula $m^{sc} + st^{sc} + 3st + 2t$, except *V. michauxii* subsp.

Table 3. Karyotype formulas, mean set lengths, indices, and Stebbins categories for *Vicia* samples

Species	Karyotype formula	Total length of haploid set (μm)	Rec index	SYi index	TF% index	Stebbins categories
V. michauxii	$m^{sc} + st^{sc} + 3st + 2t$	35.96 ± 2.68	66.69	22.45	16.49	3A
V. michauxii subsp. *stenophylla*	$m + sm^{sc} + 5st$	41.90 ± 3.75	65.65	28.13	21.24	3A
V. peregrina	$m^{sc} + st^{sc} + 3st + 2t$	42.29 ± 1.08	66.75	21.71	16.24	3A
V. aintabensis	$m^{sc} + st^{sc} + 3st + 2t$	40.57 ± 1.17	66.61	22.09	16.42	3A
V. mollis	$m^{sc} + 4st$	38.12 ± 1.41	48.31	38.12	25.81	3B
V. narbonensis [a]	$sm^{sc} + 2m + 4sm$	27.15 ± 2.28	84.60	56.05	34.57	2A
V. bithynica [a]	$st^{sc} + 6st$	24.07 ± 1.83	86.99	23.72	18.49	4A
V. faba [a]	$sm^{sc} + 3st + 2t$	58.11 ± 5.04	46.05	23.08	17.70	3B
V. sativa	$m + sm^{sc} + 2sm + 2st$	16.14 ± 0.69	78.72	34.79	24.16	3B

[a] Venora et al. 2000

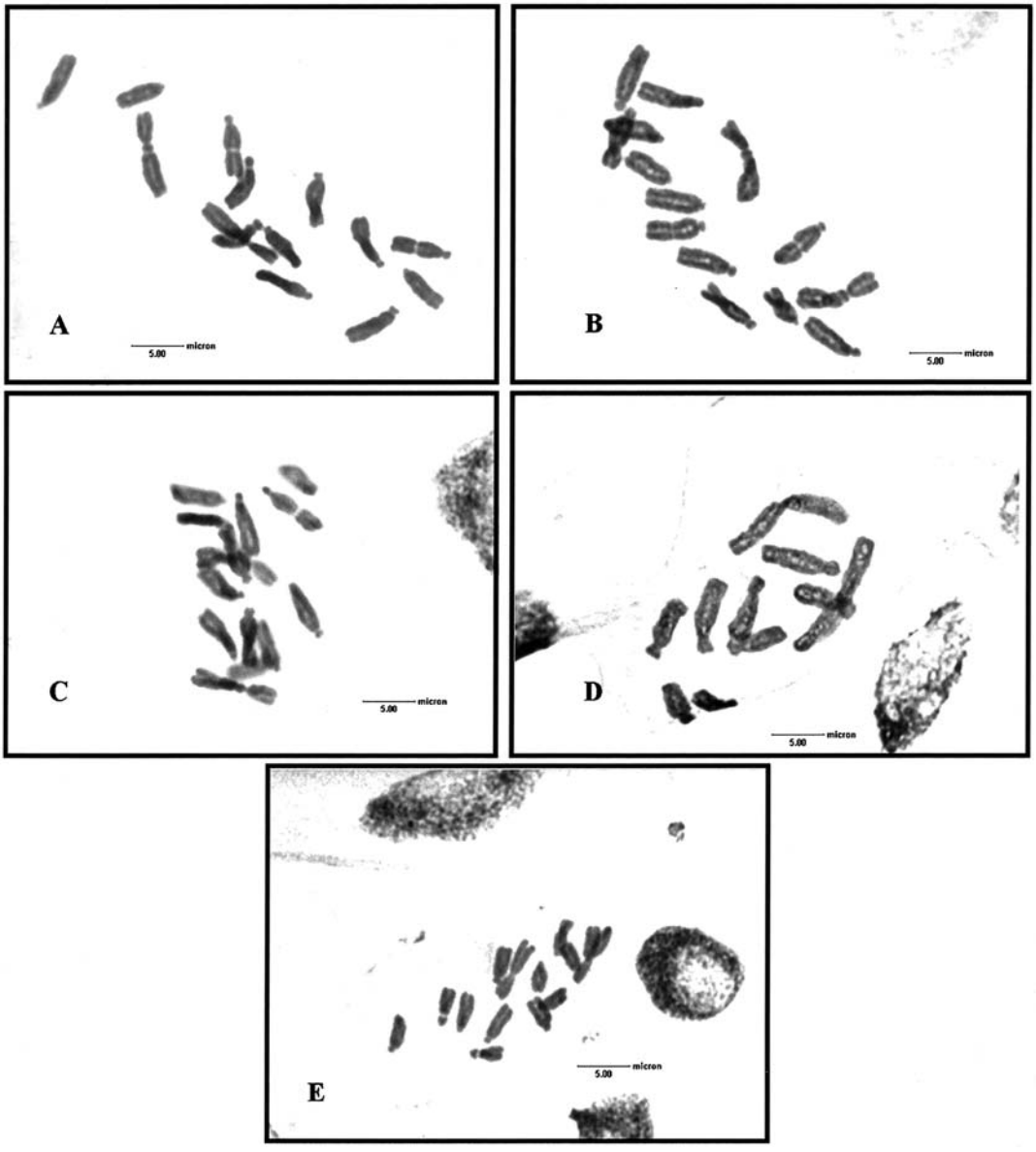

Fig. 1 A–E. Raw metaphase plates of the investigated species. **A** *Vicia michauxii* acc. 831; **B** *Vicia peregrina* acc. 12096; **C** *Vicia aintabensis* acc. 17514; **D** *Vicia mollis* acc. 62649; **E** *Vicia michauxii* subsp. *stenophylla* acc. TR3/93. Bars: 5 μm

s*tenophylla*, which presented the karyotype formula m + sm^{sc} + 5st. The karyotype of *V. mollis* was described by the formula m^{sc} + 4st, whereas that of *V. sativa*, used as outgroup, was m + sm^{sc} + 2sm + 2st.

All the species are classified in category 3A according Stebbins's classification (1971), except for *V. mollis* and *V. sativa*, which belong to category 3B.

In terms of symmetry, as shown by the Rec, Syi, and TF% indices, that of *V. mollis* is the most asymmetric karyotype and greatly differs from those of the other species; in sect. *Peregrinae*, the largest SYi and TF% indices occur in *V. michauxii* subsp. s*tenophylla* (Table 3). In Table 3, data relative to *V. faba*, *V. narbonensis*, and *V. bithynica* are also reported (Venora et al. 2000).

Figure 2 shows a spatial representation of the species of the section *Peregrinae* (*V. mollis* not included) based on the karyotype indices. All the investigated species are very similar, except for *V. michauxii* subsp. *stenophylla.* Figure 3 shows the spatial representation of the species of the section *Peregrinae* together with representative species of sections *Faba*, *Bithynicae*, and *Narbonensis*. All the species of sect. *Peregrinae* appear as intermediate (but distinct) be-

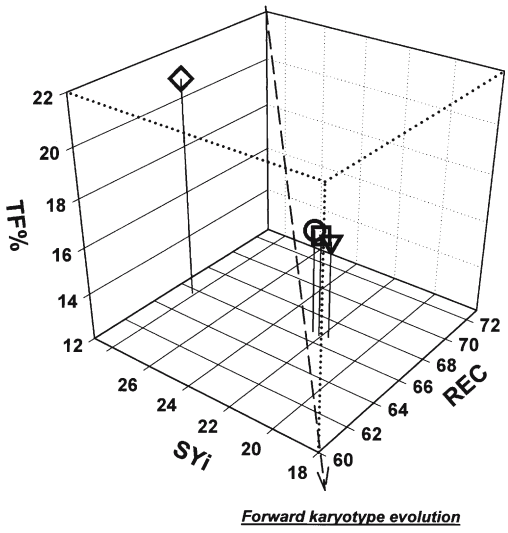

Fig. 2. Karyotype symmetry of species of sect. *Peregrinae* with Rec, Syi, and TF% indices. ○, *V. michauxii*; ▽, *V. peregrina*; □, *V. aintabensis*; ◇, *V. michauxii* subsp. *stenophylla*

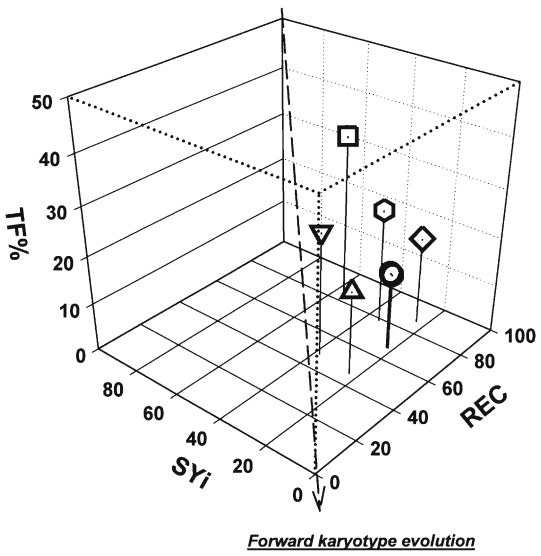

Fig. 3. Karyotype symmetry of sect. *Peregrinae*, *V. mollis*, *V. narbonensis*, *V. bithynica*, *V. faba*, and *V. sativa* with Rec, Syi, and TF% indices. ○, Section *Peregrinae* (without *V. michauxii* subsp. *stenophylla*); □, *V. narbonensis*; △, *V. faba*; ▽, *V. mollis*; ◇, *V. bithynica*; ⬡, *V. sativa*

tween *V. faba* and *V. bithynica*, whilst appearing distant from *V. mollis*, *V. sativa*, and *V. narbonensis*.

Sequence analysis

The PCR-amplified DNA fragments of all the species showed a single band when examined on agarose gel. Sequence lengths and EMBL accession numbers are reported in Table 4.

The ITS1 of the investigated *Vicia* species ranged in length from 200 (for *V. mollis*) to 236 bp (various species); the ITS2 from 208 (for *V. faba* and *V. faba* subsp.

Table 4. EMBL accession numbers and lengths of ITS and ETS sequences in *Vicia* species

Species	ITS accession nr.	Length (bp) of:		ETS accession nr.	Length (bp) of:	
		ITS1	ITS2		ETS[a]	Subrepeat unit
V. michauxii	AJ414585	236	209	AJ605720	555 (372)	94
V. michauxii subsp. *stenophylla*	AJ414586	236	209	AJ605722	648 (372)	94
V. peregrina	AJ566206	236	210	AJ585037	552 (370)	94, 93
V. aintabensis	AJ566207	236	210	AJ585035	552 (370)	94, 93
V. mollis	AJ566208	200	209	AJ585036	562 (376)	96
V. narbonensis	AJ130833[b]	235	209	AJ586812	376	
V. eristalioides	AJ010808[b]	235	209	AJ586813	376	
V. galilaea	AJ131077[b]	235	209	AJ586815	376	
V. hyaeniscyamus	AJ131073[b]	235	209	AJ586818	376	
V. johannis	AJ131080[b]	235	209	AJ586817	376	
V. kalakhensis	AJ131071[b]	235	209	AJ586814	376	
V. serratifolia	AJ131075[b]	235	209	AJ586816	375	
V. bithynica	AJ130831[b]	235	209	AJ586819	367	
V. faba	Yokota et al. 1989	235	208	Kato et al. 1990	582 (374)	67, 68, 66
V. faba subsp. *paucijuga*	AJ131078	236	208	AJ851227	586 (372)	69
V. sativa	AJ010804[b]	218	209	AY234366[c]	922	67

[a] In parentheses, length after removal of repeats
[b] Venora et al. 2000
[c] Macas et al. 2003

paucijuga) to 210 bp (for *V. aintabensis* and *V. peregrina*), whereas the 5.8S was invariably 164 bp in all the investigated species; the amplified ETS products ranged in length from 367 (for *V. bithynica*) to 648 bp (for *V. michauxii* subsp. *stenophylla*). As the literature accession of *V. sativa* ETS differed for a single, unique point mutation from the one obtained in the present paper, only the accession from literature was kept for the analysis.

The dot plot comparison showed that no repeat is present in ETS of *V. narbonensis*, *V. eristalioides*, *V. galilaea*, *V. hyaeniscyamus*, *V. johannis*, *V. kalakhensis*, *V. serratifolia*, and *V. bithynica*. In contrast, *V. michauxii* (including subsp. *stenophylla*), *V. peregrina*, *V. aintabensis*, *V. mollis*, *V. faba*, and *V. faba* subsp. *paucijuga*, as well as *V. sativa*, showed various subrepeats. The length of the unit subrepeats is 93 to 94 bp, with the exception of *V. faba*, *V. faba* subsp. *Paucijuga*, and *V. sativa*, for which the unit length is 66 to 69 bp.

The alignment of ITS1, 5.8S, and ITS2 sequences had a consensus length of 613 characters (33 of which informative within ITS1 and -2); the alignment of the ETS sequences after removal of the repeats had a consensus length of 404 characters (73 of which informative). Repeats were removed (except for their first occurrence) because a preliminary phylogenetic analysis (Fig. 4) of the repeats showed that at least in *V. sativa*, in *V. mollis*, and partly in *V. faba*, repeats are paralogous in origin. The combined matrix, with 16 terminals, had 1018 characters, 111 of which were informative. This matrix yielded a single most parsimonious cladogram (L = 323 steps, C.I. = 0.85, R.I. = 0.86; by removing uninformative characters, L = 192 steps, C.I. = 0.76, R.I. = 0.86), shown in Fig. 5. The in-group is divided in two major clades, one including *V. faba* and *V. faba* subsp. *paucijuga*, and the other including the remaining species. In this latter clade, two groups are recognizable, one including *V. mollis*, as sister group to *V. aintabensis*, *V. peregrina*, *V. michauxii*, and *V. michauxii* subsp. *stenophylla*, and the other including the remaining species. This group in turn shows *V. bithynica* as sister group to a poorly resolved clade, with *V. serratifolia* at the basis. More internally, *V. eristalioides*, *V. kalakhensis*, and *V. narbonensis* represent a basal collapse for a clade including *V. galilaea*, *V. hyaeniscyamus*, and *V. johannis*. Bootstrap percentages (out of 1000 replicas) are quite high, with few exceptions (Fig. 5; percentages of <50% not shown). By the same token, decay analysis shows that this data set includes, besides the cladogram of maximum parsimony, only another 791 trees up to 15 step longer. Very few one-step longer cladograms exist, and Bremer support (Bremer 1994) for all the major clades of this investigation is quite strong (Fig. 5).

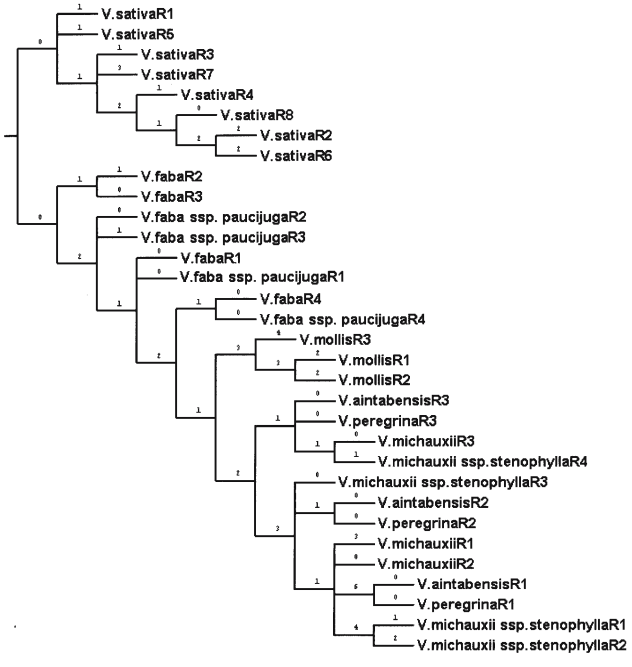

Fig. 4. Consensus tree of six equally parsimonious cladograms obtained by the cladistic analysis of the ETS repeats (L = 95 steps, C.I. = 0.75, R.I. = 0.92). Numbers of synapomorphies are indicated above internodes

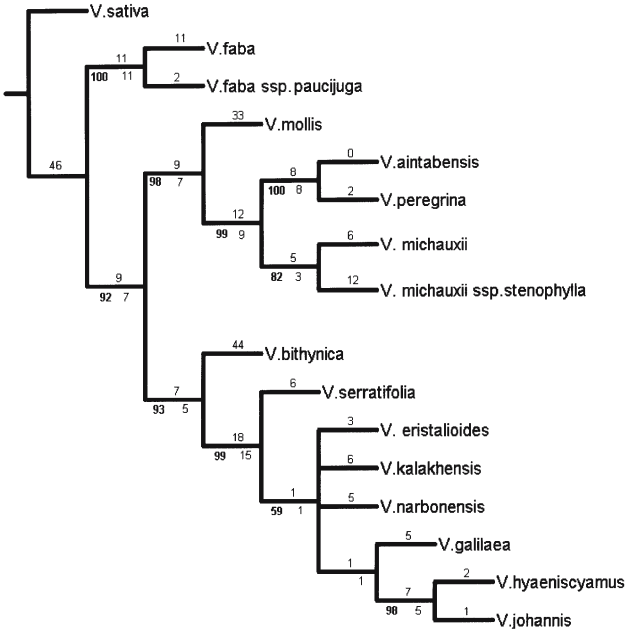

Fig. 5. Single most parsimonious cladogram for the combined data set (L = 323 steps, C.I. = 0.85, R.I. = 0.86; by removing uninformative characters, L = 192 steps, C.I. = 0.76, R.I. = 0.86). Numbers of synapomorphies are indicated above internodes; bootstrap percentages (out of 1000 replicas; those of <50% are not shown) are indicated in bold type below the internodes; Bremer support is indicated at right of bootstrap values (support for the ingroup not shown)

Discussion

In the taxonomic history of subgenus *Vicia*, the contribution by Maxted (1993) represents a turning point, especially because it produced the first evidence about the fact that *Vicia faba* is not as strongly allied to *V. narbonensis* and related species as it was thought before (see Introduction). In line with the above mentioned findings, in a previous paper we presented evidence for the remoteness of *V. faba* from the species of sect. *Narbonensis* and *V. bithynica* (Venora et al. 2000).

The results of molecular and karyological investigations concerning the species of the section *Peregrinae* and *V. mollis* reported here represent a further step in the study of all the sections of the subgenus *Vicia*, which, in our opinion, will contribute to clarify the phylogenetic relationships among the species of the subgenus.

As a first step in the characterization of the analyzed species, we determined the nuclear DNA contents (Table 1). Even if the accuracy of cytophotometric determinations of nuclear DNA content by the Feulgen method has been questioned (Greilhuber 1986), the observed differences in Feulgen absorption values do reflect real differences in the DNA content, since nuclei were analyzed in the same condition (early prophases) and all the squashes were stained together. Moreover, our data agree with the results of Chooi (1971) and Raina and Rees (1983) for *V. peregrina*, and with those by Maxted et al. (1991) for *V. mollis*; in contrast, our results do not concur with those by Raina and Bisht (1988) on *V. michauxii*. Differences are probably related to methodological issues, as Raina and Bisht (1988) hydrolyzed and stained root tips before squashing. Our determination of nuclear DNA content of *V. aintabensis* and *V. faba* subsp. *paucijuga* are the first in the literature.

As regards karyotype parameter determination (Tables 2 and 3), our method enables an accurate determination of the karyomorphological indices (TF%, Rec, and SYi) which are considered directly correlated with the evolution of the karyotype, as reported for *Cicer arietinum* (Venora et al. 1995), *Vigna* (Venora et al. 1998) and *Vicia* species (Venora et al. 2000).

The spatial representation of the species of the section *Peregrinae* (Fig. 2) shows that *V. michauxii*, *V. aintabensis*, and *V. peregrina* are grouped in a single cluster, which does not include *V. michauxii* subsp. *stenophylla.* As shown in Fig. 3, the cluster of the species belonging to the section *Peregrinae* is flanked by *V. faba* and *V. bithynica*, while *V. narbonensis* and *V. mollis*, as well as *V. sativa*, are far removed.

As far as the ribosomal DNA phylogenetic analysis is concerned, some preliminary comments are appropriate on the reason why the ETS subrepeats were not employed. The reason for their exclusion (Fig. 4) was the apparent paralogy of the repeats in *V. sativa* (all repeats), in *V. faba* (repeats nr. 2 and 3), in *V. mollis* (all repeats), and perhaps in *V. faba* subsp. *paucijuga* (repeats nr. 2 and 3; regardless of their not forming a clade). The hypothesis formulated here is related to the fact that, if repeats from the same species form a clade not including repeats from other species, the duplication event occurred after reproductive isolation; in contrast, if repeats from different species form a clade, the origin of repeats may have preceded species segregation (Sanderson and Doyle 1992). In the first case, only the one occurrence of the repeat should be included in the phylogenetic analysis, so as not to artificially increase the number of synapomorphies (Linder et al. 2000).

The single most parsimonious cladogram shown in Fig. 5 indicates that both sect. *Narbonensis* and sect. *Peregrinae* are monophyletic units. *Vicia bithynica* is a sister group to sect. *Narbonensis*, but shows a very high number of autapomorphies (44). Moreover, this species shares the absence of repeats in its ETS (Table 4) with sect. *Narbonensis*. By the cladogram of the repeats shown in Fig. 4, it is impossible, however, to assess whether the presence of repeats is an apomorphic trait in the group of species used in this study (indeed, according to Fig. 4, sequence amplification occurred independently in *V. sativa* and in the *V. faba* clade). Section *Peregrinae* has *V. mollis* as sister group. Also in this case, the latter species has a very high number of synapomorphies (33); moreover, within the limits of an investigation carried out by direct sequencing, its ETS repeats are paralogues which developed independently from those of the species in sect. *Peregrinae* (Fig. 4). The clade including *V. mollis* and sect. *Peregrinae* is a sister group to that including *V. bithynica* and sect. *Narbonensis*. This relationship is quite strong (9 synapomorphies, 92% bootstrap, BS 7), and documents a closer proximity between sect. *Peregrinae* (plus *V. mollis*) and sect. *Narbonensis* (plus *V. bithynica*) than either has with sect. *Faba*. The latter, indeed, behaves as a sister taxon to the clade including sect. *Peregrinae* and sect. *Narbonensis*.

From the comparison of molecular data with karyomorphological ones, it is possible to draw some considerations about the phylogenetic relationships among the analyzed species.

As far as sect. *Peregrinae* is involved, clear affinities are documented among its species both on a karyological and a molecular standpoint (Figs. 2 and 5); however, *V. michauxii* subsp. *stenophylla*, albeit clearly related to *V. michauxii* (Fig. 5), shows relevant differences from the latter at the karyomorphological level (Fig. 2). Moreover, the molecular investigation shown in Fig. 5 indicates that *V. michauxii*

subsp. *stenophylla* has a rather large number of autapomorphies as compared to the other taxa of the section; therefore, karyomorphological and molecular differences combined are so evident as to question its condition of subspecies.

As regards the relationships between sections, both karyotypes and ribosomal sequences (Figs. 3 and 5) confirm the remoteness of *V. faba* from *V. bithynica* and from the species of sect. *Narbonensis*. The relationships of *V. faba* with sect. *Peregrinae* are, however, somehow contradictory. Karyomorphological data (Fig. 3), in fact, would suggest that *V. faba* is more closely related to the species of sect. *Peregrinae* than to the species formerly considered as its closest wild relatives and now enclosed in the sections *Narbonensis* and *Bithynicae* (Maxted 1993). Phylogenetic analysis of DNA sequences (Fig. 5), in contrast, would indicate that *V. faba* is less related to sections *Peregrinae*, *Narbonensis*, and *Bithynicae* than they are between themselves. In this regard, however, it may be worth noting that, by computing a variety of genetic distances between the sequences of all the involved taxa (data not shown), the closest taxon to both *V. faba* and *V. faba* subsp. *paucijuga* is *V. michauxii*, i.e., a member of sect. *Peregrinae*.

We are not ready, at present, to justify the above discrepancy. However, by investigating ITS and ETS DNA sequences separately (data not shown), we observed that ITS analysis concurs to a much larger extent with karyological data than results from ETS. This may be related to the fact that ETS sequences diverge more rapidly than ITS sequences (as indicated for our species by computing the number of informative characters versus the total number of characters) and, therefore, homoplasy may obscure relationships.

Nothing can be said, at present, on the relationships between *V. mollis* and sect. *Peregrinae*. Only an expanded analysis, with a larger sample of taxa, will indicate whether the differences in terms of karyotype (Fig. 3) and the large number of autapomorphies (Fig. 5) shown by *V. mollis* justify its exclusion from sect. *Peregrinae*, as indicated by Plitman (1967) and Maxted (1994).

Our results are broadly concurring with recent literature data obtained by different methods. Indeed Van de Ven et al. (1993) suggested by nuclear and mitochondrial RFLP and PCR analysis that *V. faba* is more closely related to species of the sections *Peregrinae* and *Hypechusa* than to those of the section *Narbonensis*; the same conclusion was obtained by the analysis of chloroplast *trnL* introns by Fennell et al. (1998). Moreover, even if within the limits of isozyme analysis, Jaaska (1997) and Leht and Jaaska (2002) demonstrated that *V. faba* stands basally in a monophyletic group with the species of the section *Peregrinae* and its morphologically closest wild relatives of the section *Narbonensis* stand in a different monophyletic group.

In conclusion, we would suggest that a further reconsideration of phylogenetic relationships inside the subgenus *Vicia* is necessary. Such a revision should be the result of a wide-range analysis taking into account species of the different sections and employing various methods of analysis, so as to obtain a set of complementary data to be integrated with traditional taxonomic studies.

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