Protoplasma (2006) 228: 167–177
DOI 10.1007/s00709-006-0158-2

PROTOPLASMA
Printed in Austria

Nuclear DNA contents, rDNAs, and karyotype evolution in subgenus *Vicia*: III. The heterogeneous section *Hypechusa*

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Received August 4, 2005; accepted September 29, 2005; published online July 17, 2006

Summary. Nuclear DNA contents, automated karyotype analyses, and sequences of internal transcribed spacers from ribosomal genes have been determined in the species belonging to section *Hypechusa* of the subgenus *Vicia*. Karyomorphological results and phylogenetic data generated from the comparison of rDNA (genes coding for rRNA) sequences showed that sect. *Hypechusa* is not monophyletic; however, some monophyletic units are apparent (one including *Vicia galeata*, *V. hyrcanica*, *V. noeana*, and *V. tigridis*, another including *V. assyriaca*, *V. hybrida*, *V. melanops*, *V. mollis*, and *V. sericocarpa*), which partly correspond to morphology-based infrasectional groups. The relationships among these species and the species in sections *Faba*, *Narbonensis*, *Bithynicae*, and *Peregrinae* have been also investigated.

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

Introduction

The genus *Vicia* L. is a member of the legume tribe Vicieae (Fabaceae). Ball (1968) divided the genus into four sections: *Vicia*, *Cracca* Gray, *Ervum* (L.) Gray, and *Faba* (Mill.) Gray; and Kupicha (1976) subsequently recognised two subgenera, *Vicilla* and *Vicia*, with 17 and 5 sections, respectively. Subgenus *Vicia* contains fewer species than *Vicilla* but includes the more agriculturally important species, such as faba bean (*Vicia faba* L.), narbon vetch (*V. narbonensis* L.), and common vetch (*V. sativa* L.). The faba bean is an important grain legume across the Northern temperate zone and at higher temperatures in some subtropical regions. It is unusual among pulse crops in that no clear picture of its ancestry is yet available, and its close taxonomic relationships are imperfectly known (Khattab et al. 1988, Hanelt and Mettin 1989, Maxted et al. 1991, and references therein). Various studies suggested that the closest relatives of faba bean were to be found in sect. *Faba* sensu Kupicka (including, besides *V. faba*, *V. bithynica* and a group of species referred to as the Narbonensis complex); in particular, strong evidence was found to suggest that *V. narbonensis* and its closely related species are immediately related to *V. faba* (Ball 1968, Kupicha 1976). More recently, Maxted (1993) reexamined subgenus *Vicia* and proposed a new classification with nine sections: *Atossa* (Alef.) Asch. & Graebner, *Microcarinae* Maxted, *Hypechusa* (Alef.) Asch. & Graebner, *Peregrinae* Kupicha, *Wiggersia* (Alef.) Maxted, *Vicia*, *Narbonensis* (Radzhi) Maxted, *Bithynicae* (B. Fedtsch. ex Radzhi) Maxted, and *Faba* (Miller) Ledeb., where sections *Bithynicae* and *Faba* are monospecific units.

Various aspects of the classification proposed by Maxted (1993) have been recently confirmed (Fennell et al. 1998, Venora et al. 2000, Leht and Jaaska 2002, Frediani et al. 2005). Most notably, the remoteness of *V. faba* from *V. bithynica* and from the species of sect. *Narbonensis* has been proven by molecular methods. However, various issues remain obscure, including not only the position of *V. faba* but also the sectional membership of some species and the phylogenetic relationships among the sections.

Elucidating the taxonomic relationship between the crop and its wild relatives is important, especially in connection with the exploitation of the germplasm of wild relatives as a source of novel characteristics to be intro-

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duced into the crop by wide crosses. To improve understanding of the relationships within subgenus *Vicia*, we started the analysis of its nine sections by karyological and molecular methods. Results for sections *Bithynicae*, *Faba*, *Narbonensis*, and *Peregrinae* have been published (Venora et al. 2000, Frediani et al. 2005).

In the present report, karyomorphology and molecular phylogeny of sect. *Hypechusa* have been investigated. The concept of sect. *Hypechusa* is derived from Kupicha (1976), who in turn derived it from the genus *Hypechusa* instituted by Alefeld (1860). Maxted (1993) and Maxted and Douglas (1997) reviewed Kupicha's classification, including *V. tigridis* and *V. mollis*. As a consequence, sect. *Hypechusa* now includes 14 species (see Table 1), divided into two series, *Hyrcanicae* and *Hypechusa*, on the basis of their peduncle length, corolla shape and size, and standard pubescence.

Karyological and molecular data obtained from sect. *Hypechusa* are compared with analogous data on the other sections of subgenus *Vicia* (Venora et al. 2000, Frediani et al. 2005).

Material and methods

Plant materials

The names, sources, 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 coverslip in a drop of 45% acetic acid, after pectinase (Sigma, St. Louis, Mo., U.S.A.) treatment. All slides were then simultaneously hydrolysed in 5 N HCl at room temperature for 30 min, stained with Schiff's reagent, and washed, as described by 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 (Leitz, Wetzlar, Federal Republic of Germany).

All squashes were stained concurrently with *V. faba* preparations, which were 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 a 4C DNA content of 53.12 pg for *V. faba* (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 and 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 were 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, and the dedicated Ikaros 3.4 karyotyping software (Metasystem GmbH, Altlussheim, Federal Republic of Germany). The automated image analysis system ensures a high degree of accuracy because measurements are taken directly from the first image of the chromosomes (Venora et al. 1991). This method, therefore, allows a precise determination of the karyomorphological indices

Table 1. Accession, source, chromosome number, and mean nuclear DNA content of species of *Vicia* sect. *Hypechusa*[a]

Species	Accession nr.	Source[b]	Chromosome nr. (2n)	DNA amount 4C (pg)
Series *Hyrcanicae*				
V. assyriaca Boiss.	IG 64098	ICARDA	12	26.31 ± 0.38
V. tigridis Mout.	IG 63488	ICARDA	12	25.81 ± 0.45
V. galeata Boiss.	PI 602380	USDA	12	30.55 ± 1.10
V. hyrcanica Fisch. & Mey.	PI 561419	USDA	12	46.91 ± 0.84
V. noeana (Re. in B.) Boiss.	IG 63757	ICARDA	12	52.95 ± 0.37
Series *Hypechusa*				
V. melanops Sib. & Smith	IG 64074	ICARDA	10	27.52 ± 0.27
V. ciliatula Lipsky	IG 63373	ICARDA[c]	10	26.48 ± 0.19
V. anatolica Turrill	IG 64625	ICARDA	10	30.18 ± 0.19
V. mollis Boiss & Haus. ex Boiss.[d]	IG 62649	ICARDA	10	31.31 ± 1.42
V. pannonica Crantz	PI 369156	USDA	12	39.92 ± 0.77
V. hybrida L.	IG 60008	ICARDA	12	27.93 ± 0.49
V. sericocarpa Fenzl.	IG 64103	ICARDA	12	48.52 ± 0.51
V. lutea L.	201994	USDA	14	35.90 ± 0.26

[a] *Vicia esdraelonensis* (series *Hypechusa*) was not included in this study because seeds were not available from germplasm banks

[b] ICARDA, International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria; USDA, United States Department of Agriculture, Pullman, Wash., U.S.A.

[c] Gift from Prof. Jaaska, Dept. of Botany, Estonian Agricultural University of Tartu, Estonia

[d] Frediani et al. 2005

(TF%, Rec, and SYi). To allow comparison between raw data and the results of automated karyotype analysis, typical images are shown (see Figs. 1 and 2); as an example, the chromosome pairs of one metaphase plate (Fig. 1F) are also identified with the same numbers as reported in Table 2.

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 Karyo 95 software (Dipartimento di Botanica, Università di Catania, Italy) to improve accuracy in matching chromosome pairs, using the data for 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 the 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 the average length of the long arms.

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

DNA sequencing

Nuclear DNAs were extracted and purified from secondary roots produced after decapitation of the primary one for all the species listed in Table 1, with the exclusion of *V. mollis*, for which ITS sequences were already available (Frediani et al. 2005). The extraction was carried out by a small-scale DNA isolation method (Epicentre Biotechnologies, Madison, Wis., U.S.A.) and extracts were further purified on a S-200 Microspin column (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

The entire region including ITS1, 5.8S, and ITS2 rDNA (genes coding for rRNA) 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 and 72 °C for 2 min, and finally 72 °C for 5 min.

The primers designed with the conserved coding regions of the 18S and 26S ribosomal genes were 18Sdir, 5′-CGTAACAAGGTTTCCGTAGG-3′, and 26Scom, 5′-AGCGGGTAGTCCCGCCTGA-3′.

The PCR-amplified DNA fragments from all the species showed a single band when examined on agarose gel. All the PCR products were sequenced directly along both strands using the ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, Calif., U.S.A.). Sequence lengths and EMBL accession numbers are reported in Table 4.

Internal transcribed spacer (ITS) sequences (without intervening 5.8S, so as to render them comparable with published sequences) were aligned by the Clustal 1.83 software with default values (Thompson et al. 1994). Alignments were carried out as daughter processes of Bioedit (Hall 1999), which was also used for sequence editing and manipulation. In addition to the 13 species indicated in Table 1, all taxa belonging to sections *Peregrinae*, *Narbonensis*, *Bithynicae*, and *Faba* previously investigated by Frediani et al. (2005) were included in the alignment. Moreover, the ITS sequences of *V. ervilia* and *V. tetrasperma*, employed as outgroups for the cladistic analyses, were also aligned, giving a total of 27 taxa. The complete alignments used for all further analyses are available upon request from the senior author.

Cladistic analyses were carried out by Winclada (Nixon 1999) and Nona (Goloboff 1999). Settings included the following: a maximum storage space of 10000 trees (hold 10000); a tree storage space per iteration of 100 (hold/100); 100 iterations of an algorithm which randomises the addition order of taxa, creates a Wagner tree, and swaps its branches by tree bisection-reconnection (mult*100); and further branch swapping on all the found trees (max). Cladograms were further manipulated by Winclada, which was also used for bootstrapping the results (1000 replicas) (Felsenstein 1985).

In addition, a Bayesian analysis was attempted on the same matrix used for cladistic analysis by the MrBayes software (Huelsenbeck and Ronquist 2001), starting from random trees and running the analysis for 2 million generations, and with four simultaneous Markov Monte Carlo chains, sampling trees every 200 generations (number of generations, 2000000; number of chains, 4; printing frequency, 200; sampling frequency, 200).

As the Clustal series of alignment software does not impose any criterion of optimality during the search, a parallel strategy of "direct optimisation" (Wheeler 1996) was pursued as implemented in the POY 3.0 program (Wheeler et al. 2002). This method searches for the alignment which, under a given matrix of costs for insertion–deletion events (indels) and nucleotide changes (transversions and transitions), expresses the maximum parsimony tree topology. Parameters for the latter software were the following: number of replicates, 100; insertion–deletion cost (-gap), 1; maximum number of trees held in buffer (-maxtrees), 2; tree bisection–reconnection branch swapping (-tbr), no subtree pruning-and-regrafting branch swapping (-nospr), generation of a typology-specific multiple alignment based on the synapomorphy scheme (-impliedalignment).

Results

Cytophotometry

The chromosome numbers and the nuclear DNA contents of the investigated species are listed in Table 1. The DNA content in the sect. *Hypechusa* (picograms per 4C nucleus) ranges from 25.81 pg in *V. tigridis* to 52.95 pg in *V. noeana*.

Karyomorphometry

Karyological data from the analysed species are reported in Table 2. The karyotype formulas, total length of haploid set, Rec, Syi, and TF% indices, and the Stebbins categories are summarised in Table 3, where data relating to the species of sections *Peregrinae*, *Narbonensis*, *Bithynicae*, and *Faba* are also reported (Venora et al. 2000, Frediani et al. 2005).

The species of series *Hyrcanicae* presented the complement 2n = 12 and one couple of satellite chromosomes, except *V. assyriaca*, which had three satellite couples (Figs. 1 and 2). Three of the five species of this series (*V. tigridis*, *V. galeata*, and *V. hyrcanica*) showed the same karyotype formula (m^{sc} + 2sm + 3st), and all the species of the series were classified in Stebbins category 3A (Table 3). *Vicia assyriaca* showed the highest values for the SYi and TF% indices, and *V. galeata* the highest value for the Rec index (Table 3).

The species belonging to series *Hypechusa* showed three different chromosome numbers: four species with 2n = 10 (*V. mollis*, *V. melanops*, *V. ciliatula*, and *V. anatolica*), three species with 2n = 12 (*V. pannonica*, *V. hybrida*, and *V. sericocarpa*), and one species (*V. lutea*) with 2n = 14 chromosomes; all the species presented two couples of

Table 2. Chromosome morphometric data of species of *Vicia* sect. *Hypechusa*

Species and chromosome nr.[a]	Length (μm) of:[b]		Long/short arm ratio[c]	Species and chromosome nr.[a]	Length (μm) of:[b]		Long/short arm ratio[c]
	Chromosome[c]	Satellite			Chromosome[c]	Satellite	
V. assyriaca				*V. anatolica*			
1	9.35 ± 0.43 a		2.77 c	1*	10.38 ± 1.04 a	1.56 ± 0.13	1.09 e
2*	7.46 ± 0.35 b	2.02 ± 0.17	3.26 a	2	8.17 ± 0.79 b		2.66 c
3*	7.11 ± 0.49 c	1.61 ± 0.11	1.22 e	3*	5.77 ± 0.46 c	2.27 ± 0.21	1.35 d
4*	6.65 ± 0.31 d	1.56 ± 0.08	1.56 d	4	5.74 ± 0.44 c		3.22 a
5	6.49 ± 0.04 d		3.04 b	5	5.01 ± 0.54 d		3.01 b
6	5.51 ± 0.06 e		2.52 c				
V. tigridis				*V. mollis*[d]			
1	7.84 ± 0.40 a		2.56 d	1*	13.00 ± 0.09 a	2.47 ± 0.06	1.51 b
2*	6.07 ± 0.38 b	3.39 ± 0.20	1.35 e	2	7.59 ± 0.10 b		3.54 a
3	5.83 ± 0.52 b		2.69 d	3	6.40 ± 0.07 c		3.16 a
4	5.66 ± 0.25 c		3.84 b	4	6.11 ± 0.15 c		3.66 a
5	5.49 ± 0.18 c		3.50 c	5	5.02 ± 1.00 d		3.44a
6	4.81 ± 0.15 d		4.34 a				
V. galeata				*V. pannonica*			
1	7.44 ± 0.52 a		2.74 b	1	6.07 ± 0.45 a		3.05 b
2*	6.49 ± 0.34 b	3.67 ± 0.29	1.64 c	2*	5.23 ± 0.38 b	1.36 ± 0.11	3.25 a
3	6.10 ± 0.37 b		2.79 b	3*	5.12 ± 0.31 b	1.77 ± 0.31	1.31 d
4	5.98 ± 0.44 b		3.07 b	4	4.92 ± 0.28 c		2.73 c
5	5.30 ± 0.46 c		3.21 a	5	4.56 ± 0.31 c		2.65 c
6	5.12 ± 0.51 c		3.10 b	6	4.23 ± 0.23 d		2.81 b
V. hyrcanica				*V. hybrida*			
1	7.00 ± 0.66 a		2.54 d	1	8.11 ± 1.21 a		3.25 c
2*	5.91 ± 0.36 b	3.29 ± 0.23	1.54 e	2*	7.47 ± 0.87 b	2.05 ± 0.15	1.08 d
3	5.74 ± 0.40 b		3.48 a	3*	6.74 ± 0.80 c	1.77 ± 0.24	3.64 a
4	5.32 ± 0.28 c		3.22 b	4	6.46 ± 0.71 d		3.42 b
5	4.85 ± 0.27 d		3.29 b	5	6.17 ± 0.71 d		3.28 c
6	4.78 ± 0.26 d		2.85 c	6	5.78 ± 0.64 d		3.18 c
V. noeana				*V. sericocarpa*			
1*	8.95 ± 0.98 a	4.89 ± 0.69	1.94 c	1*	8.35 ± 0.70 a	1.13 ± 0.11	3.85 a
2	6.97 ± 0.45 b		3.66 a	2*	7.34 ± 0.64 b	1.90 ± 0.14	1.15 e
3	6.51 ± 0.35 c		2.60 b	3	6.40 ± 0.43 c		3.03 d
4	6.15 ± 0.27 d		2.64 b	4	6.09 ± 0.57 d		3.51 b
5	5.97 ± 0.30 d		3.52 a	5	5.76 ± 0.49 d		3.30 c
6	5.66 ± 0.31 d		3.64 a	6	5.50 ± 0.48 d		2.96 d
V. melanops				*V. lutea*			
1*	12.25 ± 0.94 a	2.77 ± 0.17	2.13 d	1	7.61 ± 0.87 a		2.75 c
2	7.82 ± 0.61 b		3.65 a	2*	7.26 ± 1.00 a	1.35 ± 0.18	5.49 a
3*	7.49 ± 0.48 b	2.62 ± 0.16	1.39 e	3	7.25 ± 0.85 a		3.07 c
4	6.44 ± 0.31 c		3.38 b	4	7.22 ± 0.97 a		2.50 c
5	5.91 ± 0.27 d		3.08 c	5	6.92 ± 0.91 a		3.17 b
				6	6.75 ± 0.72 a		2.71 c
				7	6.36 ± 0.63 a		3.03 c
V. ciliatula							
1*	10.33 ± 0.88 a	3.63 ± 0.43	4.40 a				
2*	7.76 ± 0.81 b	1.74 ± 0.09	1.15 c				
3	6.35 ± 0.43 c		2.94 b				
4	5.53 ± 0.44 d		2.81 b				
5	5.15 ± 0.36 d		2.87 b				

[a] Asterisk indicates satellited 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] Frediani et al. 2005

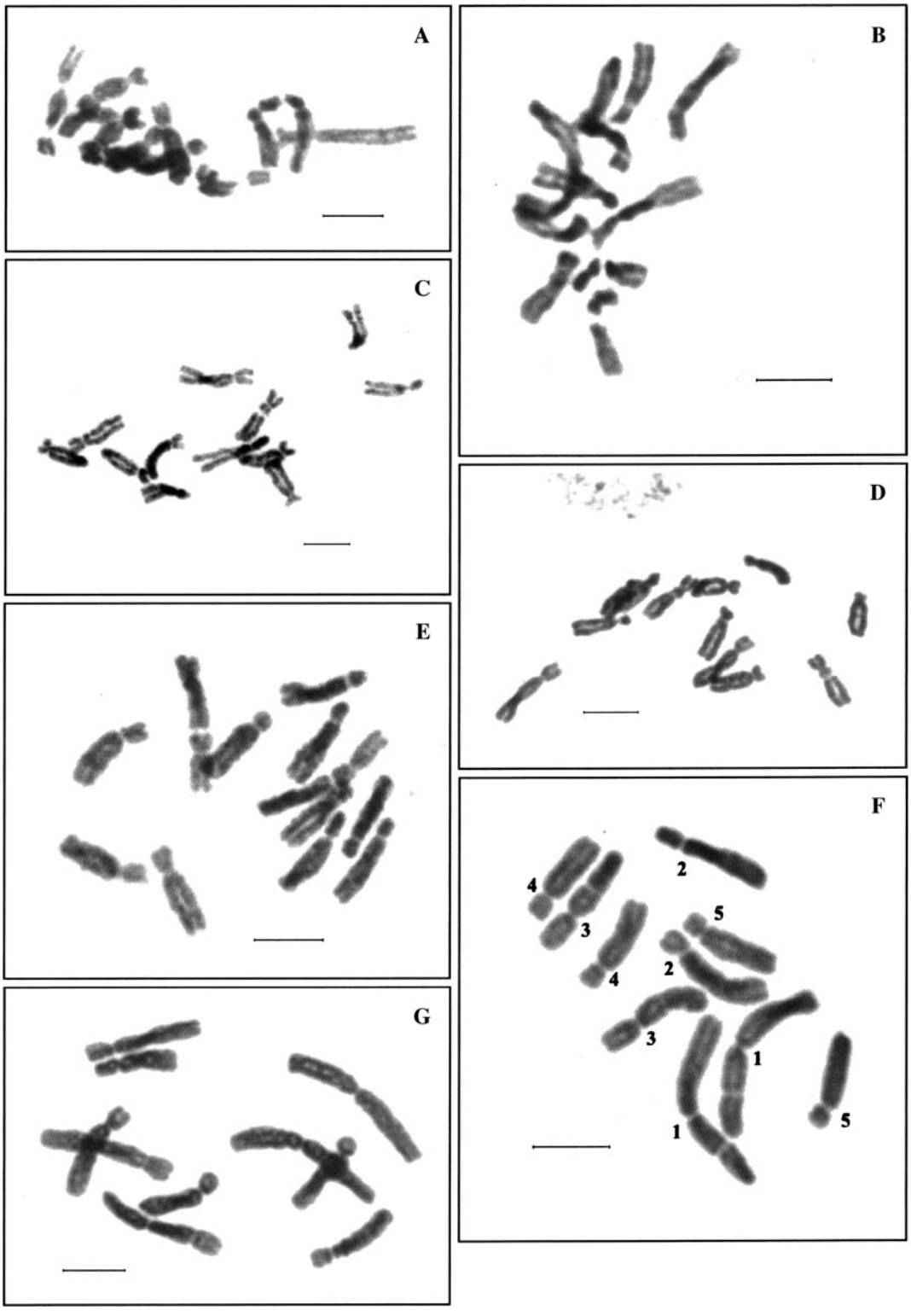


Fig. 1 A–G. Metaphase plates of species of sect. *Hypechusa*: **A** *V. assyriaca*; **B** *V. tigridis*; **C** *V. galeata*; **D** *V. hyrcanica*; **E** *V. noeana*; **F** *V. melanops*; **G** *V. ciliatula*. As an example, in metaphase plate F, the chromosome pairs are identified according to the numbering reported in Table 2. Bars: 5 μm

satellite chromosomes, except *V. mollis* and *V. lutea*, which had only one pair (Figs. 1 and 2). The species of this series showed different karyotype formulas and were classified in Stebbins categories 3A, 3B, and 4A (Table 3). *Vicia lutea* showed the highest value for the Rec index and *V. anatolica* the highest values for the SYi and TF% indices (Table 3).

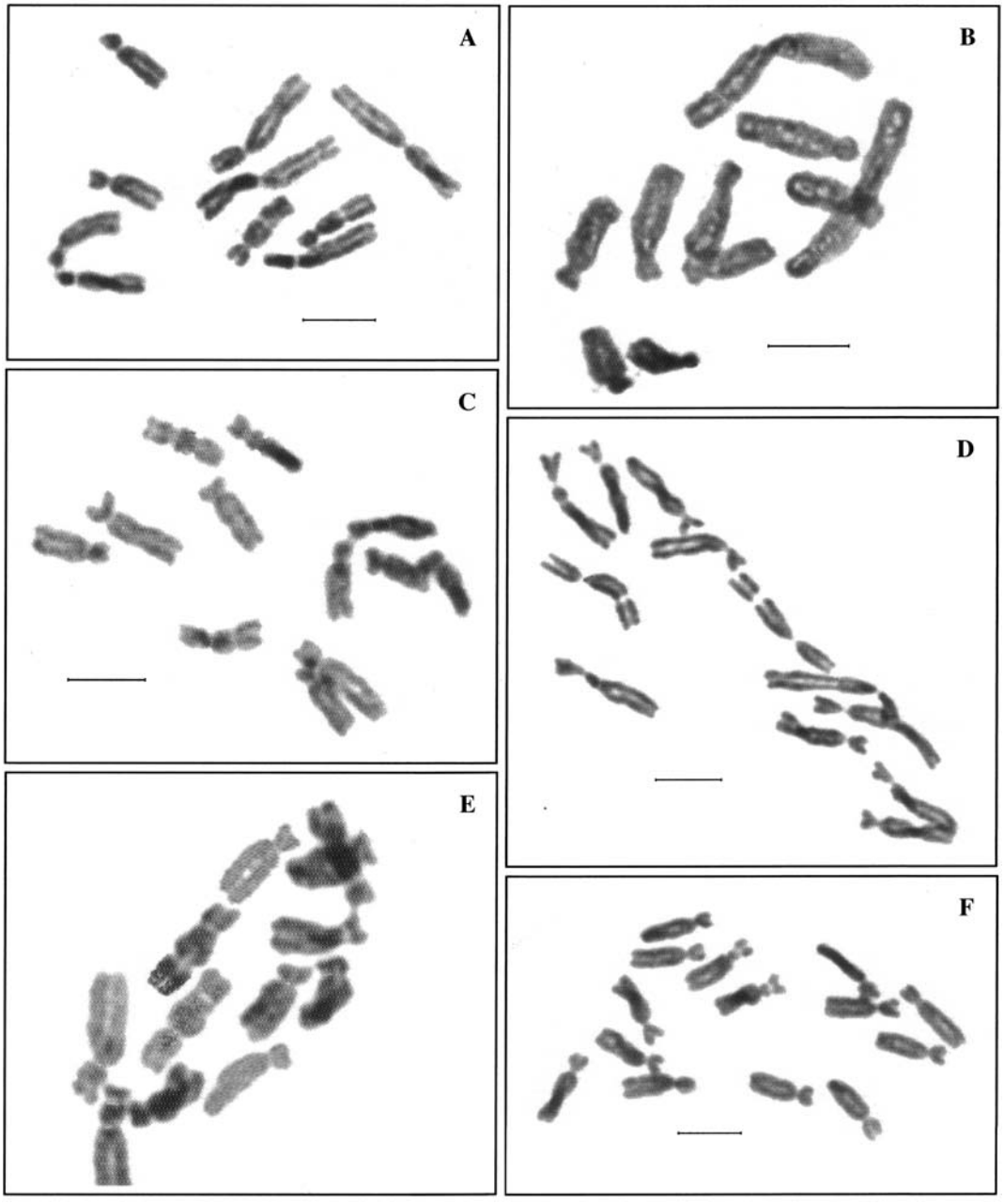


Fig. 2 A–F. Metaphase plates of species of sect. *Hypechusa*. **A** *V. anatolica*; **B** *V. mollis*; **C** *V. pannonica*; **D** *V. hybrida*; **E** *V. sericocarpa*; **F** *V. lutea*. Bars: 5 μm

The spatial representation of the thirteen species of sect. *Hypechusa* is shown in Fig. 3. All the species of series *Hyrcanicae* are very close, except *V. assyriaca*, while those of series *Hypechusa* are widely scattered.

When the species of sect. *Hypechusa* are plotted with those of sections *Peregrinae*, *Narbonensis*, *Faba*, and *Bithynicae* (Fig. 4), all the species of sect. *Hypechusa* are located in the centre of the spatial representation, far from those belonging to the other sections.

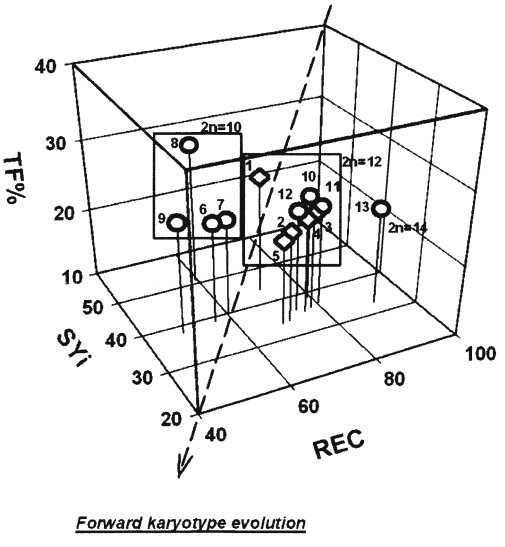
Sequence analysis

The complete ITS matrix (Table 4) included 453 characters (63 of which were informative). Phylogenetic analysis yielded 5 equally parsimonious cladograms (L = 193, C.I. = 0.77, R.I. = 0.79; by excluding noninformative characters, L = 129, R.I. = 0.65, C.I. = 0.79), the strict consensus of which is shown in Fig. 5. The ingroup, which shows extensive collapses, includes only a few detectable clades,

Table 3. Karyotype formulas, mean set length, indices and Stebbins categories in *Vicia* species

Species	Karyotype formula	Total length of haploid set (μm)	Rec index	SYi index	TF% index	Stebbins category
Sect. *Hypechusa* series *Hyrcanica*e						
V. assyriaca	$2m^{sc} + 2sm + st^{sc} + st$	42.55 ± 0.30	71.02	43.86	26.77	3A
V. tigridis	$m^{sc} + 2sm + 3st$	35.07 ± 0.30	71.07	34.07	23.00	3A
V. galeata	$m^{sc} + 2sm + 3st$	36.43 ± 2.43	77.93	35.88	23.74	3A
V. hyrcanica	$m^{sc} + 2sm + 3st$	33.59 ± 1.80	76.00	35.37	23.57	3A
V. noeana	$sm^{sc} + 2sm + 3st$	39.91 ± 2.08	69.18	33.82	22.17	3A
Sect. *Hypechusa* series *Hypechusa*						
V. melanops	$m^{sc} + sm^{sc} + 3st$	39.91 ± 2.20	56.45	38.91	24.23	3B
V. ciliatula	$m^{sc} + 3sm + st^{sc}$	35.12 ± 2.89	60.00	39.54	24.00	3B
V. anatolica	$2m^{sc} + sm + 2st^{sc}$	35.07 ± 3.04	59.47	50.99	30.08	3B
V. mollis[a]	$m^{sc} + 4st$	38.12 ± 1.41	48.31	38.12	25.81	3B
V. pannonica	$m^{sc} + 3sm + st^{sc} + st$	30.13 ± 1.82	79.28	38.75	25.02	3A
V. hybrida	$m^{sc} + st^{sc} + 4st$	40.73 ± 3.01	80.44	36.60	24.28	3A
V. sericocarpa	$m^{sc} + sm + st^{sc} + 3st$	39.44 ± 3.00	74.47	36.26	24.57	3A
V. lutea	$sm + st^{sc} + 2sm + 3st$	49.37 ± 5.83	91.46	32.73	23.98	4A
Sect. *Peregrinae*						
V. michauxii[a]	$m^{sc} + st^{sc} + 3st + 2t$	35.96 ± 2.68	66.69	22.45	16.49	3A
V. peregrina[a]	$m^{sc} + st^{sc} + 3st + 2t$	42.29 ± 1.08	66.75	21.71	16.24	3A
V. aintabensis[a]	$m^{sc} + st^{sc} + 3st + 2t$	40.57 ± 1.17	66.61	22.09	16.42	3A
Sect. *Narbonensis*						
V. narbonensis[b]	$2m + sm^{sc} + 4sm$	27.15 ± 2.28	84.60	56.05	34.57	2A
V. eristalioides[b]	$3m + sm^{sc} + 3sm$	33.38 ± 0.37	83.02	56.76	34.07	2A
V. galilaea[b]	$sm^{sc} + 6sm$	40.34 ± 1.71	85.94	47.69	31.18	3A
V. hyaeniscyamus[b]	$3m + 3sm + st^{sc}$	29.21 ± 2.85	65.99	57.64	35.32	2A
V. johannis[b]	$6m + sm^{sc}$	33.56 ± 3.19	86.33	48.12	31.31	3A
V. kalakhensis[b]	$3m + 3sm + t^{sc}$	42.28 ± 0.10	85.02	55.52	33.73	2A
V. serratifolia[b]	$m + 5sm + sm^{sc}$	38.16 ± 2.06	89.69	52.44	32.68	2A
Sect. *Bithynicae*						
V. bithynica[b]	$st^{sc} + 6st$	24.07 ± 1.83	86.99	23.72	18.49	4A
Sect. *Faba*						
V. faba[b]	$sm^{sc} + 3st + 2t$	58.11 ± 5.04	46.05	23.08	17.70	3B

[a] Frediani et al. 2005
[b] Venora et al. 2000



among which a clade with *V. bithynica* and *V. anatolica*, a group including *V. faba*, *V. michauxii*, *V. aintabensis*, and *V. peregrina*, a clade with *V. noeana* and a collapse of *V. galeata*, *V. hyrcanica*, and *V. tigridis*, a clade with *V. melanops*, *V. hybrida*, *V. assyriaca*, *V. sericocarpa*, and *V. mollis*, and a clade including a collapse of *V. eristalioides*, *V. galilaea*, *V. hyaeniscyamus*, *V. johannis*, *V. kalakhensis*, *V. narbonensis*, and *V. serratifolia* (i.e., sect. *Narbonensis*).

Results from the Bayesian analysis yielded a majority-rule consensus topology (out of 10000 generated trees)

Fig. 3. Karyotype symmetry of the species of sect. *Hypechusa*: ◊, series *Hyrcanicae* (1, *V. assyriaca*; 2, *V. tigridis*; 3, *V. galeata*; 4, *V. hyrcanica*; 5, *V. noeana*); ○, series *Hypechusa* (6, *V. melanops*; 7, *V. ciliatula*; 8, *V. anatolica*; 9, *V. mollis*; 10, *V. pannonica*; 11, *V. hybrida*; 12, *V. sericocarpa*; 13 *V. lutea*), with Rec, Syi, and TF% indices. Boxes include species with the same chromosome number

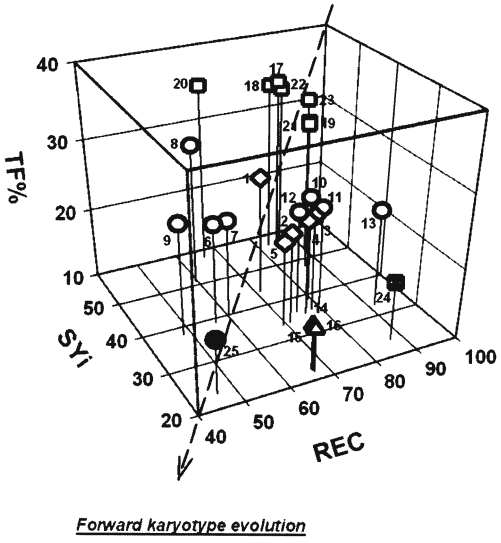


Fig. 4. Karyotype symmetry of sect. *Hypechusa*: ◊, series *Hyrcanicae* (1, *V. assyriaca*; 2, *V. tigridis*; 3, *V. galeata*; 4, *V. hyrcanica*; 5, *V. noeana*); ○, series *Hypechusa* (6, *V. melanops*; 7, *V. ciliatula*; 8, *V. anatolica*; 9, *V. mollis*; 10, *V. pannonica*; 11, *V. hybrida*; 12, *V. sericocarpa*; 13, *V. lutea*); △, sect. *Peregrinae* (14, *V. michauxii*; 15, *V. peregrina*; 16, *V. aintabensis*); □, sect. *Narbonensis* (17, *V. narbonensis*; 18, *V. eristalioides*; 19, *V. galilaea*; 20, *V. hyaeniscyamus*; 21, *V. johannis*; 22, *V. kalakhensis*; 23, *V. serratifolia*); ■, sect. *Bithynicae* (24, *V. bithynica*); and ●, sect. *Faba* (25, *V. faba*), with Rec, SYi and TF% indices

which is slightly more resolved than that obtained from maximum parsimony (Fig. 6). Results from the "direct optimisation" alignment (Wheeler 1996, Wheeler et al. 2002) yielded a consensus topology (out of 12 trees) which is almost identical to that in Fig. 6 (the only difference being a sister group relationship between *V. assyriaca* and *V. hybrida*).

Discussion

Section *Hypechusa* is the one of the few sections of subgenus *Vicia* showing three clear morphological synapomorphies. The lens in the seeds of all species of the section is located on the seed circumference opposite the hylum (Kupicha 1976), the cortical bundles at each node are only partially replaced (Kupicha 1976), and wings show a basal kink not found in other *Vicia* species (Maxted 1995). This led various authors (see Maxted 1993, Maxted and Douglas 1997) to conclude that sect. *Hypechusa* forms a cohesive taxonomic unit in comparison with other sections of subgenus *Vicia*. However, in spite of these distinctive characters, Maxted (1993) subdivided the sect. *Hypechusa* into two series, *Hyrcanicae* and *Hypechusa*, on the basis of the different lengths of flower

Table 4. EMBL accession numbers and lengths of ITS1 and ITS2 sequences from *Vicia* species

Species	EMBL accession nr.	Length (bp) of:	
		ITS1	ITS2
V. assyriaca	AJ851217	236	209
V. tigridis	AJ851216	236	209
V. galeata	AJ851219	236	209
V. hyrcanica	AJ851221	236	209
V. noeana	AJ851224	236	209
V. melanops	AJ851223	236	209
V. ciliatula	AJ851218	236	209
V. anatolica	AJ851215	219	209
V. mollis [a]	AJ566208	200	209
V. pannonica	AJ851225	236	205
V. hybrida	AJ851220	236	209
V. sericocarpa	AJ851226	200	209
V. lutea	AJ851222	236	209
V. michauxii [a]	AJ414585	236	209
V. peregrina [a]	AJ566206	236	210
V. aintabensis [a]	AJ566207	236	210
V. narbonensis [b]	AJ130833	235	209
V. eristalioides [b]	AJ010808	235	209
V. galilaea [b]	AJ131077	235	209
V. hyaeniscyamus [b]	AJ131073	235	209
V. johannis [b]	AJ131080	235	209
V. kalakhensis [b]	AJ131071	235	209
V. serratifolia [b]	AJ131075	235	209
V. bithynica [b]	AJ130831	235	209
V. faba	Yokota et al. 1989	235	208

[a] Frediani et al. 2005
[b] Venora et al. 2000

peduncles, shapes of the corolla, hairiness of the standard, and degrees of basal kinking of the wings. At the same time, Maxted (1993) also pointed out the heterogeneity of these series, stating that the enclosed species could be further subdivided by taking the morphological characters separately into account, and the segregated groups would differ depending on the examined character. In agreement with these studies, the investigation carried out in this report does not provide evidence for a monophyletic status of sect. *Hypechusa*.

As a first step in the characterisation of the analysed species, we determined the nuclear DNA contents (Table 1). Even though 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 DNA content, since nuclei were analysed in the same condition (early prophases) and all the squashes were stained together. In terms of both DNA content and chromosome complements (Table 1), the results were very heterogeneous, particularly in compari-

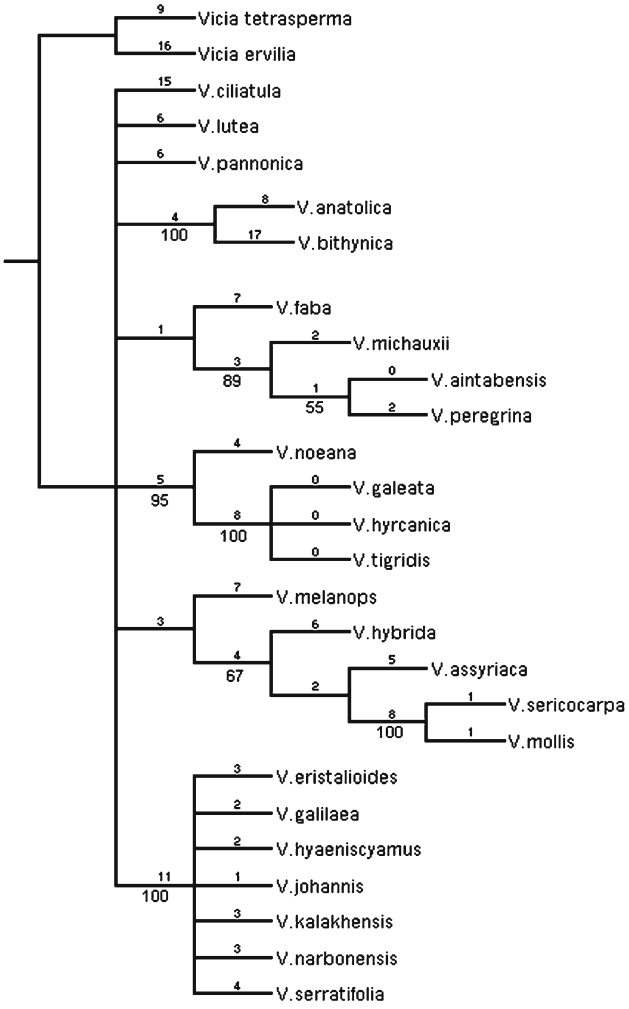


Fig. 5. Strict consensus of 5 equally parsimonious cladograms for sections *Hypechusa*, *Narbonensis*, *Faba*, *Bithynicae*, and *Peregrinae* (L = 193, C.I. = 0.77, R.I. = 0.79; by excluding noninformative characters, L = 129, R.I. = 0.65, C.I. = 0.79). *Vicia ervilia* and *V. tetrasperma* were used as outgroups. Numbers above branches indicate synapomorphies, numbers below branches indicate bootstrap support (out of 1000 replicas; values of <50% not shown). Support for the ingroup is not indicated

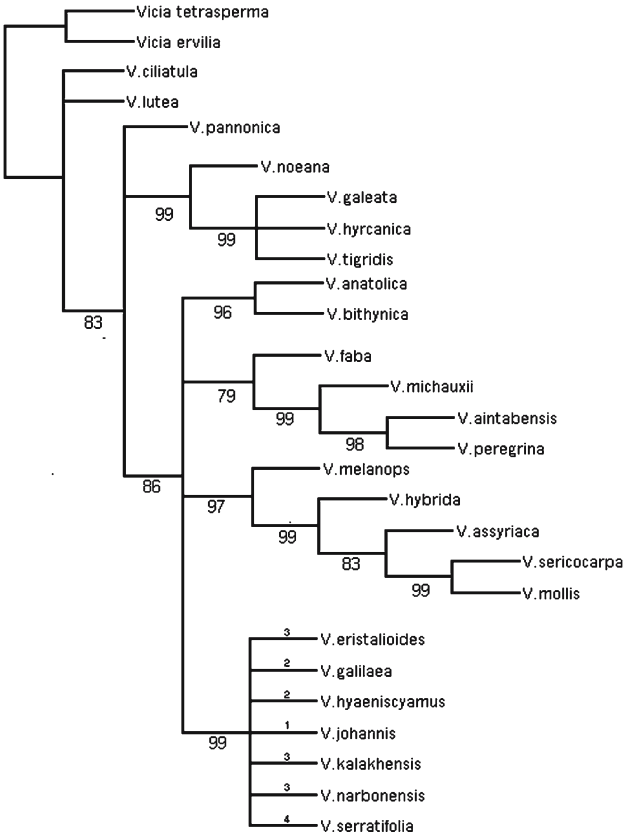


Fig. 6. Majority-rule consensus tree (out of 10000 generated trees) from a Bayesian analysis (2 million generations, four simultaneous Markov Monte Carlo chains) for sections *Hypechusa*, *Narbonensis*, *Faba*, *Bithynicae*, and *Peregrinae*. *Vicia ervilia* and *V. tetrasperma* were used as outgroups. Numbers below branches indicate percentage of occurrence of the clade on the right. Support for the ingroup is not indicated

son with sections *Narbonensis* (Venora et al. 2000) and *Peregrinae* (Frediani et al. 2005). While the DNA amount per 4C nucleus ranged from 25.08 pg to 42.22 pg in sect. *Narbonensis* and from 29.37 pg to 36.22 pg in sect. *Peregrinae*, the range is wider in sect. *Hypechusa* (from 25.81 pg to 52.95 pg). Moreover, in sections *Narbonensis* and *Peregrinae*, the chromosome number is constant for all species (2n = 14), whereas in sect. *Hypechusa*, diploid numbers vary from 2n = 10 through 2n = 12 to 2n = 14.

In terms of karyotype parameter determination (Tables 2 and 3), our method allows an accurate determination of the karyomorphological indices (TF%, Rec and SYi), which are considered to be directly correlated with the evolution of the karyotype, as reported for *Cicer arietinum* (Venora et al. 1995) and *Vigna* (Venora et al. 1998) and various *Vicia* species (Venora et al. 2000). The analysis of the indices of symmetry (Table 3) and the related spatial representation (Fig. 3) also point to the heterogeneity of sect. *Hypechusa*. Its species are clustered in three different groups, across the two series, but in accordance with the chromosome number: on the left, species with 2n = 10; in the centre, species with 2n = 12; and on the right, *V. lutea* alone, with 2n = 14.

In the spatial representation (Fig. 4) of all sections hitherto investigated (i.e., sect. *Hypechusa* together with the previously analysed sections *Peregrinae*, *Narbonensis*, *Faba*, and *Bithynicae*), the species of sect. *Hypechusa* are scattered, but located in a broadly intermediate position between the species of sect. *Narbonensis* and those of the other sections. As a consequence, on the basis of karyological parameters, the sections *Peregrinae*, *Faba*, and

Bithynicae appear to be more evolved in comparison with sect. *Hypechusa* and, especially, with sect. *Narbonensis*.

In the ITS phylogenetic analyses (Figs. 5 and 6), sect. *Hypechusa* was not found to belong to a monophyletic group which did not include sections *Narbonensis* and *Peregrinae* on any occasion (none of the five equally parsimonious cladograms obtained in the original analysis, the 12 equally parsimonious cladograms obtained from the "direct optimisation" alignment or the majority consensus tree from the Bayesian analysis). The results shown here also indicate that neither series of sect. *Hypechusa* is monophyletic. Indeed, the monophyletic unit which includes *V. galeata*, *V. hyrcanica*, *V. noeana*, and *V. tigridis* (i.e., the majority of the species of series *Hyrcanicae*) does not include *V. assyriaca*. On the contrary, the latter species appears in a monophyletic unit including *V. hybrida*, *V. melanops*, *V. mollis*, and *V. sericocarpa* (i.e., species of series *Hypechusa*). Moreover, *V. bithynica* (included in the monotypic sect. *Bithynicae*) is sister group to *V. anatolica*, which in turn is not present in the monophyletic group corresponding to series *Hypechusa* pro parte.

In conclusion, cytological, karyomorphological, and molecular data indicate that sect. *Hypechusa* is so highly heterogeneous as to raise a strong doubt about its justification and its infrasectional taxonomy (i.e., the two series). Our data confirm the results of Potokina et al. (1999), in that some species of sect. *Hypechusa* are quite remote from other species of the same section, and support the placement of *V. mollis* in this section, in agreement with Kupicha (1976) and Maxted (1993, 1994).

As far as the relationships among the sections are concerned, our data does not indicate a close alliance between sections *Peregrinae* and *Hypechusa*, as suggested by Kupicha (1976). Moreover, according to karyomorphological evidence, sect. *Hypechusa* is clearly separated from the other sections, while phylogenetic analysis of DNA sequences indicates a somewhat closer affinity among species belonging to different sections.

We are not ready at present to justify the observed discrepancy between our data and previous morphology-based evidence, especially because the position of the lens in the seeds and the characteristics of the cortical bundles at each node (Kupicha 1976), if not the basal kink in the wings, do not show a clear adaptative value, and therefore, are not easily interpretable as parallelisms; however, their potential role as synapomorphies for sect. *Hypechusa* has been challenged by the evidence presented here. Only further analyses progressively including more species and possibly the integration of different methods (i.e., more extensive nuclear DNA sequencing, inclusion of selected organellar sequences, reassessment of critical morphological characters) will elucidate the relationships among the sections of the genus *Vicia*.

References

Alefeld F (1860) *Hypechusa*, nov. gen Viciearum. Bot Z 19: 165–166

Ball P-W (1968) *Vicia* L. In: Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM, Webb DA (eds) Flora Europaea. Cambridge University Press, Cambridge, pp 129–136

Ceccarelli M, Minelli S, Maggini F, Cionini P-G (1995) Genome size variation in *Vicia faba*. Heredity 74: 180–187

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791

Fennell SR, Powell W, Wright F, Ramsay G, Wangh R (1998) Phylogenetic relationships between *Vicia faba* (Fabaceae) and related species inferred from chloroplast trn*L* sequences. Plant Syst Evol 212: 247–259

Frediani M, Caputo P, Venora G, Ravalli C, Ambrosio M, Cremonini R (2005) Nuclear DNA contents, rDNAs and karyotype evolution in subgenus *Vicia*: II. Section *Peregrinae*. Protoplasma 226: 181–190

Goloboff P (1999) Nona. Instruction manual. Published by the author, S. M. de Tucumán, Argentina

Greilhuber J (1986) Severely distorted Feulgen-DNA amounts in *Pinus* after non-additive fixations as a result of meristematic self-tanning with vacuole contents. Can J Gen Cytol 28: 409–415

Greilhuber J, Speta F (1976) C-banded karyotypes in the *Scilla hohenacheri* group: *S. persica* and *Puschkinia* (Liliaceae). Plant Syst Evol 126: 149–188

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98

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

Huelsenbeck PJ, Ronquist F (2001) MrBayes: Bayesian inference of phylogeny. Bioinformatics 17: 754–755

Huziwara Y (1962) Karyotype analysis in some genera of *Compositae* VIII: further studies on the chromosomes of *Aster*. Am J Bot 49: 116–119

Khattab AMA, Maxted N, Bisby FA (1988) Close relatives of the faba bean from Syria: a new species of *Vicia* and notes on *V. hyaeniscyamus* (Leguminosae). Kew Bull 43: 535–540

Kotseruba VV, Venora G, Blangiforti S, Ruffini Castiglione M, Cremonini R (2000) Cytology of *Vicia* species. IX. Nuclear DNA amount, chromatin organization and computer aided karyotyping of a Russian accession of *Vicia faba* L. Caryologia 53: 195–204

Kupicha FK (1976) The infrageneric structure of *Vicia*. Not R Bot Gard Edinb 34: 278–326

Leth H, Jaaska V (2002) Cladistic and phenetic analysis of relationships in *Vicia* subgenus *Vicia* by morphology and isozymes. Plant Syst Evol 232: 237–260

Levan A, Freda K, Sandberg AA (1964) Nomenclature for centromeric position chromosomes. Hereditas 52: 201–220

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

Maxted N (1994) A phenetic investigation on *Vicia* section *Peregrinae* Kupicha (Leguminosae, Papilionoideae, Vicieae). Edinb J Bot 51: 75–97

Maxted N (1995) An ecogeographical study of *Vicia* subgenus *Vicia*. Systematic and ecogeographic studies on crop genepools. 8. IPGRI, Rome, Italy

Maxted N, Douglas C (1997) A phenetic investigation of *Vicia* section *Hypechusa* (Alef.) Aschers. & Graebner (Leguminosae, Papilionoideae, Vicieae). Lagascalia 19: 345–370

Maxted N, Khattab AMA, Bisby FA (1991) The newly discovered relatives of *Vicia faba* do little to resolve the enigma of its origin. In: Phitos

D, Greuter W (eds) Proceedings of the VI OPTIMA meeting. Botanika Chronika 10. Botanical Institute, University of Patras, Athens, pp 435–465

Nixon KC (1999) Winclada (beta) ver. 0.9.9. Published by the author, Ithaca, New York, USA

Pavone P, Salmeri C, Scammacca B (1995) "Karyo 95" nuovo programma di gestione dei dati cariologici. G Bot Ital 129: 2

Potokina EK, Tomooka N, Vaughan DA, Alexandrova T, Xu RQ (1999) Phylogeny of *Vicia* subgenus *Vicia* (Fabaceae) based on analysis of RAPDs and RFLP of PCR amplified chloroplast genes. Genet Res Crop Evol 46: 149–161

Scott AJ, Knott M (1974) A cluster method for grouping means in the analysis of variance. Biometrics 30: 505–512

Stebbins L (1971) Chromosomal evolution in higher plants. Edward Arnold, London

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Venora G, Conicella C, Errico A, Saccardo F (1991) Karyotyping in plant by an image analysis system. J Genet Breed 45: 233–240

Venora G, Ocampo B, Singh K, Saccardo F (1995) Karyotype of the Kabuli-type chickpea (*Cicer arietinum* L.) by image analysis system. Caryologia 48: 147–155

Venora G, Blangiforti S, Cremonini R (1998) Karyotype analysis of twelve species belonging to genus *Vigna*. Cytologia 64: 117–127

Venora G, Blangiforti S, Frediani M, Maggini F, Gelati M-T, Ruffini Castiglione M, Cremonini R (2000) Nuclear DNA contents, rDNAs, chromatin organization and karyotype evolution in *Vicia* sect. *faba*. Protoplasma 213: 118–125

Wheeler W (1996) Optimization alignment: the end of multiple sequence alignment in phylogenetics? Cladistics 12: 1–9

Wheeler W, Gladstein D, De Laet J (2002) POY, phylogeny reconstruction via optimization of DNA and other data, version 3.0.11. American Museum of Natural History, New York

Yokota Y, Kawata T, Iida Y, Kato A, Tanifuji S (1989) Nucleotide sequences of the 5.8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. J Mol Evol 29: 294–301