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Taxonomic relationships between *V. faba* and its relatives based on nuclear and mitochondrial RFLPs and PCR analysis

W. T. G. van de Ven, N. Duncan, G. Ramsay, M. Phillips, W. Powell, and R. Waugh

Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK

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Summary. The taxonomic relationships between 52 accessions of 12 *Vicia* species and three accessions of *Lathyrus* were examined using nuclear RFLP- and PCR-generated data. Two hundred and sixty informative restriction fragments or amplification products were analysed by single linkage analysis, average cluster analysis, and the Wagner parsimony method. Dendrograms constructed from each type of analysis showed similar overall topologies and could be divided into three parts corresponding respectively to the *Lathyrus* outgroup, the species grouped in the section *Faba/narbonensis* complex, and the species belonging to the sections *Hypechusa* and *Peregrinae*. With few exceptions, the majority of accessions belonging to one species grouped together before branching to other species. An analysis of mitochondrial DNA phenotypes was both consistent with and complemented the results from the nuclear data. Overall, the species relationships show a good correlation with the classification of Maxted et al. but suggest that *V. faba* is more closely aligned to species from the sections *Hypechusa* and *Peregrinae* than to those in the *narbonensis* complex. In addition, the position of two new species, *V. kalakhensis* and *V. eristaloides*, as members of the *narbonensis* complex was supported by the molecular data, which also allowed a preliminary classification for recently collected *Vicia* accessions.

Key words: *Vicia faba* – RFLPs – Taxonomy – mtDNA

Correspondence to: R. Waugh

Introduction

The genus *Vicia* comprises approximately 166 species (Allkin et al. 1986) which are widely distributed throughout the temperate zones of both northern and southern hemispheres. There are two widely quoted intrageneric classifications. Ball (1968) divided the genus into four sections; *Vicia*, *Cracca*, *Ervum* and *Faba*. Kupicha (1976), using a wide variety of morphological and chemical characters, recognised 22 sections in two subgenera, *Vicia* and *Vicilla*. *Vicia* contains five sections: *Atossa*, *Vicia*, *Faba*, *Hypechusa* and *Peregrinae*. The section *Faba* from both schemes contains *V. faba*, *V. bithynica*, *V. narbonensis*, *V. serratifolia*, *V. galilaea*, *V. johannis* and *V. hyaeniscyamus*. The last five species are grouped collectively in the *narbonensis* complex (Schäfer 1973). *V. faba*, which contains the cultivated faba bean, contains four botanical varieties: *minor*, *major*, *paucijuga* and *equina*. Several methods have been used to assess the relatedness of the species within the section *Faba sensu* Kupicha (1986) including morphological, geographical and karyotype characters (Yamamoto 1973, 1984; Ladizinsky 1975a; Hammer et al. 1986; Khattab 1988), crossability (Ladizinsky 1975a; Cubero 1981; Ramsay and Pickersgill 1984), isozymes (Ladizinsky 1975b; Yamamoto 1984; Mancini et al. 1988), morphometrics (Birch et al. 1985), flavonoid analyses (Perrino et al. 1989), and rRNA spacer length polymorphism (Delre et al. 1988). All agree that *V. faba* is genetically distant from other species in the section *Faba* and is, therefore, probably a monophyletic species with its wild progenitor apparently extinct (Ladizinsky 1975a; Birch et al. 1985). Maxted et al. (1991a) refined Kupicha's (1976) classification of the subgenus *Vicia* by dividing the 38 species into nine sections. The new classification

gives sectional status to *V. faba*, *V. bithynica*, and the species comprising the *narbonensis* complex (previously grouped in the section *Faba*) and includes two new sections, *Microcarinae* and *Wiggersia*, erected from species unknown to Kupicha.

Recently, molecular approaches have become increasingly utilised for taxonomic and phylogenetic analyses. Restriction fragment length polymorphisms (RFLPs) in both nuclear and cytoplasmic genomes have been applied to a wide range of plant species including potato (Debener et al. 1990), tomato (Miller and Tanksley 1990), *Brassicas* (Song et al. 1988a, b, c), *Rubus* (Waugh et al. 1990), and groundnut (Kochert et al. 1992). This approach is especially informative because the markers are phenotypically neutral and not subject to environmental effects. More importantly, a large number of markers can be used for analysis. Putative phylogenies can be routed bi-parentally by examining nuclear DNA sequences or, in organisms where male transmission of organelles does not occur, through the maternal lineage, by using cytoplasmic DNA markers. In a number of cases RFLP analysis has permitted unclassified accessions to be assigned to specific taxonomic groups or else allowed accessions classified by traditional criteria to be re-classified. In this report, we have used nuclear and cytoplasmic RFLP data to examine potential phylogenetic relationships among *Vicia* species from the sections *Faba sensu* Kupicha (1976), *Hypechusa* and *Peregrinae*. Five unassigned species were included in the analysis as well as representatives from each of the *V. faba* types (*major*, *minor*, *paucijuga* and *equina*). Phenograms based on the molecular data have been constructed using three *Lathyrus* accessions as an outgroup.

Materials and methods

Fifty-two accessions of 12 *Vicia* species, belonging to the section *Faba sensu* Kupicha (1976), the section *Hypechusa* or the section *Peregrinae*, and three accessions of *Lathyrus* from different geographical origins, were selected (Table 1). The methods used for DNA isolation and the detection of nuclear RFLPs were described previously (van de Ven et al. 1990, 1991). DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII. Thirteen cloned *Vicia faba* genomic DNA and cDNA probes, total maize mitochondrial DNA (Table 2), and a pair of PCR primers, were used to generate the information for analysis. The cDNA probes were selected on the basis that they have previously been shown to detect polymorphism in *Vicia* species (van de Ven et al. 1990).

Table 1. *Vicia* cultivars, species and accessions included in the taxonomic analysis

Section (*sensu* Kupicha)	Accession no.	Chromosome no.	DNA (pg)	Location	Source
Faba					
1. *V. galilaea* Plitm. & Zoh. subsp. *faboidae*	L1	14		Bat-shlomo, Israel 1982	Ladizinsky, Israel
2. *V. galilaea*	L2			Bat-Giyora, Israel 1982	Ladizinsky
3.	44			Israel 88-53 Gatersleben	
4.	112005			Bari, Italy	
5. *V. hyaeniscyamus* Mouter	112421	14		Bari, Italy	
6.	867083			Um Jammeya, Homs, Syria	Vicieae Project, Southampton
7.	867093			Al Naoora, Homs, Syria	Vicieae Project, Southampton
8.	867152			Qalaat-al-Hosn, Homs, Syria	Vicieae Project, Southampton
9.	867215			Kafre Sneef, Tartous, Syria	Southampton
10. *V. johannis* Tamamsch. var. *johannis*	50	14	14.14	Eskiehir, Turkey	Gatersleben
11. var. *procumbens* Schäf.	64			Turkey	Gatersleben
12. *V. johannis*	106214				Bari, Italy
13. var. *procumbens* Schäf.	800278			25 km from Tokat to Camubel, Turkey	ICARDA
14. *V. narbonensis* L. var. *narbonensis*	1	14	16.11	Crete	Gatersleben
15. var. *narbonensis*	5			Sacavem, Portugal	Gatersleben
16. var. *narbonensis*	7			Japan	Gatersleben

Table 1. (*Continued*)

Section (*sensu* Kupicha)	Accession no.	Chromosome no.	DNA (pg)	Location	Source
17. var. *aegyptiaca* Korn.	8			Ankara, Turkey	Gatersleben
18. var. *jordanica* Schäf.	38			Gonner, Israel	Gatersleben
19. var. *salmonea*	43			Gilat bei Beershieba, Israel	Gatersleben
20. *V. narbonensis*	128			Portugal	ICARDA
21. *V. narbonensis*	129			Portugal	ICARDA
22. *V. narbonensis*	130			South Australia or Austria	ICARDA
23. *V. narbonensis*	556			Lebanon	ICARDA
24. *V. narbonensis*	557			Lebanon	ICARDA
25. *V. narbonensis*	566			Lebanon	ICARDA
26. *V. narbonensis*	567			Lebanon	ICARDA
27. *V. narbonensis*	568			Lebanon	ICARDA
28. *V. narbonensis*	569			Lebanon	ICARDA
29. *V. narbonensis*	570			Lebanon	ICARDA
30. *V. kalakhensis*	867095			Al-Naoora, Homs, Syria	Vicieae project, Southampton
31. *V. kalakhensis*	867166			Hwach, Homs, Syria	Vicieae project, Southampton
32. *V. sp. nov.*	584			Kfardan, Lebanon	ICARDA
33. *V. sp. nov.*	867115			Al Makula, Homs, Syria	Vicieae project, Southampton
34. *V. eristaloides*	877321			Kumluca, Antalya, Turkey	Vicieae project, Southampton
35. *V. serratifolia* Jacq.	808289	14	15.63	Malta	Vicieae project, Southampton
36. *V. serratifolia* Jacq.	810194				Vicieae project, Southampton (Coimbra, Portugal)
51. *V. faba* L. var. *paucijuga*	172	12	28.07	Afghanistan	Cordoba, Spain
52. *major*	Optica			Netherlands	
53. *minor*	AxBxC			France	Dijon, France
54. *minor*	IVS G			UK/Sudan	Durham, UK
55. *equina*	H51/3			UK	PBI, UK
Hypechusa					
37. *V. lutea*	311	14	18.03	Balkan 1941, Buchunista-Magalochol, Greece	Gatersleben
38. *V. lutea*	105801				Bari, Italy
39. *V. lutea*	106001				Bari, Italy
40. *V. lutea*	106007				Bari, Italy
41. var. *lutea*	780361			C. Crete	Vicieae project, Southampton (Kew)
42. *V. melanops* Sibth. et Sm. var. *melanops*	474	10	20.02		Gatersleben, (Nat. Hist. Museum, Paris)
43. *V. melanops*	104608				Bari, Italy
44. *V. melanops*	105037				Bari, Italy
45. *V. hybrida* L.	181	12	16.46		Gatersleben
Peregrinae					
46. *V. peregrina* L.	315	14	19.15	Yen, Cifeik, Anatolia	Gatersleben
47. *V. michauxii* Sprang.	730	14	20.68	Usbekistan, SSR	Gatersleben
Lathyrus accessions					
48. *L. latifolius* L.		14	20.78		Botanic Gardens, Dundee
49. var. *albus*					Botanic Gardens, Dundee
50. *L. vernus*					Botanic Gardens, Dundee

Table 2. Probes and enzymes used for detecting RFLPs

Probe	Function	Source	Enzymes used	Reference
pBG 35	rRNA repeat	Flax	*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII	Goldsborough and Cullis (1981)
pBG 13	5*s* RNA repeats	Flax	*Bam*HI	Goldsborough et al. (1981)
pAD 4.4	Legumin	Pea	*Eco*RI, *Eco* RV, *Hin*dIII	Lycett et al. (1984)
pC7.18	cDNA	*Vicia*	*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII	van de Ven et al. (1990)
pC8.42	cDNA	*Vicia*	*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII	van de Ven et al. (1990)
pG6	Genomic DNA	*Vicia*	*Eco*RV	This study
pG10	Genomic DNA	*Vicia*	*Hin*dIII	This study
pG75	Genomic DNA	*Vicia*	*Hin*dIII	This study
pG97	Genomic DNA	*Vicia*	*Eco*RV	This study
pG124	Genomic DNA	*Vicia*	*Eco*RV	This study
pG127	Genomic DNA	*Vicia*	*Eco*RI	This study
pG132	Genomic DNA	*Vicia*	*Hin*dIII	This study
pG134	Genomic DNA	*Vicia*	*Hin*dIII	This study
mtDNA	Mitochondrial DNA	Maize	*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII	This study

The nuclear clones were obtained from a genomic DNA library of the *V. faba* line A × B × C and represented low-copy sequences. Plasmids were isolated by the rapid-alkaline SDS procedure of Silhavy et al. (1984) and total mitochondrial DNA was isolated from *Zea mays* cv Sunrise according to the method of Leaver et al. (1982). PCR primers were designed from the conserved sequences of two pea and one *Vicia* legumin gene and synthesised on an Applied Bio-systems 391 PCR mate. The nucleotide sequences of the primers and their acronyms are: legbox, $^{5'}$AGGTATCGGTACGT$^{T}_{A}$CGAC$^{G}_{T}$T$_{C}$TTACAG$^{3'}$ and legumin, $^{5'}$CGATTCAG$^{GG}_{AA}$A$^{G}_{A}$GGTGATATCATTGC$^{3'}$. Amplification reactions were in a total volume of 100 µl and contained 1 × *Taq* buffer (5 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin), 0.2 mM dATP, dCTP, dGTP and dTTP, 1 µM of each primer, 100 ng of genomic DNA, and 1.3 units of *Taq* DNA polymerase (NBL) covered with 60 µl of mineral oil. Amplification was performed in a Techne Programmable Dri-block PHC-1 or PHC-2 programmed for 30 cycles of 1 min at 92 °C, 1.5 min at 35 °C, 2 min at 72 °C followed by 5 min at 72 °C. Reactions products were run on 1.5% agarose gels and stained with ethidium bromide.

RFLP and PCR data were recorded as the presence or absence of a restriction or amplification fragment of a given length. Each fragment was treated as a unit character and analysed using single linkage or nearest neighbour analysis, average cluster analysis using arithmetic average unweighted clustering (UPGMA) (Sneath and Sokal, 1973), and the Wagner parsimony method [phylogenetic analysis using parsimony (PAUP) developed by D. L. Swofford, Illinois Natural History Survey, Champaign/Ill.]. Data were analysed using PAUP with, hold = 5, characters unordered, addition stepwise, alternative branch swapping, rooted with outgroup (*Lathyrus*).

Results

Two hundred and sixty different restriction fragments and PCR products were scored across the 55 accessions using 24 probe-enzyme combinations and the legumin primers. Among the 260 fragments recorded, five (1.9%) were common to all accessions, 77 (29.8%) were unique, and 178 (68.4%) were phenetically informative in that an individual fragment was shared by at least two accessions but not all.

Phenetic analysis

The majority of RFLP patterns for individual probe-enzyme combinations (62.5%) distinguished a particular species or group of species from the remainder. For example, in Fig. 1a, all five *V. lutea* accessions share a unique hybridising fragment which separates them from the other accessions. *V. galilaea* (with the exception of accession 1) and *V. hyaeniscyamus* share a similar molecular profile with one of the *V. melanops* accessions, *V. michauxii* and *V. peregrina*. A different molecular profile is shared by *V. johannis*, *V. narbonensis*, *V. kalakhensis*, *V. sp. nov.*, *V. hybrida*, *V. serratifolia*, *V. faba* and two of the three *V. melanops* accessions. The remaining probe enzyme combinations (37.5%) more often identified accessions within a species with unique RFLP profiles. In Fig. 1b polymorphism was detected with a single probe-enzyme combination with *V. galilaea*, *V. hyaeniscyamus*, *V. johannis*, *V. narbonensis*, *V. serratifolia*, *V. lutea*, *V. melanops*, *V. faba* and the *Lathyrus* species. However, each probe-enzyme combination produced different groupings which made it difficult to assess phenetic relationships by comparing RFLP patterns from individual probe-enzyme combinations (e.g., compare Fig. 1a, b and c). This is similar to the situation found in *Brassica* (Song et al. 1988b). The complete set of RFLP and PCR data were therefore used for analysis.

When comparing dendrograms constructed by the three different types of analysis outlined in Materials

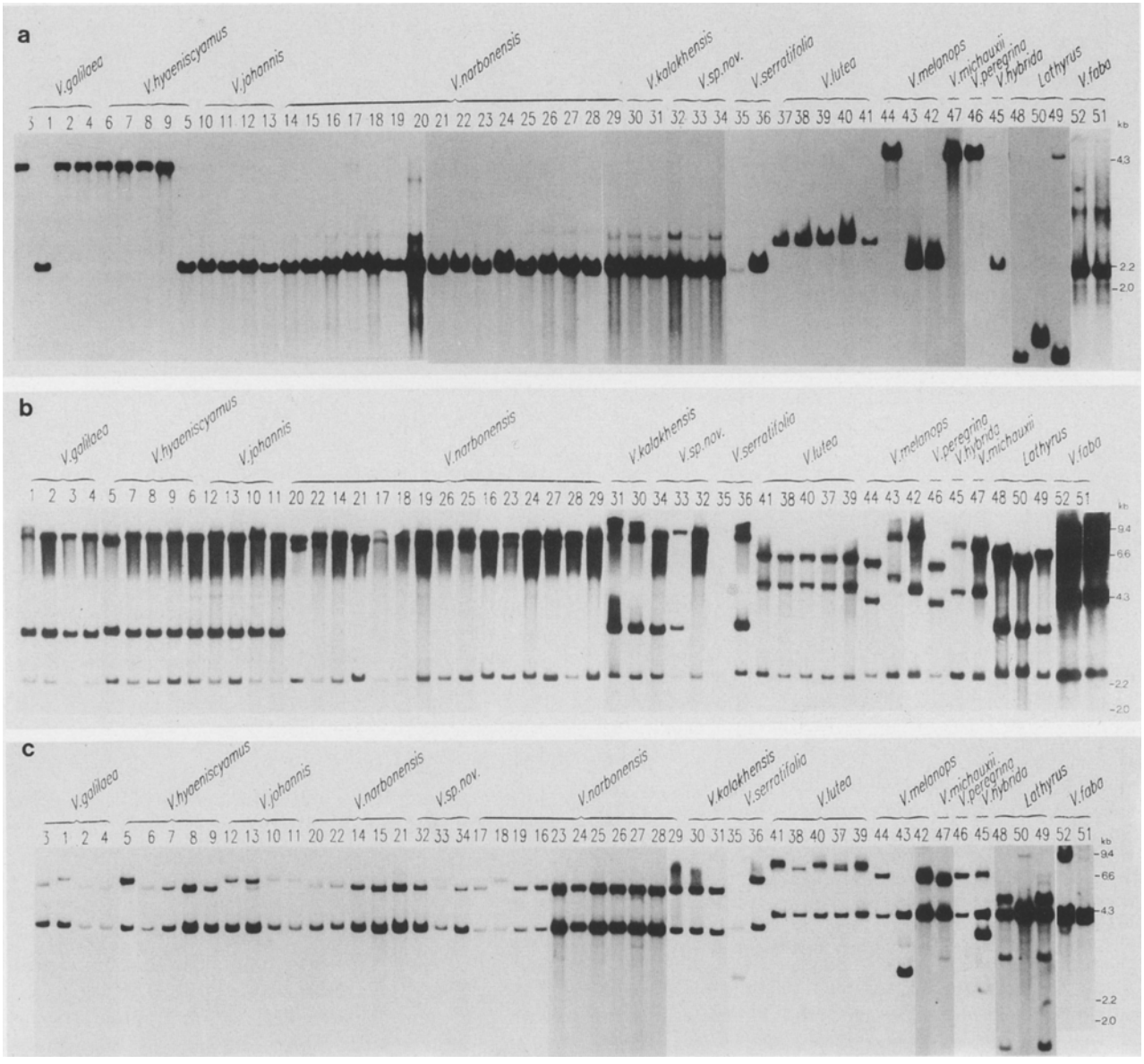

Fig. 1a–c. RFLP patterns for the accessions used in this study belonging to 12 *Vicia* and two *Lathyrus* species **a** DNA digested with *Eco*RV and probed with pC7.18. **b** DNA digested with *Bam*HI and probed with pC8.42. **c** DNA digested with *Eco*RI and probed with pBG35. *Numbers* correspond to the accessions given in Table 1

and methods, there was little difference in overall topology. In each case the trees could be divided into three parts: (1) the outgroup represented by the *Lathyrus* species; (2) the species grouped in the section *Faba/narbonensis* complex; and (3) the species belonging to the sections *Hypechusa* and *Peregrinae* (with the addition of *V. faba*). Therefore, only the results from the PAUP analysis are presented. Using PAUP a total of 24 trees were found with a length of 508 and a consistency index of 0.500. The 24 trees were similar in their topologies and contained only minor differences. One representative tree is shown in Fig. 2. The selection of this tree was based on the following criteria: (1) the frequency of individual taxa at certain positions, and (2) the frequency of a particular topology of individual clusters in all possible trees.

Section Faba

Based on the RFLP and PCR data, accessions 1 (*V. galilaea*) and 5 (*V. hyaeniscyamus*) are very similar and would appear to be related to *V. johannis* (10, 11, 12, 13). *V. serratifolia* (36) is also associated with these

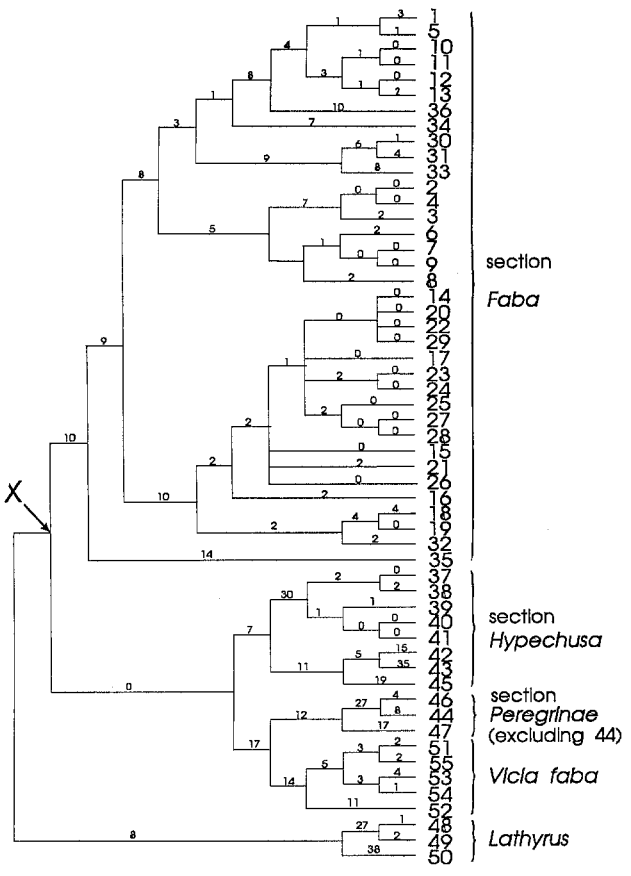

Fig. 2. Phenograms generated using PAUP, of 52 *Vicia* accessions and three *Lathyrus* accessions based on RFLP and PCR data. The numbers at the end of each branch correspond to the accessions in Table 1. The numbers on the branches indicate the minimum number of mutational steps and the length of the branch is not proportional to the number. *X*, common ancester of *Vicia* species

accessions. The other *V. serratifolia* accession (35) does not group together with genotype 36, but is located between the section *Faba* and the other *Vicia* species. The extreme divergence of the two *V. serratifolia* (35 and 36) accessions may be confounded by the missing data for genotype 35 where 104 of 260 fragments could not be scored. However consistent differences between the two accessions were apparent (e.g., Fig. 1c).

Two accessions, 30 and 31, recently classified as *V. kalakhensis* (Ehrman and Maxted, 1989) cluster together with an unclassified accession, *V. sp. nov.* 867115 (33). This accession may also be *V. kalakhensis*. This cluster is grouped between the *V. johannis* and *V. galilaea*/*V. hyaeniscyamus* clusters. The *V. galilaea* accessions (2, 3, 4) group together, as do the *V. hyaeniscyamus* accessions (6, 7, 8, 9), and these two species seem to be more closely related to each other than to the other species from the section *Faba*. *V. sp. nov.* 877321 (34) groups between several species while *V. sp. nov.* 584 (32) clusters with the *V. narbonensis* accessions (14–29) and by these criteria may be classified as a *V. narbonensis*.

The sections Hypechusa and Peregrinae

Within this group four clusters are consistently formed. The first is formed by *V. hybrida* (45) and two *V. melanops* species (42, 43). The second contains another *V. melanops* (44) species together with *V. peregrina* (46) and *V. michauxii* (47). Although formally not placed with these sections the third cluster is represented by the *V. faba* species (51, 52, 53, 54, 55). Within this group the two *minor* types group together as do the *equina* and *paucijuga* types. The *major* accession is peripheral in this cluster. The fourth cluster is represented by the *V. lutea* species (37, 38, 39, 40, 41), in which accessions 37 and 38 and accessions 39, 40 and 41 fall into separate subgroups.

All *Vicia* species share a hypothetical common ancestor at point X of the tree and eight characters (restriction fragments) distinguish them from the outgroup (*Lathyrus*). The section *Faba* group, and the group comprising the section *Hypechusa*, the section *Peregrinae* and *V. faba* are 'sister groups' and are equally distant from the outgroup in terms of evolutionary time.

Mitochondrial DNA analysis

To complement the nuclear DNA analysis a study of mitochondrial DNA RFLPs was undertaken to analyse the phylogeny through the maternal lineage. However, only phenotypes were scored from genomic blots probed with mitochondrial DNA (mtDNA) because the relationships between individual polymorphic bands were too complicated to interpret. Phenotypic data were obtained from mtDNA digested with four enzymes (*Bam*HI, *Eco*RV, *Hin*dIII and *Eco*RI). Figure 3 shows an example of the mtDNA patterns obtained using the restriction enzyme *Eco*RI and Table 3 summarises the phenotypes obtained with all four enzymes. In this analysis, *V. hyaeniscyamus* 112421 (5) and *V. serratifolia* 810194 (36) show the same mtDNA phenotype for all four enzymes used and therefore probably share a common female ancestor. All the *V. johannis* accessions (10–13) share the same mtDNA pattern and for three enzymes this pattern is the same for *V. galilaea* L1 (1), *V. hyaeniscyamus* 112421 (5) and *V. serratifolia* 810194 (36), but different from the other *Vicia* and *Lathyrus* accessions. These seven accessions were also closely related using nuclear DNA analysis (see Fig. 2). *V. galilaea* L1 (1) and *V. hyaeniscyamus* 112421 (5) do not have the same mitochondrial DNA profile as the other accessions of the same species (accessions 2–4 and 6–9 respectively). With the exception of the *V. melanops* accessions (42,

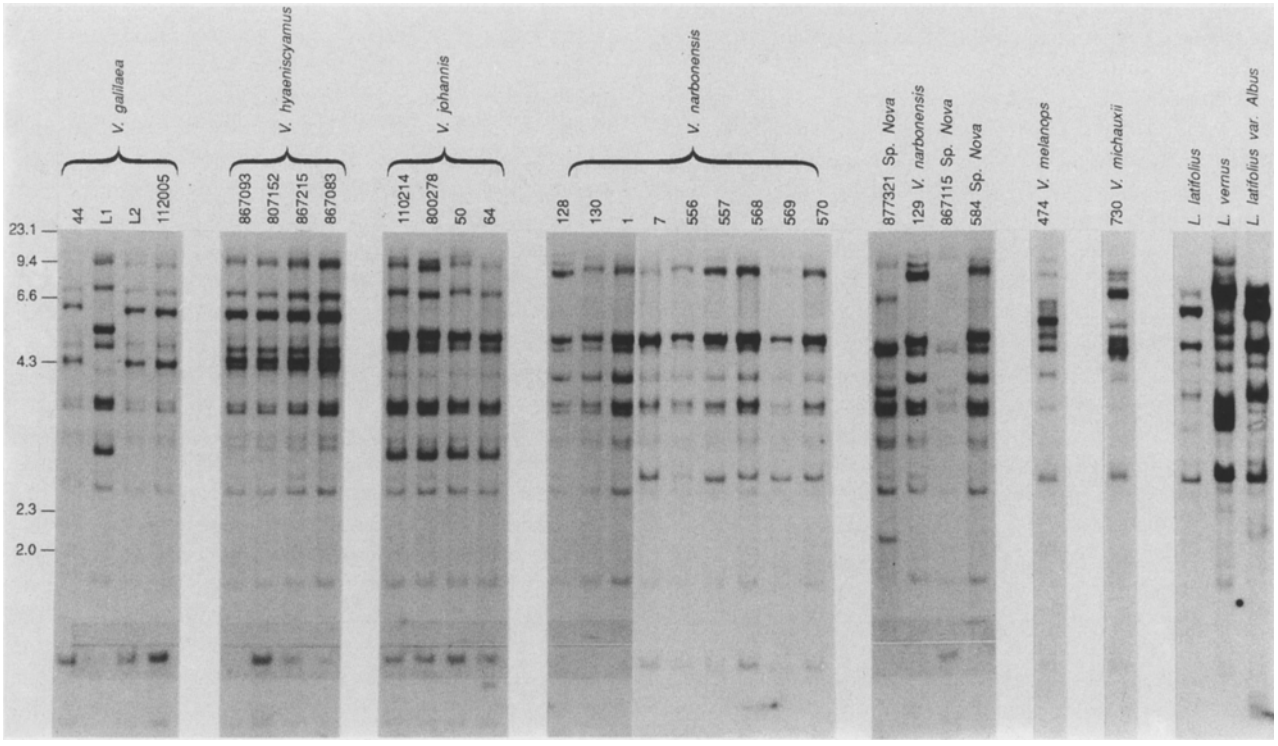

Fig. 3. Total mtDNA profiles of several *Vicia* and *Lathyrus* accessions

Table 3. List of genotypes obtained using mtDNA and four restriction enzymes

Accessions	1	2–4	5	6–9	10–13	14–29	30–31	32	33	34	36	37–41	42	43	44	45	46	47	48	49	50	51–55
*Bam*HI	A	B	A	C	A	D	E	D	F	G	A	H	I	L	K	M	K	N	O	O	P	R
*Eco*RI	V	B	G	C	A	D	H	D	H	F	G	I	N	L	K	M	K	O	P	R	S	T
*Eco*RV	A	B	A	C	A	D	F	D	F	E	A	G	I	H	I	K	I	M	N	N	O	P
*Hin*dIII	A	B	A	C	A	D	E	D	E	F	A	G	L	K	H	L	H	M	N	N	O	P

43, 44), all other accessions within a species have identical mtDNA phenotypes and the patterns between species differ. *V. melanops* 105037 (44) and *V. peregrina* (46) have similar mtDNA patterns for all four enzymes and are closely related using nuclear RFLP analysis. The two *Lathyrus latifolius* accessions (48 and 49) share three out of four mtDNA phenotypes and are clearly different from *L. vernus* (50). *V. sp. nov.* 584 (32) shares the pattern of *V. narbonensis*. The two *V. kalakhensis* accessions (30, 31) are similar and share three mtDNA phenotypes with *V. sp. nov.* 867115 (33). *V. sp. nov.* 877321 (34) has unique mtDNA patterns. The *V. faba* genotypes studied were homomorphic for mitochondrial DNA profiles and were unique amongst the samples analysed.

The species relationships based on mtDNA are therefore similar to, and complement those derived from, nuclear DNA analysis.

Discussion

In this report, we have used a combination of approaches to examine the molecular taxonomy of *Vicia* species from the sections *Hypechusa, Peregrinae* and *Faba sensu* Kupicha (1976). In our analysis it was difficult to assess the relationships between accessions by comparing the RFLP patterns of individual probe-enzyme combinations. Song et al. (1988b) reported similar findings in *B. napa* and *B. oleracea*. No conclusions were therefore drawn from single probe-enzyme combinations and the complete data set was used for analysis. Debener et al (1990) used alternative approaches to examine the reliability of their phenograms of 17 *Solanum* species. As the reliability of the analysis increases by reducing the number of accessions, they divided their data into subsets, used different statistical analyses, and ran each several times

using different input orders. To examine the reliability of the tree topologies we analysed our data using three statistical approaches and super-imposed mtDNA information.

V. galilaea L1 (1) and *V. hyaeniscyamus* 112421 (5) were more closely related to *V. johannis* accessions than to their respective species, using both nuclear and mtDNA probes. *V. galilaea* L1 on morphological ground is *V. galilaea* subsp. *faboidae*, a taxon which Ladizinsky (1975a) considers to be a form of *V. hyaeniscyamus*. The identity of *V. hyaeniscyamus* 112421 (5) has not been verified but it is conceivable that this accession was classified according to Ladizinsky's criteria and it may, therefore, be the same taxon as accession 1. The original designations of accessions 2–4 as *V. galilaea*, accessions 6–9 as *V. hyaeniscyamus*, and 10–13 as *V. johannis*, are considered to be reliable. The grouping of accessions 1 and 5 closer to *V. johannis* than other *V. galilaea* and *V. hyaeniscyamus* was unexpected and may indicate that this group is actively evolving and cannot be ascribed to three distinct species. All other individuals belonging to the same species were grouped before they joined the clusters of other species, with the exception of the two *V. serratifolia* accessions and *V. melanops* 105037. However, 40% of the data were missing for *V. serratifolia* 808289 (accession 35), which is a reasonable explanation for the *V. serratifolia* accessions being apparently fairly distantly related based on available molecular data. Alternatively, as *V. serratifolia* is easy to confuse with *V. narbonensis* on morphological grounds accession 35 may have been mis-identified.

Two unclassified species were included in the study and both could be assigned to existing groupings based on their RFLP and PCR profiles. *V. sp. no.* 867115 (33) originating in Syria, clusters together with the newly erected species *V. kalakhensis* (Khattab et al. 1988) which falls within the *narbonensis* complex as suggested by Maxted et al. (1991a). *V. sp. nov.* 584 (32) based on molecular analysis could be classified as a *V. narbonensis* accession. Accession 32 was originally erroneously classified as *V. hyaeniscyamus*. On morphological grounds it resembles an outlying form of *V. narbonensis*, with some affinities to var. *jordanica* and var. *salmonea*. The molecualr analysis also places this accession close to vars. *jordanica* and *salmonea*. Maxted et al. (1991b) considered *V. eristaloides* an outlying species within the *narbonensis* complex. Based on our data, its closest affinities are with *V. johannis* and *V. serratifolia* accession 810194. The inclusion of these novel *Vicia* species has provided an opportunity to demonstrate the power of RFLPs to classify germplasm independently of other taxonomic information.

The tree topologies derived from RFLP and PCR analysis were compared to the system of classification based on morphological, karyotype, biochemical, and other studies (Ball 1968; Kupicha 1976; Maxted et al. 1991a, b). Species belonging to *Faba sensu* Kupicha (1976), *Hypechusa* and *Peregrinae* were used in this study. There is a very good correlation between our phenogram (Fig. 2) and the classifications of both Kupicha (1976) and Maxted et al. (1991a, b). Only two exceptions are found: one of the three *V. melanops* accessions (44) falls into the wrong section and all of the *V. faba* accessions group closer to the section *Peregrinae* than the other two sections. Many authors have considered the taxonomic position of *V. faba*. Cubero (1981) summarised three possibilities: (1) the existence of a *Faba* genus different from but related to *Vicia*; (2) the inclusion of faba beans as one more

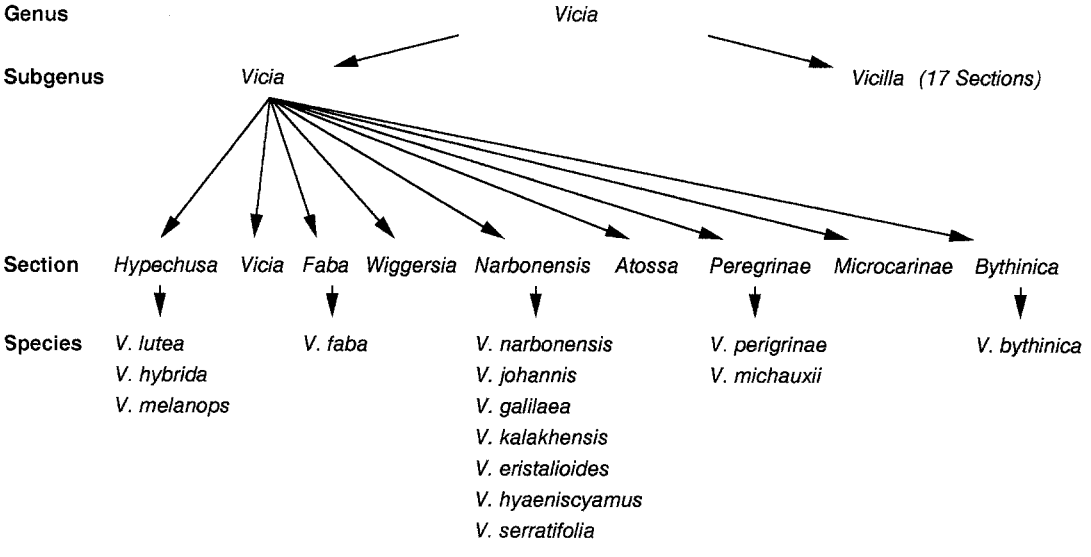

Fig. 4. Classification of several *Vicia* species according to Maxted et al. (1991a)

species of *Vicia* section *Faba* together with the *narbonensis* complex (*V. narbonensis*, *V. johannis*, *V. galilaea*, *V. hyaeniscyamus* and *V. serratifolia*) and *V. bithynica*; and (3) the faba bean as the only species of the section *Faba* of the sub-genus *Vicia*. From the molecular data obtained here, option (3) would be most likely, as *V. faba* groups with other *Vicia* species, and is more similar to species from the section *Peregrinae* as opposed to species from the section *Faba*. This is in agreement with the current taxonomic views of Maxted et al. (1991a) who divide the section *Faba* into three giving sectional status to *V. faba*, *V. bithynica* and the *narbonensis* complex species (outlined in Fig. 4). On the basis of available information these authors also suggest that the closest relative of *V. faba* in the subgenus *Vicia* is *V. narbonensis*. Based on our molecular data, *V. faba* is more closely related to species from the section *Peregrinae*. However, as the inclusion of *V. faba* in the section *Peregrinae* would be ruled out on morphological grounds (Kupicha, 1976), a better option would be to place *V. faba* in a monospecific section *Faba* and delimit a new section to include the species allied to *V. narbonensis*.

Schäfer (1973) was one of the first to divide the *narbonensis* complex into *V. narbonensis*, *V. serratifolia*, *V. galilaea*, *V. johannis* and *V. hyaeniscyamus*. This was based primarily on the results of hybridisation experiments, but also included morphological characters. On the basis of characters such as leaf shape, seeds per pod, seed colour, and flower colour, other taxonomists have not separated *V. serratifolia* and *V. johannis* from *V. narbonensis* (Ball 1968; Plitmann 1967) or *V. hyaeniscyamus* from *V. galilaea* (Plitmann 1967). Using RFLP analysis within the *narbonensis* complex all species grouped together before they clustered to any of the other species, which supports the distinctness of each species group. *V. galilaea* and *V. hyaeniscyamus* appear to be more similar than the other species. Two other species not distinguished from *V. narbonensis* by Ball (1968) and Plitmann (1967) were *V. serratifolia* and *V. johannis*. Using molecular DNA analysis the two species are closely related but different from *V. narbonensis*. Birch et al. (1985) used cluster analysis based on morphometric data and demonstrated that *V. johannis* was clearly separable from other members of the *narbonensis* complex. Khattab et al. (1988) described a new *Vicia* population found in Syria and conducted extensive morphological, chemical, geographical, and cytological analysis which resulted in the designation of this population as a new *Vicia* section *Faba* species: *V. kalakhensis*. Molecular data support this conclusion since accessions of *V. kalakhensis* cluster together close to other members of the *narbonensis* complex.

Within *V. faba* four botanical groups, based mainly on seed size, have been recognised: these are the varieties *major*, *minor*, *equina* and the variety or sub species *paucijuga*. Two *minor* types and one accession of each of the other types were used in this study. Although the grouping of the *minor*, *equina* and *paucijuga* accessions differed between analyses, the *major* type always separated before the others. These results agree with those from flavonoid studies of the botanical types of *V. faba* where Perrino et al. (1989) found *minor* most similar to *equina* and *equina* most similar to *paucijuga*. Cubero (1974) and Cubero and Suso (1981) have considered the evolution of *V. faba*. *V. faba* var. or subsp. *paucijuga* possesses a number of primitive characteristics but lacks two found in other *V. faba* populations, notably pod dehiscence and a degree of allogamy. It is likely that the *paucijuga* types represent an offshoot of a relatively primitive *minor* stock which has subsequently failed to evolve along with other *V. faba* types due to both geographical isolation and genetic isolation due to autogamy. If the phenogram in Fig. 2 is traced from the point at which all *V. faba* accessions radiate to each *V. faba* accession in turn, the smallest 'minimum number of mutational steps' is found to one of the accessions of var. *minor*, suggesting that it may be primitive. Analysis of a larger number of *V. faba* accessions with more molecular probes would be required to confirm this hypothesis.

In summary, the species relationships in the sections *Faba sensu* Kupicha (1976), *Hypechusa* and *Peregrinae* have for the first time been examined using molecular approaches. Overall, the species relationships based on molecular data concur with the classical taxonomic groupings of Kupicha (1976) and Maxted et al. (1991a, b). However, exceptions include the position of *Vicia faba* and two accessions classified as respectively *V. galilaea* (L1) and *V. hyaeniscyamus* (112421). The *V. faba* genotypes studied exhibit closest molecular affinity to species within the section *Peregrinae* rather than the section *Faba sensu* Kupicha (1976). The inclusion of new *Vicia* populations from Syria has also allowed the application of both nuclear and mitochondrial DNA analysis to confirm the position of *V. kalakhensis* and *V. eristaloides* relative to other species in the section *Faba* and allowed the classification of two previously unassigned accessions.

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