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Chloroplast DNA diversity in *Vicia faba* and its close wild relatives: implications for reassessment

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Abstract To obtain new information on phylogenetic relationships between wild and cultivated broad bean, restriction fragment length polymorphism (RFLP) analysis of chloroplast (cp) DNAs from *Vicia faba* and eight subspecies/species of its close wild relatives grouped together in the Narbonensis complex was carried out using 14 restriction endonucleases. The molecular sizes of the cpDNAs obtained were similar (122.6–123.4 kbp), indicating that they had all lost one of inverted repeats. Among the more than 300 sites surveyed, the three subspecies within *V. narbonensis*, which exhibit just as many types of karyotypes, were shown to have identical cp fragment patterns. Genetic distances between all of the pairs of species were calculated from RFLP data. The cpDNA diversity within the Narbonensis complex was found to be more extensive than expected, except for the genetic relationship between *V. hyaeniscyamus* and *V. johannis* in which a total of three mutations were detected among the 300 sites sampled, thereby showing their close relatedness. The cpDNA of *V. faba* vis-a-vis its wild relatives also exhibited startling differences, indicating a clear division of *Vicia* species into two distinct lineages. This analysis unambiguously provides new evidence that the wild species grouped in the complex did not contribute their plastomes to the evolution of *V. faba*, and hence none of the species can be considered to be putative allies of broad bean. The present study also demonstrates profound cpDNA diversity among closely related species that have lost one of inverted repeats.

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Introduction

At present about 15 species of legumes serve as staple food in various parts of the world (Summerfield and Bunting 1980; Duke 1981). Among these, broad beans (*Vicia faba*) are the fourth most important of the food legume crops (Kay 1979). However, in spite of its great potential for being one of the most important protein sources in many countries, its area of cultivation has been decreasing over the years (Torres et al. 1993), primarily because of low and highly variable yields. The utilization of germ plasm available within *V. faba* has not solved the major problems like susceptibility to various pests and diseases, mainly black bean aphid (*Aphis fabae*), chocolate spot disease (*Botrytis fabae*, *B. cinerea*), respectively, and tolerance to stress environments. In order to solve these problems effectively new types of germ plasm have to be found. Wild relatives of the crop provide additional sources of genetic variability, and it is in this context that a great deal of interest is currently being directed towards not only *V. faba*, but also the group of closely related species (*V. narbonensis*, *V. serratifolia*, *V. johannis*, *V. kalakhensis*, *V. galilaea*, *V. hyaeniscyamus*, *V. eristalioides*) that comprise the Narbonensis species complex (Maxted et al. 1991). This complex includes the taxa that are considered to be morphologically the closest wild relatives of *V. faba* (Zohary and Hopf 1973; Cubero 1984; Birch et al. 1985; Schäfer 1973), and according to Zohary and Hopf (1973) and Schäfer (1973) it shares a common ancestry with *V. faba*. Several species within the complex possess agronomically useful characters of importance. *V. narbonensis* and *V. johannis*, for example, are genetically resistant to *Aphis fabae* and *Botrytis fabae* and show a high level of winter hardiness (Birch et al. 1985); they could, therefore, be possible sources of germ plasm for *V. faba* breeding.

In comparison to *V. faba*, the Narbonensis species complex has been the focus of relatively little research in assessing interspecies' relationships within the complex and between the complex and *V. faba*: the information available does not include all of the species discovered in recent years in the Near East (area of origin of *V. faba*) and/or the patterns of variation reported so far and the interpretations based upon them have been considered exclusively in terms of nuclear events (Schäfer 1973; Raina and Rees 1983; Ladizinsky 1975; Perrino et al. 1989; van de Ven et al. 1990). None of these studies has succeeded in providing conclusive evidence for the primary aim, that of identifying the wild progenitor of *V. faba*.

The other new and, in some cases, very useful tool for assessing phylogenetic relationships at the inter-species level has been chloroplast DNA (cpDNA) restriction pattern variation (Palmer 1986). Two main features make the cpDNA molecule suitable for phylogenetic studies: (1) the chloroplast genome is evolving quite slowly at the nucleotide sequence level and therefore exhibits a highly conservative mode of evolution and (2) the genome is small (120–217 kbp) in size so that an entire array of fragments produced by many six-base restriction enzymes can be conveniently visualized on a single agarose gel in order to estimate the rates of nucleotide substitution, distribution of variant restriction sites and sequence distinctions among related DNAs.

In the study reported here the cpDNA restriction fragment pattern, using 14 restriction endonucleases, of *V. faba* and the Narbonensis complex was investigated on the basis of restriction fragment length polymorphism (RFLP) fingerprinting. Possible genetic relationships among wild and domesticated species are also discussed.

Materials and methods

Plant materials

A list of the species investigated is given in Table 1. The seeds of the species belonging to the Narbonensis complex were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria. *V. faba* seeds were obtained from the local seed company.

Table 1 List of *Vicia* species examined in the present study

Taxa	2n	Karyotype	Status	Abbreviation
V. faba L.	12		Cultivated	F
Narbonensis species complex				
V. narbonensis L.				N
ssp. *salmonea*	14	A	Wild	
ssp. *aegyptica*	14	B	Wild	
ssp. *narbonensis*	14	C	Wild	
V. serratifolia Jacq.	14		Wild	S
V. galilaea Plitm. & Zoh.	14		Wild	G
V. johannis Tamam.	14		Wild	J
V. kalakhensis Kh., Max. and Bis	14		Wild	K
V. hyaeniscyamus Mout.	14		Wild	H

Chloroplast DNA isolation and restriction endonuclease analysis

CpDNAs were extracted from seedling leaves according to Ogihara and Tsunewaki (1982), digested with 14 restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Nco*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Xba*I and *Xho*I) according to the manufacturer's instructions (Takara Shuzo Co) and fractionated by 0.8% or 1% agarose gel electrophoresis in TAE buffer (Maniatis et al. 1982). The molecular sizes of each fragment were calculated by making comparisons to molecular size markers after photographs had been taken.

Data analysis

The fragment patterns of cpDNAs from seven species related to *V. faba* were compared for all of the species pairs. Their genetic distances were estimated according to Nei (1987). On the basis of these distances, phenograms were constructed by the UPGMA method (Sokal and Sneath 1963).

Results

Consequent upon single digestion with 14 enzymes, the electrophoretic pattern of DNA fragments were clearly reproducible in all of the taxa examined (Figs. 1 and 2).

Genome size

The genome size was estimated from the restriction fragments generated by 5 enzymes (*Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I). The size for a particular species deduced from various enzyme digests was about the same (Table 2).

V. faba

The average size of the chloroplast genome was 123 kbp. The size estimated in this study is comparable to the size reported by Ko et al. (1983). Koller and Delius (1980) reported that the chloroplast genome size is 79.8×10^6 Da or approximately 120 kbp.

Narbonensis complex

The average size of chloroplast genomes in the six species within the complex corresponded with the estimates of *V. faba*, suggesting that the cpDNAs of all of the six species examined here had lost one of their inverted repeat sequences.

Restriction fragment patterns

Within species

The two types (A, C) of karyotypes found in *V. narbonensis* accessions result from segmental interchanges (Raina

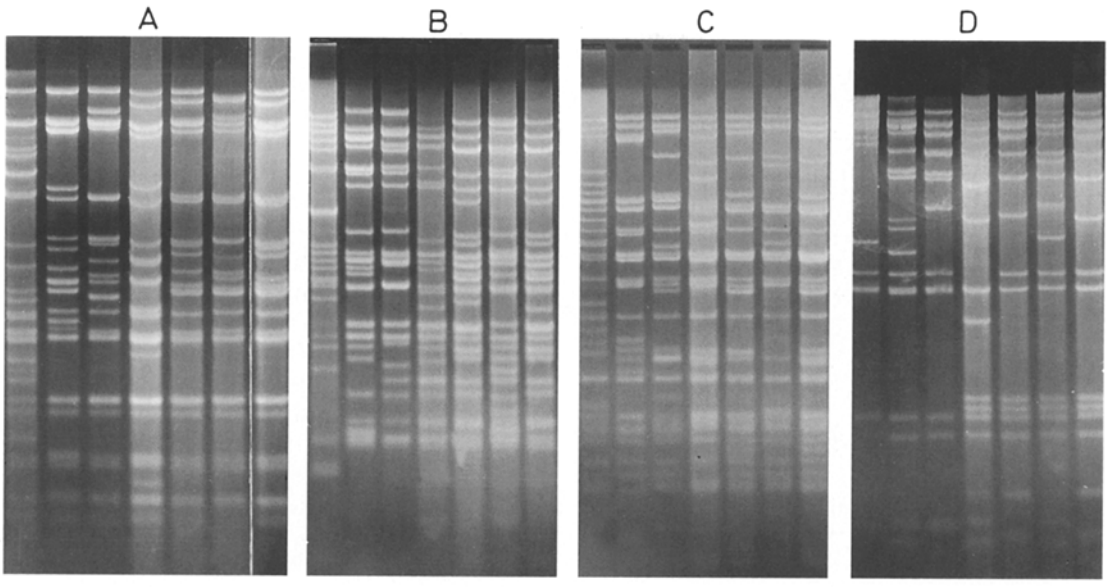

Fig. 1A–D Restriction fragment patterns of cpDNAs from *V. faba*, *V. narbonensis*, *V. serratifolia*, *V. galilaea*, *V. johannis*, *V. kalakhensis*, *V. hyaeniscyamus* (*from left to right*) digested with **A** *Hin*dIII, **B** *Xba*I, **C** *Bam*HI, **D** *Xho*I

et al. 1989). B is the normal karyotype. The complete elimination of heterozygotes and establishment of homozygotes (A, C) as a means of cytogenetic differentiation within *V. narbonensis* is the mechanism generally found at the interspecific level (Pal and Khoshoo 1973). The remaining species belonging to the complex and *V. faba* do not exhibit variation in karyomorphology within species (Schäfer 1973; unpublished data). It was, therefore, of particular interest to investigate the cpDNA of more than one accession in *V. narbonensis*. In the DNA extracted from chloroplasts of three subspecies (*salmonea*, *aegyptica*, *narbonensis*) containing the A, B and C karyotypes, respectively, no detectable variation was observed in any of the *Bam*HI, *Hin*dIII, *Xba*I and *Xho*I restriction patterns.

Narbonensis complex

Interestingly enough, *V. johannis* and *V. hyaeniscyamus* exhibited exactly similar patterns in all but the *Bam*HI, *Hin*dIII and *Xba*I digests. Even in these enzymes the gain or loss of one fragment rarely occurred (Fig. 1). When a restriction endonuclease is used to compare cpDNAs from very closely related species, such as Narbonensis complex species, it is usually necessary to establish that the band pattern encountered between species is not influenced by the change in band mobility

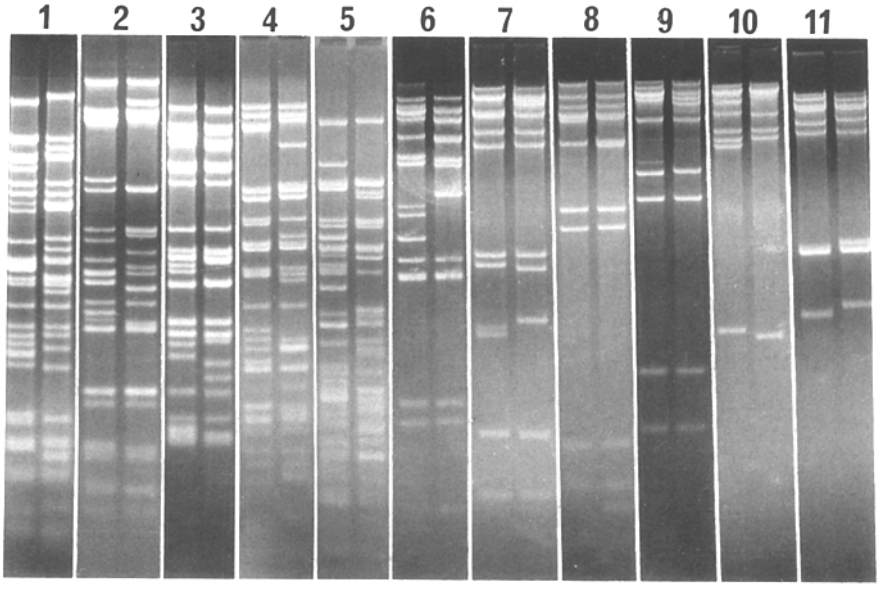

Fig. 2 Restriction fragment patterns of cpDNAs from *V. narbonensis* and *V. serratifolia* digested with *1 Eco*RV, *2 Hin*dIII, *3 Xba*I, *4 Bam*HI, *5 Dra*I, *6 Xho*I, *7 Pvu*II, *8 Kpn*I, *9 Pst*I, *10 Sal*I, *11 Sma*I

Table 2 Estimated molecular sizes (kbp) of cpDNAs

Taxa	*Kpn*I	*Pst*I	*Xho*I	*Xba*I	*Hin*dIII	Mean
V. faba	123.2	124.0	123.1	122.62	123.95	123.37
V. narbonensis ssp *narbonensis*	123.0	125.85	122.92	122.81	122.61	123.43
V. serratifolia	123.0	123.25	120.87	122.49	123.25	122.57
V. galilaea	123.75	123.25	123.35	123.41	122.47	123.24
V. johannis	122.7	123.25	122.35	122.84	122.34	122.7
V. kalakhensis	123.8	123.25	123.35	123.08	123.73	123.44
V. hyaeniscyamus	122.7	123.25	122.35	122.73	122.20	122.65

Table 3 Total numbers of restriction fragments (upper right half) and fragment similarity in percentage (lower left half) between all pairs of seven species related to *V. faba* in RFLP analysis using 14 restriction endonucleases (For abbreviations, see Table 1).

	F	N	S	G	J	K	H
F	*300*[a]	621	614	621	623	624	624
N	38.32	*321*	635	642	644	645	645
S	38.11	73.70	*314*	635	637	638	638
G	38.97	72.59	68.03	*321*	644	645	645
J	37.24	69.25	70.01	74.84	*323*	647	647
K	38.46	69.46	71.47	68.22	76.66	*324*	648
H	37.18	69.46	69.90	75.66	99.23	76.54	*324*

[a] Restriction fragment numbers in each species produced by the 14 restriction endonucleases are given by italics

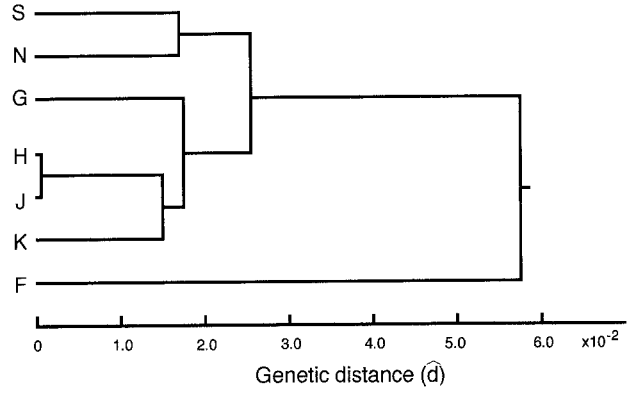

Fig. 3 Dendrogram showing the genetic relationships among nine species related to *V. faba* based on the RFLP data of cpDNA, and constructed by the UPGMA method from the Nei's genetic distance

in the agarose gels and is within the acceptable limits of error for the mobility measurements. In our results it can be assumed that on the basis of an overall relationship in mobility of the similar bands in cpDNAs between *V. johannis* and *V. hyaeniscyamus* a change in band mobility, observed in other species, actually reflects a difference in the size of a chloroplast fragment cleaved from the same region of the genome. Barring *V. hyaeniscyamus* and *V. johannis*, where the percentage of common fragments was 99.23%, the percentage in other species ranged from 68.03 between *V. galilaea* and *V. serratifolia* to 76.66% between *V. johannis* and *V. kalakhensis* (Table 3), with an overall average between six species to the extent of 73.67%.

Between V. faba and the Narbonensis complex

The most striking feature of our investigation was the considerable variation in fragment patterns between *V. faba* and its close allies within the complex that was revealed (Fig. 1 and Table 3). The percentage of common fragments was estimated to range from just 37.18% between *V. faba* and *V. hyaeniscyamus* to 38.32% between *V. faba* and *V. narbonensis* with an overall average of 38.05%.

Phylogenetic analysis

In the phenogram (Fig. 3), *V. johannis* (J) and *V. hyaeniscyamus* (H), with almost the same chloroplast genome, are closer to *V. kalakhensis* (K) than to the three other species within the complex. The cpDNA of *V. galilaea* clusters into the J-H-K group, and the cpDNAs of *V. serratifolia* and *V. narbonensis* consist of one cluster separated from the above group. The cpDNA of *V. faba* is clearly isolated from the Narbonensis complex and forms a distinct lineage (Fig. 3 and Table 4).

Discussion

Although there has been considerable disagreement regarding the number of species within the Narbonensis complex, all taxonomists recognize *V. narbonensis*, *V. kalakhensis* and *V. eristalioides* as distinct species. *V. serratifolia*, for example, has been included in *V. narbonensis* by Plitmann (1967) and Ball (1968) while it has been assigned the rank of distinct species by Schäfer (1973). Similarly, Plitmann (1967) doubted the distinctness of *V. hyaeniscyamus*, *V. johannis* and *V. galilaea*, while Schäfer (1973) and Maxted et al. (1991) are of the view that all three species should be given species status. On the basis of cytogenetic, biosystematic and chemotaxonomic evidence (Raina and Rees 1983; Raina and Bisht

Table 4 Matrix of the Nei's genetic distances ($d \times 10^2$) between all pairs of the 14 species related to *V. faba* (For abbreviations, see Table 1)

	F	N	S	G	J	K
N	5.71					
S	5.75	1.74				
G	5.60	1.82	2.21			
J	5.89	2.10	2.04	1.65		
K	5.69	2.08	1.92	2.19	1.51	
H	5.90	2.08	2.05	1.59	0.04	1.52

1988; Maxted et al. 1991; Schäfer 1973; Ladizinsky 1975), there is no doubt, however, that the nuclear genomes of the species within the complex are genetically very close to each other. The high frequency of bivalents (40–80%) and one to two quadrivalents in the F_1s between *V. narbonensis*, *V. serratifolia*, *V. johannis* and *V. hyaeniscyamus* is indicative of the fact that there is considerable chromosomal homology between the taxa and that differentiation between species might chiefly be a result of segmental interchange (Hanelt and Mettin 1989).

To our knowledge our investigation is the first to examine cpDNA RFLPs in *V. faba* and its wild relatives. The data on cpDNAs of the Narbonensis complex do not discriminate between the *V. hyaeniscyamus* and *V. johannis*: between these two there are differences in just 3 out of about 300 common fragments. On the other hand, cpDNAs from the remaining species as well as from *V. hyaeniscyamus*/*V. johannis* can be distinguished by the considerable differences in the mobility of several individual restriction fragments. To cite an example, several papers have described a very close, often indistinguishable (*V. narbonensis* var 'serratifolia', *V. narbonensis*), relationship between *V. serratifolia* and *V. narbonensis*, and yet these species share only 230 out of an average of 317 fragments scored for 14 enzymes (Fig. 2 and Table 3). Therefore, unlike the relatively very low frequency of site changes (2.1–18%, see Sytsma and Gottlieb 1986) and length mutations between species that occurs in other plant genera (but surely not close enough to include them in a complex as in the present case, see Palmer 1986; Palmer et al. 1988), considerable heterogeneity exists among cpDNAs of the Narbonensis complex species. Chloroplast DNAs from the taxa representing most of the diversity in *Lycopersicon* and the three *Solanum* species, for example, showed hardly 39 site mutations among 484 sites surveyed (Palmer and Zamir 1982), and so on. The extent of variation in the species complex approximates the estimates for ten genera within subtribe Brassicinae of family Brassicaceae (Pradhan et al. 1992).

Similarly, several hundred papers have described considerable homology and/or non-existent non-homology in cpDNA profiles between the cultivated species and their wild progenitors, including between amphidiploids and putative diploid species (see Palmer 1985, 1986; Palmer et al. 1988). In other words, if wild and cultivated species exclusively share a chloroplast genome type by showing almost similar cleavage patterns, the latter probably originated from the former (see Ogihara and Tsunewaki 1988). A comparison of *V. faba* cpDNA, however, with those from the other six wild species in the Narbonensis complex reveals startling differences in RFLP patterns. There is a case, therefore, for claiming that none of the wild species in the complex can be considered the immediate wild progenitor of *V. faba*.

V. faba is a popular species for other types of study also, but few have included the complex taxa for facilitating comparison. Wherever this has been carried out, however, *V. faba* has been shown, although not as clearly as from the cpDNA study detailed above, to be genetically distinct in its nuclear genome from its presumed putative allies (Raina 1990; Raina and Rees 1983; Schäfer 1973; Ladizinsky 1975; Perrino et al. 1989). Does this mean that the wild progenitor or a bridging species could either be extinct, not recognized for what it is, or is yet to be collected? In the light of extensive and systematic collections made in recent years throughout the Near East region, the latter option according to Maxted et al. (1991) seems highly unlikely. The present data on cpDNA in fact goes still further. The percentage of common cleavage sites analysed between the species in the Narbonensis complex and 20 other species in *Vicia* (unpublished data) ranges from about 70% to 100%, and between these and *V. faba* the percentage does not exceed beyond what has been already detailed above. This raises the question of whether the observed explicit differences between chloroplast genomes of *V. faba* and other *Vicia* species may warrant generic rank for *V. faba*? The summarized taxonomic history of *V. faba* as well as section Faba is extensive and contentious (see Maxted et al. 1991; Raina et al. 1989). Section Faba has indeed been considered to be the genus Faba but has been recently classified as a section of *Vicia*. Several others, on the other hand, are of the view that *V. faba* could be either considered as a separate genus (*Faba bona* Medikus/*Faba vulgaris* Moerich) or given subgeneric rank placing faba bean as the only species of the subgenus Faba of genus *Vicia*. The present data indicates that because of the unique characteristics of its chloroplast genome vis-a-vis other *Vicia* species, *V. faba* should be placed in a separate genus.

V. faba is a member of a small group of legumes that has lost one of its inverted repeated sequences (Koller and Delius 1980; Palmer and Thompson 1982; Ko et al. 1987). This event has reduced the genome size of *V. faba* to 123 kbp. The size estimated in this study for the species within the Narbonensis complex is comparable to the size of the *V. faba* genome. It appears, therefore, that these species have also lost the repeat structure. It has been established beyond doubt that, in strong contrast to chloroplast genomes which have retained the inverted repeat structure (characteristic of most chloroplast genomes) and are highly conserved, there is an extremely scrambled arrangement of sequences in genomes that have lost one entire copy of duplication element (Palmer and Thompson 1982; Ko et al. 1987). Hence the latter represents one of the most interesting results of chloroplast genomic evolution. Since comparative RFLP analysis of cpDNA between closely related species in *Pisum* revealed a high incidence of length mutations rather than base substitutions (Palmer et al. 1985), the molecular nature of a large number of mutations resulting in a high incidence of fragment changes among Narbonensis complex species could be attributed either to the illegitimate recombination through oligonucleotides or slippage of DNA poly-

merase during replication (Ogihara et al. 1992). Notwithstanding the real cause of such an event, this investigation revealed extreme forms of changes in the chloroplast genomes of very closely related species.

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