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Genetic diversity and relationships in some *Vicia* species as determined by SDS-PAGE of seed proteins

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Abstract

To evaluate the genetic diversity of some *Vicia* species, seed proteins of 160 accessions (30 of *Vicia faba*, 15 of *V. narbonensis*, 82 of *V. sativa* and 25 of *V. ervilia* and 8 accessions of other *Vicia* species) were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The dendrogram showed that the two outcrossing species *V. faba* and *V. villosa* were the most distant among all species (average percent disagreement value PDV 0.47 and 0.45, respectively). The tree was divided into small clusters of two species each. *V. narbonensis* fell in one cluster with *V. michausai* (at PDV = 0.35) and *V. lutea* (var. *hirta*) fell in one cluster with *V. serococorpes* (at PDV = 0.32) whereas, *V. ervilia* fell in one cluster with *V. sativa* (at PDV = 0.27). The *V. sativa* subspecies, however, were closely related (PDV < 0.1). In general, this study did not prove any relationship between the studied storage proteins and the geographical distribution or ecological needs of the studied accessions.

Additional key words: electrophoresis, storage proteins, UPGMA dendrogram.

Introduction

Characterization of the plant genetic resources is a key factor in crop improvement programmes. The current characterization of plant germplasm collections relies strongly on morphological descriptors which are regarded as reliable, easy to study and relatively of low cost to evaluate. However, the use of morphological descriptors has some limitations such as their limited polymorphism and their tendency to be affected by the environment. These problems have been overcome to some extent by the use of electrophoresis in the characterization of genotypes on the basis of genotype-specific protein markers. For that, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of seed storage proteins proved to be both simple and effective for distinguishing among cultivars of the largely cross-fertilised pasture grasses and legumes despite their high innate genetic variability (Gardiner and Forde 1992).

The seed proteins are distinguished from other proteins by some characteristics: *1*) they have high accumulation rates in the seed during mid-maturation stage of development; *2*) they are used up during germination and are synthesized only in the seed and not in other tissues; *3*) they are deposited mostly in special storage organelles called protein bodies and; *4*) they lack any other functional activity (MirAli 1987). Unlike the cereals in which prolamins and glutelins are the major storage proteins, albumins and globulins comprise the major storage proteins of legumes.

The legumes family (*Fabaceae*) contains more than 650 genera and 18 000 species and is considered as the third largest family of higher plants and is second to grasses in agricultural importance (Young *et al*. 2003). Some genera like *Vicia* have genome sizes that vary by a factor of 10 or more. This is associated with extensive differences in the abundance of retroelements which account for substantial proportions of these genomes (Pearce *et al.* 1996).

Seed protein electrophoresis has become a useful tool in evolutionary studies to determine species relationships. In their study on the seed proteins electrophoretic patterns of 12 taxa that belong to 9 species of *Vicia* endemic to East Asia. Potokina *et al*. (2003) found the resultant phylogenetic implications to be consistent with those from morphological or DNA data and suggested that the

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Abbreviations: PVD - percent disagreement value; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis; UPGMA - unweighted pair group method with arithmetic averages.

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electrophoretic patterns of seed proteins is useful to clarify inter- and intra- specific phylogenetic relationships among the East Asian endemic taxa of *Vicia*. In an earlier work, Ladizinsky and Wains (1982) studied the seed protein polymophism of 42 accessions of *Vicia sativa* representing five different karyotypes with 2n=10, 12 and 14. They found that all accessions, except those with 2n=14 have highly variable protein profiles and the variation within and between karyotypes was of the same magnitude and no protein bands could be correlated to a particular karyotype. Haider and El-Shanshoury (2000) studied the relationships among 20 samples belonging to 6 subspecies of *Vicia sativa* based on the variability of storage proteins and esterase isozyme electrophoretic patterns. Their results showed that some accessions had identical electrophoretic profiles within the subspecies (*e.g. macrocarpa* and *cordata*), similar (*e.g. amphicarpa*) or very variable profiles (*e.g. nigra* and *sativa*).

The objective of this study was to analyse the genetic diversity among some *Vicia* species endemic to Syria and to determine the possibility of accession identification and/or the relationship between the observed genetic variation and seed protein electrophoretic patterns using SDS-PAGE.

Materials and methods

Plants: The study covered a total of 160 accessions that belong to different *Vicia* species (82 of *V. sativa*, 25 of *V. ervilia*, 15 of *V. narbonensis*, 30 of *V. faba*, 4 of *V. villosa* in addition to 4 accessions belong to other *Vicia* species). The studied accessions were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

Sample preparation and electrophoresis: Total seed storage proteins were extracted from bulk crushed seeds. Each sample was suspended in a medium containing 2 % (m/v) SDS, 5 % (m/v) 2-mercaptoethanol, 0.001 % (m/v) pyronin, 10 % (v/v) glycerol and 1 M Tris-HCl (pH 6.8). The samples were left for 3 h at room temperature and shaken every 15 min. Then, they were placed in a boiling water bath for 2 min and allowed to cool and were put in an Eppendorf microcentrifuge for 15 min at 21 000 *g*. 0.015 cm^3 from each sample were placed into each slot of a vertical slab gel electrophoresis unit (160 × 180 × 0.75 mm, *Hoefer SE-600*, Groton, USA) with a 15-well comb.

Sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 17.5 % slab gels in a discontiuous buffer system according to Laemmli (1970) as described by MirAli (2000) except for the molarity of Tris-HCl (pH 8.8) in the main gel which was 3.5 M. A constant electric current of 25 mA was used to run two gels for 20 h.

Data analysis: Relative molecular mass (M_r) of polypeptide bands was estimated by SDS-PAGE using a low molecular mass marker kit from *Amersham Pharmacia Biotech* (Uppsala, Sweden) that contains the following standard proteins: phophorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

SDS-PAGE assays were performed in duplicate and only those bands obtained clearly twice were scored either 1 as present or 0 as absent. The unweighted pair group method with arithmetic averages (UPGMA) and percent disagreement values of the *Statistica* program were used to construct the matrix and the phylogenetic tree (*Statsoft, Inc*. Tulsa, USA).

Results

We have used SDS-PAGE to detect intraspecific and interspecific genetic variation in some species of the subgenus *Vicia* that were collected from various regions of Syria. Since both seed storage protein classes legumin and vicilin partly overlap under the SDS-PAGE reducing conditions, we did not attempt to assign the recorded polypeptides to either class. Rather, we divided the gels into four regions according to the mobilities of its proteins. Region I contains proteins with molecular mass of more than 70 kDa, region II contains proteins between 45 and 70 kDa, region III contains proteins between 30 and 45 kDa and region IV contains proteins with less than 30 kDa.

The highest number of accessions tested (82) belonged to *V. sativa* aggregate represented here by three subspecies namely ssp. *amphicarpa*, *nigra* and *sativa*. Region I was the highest in polymorphism (10 biotypes) followed by region II (9 biotypes) and region III (4 biotypes) and lastly region IV which contains two biotypes one of which contains only one accession (3478) (Fig. 1).

In *V. ervilia* the number of bands varied between 21 (accession 2975) and 25 (accession 4288). Four biotypes were detected in regions I, II and III with no variability being observed in region IV (Fig. 2). Region I was distinguished by high frequencies for biotypes 1 and 4 (11 and 9 accessions, respectively). The other two biotypes, however, had much lower frequencies: biotype 3 appeared in four accessions and biotype 2 appeared in one accession only (accession 3477). The same trend was realyzed in regions II and III where a single biotype repeated in 22 accessions and the three other biotypes

appeared once in each of the accessions 5160, 3477, and 3400 in region II and 3904, 5160 and 4288 in region III, allowing to distinguish these accessions by these unique bands. Region IV, on the other hand, did not show any polymorphism.

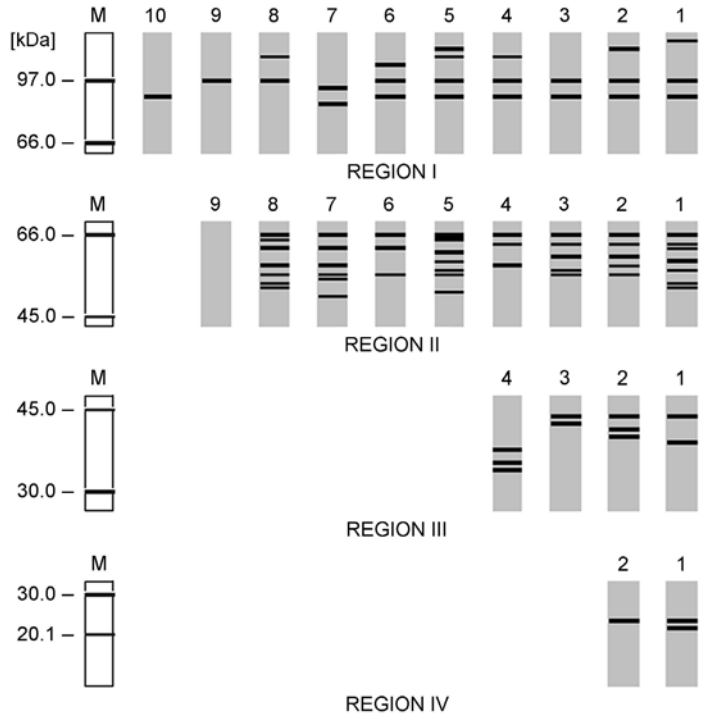

Fig. 1. The biotypes observed in the four electrophoretic regions in *Vicia sativa* subspecies *sativa.*

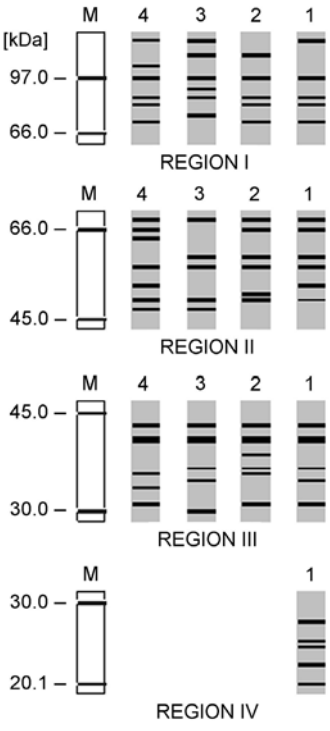

Fig. 2. The biotypes observed in the four electrophoretic regions in *Vicia ervilia.*

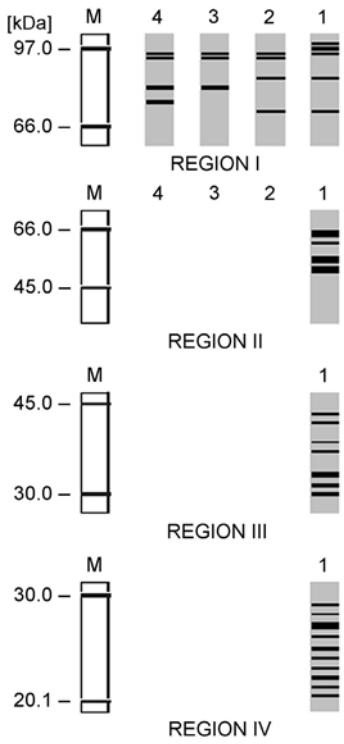

Fig. 3. The biotypes observed in the four electrophoretic regions in *Vicia narbonensis.*

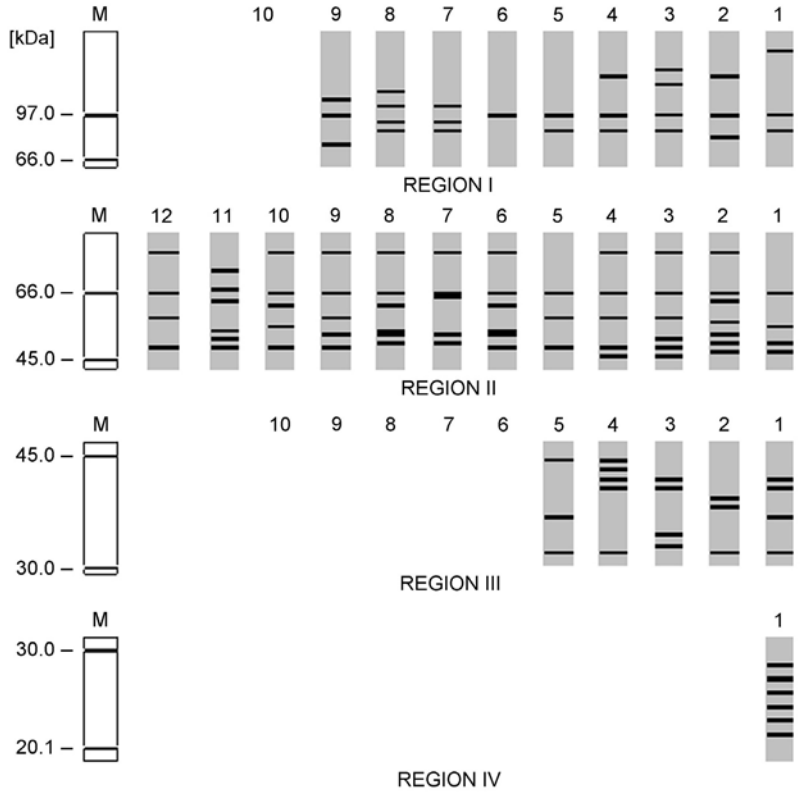

Fig. 4. The biotypes observed in the four electrophoretic regions in *Vicia faba.*

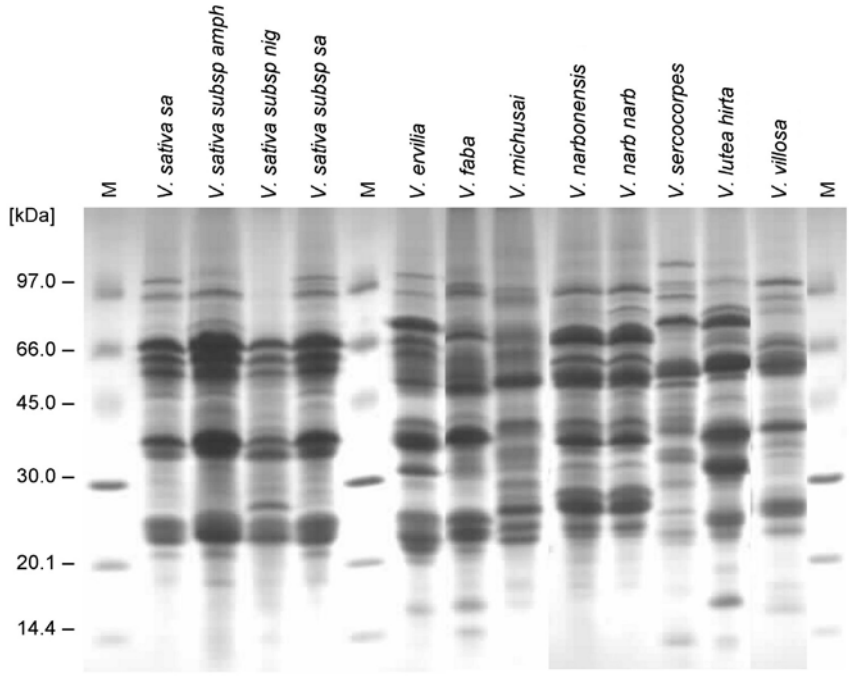

Fig. 5. Electrophoregram of bulk representative samples of studied taxa of subgenus *Vicia*.

The range of band number in *V. narbonensis* as very little, varying between 27 (accession 2383) and 29 (accession 2387). In general, this species showed no polymorphism in regions II, III and IV and very little polymorphism in region I where four biotypes were detected (Fig. 3). Two biotypes (1 and 4) appeared once

(2392 and 2387 respectively), biotype 3 was repeated in 5 accessions, and biotype 2 was the most frequent one with appearance in 8 accessions.

In *V. faba* the number of bands ranged from 17 (accession 3434) to 24 (accession 1070). Unlike all previous species, region II was the most polymorphic region with 12 biotypes, followed by region I with 9 biotypes, region III with 5 biotypes and lastly region IV which did not show any polymorphism (one biotype) (Fig. 4). Four accessions could be distinguished based on unique bands in region I, namely accessions 3581, 3616, 650103 and 65044. From the banding patterns of region II, it was possible to identify six accessions, namely accessions 65044, 3627, 3618, 3614, 3554 and 3639. Whereas, only one accession could be distinguished based on its unique banding pattern in region III (accession 3571).

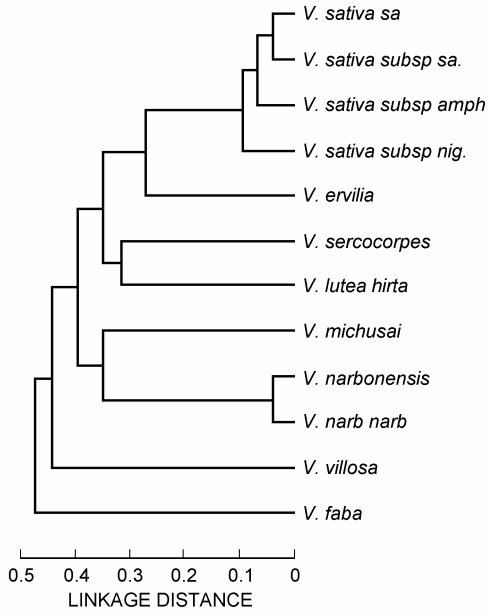

Fig. 6. The dendrogram based on the presence or absence of seed protein bands in bulked seed samples representing the studied taxa of *Vicia*.

Mature seeds representing most or all studied accessions of each species/ subspecies were crushed together and its proteins were extracted and electrophoresed as mentioned earlier. From Fig. 5, it can be stated that the SDS-PAGE method used herein could distinguish all studied taxa from its electrophoregrams. However, there were considerable differences between the PDV for inter versus intra- specific relationships. When the data was put to construct the dendrogram (Fig. 6), it was shown that the two outcrossing species *V. faba* and *V. villosa* were most distant among all species (average percent disagreement value PDV 0.47 and 0.45, respectively). The tree was then divided into small clusters of two species each. *V. narbonensis* fell in one cluster with *V. michausai* (at PDV = 0.35) and *V. lutea* var. *hirta* fell in one cluster with *V. serococorpes* (at PDV = 0.32) whereas, *V. ervilia* fell in one cluster with *V. sativa* (at PDV = 0.27). The *V. sativa* subspecies, however, were closely related (PDV < 0.1).

Discussion

The main objective of this study was to measure intra and interspecific genetic variation and relationships in subgenus *Vicia* by using electrophoretic variation of seed proteins. Our data show that the studied *Vicia* taxa exhibited seed proteins with molecular mass ranging from 14 kDa to over 100 kDa. Based on M_r, we found that there are four main regions in the electrophoregram which appeared to support the existance of four different encoding regions determining the synthesis seed proteins in *Vicia*. Vargas *et al*. (2001) found four banding regions in phaseolins with some banding patterns being more abundant than others. These authors attributed the differences between these regions to polymorphism in the coding regions involved in their synthesis. These polymorphisms may result from either differences in the DNA sequences that code for the peptides or from differences in the genes involved in post-transcriptional changes.

The four regions had different polymorphism levels (as defined by the number of patterns divided by the number of accessions in each region). In general, region I with the highest M_r subunits (more than 70 kDa) was the most polymorphic region, whereas, region IV with the M_r subunits (with less than 30 kDa) was the least polymorphic one. Regions II and III with intermediate M_r (between 30 and 70 kDa) had, on average, similar and intermediate polymorphism level. Although both outcrossing and self-crossing species studied had the same trend regarding these regions, the polymorphism levels obtained were much higher in the former as compared with the latter species. The different regions may have variable degrees of polymorphism in different species. Thus, in contrast to our results on *Vicia*, Bertozo and Valls (2001) working on *Arachis* detected polymorphism only in the basic arachin group (the lowest M_r 14 - 24 kDa). Polignano *et al.* (1990) studied seed storage protein diversity in *Vicia faba* entries from Ethiopia and Afghanistan. Although, their results did not allow them to determine the origin of the species, ample polymorphisms (16 different patterns) were found in the middle region of the gels (Rf 0.43 - 0.56). Our results were at conformity with those of Polignano *et al*. (1990) regarding the region with most polymorphisms in *V. faba*. *V. faba* was the sole species studied which had highest polymorphism in region II, all other species showed that region I was the highest and region IV the lowest in polymorphism.

Electrophoresis of seed proteins of a bulked seed sample has proved to be a more effective method for

distinguishing cultivars of cross-polinating crops (Gardiner and Forde 1992). A bulk sample is a composite seed sample that represents the mixture of electrophoretic phenotypes present in a certain cultivar, population or species (Rogal and Javornik 1996).

Our results showed that we were able to distinguish the different species studied and to construct their phylogeny tree based on the ratio of shared protein subunits to all subunits. The dendrogram showed the two outcrossing species *V. faba* and *V. villosa* as the most distant among all species (PDV 0.47 and 0.45, respectively).

Based on the seed protein electrophoregram, *V. faba* was not as closely related to its proposed wild relative *V. narbonensis* as previously assumed from morphological similarity. Similar finding was reported by Jaaska (1997) who placed cultivated *V. faba* and its morphologically closest wild relatives of the section *narbonensis* in different monophyletic clades indicating more remote relationship between them. Further support for this comes from the molecular data of both genomic DNA (RAPDs) and chloroplast DNA (PCR-RFLP) obtained by Potokina *et al*. (1999) which indicate that *narbonensis* should be considered a well separated section which may be related to section *Vicia*.

Potokina *et al*. (2000) found considerable genetic divergence between and within taxa of *V. sativa* aggregate which were not always reflected at the phenotypic level. The same group, however, pointed out that seed protein patterns are an effective tool for identifying accessions that can not be identified clearly by morphological criteria alone. In his argument concerning morphological and cytogenetic variations between *Lens* species which were not reflected in the electrophoretic patterns, Ladizinsky and Hymowitz (1979) claimed that these variations are governed by a small number of genes that have not altered the seed protein profiles. Since the data on the agronomical and/or morphological characteristics of the accessions studied are lacking, it is not possible to relate any particular seed protein pattern to any of these characters. Nevertheless, in conformity with the results reported by Ahmad *et al*. (1997) on *Lens,* we were able to make use of SDS-PAGE as a tool for determining genetic relationships within *Vicia*.

Potokina *et al*. (2000) used both seed protein and RAPDs to study intraspecific variation between taxa in *V. sativa* aggregate. Their results support the view that *V. sativa* aggregate consisted of 8 taxa warranting recognition at the species level. These authors suggested that *V. sativa* aggregate comprises a complex of well separated taxa and derivated forms representing various degrees of phylogenetic divergence. Our results showed that the four studied subspecies of *V. sativa* are much more closely related to each other (not more than PDV = 0.15) than to either any species of *Vicia* (the most closely related species *V. sativa* and *V. ervilia* had a PDV of 0.33). It has been suggested that seed protein profiles reflect genetic affinities within a taxon and even between different biological entities based on the assumption that closely related species show more similar electrophoretic protein patterns than those that are phylogenetically less related (Crawford 1990). The results of the current study showed that, among all species studied, mean PDV values were higher in cross-fertilized species (*V. faba* and *V. villosa*) compared with those of self-fertilized species. This is in line with the generally accepted concept that in populations that have high levels of selfing, heterozygosity level is much lower than that of cross-fertilized species (Maquet *et al*. 1996).

In conclusion, the analysis of distribution of variation between *Vicia* species studied showed that: *1*) there were major differences in the frequencies of the electrophoretic profiles in the different species; *2*) the technique employed is species specific; *3*) the analysis showed that the abundance of polymorphisms in cross-fertilised species is much higher than those of self-fertilised ones.

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