Molecular characterization of some species of the genus *Vicia*

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ABSTRACT

The present study was carried out to determine the genetic diversity between faba bean and its related Vicia species. Thirteen taxa representing six Vicia species (V. sativa, V. villosa, V. monantha, V. narbonensis and V. cinerea, in addition to V. faba) were collected from the north-west coastal region of Egypt. Electrophoretic protein patterns indicated clear differences among different Vicia species as well as within the taxa of the same species. Three isozyme systems (glutamate oxaloacetate transaminase, amylase and esterase) were examined in dry seeds and showed that V. faba and V. narbonensis have specific isozyme patterns different from those of other species. Variability on the DNA level was investigated through RAPD-PCR analysis, which indicated clear differences among different Vicia species and considerable similarities within the taxa of the same species. The results indicated that V. monantha and V. villosa are most closely related to V. cinerea. It was also found that V. faba and V. narbonensis are completely distant. The marked differences between Vicia faba and other wild species indicated that none of the latter can be considered as the wild progenitor of V. faba.

***Key words:** Faba bean, genetic diversity, storage proteins, SDS-PAGE, isozymes, RAPD markers.*

INTRODUCTION

The genus *Vicia* includes a number of species ranging from 180 to 210, distributed throughout the temperate zones of the world (Hanelt and Mettin, 1989). In Egypt, broad bean (*Vicia faba* L.) is one of the most important food legumes cultivated as a human and animal food. The level of morphological variation implied the division of this genus into several sections, each containing a more homogenous group of taxa. Besides protein polymorphism, additional information can be obtained from the analysis of isozyme patterns, which have been widely used for evaluation and characterization of genetic resources in different plant species (Andersen and Fairbanks, 1990). In *Vicia faba* L., isozyme polymorphism has been used for taxonomic studies (Yamamoto *et al.*, 1983), varietal identification (Gates and Boulter, 1980) and for other aspects of breeding activities such as genetic control of some enzymatic systems (Suso and Moreno, 1982). Work on isozyme polymorphism in *Vicia* species by Yamamoto and Plitmann (1980) has identified four variable isozymes; glutamate oxaloacetate transaminase (GOT),

phosphoglucose isomerase (PGI), amylase and esterase.

DNA-based genetic markers have been recently integrated into several plant systems and are expected to play a very important role in the future of molecular genetics and plant breeding. Over the last decade, the revolution of polymerase chain reaction (PCR) technology has been initiated as a novel genetic assay based on selective DNA amplification (Saiki *et al.*, 1983). This new assay is more amenable to automation than conventional techniques, simple to perform and preferable in experiments where genotypes of a large number of individuals are determined at a few genetic loci. Recently, a new genetic assay was developed, independently, by two different laboratories. This assay is called randomly amplified polymorphic DNA (RAPD) by Williams *et al.* (1990), while called arbitrarily primed-PCR (AP-PCR) by Welsh and McClelland (1990). This procedure is rapid, requires only small amounts of DNA and provides a quick method for generating genetic maps targeting genetic markers, pooling strategies and analyzing populations. Therefore, the RAPD technology has quickly gained a widespread acceptance and applications. Genetic diversity determined with molecular markers has been explored in the genus *Vicia* mainly at the inter-specific level (Van de Ven *et al.*, 1993).

This work describes the inter- and intra-specific relationships within the genus *Vicia*, based upon storage proteins of dry seeds, three isozyme systems (GOT, amylase and esterase) and RAPD-PCR markers. The study involved one local cultivar of *Vicia faba* L. (Giza 5), taxa of four wild *Vicia* species (*V. sativa, V. monantha, V. villosa* and *V. cinerea*) and one exotic species namely *V. narbonensis*.

MATERIALS AND METHODS

Plant material

Seeds of *Vicia faba* L. local cultivar Giza 5 were obtained from the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt. Taxa representing different wild *Vicia* species (*V. sativa, V. monantha, V. villosa* and *V. cinerea*) were collected from different locations at the north west coastal region of Egypt. Seeds of *V. narbonensis* were kindly provided by Prof. Muntz, Institute Für Pflanzengenetik und Kluturpflanzenforschung (IPK), Gatersleben, Germany.

Protein electrophoresis

Seeds representing different *Vicia* species were ground to a fine powder and total proteins were extracted for 1 h at 4°C in 0.2 M Tris/HCl, pH 8.0, 10 % sucrose (w/v), 2 % SDS (w/v), 0.1% β-mercaptoethanol (v/v) added in a ratio of 0.2 g powder : 1 ml buffer. After centrifugation at 10,000 rpm for 10 min, protein extract was mixed with equal volume of loading buffer (0.125 M Tris/HCl, pH 6.8, 2% SDS (w/v), 10% glycerol, 1% β-mercaptoethanol (v/v) and 1% bromophenol blue (w/v) and denatured by heating on a boiling water bath for 3-5 min. Protein samples were then loaded onto 12% polyacrylamide slab gels (0.15 x 11 x 16 cm). Electrophoresis was carried out initially at 20 mA for 1 h and then subsequently at 30 mA until the tracking dye reached the bottom of the gel and gels were stained as described by Laemmli (1970).

Detection of isozymes

Extraction of the three isozymes (GOT, amylase and esterase) was carried out by mixing 5 g seed powder with 10 ml extraction

buffer (1.21% Tris, pH 7.0, 0.1% boric acid, 0.1 M EDTA, 10% sucrose, 5% polyvinylpyrrolidone (PVP). The extract was centrifuged at 10,000 rpm for 5 min at 4°C, then the supernatant was mixed with an equal volume of loading buffer (1.21% Tris, pH 7.0, 0.1% boric acid, 0.1 M EDTA, 10% sucrose, 5% PVP, 0.01% β-mercaptoethanol and 1% bromophenol blue). Protein samples were loaded onto native polyacrylamide (8%) slab gels (0.15 x 11 x 16 cm) without SDS neither in running buffer nor in the matrix, and electrophoresis was carried out as described above. The detection of different isozymes was carried out according to Gabriel (1971).

Extraction of DNA

Total genomic DNA was isolated from 1 g of *Vicia* leaves using cetyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris/HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, 1% PVP) as described by Doyle and Doyle (1990). DNA was precipitated in equal volume of isopropanol and pellets were resuspended in 200 µl TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA). The concentration of DNA was determined spectrophotometrically and adjusted to 50 ng /µl.

PCR amplification

PCR reactions were conducted according to Williams *et al.* (1990) to detect RAPD markers using seventeen arbitrary 10-mer primers (Table 1) synthesized at AGERI

The reaction conditions were optimized using some combinations of reaction constituents under two annealing temperatures (35°C and 37°C) to fit for the RAPD analysis of *Vicia*. The PCR reactions were performed in 50 µl total volumes of 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 2.5 mM MgCl2, and 200 µM of each of dATP, dGTP, dCTP and DTTP (Promega), containing 100 pmol primer, 1.25 U *Taq* DNA polymerase (Promega) and 50 ng template DNA overlaid with a drop of mineral oil. Amplification was carried out in a DNA thermocycler (Perkin Elmer Cetus 480) programmed for 35 cycles with the following temperature profile: 1 min at 94°C, 1 min at 35°C and 2 min at 75°C followed by a final extension of 72°C for 7 min. Controls lacking template DNA were included for each primer reaction mixture. Amplification products were visualized along with a DNA marker (λ phage DNA digested with BstEII) on 1.2% agarose gel with 1X TAE buffer (10 mM Tris/acetic, pH 7.4 and 1 mM EDTA) and detected by staining with ethidium bromide (10 µg/µl) for 30 min. Gels were then destained in deionized water for 10 min and photographed on Polaroid films under UV light.

Table (1): Names and Sequences of the seventeen 10-mer primers used in this study.

No.	Primer name	Sequence (5`-3`)	No.	Primer name	Sequence (5`-3`)
(a)	F01	ACGGATCCTG	(j)	F11	TTGGTACCCC
(b)	F03	CCTGATCACC	(k)	F12	ACGGTACCAG
(c)	F04	GGTGATCAGG	(l)	F13	GGCTGCAGAA
(d)	F05	CCGAATTCCC	(m)	F14	TGCTGCAGGT
(e)	F06	GGGAATTCGG	(n)	F16	GGAGTACTGG
(f)	F07	CCGATATCCC	(o)	F17	AACCCGGGAA
(g)	F08	GGGATATCGG	(p)	F19	CCTCTAGACC
(h)	F09	CCAAGCTTCC	(q)	F20	GGTCTAGAGG
(i)	F10	GGAAGCTTGG			

Data scoring and cluster analysis

Data were scored for computer analysis on the basis of presence or absence of protein bands, isozyme bands or amplified products for each primer. To ease computer analysis, a product (or a marker band) was designated "1" when present in any given genotype, while designated "0" when absent, after excluding non-reproducible bands. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic bands, were used to generate similarity coefficients. The similarity coefficients were then used to construct a dendrogram by UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) using NTSYS-pc (Rohlf, 1993).

RESULTS AND DISCUSSION

SDS-PAGE of storage proteins

Storage proteins contained in dry seeds of 13 samples representing six *Vicia* species were analyzed using SDS-PAGE. Electrophoretic protein patterns of the different taxa are shown in Fig. (1). The protein profile showed a maximum of 52 protein bands distributed over a wide range of molecular weights. In all tested genotypes, protein patterns revealed a polymorphism among all polypeptides. Electrophoreic patterns also indicated a considerable variation between different *Vicia* species and also among different taxa of the same species. Naidu and Chabra (1985) and Perrino and Pignone (1981) observed some differences between the faba bean forms representing different botanical varieties and/or geographical origins. Similar results were found by Salih and El-Hardallou (1986) when they analyzed 12 cultivars of faba bean grown at five sites in the northern region of Sudan. They found that the variation among taxa of the same species is less than that between different species.

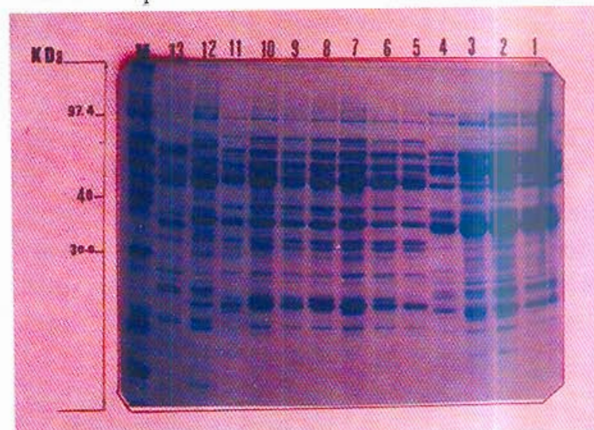

Fig. (1): SDS-PAGE protein profile from dry seeds of different Vicia species; lanes 1-4: V. sativa, 5-7: V. villosa, 8-10: V. monantha, 11: V. cinerea, 12: V. faba, 13: V. narbonensis, and M: marker proteins.

The data also showed that *V. monantha* and *V. villosa* have similar protein patterns, which are slightly similar to *V. cinerea*. *V. faba* showed a distinct protein pattern, different from the wild species. This could be correlated with the sensitivity to stress conditions in natural habitat. The marked difference in seed protein profile between *V. faba* and other wild species may indicate that none of the latter can be considered as the wild progenitor of *V. faba* as suggested by Salmanowicz and Przybylska (1997). They reported that the differences in seed albumin composition of *V. faba* and wild *V. kalakhensis* are consistent with other taxonomical data indicating that *V. faba* is genetically distant from its wild relatives, although Kupicha (1976) and Maxted (1991) reported that *Vicia narbonensis* is considered to be the morphologically nearest wild relative to the faba bean.

Isozyme analysis

The occurrence of GOT isozyme banding pattern is shown in Fig. (2a) in which a total of eight bands were detected. These bands are designated *Got-1* through *Got-8*, in which *Got-8* is the fast moving band, while *Got-1* is a the slow moving band. The *Got-5* isozyme band was detected in all samples, except in *V. faba* where it was replaced by *Got-6*. Taxa representing *V. sativa* appeared to be closely similar to each other as well as those of *V. monantha* and *V. villosa*. Each of *V. cinerea, V. narbonensis* and *V. faba* gave specific patterns different from those of other species. These data support the suggestion of Gates and Boulter (1979), who reported that GOT isozyme patterns are genotype-specific within faba bean cultivars.

Amylase banding pattern presented in Fig. (2b) shows a total of 11 bands with no common bands observed in all studied taxa, except for *Amy-5* band which was detected in most *Vicia* species. A clear similarity was observed between taxa within the same species as in case of *V. sativa, V. monantha* and *V. villosa*. Each of *V. faba* and *V. narbonensis* has specific banding pattern different from other species.

Zymogram of esterase pattern of *Vicia* samples is shown in Fig. (2 c). A total of 18 polymorphic bands (*Est-1* through *Est-18*) were detected. The maximum band number (9) was detected in *V. monantha, V. villosa and* and *V. cinerea*. The minimum number of bands (3) was detected in *V. narbonensis*. There were five common bands in each of *V. monantha, V. villosa,* and *V. cinerea* designated as *Est-3, Est-7, Est-9, Est-12* and *Est-18*. *Vicia sativa* has four specific bands, while *V. faba* gave a specific pattern different from those of the other species.

Yamamoto and Plitmann (1980) proposed that some isozyme systems such as intraspecific level, whereas others (e.g.

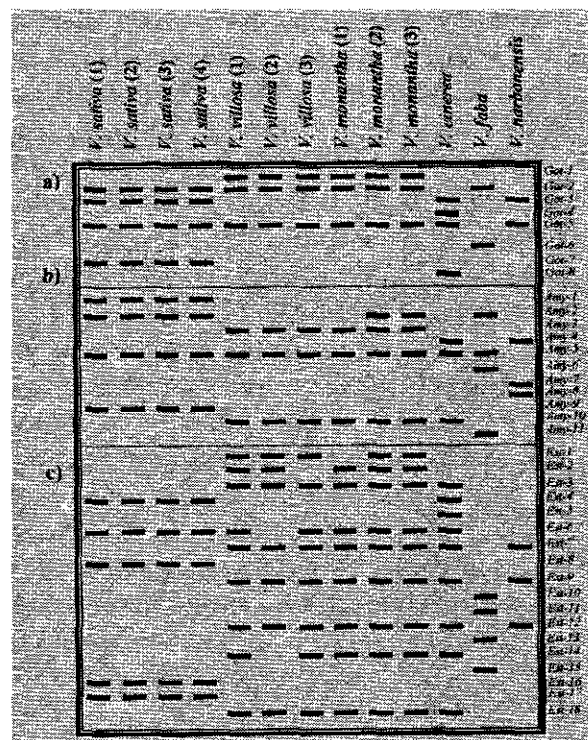

Fig. (2): Banding patterns of GOT (a), amylase (b) and esterase (c) isozymes from germinated seeds of different Vicia species.

esterase) might be helpful in defining intraspecific entities. In addition, Barratt (1980) and Gates and Boulter (1980) indicated that esterases were among the most suitable systems for cultivar identification. The data from this study indicated that only 8 bands of zymogram were scored for GOT isozyme, while esterase scored a total of 18 bands. It also indicated that GOT system was insensitive to the genetic variations existing among *Vicia* genotypes. Our results are consistent with those of Ehrman and Maxted (1989), who reported that *V. villosa* and *V. monantha* have a high degree of resemblance in their GOT, amylase and esterase isozyme patterns. This is also in accordance with karyotypic similarity reported by Yamamoto (1973), in which each of those two species has

2n =14. Results of this study also indicated that *V. faba* is genetically distinct from *V. narbonensis,* which is correlated with the difference in their chromosome number; *V. faba* (2n=12) and *V. narbonensis* 2n=14 (Yamamoto, 1973). However, *V. narbonensis* is considered by many authors (Schafer, 1973; Kupicha, 1976; Khattab, 1984) to be morphologically the closest wild relative of *V. faba*. It is noticed that *Got-5* and *Est-8* bands are significantly common in wild species, but absent in *V. faba* cultivar. Thus, these bands could be correlated to the stress environmental conditions in wild habitat. This principle has been adhered to transferring stress related genes from many diverse geographic and climatic zones into several crop plants (Amet, 1986).

RAPD-PCR and cluster analysis

Preliminary amplification reactions were conducted to optimize conditions for RAPD analysis of *Vicia*. One factor known to affect the stringency of PCR amplification is the magnesium concentration. The concentration of magnesium, as $MgCl_2$, was varied between 1.5 and 3 mM. A concentration of 2.5 mM $MgCl_2$ produced the most reliable results. A constant banding pattern was obtained with the concentration of template DNA ranging from 10 to 60 ng/50 µl reaction mixture, and the concentration of 50 ng/50 µl reaction mixture produced the most intensive product of PCR from *Vicia*. Primer concentration of 100 pmol/50 µl reaction mixture gave the most reproducible amplification. Both tested annealing temperatures of 35°C and 37°C produced the same banding pattern, however, few bands either fadded or disappeared when the annealing temperature was raised to 37°C.

The 13 samples representing six *Vicia* species were compared using 17 random 10-mer primers and generated a total of 150 polymorphic bands, after excluding non-reproducible bands (Fig. 3). Band profiles for individual primers ranged from 7 to 12 bands across *Vicia* samples. RAPD-PCR patterns indicated clear differences between *Vicia* species and low level of polymorphism within accessions of the same species. This is consistent with Whitty and Powell (1994) and Haley *et al.,* (1994), who found that RAPD-PCR generated high polymorphism between populations from different geographic regions more than that within the same population.

The dendrogram generated on the basis of seed protein, isozymes and RAPD markers (Fig. 4) indicated five clusters, which could be distinguished among the examined *Vicia* genotypes. One of these clusters is formed by the four closely associated botanical varieties of *V. sativa* in which each of *V. sativa (1)* with *V. sativa (2)* and *V. sativa (3)* with *V. sativa (4)* are phylogenetically close by 0.94 and 0.95, respectively. Each of *V. villosa* and *V. monantha* are grouped together in the second cluster in which *V. villosa (1)* and *V. villosa (3)* gave a high level of similarity (0.93). *Vicia cinerea, V. faba* and *V. narbonensis* were clustered in separate groups in which *V. faba* showed a 0.55 level of similarity with *V. narbonensis*. *Vicia cinerea* appeared to be closely related to *V. villosa* and *V. monantha* of the second group by about 0.77 level of similarity. *Vicia narbonensis* group showed the lowest similarity level (0.47) with the group of *V. sativa* in the first cluster.

Our results indicated that *V. faba* and *V. narbonensis* are different from each other and different from other species, which is in agreement with the molecular DNA data found by van de Ven *et al.* (1993). They reported that *V. faba* is genetically distinct *from V. narbonensis* complex.

The present study helped to solve the complexity among *V. villosa* and *V. monantha,*

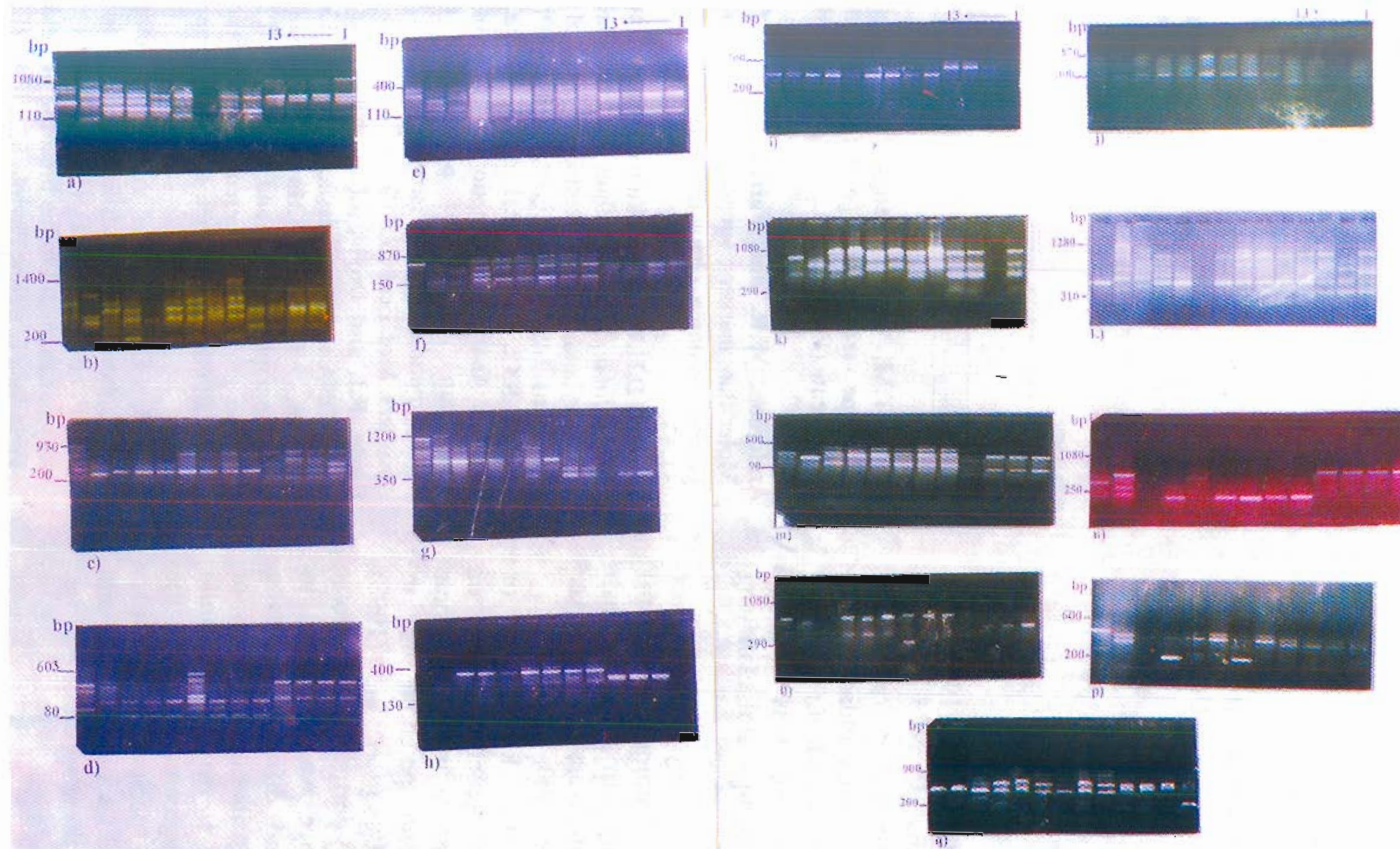

Fig. (3): Electrophoretic pattern of RAPD markers using the seventeen 10-mer random primers as numbered in Table 1; lanes 1-4: V. sativa, 5-7: V. villosa, 8-10: V. monantha, 11: V. cinerea, 12: V. faba and 13: V. narbonensis.

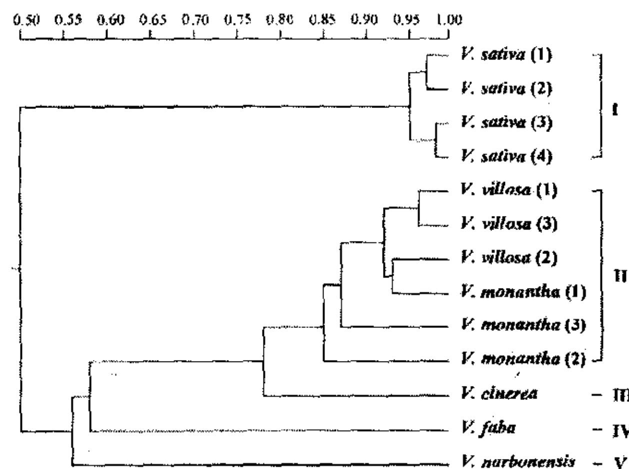

Fig. (4): Clustering of different Vicia species using UPGMA analysis.

described by Hanelt and Mettin (1989). Our results also indicated that *V. villosa* and *V. monantha* could be grouped in one single species. In general, this study indicated that RAPD may be useful in the identification of different *Vicia* species and may help in studying genetic relationships and clarifying taxa of the same species whose taxonomy is still in dispute. Even at very low level of polymorphism, RAPD technique could still detect enough polymorphism to distinguish among species by at least one marker difference (Quiros *et al.*, 1991).

In addition, the knowledge of population structure and genetic diversity of wild relatives is needed to investigate their evolutionary history and potential use in breeding programs. Buso *et al.* (1998) indicated that a large portion of the total genetic variation of rice was attributed to regional divergence and differences among populations. This distribution pattern of genetic variation of *Oryza glumaepatula* populations is in agreement with the expectation for *Vicia* species and provides important baseline data for conservation and collection strategies for these species.

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الملخص العربي

التوصيف الجزيئى لبعض الأنواع من جنس الفيشيا

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تهدف الدراسة إلى تحديد التنوع الوراثى فيجنس الفيشيا و التى منها الفول البلدى وبعض الانواع القريبة. وقد استخدم فى الدراسة ثلاثة عشر وحدة تقسيمية (Taxon) ممثلة لستة انواع من جنس الفيشيا هى:
(*V. sativa, V. villosa, V. monantha, V. narbonensis and V. cinerea*) بالإضافة إلى الفول البلدى *V. faba*. والتي تم تجميعها من مناطق الساحل الشمالى الغربى لجمهورية مصر العربية. وبعمل التفريد الكهربى لنماذج البروتين أمكن تحديد اختلافات واضحة بين الأنواع المختلفة وكذلك بين الوحدات التقسيمية داخل النوع الواحد. وقد تم ايضاً استخدام ثلاثة انظمة من مشابهات الإنزيم هى جلوتاميت أوكسالو اسيتيت ترانس امينيز ، الأميليز ، الإستريز. وتم إختبار هذه الأنظمة فى المستخلص البروتينى للبذور الجافة، وأظهرت التجارب أن الفول البلدى *V. faba* والنوع *V. narbonensis* لهما أنماط إنزيمية مختلفة عنها فى الأنواع الأخرى تحت الدراسة. وأظهرت الدراسة على مستوى الـ د ن أ DNA بإستخدام تحليل الـ RAPD-PCR وجود إختلافات واضحة بين الأنواع المستخدمة وكذلك درجة عالية من التشابه داخل النوع الواحد. ومن النتائج يمكن القول أن النوع *V. monantha* و *V. villosa* كانا أكثر قربا مع النوع *V. cinerea*. ومن دراسة علاقات القرابة بين الفول البلدى والنوع البرى *V. narbonensis* وجد انهما أكثر بعداً. وعموماً فإن الإختلافات الكبيرة بين الفول البلدى وأنواعه القريبة توضح أن هذه الأنواع ليس لها دور فى نشأة الفول البلدى.