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Specific differentiation in *Vicia* genus by means of capillary electrophoresis☆

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Abstract

Capillary electrophoresis has been applied to the discrimination of 13 *Vicia* species, belonging to four sections of *Vicia* genus. The studied species necessitate of plant growing tests or DNA molecular markers to be distinguished being their seeds morphologically very similar. Alcoholic/saline extracts from dry cotyledons were separated in uncoated fused-silica capillary with iminodiacetic acid isolectric buffer containing hydroxypropylmethylcellulose and acetonitrile. The low intra-specific variation observed for 11 species, suggests that this approach is suitable to carry out species discrimination. Species-specific peaks were identified for *V. articulata*, *V. atropurpurea*, *V. bithynica*, *V. benghalensis*, *V. disperma*, *V. ervilia*, *V. monantha*, *V. sativa* and *V. villosa*. Conversely, *V. lutea*, *V. melanops* and *V. peregrina*, showing very similar electrophoregrams, require other methodological approaches to be discriminated. The discussed CE method appears to have a potential to be regarded as an alternative tool to identify some *Vicia* species being far less expensive and time consuming than plant growing tests and DNA molecular markers.

Keywords: Genetic resources; Isoelectric buffer; Protein separation; *Vicia* spp.

1. Introduction

The genus *Vicia* comprises about 200 species [1], widely distributed in the temperate zones of the world. Nowadays, only a few species have many profitable uses such as forage [2], nitrogen-fixing organisms, cover crops for saline soils [3], animal feed [4,5] and occasionally human nutrition [6]. The high level of morphological variation implied the division of this genus in sections, each one containing a more homogeneous group of taxa. Within some sections, species sharing some traits are considered as aggregates such as the *V. narbonensis* group, while subspecies have been established within those species showing significant intra-specific variation such as *V. villosa* [7]. The classification of lots of seeds based only on the seed morphology relies on relatively few traits (i.e. shape, colour, circumference, relative hilum length, and hilum shape) that are generally common to several species. For this reason, the field examination of growing plants was widely used in the past in order to classify unknown *Vicia* samples. However, the morphological approach is insufficient to account for the entire genetic variation existing in the *Vicia* genus.

In the last decades, cytological [8,9], biochemical [10,11] and molecular [12–14] approaches have been proposed as alternatives to the field examination. The reduction of both time and resources necessary to catalogue seed samples is a very important goal for the institutions (i.e. gene-banks) that usually manipulate a high number of samples.

Electrophoretic analysis of seed storage proteins, being a simple and reproducible method, has been widely applied for the reliable seed identification and much pertinent literature exists for the most important crops. Examples of polyacrylamide gel electrophoresis applied to the discrimination of some *Vicia* species are reported in literature [15–17].

During the last decades, capillary electrophoresis (CE) has attracted the attention being a method potentially alternative to polyacrylamide gel electrophoresis and complementary to

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HPLC. It allows rapid and high-resolution separation of seed storage proteins with full automation. Nowadays, CE is commonly used to study the variation of gliadins and glutenins in cereals as well as to identify wheat varieties. Conversely, only a few studies were devoted to the development of CE methods suitable for comparative analysis of legume proteins. The potentialities of CE in the varietal identification were studied in common bean [18], while the species dis-

Table 1
List of tested accessions

Section	Species	Donor code	Origin
Cracca S.F. Gray	*V. articulata* Hornem.	MG 105616	Italy
		MG 105776	Sweden
		MG 107371	Spain
		pop. 1	Italy, Sardinia
		pop. 2	Spain, Canarie
	V. atropurpurea Desf.	MG 105596	Portugal
		MG 105597	Italy
		MG 105647	France
		MG 105832	Unknown
	V. benghalensis L.	MG 104746	Latvia
		MG 105608	Italy
		MG 105912	Unknown
		MG 114087	Unknown
	V. monantha Retz.	MG 105621	Australia
		MG 105652	Unknown
		MG 106221	Egypt
	V. villosa Roth subsp. *eriocarpa*	vic 615/79	Germany
		vic 616/78	Turkey, Anatolia
		vic 621/73	Turkey, Anatolia
		vic 843/79	Greece
	V. villosa Roth subsp. *varia* (Host) Corb.	vic 510/79	Canada
		vic 608/73	Turkey, Anatolia
		vic 609/79	Turkey, Anatolia
		vic 872/82	Italy
	V. villosa Roth subsp. *villosa*	vic 506/83	Ex URSS
		vic 515/83	Germany
		vic 518/73	Turkey, Anatolia
Ervum (L.) S.F. Gray	*V. disperma* DC	MG 106195	Denmark
		vic 792/75	Portugal
	V. ervilia (L.) Willd.	MG 105623	Germany
		MG 105624	Australia
		MG 105625	Germany
		MG 112372	Cyprus
Faba (Miller) S.F. Gray	*V. bithynica* L.	MG 105593	Italy
		vic 793/77	Italy
		vic 855/79	France
Vicia	*V. lutea* L.	MG 103293	Italy
		vic 738/80	France
	V. grandiflora Scop.	MG 105220	Italy
	V. grandiflora Scop. var. kitalbeliana Kock.	vic 736/76	Hungary
	V. grandiflora Scop. var. grandiflora	vic 741/78	Turkey, Anatolia
	V. melanops Sibth. & Smith	MG 112511	Unknown
	V. melanops Sibth. & Smith var. loiseau d'Allz.	vic 475/78	France
	V. peregrina L.	MG 105613	Malta
		vic 747/78	Turkey
		vic 787/77	Spain
	V. sativa L. subsp. *cordata*	MG 103292	Italy
		MG 103327	Italy
		MG 104383	Italy

The subspecies and the origin are indicated for the species having them.

crimination was investigated for the *Lupinus* genus [19]. As concerns the *Vicia* genus, Lucchese et al. [17] reported the distinction of *V. sativa* and *V. villosa* varieties by using both isoelectric focusing–polyacrilamide gel electrophoresis (IEF–PAGE) and CE data. Salmanowicz [20] discriminated three *Vicia* species by analysing the albumin fraction with coated capillaries.

This study was aimed to the development of a reliable method to discriminate several *Vicia* species selected among those that necessitate field tests or other methodologies to be distinguished being their seeds morphologically very similar. *V. ervilia* represented the exception being characterised by a seed shape easily recognisable.

2. Materials and methods

2.1. Plant materials

From 4 to 9 accessions belonging to 13 *Vicia* species distributed among four sections were studied (Table 1). Accessions from different geographical areas were analysed for each species. Seed samples were obtained from the Istituto di Genetica Vegetale-CNR (IGV) (Bari, Italy) and from the Institut fűr Pflanzengenetik und Kulturpflanzenforschung (IPK) (Gatersleben, Germany). The pop. 1 of *V. articulata* was collected in Sardinia (Italy) by the exploration team of IGV [21], while the pop. 2 was received from the Servicio de Patrimonio Historico (Arrecife Lanzarote, Islas Canarias, Spain).

2.2. Extraction of proteins for CE

From 3 to 5 dehulled seeds for each accession were manually milled. The extraction of proteins for CE was carried out according to Lucchese et al. [17] with some modifications. A pre-extraction was done by mixing the meal with a solvent (1:10, w/v) at room temperature. Acetone, diethyl ether, chloroform and hexane were tested. The extraction was repeated twice for 20 min. Longer extraction time did not improve the effectiveness of the extraction. The suspension was vortexed periodically, centrifuged at 11 000 rpm for 10 min and air dried. The two fractions, collected for each solvent, were mixed and the visible spectrum was recorded in the range 380–500 nm with a spectrophotometer DU 680 (Beckman-Coulter, USA). The highest absorbances were detected for acetone. The defatted meal (20 mg) was suspended in 70% (v/v) ethanol/0.5 M NaCl buffer (1:8, w/v) for 2 h at room temperature with brief vortexing every 20 min. The suspension was centrifuged at 11 000 rpm for 10 min, the supernatant was used for CE and analysed on the day of extraction.

2.3. Capillary zone electrophoresis

Beckman P/ACE model MDQ equipment (Beckman Coulter, USA) was used to separate the protein extracts. Separations were achieved using 30 cm long (22 cm to detector), 50 μm I.D. uncoated fused-silica capillaries. The extracts were pressure injected (Table 2) and the separated proteins were detected by UV absorbance at 214 nm. In order to obtain fast, high-resolution and reproducible separations, different CE buffers, applied voltage and capillary temperature were tested. The optimal conditions for each buffer are shown in Table 2. Buffers were prepared with HPLC-grade water obtained with a Milli-Q water purification system (Millipore, USA), the chemicals were of analytical grade. The cleaning procedure of capillary between consecutive runs carried out with iminodiacetic acid (IDA) buffer was according to Olivieri et al. [22]. It consisted of successive rinsing with the electrophoretic buffers and water. Then a 6 kV voltage was applied for 6 min and reverse polarity between a solution 25 mM of sodium phosphate pH 7 and 7 M urea placed at the anode and the same buffer containing 60 mM sodium dodecyl sulfate (SDS) placed at the cathode. After washing with water the capillary was reconditioned with the electrophoretic buffer. An acceptable run-to-run reproducibility was obtained under the described conditions. Beckman Karat 32 software was used for the acquisition and elaboration of electropherograms.

Table 2
Description of separation buffers tested in this study

Separation buffer composition	Operating conditions
0.1 M phosphate pH 2.5, 0.05% HPMC, 20% acetonitrile	20 kV for 15 min, 45 °C, injection 3447 Pa for 6 s
0.1 M phosphate–glycine pH 2.5, 0.05% HPMC, 20% acetonitrile	15 kV for 18 min, 40 °C, injection 3447 Pa for 6 s
40 mM aspartic acid, 4 M urea, 0.5% HEC, 20% acetonitrile	25 kV for 15 min, 35 °C, injection 4137 Pa for 8 s
50 mM IDA, 0.05% HPMC, 20% acetonitrile	25 kV for 8 min, 35 °C, injection 3447 Pa for 6 s

Only the best operating conditions for each buffer are reported. HEC, hydroxyethylcellulose; HPMC, hydroxypropylmethylcellulose.

2.4. Protein extraction for polyacrilamide gel electrophoresis

Total proteins for SDS–PAGE were extracted as previously described [16] from the same pool of seeds used for CE. The undefatted meal (10 mg) was mixed with Tris–HCl 0.3 M buffer at pH 6.8 containing 1% SDS, 0.3% 2-mercaptoethanol and 8% glycerol (1:20, w/v). One dimensional SDS–PAGE was performed using 17% polyacrilamide gel. Electrophoresis was carried out at a constant current 60 mA for 5–6 h [16].

3. Results and discussion

3.1. Protein extraction and optimisation of CE separation

Lucchese et al. [17] reported that CE analysis of alcoholic/saline extracts in uncoated fused-silica capillary is a

reliable method to discriminate *V. sativa* and *V. villosa* accessions. Unfortunately, some troubles are associated to the application of this method to other *Vicia* species. In fact, we observed that increasing the number of consecutive analyses a significant loss of reproducibility of current intensity took place despite extensive flushing with the background electrolyte (BGE) between subsequent runs. Rinses with basic (0.1 M sodium hydroxide) or acidic solutions (1 M phosphoric acid or 0.1 M hydrochloric acid) followed by BGE were little effective to confer high reproducibility to the analyses.

A poor repeatability of CE analyses was described by Salmanowicz [20], who analysed the albumins of three *Vicia* species by using phosphate and borate buffers embracing a range of pH from 6.5 to 9.0. Changes of the ξ potential, attributable to the irreversible adsorption of proteinaceous and/or non proteinaceous compounds onto the capillary inner walls, were indicated as the reasons of the poor repeatability of analyses. To obtain a good reproducibility, Salmanowicz [20] proposed the use of surface-modified capillaries. However, this procedure showed some limitations attributable to the partial washing out of hydrophobically adsorbed surfactant from the capillary inner walls during the washing steps.

The possibility that non proteinaceous compounds could be involved in the reduction of capillary efficiency would have to be carefully considered because the alcoholic/saline extracts, obtained according to Lucchese et al. [17], are intensely coloured. In order to improve the reproducibility of CE analysis, the meals were treated with organic solvents before the protein extraction. Acetone, diethyl ether, chloroform and hexane were tested. This step removes fats, pigments, etc. that, being co-extracted with proteins, could interfere with CE separations. The effectiveness of the pre-extraction was monitored spectrophotometrically. The highest absorbances, which indicate higher amounts of interfering compounds removed from the matrix, were recorded for acetone. Visually the alcoholic/saline extracts obtained after the meal pre-extraction were colourless, only those from *V. villosa* and *V. monantha* retained a pale yellow coloration. The analysis reproducibility of these 'clean' extracts really increases, but the progressive loss of separation efficiency is not completely removed.

It is known that the interaction between proteins and capillary walls is strongly reduced by using BGE at acidic pH. For this reason, the buffers listed in Table 2, were tested in substitution of borate buffer used by Lucchese et al. [17]. As shown in Fig. 1, the influence of BGE on the electroelution time, the electrophoretic pattern and the shape of peaks was significant. The run required 12–14 min to be performed when phosphate based buffers were used, while less than 8 min were sufficient with IDA buffer. Moreover, phosphate based buffers adversely affected other important analytical parameters. Peaks appeared broader (Fig. 1, traces B and C) as compared to IDA buffer (Fig. 1, trace A) and the current intensity was plagued, mainly in the initial stages of run, by significant fluctuations that were not reproducible from run-to-run. At the opposite, IDA buffer was characterised by high

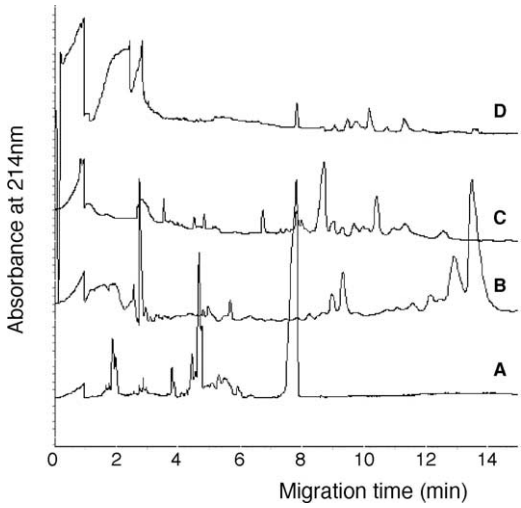

Fig. 1. Capillary electrophoretic patterns of the alcoholic/saline extract from *V. atropurpurea* (MG 105647) separated in: (A) IDA buffer; (B) phosphate buffer at pH 2.5; (C) phosphate–glycine buffer at pH 2.5; (D) aspartic buffer. The buffer composition and the run conditions are according to Table 2.

stability of the current during the run and good reproducibility of the current among subsequent runs. This is not surprising being capillary electrophoretic analysis in isoelectric acidic buffers routinely used for cereal prolamins analysis [23]. A remarkable difference was observed between the tested isoelectric buffers. The aspartic acid buffer resulted unsuitable for the analysis of *Vicia* extracts being characterised by long electroelution times and very poor peak shape (Fig. 1, trace D).

The tendency for the whole train of peaks to lengthen a bit the transit times in the repeated runs was observed even with IDA buffer. The replenishment of the buffer reservoirs before each run to avoid changes of buffer composition due to both acetonitrile (ACN) volatility and pH variations, in response to oxidation/reduction processes at the electrodes, was little effective. This suggests that a gradual absorption of proteins on the capillary inner walls take place even at acidic pH. Among the tested methods to regenerate the capillary the most efficient desorption of bound material was obtained via sweeping the silica surface with SDS micelles, driven electrophoretically into the lumen from the cathodic reservoir. Previous studies showed that as the SDS micelles move in the capillary they would displace any bound proteinaceous material in an electrophoretic desorption process analogous to displacement chromatography [22]. Applying this washing procedure the overall variation of migration time, measured on the middle peak of electrophoregrams, was 4–5%.

3.2. CE analysis of alcoholic/saline extracts from different Vicia species

In order to explore the potential of described method applied to *Vicia* species discrimination, the intra-specific variation was evaluated. The analysis for each species, of accessions from different countries, evidenced a very low

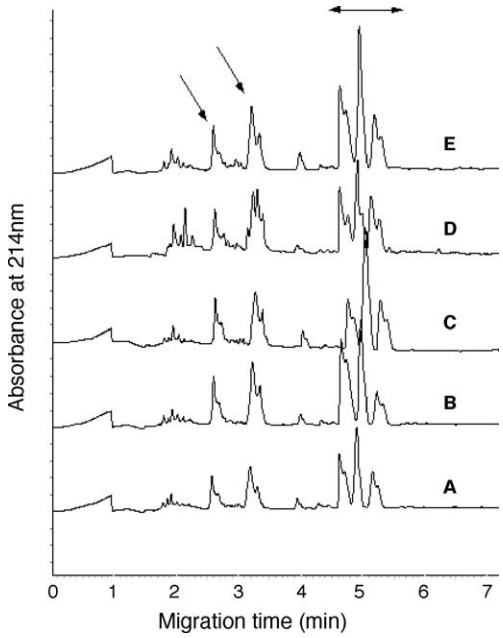

Fig. 2. Electrophoretic pattern of *V. articulata* accessions: (A) pop. 1; (B) MG 105776; (C) MG 107371; (D) pop. 2; (E) MG 105616. The separation was performed in 50 mM IDA, 0.05% HPMC, 20% ACN at 35 °C and 25 kV.

intra-specific variation for 11 species. The electrophoregrams of *V. articulata* (Fig. 2) and *V. bithynica* (Fig. 3) are shown as examples. Three main groups of peaks were detected from 2.5 to 5.2 min (see arrows in Fig. 2) for the five accessions of *V. articulata*. The discrimination of the accessions can be attempted by comparing the relative heights of peaks. For example, the protein fractions migrating from 1.8 to 2.2 min were detected in large amount only in the pop. 2 (Fig. 2, trace D). The very high similarity observed by CE was confirmed by submitting the total seed storage proteins, extracted from the same pool of seeds used for CE, to SDS–PAGE (Fig. 4).

The electrophoregrams relative to *V. bithynica* accessions are shown in Fig. 3. The pattern associated to this species

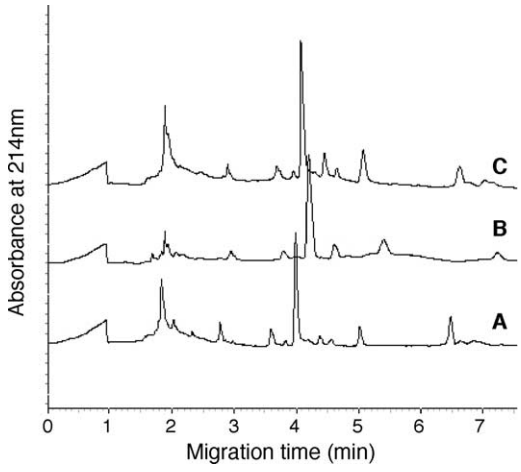

Fig. 3. Electrophoretic pattern of *V. bithynica* accessions: (A) vic 855/79; (B) MG 105593; (C) vic 793/77. Separation conditions as in Fig. 2.

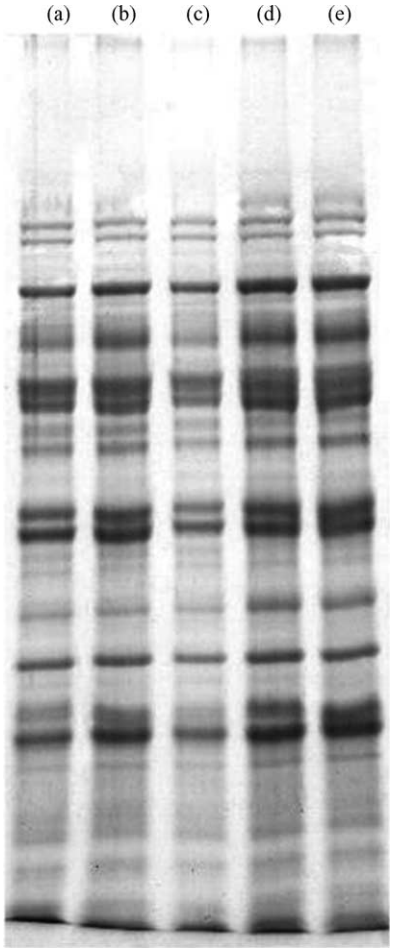

Fig. 4. SDS–PAGE profiles of *V. articulata* accessions lane: (a) pop. 1; (b) MG 105776; (c) MG 107371; (d) pop. 2; (e) MG 105616.

consisted of a main peak with migration time around 4.2 min and minor fractions, from 8 to 11, with shorter or longer migration time.

Very similar electrophoregrams were obtained also for the 11 accessions *V. villosa* though they belonged to three sub-species (Table 1). Only differences in the height of some peaks were detected within and among the sub-species (Fig. 5, traces A–D). For example, the protein fractions grouped in the cluster with migration time around 3.4 min were found abundant only in the accession vic 506/83 (Fig. 5, trace C), while the relative height of the two peaks from 3.80 to 3.95 min characterised the accession vic 609/79 (Fig. 5, trace D). Lucchese et al. [17] used the minor differences detectable among the electrophoregrams to differentiate *V. villosa* accessions. The present study showed that these differences, being unrelated to the sub-species, make CE analysis of alcoholic/saline extracts not suitable to categorise the accessions into the sub-species recognised in *V. villosa*. On the other hand, SDS–PAGE of accessions analyses in this study evidenced that they share a very high number of bands (data not shown).

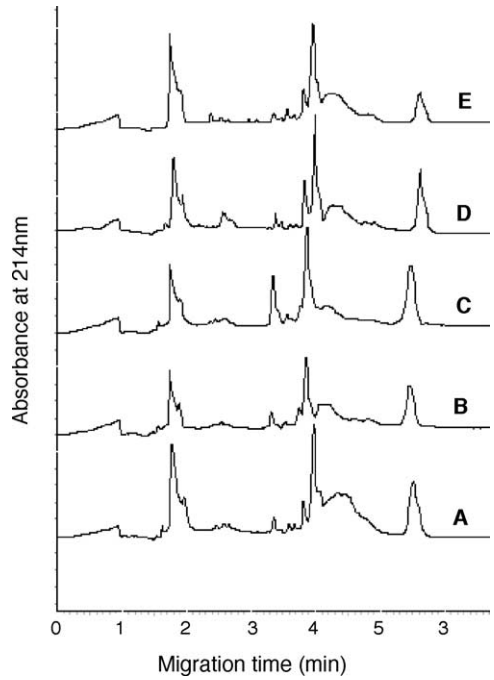

Fig. 5. Electrophoretic pattern of *V. villosa* accessions: (A) vic 615/79; (B) vic 515/83; (C) vic 506/83; (D) vic 609/79; (E) *V. monantha*, MG 105621. Separation conditions as in Fig. 2.

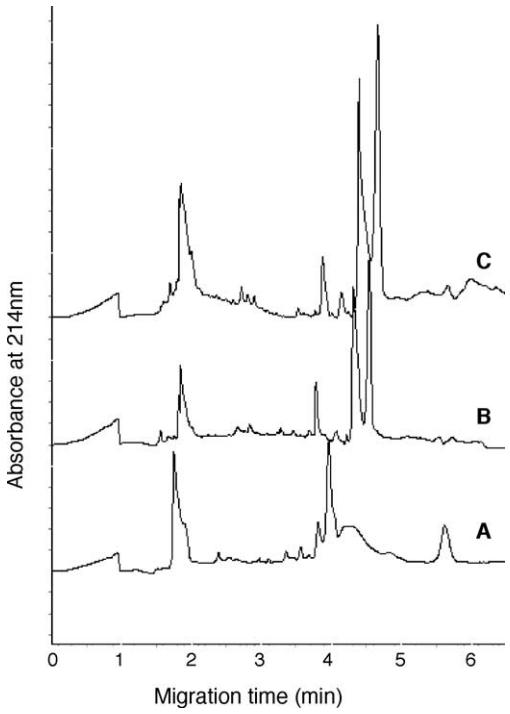

Fig. 7. Electrophoregrams of *V. monantha* accessions: (A) MG 105621; (B) MG 105652; (C) MG 106221. Separation conditions as in Fig. 2.

Intra-specific variation of alcoholic/saline extracts was observed for *V. grandiflora* and *V. monantha*. As shown in Fig. 6, qualitative and quantitative differences can be detected by comparing the electrophoregrams of *V. grandiflora* accessions. The accession MG 105220 (Fig. 6, trace C), besides a sharp difference in the height of peak at 6 min, was characterised by a very low number of peaks from 2 to 5.5 min. Conversely, accessions vic 736/76 and MG 105220 shared several peaks. As concerns *V. monantha*, two of tested accessions showed the same pattern (Fig. 7, traces B and C), whereas the electrophoregram of the third accession (Fig. 7, trace A) resulted completely different. However, we have doubts if this result actually indicates intra-specific variation. In fact, the misclassification of the accession MG 105621 should be taken in account because its electrophoregram has a very high resemblance with those of *V. villosa* accessions (Fig. 5). Moreover, this accession comes from Australia and this is unusual being *V. monantha* a species typical of Mediterranean basin [24].

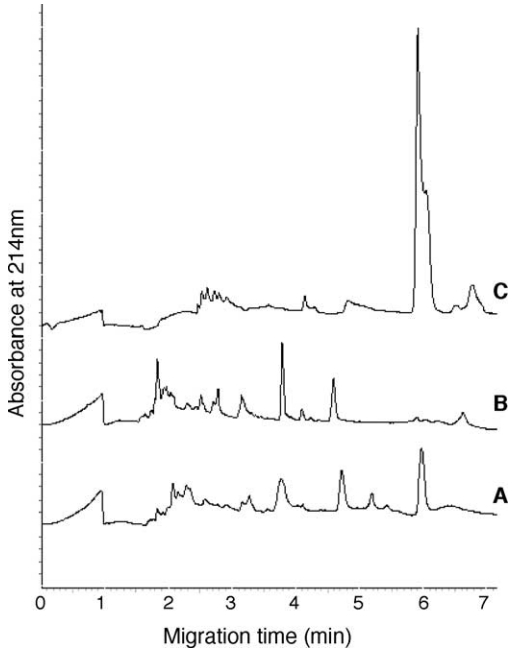

Fig. 6. Electrophoregrams of *V. grandiflora* accessions: (A) vic 741/78; (B) vic 736/76; (C) MG 105220. Separation conditions as in Fig. 2.

The effectiveness of CE to differentiate *V. sativa* and *V. villosa* [17] or *V. faba*, *V. narbonensis* and *V. serratifolia* [20] has been reported in literature. The present investigation showed that CE is suitable to discriminate several species. In fact, the electrophoregrams of tested species greatly differed for the number and migration time of separated proteins. This indicates that the protein fraction soluble in the alcoholic/saline buffer, being sufficiently variable, can be a useful tool for the discrimination of *Vicia* species. Species-specific peaks were identified for *V. atropurpurea* (Fig. 1, trace A), *V. articulata* (Fig. 2), *V. benghalensis*, *V. disperma*, *V. ervilia*, *V. bithynica*, *V. monantha* and *V. sativa* (Fig. 8). The electroelution times of these peaks are listed in Table 3. The presented method did not allow attempting the identification of *V. grandiflora* due to the observed intra-specific variation, while the differences among *V. lutea*, *V. peregrina* and *V. melanops*, being

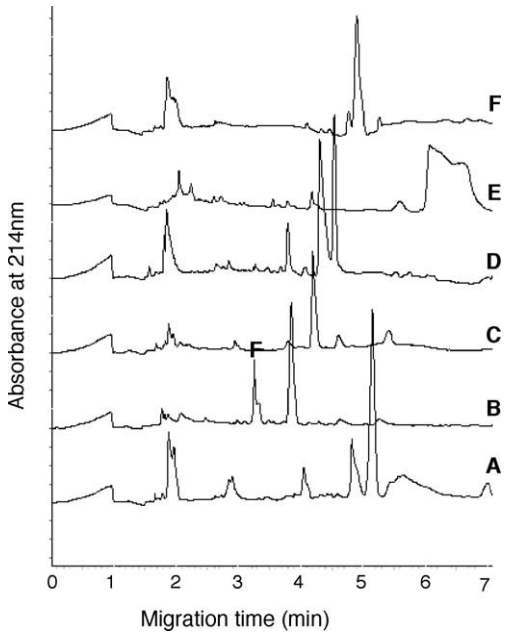

Fig. 8. Electrophoregrams of: (A) *V. benghalensis*, MG 105608; (B) *V. ervilia*, MG 105625; (C) *V. bithynica*, MG 105932; (D) *V. monantha*, MG 105652; (E) *V. sativa*, MG 103327; (F) *V. disperma*, MG 106195. Separation conditions as in Fig. 2.

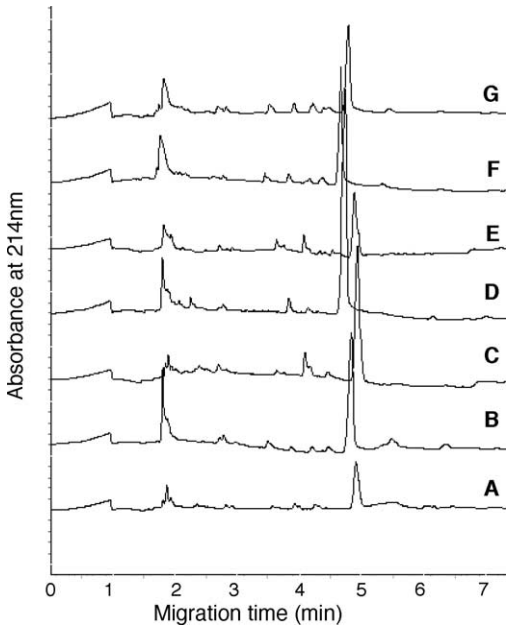

Fig. 9. Electrophoregram of: *V. lutea* MG 103293 (A) and vic 738/80 (B); *V. melanops* MG 112511 (C) and vic 475/78 (D); *V. peregrina* MG 105613 (E), vic 787/77 (F) and vic 747/78 (G). Separation conditions as in Fig. 2.

associated only to minor peaks, are useless to achieve their discrimination (Fig. 9).

Some interesting considerations can be done on *V. benghalensis* and *V. atropurpurea*. The classification of these species is still today object of debate. In the opinion of some authors these names should be considered synonymous because the existence of two entities is not supported from sufficient morphological variation of plant traits. Recent studies, based on different methodological approaches, evidenced that within the material currently classified as *V. benghalensis* or *V. atropurpurea* there are two well distinguishable entities [11,16]. Data collected in this study agree with this hypothesis. In fact, similarly to SDS–PAGE analysis of the total seed storage proteins [16], the alcoholic/saline extracts of *V. atropurpurea* (Fig. 1, trace A) and *V. benghalensis* (Fig. 8, trace A) showed different patterns.

Table 3
Migration time relative to the species-specific peaks identified for each species

Species	Average migration time
V. articulata Hornem.	2.6 (sp + sh); 3.2–3.3 (cl); 4.6–5.3 (cl)
V. atropurpurea Desf.	1.8–2.0 (cl); 4.4–4.7 (cl); 7.9 (sp)
V. benghalensis L.	1.8–2.0 (cl); 4.1 (sp + sh); 4.8 (sp); 5.1 (sp)
V. monantha Retz.	1.8–2.0 (cl); 4.3 (sp); 4.6 (sp)
V. villosa Roth.	1.8–2.0 (cl); 3.8 (sp); 3.9 (sp); more than 4.0 unresolved peaks
V. disperma DC	1.8–2.0 (cl); 4.8 (sp); 4.9 (sp + sh)
V. ervilia (L.) Willd.	3.2 (sp + sh); 3.8 (sp)
V. bithynica L.	4.1 (sp)
V. sativa L.	More than 6.0 unresolved peaks

cl, cluster of peaks; sh, shoulder; sp, single peak.

4. Conclusions

Different buffers and other CE parameters were optimised for analysing alcoholic/saline soluble proteins of some *Vicia* species. This protein fraction can be separated and characterised by CE with an isoelectric buffer based on IDA. The alcoholic/saline soluble proteins, having a high variability among the tested species, allowed the identification of species-specific peaks for 11 tested species. These results encourage substituting slab gel electrophoresis, historically extensively used to analyse seed storage proteins, with CE that affords a faster time of analysis and is a full automated procedure. Moreover, capillary electrophoresis, being far less expensive and time consuming than plant growing tests and DNA molecular markers, has a good potential to be considered as an alternative method to identify some *Vicia* species.

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References

[1] P. Hanelt, D. Mettin, Ann. Rev. Ecol. Syst. 20 (1989) 199.
[2] J.D. Berger, L.D. Robertson, P.S. Cooks, Genet. Res. Crop Evol. 49 (2002) 313.
[3] J.P. Mitchell, C.D. Thomsen, W.L. Graves, C. Shennan, J. Agron. Crop Sci. 183 (1999) 167.

[4] S. Jaques, R.M. Dixon, J.H.G. Holmes, Small Rum. Res. 15 (1994) 39.
[5] J.D. Berger, L.D. Robertson, P.S. Cooks, Genet. Res. Crop Evol. 50 (2003) 201.
[6] G. Laghetti, A.R. Piergiovanni, I. Galasso, K. Hammer, P. Perrino, Genet. Res. Crop Evol. 47 (2000) 461.
[7] P.W. Ball, Leguminosae, in: T.G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, D.A. Webb (Eds.), Flore Europaea, vol. 2, Cambridge University Press, 1968, p. 80.
[8] N. Maxted, M.A. Callimassia, M.D. Bennett, Pl. Syst. Evol. 177 (1991) 221.
[9] R. Cremonini, S. Funari, S. Mazzucca, Chromatin 1 (1992) 135.
[10] P. Perrino, G. Maruca, R.N. Lester, V. Linsalata, V. Lattanzio, P. Hanelt, Feddes Repert. 102 (1991) 319.
[11] G. Sonnante, A.R. Piergiovanni, D. Pignone, Pl. Syst. Evol. 207 (1997) 99.
[12] W.T. Van de Ven, N. Duncan, G. Ramsay, M. Phillips, W. Powell, R. Waugh, Theor. Appl. Genet. 86 (1993) 71.
[13] S.N. Raina, Y. Ogihara, Theor. Appl. Genet. 88 (1994) 261.
[14] M. Nouzova, P. Neumann, A. Navratilova, D.W. Galbraith, J. Macas, Plant Mol. Biol. 452 (2001) 229.
[15] P. Perrino, G. Colaprico, D. Lafiandra, Genet. Agric. 41 (1987) 375.
[16] A.R. Piergiovanni, I. Galasso, P. Perrino, D. Pignone, Genet. Res. Crop Evol. 42 (1995) 347.
[17] C. Lucchese, G. Dinelli, A. Bonetti, A. Lovato, Seed Sci. Technol. 25 (1997) 253.
[18] G. Dinelli, A. Bonetti, Seed Sci. Technol. 20 (1992) 241.
[19] B.P. Salmanowicz, J. Chromatogr. A 894 (2000) 297.
[20] B.P. Salmanowicz, Chromatographia 41 (1995) 99.
[21] G. Laghetti, P. Perrino, S. Cifarelli, S. Bullitta, K. Hammer, Plant Genet. Res. Newslett. 120 (1999) 30.
[22] E. Olivieri, R. Sebastiano, A. Citterio, C. Gelfi, P.G. Righetti, J. Chromatogr. A 894 (2000) 273.
[23] P.G. Righetti, C. Gelfi, A. Bossi, E. Olivieri, L. Castelletti, B. Verzola, A.V. Stoyanov, Electrophoresis 21 (2000) 4046.
[24] F.W. Ball, *Vicia*, in: T.G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, D.A. Webb (Eds.), Flora Europaea, Cambridge University Press, London, 1968.