

## ELECTROPHORETIC ANALYSIS OF SEED PROTEINS IN ARGENTINEAN SPECIES OF PHASEOLINAE (FABACEAE)

SHIRLEY MARY ESPERT<sup>1</sup> and ALICIA DIANA BURGHARDT<sup>1</sup>

**Summary:** The relationships among Argentinean species of four genera of the subtribe *Phaseolinae* (Phaseoleae, Fabaceae): *Phaseolus*, *Vigna*, *Dolichopsis* and *Macroptilium*, had never been analyzed together using molecular markers. In this paper, twenty species of these genera were studied by means of electrophoresis of seed storage proteins on a SDS-PAGE system. Seed protein electrophoretic banding patterns allowed the recognition of a genetic basis of the generic divisions, which were proposed using morphological and palinological data. Polypeptidic profiles provided interesting data for the analysis the subgeneric divisions. Moreover, the presence of marker bands allows the unequivocal characterization of most of the Argentinean species here studied.

**Key Words:** Systematics, Phaseolinae, Fabaceae, seed protein electrophoresis

**Resumen:** Análisis electroforético de las especies argentinas de Phaseolinae (Fabaceae). Las relaciones entre las especies argentinas de cuatro géneros de la subtribu *Phaseolinae* (Phaseoleae, Fabaceae): *Phaseolus*, *Vigna*, *Dolichopsis* y *Macroptilium*, fueron analizadas por primera vez en conjunto usando marcadores moleculares. Se estudiaron veinte especies de estos géneros utilizando electroforesis de proteínas seminales, en un sistema SDS-PAGE. Los patrones de bandas proteicas evidenciaron la existencia de una base genética para las divisiones genéricas, que fueron propuestas mediante el uso de datos morfológicos y palinológicos. Los perfiles polipeptídicos proveen información interesante para analizar las divisiones subgenéricas. Además, la presencia de bandas marcadores permite la caracterización inequívoca de la mayoría de las especies argentinas aquí estudiadas.

**Palabras clave:** Sistemática, Phaseolinae, Fabaceae, electroforesis de proteínas seminales.

### INTRODUCTION

The Subtribe *Phaseolinae* Benth. (Phaseoleae, Fabaceae) has 23 genera, four of them growing in Argentina: *Phaseolus* L. emend Verdc., *Vigna* Savi, *Macroptilium* (Benth.) Urb., and *Dolichopsis* Hassl.

There are several morphological (Verdcourt, 1970; Marèchal *et al.* 1978; Drewes, 1995; Palacios & Hoc, 2001), biochemical (Zalocchi, 1992) and molecular studies (Becerra Velasquez & Gepts, 1994; Fofana, 1999; Ruffini Castiglione *et al.* 1998; Goel *et al.* 2002), which have attempted to solve taxonomic problems in this group. However, only partial analyses have been made and most of the studies have exclusively involved wild species of Mesoamerican origin (Maquet *et al.* 1999).

Marèchal *et al.* (*op. cit.*) developed the morphological

taxonomic system accepted at present, including delimitations of the genera and their subdivisions in subtribe *Phaseolinae*. This classification is based on morphological and palinological data. These authors emphasized the fact that there are still too many problems to be solved due to the great amount of taxa involved.

All of the *Phaseolinae* species growing in Argentina had never been analyzed together.

*Phaseolus* L. has three sections: sect. *Phaseolus*, the only section with species in Argentina, *Alepidocalyx* (Piper) Marèchal *et al.* and *Minkelersia* (Mart et Gall.) Marèchal *et al.* The "common bean" *P. vulgaris* is very important in the economy of Northwestern Argentina region. The study of the natural populations of *P. vulgaris* var. *aborigineus*, the wild relative of the common bean, is crucial for crop breeding (Palacios & Hoc, 2001).

Many species of *Vigna* are used as food and forage (Smartt, 1990), so the knowledge of the group is important for their biotechnological improvement. Results reported by Delgado Salinas *et al.* (1993) are consistent with the hypothesis of a polyphyletic ori-

<sup>1</sup>Lab. Plantas Vasculares, Depto. Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Cantilo y Costanera Norte, 4° piso, Pabellón 2, Ciudad Universitaria (C1428EHA), Buenos Aires, ARGENTINA. E-mail: [alibu@bg.fcen.uba.ar](mailto:alibu@bg.fcen.uba.ar)

gin of the genus *Vigna*. They divided this genus in two subclades: one including the Old World subgenera and the American subgenus *Lasiospron*, and the other subclade strictly of the New World. Here, species of the subgenera *Sigmoidotropis*, *Lasiospron* and *Vigna* growing in Argentina have been analyzed. Only *V.hookeri* and *V.caracalla* have not been included in the analysis. The latter because we couldn't collect mature legumes, and *V. hookeri* because it is believed that is extinct, for the last collection was in the 1950's (Troncoso de Burkart & Bacigalupo, 1987).

The third genus here studied is *Macroptilium*, with nine species in Argentina, all of them included in the analysis. Cladistic analysis using morphological characters suggests the monophyly of both sections of the genera: *Macroptilium* and *Microcochle* (Drewes, 1995). Studies using molecular techniques had never been carried out in this group.

*Dolichopsis* is a monotypic genus. The only species *D. paraguariensis* Hassler is endemic to Paraguay and Argentina (Palacios & Hoc, 2001).

In the present work, species of *Phaseolus*, *Vigna*, *Macroptilium* and *Dolichopsis* have been studied by means of electrophoresis of seed storage proteins. This technique has proved to be useful in legume systematics (Burghardt & Palacios, 1997; Burghardt, 2000a and b; Fotso *et al.* 1994; Przybylska, 1995; Przybylska & Zimniak-Przybylska, 1997; Maquet *et al.* 1999). It has been used to clarify species delimitations, and to perform many supra- and intraspecific systematic studies (De Bustos *et al.* 1999; Sammour, 1994).

The main objective of this work is to find useful molecular markers of the Argentinean species of the genera *Vigna*, *Phaseolus*, *Dolichopsis* and *Macroptilium*, which will allow the identification of each species, and to analyze the possible genetic variability between and within them. Many of the species of these genera are economically important, so a greater knowledge of their variability and relationships is crucial in order to establish strategies for plant breeding and germplasm management.

## MATERIALS AND METHODS

**Plant Material.** Sixty-one accessions of twenty Argentinean species of *Phaseolus*, *Vigna*, *Dolichopsis* and *Macroptilium* were studied. Collection data of the material examined in this study are indicated in Table 1. They are all deposited in the Herbarium of the Facultad de Ciencias Exactas y Naturales (University of Buenos Aires), BAFC (abbreviation according to Holmgren *et al.* 1981).

**Seed protein analysis.** Storage proteins were extracted by grinding one or more mature seeds, and mixing 100 mg of the powder with 0.4 ml of 0.5M NaCl. The suspension was rest at room temperature for two hours and centrifuged at 10000 rpm for 10 min. The supernatant was mixed with an equal volume of cracking buffer (0.125M Tris-HCl pH 6.8; 4% w/v SDS; 20% w/v Glycerol; 10% w/v 2-mercaptoethanol; 0.001% w/v bromophenol blue) and boiled for 2 min.

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). The running gel contained 15% of polyacrylamide in 1.5M Tris-HCl pH 8.8 buffer, with 10% w/v SDS, 0.05% w/v ammonium persulfate and 0.0005% TEMED. The stacking gel had 4.5% of polyacrylamide in a 0.5M Tris-HCl pH 6.8 buffer, with 10% w/v SDS, 0.005% w/v ammonium persulfate and 0.0001% TEMED. The electrode buffer had 0.025M Tris, 0.192M Glycine at pH 8.3, and 0.1% w/v SDS.

The running was conducted for 3 - 5 hours at 40 mA and 120 V in a BioRad Protean II apparatus. The gel was stained with 0.05% w/v Coomassie Blue and 12% w/v trichloroacetic acid, overnight, and then washed with 7% v/v acetic acid solution.

**Numerical analysis.** Basic data matrix was made considering protein bands in the electrophoretic runnings as absence/presence characters. UPGMA, Minimum Spanning Tree and Principal Coordinates Analysis methods were applied. The analysis was performed using the NTSYS-pc 1.7 (Numerical Taxonomy and Multivariate Analysis System) computer program designed by F. James Rohlf (1988)

## RESULTS

A hundred and thirty five protein bands are found in the 20 taxa analyzed. Figure 1 shows some of the protein patterns obtained in this study. Within each species, all seeds had the same protein pattern. The only exception is *Phaseolus vulgaris*: *P. vulgaris* var. *vulgaris* and *P. vulgaris* var. *aborigineus* have in common 16 of their 17 bands.

Three marker bands (i.e., exclusive and constant bands) are found in *Macroptilium* and five in *Phaseolus*, while no marker bands are detected in *Vigna* as a whole. Species of the four genera share one band at 66kDa. Species of *Phaseolus*, *Vigna* and *Macroptilium* share two bands at 116 and 21 kDa. All the species of *Macroptilium* and *Vigna* share three bands, two of them around 200 and one around 18kDa (Fig.1).

**TABLE 1.** Taxonomic classification (sensu Maréchal *et al.* 1978) of the species analyzed. RP=Ramón Palacios, HOC= Patricia Hoc, SD= Susana Drewes, MMS= M. del Carmen Menéndez Sevillano.

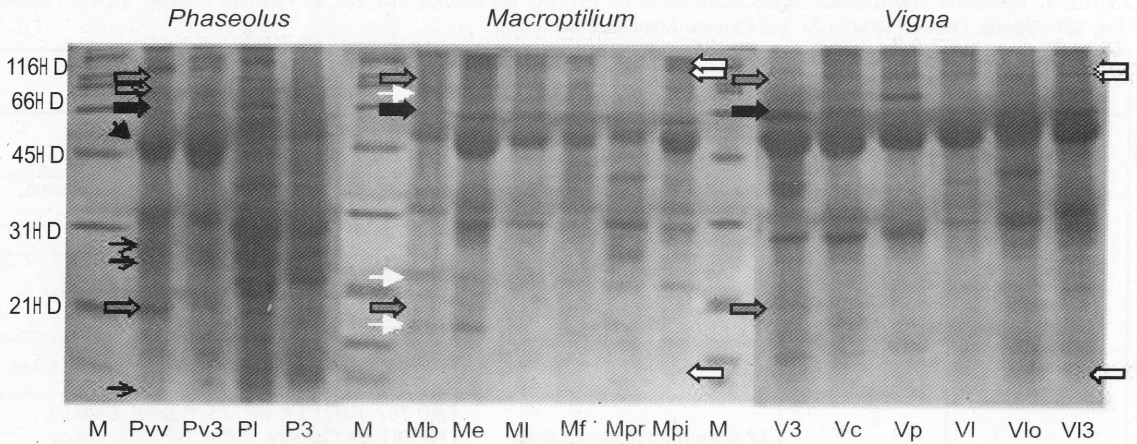
Genus	Section or subgenus	Species	Id. Number and Origin
<b>Macroptilium</b>	Section <i>Macroptilium</i>	<i>M. bracteatum</i> (Nees et Mart.) Maréchal et Baudet	RP769: Capital, Salta; RP1275 & SD503: Concordia, Entre Ríos.
		<i>M. panduratum</i> (Mart. ex Benth.) Maréchal et Baudet	RP1357: Guemes, Salta; HOC341: Chicoana, Salta
		<i>M. lathyroides</i> (L.) Urban	RP891: San Fernando, Chaco
		<i>M. erythroloma</i> (Mart. ex Benth.) Urban	RP892: San Fernando, Chaco; RP878, RP1100 & RP1284: San Ignacio, Misiones; RP1286: Candelaria, Misiones
		<i>M. longepedunculatum</i> (Benth.) Urban	RP 902, RP908 & RP910: Esquina, Corrientes
	Section <i>Microcochle</i>	<i>M. fraternum</i> (Piper) Juárez	RP 767, RP773 & RP775: Capital, Salta; RP781: La Caldera, Jujuy; RP1014: Jujuy, Jujuy
		<i>M. arenarium</i> (Bacigalupo) Drewes et Palacios	RP1299: Gualeguaychu, Entre Ríos; HOC373 & HOC374: Concepcion, Entre Ríos
		<i>M. psammodes</i> (Lindm.) Drewes et Palacios	RP1090 & RP1280: Gral. Alvear, Corrientes; RP1279: San Martin, Corrientes; RP1282: Capital, Misiones
<i>M. prostratum</i> (Benth.) Urban		RP844 & RP1205: Colon, Entre Ríos; RP856: Concordia, Entre Ríos; RP1073: Santo Tome, Corrientes; RP1287: San Javier, Misiones	
<b>Phaseolus</b>	Section <i>Phaseolus</i>	<i>P. augusti</i> Harms	HOC281 & PH282: Rosario de Lerma, Salta
		<i>P. lunatus</i> L var. <i>sylvester</i> (Burk.) Baudet	RP1210: Ledesma, Jujuy
		<i>P. vulgaris</i> L var. <i>aborigineus</i> (Burk.) Baudet	HOC347, HOC349, HOC351, HOC353, HOC 354, HOC359 & HOC360: Rosario de Lerma, Salta; RP 1010: Capital, Jujuy
		<i>P. vulgaris</i> L var. <i>vulgaris</i>	MMS110: Iruya, Salta
<b>Vigna</b>	Subgenus <i>Sigmoidotropis</i>	<i>V. adenantha</i> (G. Mey) Maréchal <i>et al.</i>	RP896: San Fernando, Chaco; RP1048: Famailá, Tucumán; RP1092: Gral. Alvear, Corrientes; RP1209: Ledesma, Jujuy; SD507: Isla Talavera, Buenos Aires
		<i>V. candida</i> (Vell.) Maréchal <i>et al.</i>	RP880: San Ignacio, Misiones; RP1288 & RP1289: San Javier, Misiones
		<i>V. peduncularis</i> (Kunth) Faluc. et Rendle	RP881 & RP1296: San Ignacio, Misiones; RP1294: San Javier, Misiones
	Subgenus <i>Vigna</i>	<i>V. luteola</i> (Jacq.) Benth.	RP956: Delta, Buenos Aires; RP1300: Gualeguaychu, Entre Ríos; RP1378: Predelta, Entre Ríos
	Subgenus <i>Lasiospron</i>	<i>V. longifolia</i> (Benth.) Verdcourt	RP1297: Paso de los libres, Corrientes
		<i>V. lasiocarpa</i> (Benth.) Verdcourt	RP1285 & RP1295: San Ignacio, Misiones
<b>Dolichopsis</b>		<i>D. paraguariensis</i> Hassler	RP1513: Asunción, Paraguay

Genus *Macroptilium* has three exclusive and constant bands: one around 116 kDa, one at 25 and another at 19 kDa. There aren't any marker bands characterizing each section. Only four of the nine species of *Macroptilium* here analyzed have marker bands: *M. bracteatum* and *M. panduratum* (section *Macroptilium*), and *M. fraternum* and *M. prostratum*

(sect. *Microcochle*).

Twenty-eight bands are identified in polypeptidic patterns of *Phaseolus augusti* and *P. lunatus*; twelve of these bands are shared by both species. *P. augusti* has seven marker bands, and *P. lunatus* has three. *P. vulgaris* has four exclusive and constant bands, three of these conforms the phaseolin protein placed around 45kDa. Accessions of





**Fig. 1.** Seed protein patterns (SDS-PAGE) of some species of *Phaseolus*, *Vigna* and *Macroptilium*: M Protein ladder, Pvv *P. vulgaris* var *vulgaris*, Pva *P. vulgaris* var *aboriginus*, Pl *P. lunatus*, Pa *P. augusti*, Mb *M. bracteatum*, Me *M. erythroloma*, Ml *M. lathyroides*, Mf *M. fraternum*, Mp *M. prostratum*, Mps *M. psammodes*, Va *V. adenantha*, Vc *V. candida*, Vp *V. peduncularis*, Vl *V. luteola*, Vlo *V. longifolia*, Vla *V. lasiocarpa*. The arrows indicate:  $\blacktriangle$  Phaseolin;  $\rightarrow$  *Phaseolus* marker bands;  $\blacksquare$  *Macroptilium* marker bands;  $\leftarrow$  Bands shared by *Macroptilium* and *Vigna*;  $\Rightarrow$  Bands shared by *Macroptilium*, *Vigna* and *Phaseolus*;  $\blackrightarrow$  Bands shared by the four genera.

*P. vulgaris* here studied show a phaseolin pattern type T, according to the phaseolin classification of Brown *et al.* (1981). This fraction doesn't appear in any of the accessions of *P. augusti* or *P. lunatus* (Fig. 1).

Electrophoregrams of the species of *Vigna* show thirty-four bands. Subgenus *Sigmoidotropis* has only one marker band, and subgenus *Vigna* has four. These subgenera have two bands in common, while *Vigna* and *Lasiospron* share three peptidic fractions. In subgenus *Lasiospron*, six exclusive and constant bands are observed in all their species. Each *Vigna* species have marker bands by which it can be distinguished.

*Dolichopsis* presents 12 exclusive protein bands, sharing another one with *Macroptilium* and *Vigna*.

The result of the UPGMA, based on the Jaccard coefficient, is shown in Figure 2. The clustering produces a very low distortion (cophenetic correlation value of 0.95). A minimum spanning tree (Fig. 3) has been derived from a matrix of taxonomic distances. Figure 4 shows the distribution of the species in a three-dimensional space, delimited by the first three principal coordinates derived from a Principal Coordinates Analysis.

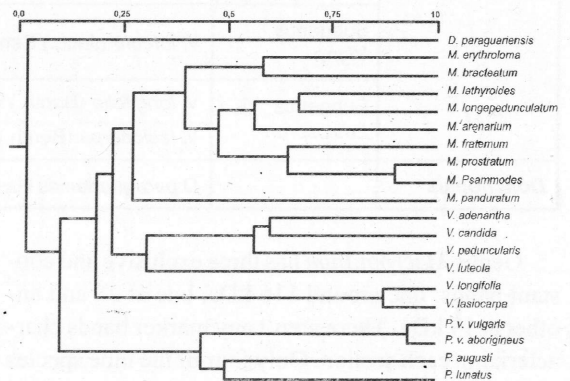
## DISCUSSION

Protein patterns corroborate genetic and taxonomic identities of the four genera analyzed, and con-

tribute with evidence to establish their relationships. The results here reported are in agreement with morphological observations previously performed (Verdcourt, 1971; Maréchal *et al.* 1978).

### Generic differentiation and relationships.

Protein profiles of four genera allow their clear identification by differences in the patterns of presence/absence of their polypeptidic bands. The phenogram shows the species of each genus included in unique clusters. Nevertheless, species of *Vigna* are included in two different clusters in the phenogram (Fig. 2). The identity of the genera can also be inferred viewing the Minimum Spanning Tree



**Fig. 2.** UPGMA cluster phenogram based on the Jaccard Coefficient:  $r$  (cophenetic value) = 0.95



(Figure 3). The position of the species on the space delimited by the three first Principal Coordinates (Fig. 4) shows that each genus constitutes a defined group. The species belonging to a particular genus are positioned as a well-defined constellation in different regions of the diagram. This indicates a greater similarity between species from the same genus than between species of different genera.

*Phaseolus* species compose a well-defined group in all the analysis here performed. Ordination analyses show that *Phaseolus lunatus* is the *Phaseolus* species that seems to be most closely related with the other genera (Fig. 3 and 4). This result is in agreement with the observations on flowers: *P. lunatus* ones are smaller than those of other species of *Phaseolus*, being its size similar to the flowers of *M. lathyroides* and *V. peduncularis* (Marèchal *et al. op. cit.*).

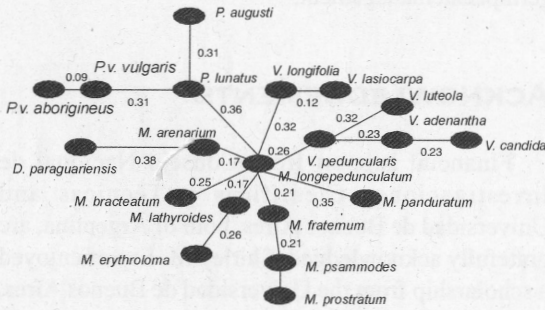


Fig. 3. Minimum Spanning Tree derived from a matrix of taxonomic distances.

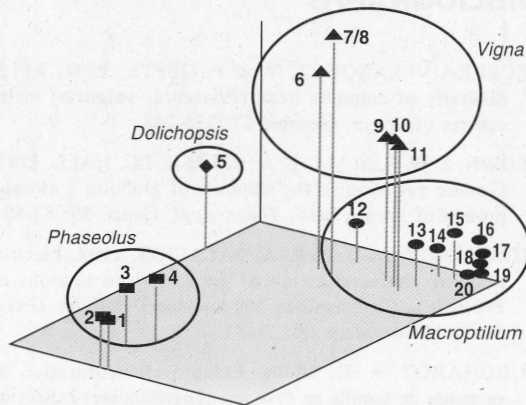


Fig. 4. Distribution of the species in a three-dimensional space, delimited by the first three principal coordinates, derived from a Principal Coordinates Analysis. (1 *P. vulgaris* var *vulgaris*, 2 *P. vulgaris* var *aborigineus*, 3 *P. augusti*, 4 *P. lunatus*, 5 *D. paraguayensis*, 6 *V. luteola*, 7 *V. longifolia*, 8 *V. lasiocarpa*, 9 *V. candida*, 10 *V. adenantha*, 11 *V. peduncularis*, 12 *M. panduratum*, 13 *M. erythroloma*, 14 *M. bracteatum*, 15 *M. arenarium*, 16 *M. longepedunculatum*, 17 *M. lathyroides*, 18 *M. fraternum*, 19 *M. psammodes* & 20 *M. prostratum*).

Numerical analysis shows a greater affinity between *Macroptilium* and *Vigna*. This fact is in agreement with morphological (Marèchal *et al. op. cit.*; Verdcourt 1970) and molecular data (Caicedo *et al.* 1999).

In UPGMA and Minimum Spanning Tree diagrams (Fig. 2 and 3), the species of *Vigna* appear in separate groups, showing that individuals of subgenera *Vigna* and *Sigmoidotropis* are more similar to *Macroptilium* than to subgenera *Lasiospron*; as was pointed out by Marèchal *et al.* (1978) based on morphological data.

*Dolichopsis* is the more distant genus. Previous works place *D. paraguayensis* closer to *Vigna* subgenus *Vigna* (Marèchal *et al. op. cit.*) or to *Vigna* subgenus *Sigmoidotropis* and another genus of Phaseolinae, *Strophostyles* Elliot, not analyzed here (Delgado Salinas *et al.* 1999; Goel *et al.* 2002). The inclusion of genera and species that don't grow in Argentina will improve the understanding of the placement of this monotypic genus.

*Infrageneric differentiations and relationships. Phaseolis L. emend Verdcourt.*

This genus is the focus of many works because of its economic importance. This is in contrast to the other three genera analyzed here, which are less studied than *Phaseolus*.

The phenogram based on seed protein analysis shows that *P. lunatus* constitutes a cluster with *P. augusti*, distant from *P. vulgaris* (Fig. 2). One of the greatest differences of *P. vulgaris* profiles is the presence of a conspicuous group of bands (phaseolin protein), which is absent in the other *Phaseolus* species (see black arrow in Fig. 1). Other studies also showed that Andean wild species of *Phaseolus* (like *P. augusti*), don't present this phaseolin (Maquet *et al.* 1999). Also, Maquet (*op. cit.*) reported that these species are more closely related to *P. lunatus*, as is shown here. This result agrees with other molecular data (Caicedo *et al.* 1999; Delgado Salinas *et al.* 1999).

The two varieties of *P. vulgaris* (var *vulgaris* and var *aborigineus*) differ only in one polypeptidic band. This high affinity supports the inclusion of both taxa in the same species, in agreement with the criteria adopted by Baudet, who studied morphological data (Palacios & Vilela, 1993).

Only cultivated forms of *P. vulgaris* var *vulgaris* are known. These cultivated forms of beans are the result of a long domestication process. This practice could lead to a reduction of the variability because of genetic drift, and an increase in the differentiation, so one can expect a different pattern between cultivated

forms and related wild species. This would be the reason of the great differences observed between *Phaseolus* species, which are similar to those found between sections and subgenera in *Macroptilium* and *Vigna* (Fig.2)

*Macroptilium* (Bentham) Urban.

There are very few previous studies on species from this genus, and almost none using molecular techniques. Drewes (1995 & 1996) pointed out the separation of *Macroptilium* in the sections *Microcochle* and *Macroptilium*, as proposed by Lackey (1983) based on morphological observations. The phenogram here performed do not show clearly this separation, neither do the Minimum Value Tree and the Principal Coordinates Analysis.

Species of section *Macroptilium* are highly different between each other, regarding morphological data. Phenogram based on electrophoretic results in particular shows that *M.lathyroides* and *M.longepedunculatum* belonging to sect. *Macroptilium* clearly have more closely relationships with species of sect. *Microcochle*. It is not possible to make comparisons about infrageneric data with previous works based on DNA sequence data. Delgado Salinas *et al.* 1999 and Goel *et al.* 2002 are the only two molecular systematic researchers who included species of *Macroptilium*, but they only analyzed two species.

*Vigna* Savi.

Electrophoretic results show that *V. luteola* forms a group with species of subgenus *Sigmoidotropis*, and presents low affinity with species of subgenus *Lasiospron* (Fig. 2, 3 and 4). In all the analyses, *V. luteola* is well separated from the other species. This result is in agreement with the subdivision of the genus performed by Marèchal *et al.* (1978), who placed *V. luteola* in a different subgenus (*Vigna*), while it doesn't support Lackey's system (1983), that groups *Vigna luteola*, *V. longifolia* and *V. lasiocarpa* in the same subgenus.

There is a great differentiation between protein profiles of *Lasiospron* and *Sigmoidotropis* species. This feature is in agreement with *Vigna* polyphyletic origin hypothesis, proposed by Delgado Salinas *et al.* (1993) using cpDNA sequences. The absence of marker bands that characterize the genus also support this hypothesis.

## CONCLUSIONS

In this work, it has been possible to study the relationships between Argentinean species belong-

ing to four genera of Phaseolinae (*Phaseolus*, *Dolichopsis*, *Vigna* and *Macroptilium*), which had never been analyzed together by using molecular markers. Seed protein electrophoretic banding patterns allowed the recognition of a genetic basis of the generic divisions, which were proposed using morphological and palinological data. This work supports the subgeneric divisions in *Phaseolus* and *Vigna* (*sensu* Marèchal *et al.*), but not in *Macroptilium*. Besides, the identification of all species of the genera *Vigna*, *Dolichopsis* and *Phaseolus*, and most of *Macroptilium* is possible using marker bands. An electrophoretic analysis including species of other American countries is in progress in our laboratory, and will be very important in order to get a greater knowledge of the genetics of the group, which is essential for its biotechnological improvement and germplasm management.

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