# Protein composition of *Cucurbita maxima* and *C. moschata* seeds

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## Abstract

Seeds of *Cucurbita maxima*, *C. moschata* and their interspecific hybrids were used to evaluate the intrapopulational and interpopulational variation of their protein composition. Three immunoprecipitating systems common to all the studied samples were detected by the Ouchterlony technique. Fourteen protein bands were identified by polyacrylamide gel electrophoresis (PAGE) whereas 23 bands were identified by sodiumdodecylsulfate (SDS)-PAGE. Using Western blotting (WB) also 23 bands were detected. The Jaccard's index of similarity calculated from SDS-PAGE and WB varied between 91 and 100 % for all the compared pairs of samples. These results demonstrate a high uniformity in the protein composition of all the samples and do not allow for their clear characterization.

*Additional key words*: antigenic composition, polyacrylamide gel electrophoresis, Western blotting.

## Introduction

The *Cucurbitaceae* are a highly specialized family of dicotyledons whose relationships with other angiosperms have been, and continue to be, controversial (Melchior 1964, Takhtajan 1980, Thorne 1983, Dahlgren 1983, Cronquist 1988), although most modern authors consider the family as an isolated evolutionary unit (Heywood 1993).

In order to explain the relationships between plant taxa biochemistry has been used to complement morphology, embryology, ecology and palinology (Giannasi and Crawford 1986, Stuessy 1990). In each particular case the variability of the composition of the proteins within any taxonomic group should be evaluated so that these data can be used to compare taxa of higher level.

The two species used in this investigation represent two different intrapopulational situations within the genus in reference to the intraspecific morphological variability seen in seeds and fruits. In the case of *C. moschata*, notable morphological uniformity has been maintained in the crop over the years. In contrast the variability observed in the size, shape, texture, and colour of the skin of the fruits of *C. maxima* is considerable, and there is also great variability in the size and colour of the seeds. The intraspecific and interspecific variability in the storage proteins in the seeds of the two *Cucurbita* species were studied by four methods in order to evaluate the genetic diversity.

## Materials and methods

Seeds of mature fruits of *Cucurbita moschata* Duch. ex Poir. (cv. Anquito) and of *Cucurbita maxima* var. *maxima* Duch. (cv. Criollo Plomizo) obtained from the horticultural belt of Médanos, partido of Villarino, province of Buenos Aires, were used. The seeds of *C. moschata* were collected from a crop where fourth generation seed from the original certified seed had been sown. The fruits were very homogenous in size, colour, shape and taste, and conform with the standard of the cultivar. The seeds of *C. maxima* were collected from a local crop which consisted of at least the tenth generation since the original commercial seed lot. In contrast to the former case the fruits and seeds of this population showed considerable variation (Fig. 1). For the interpopulational

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*Abbreviations*: PAGE - polyacrylamide gel electrophoresis; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; SI - Jaccard's index of similarity; WB - Western blotting.
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comparisons three samples of *C. maxima* (cvs. Zapuco and Redondo de Tronco, lines 551-557 and 569-574), two samples of C. *moschata* (cvs. Frontera and Paquito) and two interspecific hybrids (Veronés and an unnamed hybrid) were added. All seven samples of certified seed were obtained from INTA (Instituto Nacional de Tecnología Agropecuaria, Estación Experimental La Consulta, province of Mendoza).

Individual embryos of a single population of *C. maxima* and one of *C. moschata* were ground separately and the paste was washed several times with petroleum ether and then with acetone (1:5 m/v; 4 ºC; 30 min). The powder obtained (acetonic powder) was left to dry in the air and was then passed through a calibrated brass sieve (mesh Nº 60) and kept at 4 ºC until required. Alternatively, pools of ten seeds taken from a sample of certified seed were processed in the same way. Acetonic powder was suspended in Tris-glycine buffer (0.025 M, pH 8.3; 1:20 m/v) at 4 ºC for 24 h and centrifuged (10 min; 5000 *g*). The supernatants obtained (crude extracts) were separated and stored at -20 ºC until required. The protein content of the extracts was determined by the Coomassie Blue method (Spector 1978).

For antisera production the inoculants were prepared by emulsifying equal parts of crude extract of *Cucurbita maxima* with complete Freund's adjuvant. Rabbits (New Zealand race) were injected with 0.5 $cm^3$ every week until enough antibodies were obtained. The antisera were kept at -20 ºC until required (Castro *et al*. 1999).

The method described by Villamil and González (1993) was followed to prepare the plates for double diffusion in two dimensions (Ouchterlony's micro-method). As concern polyacrylamide gel electrophoresis (PAGE), the method described by Margni *et al*. (1996) was followed using a *Hoefer Mighty Small SE 245* apparatus (*Amersham Pharmacia Biotech*, San Francisco, USA) with plates of 9 cm × 10 cm × 1.5 mm, prepared according to Villamil and Fairbrothers (1974). PAGE was performed in a *SE 280 Hoefer* chamber (300 V, *ca*. 18 mA) during 90 min. For sodiumdodecylsulphate (SDS)-PAGE the Schägger and von Jagow (1987) method was followed, using 13.5 % acrylamide for the separating gel and 8 % for the stacking gel. One part of the protein extract was treated with Laemmli buffer (Laemmli 1970). The run was carried out (90 V, *ca*. 44 mA) for 6 h. For both methods staining was carried out with Amido Schwartz.

For Western blotting (WB) the procedure described by Towbin (1979) was followed. The proteins separated by SDS-PAGE were transferred to a pore size 0.45 μm nitrocellulose membrane (*Sigma*, St. Louis, USA) in a *Hoefer TeSERIES* chamber (350 V, *ca*. 75 mA) for 90 min. After blocking and washing the membrane was then incubated with anti-*Cucurbita maxima* polyclonal serum at room temperature for 2 h, washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (*Sigma A-6154*) as the secondary antibody at a 1:1000 dilution. The colour was developed by treatment in the dark with 4-chloro-naphthol and hydrogen peroxide.

The obtained gels (PAGE and SDS-PAGE) and membranes (WB) were scanned on a *G-710* equipment (*Bio-Rad*, Hercules, California) and the corresponding densitometric graphs were traced using the *Quantity One 4.0.2* programme (*Bio-Rad*). The bands were considered present when seen on the gel and as peaks in the densitometric graphs. The degree of similarity between samples was determined by Jaccard´s Similarity Index [SI = No. of the common bands/(No. of common bands + No. of non-common bands)].

## Results

Only three immunoprecipitating systems could be detected by the Ouchterlony method, using the antiserum obtained against *C. maxima* as a reference. No significant differences were seen in the composition of antigens obtained from various populations of *C. maxima* and *C. moschata*.

With PAGE sharp patterns and good repeatability was obtained. Nevertheless, in certain parts of the gel, particularly in the vicinity of the more intense bands, detection of the weaker bands became difficult. Protein extracts of individual seeds from the same fruit showed very uniform qualitative patterns, with only minimal differences in the intensity of the same band for different individuals. A maximum number of 14 bands was detected for all the samples, lower than that detected by the two following methods. Consequently, this method was not used further for comparative studies in this investigation.

Using SDS-PAGE also no significant differences were found in the protein composition of individual seeds of the same fruit, nor between pools of seeds from different fruits of the same population in either of the two species. A great homogeneity in the different populations was also found (Figs. 2 and 3). A total of 23 bands were detected by this method. Only two weak bands were not detected in all samples: band No. 15 was not found in one sample, while band No. 22 was not seen in three samples (Fig. 2). Jaccard's Similarity Index varied between 91 and 100 % for all pairs of samples compared (Table 1).

The application of WB showed similar qualitative results. However, some minor quantitative differences between pools of seeds obtained from the same population were detected. These variations, nevertheless, could not be proved significant for a clearcut characterization of samples within either species.

The results obtained comparing seed pools from different populations of *C. maxima*, *C. moschata* and their hybrids are shown in Fig. 4. At this level also the

patterns obtained for all samples showed great similarity (Fig. 5). A total of 23 bands were detected, of which 21 were common to all samples. Band No. 6 was only present in three samples, while band No. 8 was detected in four samples (Fig. 4). Jaccard´s Index between pairs varied from 91 to 100 % (Table 2).

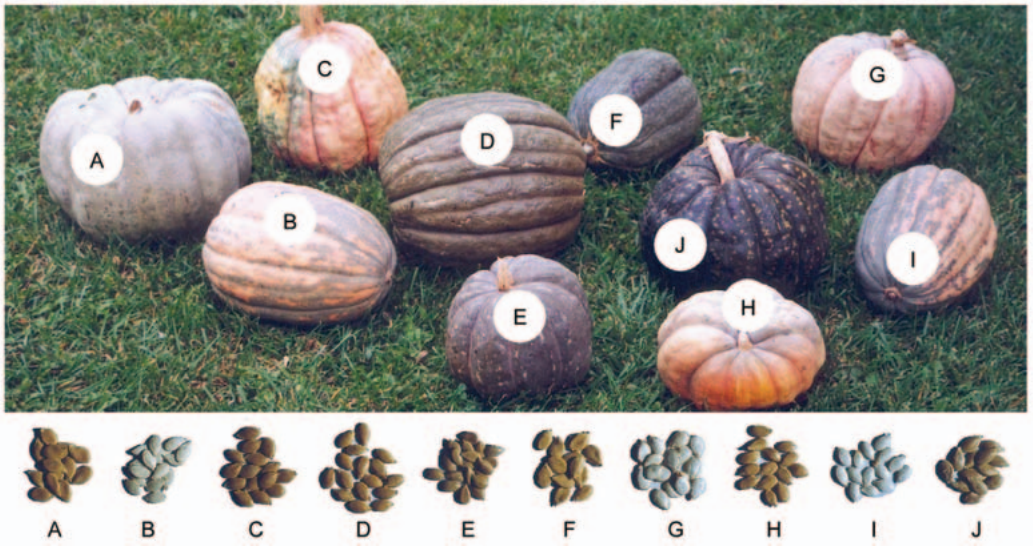


Fig. 1. Morphological variability within one single population of *Cucurbita maxima* cv. Criollo Plomizo. The seeds were obtained from the fruit indicated with the corresponding letter.

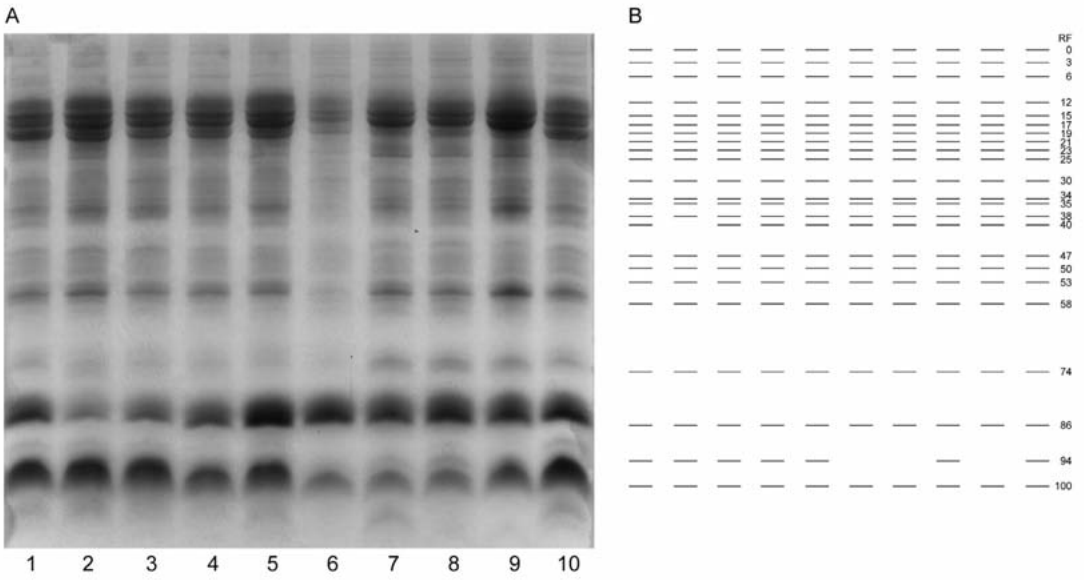


Fig. 2. *A*: Patterns of general proteins (SDS-PAGE) obtained from four cultivars of *C. maxima* (*lanes 1* and *10*: cv. Zapuco; *lane 2*: cv. Redondo de Tronco lines 569-574; *lane 3*: cv. Redondo de Tronco lines 551-557; *lane 4*: cv. Criollo Plomizo), two hybrids of *C. maxima* × *C. moschata* (*lane 5*: cv.Veronés; *lane 6*: unnamed hybrid) and three of *C. moschata* (*lane 7*: cv. Frontera; *lane 8*: cv. Paquito; *lane 9*: cv. Anquito). *B*: Diagramatic representation of *A*.

## Discussion

The two species included in this study are diclino-monoecious plants with exalbuminous seeds and outcross pollination. It can therefore be supposed that each seed represents an individual genotype, whereas the pools obtained from different fruits partially represent the genetic pool of each population.

Double diffusion in two dimensions in semisolid media (Ouchterlony 1964) has been frequently used in

plant serosystematics and there is an extensive bibliography on the subject (Fairbrothers 1968, 1969, Stuessy 1990). In this study the same antisera were used for this technique and for WB. Although the amount of information obtained by the latter was larger, the results using both methods were coincident.

Enzymatic activity in vegetative tissues has been used to assign *C. maxima* and *C. moschata* to different groups of species (Puchalski and Robinson 1990, Decker and Wilson 1987), and esterase and peroxidase activity has been mentioned in seeds of Cucurbitaceae (Jacks 1990). However it could not be detected by PAGE in our study when using dormant seeds as the protein source.

SDS-PAGE and WB runs resulted in a larger number of bands and sharper patterns than PAGE. The data obtained from electrophoresis and from WB were congruent and demonstrated great uniformity between all the samples, even those belonging to two different species (*C. maxima* and *C. moschata*). No bands were found in the samples of seed pools that were not present in some of the individual genotypes of the corresponding population, and there were no significant differences in the seed pools from different fruits of the same population.

Each crude protein extract triggers a polyclonal humoral response, resulting in a population of antibodies capable of recognizing the common immunogenic components (Benjamin *et al.* 1984), making it possible to confirm whether two bands with the same mobility also share the same antigenic properties (Chase and Kuhns 1970). The intensity of the bands as revealed by SDS-PAGE depends on their concentration in the extract, and those detected by WB depend on this, and on their specific antigenicity.

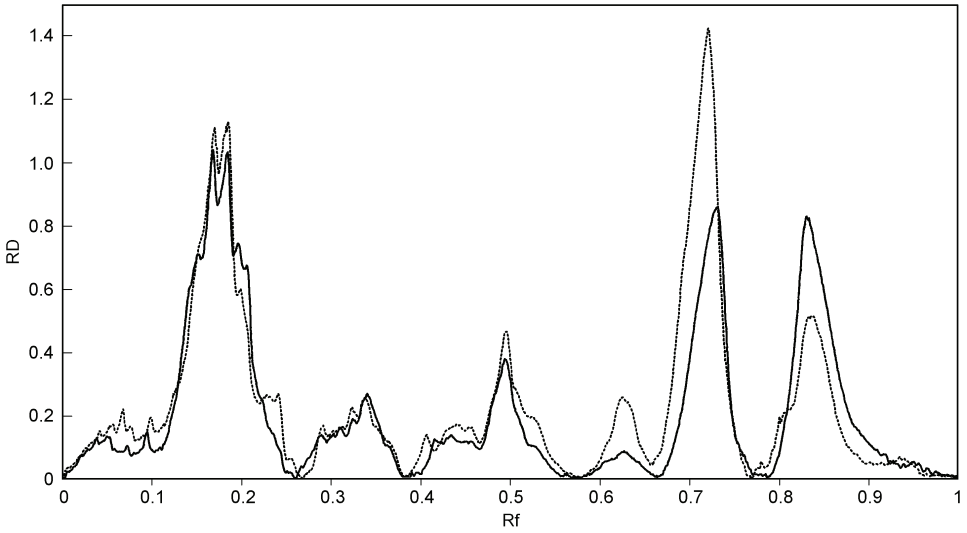


Fig. 3. Densitographic traces obtained from Fig. 2 (SDS-PAGE) corresponding to two cultivars of *Cucurbita* (*solid line*: *C. maxima* Criollo Plomizo, *dotted line*: *C. moschata* Anquito)

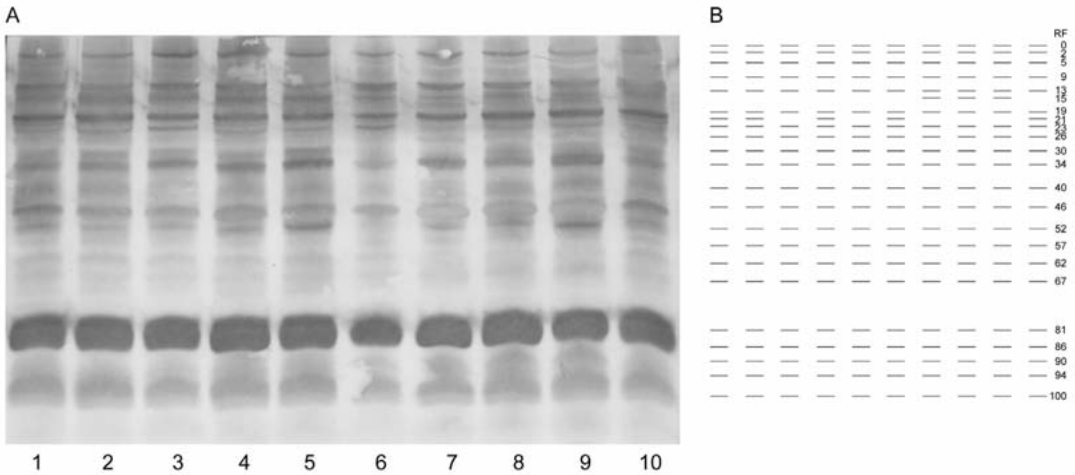


Fig. 4. *A*: Patterns of antigenic activity (WB) obtained from four cultivars of *C. maxima* (*lanes 1* and *10*: cv. Zapuco; *lane 2*: cv. Redondo de Tronco lines 569-574; *lane 3*: cv. Redondo de Tronco lines 551 - 557; *lane 4*: cv. Criollo Plomizo), two hybrids of *C. maxima* × *C. moschata* (*lane 5*: cv. Veronés; *lane 6*: unnamed hybrid) and three of *C. moschata* (*lane 7*: cv. Frontera; *lane 8*: cv. Paquito; *lane 9*: cv. Anquito). *B*: Diagramatic representation of *A*.

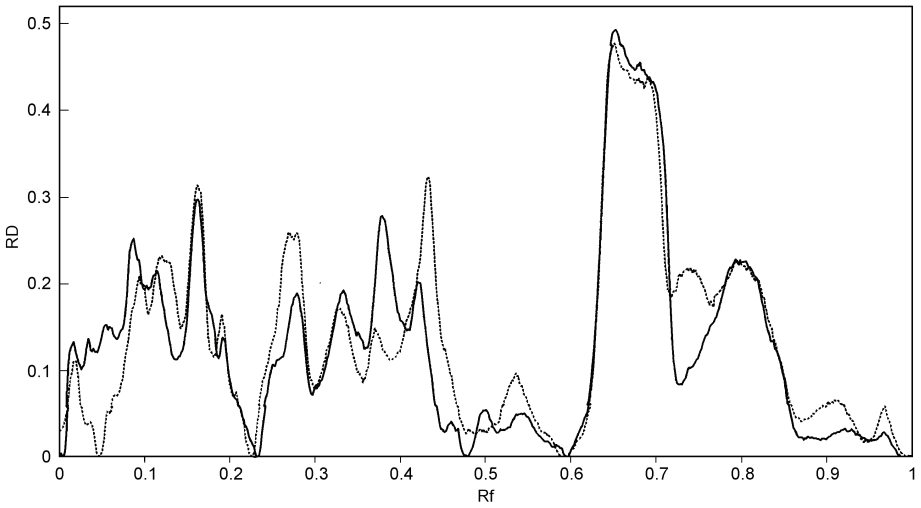


Fig. 5. Densitographic traces obtained from Fig. 4 (WB) corresponding to two cultivars of *Cucurbita* (*solid line*: *C. maxima* cv. Zapuco, *dotted line*: *C. moschata* cv. Anquito).

Table 1. Similarity Index for pairs of cultivars of *Cucurbita maxima* (1 and 10: cv. Zapuco; 2: cv. Redondo de Tronco lines 569 - 574; 3: cv. Redondo de Tronco lines 551-557; 4: cv. Criollo Plomizo), hybrids of *C. maxima* × *C. moschata* (5: *cv.* Veronés; 6: unnamed hybrid) and *C. moschata* (7: cv. Frontera; 8: cv. Paquito; 9: cv. Anquito) using SDS-PAGE.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |
| 2 | 96 | 100 | 96 | 96 | 96 | 91 | 91 | 96 | 91 | 96 |
| 3 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |
| 4 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |
| 5 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |
| 6 | 96 | 91 | 96 | 96 | 96 | 100 | 100 | 96 | 100 | 96 |
| 7 | 96 | 91 | 96 | 96 | 96 | 100 | 100 | 96 | 100 | 96 |
| 8 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |
| 9 | 96 | 91 | 96 | 96 | 96 | 100 | 100 | 96 | 100 | 96 |
| 10 | 100 | 96 | 100 | 100 | 100 | 96 | 96 | 100 | 96 | 100 |

The extracts obtained for ten individual genotypes from the same fruit in *C. moschata* and *C. maxima* did not show any significant differences regardless of the intraspecific morphological variability of each taxon. Also a great similarity between the seed protein components of samples taken from co-specific populations, and even between two systematically well characterized species of *Cucurbita* (Millán 1968, Della Gaspera 1994, Lira Sade 1995), has been demonstrated. It should be noted that the only differences observed between the samples correspond to bands of very low intensity in the patterns obtained. Therefore we have not considered these differences as of systematic significance, but have rather attributed them to experimental difficulties of detecting the presence of components in very low concentrations. On the other hand, differences of intensity between bands in the same position in different extracts suggest quantitative differences between the corresponding samples. Results similar to these were obtained comparing cultivars of *Cucumis melo*, another species of economic importance in the same family (Bonfitto *et al.* 1999) and in a species belonging to a distant family of dicots (Haider and El-Shanshoury 2000, Jha and Ohri 2002).

Table 2. Similarity Index for pairs of cultivars of *Cucurbita maxima* (1 and 10: cv. Zapuco; 2: cv. Redondo de Tronco lines 569-574; 3: cv. Redondo de Tronco lines 551 - 557; 4: cv. Criollo Plomizo), hybrids of *C. maxima* × *C. moschata* (5: cv. Veronés; 6: unnamed hybrid) and *C. moschata* (7: cv. Frontera; 8: cv. Paquito; 9: cv. Anquito) using WB.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 100 | 95 | 100 | 95 | 100 | 91 | 91 | 91 | 100 |
| 2 | 100 | 100 | 95 | 100 | 95 | 100 | 91 | 91 | 91 | 100 |
| 3 | 95 | 95 | 100 | 95 | 100 | 95 | 95 | 95 | 95 | 95 |
| 4 | 100 | 100 | 95 | 100 | 95 | 100 | 91 | 91 | 91 | 100 |
| 5 | 95 | 95 | 100 | 95 | 100 | 95 | 95 | 95 | 95 | 95 |
| 6 | 100 | 100 | 95 | 100 | 95 | 100 | 91 | 91 | 91 | 91 |
| 7 | 91 | 91 | 95 | 91 | 95 | 91 | 100 | 100 | 100 | 91 |
| 8 | 91 | 91 | 95 | 91 | 95 | 91 | 100 | 100 | 100 | 91 |
| 9 | 91 | 91 | 95 | 91 | 95 | 91 | 100 | 100 | 100 | 91 |
| 10 | 100 | 100 | 95 | 100 | 95 | 100 | 91 | 91 | 91 | 100 |

Data obtained in this work support the hypothesis that the evolution of the storage proteins in the *Cucurbitaceae* has followed more conservative patterns (Jensen and Penner 1980, Jensen and Büttner 1981) than that of the proteins with enzymatic activity extracted from vegetative organs (Puchalski and Robinson 1990) and than the morphological characters.

The uniformity observed among samples of these two

species appears to be significant for the characterization of the genus *Cucurbita*. Further studies including more species of this and other genera would demonstrate if the seed storage proteins offer a suitable tool for comparative studies at higher taxonomic levels in the family *Cucurbitaceae*.

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