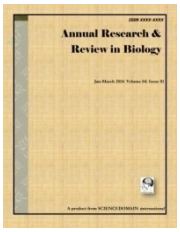

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Morphological Seeds Descriptors for Characterize and Differentiate Genotypes of *Opuntia* (Cactaceae, Opuntioideae)

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Autores' contributions

This work was carried out in collaboration between both authors. Author EVM wrote the protocol and supervised the work in all its aspects. Author SS collected the samples, worked in the practical part and written the draft of the manuscript. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: In this paper, a morphometric study was carried out to analyze the variation of seeds of *Opuntia* accessions using several statistical approaches. The main objective was to select morphological seeds variables for characterization and differentiation of *Opuntia* genotypes.
Place and Duration of Study: The research was conducted in Crops Science Department of the Chapingo Autonomous University, Mexico. The sample collection was down in 2012. Seed data was obtained during 2013.
Methodology: A total of 110 *Opuntia* accessions (some classified and other ones with no specific taxonomic assignation), one accession of *Cylindropuntia* sp. (*Cactaceae*, *Opuntioideae*) and two other outgroups (*Cactaceae, Pachycereae*) were used. Nineteen internal and external seeds variables were obtained using image analysis. Basic statistical analysis, analysis of variance, principal component analysis, cluster and discriminant analysis were performed.
Results: Highly significant differences among accessions for all seed characters were showed. The most of the variables showed a coefficient of variation less than 10%. From de 19 variables studied, two variables did not contribute significantly to discriminate between accessions as determined by Step wise Discriminant Analysis. The principal

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components analysis showed that the first three components accounted for 83.35% of the variability; the first component contributed twice the variability (48.12%) respect to the second one (23.77%). Tukey's test determined that the Feret diameter and seed area were the most discriminating variables between the 7 groups resulting from de cluster analysis.
Conclusion: The selected variables, using several statistical approaches, were of interest for the characterization and identification of the different *Opuntia genotypes*. The morphological seed characteristics responsible for the separation between genotypes were Area, Major Axis Length, Minor Axis Length, Feret Diameter and Weight. These variables have a high discriminatory power and can be taken into account as potential parameters for genotypes assignation within the *Opuntia* genus.

Keywords: Opuntia; seed morphology; longitudinal section; embryo; multivariate analysis.

1. INTRODUCTION

The *Opuntia* genus (*sensu stricto*; *Cactaceae, Opuntioideae*) refers to cacti with flat pseudostems or cladodes, cyathiform tubular perianths with shorter stamens than the tepals [1]. This genus includes191-215 species [2,3], originating in the north and south of the American continent; some of them were relatively new distributed worldwide. The difference in the number of species is mainly due to the nomenclature problems occurred not only in *Opuntia* but also within the other genera of Opuntioideae subfamily [4]. In Mexico, about 83 species are recognized which renamed "nopal" [5]. *Opuntia* plants are closely associated with the Mexican culture development; since they were used for human food, such as vegetables and fruit, in semiarid regions of the southwestern areas of Tamaulipas and Tehuacan Valley from 9.000 to 11.000 years ago [6]. The tender cladodes are also used to prepare juice, jelly, honey, jam and pasta, and the oil is extracted from its seeds. *Opuntia* plants are also used as fodder and for the restoration and vegetation in arid and semi-arid environments. The cultivated *Opuntia* species include: *O. megacantha*, *O. streptacantha*, *O. albicarpa*, *O. amyclaea*, *O. robusta*, *O. hyptiacantha*, *O. cochinillifera*, *O. joconostle*, and *O. matudae*, among others [4,7].

Today, commercial varieties are generally octaploid but the ploidy level is varied from 2X to 8X [8], although their ancestry is unknown. Moreover, many authors report the difficulty of the correct assignment of cultivated genotypes in a defined taxon [4,9]. The continuous morphological variation, the lack of clear descriptors for each specie, the high phenotypic plasticity and the ploidy variation numbers have led to problems in species delimitation and genotypes assignation [4]. As a result of incorrect assignments, the same varieties are often classified as belonging to different species, and in other cases they are considered to be hybrids among unknown parentals.

The classification of *Opuntia* genotypes has been based only on morphological characteristics, especially fruits and cladodes variation; and the specie determination is based on taxonomic keys by comparing few wild individuals [10]. However, the differences that may exist at the time of the identification can be inconsistent and resulting form environmental variation. To overcome this, alternatives approaches are suggested, one of them is based on quantitative approaches to grouping genotypes by similarities between traits measured in cladodes, fruits and flowers [11,12]. Valdez-Cepeda et al. [13] reported that the presence/absence of spines and their lengths are useful traits for morphological

characteristics. However, Felker et al. [8] suggested that the absence of spines should not be considered as basis for taxonomic classification, because this character has simple inheritance. In this regard, several features of the spine such as length, thickness, inclination, color and layout, as well as their number by areola are partially dependent on the environment conditions, such as availability of nutrients and moisture [14]. For these reasons, spineless genotypes have been classified as *O. ficus-indica* and genotypes with spines as *O. megacantha*, *O. streptacantha* and *O. amyclaea* [15]. Unlike at other times, genotypes with spines have been classified as *O. ficus-indica* [16]. Kiesling [6] considered to *O. amyclaea*, *O. megacantha* and *O. streptacantha* as synonyms of *O. ficus-indica*, and he divided this latter species into two botanical forms: a) *O. ficus-indica* f. *Amyclaea*, with presence of spines; b) *O. ficus-indica* f. *Ficus-indica*, spineless. Actually, the presence of spines in the cladodes is an inadequate feature to classify *Opuntia* species [16]. Caruso et al. [4] reported that the character of spinescence might have been developed multiple times during the evolution of the genus, and might have been selected from different populations. In other researches, *Opuntia* varieties have been differentiated and described using molecular markers such as RAPD [17], ISSR [18,19] and SSR [4,20]. Based on molecular data, morphological and biogeographic distribution, Labra et al. [9] suggest that *O. ficus-indica* should be regarded as the domesticated form of *O. megacantha*. Furthermore, based on Bayesian phylogenetic analyzes of nrITS sequences, Griffith [21] affirmed the hypothesis that *O. ficus-indica* is a close relative of an arborescent group with fleshy fruits of central and southern Mexico, and the taxonomic concept of *O. ficus-indica* may include clones derived from multiple lineages. However, using SSR markers, Caruso et al. [4] could not separate *O. ficus-indica* from other arborescent species. Moreover, Helsen et al. [20] attempted to distinguish two varieties of *O. echios* (echios and gigantea) using SSR markers, but the results again emphasized that the current taxonomic differentiation was not supported by molecular data.

Despite the rapid advances in molecular techniques and the interest for the characterization of plant genetic resources with these tools, the morphological characterization should always be considered as useful for the use in collections and description studies [22]. Morphological characterization is necessary because it provides to users valuable information about individual accessions, the relationship between the characters, and the structure of the collections [23]. Meanwhile, statistical methods, including principal component analysis and cluster, can be used as effective tools to assess variability among genotypes. The lack of a general consensus on the taxonomy of *Opuntia* genus makes difficult the correct assignation of genotypes in the collections. Furthermore, the identification of highly discriminating descriptors is important to obtain an efficient and reproducible classification of the species and varieties and to adapt the list of descriptors for specific purposes.

In none of the characterization studies in *Opuntia* it has been taken into account the differences that may exist between *Opuntia* seed and its possible discriminatory potential. The potential taxonomic significance of seed morphology has been recognized in several groups of plants [24-26], and the delimitation of the genera based on these characters was in agreement with the results of molecular studies. Meanwhile, the seed image analysis has gained great importance for the species identification of wild plants and as well as seeds of species and varieties of agronomic importance [27], proving, thus, be a useful tool for taxonomic studies.

Therefore, the objectives of the present research were to: (1) Investigate the discriminatory potential of variables of *Opuntia* seeds, accurately measured using reliable and repeatable

method such as image analysis, and (2) Determine the potential use of these variables for classification and taxonomic position in this genus.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fruit samples of 110 *Opuntia* accessions were collected at two locations; CRUCEN-UACh, Zacatecas and "Nopalera" UACh, Texcoco germplasm banks, Mexico (Table 1). Ten fruits from at least three individuals plants of each accession were harvested at commercial maturity, from which all mature seeds were removed manually, then dried in the open air, cleaned off any remaining pulp and only viable seeds were stored in paper-bags at room temperature until use. One sample of *Cylindropuntia* sp. one other of pitahaya (*Hylocereus undatus)* and one pitaya (*Stenocereus thurberi*) were included as out groups. Some of *Opuntia* accessions are classified in delimited species but other ones have no specific assignation (Table 1).

2.2 Seed Measurements

A total of 19 characteristics of seeds and 113 accessions were used to build the data set and statistical analysis to characterize *Opuntia* accessions and to determine the potential use of these characteristics for taxonomy. For external morphology, 24 seeds/repetition (3 repetitions) of each sample were randomly chosen to take pictures of them with a digital camera. For internal morphology, the technique developed by Guerrero-Muñoz et al. [28] was applied. Five clean and viable seeds/repetition (3 repetitions) were adhered to the surface of a glass slide and oriented parallel to the median section. These seeds were polished symmetrically and parallel to median section (longitudinal section) with fine sandpaper until the mid-section and they were viewed and photographed individually under a Leica EZ4 stereoscope (Leica Microsystems, Switzerland) with an integrated camera.

All obtained images were processed using Photoshop CS5 12.0 program to define the area of seed, embryo, perisperm and funicular seedcoat (testa). The seed variables were then obtained by UTHSCSA Image Tool software v 3.00. The methodology described by Mebatsion et al. [29] was adopted to improve the contrast. To determine the weight of seeds, 100 fully developed seeds (three replicates) were counted and weighed with analytic balance (220g/0.1mg) (ABS 220-4; Karn and GmbH).

The variables obtained from entire seeds were: Area = the area of the object measured as the number of pixels in the polygon; Perimeter = the length of the outside boundary of the object; Major Axis Length = the length of the longest line that can be drawn through the object: Minor Axis Length = the length of the longest line that can be drawn though the object perpendicular to the major axis; Elongation = the ratio of the length of the major axis to the length of the minor axis (if the value is 1, the object is roughly circular or square, whereas it is more elongated when the ratio decreases from 1); Roundness = if the ratio is equal to 1, the object is a perfect circle, when the ratio decreases from 1, the object departs from a circular shape, calculated as $R = [(4\pi \times \text{area})/\text{perimeter}^2]$; Feret Diameter = the diameter of a circle having the same area as the object, calculated with the formula: FD=square root of $[(4 * \text{area})/\pi]$; Compactness = provides a measure of the object's roundness: at 1 the object is roughly circular, when it decreases from 1, the object results less circular, calculated as C = FD/Major Axis Length.

Table 1. List of prickly pear accessions from Mexico evaluated to study seed morphometric diversity of *Opuntia* spp

N	Accessions	*Opuntia*species	N	Accessions	*Opuntia*species
1	Alfajayucan	*O. albicarpa* Scheinvar	58	Liso Forrajero	*Opuntia* sp.
2	Alteña Blanco	*Opuntia* sp.	59	Mango	*O. albicarpa* Scheinvar
3	Alteña Rojo	*Opuntia* sp.	60	Mansa Amarilla	*Opuntia* sp.
4	Amarilla 2289	*Opuntia* sp.	61	Memelo	*O. affinis hyptiacantha*
5	Amarilla 3389	*Opuntia* sp.	62	Milpa Alta	*O. ficus-indica* (L.) Mill.
6	Amarilla China	*Opuntia* sp.	63	Montesa	*Opuntia* sp.
7	Amarilla Jalpa	*O. ficus-indica* (L.) Mill.	64	Morada	*O. megacantha* Salm-Dyck.
8	AmarillaJarro	*O. megacantha* Salm-Dyck.	65	Morada T10	*O. megacantha* Salm-Dyck.
9	Amarilla Milpa Alta	*Opuntia* sp.	66	Naranjón Legítimo	*O. albicarpa* Scheinvar
10	Amarilla Miquihuana	*O. lasiacantha* Pfeiffer	67	Naranjona	*Opuntia* sp.
11	Amarilla Montesa	*O. megacantha* Salm-Dyck	68	*O. cochillinifera*	*O. cochillinifera*
12	Amarilla Oro	*O. albicarpa* Scheinvar	69	Oreja de Elefante	*O. undulate* Griffiths
13	Amarillo Plátano	*O. megacantha* Salm-Dyck.	70	Pabellón	*O. ficus-indica* (L.) Mill.
14	Amarilla San Elías	*Opuntia* sp.	71	Pachón	*Opuntia* sp.
15	Amarilla Zacatecas	*O. megacantha* Salm-Dyck.	72	Pelón Rojo	*O. ficus-indica* (L.) Mill.
16	Amarillo	*O. megacantha* Salm-Dyck.	73	Pico Chulo	*O. megacantha* Salm-Dyck.
17	Amarillo Aguado	*Opuntia* sp.	74	Pico de Oro	*Opuntia* sp.
18	Atlixco	*O. ficus-indica* (L.) Mill.	75	Pitahaya	*Hylocereu sundatus*
19	Bam	*Opuntia* sp.	76	Pitaya	*Stenocereus thurberi*
20	Blanca de Castilla	*O. puntia*sp.	77	Plátano	*Opuntia* sp.
21	Blanca del cerro	*Opuntia* sp.	78	Princesa	*Opuntia* sp.
22	Blanca San José	*O. albicarpa* Scheinvar	79	Red Villa Puebla	*O. puntia*sp.
23	Blanco Atlacomulco	*Opuntia* sp.	80	Reyna	*O. albicarpa* Scheinvar
24	Blanco Huexotla	*Opuntia* sp.	81	Reyna Crucen	*Opuntia* sp.
25	Bola de Masa	*O. albicarpa* Scheinvar	82	Roja Azteca	*O. megacantha* Salm-Dyck.
26	Burrona	*O. albicarpa* Scheinvar	83	Roja San Martín	*O. megacantha* Salm-Dyck
27	Cacalote	*O. cochinera* Griffits	84	Rojo 3589	*Opuntia* sp.
28	Camuezo	*O. megacantha* Salm-Dyck.	85	RojoLirio	*O. megacantha* Salm-Dyck.
29	Cardón	*O. streptacantha* Lem.	86	RojoLiso	*Opuntia* sp.
30	Cardón Blanco	*O. streptacantha* Lem.	87	RojoUACh	*Opuntia* sp.
31	Cardona de Castilla	*O. streptacantha* Lem.	88	Rojo Vigor	*O. ficus-indica* (L.) Mill.
32	Cascarón	*O. chaveña*	89	Rosa de Castilla	*O. megacantha* Salm-Dyck.
33	Chapeada	*O. albicarpa* Scheinvar	90	Rubí Reyna	*O. megacantha* Salm-Dyck.
34	CharolaTardía	*O. streptacantha* Lem.	91	San Juan	*Opuntia* sp.
35	Chicle	*O. ficus-indica* (L.) Mill.	92	Sangre de Toro	*Opuntia* sp.
36	Col. Barr. Chica	*Opuntia* sp.	93	Sanjuanera	*O. lasiacantha* Pfeiffer
37	Col. Barr. Grande	*Opuntia* sp.	94	Solferino	*Opuntia* sp.
38	Color de Rosa	*O. albicarpa* Scheinvar	95	Tapón Aguanoso	*O. robusta* H.L. Wendland
39	Colorada	*Opuntia* sp.	96	Tapónrojo	*O. robusta* H.L. Wendland
40	Copena CEII	*O. ficus-indica* (L.) Mill.	97	Tapona de Mayo	*O. robusta* H.L. Wendland
41	Copena F1	*O. ficus-indica* (L.) Mill.	98	Tobarito	*Opuntia* sp.
42	Copena T12	*O. ficus-indica* (L.) Mill.	99	Toluca	*Opuntia* sp.
43	Copena T5	*O. ficus-indica* (L.) Mill.	100	Torreoja	*O. megacantha*Salm-Dyck.
44	Copena V1	*O. ficus-indica* (L.) Mill.	101	Trompa Cochino	*Opuntia* sp.
45	Copena Z1	*O. albicarpa* Scheinvar	102	Tuna Mansa	*O. albicarpa*Scheinvar
46	Cristalina	*O. albicarpa* Scheinvar	103	Tuna Rosa	*O. albicarpa* Scheinvar
47	Cylindropuntia	*Cylindropuntia* sp.	104	Tuna Sandia	*Opuntia* sp.
48	Fafayuca	*O. albicarpa* Scheinvar	105	Var S/I	*Opuntia* sp.
49	Gavia	*O. albicarpa* Scheinvar	106	Verdulero	*Opuntia* sp.
50	Green Guanajuato	*Opuntia* sp.	107	Villanueva	*O. albicarpa* Scheinvar
51	Huatusco	*Opuntia* sp.	108	X_Blanco	*O. joconostle* F.A.C. Weber
52	INIFAP	*Opuntia* sp.	109	X_Chivo	*Opuntia* sp.
53	Jade	*Opuntia* sp.	110	X_Colorado	*O. joconostle* F.A.C. Weber
54	Jarilla Grande	*Opuntia* sp.	111	X_Cuaresmero	*O. matudae* Scheinvar
55	Laltus	*Opuntia*s p.	112	X_Manzano	*O. joconostle* F.A.C. Weber
56	Larreguin	*Oficus-indica* (L.) Mill.	113	X_Rojo	*Opuntia* sp.
57	Liso Amarillo	*Opuntia* sp.			

The variables obtained from the median section of the seeds (internal morphometric) were: Area and Perimeter of embryo, Area and perimeter of perisperm and funicular seed coat. Ratios between variables were also calculated (Table 2).

2.3 Statistical Analysis

A total of 19 quantitative variables were analyzed (Table 2). Both internal and external morphometric seed variables were analyzed together because both types of variables may respond in similar ways to environmental and genetic conditions; therefore the two types of data are similar. Descriptive statistics were performed for all variables, and the following parameters were obtained: Mean, minimum, maximum and coefficient of variation. The analysis of variance (ANOVA) was applied to detect discriminant variables among genotypes, and multiple comparisons (Tuckey's test) were computed to identify the difference between each pair of accessions (P<.001). A variable reduction technique was used to select the most discriminating variables among the 19 measured traits. Stepwise discriminant analysis was used to select traits that were included in the classification model. A significance level of 0.001 of an F test from an analysis of covariance was imposed to choose the most discriminating traits. Wilk's lambda (λ) was used as the criterion to determine the classification efficiency with the entry of each trait. The selected traits were then used in the subsequent analyses. To find out the relevant variables for morphological seed description, a correlation matrix was built using Pearson correlation coefficients to aid in interpretation of the analysis, and thereafter a principal component analysis (PCA) was performed. PCA was used on the ranged data as a linear dimensionality reduction technique to identify orthogonal directions of maximum variance in the original data set and to project the data into lower dimensions of the highest variance components, and to examine the percentage contribution of each trait to variation. Then, the cluster analysis using the squared Euclidean distance and Ward's minimal variance method was performed. The relationships among the clusters were elucidated. To facilitate the identification of diagnostic variables, significant differences among means of groups were evaluated by variance analysis under the general linear model because there were unequal numbers of accessions per cluster. Differences between means of groups were compared using Tukey's post hoc test. Finally, Stepwise Linear Discriminant Analysis (LDA) algorithm was performed to predict the membership of each accession to the corresponding group resulting from cluster analysis. When several variables are available, the stepwise method can be useful by automatically selecting the best characters on the basis of three statistical variables: Tolerance, F-to-enter and F-to-remove. The Tolerance value indicates the proportion of a variable variance not accounted for by other independent variables in the equation. A variable with very low Tolerance value proves little information to a model. F-to-enter and F-to-remove values define the power of each variable in the model and they are useful to describe what happens if a variable is inserted and removed, respectively, from the current model [27]. This approach is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables. The best features for seed sample identification were detected implementing a stepwise LDA method and a statistical classifier to discriminate and classify the seeds on the basis of the selected characters. This method starts with a model that does not include any of the variables. At each step, the variable with the largest F to enter value that exceeds the entry criteria chosen (F≥3.84) is added to the model. The variables left out of the analysis at the last step have F to enter values smaller than 3.84, so no more are added. The process was automatically stopped when no remaining variables increased the discrimination ability [27]. A cross-validation procedure was applied to verify the performance of the classifiers. All calculations were done using SAS 9.2 software [30] and/or SPSS 20.0 for Windows [31].

3. RESULTS

Using the wear technique to display the median plane of seeds, together with the variables derived from the external morphology, 19 quantitative morphometric data were obtained from internal and external features of seeds of 110 accessions of *Opuntia* and tree out groups of the Cactaceae family.

3.1 Seed Variables Variation

Analysis of variance showed highly significant differences (P<.05) among *Opuntia* accessions for all characters studied, indicating the existence of a high degree of morphological diversity of seeds. In this regard, the Seeds Weight ranged between 0.10 and 0.26g, thereof the seed surface between 7.83 and 20.80 mm^2, the Major Axis Length between 3.57 and 5.78mm and the Embryo Area varied from 3.30 to 6.52 mm^2. Mean values and the amplitude of the other variables are summarized in Table 2. The coefficient of variation ranged from 0.98 (C) to 19.40% (PA/SA). However, the most of the variables showed a coefficient of variation less than 10% (Table 2). Tukey's post hoc test (P<.05) separated the accessions into different groups depending on the variable (data not shown). However, the variety Oreja de Elefante was separated (P<.05) from other accessions; since it had greater SA (20.80 mm^2), SP (18.10 mm), MjA (5.78 mm) and FD (0.52). The Larreguin (*Opuntia ficus-indica*) accession was characterized by (P< .05) their high PA/SA (0.04). The variables SW, MjA, FD, SA, SP and MnA were the most different (P<.05) among the studied characteristics (Fvalues, Table 2).

3.2 Stepwise Discriminant Analysis

The discriminating power of 17 morphological seed variables was sufficient to differentiate the *Opuntia* accessions (Table 2). The significant results (P=.05) using fewer variables, confirmed the usefulness of the STEPDISC procedure in selecting a critical subset of features. Considering the variables selected by this statistical method, this analysis could reduce the cost and time for investigating *Opuntia* morphological relationships without compromising information gained. The variables PP and PA/EA did not contribute significantly to discrimination of accessions and were eliminated in the STEPDISC procedure. This method can detect redundant characters as reported by Yada et al. [32].

According to the results of the linear correlations, a high positive correlation was obtained between the variables Area and Weight of Seeds (SA *vs.* SW), Seed Area and Major Axis Length (SA *vs.* MjA), Seed Area and Minor Axis Length (SA *vs.* MnA), Seed Weight and Major Axis Length (SW *vs.* MjA), Seed Weight and Minor Axis Length (SW *vs.* MnA); while the Area of the Embryo and Perisperm were not associated with either the Weight or Area of Seeds (Fig. 1). These results suggest that developmental increases in seed size (weight and area) correspond to increases in the width thereof, as well as in its length.

Table 2.Seed variables variation among 110 *Opuntia* accessions (Mean: mean value of the continuous variable, Max: maximum value, Min: minimum value, CV: coefficient of variation, g: gram, mm: millimeter, mm^2: square millimeter)

Variables	Abbreviation	ANOVA and descriptive analysis					STEPDISC Procedure					
		Min	Max	Mean	CV (%)	F value	Step	partial R-square[1]	F Value	Pr> F	Wilks' Lambda[2]	Pr< Lambda
100 Seeds Weight (g)	SW	1.03	2.61	1.66	4.57	52.7***	3	0.932	27.1	<.0001	0.00002996	<.0001
Seed Area (mm^2)	SA	7.83	20.8	13.5	4.21	46.8***	2	0.981	99.5	<.0001	0.00044185	<.0001
Seed Perimeter (mm)	SP	11.2	18.1	14.6	2.42	36.2***	12	0.739	5.37	<.0001	0.00000000	<.0001
Major Axis Length (mm)	MjA	3.57	5.78	4.66	2.23	48.7***	4	0.898	17.2	<.0001	0.00000307	<.0001
Minor Axis Length (mm)	MnA	2.90	4.74	3.78	2.58	35.0***	14	0.641	3.35	<.0001	0.00000000	<.0001
Elongation	Elg	1.12	1.35	1.24	2.33	6.99***	15	0.570	2.47	<.0001	0.00000000	<.0001
Roundness	R	0.70	0.86	0.79	2.50	5.34***	11	0.721	4.91	<.0001	0.00000000	<.0001
Feret Diameter	FD	0.31	0.51	0.41	2.14	47.6***	1	0.977	86.1	<.0001	0.02261176	<.0001
Compactness	C	0.85	0.93	0.88	0.98	8.49***	5	0.980	95.7	<.0001	0.00000006	<.0001
Embryo Area (mm^2)	EA	3.30	6.52	5.18	7.56	7.80***	13	0.695	4.30	<.0001	0.00000000	<.0001
Embryo Perimeter (mm)	EP	8.80	13.9	11.2	5.53	6.80***	10	0.715	4.79	<.0001	0.00000000	<.0001
Perisperm Area (mm^2)	PA	0.08	0.41	0.22	16.1	11.5***	7	0.899	17.3	<.0001	0.00000000	<.0001
Perisperm Perimeter (mm)	PP	1.74	4.14	2.93	10.3	8.20***	Removed (no entered)					
Embryo Area/Seed Area	EA/SA	0.20	0.56	0.39	9.06	8.56***	9	0.782	6.90	<.0001	0.00000000	<.0001
Perispem Area/Seed Area	PA/SA	0.01	0.04	0.02	18.3	10.7***	6	0.863	12.2	<.0001	0.00000001	<.0001
Perisperm Area/Embryo Area	PA/EA	0.02	0.12	0.04	19.4	9.00***	Removed (no entered)					
Embryo Perimeter/Seed Perimeter	EP/SP	0.55	0.94	0.77	6.03	6.10***	8	0.803	7.86	<.0001	0.00000000	<.0001
Perisperm Perimeter/Seed Perimeter	PP/SP	0.12	0.30	0.20	10.7	6.26***	17	0.491	1.78	0.0002	0.00000000	<.0001
Perisperm Perimeter/Embryo Perimeter	PP/EP	0.15	0.37	0.26	11.8	5.20***	16	0.543	2.20	<.0001	0.00000000	<.0001

**** Indicates significant difference at 0.001 level*

[1] The marginal variability accounted for by a variable when all others are already included in the model

[2] The likelihood ratio measure of a trait's contribution to the discriminatory power of the model

Principal component analysis (PCA) was used before cluster analysis to determine the relative importance of the 17 traits. PCA revealed that the first four components explained 90.97% of the total variability (Fig. 2). The first three components accounted for 83.35% of the variability, of which the first component contributed twice the variability (48.12%) respect to the second component (23.77%). The variables that defined, according to their eigenvectors (value in parentheses), the first component in the positive direction were MnA (0.98), FD (0.98), SA (0.95), SP (0.95), MjA (0.94), SW (0.84), EA (0.77), EP (0.73), and in the negative direction EA/SA (0.64). The second component was related to the variables PA/SA (0.95), PA/EA (0.90), PP/SP (0.89), PA (0.83) and PP/EP (0.81) in the positive sense. The third component was determined by the variables C (0.69) in the positive direction and by Elg (0.64) in the negative one. These results revealed that the first component was defined by the variables measured directly on the seeds (weight, length, area and perimeter), while the remaining components were defined by the rations between different variables.

The projection of all 113 studied accessions on the first two components (CP 1 and CP 2) showed high dispersion around the origin of the plot (Fig. 3). However, Larreguin (55) and *Cylindropuntia* sp. (67) accessions were separated from the remaining ones on the positive sense of the second component; since they have greater Perisperm Area (0.405 and 0.377 mm^2, respectively). In turn, the pitahaya (75) and pitaya (76) accessions were separated on the negative sense of the first component to having small seeds. It is noteworthy that the genotypes corresponding to xoconostles, acidic prickly pear (108 to 113), were placed together, since their seed dimensions were lower than the most of the other opuntias. However some prickly pear genotypes such as 20, 32, 38, 71 and 98 were placed together with xoconostles, indicating the need to integrate other data such as fruit characters and/or molecular markers to separate these two *Opuntia* groups.

Cluster analysis separated the 113 accessions studied in seven main groups, of which the group 7 included the two outgroups pitahaya and pitaya. Variance analysis was used to select diagnostic variables between groups, previously defined by cluster analysis. Tukey's test was applied to determine the variables that discriminate between these groups (Table 3). With the exception of the variables PA/SA, PA/EA, and PP/SP, all 14 remaining ones separated the Opuntioideae accessions (groups 1 to 6) from the *Pachycereae* ones (group 7; *Hylocereus undatus* and *Stenocereus thurberi;* Pitahaya and Pitaya, respectively). Among *Opuntioideae* accessions, the 6 obtained groups contained different number of accessions (14, 14, 31, 10, 31, 11 in groups 1 to 6, respectively; Table 3). Most of the variables (SW, SA, SP, MjA, MnA, FD, EA, EP, EA/SA) contributed to the separation between the 6 *Opuntioideae* groups resulting from cluster analysis. Groups 4 (10 genotypes) and 6 (11 genotypes) were characterized by extreme values (highest and lowest, respectively) for the variables SA, SP, MJA, MnA and FD (Table 3). Group 1 (14 genotypes) was characterized by genotypes with high EA and EP. Groups 2 (14 genotypes), 3 (31 genotypes) and 5 (31 genotypes) were characterized by genotypes with intermediate values, in order from lowest to highest, of the variables SA, SP, MjA and MnA.

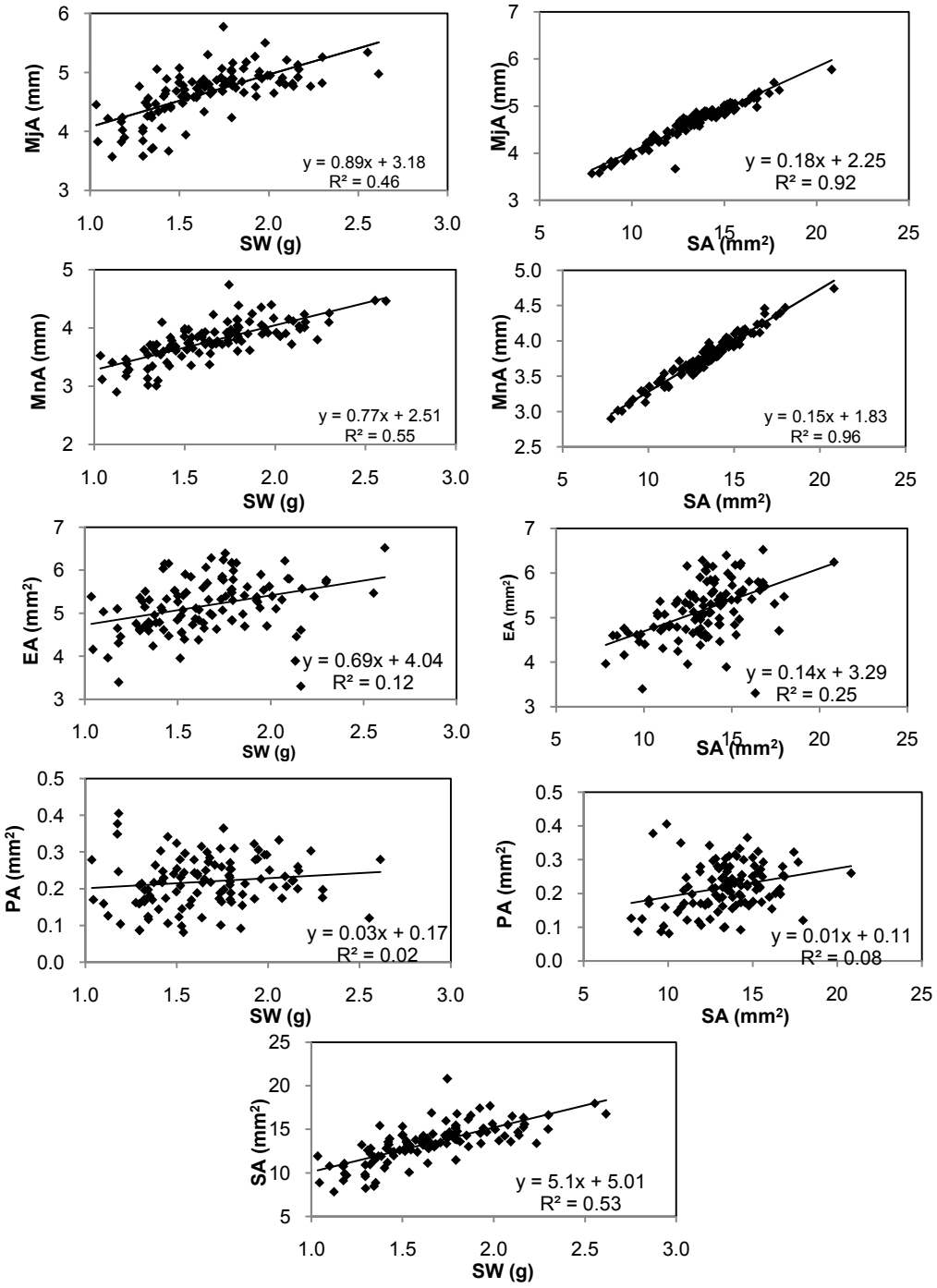

Fig. 1. Linear correlation between seed morphometric variables from *Opuntia* accessions. SA: Seed Area, SW: Seed Weight, MjA: Major Axis Length, MnA: Minor Axis Length, EA: Embryo Area, PA: Perisperm Area, g: gram, mm: millimeter, mm^2: square millimeter. Positive correlation: SA *vs.* SW, SA *vs.* MjA, SA *vs.* MnA, SW *vs.* MjA, SW *vs.* MnA

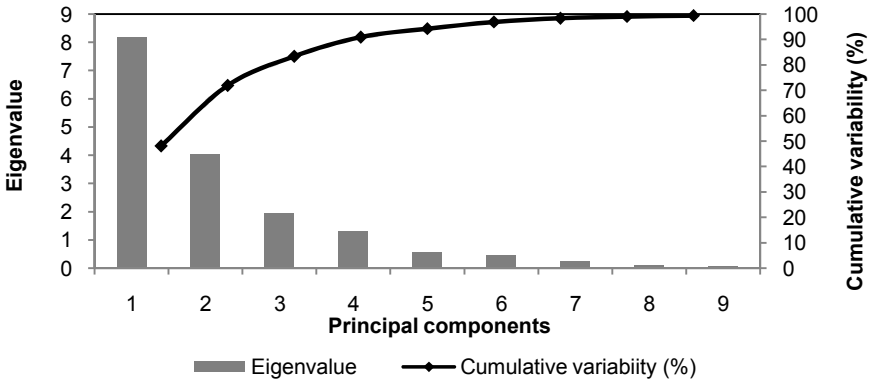

Fig. 2. Representative plot of the cumulative variability and eigenvalues of the first ten PCA components resulting from 17 seed morphometric variables measured on 110 *Opuntia* accessions, one sample of *Cylindropuntia* sp. and two samples of pitahya and pitaya

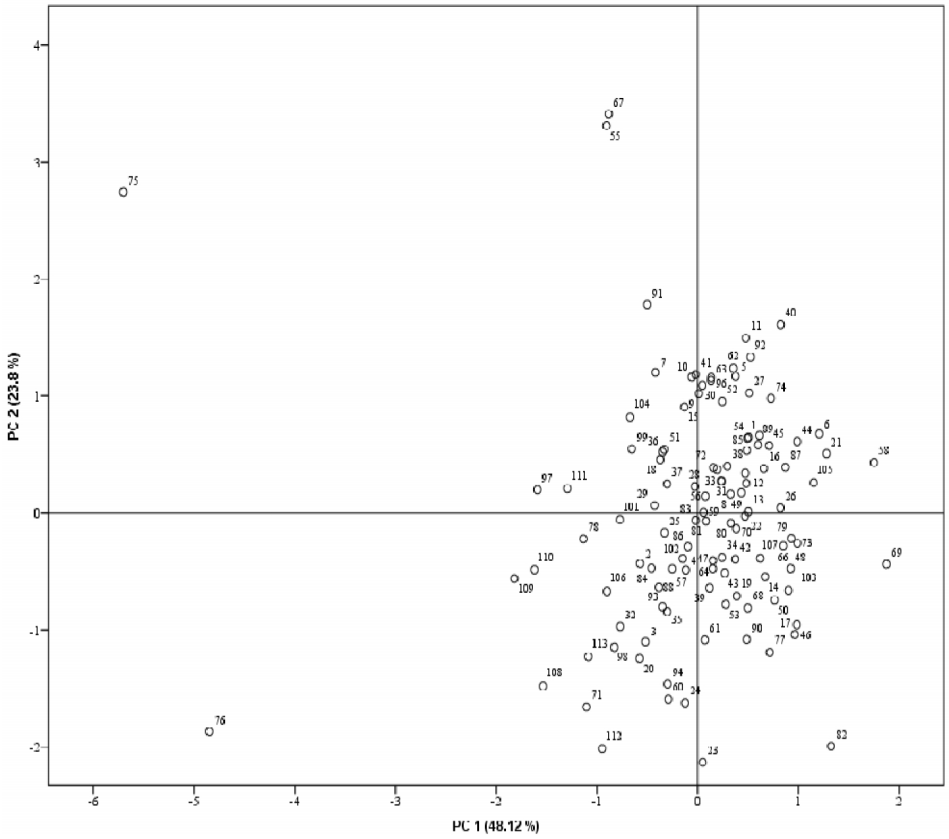

Fig. 3. Plot distribution of the 110 *Opuntia* accessions, one sample of *Cylindropuntia* sp. (47) and two samples of pitahya (75) and pitaya (76) based on 17 external and internal seed quantitative variables

Table 3. Quantitative variables used to investigate the morphological variation between the seeds groups resulting from the cluster analysis

Groups (accessions number)	Cluster accessions	SW	SA	SP	MjA	MnA	Elg	R	FD	C	EA	EP	PA	EA/SA	PP/EP
Grp. 1 (14)	1, 5, 11, 27, 31, 38, 52-54, 59, 61, 64, 81, 95	1.67bc	13.8c	14.8bc	4.77bc	3.82c	1.26b	0.79a	0.42c	0.88a	5.79a	12.4a	0.26a	0.42bc	0.26a
Grp. 2 (14)	2, 3, 20, 29, 32, 36, 37, 57, 91, 98, 99, 101, 104, 106	1.38cb	11.3d	13.4d	4.29d	3.49d	1.24b	0.79a	0.38d	0.89a	4.73cd	10.5cd	0.19a	0.42bc	0.25a
Grp. 3 (31)	4, 7, 9, 10, 15, 18, 24, 25, 28, 30, 33-35, 39, 41, 51, 56, 60, 62, 63, 70, 72, 83, 84, 86, 88, 93, 94, 96, 100, 102	1.57bcd	13.0c	14.5c	4.60c	3.71c	1.26b	0.78a	0.41c	0.88a	5.09bc	11.0bcd	0.22a	0.39cd	0.27a
Grp. 4 (10)	6, 21, 46, 48, 58, 69, 73, 79, 82, 105	2.05a	17.4[a]	16.7a	5.30a	4.37a	1.22b	0.78a	0.47a	0.89a	5.69ab	12.0ab	0.24a	0.33d	0.27a
Grp. 5 (31)	8, 12, 13, 14, 16, 17, 19, 22, 23, 26, 40, 42, 43, 44, 45, 47, 49, 50, 65, 66, 68, 74, 77, 80, 85, 87, 89, 90, 92, 103, 107	1.87ab	15.0b	15.4b	4.92b	4.01b	1.23b	0.79a	0.44b	0.89a	5.32abc	11.3bc	0.23a	0.36cd	0.27a
Grp. 6 (11)	55, 67, 71, 78, 97, 108-113	1.26d	9.14e	12.2e	3.81e	3.15e	1.22b	0.77a	0.34e	0.89a	4.38d	10.1d	0.17a	0.48ab	0.24a
Grp. 7 (2)	75, 76	0.14e	2.42f	6.69f	2.20f	1.44f	1.55a	0.68b	0.17f	0.79b	1.28e	6.74e	0.05b	0.54a	0.17b

Label in the cluster accessions case refers to the corresponding accession mentioned in the table 1. Different letters indicate significant differences between groups resulted of Tukey's post hoc comparison, P<.05

Data analyzed by Stepwise Linear Discriminant and statistical classifiers were developed in order to distinguish the obtained groups from de the cluster analysis. The best discriminating variables selected by the stepwise method among the 17 variables were FD, SA, EP, EA/SA, EA, Elg, Mn A and C. The first two variables selected by the model were the same in cluster analysis. These were Feret Diameter (FD) and Seed Area (SA) that moreover showed values of F-to-remove clearly higher than other selected features. Using this model, 96.5% of original grouped cases correctly classified and 92.9% of the cross-validated samples of the seven clusters were correctly classified (Table 4). Accessions of the group 7 were correctly identified in 100% of the cases and none of the seeds of other studied accession was mistaken for it. Contrastingly, group 1 showed a lower percentage of correct identification (78.6%), as accessions were mainly misclassified among those of group 3. The other groups had higher percentages of correctly identification upper of 90% and only one genotype of each group was wrongly placed (Table 4).

Table 4. Predicted groups membership and cross-validated of correct classification of the *Opuntia* accessions resulting from the cluster analysis. The number of accessions is indicated in brackets

		Predicted Group Membership							Total
Classification Results	**Groups**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	
Original	1	85.7% (12)		14.3% (2)					14
	2		92.9% (13)	7.1% (1)					14
	3			100% (31)					31
	4				90% (9)	10% (1)			10
	5					100% (31)			31
	6						100% (11)		11
	7							100% (2)	2
Cross-validated	1	78.6% (11)		21.4% (3)					14
	2		92.9% (13)	7.1% (1)					14
	3	3.2% (1)		96.8% (30)					31
	4				90% (9)	10% (1)			10
	5	3.2% (1)				96.8% (30)			31
	6		9.1% (1)				90.9 % (10)		11
	7							100% (2)	2

4. DISCUSSION

One of the distinguish characters subfamily *Opuntioideae* from other subfamilies in *Cactaceae* is the seed structure. The Opuntioideae seeds are unique, not just in the *Cactaceae* or even the Caryophyllales but in the whole of the Angiospermae, in being entirely encased by a hard aril derived from the funiculus [1]. Surprisingly, in view of their uniqueness, the *Opuntioideae* seed have received little attention. The most remarkable character of *Opuntioideae* seeds is that they are completely covered by a tissue derived from the funiculus. Seeds have a thick white funiculus surrounding them, well-developed perisperms and curved embryos. The curvature of the embryo is the result of the campylotropous curvature of the ovule [1]. Seeds of Opuntia species have hard (to-the-touch) seed covers [33], and pressures of 440daN may be required to break them.

In this study, seeds of *Opuntia* accessions are studied to obtain quantitative variables related to external and internal morphology. These variables were obtained by image analysis and investigated using several statistical analyses. Sassone et al. [34] reported the importance of uni and multivariate analysis to obtain new association between accessions and species, and supported the importance of these tests to evaluate the taxonomic entities.

The obtained results showed that the seeds of *Opuntia* have a high range of variation in size (major and minor length) in weight and also in the area. All studies variables were able to discriminate accessions, since the analysis of variance showed highly significant variation. The most discriminating variables were Seed Weight, Major Axis Length, Feret Diameter, Area and Perimeter of the seed, and Minor Axis Length. In addition, low coefficient of variation values suggests discriminatory stability of these variables, as well as reported by Guerrero-Muñoz et al. [28]. According to Andrés-Agustín et al. [35], coefficients of variation of 12%, or less, are acceptable in characterizing plant organs in horticultural species and would be desirable to increase the sample size if this ratio is higher. In our case, out of the 19 studied variables, only three showed high coefficients of variation (PA/SA (19.4%), PA/SA (18.3%), PA (16.1%); while the remaining ones had values lower than 10%. This indicates that the number of used seeds here was appropriate to obtain stable and useful variables for characterization and differentiation purposes.

Estimation of the measured parts of the seed (embryo, testa, perisperm and total area) revealed that the embryo and perisperm area represent 38.4% and 1.63% of the total seed area, respectively. Similar values were reported by Stuppy [1] and Guerrero-Muñoz et al. [28]. A large embryo (whose function is to storage the reserves) produces a seedling with higher photosynthetic productivity and being able to grow faster and compete more successfully [36]. Stuppy [1] reported that the *Opuntia* seed has small sized, oval, and the embryo has a spiral shape around a folded perisperm strongly reduced, since embryo length increases the storage capacity is increased too.

Out of 19 variables, 17 had a high discriminative power as stepwise discriminant analysis showed, with the exception of two variables (Perisperm Perimeter and Perisperm Area/Embryo Area ratio). Yada [32] reported the usefulness of this statistical technique to reduce the number of characters to be measured; which implies savings in time, effort and expense, without compromising results gain; besides detecting redundancy in the variables.

The PCA, based on 17 variables, was performed to study the combination of traits that best explain the variability. The usual procedure to identify the components is to detect the first components that explain the largest proportion of the total variance [37]. In our case, the

components considered with eigenvalues above than 1 (8.18, 4.04, 1.95 and 1.30 for the components 1, 2, 3 and 4, respectively (Fig. 2). The PCA results showed the usefulness of the variables Minor Axis Length (MnA), Feret Diameter (FD), Seed Area (SA), Seed Perimeter (SP), Major Axis Length (MjA), Seed Weight (SW) for their ability to differentiate between accessions. The projection of all studied accessions on the first two components (CP 1 and CP 2) showed high dispersion around the origin of the plot, indicating a continuity of variables among accessions without clear boundaries between them. This is due to all the variables used are quantitative.

Cluster analysis separated the 113 accessions studied in seven main groups. Group 7 was composed of two genotypes of Pachycereae included as out groups. Group 6 was composed mainly of genotypes belonging to xoconostles. Most variables (SW, SA, SP, MjA, MnA, FD, EA, EP and EA/SA) contributed to the separation of the 6 *Opuntia* groups, resulting from the cluster analysis. However, SA, SP, MjA, MnA, AD and PD and FD variables had the greater power to define this grouping.

The pattern grouping of genotypes did not fit the actual species assignment, nor in PCA neither in cluster analysis. Similar results were found by Reyes-Agüero et al. [15] and Gallegos-Vázquez et al. [38] using morphological markers as variables derived from cladodes and fruits. This is probably related to the high level of phenotypic plasticity and polyploidy, and also due to the morphological diversity of these accessions. These genotypes had several end use; as fruits, vegetables and/or as forage. For these reasons, many studies have suggested the revision of the classification of the *Opuntia* genus [4,19,20]. Moreover, the geographical accessions origin affects their morphological variation, and this has led to very narrow use of the concept of species. Often the location of an accession in a species is arbitrary and lack of solid descriptors; many of the accessions considered in our study have not yet been taxonomically assigned (Table 1). However, accessions representatives of xoconostles were grouped together (in both analyses), thus showing its distinction from other accessions because them having smaller size for seeds. Studies based on fruit morphology [12] and molecular markers [18], placed to the xoconostles as sister groups of prickly pears. According to Gallegos-Vázquez et al. [38], the absence of the pulp and the presence of an edible pericarp are the most significant differences between prickly pears and xoconostles accessions. However, the presence of some prickly pears genotypes grouped together with xoconostles suggests the need to use other plant organs and/or molecular markers to differentiate these two *Opuntia* plants.

The classification test of genotypes to clusters by linear discriminant analysis showed a cross-validation of 92.9%. Similar results were found by Bacchetta et al. [27], where cross-validation of 92.7% was found in samples from five taxa of *Lavatera*. This statistical technique approved the discriminating power of the image analysis derived variables from *Opuntia* seed obtained.

For the *Opuntia* genus, the use of plant height, cladodes, fruit and the flower is the traditional way for classifying the genotypes and assign them in their respective species [7,10]. These descriptors are considerably affected by the environment geographical conditions and show a low discriminating power. Similarly and although flower attributes are considered stable, Fuentes-Pérez et al. [39] reported that the floral anatomical characteristics of five species of the *Opuntia* genus was not decisive in the taxonomic separation between species. In the present study, we demonstrated that many of seeds variables analyzed with images are of potential candidates for use in this complex taxonomic genus. These results can be transferred to state characters useful for cladistics analysis and can be used as guide

selection of taxonomic characters. Seed *Opuntia* variables are little influenced by environmental pressure and are more affected by the genetic control, which is likely due (i) to the hardness of the seed; (ii) The protective effect offered by the pulp and seeds testa and (iii) The short period of exposure the fruits to environmental factors.

Morphometric characterization of seeds is rapid, reproducible and reliable that accurately identifies the seeds of species from wild plants. Their usefulness in taxonomic studies is promising, due to its efficiency in discriminating between accessions at the level of inter population [27]. This provides new insights into plant taxonomy, and also offers the opportunity to the germplasm banks to identify their accessions through standardized and quickly methods. Our results demonstrated that the image analysis allows estimating the principal dimensions of the seeds (length, width and elongation) with high accuracy. Since the manual measurements are difficult due to the small size of these seeds. Another advantage of this type of analysis is to provide additional features, to be determined objectively and with good discriminating power, such as FD and Elg. Moreover, they are continuous variables, which allow the use of ANOVA statistics [40].

Despite the lower costs associated with the analysis of morphological variables of seeds, molecular analysis remains an essential tool for the investigation of the variability within and between genotypes, and for estimating genetic relationships and assigning genotypes to a defined species.

5. CONCLUSION

The results presented here proved the utility of the seed variables for characterize and differentiate genotypes of *Opuntia* such Seed Area, Major Axis Length, Minor Axis Length, Feret Diameter and Seed Weight. These variables, derived from image analysis, have a high discriminatory power and can be taken into account as potential descriptors for genotypes assignation within the *Opuntia* genus. On the other hand, the grouping of the accessions resulting from PCA and cluster analysis did not consistent with the current taxonomy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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