Genetic diversity of squash landraces (*Cucurbita maxima*) collected in Andean Valleys of Argentina

Diversidad genética de poblaciones de zapallo (*Cucurbita maxima*) colectadas en los valles andinos de la Argentina

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Originales: *Recepción:* 01/03/2018 - *Aceptación:* 28/07/2018

Abstract

Squash landraces (*Cucurbita maxima*) are maintained by small farmers as a major nutritional food. Twenty seven of these landraces were collected in Argentinian Andean Valleys and morphologically characterized. Genetic diversity was evaluated with microsatellite markers designed for *Cucurbita pepo* and *Cucumis melo* and evaluated for the first time in *C. maxima*. Seven microsatellite primers detected 26 alleles with 3.10 average alleles per locus. The genetic diversity reached an average of 0.26; a Polymorphic Information Content (PIC) of 0.20 and 45.5% of polymorphic *loci*. Higher diversity was found at intra population level. No evidence of lineal correlation between the observed diversity and the geographical distribution of squash landraces was found. Results demonstrate a moderate genetic diversity for all populations, with a wide range of variation in different groups. A subgroup of 10 populations with the highest levels of genetic diversity was considered for maintenance within core collections in the Vegetable Crop Germplasm Bank of Agricultural Research Station (EEA) La Consulta, Mendoza, National Institute of Agricultural Technology (INTA). Anthropogenic and environmental processes, mainly abandonment of cultivated areas and frequent droughts could erode squash landraces diversity. Conservational strategies and new collecting expeditions can be decided based on the genetic diversity found.

Keywords
Microsatellite markers • genetic diversity • germplasm banks • genetic resources. squash landraces • *Cucurbita maxima*

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Resumen

En Argentina los pequeños agricultores mantienen poblaciones de zapallo (*Cucurbita maxima*) de gran importancia nutricional. Veintisiete de estas poblaciones fueron recolectadas en los valles andinos y caracterizadas morfológicamente. Marcadores microsatélites diseñados para *Cucurbita pepo* y *Cucumis melo* se aplicaron por primera vez en *C. maxima* para evaluar diversidad genética. Siete cebadores detectaron 26 alelos con 3,10 alelos promedio por *locus*. La diversidad genética alcanzó una media de 0,26; el contenido de información polimórfica (PIC) de 0,20 y el 45,5% de los *loci* resultaron polimórficos. La diversidad a nivel intrapoblacional fue mayor que entre poblaciones. No se encontró correlación lineal entre la diversidad observada y la distribución geográfica poblacional. La diversidad genética fue moderada para el conjunto de poblaciones, con un amplio rango de variación. Un subgrupo de 10 poblaciones con los mayores valores de diversidad genética fue considerado para su mantenimiento dentro del Banco de Germoplasma de la Estación Experimental Agropecuaria (EEA) La Consulta, Instituto Nacional de Tecnología Agropecuaria (INTA). Los procesos antropogénicos y ambientales, principalmente el abandono de áreas cultivadas y las frecuentes sequías, estarían erosionando la diversidad de estos recursos. Sobre la base de los resultados obtenidos se pueden plantear estrategias de conservación y nuevas expediciones de colecta.

Palabras clave

Marcadores microsatélites • diversidad genética • bancos de germoplasma • recursos genéticos • zapallo • *Cucurbita maxima*

Introduction

Squash (*Cucurbita maxima* Duchesne) is a native species of South America traditionally used for its fruit nutritious value, and is cultivated worldwide mostly in temperate zones. *Cucurbita maxima* Duchesne subsp. *andreana* (Naudin) Filov, from humid lowlands of Bolivia and warm temperate zones of Argentina and Uruguay, is considered as the putative ancestor of *C. maxima* (47, 51, 61). Archeological studies indicate that *C. maxima* was brought from Peru to northern Argentina, with evidence of domestication about 1800 years B.C. in Peru and between 500 to 1000 years A.C. in Argentina (38). Squash was domesticated and adapted to different environmental conditions in Andean areas, where diversity of local landraces is found.

The Andean Valleys of Argentina are part of the Peruvian-Bolivian center of origin of different cultivated species, and considered the southern limit of many primitive cultivars and/or crop species used since pre-Columbian times (58). These valuable genetic resources are maintained by local communities using their traditional agriculture, contributing to farmer sustainability. Squash is a fundamental food in their diet, since is highly digestible and provides valuable antioxidant nutrients such as alpha and beta carotenes, precursors of vitamin A (18, 22, 31, 59).

Squash landraces are extremely variable in fruit shape, size and color, characteristics traditionally used for their classification (6, 34).

Cucurbita maxima is a diploid (2n = 2x = 40), decline, allogamous species with a broad genetic base (10). However, conservation of crop diversity is threatened due to environmental and socioeconomic factors, such as frequent droughts and farms abandonment. In this context, landraces preservation is a priority in Argentina. Recuperation of Andean squashes was part of a first systematic effort to preserve the diversity of local crops (2, 3, 41, 43, 56).

Molecular tools are currently widely used for evaluating crop diversity (14, 32, 34, 37). genetic diversity of *C. maxima* collections has been determined with different markers (4, 20, 21, 30, 64) like microsatellites (33, 36, 65, 68).

This study aimed to evaluate 27 populations of *C. maxima* collected in Andean Valleys of Argentina (56), using microsatellites markers (65, 68) for the first time in Argentinian *C. maxima* germplasm. These landraces were previously morphologically characterized (43).

The results obtained are strategic to preserve squash landraces diversity in germplasm banks.

Materials and Methods

Collecting expeditions and morphological characterization

Eight collecting expeditions, between 2005 and 2008, were conducted covering agro-ecological regions of northwestern Argentina (NOA), Cuyo and Patagonia (figure 1, page 296). An exhaustive collection of squash accessions was performed in Andean communities where local farmers maintained their crops in different environments using diverse crop managements (41, 56). Twenty seven accessions of various morphotypes (figure 2, page 297) collected in different localities during the first two expeditions to Valle Fértil (Province of San Juan), and Northwestern Argentina (table 1, page 298), were selected for field evaluation (41, 43), and molecular characterization. During two seasons 49 plant, flower, fruit, and seed traits were measured using morphological traits for the genus *Cucurbita* (27, 43). As a control, *C. maxima* 'Marino FCA' and 'Veronés INTA' were used (43, 56).

DNA extraction

Genomic DNA of five seedlings per accession of the 27 selected landraces (table 1, page 298) was extracted (13). In addition, two cultivars of *C. maxima* 'Marino FCA' and 'Veronés INTA', one of *Cucurbita moschata* Duchesne 'Paquito INTA' and one of *Cucurbita pepo* L. were included.

Amplification and visualization of microsatellite markers

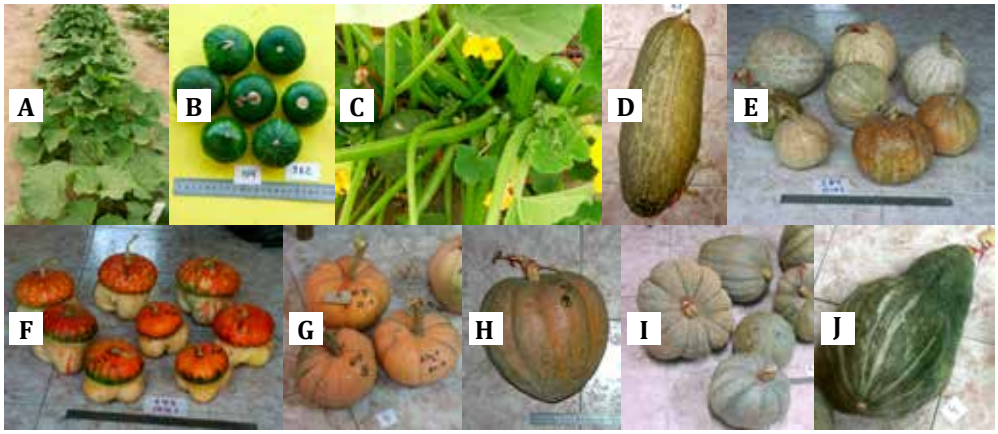
At the time this study was conducted, no reports of microsatellite markers specifi- cally designed for *C. maxima* were available. Therefore markers developed from microsatellites designed for *Cucumis melo* L. (65) and *C. pepo* (68) were used for the first time in *C. maxima*. Two hundred fifty-seven primer pairs designed for *C. melo* were evaluated in five *C. maxima* accessions and two controls of *C. melo* species. Eight primers designed for *C. pepo* and recommended as polymorphic for *C. maxima* (68) were first evaluated in five *C. maxima* accessions and one accessions of *C. pepo* species as control. PCR experiments were performed on a PTC-100 Thermocycler MJ Research Inc. (Bio-Rad, Foster City, California, USA).

Ecoregions are highlighted with different colors: NEA: Northestern Argentina (Chaco, Formosa, Entre Ríos, Corrientes, Misiones); NWA: Northwestern Argentina Jujuy, Salta, La Rioja, Catamarca, Tucumán, Santiago del Estero); Cuyo (San Juan, Mendoza, San Luis); Pampas (Córdoba, Buenos Aires, La Pampa, Santa Fe); Patagonia (Río Negro, Neuquén, Chubut, Santa Cruz, Tierra del Fuego).

Las ecorregiones se destacan con diferentes colores: NEA noreste argentino (Chaco, Formosa, Entre Ríos, Corrientes, Misiones);NOA noroeste argentino (Jujuy, Salta, La Rioja, Catamarca, Tucumán, Santiago del Estero); Cuyo (San Juan, Mendoza, San Luis); Pampa (Córdoba, Buenos Aires, La Pampa, Santa Fe); Patagonia (Río Negro, Neuquén, Chubut, Santa Cruz, Tierra del Fuego).

Figura 1. Geo-referenced collection points of *C. maxima* landraces in Argentina.

Figure 1. Puntos de colecta de poblaciones de *C. maxima* en Argentina georreferenciados.



a, b, c "Zapallito redondo del tronco" (*C. maxima* Duch. var. zapallito (Carrière) Millán) (variety consummed only in Argentina: bushy plants, with fruits growing near the principal stem, edible immature fruit). **d-j** Winter squashes (plants with long trailing vines, edible mature fruit): **d** Elongated oblong. **e** Globular show type. **f** Turban. **g** Flattened. **h** Acorn delicious type. **i** Flattened and/or globular creole gray squash type. **j** Elliptical or oval Hubbard type (27).

a, b, c "Zapallito redondo del tronco" (*C. maxima* Duch. var. zapallito (Carrière) Millán) (variedad consumida solo en Argentina. Desarrolla plantas sin guías, sus frutos crecen cerca del tallo principal y se consumen inmaduros). **d-j** Zapallos de invierno (plantas con guías largas, fruto comestible a la madurez): **d** oblongo alargado. **e** Tipo globular. **f** Turbaniforme **g** Achatado. **h** Tipo delicioso. **i** Zapallo gris criollo aplanado y/o globular**. j** Tipo Hubbard elíptico u oval (27).

Figure 2. Fruit morphotypes found among *C. maxima* landraces.

Figura 2. Morfotipos de frutos encontrados en las poblaciones de *C. maxima*.

The following reaction mixture to 15 µl final volume was formulated: 40ng DNA, 5x buffer with magnesium chloride (final concentration of 1.5mM $MgCl_2$), 0.16 mM dNTPs, 0.03µM of each primer; 1 unit Taq polymerase (Promega, Madison, Wisconsin, USA,). Samples were subjected to the following thermal profile for amplification: four minutes of denaturing at 94°C, 35 cycles of denaturing at 94°C for 45 seconds, 45 seconds of annealing at 48°C and 30 seconds of elongation at 72°C, with a final elongation step of 72°C for five minutes. PCR products were evaluated by electrophoresis on a 6% denaturing polyacrylamide gels (PAGE). Amplification fragments were visualized on a 3130 ABI Genetic Analyzer (Applied Biosystems, Foster City, USA), primers that amplified polymorphic fragments were synthesized attaching a DNA fragment known as M13 (-21) universal tail (62) on the forward primer 5' end. This fragment is complementary to the universal tail M13 (-21) which is labeled with a fluorescent dye and added to the reaction mixture of PCR. Thus, during the polymerase chain reaction, fragments are labeled by incorporating a tail sequence and can be evaluated by capillary electrophoresis and laser detection. The new mixture of PCR included the forward primer synthesized with M13 (-21) tail, reverse primer and tail labeled M13 (-21) with each of the following fluorescent dyes: FAM, JOE, HEX or TAMRA. Mix 15 µl reaction was formulated by the following way: 40ng DNA, 5x buffer, 2mM $MgCl_2$, 0.16mM dNTPs, 0.05µM forward tailed primer, 0,2µM reverse primer, 0.2µM universal M13 tail (-21) marked, 0.5 units Taq polymerase (Promega, Madison, Wisconsin, USA).

Table 1. Squash accessions.
Tabla 1. Entradas evaluadas.

Acc.	Alt.	S Lat.-W Long.	Locality-Department-Province
13 (687)	961	30°29.843-'67°34.514'	Puesto San Marcos. San Agustín del Valle Fértil. San Juan
14 (688)	929	30°31.768-67°33.701'	Usno. San Agustín del Valle Fértil. San Juan
17 (678)	1133	30°19.930'-67°39.299'	Baldes Del Rosario. San Agustín del Valle Fértil. San Juan
19 (694)	653	30°55.544'-67°15.346'	Baldes Del Rosario. San Agustín del Valle Fértil. San Juan
20 (696)	1249	30°13.275'-67°41.736'	Baldecitos. San Agustín del Valle Fértil. San Juan
22 (697)	906	30°33.974'-67°32.288'	Usno. San Agustín del Valle Fértil. San Juan
24 (699)	722	30°47.871'-67°19.641	Agua Cercada. Valle Fértil. San Juan
36 (681)	640	30°55.750'-67°15.54'	Baldes De Astica. San Agustín del Valle Fértil. San Juan
53 (348)	1208	27º56.493'-65º41.887'	La Higuera. Balcosna. Catamarca
62 (361)	1274	27°51.769'-65°45.830'	Balcosna de Afuera. Catamarca
76 (620)	1906	26°43.865'-66°03.179'	Loro Huasi. Santa María. Catamarca
127 (309)	1215	27°43.082'-67°08.234'	Londres. Catamarca
143 (387)	1246	27º39.160'-67º1.358'	Artasa. Belén. Catamarca
161 (673)	1263	27º37.895'-67º01.580'	Belén. Catamarca
173 (2482)	2083	27°09.279'-66°42.368'	Los Nacimientos. Catamarca
185 (676)	2131	26º21.446'-66º01.552'	Pichao. Tucumán
195 (418)	1951	25º59.672'-66º1.686'	San Antonio. Cafayate. Salta
210 (424)	2004	25°59.749'-66°01.681'	San Antonio. Cafayate. Salta
215 (425)	1999	25º59.611'-66º1.498'	San Antonio. Cafayate. Salta
225 (428)	1811	26°05.982'-66°00.953'	Divisadero. Salta
233 (430)	2432	25º22.507'-66º26.134'	Refugios. Luracatao. Salta
244 (431)	2400	25º22.195'-66º25.994'	Refugios. Luracatao. Salta
284 (434)	1663	24°02.022'-65°26.015'	Cabrerías. Luracatao. Salta
324 (440)	2293	23°41.405'-65°27.011'	Chañarcito. Jujuy
367 (441)	2370	23º39.246'-65º25.851'	Hornillos. Jujuy
382 (443)	2761	23°30.892'-65°24.643'	Juella. Tilcara. Jujuy
523 (445)	3169	23º5.324'-65º22.926'	Hornaditas. Humahuaca. Jujuy

Acc. accession number, between brackets number of passport of INTA EEA La Consulta Germplasm Bank; S Lat.-W Long.: Geographical coordinates of collection points; political location.

Acc.: número de entrada, entre paréntesis número de pasaporte, Banco de Germoplasma EEA La Consulta, INTA; S Lat.-W Long.: coordenadas geográficas del punto de colecta; ubicación política.

PCR conditions were the following: 5 minutes of denaturing at 94°C, 40 cycles of five minutes of denaturing at 94°C, 45 seconds of annealing at 48°C and 45 seconds of elongation at 72°C; in the following 11 cycles, the annealing temperature was increased to 53°C, with a final elongation of 10 minutes at 72°C. Table 2 (page 299) lists used primers. The results were analyzed using GeneMapper 4.0 software (Applied Biosystems, Foster City, USA).

Table 2. Microsatellite primers selected.

Tabla 2. Cebadores microsatélites seleccionados.

Name	Expected size (pb)	SSR motif	Forward	Reverse
cm 22	177	(AG)24	5´-TGT AAA ACG ACG GCC AGT CCA AAA CGA CCA AAT GTT CC-3´	5´-ATA CAG ACA CGC CTT CCA CC-3´
cp 24	130-150	(AG)4	5´-TGT AAA ACG ACG GCC AGT GTG CTG CAT GTT GGA TGT CT-3´	5'- GTG ACC ATG GAC AAC ACG TC-3´
cp 25	100-120	(CACC)4	5´-TGT AAA ACG ACG GCC AGT CTC TTC CGA TTC TCC GCT TA-3´	5´-TTC GAA CTT GAG CAA GCA AA-3´
cp 33	190-200	(TC)3(CACC)4	5´-TGT AAA ACG ACG GCC AGT CTC TTC CGA TTC TCC GCT TA-3´	5´- CCG ATC AAG AAC AGC ACA GA -3´
cp 46	160-180	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCT TCC GAT TCT CCG CTT AG-3´	5´-GCA CAG AAA ACG GGG TAA AA-3´
cp 52	180-195	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCA CTT CTC CCC TTC TCT GC-3´	5´-TTC GAA CTT GAG CAA GCA AA -3´
cp 56	130-150	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCC ATT TCC ACT CAT TTT TC-3´	5´-GAT CCA GTT GAA GCG ATT AC-3´

Primer name, expected fragment size expressed in base pairs (bp), microsatellite motif and nucleotide sequence of the forward and reverse primers used for microsatellite amplification. In red: universal fragment sequence M13 (-21) coupled to the forward primer in its 5 'end.

Denominación del cebador, tamaño del fragmento esperado expresado en pares de bases (pb), motivo del microsatélite y secuencia nucleotídica de los cebadores Forward y Reverse utilizados para la amplificación de los fragmentos microsatélites. Se destaca en rojo la secuencia del fragmento universal M13 (-21) anexada al cebador Forward en su extremo 5´.

Data Analysis

Genetic variability from population allele frequencies and genotype was determined. Genetic diversity parameters were estimated for each *locus* and for multiple *loci*: polymorphic *loci* proportion and average genetic diversity, using the formula:

$$D = 1 - \frac{1}{m} \sum_{j=1}^{m} \sum_{i=1}^{l} \rho_{ij}^2$$

where
m = number of *loci*
pij = frequency of allele
i = locus *j*

Genetic diversity per *locus* (52), average observed heterozygosity (Ho), unbiased or expected heterozygosity of Nei (He) (53), number of alleles per *locus* and number of effective alleles per *locus* (35) were also calculated. Squash accessions were compared based on the different measures of genetic variability by non-parametric Friedman test (23) under a classification criterion. Population structure was evaluated by calculating allele fixation index *F* that describes the reduction of population heterozygosity:

$$F = \frac{(He - Ho)}{He} = 1 - (Ho / He)$$

Wright's F statistics (70) were measured following the definition of Nei (1973), and the gene flow among populations was estimated from Fst statistic using the formula of Wright (1951). For measuring biological diversity, allelic richness (r) by direct counting and the Shannon-Weaver index (H) (61) were calculated according to the following formula:

$$H = -\sum_{i}^{S} p_i x \log_{2pi}$$

where

S = total number of types of traits studied

pi = measure of relative abundance of each of these types.

Genetic distances between accessions were established by the simple matching index of similarity (66). Similarity was converted to distance by the transformation:

$$\sqrt{1-S}$$

where

S = the similarity coefficient (simple matching)

Analysis of molecular variation (19) was applied to evaluate population structure using squared Euclidean distances. Principal Coordinates Analysis (25) was carried out on the proximity matrix to order populations by similarity. Molecular and morphological data were correlated by Mantel test (45) and the Generalized Procrustes Analysis (15, 26). Percentage of consensus between the two ordinations (molecular and morphological) was calculated as a measure of association between the two groups of markers.

Accessions were grouped according to their morphological characteristics and molecular profiles by cluster analysis, implicating genetic distances of Gower and unweighted pair-group arithmetic average method (UPGMA) algorithm. The InfoGen version 2011 (7) and Genalex 6 (55) softwares were used.

Results

Six from eight designed primers for *C. pepo* were selected. Two hundred fifty-four primer combinations designed for *C. melo* were monomorphic and three polymorphic in *C. maxima*.

Only one primer combination of the three polymorphic ones, amplified consistently according to the conditions detailed in materials and methods. These results demonstrate the difficulty to transfer microsatellite marker between *Cucumis* and *Cucurbita* species.

Seven primer combinations (table 2, page 299) were finally selected to analyze all *C. maxima* accessions and controls (table 1, page 298). Thirty-one alleles with an average of 4.4 alleles per primer were obtained. Twenty six alleles were detected in *C. maxima*, and only five amplified exclusively in *C. moschata* and *C. pepo* accessions used as controls (table 1, page 298). One microsatellite marker (CP24) was monomorphic for all *C. maxima* accessions. However, it gave a differential band for *C. pepo,* becoming useful for discriminating this species (41).

Genetic variability

Genetic diversity estimated for all accessions of *C. maxima* reached an average value of 0.26 (0.007-0.66), Ho average for all seven *loci* was 0.17 (0-0.54), He of 0.23 (0-0.69), *loci* polymorphic percentage of 45.5%, and PIC 0.2 (0.007-0.61). Ho per *locus* was lower than He in all cases. Nevertheless, accessions 17, 53, 76, 127, 143, 195, 215, 225, 233, 244, 382 (table 1, page 298) had allele fixation indices (F) with average negative values over all seven *loci*, indicating a slight excess of heterozygous individuals.

Around eighty percent of the evaluated accessions were homozygous at five *loci* (CP25, CP33, CP46, CP52 and CP56). The difference between populations was given by the presence of certain combinations of fragments instead of being defined by unique fragments.

Forty eight percent of alleles were unique or rare, with frequencies below 5%.

One allele was exclusive for *C. moschata*, four for *C. pepo* and three for *C. maxima*. Moreover, seven alleles resulted rare for *C. maxima* accession set (table 3). Some of these unique alleles allowed adjusting a routine technique in seed lots to discriminate an interspecific commercial hybrid between *C. maxima* and *C. moschata* (40).

Populations 22, 36, 53, 62, 127, 161, 225, 233, 382 and 523 significantly overcame the others in their values of genetic diversity, PIC, Ho and He (figure 3, page 302).

In terms of population structure, a moderate differentiation in population allele frequencies (Fst 0.199) with a small to moderate non-random effect mating within populations (Fis 0.099) was observed.

Molecular analysis of variance (AMOVA) showed that the genetic variability ($p < 0.05$) was large within accessions (82%). Coefficients Phi pop and Phi st (0.18) indicated a moderate differentiation among accessions and individuals from the same population, considering as high values those above 0.25. Regarding accessions diversity and their geographic origin in Argentina (Provinces), only 2.03 % of the variability is explained by this distribution, being this value not significant ($p = 0.18$). The degree of gene flow (Nm) was close to 1 (Nm = 0.88).

Biodiversity

Biodiversity indices showed Catamarca as the Argentinean Province with the highest allelic richness, with 22 of the 26 amplified alleles for *C. maxima*, followed by Salta and San Juan, both with 19 alleles. Catamarca also had the greatest value for the Shannon-Weaver index, surpassing the total mean value, followed by Jujuy and Salta in order of magnitude (table 4). The results found in Catamarca, Salta and Jujuy agree with values obtained in the number of polymorphic *loci*, genetic diversity, He and average number of alleles (figure 4, page 303).

Table 4. Allelic richness (r) and Shannon-Weaver index (Shaw) by province.

Tabla 4. Riqueza alélica (r) e índice de Shannon-Weaver (Shaw) por provincia.

Province	r	ShaW
Total	26	2-61
San Juan	19	2.44
Catamarca	22	2.72
Tucumán	12	2-28
Salta	18	2.45
Jujuy	16	2.54

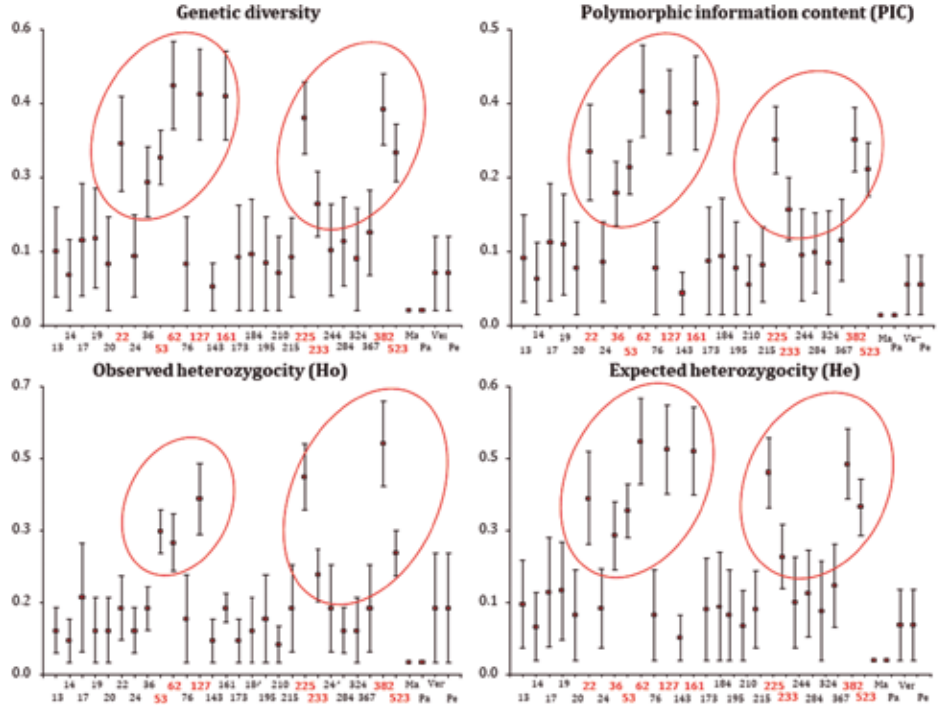
Bootstrap cycles = 1150; confidence 0.95.
Ciclos Bootstrap = 1150; confianza 0,95.

Table 3. Unique or rare alleles found in the different species evaluated.

Tabla 3. Alelos raros o únicos encontrados en las diferentes especies evaluadas.

SSR name	*Cucurbita maxima*	*Cucurbita moschata*	*Cucurbita pepo*
CP24		183 pb (unique)	
CP25	113 pb (rare)		
CP33	192 bp (rare)		200 pb (unique)
CP46	176 bp (rare)		186 bp, 169 bp (unique)
CP52	158 bp (rare)		
CP56	164 bp (rare) 171 pb (unique, acc. 22)		
CM22	170 bp (rare) 168 pb (rare) 184 bp (unique, acc. 184) 162 pb (unique, acc. 215)		171 bp (unique)

Acc.: accession number. / Acc.: número de entrada.



Numbers in the abscissa indicate *C. maxima* accessions (table 1, page 298); accessions used as controls: Ma 'Marino FCA' and Ve 'Veronés INTA' *C. maxima*, Pa: 'Paquito INTA' *C. moschata*, Pe: 'Pepo angola' *C. pepo*.

En la abscisa se indican las poblaciones de *C. maxima* (tabla 1, pág. 298) y las variedades utilizadas como controles: Ma 'Marino FCA' y Ve 'Veronés INTA' *C. maxima*, Pa: 'Paquito INTA' *C. moschata*, Pe: 'Pepo angola' *C. pepo*.

Figure 3. Average population profiles based on: Genetic Diversity, PIC, Ho, He and their corresponding standard errors.

Figura 3. Perfiles poblacionales promedio y sus errores estándar basados en los parámetros de Diversidad Genética, PIC, Ho, He.

Genetic distances

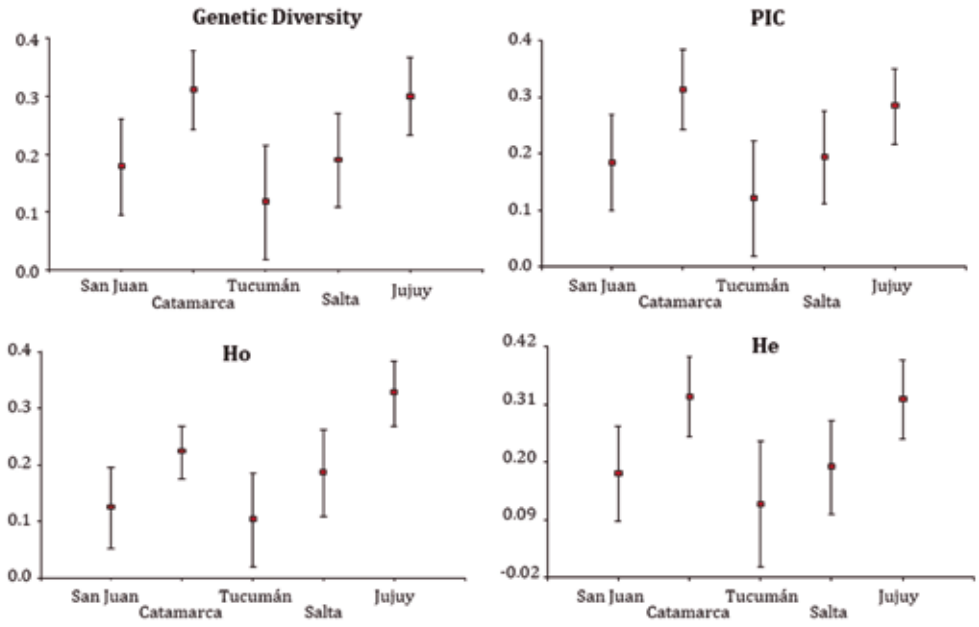
Genetic distances among populations reached values ranging from 0.18 to 0.82, with an average of 0.50. In many cases, genetic distances between *C. maxima* populations were greater than the average distance between *C. maxima* and *C. moschata* species, used as control.

A subset of squash populations were dissimilar to the majority of the accessions and exceeded the average distance for all populations evaluated. This special subgroup included accessions 22, 36, 53, 62, 127, 161, 225, 233, 382 and 523 (figure 3).

Cluster analysis

The first three coordinates in Principal Coordinates Analysis (PCoA) explained 56% of observed variability (figure 5, page 304) and produced four accessions clusters.

First coordinate grouped accessions based on genetic diversity parameters: group number 1 (G1) included accessions 62, 127 and 161 from Catamarca, which recorded the highest number of alleles (21), greater number of effective alleles (2.04), genetic diversity (0.43) and He (0.48).



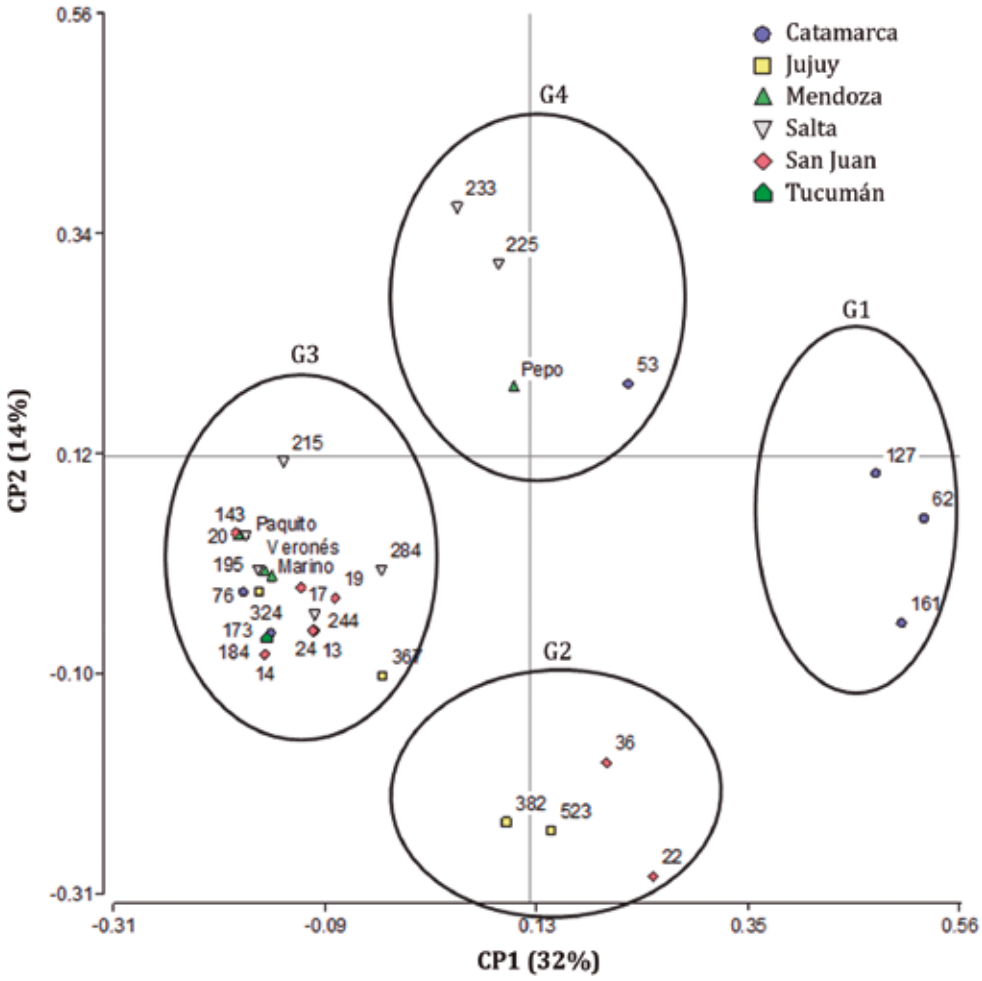
Province names in the abscissa, indices values in ordinates.

Nombre de las provincias en las abscisas, valores de los índices en las ordenadas.

Figure 4. Average provincial profiles based on genetic diversity, PIC, Ho, He, and their corresponding standard errors.

Figura 4. Gráfico de perfiles promedio provinciales, con sus errores estándar, de diversidad genética, PIC, Ho, He.

A core group (G2) with intermediate characteristics of diversity included accessions 22, 36, 523, 382; with 15 alleles and average genetic diversity values of 0.3, Ho of 0.31, He of 0.34, an average number of 2.1 alleles and 1.56 effective alleles. The third group (G3) was the least diverse including the majority of the accessions and controls of *C. maxima* and *C. moschata* species. This group had 10 alleles and a lower value of polymorphic *loci* of 0.22 % *versus* 0.86 % found in the previous groups. The genetic diversity of G3 was 0.1, Ho of 0.09, He of 0.11, average number of alleles was 1.45 and 1.27 effective alleles. The second coordinate ordered the depending on the number of shared alleles. Accessions 233, 225, 53 and *C. pepo* accession formed group 4 (G4), differing from the previous clusters and sharing five of the eight alleles present in *C. pepo.* However, this last control presented lower diversity values than the rest of the accessions of G4. The third coordinate separated accessions 17, 62, 173 and 184 for sharing a rare heterozygous genotype for the CM22 marker with two alleles of 168 and 185 bp (figure 5, page 304).



Marino FCA and Veronés INTA (C. maxima), Paquito INTA (C. moschata) and Pepo (C. pepo) showing the first two coordinates of the Principal Coordinates Analysis (ACoorP) and four groups (G1-G4). Numbers indicate accessions according to table 1 (page 298); icons indicate Argentinean Provinces.

Marino FCA y Veronés INTA (C. maxima), Paquito INTA (C. moschata) y Pepo (C. pepo) según las dos primeras coordenadas del Análisis de Coordenadas Principales (ACoorP) y cuatro grupos (G1-G4). Los números en el gráfico indican las poblaciones de acuerdo a la tabla 1 (pág. 298); los íconos indican la provincia de procedencia.

Figure 5. Scatter plot of 27 populations of *C. maxima* and 4 controls.

Figura 5. Gráfico de dispersión de 27 poblaciones de *C. maxima* y 4 testigos.

Generalized Procrustes Analysis, based on previous morphological data (41, 42, 43, 56) and molecular information, explained 78.7% of the variability in the first two axes. Ordering consensus between molecular and morphological markers was moderate (73.65%), in addition 14 of the 27 populations showed a consensus order below means (73.65%). Both, molecular and morpho- logical markers discriminated against the two controls belonging to *C. maxima* from the set of populations: Marino FCA differed to a greater extent at morphological level and Veronés INTA at molecular level.

A cluster analysis was also performed using morphological and molecular data. Interestingly, four clusters of accessions were also generated, mainly explained by fruit traits, growth habit, and type of consumption (figure 6, page 306).

Molecular genetic variability was taken in consideration in second place.

Cluster 1 included accessions from *C. maxima* var. *zapallito* "zapallito redondo del tronco", with an average genetic diversity of 18%, 40% of polymorphic *loci* and 10.75 average alleles per accession.

Cluster 2 included entries with mixtures of commercial types; genetic diversity reached 7%, 14.3% polymorphic *loci* and eight average alleles per accession.

Cluster 3 included accessions with long vines and winter fruits type with a genetic diversity average of 11.3 %, 26 % polymorphic *loci* and 10.41 alleles per accession.

Cluster 4 included those entries with larger winter fruits and greatest genetic variability; genetic diversity averaged 35.7%, 85.75% of the *loci* were polymorphic and amplified 17 alleles per accession on average.

Discussion

The Argentinean squashes landraces from different Andean environments are characterized for the first time by molecular markers and now preserved in the Vegetable Crop Germplasm Bank of EEA La Consulta, Mendoza, which belongs to the National Network of Germplasm Collections, INTA. An initial screening of microsatellite from *C. pepo* and *C. melo* allowed the selection of proper markers to use in *C. maxima* diversity analysis. A technique for generating amplification products and detecting useful markers for conservation of C. maxima genetic resources in germ- plasm banks, was developed. These results will facilitate future work in molecular studies.

The transferability of microsatellite markers between *C. maxima* and *C. melo* was very low, but one selected microsatellite effectively segregated *C. moschata* accession, and was useful in interspecific hybrid detection for breeding programs (39).

Most, diversity values were similar to the ranges found in other *Cucurbitaceae* studies (8, 20, 60, 67), and lower than those obtained by Lv *et al*. 2012, Mashilo *et al*. (2016) and Kong *et al*. 2014.

Meanwhile, Hamrick and Godt (1996) suggested a polymorphic *loci* ratio of 40% and an expected heterozygosity of 0.168 for *Cucurbitaceae*, similar to those found in the Argentinean accessions evaluated.

High frequency of homozygous genotypes for *loci* CP25, CP33, CP46, CP52 and CP56 suggests a tendency of allele fixation by mating between relatives. The small size of *C. maxima* populations grown in orchards of Andean farmers could favor inbreeding.

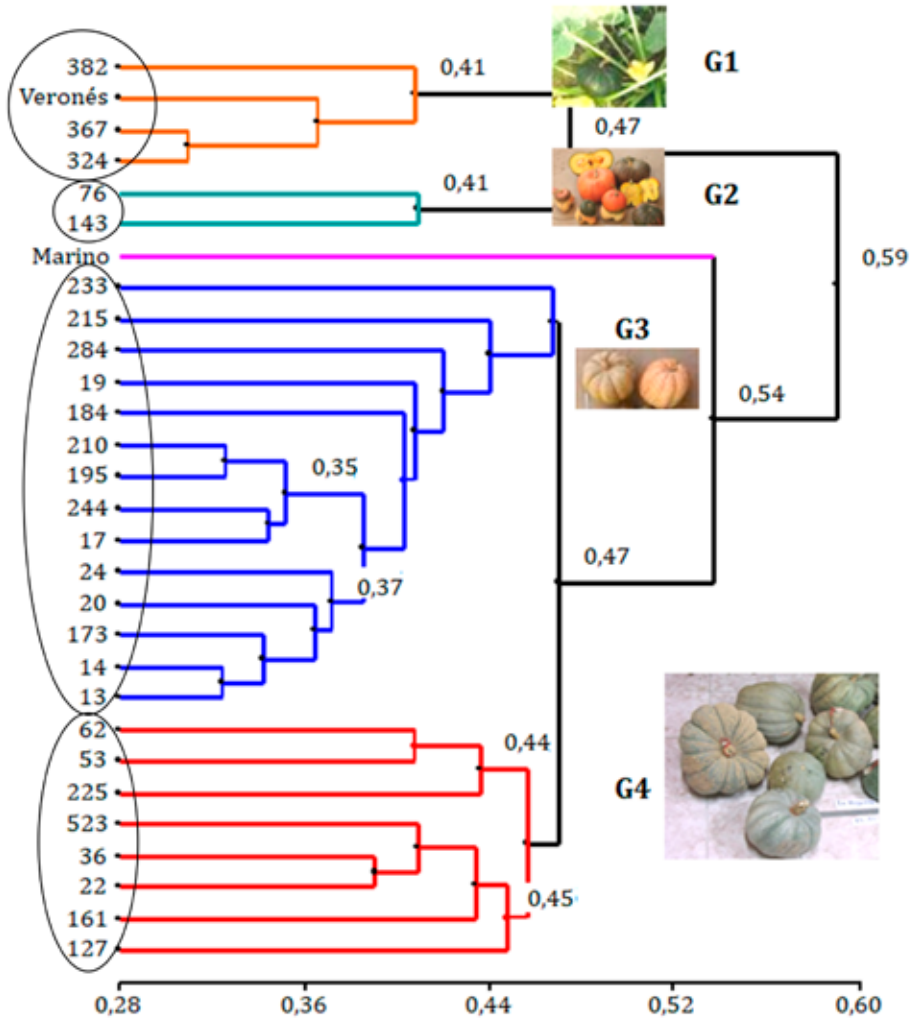


Figure 6. Cluster analysis based on morphological and molecular traits of 27 *C. maxima* accessions and two controls: 'Marino FCA' and 'Veronés INTA' *C. maxima*.

Figura 6. Análisis de conglomerados de 27 entradas de *C. maxima* y dos testigos de la misma especie: Marino FCA y Veronés INTA, utilizando caracteres morfológicos y moleculares.

Rare alleles found are of great interest for the improvement and preservation of biodiversity given that they match with genetically diverse entries. Moreover, the presence of unique alleles allowed differentiating related species such as *C. maxima*, *C. moschata* and *C. pepo*. These rare alleles may also be useful to assess gene flow, pollen, and seed contamination, and to facilitate identification of duplicates and unique accessions in gene banks. Species-specific alleles for Cucurbita species were also observed by Ferriol *et al.* (2003) and Kazminska *et al.* 2017.

Genetic differentiation among entries and among individuals within the same accession was moderate (Phi st and Phi pob 0.18), coinciding with the results obtained by Andrés (1990), Cerón González *et al*. (2010), and Decker-Walters *et al*. (1990) within the genus *Cucurbita*. The degree of gene flow, close to one, indicates that genetic drift acts independently and may generate population differentiation. Migration rate and genetic drift act in equivalent magnitudes. Genetic drift is favored by the gradual reduction in the number and size of *C. maxima* cultivated populations as well as by anthropic selection. On the other hand, gene flow acts as a force that maintains genetic cohesion between populations (17, 57). Different results were found by Mashilo *et al*. (2016) in *Lagenaria siceraria* (Molina) Standl. with low differentiation levels justified by the high levels of genetic flow.

Observed variability was concentrated at intra population level (82%), coinciding with previous studies in related species as *Citrullus lanatus* L. (49) and *C. moschata* (9). Reproductive system of *C. maxima* propitiates intra population diversity because of its flower protandry, high fertility, monoecious structure and entomophily pollination. Moreover, *Cucurbitaceae* natural seed dispersal is carried out by animals that consume their fruits and disperse them with their feces. Farmer's management includes diversification strategies for food production supporting the observed intra population diversity observed. These strategies include cultivating diverse *C. maxima* varieties and other cucurbit species simultaneously inside the orchard without reproductive barriers, and selecting cultivars for their different qualities for consumption, conservation features, and resistance to pests, drought and diseases.

Closest populations generally tend to be similar as geographical proximity favors gene flow. However, a consistent pattern for this statement, is not observed. There is no linear spatial structuring of the observed genetic diversity and no evidence of isolation by distance, meaning that population genetic differentiation, is not explained by geographic distance. These results agree with those obtained by Montes-Hernández and Eguiarte (2002), who pointed out the high potential for pollen dispersion of *Cucurbita* genus despite having specific pollinators.

In addition, the farmer's tradition of seed exchange, deeply anchored in Andean culture, contributes to generate genetic diversity in family orchards. Thus, geographical isolation of farmers in some high Andean valleys of north-western Argentina, mainly in Salta and Jujuy, seems to lack the effect of promoting greater differentiation among accessions found in different areas. The results differ from those of Nanoumé *et al.* (2013), who studied watermelon landraces from Mali founding positive correlation between genetic distances and geographical ones, indicating "that seed exchange has not been so widely used that it can overcome local adaptation".

Biodiversity indices point Catamarca and Jujuy as the Provinces with the greatest genetic diversity for cultivated squashes. These results are consistent with Vavílov (1931), who postulate the region of the tropical Andes, including NW Argentina, as the center of origin of *C. maxima*. Higher genetic diversity at molecular level in NWA region was also evident at the morphological level (41, 43); particularly in Calchaquíes valleys region (Catamarca, Jujuy) where probably some factors allow greater genetic differentiation. Environmental conditions where temperate climate prevails, with an average altitude of 1200 m and access to irrigation, may promote the establishment of larger orchards where the effects of genetic drift are attenuated.

Values of biological diversity observed for 27 accessions of *Cucurbita maxima* were higher than those found by Ceron González *et al*. (2010) for *Cucurbita argyrosperma* Huber, *Cucurbita ficifolia* Bouché, *C. moschata,* and *C. pepo*. Indices of genetic and biological diversity, in general, had a wide range of variation among entries. Most accessions showed an excess of homozygotes relative to what would be expected if populations were in Hardy-Weinberg equilibrium, revealing loss of genetic variability. This phenomenon favors fertility decline, and lower adaptative capacity.

However, a subset of ten entries (22, 36, 53, 62, 127, 161, 225, 233, 382 and 523) showed a slight excess of heterozygotes. These entries had significantly higher diversity indices, showed rare and unique alleles and constituted unique gene pools. These features place them in an advantageous position against environmental changes that exert selective pressure on *C. maxima*. Also, these entries result interesting for suggesting progenitors for hybrid varieties, and inferring evolutionary and crop management phenomena.

Genetic distances were similar to those found by Ferriol *et al*. (2003) and higher than the ones in Baranek *et al*. (2000) and Gong *et al.* (2013) for *C. maxima*. They were also higher than the ones of Ntuli *et al*. (2015) for *C. pepo*, Kong *et al*. (2014) for *C. maxima* and *C. moschata*, and Wu *et al*. (2011) for *C. moschata*.

In many cases, genetic distances between populations of *C. maxima* was greater than the average distance between *C. maxima* and the control (*C. moschata* species). These results may indicate the presence of interspecific gene flow between *C. maxima* and *C. moschata* due to the tradition of cultivating different different, altogether in orchards. Decker-Walters *et al*. (1990) mentioned the natural formation of interspecific hybrids between *C. maxima* and *C. moschata*. Therefore the existence of these hybrids within the set of studied populations, is feasible.

No clear association was found between molecular and morphological markers. This lack of correlation corroborates that the used molecular markers, were designed from genomic libraries enriched in microsatellites (65, 68). Markers obtained by this method are generally found in unexpressed genomic regions.

Generalized Procrustes Analysis allowed a wide interpretation of the relationships between accessions and to get a better description of the genetic diversity (12). Molecular markers discriminated populations of *C. maxima* better than morphological ones. There is a tendency that the most vigorous and larger fruit populations express higher genetic diversity. However, the population number 382 resulted to be one of the most diverse of the collection, even though it belongs to *C. maxima* var. *zapallito* ("zapallito redondo del tronco"). This result may be due to the

significantly lower number of populations of this commercial type, representing 3 out of the 27 populations evaluated.

Molecular diversity was lower than morphological diversity. Despite of the great range of morphotypes and agro-ecological adaptation of *C. maxima* native populations, its genetic base is not as wide as expected. These results agree with other native plant species of South America, such as bean (*Phaseolus vulgaris* L.) (5), peanut (*Arachis hypogea* L.) (28), and maize (*Zea mays* L.), where many races with different morphological characteristics are observed, but are genetically closely related (11). Results indicate that the Andean populations of *C. maxima* derived from one or a few wild populations of *C. maxima* subsp*. andreana*, possibly domesticated in the humid lowlands of Bolivia and in warm temperate areas of South America (61). The morphological diversity observed would be the result of species adaptation to Andean heterogeneous ecological environments, and anthropic selection.

Conclusion

Microsatellite markers revealed moderate genetic diversity among 27 *C. maxima* landraces from different Andean environments. Genetic diversity, both between (18%) as within populations (82%) was found. A subset of ten entries showed significantly higher diversity indices, constituiting unique gene pools.

This evaluation will allow the incorporation of *C. maxima* populations in breeding programs; facilitate its management in the Vegetable Crop Germplasm Bank of INTA EEA La Consulta; enable broadening the species genetic base, raise core collections, and aid in planning future collecting expeditions. Moreover, it will enable to establishing *in situ* and *ex situ* conservation strategies and promote its use by the Andean communities.

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Acknowledgments

We thank Rafael Bottero for elaborating the map used in this work and Cecilia Bruno for statistical advice.

We also thank Gláucia Salles Cortopassi Buso who kindly provided some primer sequences, Pedro Della Gaspera for sharing his huge knowledge about squash culture and María Virginia Sánchez Puerta for their contributions to the manuscript.

The following projects financed this work: Proyecto de Investigación Científica y Tecnológica Orientado (PICT-O 2002 N ° 08-12903) Recuperación, Evaluación y Aprovechamiento Social del Germoplasma "Criollo" de Hortalizas en Regiones Andinas. Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Secretaría de Ciencia, Tecnología e innovación Productiva (SECTYP), UNCuyo and INTA. Filogeografía e implementación de un sistema de monitoreo molecular de la identidad genética de cultivos andinos (INTA-AEBIO 2443 (EPPR), 2006-2008). This paper is part of Inés María Lorello Ph D. thesis in Biological Sciences, UNCuyo. August 2012.