Structure and Genetic Diversity of Three *Calibrachoa caesia* Populations by ISSR Markers

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Abstract

***Calibrachoa caesia* is one of thirteen native species of the *Calibrachoa* Cerv. (ex La Lave & Lexarza) genus, Solanaceae family, growing in Argentina. The genus has a high ornamental value due to the variability that it shows for different genetic characters, including the flower colors. The structure and variation of 29 accessions from three departments in the province of Misiones (Oberá, Guaraní and San Ignacio) were analyzed by ISSR markers. Thirteen ISSR primers produced a reproducible banding pattern, with 652 amplified *loci* and 97.4% polymorphism value. The polymorphism information content ranged from 0.144 to 0.170 for accessions from San Ignacio and Oberá departments, being the average 0.158, whereas the Shannon Index showed an average of 0.256. The principal coordinate analysis showed that the San Ignacio individuals were more disperse than the Guaraní and Oberá accessions. The AMOVA test for Guaraní, Oberá and San Ignacio populations, showed highest genetic variation within populations (93.59%), meanwhile the F_{st} coefficient was 0.064, indicating a low to medium differentiation between populations. These results showed a great intrapopulation genetic diversity but no significant difference was detected among San Ignacio, Oberá and Guaraní populations. According to these results, the highly polymorphic level of the 29 analyzed individuals from the three locations represents an important source of genetic variability for future breeding programs.**

INTRODUCTION

Calibrachoa caesia is a native species from Misiones province, and it one of the thirteen native Argentine species of the *Calibrachoa* Cerv. (ex La Lave & Lexarza) genus, which belongs to the *Solanaceae* family (Hunziker, 2001). In terms of the ornamental characteristics of the genus, the variability in size and color of the flowers, and the diversity in the form and size of the leaves are worth mentioning (Facciuto et al., 2006). Knowledge of genetic variability of available material, represents a key strategy for the generation of tools to assist breeding programs (Cubero, 2003). Molecular markers have proved valuable in crop breeding, especially in studies on genetic diversity and gene mapping (Varshney et al., 2005) In this context, the ISSR are a valuable tool to analyze the genetic variability in a plant collection or wild species (Escandón et al., 2007), and they are also useful to identify accessions, even in highly related individuals (Pérez de la Torre et al., 2003; Hundsdoerfer and Wink, 2005).

Inter-simple sequence repeat (ISSR) analysis involves the PCR amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR) containing primer (Zietkiewicz et al., 1994).
The major advantage of this method is the fact that it does not require an expensive time-consuming step of genomic library construction (Rakoczy-Trojanowska and Bolibok, 2004). In addition, the technique has high reproducibility and low cost (Weising *et al*., 2005).
In the present work we report the use of 13 ISSR to determine the structure and genetic diversity of three *C. caesia* populations from different regions of Misiones province.

MATERIALS AND METHODS

Plant material and DNA isolation

Lyophilized young leaves of 29 native *C. caesia* from three departments in the province of Misiones (Oberá, Guaraní and San Ignacio), were used for the total DNA extraction, following a modified CTAB protocol (Pérez de la Torre et al., 2010). Qualitative and quantitative measures of DNA were determined by electrophoresis in 0.8% agarose-TAE gels stained with Ethidium Bromide (0.01 mg/mL), by using l /Hind III (Pb-L) as a molecular weight marker.

ISSR analysis

For PCR reactions 13 ISSR primers were used (Table 1). These reactions were carried out in a final volume of 25 mL, containing 30 ng of DNA, 0.5 U Taq polymerase, 2.5 mL of 10X reaction buffer, 3.0 mM $MgCl_2$ (Kit Inbio Highway), 0.2 mM of each dNTP (Inbio Highway) and 0.8 mM primer (Qiagen Operon). DNA amplifications were performed in My Cycler of BioRad thermocycler, under the following conditions: preliminary step of 10 min at 94ºC, followed by 40 cycles of 40 sec denaturation at 90ºC, 45 sec to annealing temperature by primer (Table 1) and 90 sec extension at 72ºC with a final 10 min extension at 72ºC. PCR products were analyzed on 2.5% agarose-TAE gels stained with Ethidium Bromide (0.05 mg/mL). The obtained bands were compared (in base pairs – bp) with the 100 bp molecular marker (Pb-L). In order to evaluate the reproducibility of the DNA profile, DNA isolation and PCR reactions were carried out 3 times, and only well-defined and reproducible bands were scored. Bands with the same migration were considered homologous fragments, independently of their intensity.

Molecular data analysis

Each amplification fragment was considered as a dominant allele for a given *locus*. Presence or absence of the band was scored as 1 or 0, respectively, obtaining the molecular identification profile for each individual. The capacity of each ISSR primer to distinguish among the studied genotypes was evaluated by: the Shannon's index (H'), the polymorphic information content (PIC) and the unbiased expected heterozigosity (Uhe). PIC of dominant biallelic data was estimated by the formula: $PIC = 1-p_i^2-q_i^2$, where *"p"* is frequency of visual alleles and *"q"* is the frequency of null alleles (Hardy-Weinberg equilibrium was assumed, where: $q = (1-\text{band frequency})^{1/2}$ and $p = 1-q$). Shannon's index was calculated by the formula: $H' = -\Sigma p_i \ln p_i$.
The molecular analysis of the variance (AMOVA) based in 1023 permutations implemented in Arlequin (Excoffier and Lischer, 2010) and GenAlEx (Peakall and Smouse, 2006), was used to evaluate the variance among and within populations. The F value (F_{st}) from the AMOVA analysis was calculated to estimate the genetic differentiation of the three populations

RESULTS

Thirteen ISSR primers produced a reproducible banding pattern, with 652 amplified *loci* and 97.4% polymorphism value (Table 2).

Oberá population showed the highest PIC (0.170, Table 3), meanwhile Guaraní accessions revealed the highest percentage of polymorphic *loci* (68.25%, Table 2).

Shannon's Index showed an average of 0.256, ranging from 0.235 to 0.271 for accessions from San Ignacio and Oberá departments (Table 3).

The principal coordinate analysis (PCA) showed that the San Ignacio individuals were more disperse than the Guaraní and Oberá accessions (Figure 1).

The AMOVA test for Guaraní, Oberá and San Ignacio accessions, calculated to examine the differences among and within populations was found to be statistically significant ($p < 0{,}001$); the test showed highest genetic variation within populations (93.59%), whereas the variance between the populations was only 6.41% (Table 4, Figure 2)

The F_{st} coefficient was 0.064, indicating a low to medium differentiation among populations (Franco et al., 2001) (Table 4).

These results showed a great intrapopulation genetic diversity but no significant difference was detected among San Ignacio, Oberá and Guaraní populations.

DISCUSSION

The study of genetic diversity for the genomic characterization of germplasm is important not only for conservation, evaluation and utilization of genetic resources, but also to assist the distinctness of specific genotypes for property aims of public and private breeders (Langridge and Chalmers, 2004; Jorasch, 2004).

In this study, we demonstrated that the ISSR primers revealed high genetic variation among individuals, and also revealed low-moderate genetic differentiation among the three studied populations (Franco et al., 2001) These results could suggest that the homogeneity among San Ignacio, Oberá and Guaraní populations could be due to genetic flux or that they had a common origin (Salhi-Hannachi et al., 2005).

The PCA analysis show the samples grouped by region, with a major dispersion for San Ignacio and Oberá accessions.

The primers 5'CT, 5'CA, 5'GA, 3'GA, 3'AC, and 3'AG and 3'TG analyzed in this study were used in another ornamental genus, *Mecardonia* (Pérez de la Torre et al., 2010), as an example . In *Mecardonia*, these ISSR primers were useful to characterize 25 accessions of the genus. In *Mecardonia* 100% polymorphism was found, while in *Calibrachoa* the value was 97.4%. In the same way, in *Nierembergia linearіaefolia* (Escandon et al., 2007), all the primers used in this work were employed to analyze six new varieties of the species. In this case, the percentage of polymorphism for *N. linearіefolia* was 96.39%. These results suggest that the high degree of polymorphism detected by ISSRs could be the result of the high genome coverage, considering that microsatellites are widely distributed through the whole plant genome (Rakoczy-Trojanowska and Bolibok, 2004). However, it is important to mention that the level of polymorphism depends not only on the motifs distribution along the species genome, but also on the type of repetitive sequence incorporated into the primer used to generate the amplification products (González et al., 2005; Pérez de la Torre et al., 2010).

In conclusions, in the present work it was possible to distinguish unequivocally every individual of C. caesia and to obtain the molecular profile of the 29 accessions.The highly polymorphic level of the 29 analyzed individuals from all the studied locations represents an important source of genetic variability for future breeding programs.

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Literature cited

Blair, M.W., Panaud, O. and Mc Couch, S.R. 1999. Inter-simple sequence repeat amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.) Theoretical Applied Genetics 98: 780-792.

Cubero, J. I. 2003. Introducción a la mejora genética vegetal. Mundi-Prensa Libros. ISBN 8484760995, 9788484760993. 565 pp.

Escandón, A., Zelener, N., Pérez de la Torre, M. and Soto, S. 2007. Molecular identification of new varieties of *Nierembergia linariaefolia*, a native Argentinean ornamental plant. Journal of Applied Genetics 48(2), 115-123.

Excoffier, L. and H.E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources. En prensa. http://cmpg.unibe.ch/software/arlequin3.

Facciuto, G., Pannunzio, M.J., Coviella, M.A., Soto, S., Hagiwara, J.C. and Borja M. 2006. Characterizaation of the ornamental value of *Calibrachoa spp* native to Argentina. Act Hort (ISHS) 714: 37-42.

Franco, J., Crossa, J., Ribaut, J.M. and Bertran, J. 2001. A method for combining molecular markers and phenotypic attributes for classifying plan genotypes. Theor Appl Genet. 103:944-952.

González, A., Wong, A., Delgado-Salinas, A., Papa, R. and Gepts, P. 2005. Assessment of Inter Simple Sequence Repeat Markers to Differentiate Sympatric Wild and Domesticated Populations of Common Bean. Crop Science 45: 606–615.

Hundsdoerfer A. and Wink, M. 2005. New Source of Genetic Polymorphisms in Lepidoptera? Z. Naturforsch. 60c, 618-624. 0939-5075/2005/0700-0618.

Hunziker, A. 2001. Genera Solanacearum. The genera of Solanaceae illustrated, arranged accoding to a new sistem. A.R.G. Gantner Verlag K.-G. ISBN 3-904144-77-4.

Jain, A., Apparanda, C. and Bhalla, P. 1999. Evaluation of genetic diversity and genome fingerprinting of *Pandorea* (*Bignoniaceae*) by RAPD and inter-SSR PCR. Genome 42: 714-719.

Jorasch, P. 2004. Section IV. Legal Aspects. IV.1. Intellectual Property Rights in the Field of Molecular Marker Analysis. En: Biotechnology in Agriculture and Forestry 55.

Molecular Marker Systems in Plant Breeding and Crop Improvement. Springer-Verlag. ISSN 0934-943X. ISBN 3-540-20689-2. 478 pp.

La LLave, P. and Lexarza, J.J.M. 1825. *Calibrachoa*. Novorum Vegetabilium Descriptiones 2: 3. 1825.

Langridge, P. and Chalmers, K. 2004. Secction I. Basics. I.1: The Principle: Identification and Application of Molecular Markers. En: Biotechnology in Agriculture and Forestry 55. Molecular Marker Systems in Plant Breeding and Crop Improvement. Section 1. Basics. Springer-Verlag. ISSN 0934-943X. ISBN 3-540-20689-2. 478 pp.

Peakall, R. and Smouse P. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288-295. Versión: 6.3, 2009. http://www.anu.edu.au/BoZo/GenAlEx/

Pérez de la Torre, M., Acevedo, A., Serpa, J.C., Miyajima, I. and Escandón, A.S. 2003. Puesta a punto de la técnica de microsatélites anclados para la caracterización de individuos selectos de Jacarandá. En: Floricultura en la Argentina. Investigación y Tecnología de producción. Mascarini, L. Vilella, F. y Wright. E, eds. pp: 3-12.

Pérez de la Torre, M., Zirilli, P., Ulrich, N., Setten, L. and Escandón, A. 2010. Caracterización molecular en el género *Mecardonia* Ruiz y Pav. (*Plantaginaceae*) utilizando marcadores ISSR. Rev.Fac.Agron. Vol 109 (1): 23-29.

Pradeep Reddy, M.; Sarla, N. y Siddiq E.A. 2002. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128: 9-17.

Rakoczy-Trojanowska M. and Bolibok H. 2004. Characteristics and comparison of three classes of microsatellite-based markers and their application in plants. Cell. Mol. Biol. Lett. 9: 221–238.

Salhi-Hannachi, A., Khaled, C., Messaoud, M., Mohamed, M. and Mokhtar, T. 2005. Comparative analysis of genetic diversity in two Tunisian collections of fig cultivars based on random amplified polymorphic DNA and inter simple sequence repeats fingerprints. Genetic Resources and Crop Evolution 52: 563–573. DOI: 10.1007/s10722-003-6096-3.

UBC. Primer set #9. University of British Columbia, Vancouver, Canada.

Varshney, R., Graner, A. and Sorrells, M. 2005. Genic microsatellite markers in plants: features and applications. Trends in Biotechnology Vol.23 No.1. DOI:10.1016/j.tibtech.2004.11.005.

Weising, K., Nybon, H., Wolff, K. and Kahl, G. 2005. Applications of DNA Fingerprinting in Plant Sciences. In DNA Fingerprinting in Plants. Principles , Methods

and Applications (2nd edition). Taylor and Francis, Eds. CRC Press Boca Raton, London, New York, Singapore. 444 pp.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymorphism chain reaction amplification. Genomics: 20: 176-183.

Tables

Table 1. ISSR primers used in this study. Tº: Annealing temperature, ºC: Centigrade degree.

Primer	Sequence (5'-3')	Reference	T° (ºC)
5'CT	$CCCGGATCC(CT)_9$	Blair et al., 1999	57
5'CA	$CCCGGATCC(CA)_9$	Blair et al., 1999	57
5'GT	$CCCGGATCC(GT)_9$	Blair et al., 1999	57
5'GA	$CCCGGATCC(GA)_9$	Blair et al., 1999	60
5'GACA	$TC(GACA)_4$	Jain et al., 1999	52
3'CAC	$(CAC)_5GT$	Jain et al., 1999	57
3'CAG	$(CAG)_5AT$	Jain et al., 1999	55
3'GGG	$GGG(TGGGG)_2G$	UBC	60
3'GA	$(GA)_9T$	Blair et al., 1999	57
3'AG	$(AG)_8C$	UBC	53
3'AC	$(AC)_8G$	UBC	53
3'TG	$(TG)_8A$	UBC	51
3'TC	$(TC)_8A$	UBC	50

Table 2: Number of *loci* and polymorphic *loci* per population.

Population	**San Ignacio**	**Oberá**	**Guaraní**	**Mean**
Total *Loci* (652)	437	467	472	458,66
Polymorphic *loci*	405	433	445	427,67
% Polymorphic *loci*	62.12%	66.41%	68.25%	65.59%

Table 3: N = General Mean in each *loci* by departament. N = No. of accessions. H' = Shannon's Information Index. PIC = Polymorphic information content. UHe = Unbiased Expected Heterozygosity.

Population	**N**	**PIC**	**UHe**	**H'**
San Ignacio	9	0.144	0.152	0.235
Oberá	10	0.170	0.179	0.271
Guaraní	10	0.162	0.171	0.263
Mean	**-**	**0.158**	**0.167**	**0.256**

Table 4. Analysis of molecular variance (AMOVA) summary for *C. caesia* populations from Guaraní. Oberá and San Ignacio departments of Misiones province.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	2	250.853	5.17066	6.41%
Within populations	26	1963.078	75.50299	93.59%
Total	28	2213.931	80.67365	100%
Fixation Index	**Fst:**	0.06409	P(rand ≥data)=0.001	

Figures

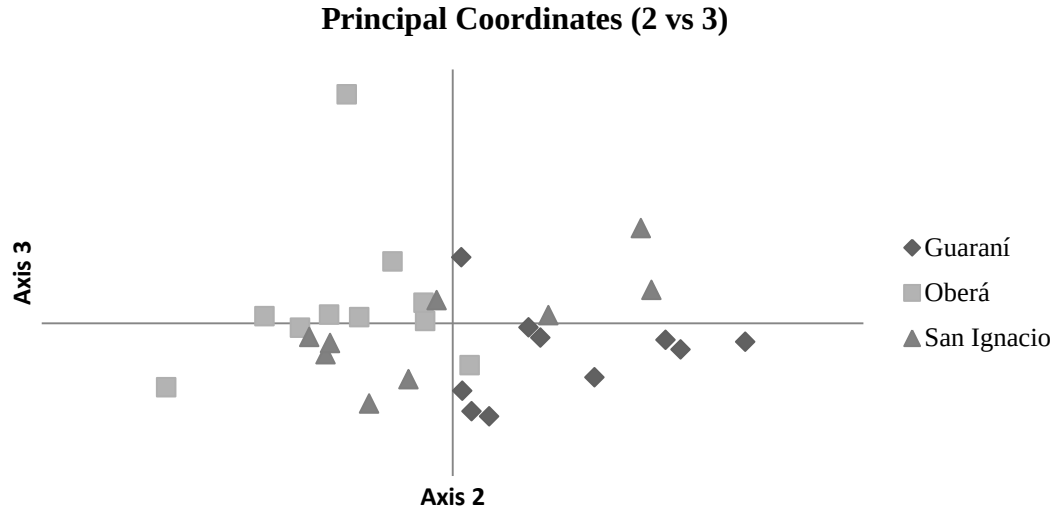

Fig. 1. Principal coordinates analysis (PCA) (axis 2 vs 3) derived from ISSR analysis of 29 *C. caesia* accessions.

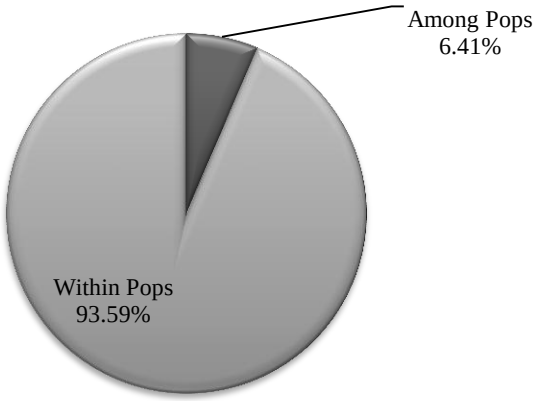

Fig. 2. Percentages of molecular variance (AMOVA)