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Analysis of genetic variability by ISSR markers in *Calibrachoa caesia*

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Abstract

Background: *Calibrachoa* Cerv. (ex La Llave & Lexarza) is a genus of the Solanaceae family (La Llave and Lexarza, 1825). This genus has a high ornamental and economic value due to its intrinsic variability and multiplicity of flower colours. In Argentina there are eight native species, and one of them is *Calibrachoa caesia*. The genetic diversity among 35 accessions of *C. caesia,* from five departments in the province of Misiones, was analyzed using ISSR markers. **Results:** Thirteen ISSR primers yielded a reproducible banding pattern, with 701 amplified loci and 98% of polymorphism. The ISSR primers 5'CT, 5'CA, 5'GA, 5'GACA, 3'CAC, 3'TG and 3'TC generated 100% polymorphic patterns. The Rp values ranged from 23.20 to 10.29 for 5'GACA and 3'AG primers, respectively, while the average values for MI and PIC were 0.367 and 0.231, respectively. The more informative primers were 5'GACA and 5'GA, and the less informative was 3'AC. Simple matching coefficient of similarity varied from 0.8875 to 0.6659, indicating high levels of genetic similarity among the genotypes studied. The UPGMA cluster analysis indicated three distinct clusters; one comprised genotypes of the five departments, while the second included individuals from Guaraní and Oberá regions and the third cluster included the San Pedro individuals. The overall grouping pattern is in agreement with principal coordinate analysis (PCoA). **Conclusions:** The Bayesian cluster analysis revealed structuring of the *C. caesia* population and two clusters were identified, which correspond to UPGMA major clades. The AMOVA test for all populations showed highest genetic variation within populations (90%), meanwhile the F_{st} coefficient was 0.098, indicating a medium differentiation between populations. These results showed a great intrapopulation genetic diversity but no significant difference was detected among populations. In this work the use of thirteen ISSR markers, allowed the characterization of every individual examined. Therefore, the possibility of using molecular data for varietal identification is a promising tool for application in future breeding programs in the genus *Calibrachoa*.

Keywords: anchored microsatellites, molecular markers, ornamental plants

INTRODUCTION

Calibrachoa Cerv. (ex La Llave and Lexarza), belongs to the Solanaceae family (La Llave and Lexarza, 1825; Hawkes et al. 1991). In Argentina there are eight native species, with a North-West distribution, specifically in the Mesopotamia region, with the exception of *C. parviflora*, which is distributed throughout the whole country (Stehmann and Greppi, 2011). *C. caesia* is a native species from Misiones province. Among the ornamental characteristics of the genus, the size and colour of the flowers and the different shapes of the leaves are worth mentioning. The different habits of the species enable it to be used as pot plant, for landscaping and bordures. The full bloom period is in spring and

summer. The native species of *Calibrachoa* are long-day plants, except for *C. caesia* that is neutral day (Facciuto et al. 2006).

An understanding of population diversity is a pre-requisite for the effective utilization of the genetic variability available to breeders (Jabbarzadeh et al. 2010). Furthermore, it is essential to have an unequivocal identification method to verify the material obtained. The traditional methods are now being complemented by molecular techniques, enabling breeders to make better decisions when choosing the germplasm used in breeding programs (Jain et al. 1999; Cubero, 2003). In this context, the ISSRs are a valuable tool to analyze the genetic variability in a plant collection or wild species, and are useful to identify genotypes, even in highly related individuals (González et al. 2002; Pérez de la Torre et al. 2003; Hundsdoerfer and Wink, 2005; Pérez de la Torre and Escandón, 2006; Escandón et al. 2007; Martins-Lopes et al. 2007; Christopoulos et al. 2010; Pérez de la Torre et al. 2010).

Microsatellites (SSRs) are tandem repeat motifs of 1-6 bp which have a frequent occurrence in all prokaryotic and eukaryotic genomes analyzed (Zane et al. 2002). ISSR markers are DNA sequences delimited by two inverted SSR composed of the same units which are amplified by a single PCR primer. This primer is constituted by few SSR units with an anchored end, to avoid unspecific hybridization to the microsatellite region and minimize the slippage (Zietkiewicz et al. 1994; Blair et al. 1999; Weising et al. 2005). ISSR-PCR gives multi locus patterns which are very reproducible, abundant and polymorphic in plant genomes (Bornet and Branchard, 2004). The major advantage of this method is its universality and ease of development (no need for sequence data) (Agostini et al. 2008; Jabbarzadeh et al. 2010), in addition to the reproducibility and low cost of the technique (Weising et al. 2005; Li et al. 2010).

The genetic diversity of the genus *Calibrachoa* can be assessed by neutral ISSR markers, using universal primers based on microsatellite motifs. The aim of the present work is to determinate the genetic variability of 35 accessions from a Floriculture Institute collection using 13 ISSR primers.

MATERIALS AND METHODS

Plant material and DNA isolation

Lyophilized young leaves of 35 *C. caesia* genotypes of the Floriculture's Institute collection (Table 1) were used for DNA extraction, following the Pérez de la Torre et al. (2010) protocol. Victoria INTA-JICA (*Tecoma* hib, Solanaceae) was used as out-group.

Qualitative and quantitative measures of DNA were determined by electrophoresis in 0.8% agarose-TAE gels stained with Ethidium Bromide (0.01 mg/mL), using λ/Hind III (Pb-L) as a molecular weight marker.

ISSR analysis

For PCR reactions, 13 ISSR primers were used (Table 2). These were carried out in a final volume of 25 µL, containing 30 ng of DNA, 0.5 U Taq polymerase, 2.5 µL of 10X reaction buffer, 3.0 mM $MgCl_2$ (Kit Inbio Highway), 0.2 mM of each dNTP (Inbio Highway) and 0.8 µM primer (Qiagen Operon). DNA amplifications were performed in My Cycler of Bio-Rad thermo cycler, 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 2) and 90 sec extension at 72ºC with a final 10 min extension at 72ºC. PCR products were resolved electrophoretically on 2.5% agarose gels run at 120 V in TAE 1X buffer and visualized by staining 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.

Table 1. Geographic distribution of 35 individuals of *C. caesia* studied.

Denomination	Departments	Latitude	Longitude	Altitude (m)
Ccae4	Cainguas	27.284	54.934	517
Ccae5	Cainguas	27.284	54.934	517
Ccae3	Candelaria	27.391	55.718	186
Ccae21	Candelaria	27.391	55.718	186
Ccae14	Guaraní	26.903	54.241	535
Ccae15	Guaraní	26.903	54.241	535
Ccae16	Guaraní	26.903	54.241	535
Ccae17	Guaraní	26.903	54.241	535
Ccae18	Guaraní	26.903	54.241	535
Ccae19	Guaraní	27.210	54.157	360
Ccae25	Guaraní	26.903	54.241	535
Ccae26	Guaraní	26.903	54.241	535
Ccae27	Guaraní	27.210	54.157	360
Ccae28	Guaraní	27.210	54.157	360
Ccae2	Oberá	27.267	55.550	152
Ccae8	Oberá	27.270	55.550	152
Ccae10	Oberá	27.270	55.583	152
Ccae11	Oberá	27.270	55.583	152
Ccae12	Oberá	27.270	55.550	152
Ccae23	Oberá	27.270	55.567	152
Ccae30	Oberá	27.270	55.550	152
Ccae31	Oberá	27.270	55.550	152
Ccae32	Oberá	27.270	55.570	152
Ccae33	Oberá	27.270	55.580	152
Ccae1	San Ignacio	27.250	55.550	197
Ccae6	San Ignacio	27.275	55.555	160
Ccae7	San Ignacio	27.254	55.538	163
Ccae9	San Ignacio	27.250	55.550	197
Ccae13	San Ignacio	27.250	55.550	197
Ccae20	San Ignacio	27.250	55.550	197
Ccae22	San Ignacio	27.250	55.550	197
Ccae24	San Ignacio	27.250	55.550	197
Ccae29	San Ignacio	27.250	55.550	197
Ccae34	San Pedro	26.838	54.348	592
Ccae35	San Pedro	26.838	54.348	592

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 binary matrix was used to calculated the Simple Matching coefficient: SM = (a + d)/(a + b + c + d), where: "a" is the number of bands present in both individuals, "b" and "c" are the number of bands present in only one individual, and "d" is the number of bands absent in both individuals. Cluster analyses were implemented by UPGMA method, and the corresponding dendrogram was constructed. In order to estimate the goodness of fit between similarity matrix and the dendrogram, the coefficient of cophenetic correlation was calculated using the Mantel test with the NTSYS PC software. The capacity of each primer to distinguish among the genotypes studied was evaluated by the Resolving power (Rp) (Prevost and Wilkinson, 1999), Marker index (MI) (Powel et al. 1996), Shannon's index (H') (Shannon and Weaver, 1949) and the polymorphic information content (PIC) (Weising et al. 2005). PIC of dominant bi-allelic data was estimated by the formula: PIC = $1-p_i^2-q_i^2$, were *"p"* is frequency of visual alleles and *"q"* is the frequency of null alleles (Hardy-Weinberg equilibrium was assumed, where: q = (1- band frequency)$^{1/2}$ and p = 1-q). MI was calculated as: MI = PIC x number of polymorphic loci. Shannon's index was calculated by the formula: H' = $-\Sigma p_i \ln p_i$. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, PIC, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall and Smouse, 2006).

Table 2. ISSR primers used in this study.

Primer	Sequence (5'-3')	Reference	Tº (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	$(CAC)_5$GT	Jain et al. 1999	57
3'CAG	$(CAG)_5$AT	Jain et al. 1999	55
3'GGG	GGG$(TGGGG)_2$G	UBC*	60
3'GA	$(GA)_9$T	Blair et al. 1999	57
3'AG	$(AG)_8$C	UBC	53
3'AC	$(AC)_8$G	UBC	53
3'TG	$(TG)_8$A	UBC	51
3'TC	$(TC)_8$A	UBC	50

Tº: Annealing temperature, ºC: Centigrade degree.
*UBC. Primer set #9. University of British Columbia, Vancouver, Canada.

To assess the population structure of the *C. caesia* accessions, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007; Hubisz et al. 2009) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard et al. 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno et al. (2005).

The molecular analysis of the variance (AMOVA) based in 1023 permutations implemented in 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. This index measures the genetic differentiation of the six populations.

RESULTS

The 13 ISSR primers employed revealed a total of 701 loci, with 98% of polymorphism, and an average of 53.92 loci by primer. An example of ISSR pattern obtained with two primers is shown in Figure 1. Table 3 summarizes the data obtained for all the analyzed loci. The polymorphism percentages ranged to 100% to 91.11% for 3'AC. Maximal Rp values were 23.20 and 22.34 for 5'GACA and 5'GA primers, respectively; whereas the 3'AG and 3'TG primers showed the lesser values (10.29 and 10.51 respectively). Major PIC average was 0.231 for 5'CT and minor for 3'TG (0.105). MI values oscillated from 11.69 for 3'GACA to 5.87 for 3'TG primer. The Shannon's index average was 0.278 for all primers, the lesser value was to 3'TG (0.196), and the major was 0.596 (3'GA). The most informative primers for this data set were 5'GACA, 5'GA, 5'CA' 5'CT, 3'CAC, 3'CT and 3'TG, while the primers 3'AC and 3'CAG were less informative ones.

Dendrogram derived from an UPGMA cluster analysis of the ISSR results and the clustering of the 35 accessions according to a model-based Bayesian algorithm are shown in Figure 2. The 35 individuals of *C. caesia* were separated into two large clusters and a minor group, one of them (larger cluster) constituted by genotypes from five departments, while the other grouped individuals from Guaraní and Oberá. San Pedro individuals were grouped together forming the smaller group. Population memberships (expressed as %) for each accession are shown as estimates based on hypothetical subpopulations. Each bar in the graph represents a single accession and its inferred proportion of admixture. The colours represent two different clusters corresponding to inferred unstructured subpopulations, red bars represent the cluster were included the accessions from Guaraní and Oberá, while the blue bars included the rest of the accessions.

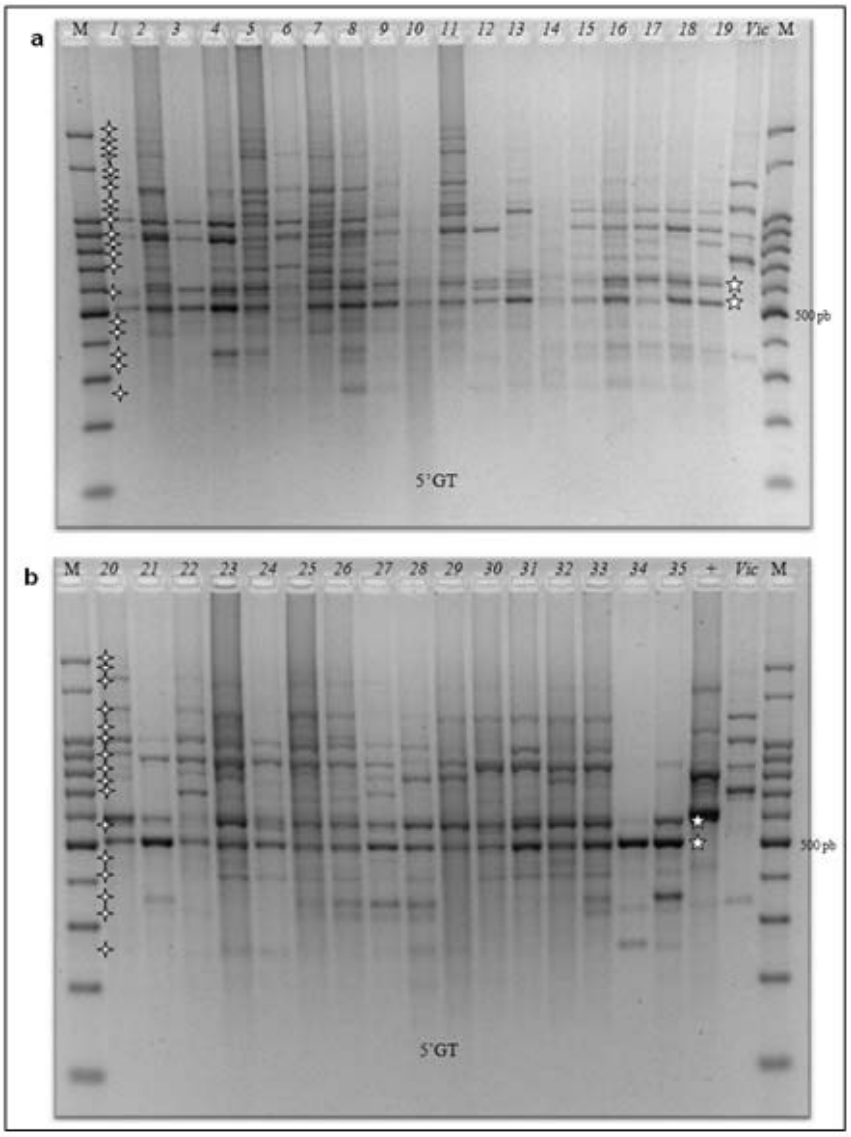

Fig. 1 Amplification profile of *Calibrachoa caesia* accessions. Primer 5'GT. ☆ : Monomorphic loci. ✦ : Examples of polymorphic loci. M: 100 bp molecular marker. +: positive control.

The true number of subpopulations was calculated according to the formula: $\Delta K = m\,|L''(K)|\,/\,s[L(K)]$ following the Evanno et al. (2005) method. For this data set, the *K* value was two, indicating a structure with two clusters (Figure 3).

Simple matching genetic distances between all genotypes were calculated. The mean distance for all comparisons was 0.8404, ranging from 0.8875 (between *Ccae30* and *Ccae31*) and 0.7665. Victoria INTA-JICA joined at 0.6659 level. The correlation cophenetic coefficient was 0.91586 ($p = 0.002$), suggesting a goodness of fit between the similarity matrix and the matrix derived from dendrogram.

Principal coordinates (PCoA) for the 35 *C. caesia* genotypes was carried out using the same set of ISSR markers (Figure 4). The PCoA graphic based in geographic regions was used to identify the accessions on the axes 1, 2 and 3.

Table 3. Results obtained of genetic diversity estimators of *C. caesia* genotypes.

Primer	Amplified loci			%P	Rp	MI	H'	PIC
	TL	ML	PL					
5'CT	40	0	40	100.00	14.80	9.24	0.367	0.231
5'CA	63	0	63	100.00	20.34	10.80	0.291	0.172
5'GT	46	2	44	95.65	17.49	8.26	0.315	0.188
5'GA	71	0	71	100.00	22.34	10.90	0.266	0.153
5'GACA	65	0	65	100.00	23.20	11.70	0.302	0.180
3'CAC	49	0	49	100.00	11.83	7.62	0.258	0.155
3'CAG	55	4	51	92.73	13.71	6.37	0.269	0.125
3'GGG	63	1	62	98.41	21.77	11.70	0.312	0.188
3'GA	56	1	55	98.21	18.51	10.30	0.309	0.188
3'AG	47	2	45	95.74	10.29	7.41	0.273	0.165
3'AC	45	4	41	91.11	14.17	6.79	0.279	0.166
3'TG	56	0	56	100.00	10.51	5.87	0.196	0.105
3'TC	45	0	45	100.00	12.29	5.95	0.236	0.132
Total	701	14	687					
Mean	53.92	1.08	52.85	98.00	-	-	0.278	0.164

The first two principal components explained 22.7% and 20.29% of the genetic variance, respectively, and the 63.39% was explained by the first three components. San Ignacio and Guaraní genotypes showed similar dispersion along the first component while Oberá genotypes clustered in two groups (Figure 4a). Within Guarani genotypes, *Cceae14* located remote from the rest of the same location on axe 3 (Figure 4b). Individuals from San Pedro clustered together, distant from the rest of the collection (Figure 4a and 4b). These results are in agreement with the dendrogram clustering.

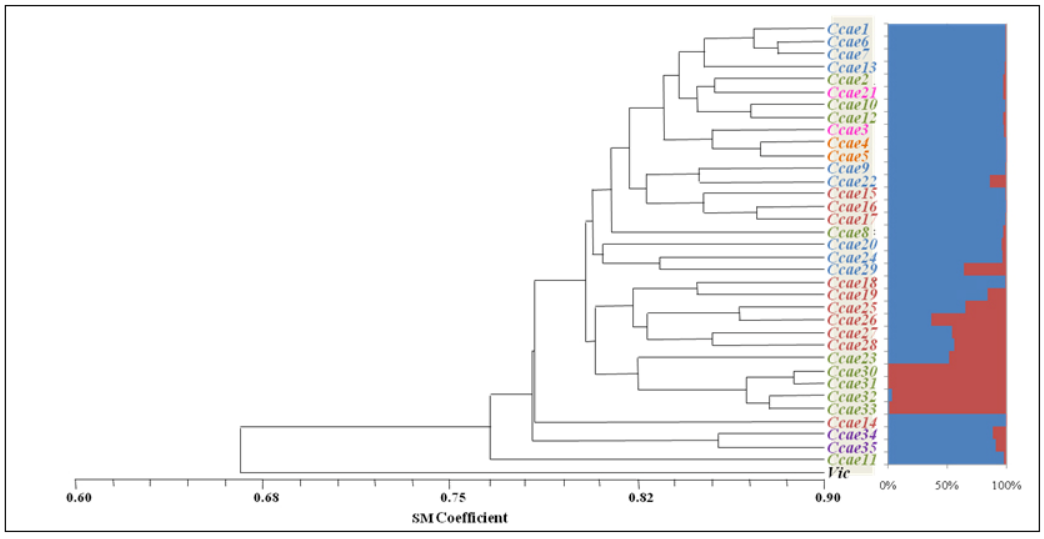

Fig. 2 Dendrogram of 35 *C. caesia* genotypes based in UPGMA clustering using a SM coefficient. A different colour represents each one of the six populations: Cainguas (orange), Candelaria (pink), Guaraní (red), Oberá (green), San Ignacio (blue) and San Pedro (purple). On the right side, clustering according to a model-based Bayesian algorithm implemented in the program STRUCTURE. The two colours represent two different unstructured subpopulations.

The AMOVA test for all accessions, calculated to examine the differences among and within geographical populations was found to be statistically significant ($p < 0,001$); the test showed highest genetic variation within populations (90%), whereas the variance between the populations was only

10% (Table 4, Figure 5). The F_{st} coefficient was 0.098, indicating a 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 the populations.

Table 5 summarizes the average of polymorphic bands according to the nucleotide repeat motifs and significance level, calculated using the Tukey test, among the different primers used.

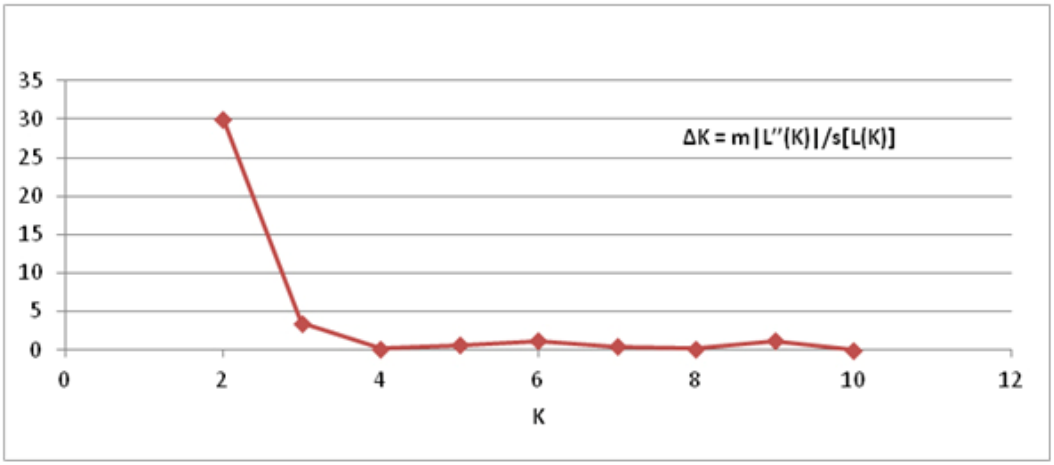

Fig. 3 Graphic showing the modal value of this distribution, this was the true K or the uppermost levels of structure, here two clusters.

DISCUSSION

The use of molecular markers has become a common practice in studies of population structure, genetic diversity for pre-breeding and breeding germplasm and in distinguishing one individual genotype to preserve the property of breeding rights (Langridge and Chalmers, 2004). In addition, ISSR markers are useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species (Christopoulos et al. 2010; Jabbarzadeh et al. 2010).

In this study, we found a high degree of polymorphism in the genotypes studied, with values above 91% and a mean of 98%. Furthermore, ISSR markers revealed high genetic variation among 35 genotypes, allowing an unequivocal identification for each one.

In this work, we used the same primers reported by Escandón et al. (2007), who identified six new varieties of *Nierembergia linariaefolia.* In that work, the six varieties revealed 443 polymorphic loci, with a 96.39% of polymorphism and a mean of 32.80 loci per primer. For *C. caesia*, the 35 genotypes showed 701 loci, 98% polymorphic (687 loci), and a mean of 52.85 loci per primer. The primers that were 100% polymorphic in *N. linariaefolia* were: 5'CT, 5'GT, 5'GA, 5'GACA and 3'TC, while for *C. caesia* these were 5'CA, 3'CAC and 3'TG. In comparison, the coincidental primers were 5'CT, 5'GA, 5'GACA and 3'TC. For Escandón et al. (2007) in *N. linariaefolia*, the most informative primers, considering percentage of polymorphism and Rp values, were 5'GT (% P = 100 and Rp = 12.67) and 5'CA (% P = 98.25 and Rp = 13.67), followed by the primers 5'GACA and 3'AC (Rp around 7). In our work the most informative primers, following the same criteria and a 100% of polymorphism, were 5'GACA, 5'GA, 5'CA and 5'CT (Table 3).

In another ornamental genus, *Mecardonia*, and using the primers 5'CT, 5'CA, 5'GA, 3'GA, 3'AC, 3'AG and 3'TG, it was possible to characterize molecularly 25 genotypes of two species of the genus, *M. procumbens* var. *flagellaris* and *M. procumbens* var. *tenella* (Pérez de la Torre et al. 2010). In this

case, the percentage of polymorphism was 100%. For *Mecardonia* the Rp values were similar to *Calibrachoa,* ranging from 11.92 for 3'TG to 25.23 for 5'CT and 5'GA, while the values for *C. caesia* were 10.29 to 23.20 for 3'AG and 5'GACA, respectively (Table 3). PIC and MI values for *Mecardonia* were also similar to this study, with a mean of 0.130 for PIC and MI values ranging from 12.160 to 5.875. For *C. caesia*, the PIC mean was 0.164 and MI values ranged from 11.70 to 5.87. Noticeably, the Rp is a measure that takes into account the proportion of individuals that contain the band, therefore, when dealing with a larger number of samples, the RP value increases. It should also be taken into consideration that the statistics calculated here (*i.e*. Rp, PIC, MI, H') correspond to this data set, and although they may be extrapolated to other species of the genus, we cannot infer beyond the genus, even to another genus highly related to *Calibrachoa*, such as *Petunia*.

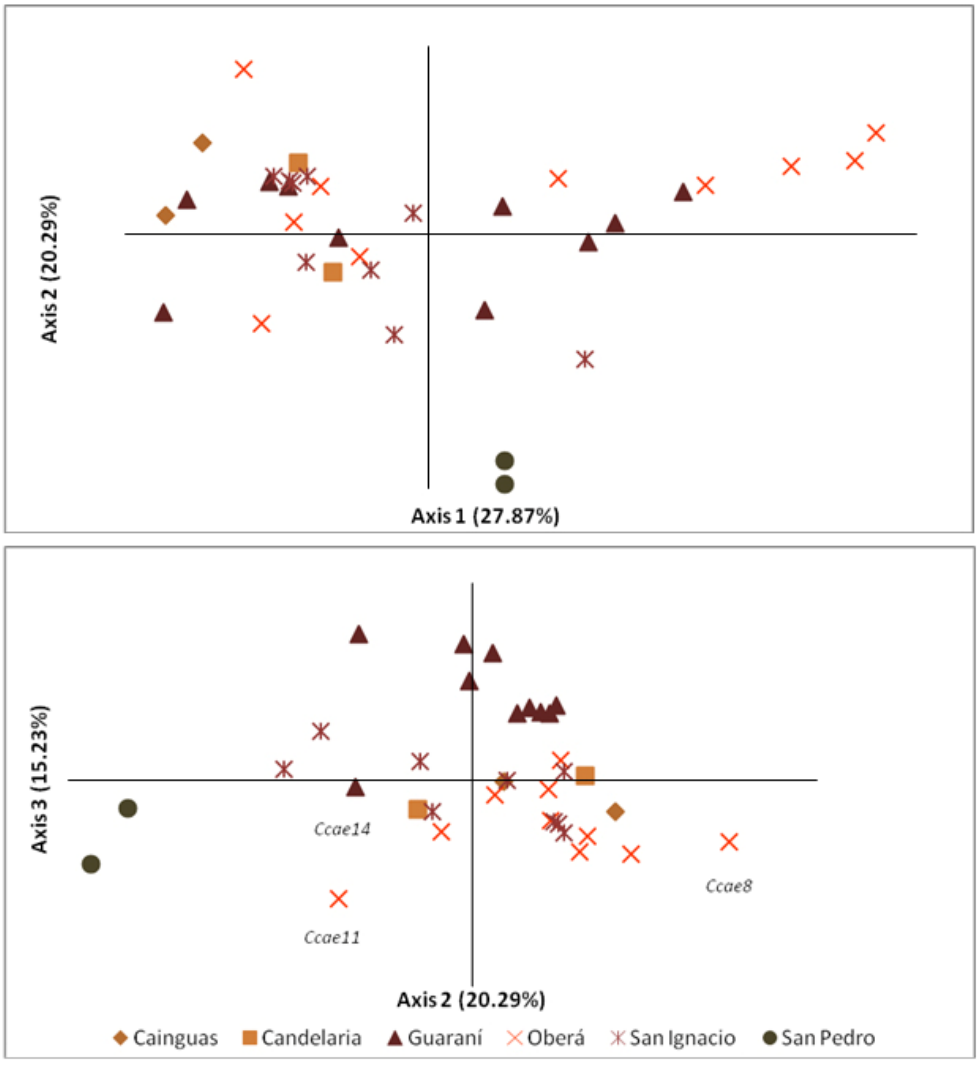

Fig. 4 Principal coordinates analysis (PCoA): (a): (axis 1 vs 2), (b): (axis 2 vs 3) derived from ISSR analysis of *C. caesia* genotypes.

The high level of polymorphism observed (98%) with the primers used in this study, indicate a high level of genetic variation among the 35 genotypes analyzed, in agreement to Rakoczy-Trojanowska and Bolibok (2004), who reported highly polymorphic patterns when reaction primers based on microsatellite sequences in plants were employed.

Table 4. Analysis of molecular variance (AMOVA) summary for *C. caesia* accessions.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	5	581.535	8.005	10%
Within populations	29	2136.522	73.673	90%
Total	34	2718.057	81.678	100%
Fixation Index	**Fst:** 0.098 P(rand ≥ data)=0.001			

In relation to genome coverage, calculated according to the Bhatia's criterion (Bathia et al. 2009), we did not find significant differences in the use of 5' or 3' anchoring primers, or with di-tri or tetra nucleotides primers (Table 5). However, in this study, as in Blair et al. (1999), primers with poly (GA) motifs generate on average, a larger number of amplified loci. In the present work, primers with 3' anchoring showed more monomorphic loci (six from eight primers) than 5' anchoring ones (one from five primers) (Table 3). These results could be possible because primers anchoring in 5' include in the amplified product the complete microsatellite sequence, and thus, the variability in the number of nucleotides within a microsatellite repeat would result in length polymorphism when using a 5' anchored primer (Pradeep Reddy et al. 2002).

These results, together with those obtained by Ruas (2003), González et al. (2005) and Pérez de la Torre et al. (2010) indicate that the level of polymorphism detected by ISSR primers depends on the species or genus and the repetitive SSR used in the primer utilized to generate the amplification profiles, in addition to the high genome-primer relationship.

The dendrogram analysis allowed the identification of two major groups (Figure 2), constituted by several subgroups, one of them formed by genotype from all the regions, while the other one only had individuals from Guaraní and Oberá. Similar results were obtained by Neighbour joining analysis performed by NTSYS PC software (data not shown). These results are in agreement with the two clusters obtained by STRUCTURE software, population determination is usually based upon geographical origin of samples or phenotypes. However, the genetic structure of populations is not always reflected in the geographical proximity of individuals. Populations that are not discretely distributed can nevertheless be genetically structured, due to unidentified barriers to gene flow. In addition, groups of individuals with different geographical locations, behaviour and patterns or phenotypes are not necessarily genetically differentiated (Evanno et al. 2005).

In this study, we demonstrated that the ISSR primers revealed high genetic variation among individuals and also revealed moderate genetic differentiation among the six populations studied (Franco et al. 2001). These results could suggest that the homogeneity among Cainguas, Candelaria, San Pedro, San Ignacio, Oberá and Guaraní populations could be due to genetic flux or that they had a common origin (Amel et al. 2005), alternatives that needs be further explored.

The PCoA graph (Figure 4) exhibits the dispersal distribution of genotypes from different populations. The analysis shows that the San Pedro individuals group together and appear separate from other populations, and that the San Ignacio and Oberá individuals are widely scattered.

In the same way, the most distanced genotypes (*Ccae8, Ccae11* and *Ccae14*) in the PCoA graphic (Figure 4) are grouped outside of the clusters in the dendrogram (Figure 2). The individual *Ccae8* differed at the phenotypic level, presenting a corolla colour lighter than the other individuals of the same region. However, the distribution of *Ccae11* cannot be associated with any morphological characteristic observed *in situ*, since this individual presents phenotypic traits similar to the rest of the population. For *Ccae14*, there is also no phenotypic distinction, because the individual exhibits characteristics that are similar to all the genotypes of the region; however, the genetic amplification pattern was clearly different from other individuals of the population in the region. In this case, a study with different primers and the inclusion of more individuals would be necessary to verify the clustering.

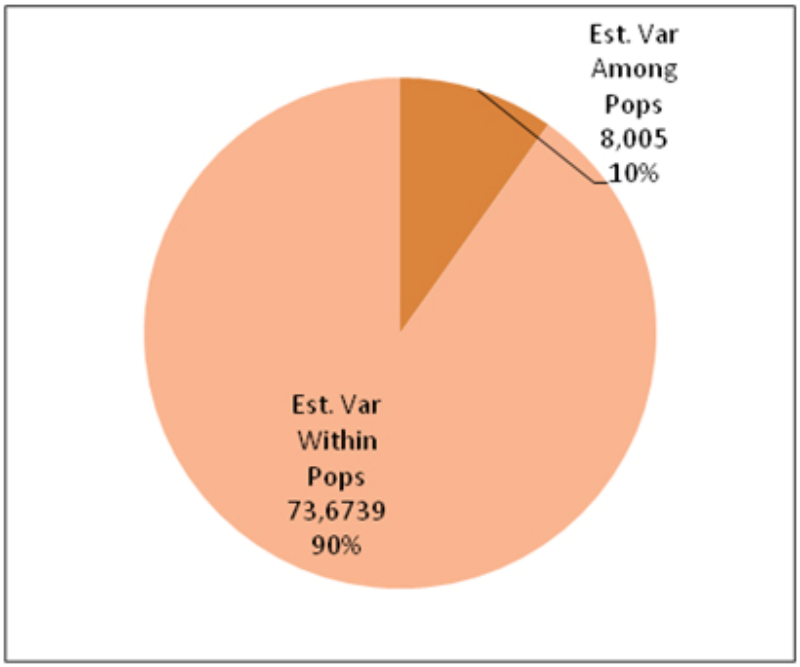

Fig. 5 Percentages of molecular variance (AMOVA).

Table 5. Average of polymorphic bands according to the nucleotide repeat motifs and significance level.

Tandem repeat	Mean polymorphic bands	Nº of primers	Signification
CT-TC	42.50	2	a
GT-TG	50.00	2	a
CA-AC	52.00	2	a
GA-AG	57.00	3	a
Tri-Tetra nt	56.75	4	a

The results obtained in this study, validate once more that ISSR are useful markers in genetic diversity studies, due to the very high polymorphism level detected by the primers. In addition, when the genetic basis of the species or genus to be analyzed is not well-known and rapid and robust results are necessary, this technique is particularly useful because it is simple and has a low cost. The possibility of characterization of every individual examined, offers a promising perspective as a molecular tool for varietal identification and breeding program applications.

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