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Assessment of genetic variation on four genera of *Cactaceae* using taxonomic, cytological and molecular markers methods

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Abstract

The genetic diversity for twenty species belonging to four genera of *Cacataceae* (*Rebutia, Aylostera, Mediolobivia* and *Sulcorebutia*) was analyzed employing taxonomic methods, chromosomes number and RAPD markers. The botanical classification that describes the phenotypic aspects of different characters, such as plant diameter, number of spines/areole, mean spines length, flower diameter, and flower color, was employed. Chromosome analysis revealed both diploids and polyploids in the studied species. Polyploidy was represented by two tetraploids species (*S. crispata* and *R. kupperiana* var. *spiniflorum*, 2*n* =4*x* =44) and by five triploids species (*R. pseudodeminuta* var. *schumaniana, R. senilis* var. *liliacinorosea, R. calliantha, R. donaldiana* and *M. diersiana*, 2*n* =3*x* =33). Thirteen species were diploid with 2*n* =2*x* =22. The total number of amplified bands was 448, and 400 bootstrap samples for generating the dendrogram, were used. The dendrogram indicates the diversity of the genotypes, which are grouped into four distinctive large groups. Almost all groups include species from the *Rebutia, Aylostera, Mediolobivia* and *Sulcorebutia* genera, which clearly share a common ancestor. *M. diersiana* and *R. donaldiana*, two triploid species (2*n*=3*x*=33) formed the same subgroup in the frame of the dendrogram. In addition, those species also present common phenotypical peculiarities like: colour of flower (orange), diameter of flower (2.5 cm). The taxonomic methods were less precisely, regarding to assess genetic variation, comparing with cytological methods, and molecular markers methods.

Keywords: cacti species, dendrogram, DNA markers, phenotypical peculiarities, chromosomes.
Abbreviation:
A: *Aylostera* genus;
RAPD: *Random Amplification of Polymorphic DNA*,
R: *Rebutia* genus, M: *Mediolobivia* genus;
S: *Sulcorebutia* genus, var: variety.

Introduction

The greatest diversity of the family *Cactaceae* is recorded in Mexico, with 586 species, followed by Brazil, Argentina, Bolivia, and Peru (Ramawat, 2010). Cacti are used as food and fodder, as medicinal and ornamental plants, and as a source of wood. The plants architecture determines their different life forms, which include arborescent, columnar, globular, barreliform, and articulated forms (Gibson and Nobel, 1986; Terrazas-Salgado and Mauseth, 2002). The species in the genera *Rebutia*, *Mediolobivia*, *Aylostera* and *Sulcorebutia* were pulled together in a single genus, *Rebutia* (Anderson, 2001; Barthlott and Hunt, 1993; Hunt, 1999; Hunt et al., 2006). Instead Backeberg (1968-1977) and Krainz (1967) divided *Rebutia* genus into three genera: *Aylostera*, *Rebutia* and *Mediolobivia* and suggests synonymies among *Sulcorebutia* and *Weingartia* and, therefore, recommends merging them into one (Ritz et al., 2007). Therefore it is required more accurate assessment of this species and varieties both to phenotypical, molecular and cytological level. Molecular biological tools can accelerate artificial breeding processes (Smolik et al., 2009) and clarify the genetic mechanisms that cannot be easily dissected with plant breeding techniques (Staub et al., 1996; Gupta, et al., 2010). Molecular tools can also give important information about the genetic distances between species. DNA isolation from cacti is difficult because they contain high levels of polysaccharides and secondary metabolites, which form insoluble complexes with nucleic acids during their extraction (Guillemaut and Maréchal-Drouard, 1992). As in other groups of plants, the secondary metabolites and polysaccharides in cacti inhibit enzyme activities (Porebski et al., 1997). Previous chromosome number reports have established the utility of knowing ploidy level differences in making taxonomic interpretations in certain cactus genera, for example *Echinocereus* (Cota and Philbrick, 1994) and *Opuntia* (Baker and Pinkava, 1999; Pinkava et al., 1985; Rebman and Baker, 1998). That objective has been continued in the current work, but considerably more attention has been directed toward the genera *Rebutia*, *Aylostera*, *Mediolobivia*, and *Sulcorebutia* to the phenotypical level, cytological and molecular. Also, a conscientious effort was made to obtain chromosome numbers and to assess the markers Random Amplification of Polymorphic DNA (RAPD) ability to detect polymorphisms in this species.

Materials and methods

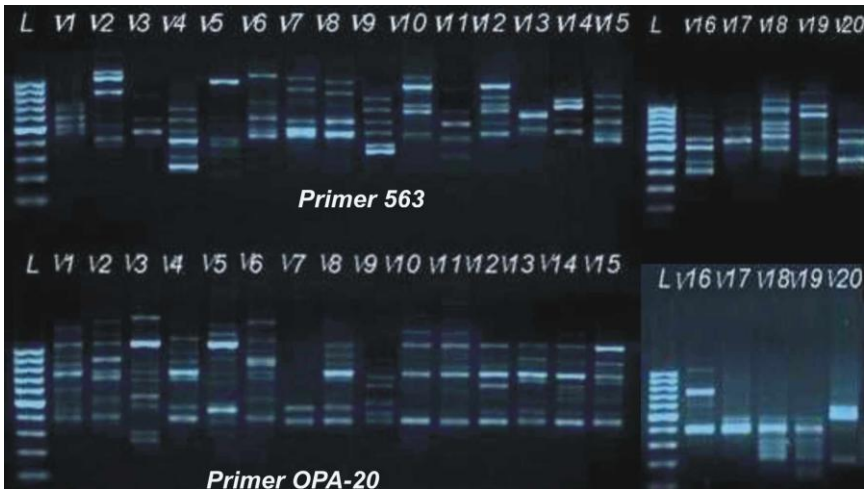
Plant material and growth conditions

The material investigated was represented by 20 species of cacti belonging to four genera from the *Chaetolobiviae*

Table 1. Phenotypic aspects of the studied cacti species and the quantity of the extracted DNA samples

The genotypes	Plant diameter (cm)	Radial (external) spines		Flower		Quantity ng/µlds DNA*
		Number /areoles	Length (mm)	Diameter (cm)	Colour	
Aylostera fiebrigii	> 6	30-40	> 20	3.5	*Red*	65.30
Aylostera narvaecensis	2.5-3.5	10-20	2-3	4	*Pink*	76.50
Aylostera vallegardensis	4-5	> 30	3-10	2	*Red*	131.60
Aylostera flavistyla	4-5	15-22	5-10	3	*Orange pink*	149.90
Mediolobivia ritteri	3	8-10	10	2.5	*Red violet*	199.10
Mediolobivia diersiana	6	10	10	2.5	*Orange*	147.20
Rebutia kupperiana var. *spiniflorum*	3	13-15	5	3.5	*Orange*	183.70
Rebutia cajasensis	3	10-11	2-3	2.5	*Red light*	83.20
Rebutia pygmaea	2	9-11	2-3	2.0	*Red*	80.80
Rebutia graciliflora	2.5	15	2-3	1.7	*Red*	163.20
Rebutia marsoneri	5	30-35	> 5	4.5	*Yellow*	69.60
Rebutia buiningiana	> 5	14-16	6-10	3	Yellow	53.20
Rebutia pseudodeminuta var. *schumaniana*	3.5	7-14	3-5	2.5	*Orange*	441.40
Rebutia violaciflora var. *luteispina*	2	20	25	3	*Pink*	63.50
Rebutia senilis var. *liliacinorosea*	7	> 25	>30	3.5	*Red*	50.90
Rebutia calliantha	2.7	12-21	5-10	4.5	*Red*	136.20
Rebutia donaldiana	8	10-12	15	3	*Orange*	341.20
Sulcorebutia candiae	5	7-10	3-7	3	*Yellow*	129.40
Sulcorebutia markusii	6	12	> 5	>3.5	*Dark red*	116.10
Sulcorebutia crispata	2.5	> 50	8-10	4	*Red light*	54.20

Note: all the values represented the arithmetic mean; *The DNA amount was provided with the NanoDrop Product "Spectrophotometer nd-1000".



V1- Rebutia violaciflora var. *luteispina*
V2-Sulcorebutia candiae
V3- Rebutia pseudodeminuta v. *schumaniana*
V4-Mediolobivia ritteri
V5- Rebutia calliantha
V6-Sulcorebutia crispata
V7- Rebutia buiningiana
V8-Rebutia pygamea
V9-Aylostera fiebrigii
V10- Rebutia cajasensis
V10-Aylostera narvaecensis
V12- Aylostera flavistyla
V13- Rebutia senilis var. *liliacinorosea*
V14- Rebutia donaldiana
V15- Rebutia graciliflora
V16- Rebutia kupperiana v. *spiniflorum*
V17-Aylostera vallegardensis
V18- Rebutia marsoneri
V19- Sulcorebutia markusii
V20- Mediolobivia diersiana

Fig 1. The amplification of the reaction products with OPA-20 and 563 primers.

subgroup*: Rebutia*, *Aylostera*, *Mediolobivia*, and *Sulcorebutia* (Table 1). The plants were grown in the Botanical Garden "Alexandru Borza" Cluj-Napoca, Romania, in a greenhouse, with a minimum of 5°C (in December) and a maximum of 23.5°C in July. The plants were analyzed at an early age (three years old).

Taxonomic methods

The plants evaluated for taxonomic characters relied on a botanical classification that describe the phenotypic aspects of different characters, such as plant diameter, number of spines/areoles, length of spines, flower diameter, and flower colour. These characters were the same as described by the UPOV normative (UPOV, 1987).

Cytological methods

Adventitious root tips were collected between 07:00 and 09:00 a.m. and were used for mitotic counts following Briones et al. (2004); Las Peñas et al. (2008); Negrón-Ortiz (2007). The root tips were treated with colchicines (0.01%, for 3 hours) to inhibited chromosome segregation during meiosis, and then were fixed in a 3:1 ethanol: glacial acetic acid mixture for 24 hours in the refrigerator (Cota and Philbrick, 1994; Cota and Wallace, 1995).

Table 2. The primers used for RAPD analyses at species from *Aylostera*, *Rebutia*, *Mediolobivia* and *Sulcorebutia* genera

No. of entry	Primer	Nucleotidic sequence(5'-3')	Molecular weight	Amplified products
1.	OPA-17	GAC CGC TTG T	3019	+
2.	OPA-18	AGG TGA CCG T	3044	+
3.	OPA-20	GTT GCG ATC C	3019	+
4.	270	TGC GCG CGG G	3085	+
5.	563	CGC CGC TCC T	2940	+
6.	OPA-11	CAA TCG CCG T	2988	–
7.	OPA-16	AGC CAG CGA A	3046	–
8.	OPC-04	CCG CAT CTA C	2948	–
9.	OPC-08	TGG ACC GGT G	3084	–
10.	OPC-09	CTC ACC GTC C	2924	–
11.	OPC-13	AAG CCT CGT C	2988	–
12.	OPC-20	ACT TCG CCA C	2948	–
13.	MIC-07	TGT CTG GGT G	3090	–
14.	MIC-13	TTC CCC CCA G	2924	–
15.	MIC-14	TGA GTG GGT G	3139	–

Note: "+" means the presence of amplified products; "–" means the absence of amplified products.

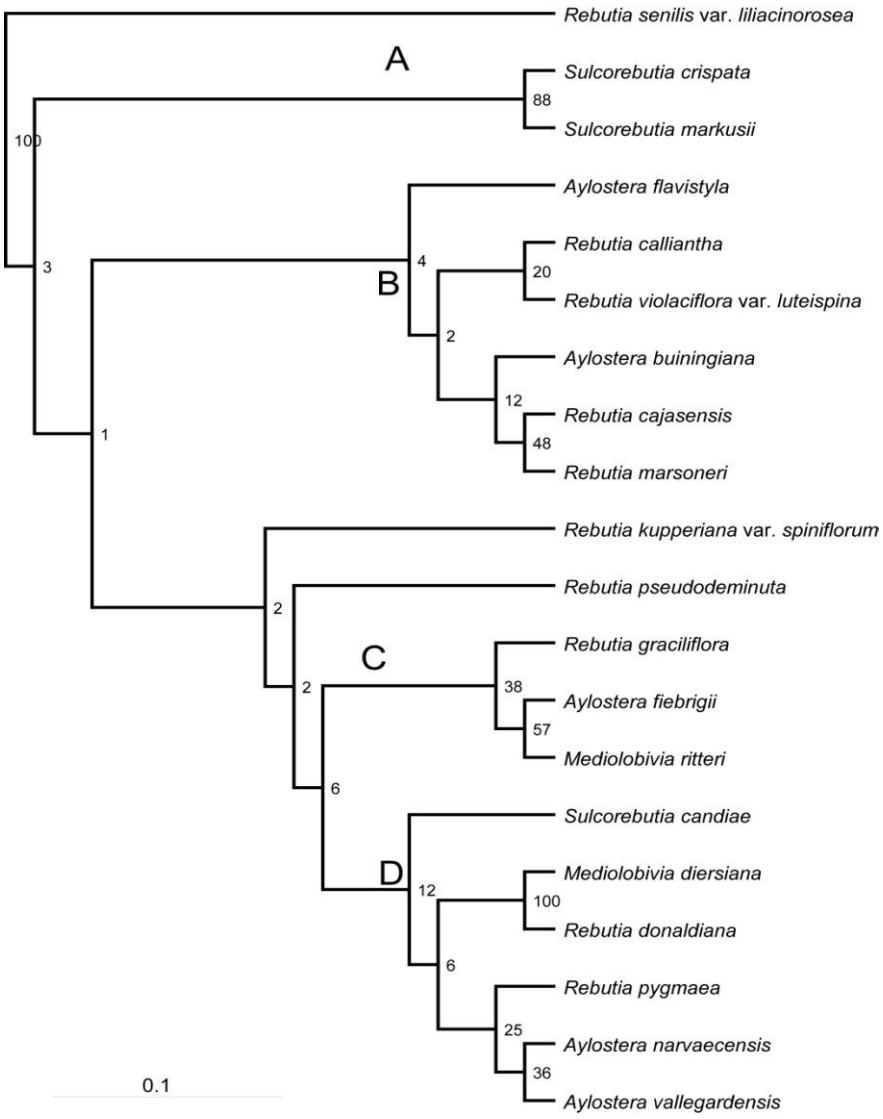


Fig 2. Dendrogram of the genotypes of cacti plants from *Rebutia*, *Aylostera*, *Sulcorebutia* and *Mediolobivia* genera.

Table 3. The number of somatic chromosomes at different cacti species

The genotype	Number of somatic chromosomes (2*n*)
S. candiae, M. ritteri, A. fiebrigii, A. narvaecensis, A. vallegardensis, R. cajasensis, R. pygmaea, A. flavistyla, R. graciliflora, R. marsoneri, A. buiningiana, R. violaciflora var. *luteispina, S. markusii*	2*n*=2*x*=*22*
R. pseudodeminuta var. *schumaniana, R. senilis* var. *liliacinorosea, R. calliantha, R. donaldiana, M. diersiana*	2*n*=3*x*=33
R. kupperiana var. *spiniflorum, S. crispata*	2*n*=4*x*=44

For slide preparation, root tips were hydrolyzed with hydrochloric acid (1N) for 11 min at 60°C and transferred to Feulgen reagent for 2 hours (Palomino et al., 1999) and were prepared using the "squash" technique. For a better view, it has been, also used a Leica CM1850 cryotome, which achieved thin sections (commonly performed in histology, pathology). Protocol included the following steps: root fragments (50 mm long) were embedded in the cryosection Jung medium (Brown et al., 2005) and then frozen at -20^{o}C. Sections of 30 µm, were taken on "Star Frost" slides and examined at Olympus DX 51 microscope with 40X, 100X and with immersion objectives and photographed with Olympus DP25 camera, using CellB program. For the observation of chromosome numbers (*2n*), 9 to 15 mitotic cells at metaphase stage from three plants of each species were observed.

Molecular marker methods

The fresh tissues of the cactus contain large amounts of polyphenolic compounds and polysaccharides, which co-precipitate with DNA and affect subsequent PCR amplification (Cruz et al., 1997). An efficient method to reduce the amount of this contaminants was the protocol of Lodhi et al. (1994), modified by Pop et al. (2004) and this method was used to isolate DNA from the studied species. This protocol also requires only a few grams of tissue to produce total genomic DNA. RAPD fragments were amplified from genomic DNA in a total reaction volume of 25 µL containing 50 ng of genomic DNA, 2.5 mM 10 X Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0,2 µM of decameric primer, and 1 U Taq DNA polymerase (Promega). Each reaction was overlaid with sterile oil. Amplifications were performed in a thermocycler programmed for 45 cycles of 1 min at 94 °C, 1 min at 38 °C, 30 s at 54 °C, 2 min at 72 °C, and a final 15 min extension at 72 °C. The amplification products were separated on 2% agarose-TAE gels run at 80 V/cm for 1 h. The gels were stained with ethidium bromide (0.5 µg/µl) and photographed under UV light. The RAPD was performed on all 41 samples with 15 decameric primers (Table 2). The total number of binary RAPD character data was 448 (presence/absence of the bands; Abdulla and Gamal, 2010). The program FreeTree (Hampl et al., 2001) was used for the construction of a phylogenetic tree and for the bootstrap analysis (Nei and Li distances; Neighbour-Joining tree-construction method; 400 resample datasets).

Results and discussion

Phenotypic evaluation

In the genera *Rebutia, Aylostera, Mediolobivia,* and *Sulcorebutia*, few (1-2) internal spines were present. The plant diameter varied between 2 cm (*R. pygmaea*, *R. violaciflora* var. *luteispina*) to 7-8 cm (*R. senilis* var. *liliacinorosea, R. donaldiana*; Table 1). The number of radial spines/areoles varied between large limits 10-11 in *R. cajasensis* to 50 in *S. crispata*. The limits of the radial spines length were smaller, comparing with the other pecularities and ranged from 2-3 mm (*R. cajasensis, R. pygmaea, R. graciliflora*) to 30 mm (*R. senilis* var. *liliacinorosea*). All the genera present sessile and solitary flower and commonly only one flower is produced per areole, which increases the fruit set of the cacti (Ramirez and Berry, 1995). In all the species of the genera *Rebutia*, *Aylostera*, *Mediolobivia*, and *Sulcorebutia*, the hairs, foliar organs, reproductive organs, glochids, and roots develop from areoles (Booke, 1980). Most of the cactus plants develop flowers at 3-5 years old. The most fundamental characteristic of a cactus flower is its inferior ovary, meaning that the ovary occurs below the perianth and the stamens (Weiss et al., 1995). The floral diameter varied between small limits: 1.7 cm (*R. graciliflora)* to 4.5 cm in *R. calliantha* (the greatest floral diameter from all the studied species). The colour of the flowers varied from pink (*A. narvaecensis*) to red (*A. vallegardensis, R. pygmaea*) to yellow (*S. candiae*).

Molecular evaluation

The DNA yields per individual ranged from 53.20 ng/µl (*R. pseudodeminuta* var. *schumaniana*) to 441.40 ng/µl (*R. violaciflora* var. *luteispina*; Table 1). DNA content for the cactus species included in the study was similar to that of *Mammillaria* species (Del Angel, 2006). Of the 15 decameric primers used for amplification, only five primers generated polymorphic bands: OPA-17, OPA-18, OPA-20, 270, and 563 (Table 2). These primers were also reported by Mihalte et al. (2010) to be successful in amplification when 21 decameric primers were tested. The capacity to produce RAPD fragments varied with the primer and the species (Fig. 1).The dendrogram (Fig. 2) calculated from the RAPD data is a precise representation of the relationships among the different species of cacti. The samples separated by the smallest genetic distances on the dendrogram are classified as closely related, whereas the samples separated by greater genetic distances are classified as remotely related (Mitre et al., 2009; Erturk and Akcay, 2010). The dendrogram indicates the diversity of the genotypes, which are grouped into four distinctive large groups, designated A-D. The group, A, includes species from the *Sulcorebutia* and *Rebutia* genera, and the group B includes species from *Rebutia* and *Aylostera* genera, which clearly share a common ancestor. This hypothesis is also in according with Ritz et al. (2007). According to Grant and Grant (1981) there are a lot of forms with intermediates between apparently distinct groupings. The most convincing example in this way is the group named D, which includes species from all the four genera. The dendrogram has several features that support the taxonomic classification of the genera *Rebutia*, *Aylostera, Mediolobivia*, and *Sulcorebutia* into one genus , *Rebutia* (Hunt et al. , 2006 ; Pilbeam and Hunt, 2004), fact that will be a huge

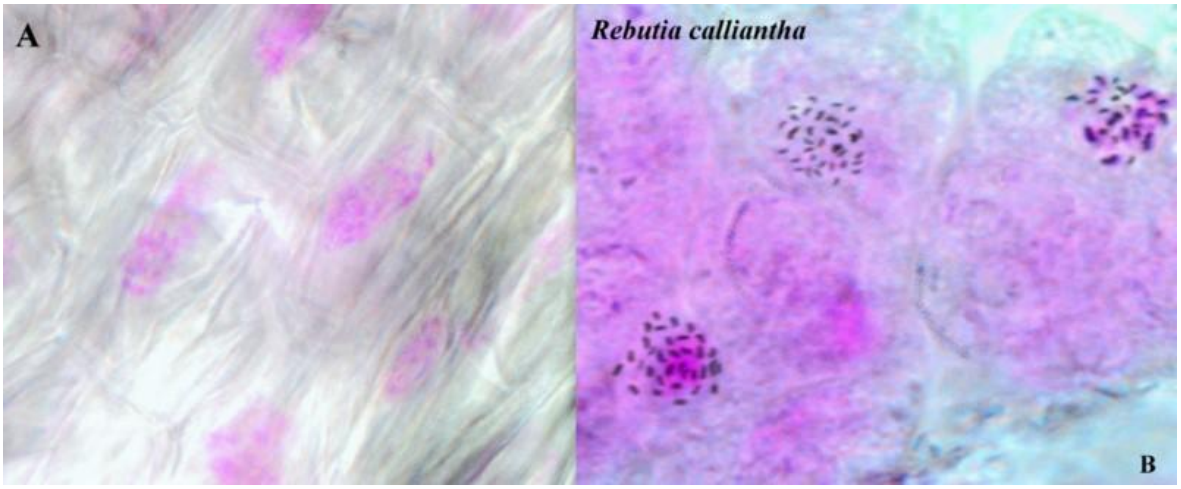


Fig 3. Microscopic samples at *Rebutia calliantha* (2*n*=3*x*=33); „squash" method (A, 40x) and seriate sections method (B, 100x).

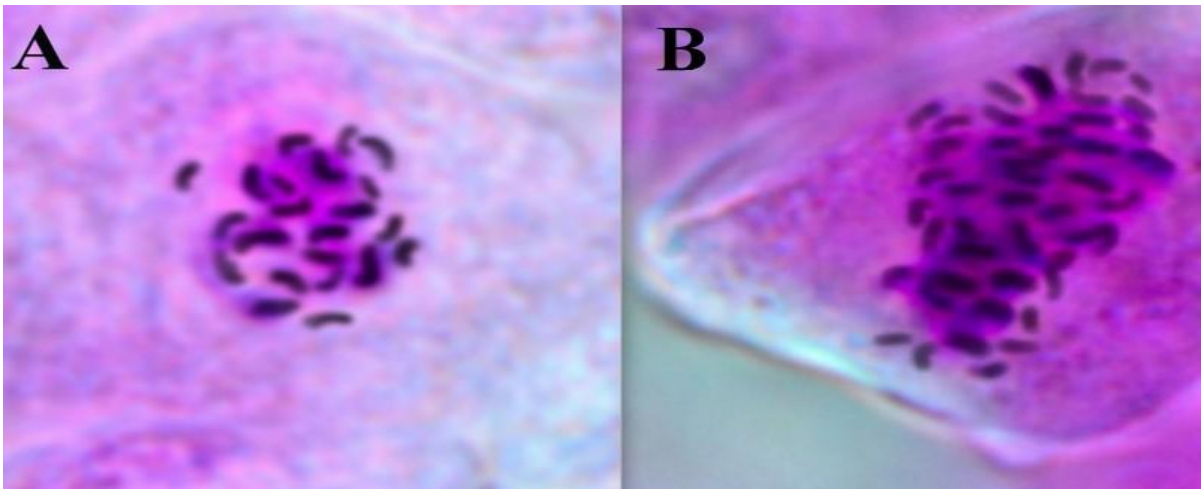


Fig 4. Microscopic samples at *Rebutia graciliflora* (2*n*=2*x*=22) and *Sulcorebutia crispata*(2*n*=4*x*=44); seriate sections method (100x, immersion objective).

simplification for the taxonomy. The present results support the recently findings, which provide the *Rebutia*, *Sulcorebutia*, *Aylostera* and *Mediolobivia* in one genus, named *Rebutia*, and came in contradiction with Backeberg classification (Backeberg, 1968-1977).

Cytological evaluation

The basic number of chromosomes in the family *Cactaceae* is *x=11* (Johnson, 1978; Palomino, 1999). Also, in the experience was found that all species which were investigated, the number of chromosomes was *x=11* (Table 3). Creating and using "squash" samples showed less clear images, because of overlapping cells (Fig. 3 A), compared with the images obtained using the cryotome (Fig. 3 B, Fig. 4). In general, at cacti the "squash" method is hard to perform, because the root tissue presents a high content of silicates (Copacescu, 2001), leading to a poor display. The length of chromosomes did not exceed 2 μm at the studied species. Achieving the length of the long arm and short arm of the chromosome was difficult; therefore, this study is limited to count the number of chromosomes in somatic cells, without developing the karyotype (Table 3). As shown in Table 3 both diploids (2*n*=2*x*=22) and polyploids were identified. The species *R. senilis* var. *liliacinorosea*, *R. pseudodeminuta* var. *schumaniana*, *M. diersiana*, *R. calliantha*, *R. donaldiana* were triploids (2*n*=3*x*=33). Two species were identified with 2*n*=4*x*=44: *R. kupperiana* var. *spiniflorum* and *S. crispata*. It is assumed that the variation in the number of chromosomes, especially polyploidy, has played a fundamental role in the *Cactaceae* evolution, being known that there are several degrees of ploidy to these species responsible for speciation (Acosta, 2005). Due to the multiplication of the number of chromosomes, the polyploid forms are more resistant to the arid and semiarid areas, than the diploid forms (Briones et al., 2004). The polyploid forms are commonly found in cold mountainous regions, in desert regions, or other areas with an arid climate (Briones et al., 2004). Unlike polyploids, diploid species are usually distributed in areas with favorable climatic conditions.

Correlations between phenotypic, molecular and cytologic evaluation

Polyploidy modifies both the genotype and the phenotype of an organism, generating morphological and physiological changes (Moraes et al., 2005; Thompson et al., 2004; Roche et al., 2001). Figueredo (2010) demonstrated that tetraploid and hexaploid taxa have larger and heavier seeds while producing fewer flowers, fruits and seeds; Oselebe and

Tenkouano (2009) shown that the diploids, in comparison with the triploids, are smaller plants. Correlating the results obtained from chromosomal counts with dendrogram (Rohami et al., 2010) can be observed that two triploid species *M. diersiana* and *R. donaldiana* formed the same subgroup. In addition this species also present common phenotypic peculiarities like: colour of flower (orange), diameter of flower (2.5 cm). The species *R. violaciflora* var. *luteispina* and *R. calliantha* presented similarity between morphological traits (number of spines/areolas, length of spines, flower diameter), presented the same number of chromosomes (2*n*=3*x*=33*)* and formed a common subgroup in the frame of the dendrogram.

Conclusions

Assessing the genetic variation through taxonomic methods provide poor and imprecisely results. All the studied species presented the plant diameter varying between 2 cm and 7-8 cm, and all had sessile and solitary flower. The molecular marker method provided valuable results. The molecular polymorphism was obvious (448 amplified products). The dendrogram has several features that suggest classifying the genera *Rebutia*, *Aylostera*, *Mediolobivia*, and *Sulcorebutia* like one genus, fact that will be a huge simplification for the taxonomy. Combining the information obtained in RAPD analyses with information from botanical classification and from cytological evaluation allows valuable conclusions to be drawn regarding the success of breeding programs. In intra-species and inter-species hybridization patterns, will be not present obstacles, unless there will be use genitors species like: *Rebutia senilis* var. *liliacinorosea and Aylostera vallegardensis.*

Acknowledgments

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