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Full Length Research Paper

Morphological characterization and phylogenetic distance among several genotypes of *Rebutia, Aylostera, Mediolobivia* and *Sulcorebutia* (*Cactaceae*)

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Four genera and 75 species belonging to *Cactaceae* family were investigated regarding their morphology and their molecular polymorphism. The botanical classification that described the phenotypic aspects of different characters, such as number of spines/areoles, length of spines and flower diameter, was used to describe the main peculiarities (morphological method). In *Rebutia* genus, the floral diameter varied between small limits: 2 cm (*R. xanthocarpa* v*. splendens,* and *R. brachyantha)* to 4.5 cm in *R. calliantha* and *R. marsoneri* (the greatest floral diameter from all the studied species). Of the studied species of *Aylostera* genus, *A. fiebrigii* has the greatest length of the spines and *A. narvaecensis* the smallest one. The analysis of the plants morphology showed a relatively low variability of biological material, according to genus and species. The genetic diversity was calculated with Nei and Li's index, and the phylogenetic tree (dendrogram) was generated with a neighbor-joining program. The dendrogram indicates the diversity of the genotypes, which are grouped into three distinctive large groups. The largest group includes species from the *Mediolobivia* and *Rebutia* genera, which clearly share a common ancestor; the group shares a common ancestor with B and C as well; A includes some but all not descendents. Species from *Rebutia* genus were present in all the described groups. The genetic distance between species from *Rebutia*, *Mediolobivia, Aylostera* and *Sulcorebutia* genera is small and the differences between the main characters was also quite small, so the trend of combining these species in one genus is justified.

Key words: *Cactaceae* family, DNA isolation, dendrogram, phenotypic traits, genetic distance.

INTRODUCTION

The family of cacti has been hypothesized to be of relatively recent origin (Gibson and Nobel, 1986; Mauseth, 1990). It comprises about 100 genera and 1500 to 1800 species native to temperate and tropical regions of the New World, especially in warm and dry environments (Barthlott and Hunt 1993; Anderson, 2001). The greatest diversity of the family *Cactaceae* is recorded in Mexico with 586 species, followed by Brazil, Argentina, Bolivia and Peru (Ramawat, 2010). Cacti present a wide range of shapes and sizes; cylindrical, globular, or flat (cladode) stems. These traits and the plants' architecture determine their different life forms, which include arborescent, columnar, globular, barreliform, and articulated forms (Gibson and Nobel, 1986; Terrazas-Salgado and Mauseth, 2002).

The studied genera in this present work are mostly high mountain plants native to Bolivia and Northern Argentina.

The species of *Rebutia* genus are generally small, colorful cacti, globular in form (Pilbeam, 1997). The genus *Rebutia* has been a popular one with collectors for many years because it blooms at an early age. There has been considerable debate about the extent of the genus. Buining and Donald (1963, 1965) divided *Rebutia* in two subgenera: *Rebutia* and *Aylostera*, based on the flower peculiarities. Krainz (1967) rejected the two subgenera because he assigned little importance to this character. Backeberg (1968 to 1977) recognized three genera:

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Aylostera, Mediolobivia, and *Rebutia*, based on the following characters: presence of hairs and bristles at the flower tube, the ability to self-fertilization and the globose or cylindrical body shape. Moreover, Anderson (2001), Barthlott and Hunt (1993), Hunt (1999) and Hunt et al. (2006) pulled together the species in the genera *Rebutia*, *Mediolobivia*, *Aylostera* and *Sulcorebutia* in a single genus, named *Rebutia*, while Ritz et al. (2007) suggests synonymies among *Sulcorebutia* and *Weingartia* and therefore recommends merging them into one.

Therefore it is required to do a more accurate assessment of these species and varieties both to phenotypical and molecular level and that is the goal of this research. Molecular tools can give important information about the genetic distances between species (Smolik et al., 2009; Staub et al., 1996; Gupta et al., 2010; Erturk and Akcay, 2010); ideally this will be to merge the systematic of the group based on morphological and molecular data, and this was the main objective of this research.

MATERIALS AND METHODS

Plant material and growth conditions

The material investigated was represented by 75 species of cacti belonging to four genera from the *Chaetolobiviae* subgroup*: Rebutia*, *Sulcorebutia, Mediolobivia* and *Aylostera*. The plants were grown in the Botanical Garden "Alexandru Borza" Cluj-Napoca, Romania, in a greenhouse with a minimum of 5.0°C (in December) and a maximum of 23.5°C in July. The plants were analyzed at an early age (three years old).

Morphological method

The plants evaluated for characters relied on a botanical classification that describes the phenotypic aspects of different characters, such as number of radial spines/areoles, length of spines and diameter of flower. These characters were the same as described by the UPOV normative (UPOV, 1987) and ten plants were used for these measurements, represented as arithmetic mean.

Molecular marker method

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; Guillemaut and Marechal-Drouard, 1992). 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.

Random amplified polymorphic 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. RAPD analysis was performed on all the 75 samples with 20 decameric primers (Table 1). The total number of binary RAPD character data was 649 (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; neighbor-joining tree-construction method; 400 resample datasets).

RESULTS AND DISCUSSION

Phenotypic evaluation of species belonging to the *Rebutia* genus

The number of radial spines/areoles (Figure 1) varied between large limits: 7 in *R. boliviensis* and *R. brachyantha* and 30 to 35 in *R. albipilosa, R. chrysacantha* var*. elegans,* and *R. marsoneri.* The longest radial spines were recorded in *R. chrysacantha* (14 mm), *R. chrysacantha* var*. elegans* (12 mm), and *R albipilosa* (11 mm); and the shortest in *R. horstii* and *R. cajasensis*.

The floral diameter varied between narrow limits: 2.0 (*R. xanthocarpa* var*. splendens, R. brachyantha)* to 4.5 cm in *R. calliantha* and *R. marsoneri* (the greatest floral diameter from all the studied species). The species from *Rebutia* genus does not present distinctive ribs, but they have regularly arranged small tubercles and they are distinctive because of their small and globular forms (Hewitt, 1993).

Phenotypic evaluation of species belonging to the *Sulcorebutia* genus

The length of the spines (Figure 2) varied between the limits; 2.0 to 11.0 mm (*S. rauschii* WR 2295; *S. cuprea PCWR 476*). *S. grandiflora* presented the biggest number of spine/areoles (25), while *S. steinbachii, S. heinzii* HS 151 presented a smaller value (6). Also, the floral diameter of *Sulcorebutia* species was similar with the floral diameter of *Rebutia* species. A distinct particularity of the *Sulcorebutia* genus, a small Bolivian genus spread at elevations between 2400 to 3600 m is that the species tend to be more rot-prone and they are not as frost resistant as the *Rebutias* (Grant, 2009).

Phenotypic evaluation of species belonging to the *Aylostera* genus

In the *Aylostera* genus, the number of spines/areoles varied greatly: 12 for *A. brunescens* and more than 35 for *A. muscula.* Of the studied species of *Aylostera* genus, *A. fiebrigii* has the greatest length of the spines and *A. narvaecensis* the smallest one (Figure 3). The floral

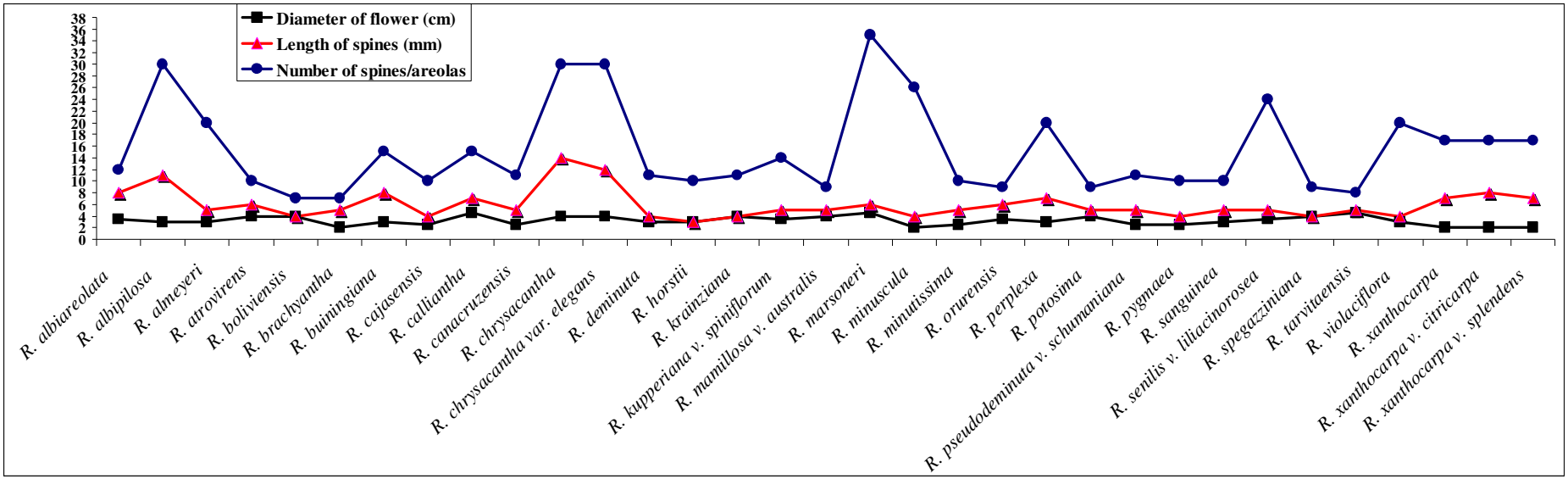

Figure 1. Number of spines/areolas, length of spines and diameter of flower of the analyzed cacti species belonging to *Rebutia* genus.

diameter were similar with the species from *Rebutia* and *Sulcorebutia* genera.

Phenotypic evaluation of species belonging to the *Mediolobivia* genus

The radial spines (Figure 4) in the genus *Mediolobivia* varied between eight to nine (*M. tarvitaensis* and *M. brachyantha*) and 24 (*M. rosalbiflora, M. diersiana* f. WR 631) and the largest flower was noted on *M. tarvitaensis* (4.0 cm).

All the studied genera presented sessile and solitary flowers and commonly only one flower was 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 developed from areoles (Booke, 1980). The analysis of the plants peculiarities therefore showed a relatively low variability of biological material according to genus and species. The differences between the main characters of species belonging to *Rebutia, Aylostera, Sulcorebutia* and *Mediolobivia* genera (classified by Backeberg system) are quite small, so the trend of combining these species in one genus is justified.

Molecular evaluation

Of the 20 decameric primers used for amplification, only six primers generated polymorphic bands: OPA-17, OPA-18, OPA-20, 270, 563 and OPAL-20 (Table 1). The capacity to produce RAPD fragments varied with the primer and the species (Baciu et al., 2010; Mihalte et al., 2011). The dendrogram (Figure 5), calculated from the RAPD data, indicates the diversity of the genotypes, which were grouped into three distinctive large groups, designated A to C. The group, A, included only species from the *Aylostera* and *Rebutia* genera, which clearly share a common ancestor. Just one species from *Sulcorebutia* genus was included in this group. These aspects are in accordance with the new findings which consider the name *Aylostera* and *Rebutia* being synonymy and include the genus *Aylostera* in *Rebutia* genus (Ritz et al., 2007). Regarding the species *S. canadiae*, Pilbeam (1997) described this nomenclature being synonymy with: *Rebutia arenacea* var. *candiae*, *Weingartia candiae, Rebutia candiae,* and *Sulcorebutia candiae.* In addition, the group

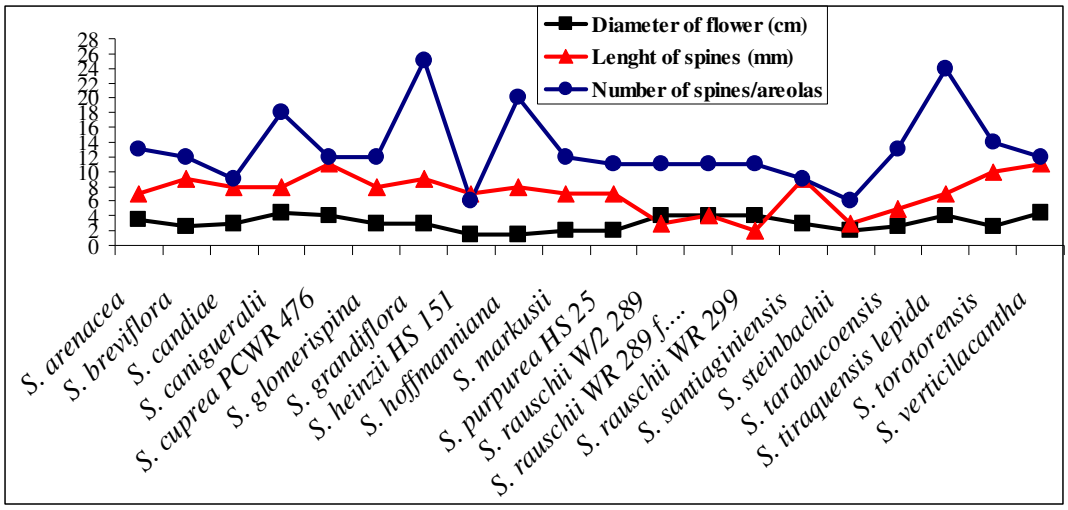

Figure 2. Number of spines/areolas, length of spines and diameter of flower of the analyzed cacti species belonging to *Sulcorebutia* genus.

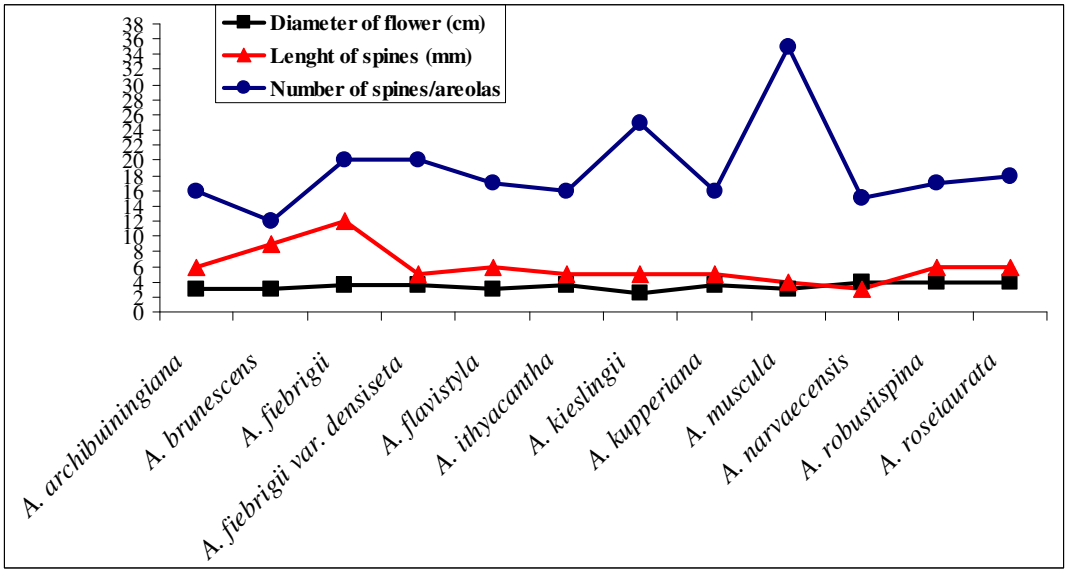

Figure 3. Number of spines/areolas, length of spines and diameter of flower of the analyzed cacti species belonging to *Aylostera* genus.

designated B included species from *Sulcorebutia* and *Rebutia* genera. All the species have similar peculiarities and according to Grant and Grant (1981) there are a lot of forms with intermediates between apparently distinct groupings. The group designated C included only species of the *Rebutia* and *Mediolobivia* genera, which have a common parent.

It can be concluded that species from *Rebutia* genus were present in all the described groups, meaning that the genetic distance based on six RAPD markers between species from *Rebutia*, *Mediolobivia, Aylostera* and *Sulcorebutia* genera is small. The hypothesis that classifies these entire species as one genus, considering that their descriptive traits are similar, is quite justified. The genera and species did not segregate into distinct groups in this research, and all the studied species presented similar phenotypic peculiarities. The dendrogram also have 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). In fact, that will be a huge simplification for the taxonomy. The results of this study support the recent findings, which grouped the *Rebutia*, *Sulcorebutia, Aylostera* and *Mediolobivia* in

Table 1. The primers used for RAPD analyses at species from *Rebutia, Mediolobivia, Aylostera* and *Sulcorebutia* genera.

Number	Primer	Nucleotide sequence (5'-3')	Molecular weight	Amplified product
	OPA- 17	GAC CGC TTG T	3019	+
	OPA-18	AGG TGA CCG T	3044	+
	OPA-20	GTT GCG ATC C	3019	+
	270	TGC GCG CGG G	3085	+
	563	CGC CGC TCC T	2940	+
	OPAL-20	CGC CGC TCC T	3085	+
	OPA-16	AGC CAG CGA A	3046	–
	OPC-04	CCG CAT CTA C	2948	–
	OPC-08	TGG ACC GGT G	3084	–
	OPC-09	CTC ACC GTC C	2924	–
	OPC-13	AAG CCT CGT C	2988	–
	OPC-20	ACT TCG CCA C	2948	–
	MIC-07	TGT CTG GGT G	3090	–
	MIC-13	TTC CCC CCA G	2924	–
	MIC-14	TGA GTG GGT G	3139	–
	70-03	ACG GTG CCT G	3044	–
	70-04	CGC ATT CCG C	2964	–
	70-08	CTG TAC CCC C	2924	–
	594	AGG AGC TGG C	3093	–
	595	GTC ACC GCG C	2989	–

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

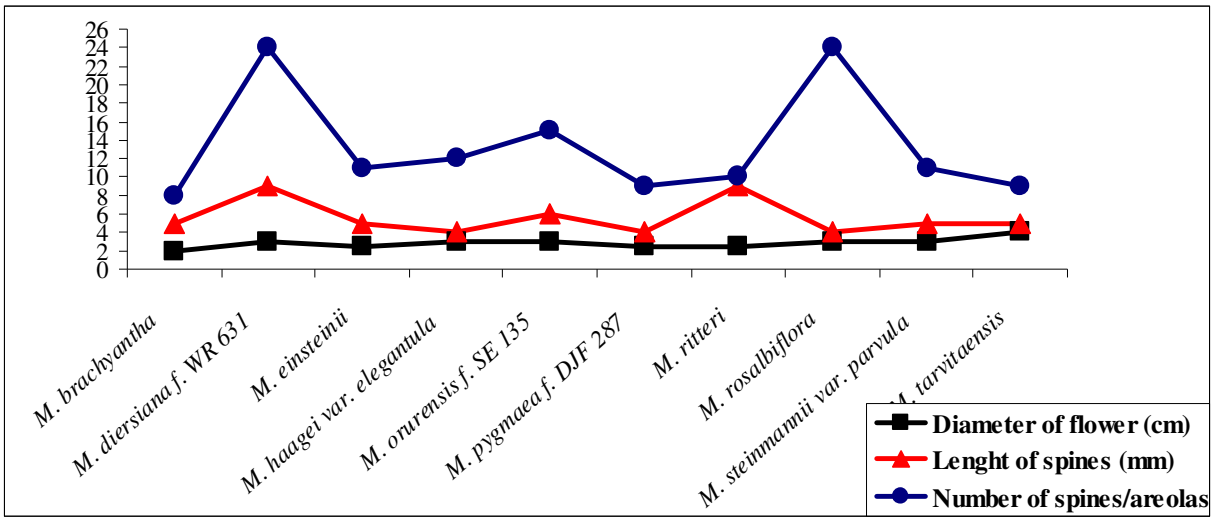

Figure 4. Number of spines/areolas, length of spines and diameter of flower of the analyzed cacti species belonging to *Mediolobivia* genus.

one genus, named *Rebutia*, and thus came in contradiction with Backeberg classification (Backeberg, 1968 to 1977).

There has been considerable debate about the extent of the genus *Rebutia*. The list of "Vascular Plant Families and Genera" maintained at the "Royal Botanic Gardens, Kew*", shows that the following genera should be regarded as synonyms of *Rebutia*: *Aylostera* Speg., *Bridgesia* Backeb., *Cylindrorebutia* Fric and Kreuz., *Digitorebutia* Fric and Kreuz., *Eurebutia* Fric, *Gymnantha* Y. Itô, *Mediolobivia* Backeb., *Mediorebutia* Fric, *Neogymnantha* Y. Itô, *Echinorebutia*, *Reicheocactus* Backeb., *Setirebutia* Fric and Kreuz., *Spegazzinia* Backeb., *Sulcorebutia* Backeb., *Weingartia* Fric and

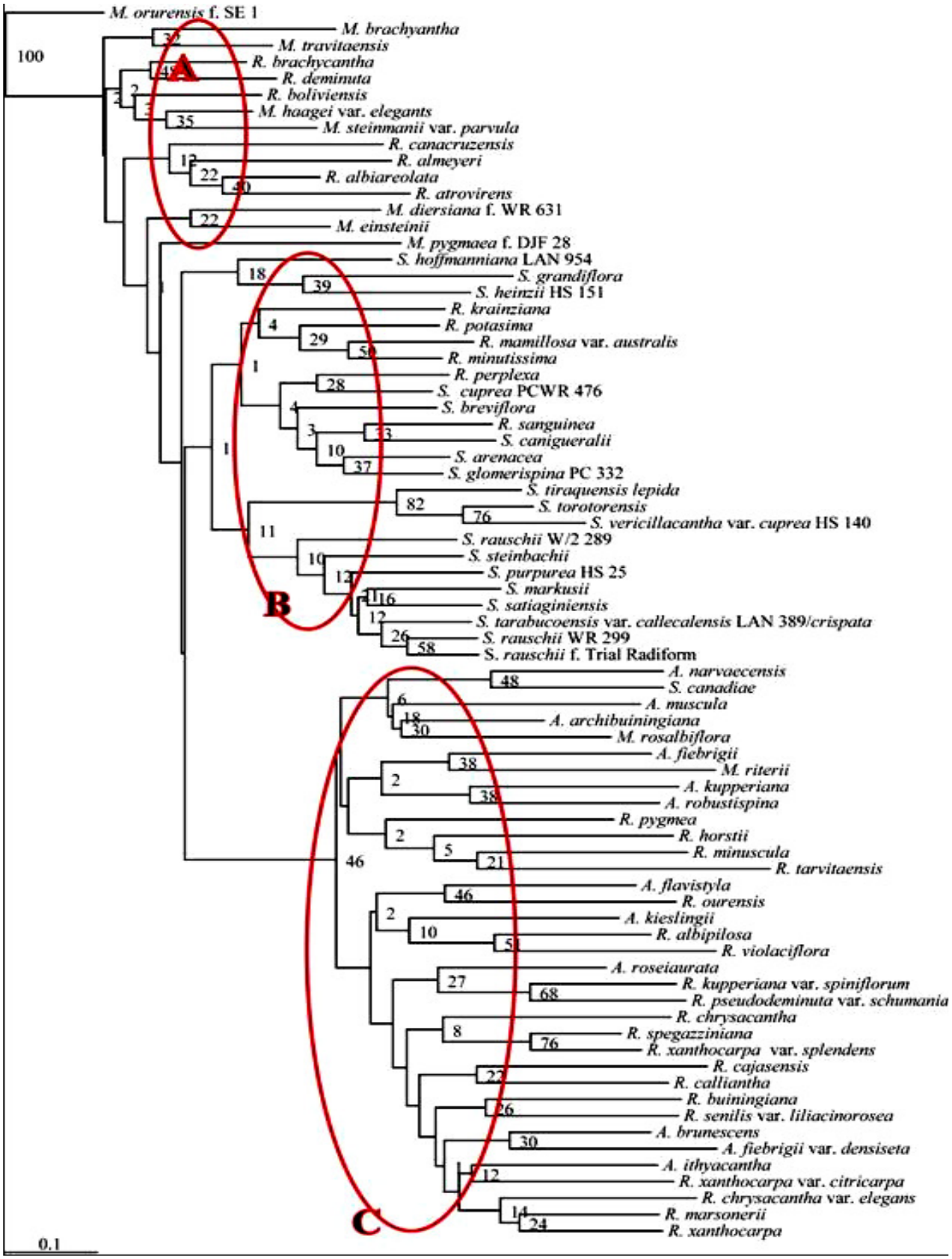

Figure 5. Dendrogram of the genotypes of cacti plants from *Rebutia, Aylostera, Sulcorebutia* and *Mediolobivia* genera based on six RAPD markers.

Weingartia Werderm. Also, the number of species is similarly debatable because of disagreement both over what constitutes the genus and what constitutes a species. A very large number of plants are circulated as species, but most of this are regarded as varieties, or forms (*S. cuprea*, versus *S. verticilacantha cuprea*; *A. fiebrigii* var. *densiseta* versus *A. densiseta*) (Anderson, 2001).

Conclusion

RAPD cluster analysis is clearly an efficient method for determining the genetic proximities of the different species of the genera *Rebutia*, *Sulcorebutia, Aylostera* and *Mediolobivia.* Combination of the information obtained in RAPD analyses with information from botanical classification (taxonomy) allows valuable conclusions to be drawn. All the cacti shown in this research appear as a monophyletic group using RAPDs; group A included groups B and C, and group B also included C. These are the facts that suggest classifying the genera *Rebutia*, *Aylostera*, *Mediolobivia* and *Sulcorebutia* as one genus, which will be a huge simplification for the taxonomy. This research is only an initial step toward the development and characterization of cacti at the molecular level. The localization of phenotypic traits on the genetic map will allow breeders to use molecular techniques in their programs.

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