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ISSR as a tool to support taxonomic decisions: a first approach for *Chascolytrum* species complexes (Poaceae)

ABSTRACT

Chascolytrum Desv. is a South American grass genus, with extension up to Central America, which presents several taxonomic controversies concerning genera and species circumscriptions. Morphological studies were not able to provide robust elements for taxonomic decisions in some species complexes. It was the case of *Chascolytrum subaristatum* and morphological allies, and of *Chascolytrum rufum* plus the acceptance (or rejection) of two varieties. In order to provide additional elements for taxonomic decisions in these species complexes, it was performed a preliminary survey using Inter Simple Sequence Repeat (ISSR) markers. Nine primers were used to build similarity dendrograms, and 25 collections were included, analysed in two separated complexes. ISSR were able to separate the two varieties of *Chascolytrum rufum*, supporting their acceptance. Two species recently described could be clearly separated from their morphologically related taxa, but the species *Briza erecta*, *Briza macrostachya* and *Chascolytrum subaristatum*, as well as *Briza subaristata* var. *interrupta*, could not be separated, adding elements to the synonymization of these three taxa under *Chascolytrum subaristatum*. The ISSR contributed to solving some controversies on genus *Chascolytrum*, but its use as an exclusive species-marker is limited in this genus due to high band polymorphism.

Keywords: *Briza*, molecular marker, Poaceae, systematics.

ISSR como uma ferramenta para apoiar decisões taxonômicas: uma abordagem preliminar para complexos de espécies em *Chascolytrum* (Poaceae)

RESUMO

Chascolytrum Desv. é um gênero sul-americano de gramíneas, com extensão da distribuição até a América Central, o qual apresenta diversas controvérsias taxonômicas com relação à circunscrição de gêneros e espécies. Estudos morfológicos não foram capazes de fornecer elementos robustos para a tomada de decisões taxonômicas em relação a alguns complexos de espécies. Esse foi o caso de *Chascolytrum subaristatum* e espécies morfológicamente afins, e de *Chascolytrum rufum* com relação à aceitação (ou rejeição) de duas variedades. Para fornecer elementos adicionais para decisões taxonômicas nesses complexos de espécies, foi realizado um estudo preliminar utilizando marcadores do tipo Inter Simple Sequence Repeat (ISSR). Foram utilizados nove primers para construir dendrogramas de similaridade, e 25 coletas foram incluídas, analisadas em dois complexos distintos. Os marcadores ISSR foram capazes de separar as duas variedades de *Chascolytrum rufum*, apoiando a aceitação das mesmas. Duas espécies recentemente descritas puderam ser claramente separadas de seus táxons morfológicamente relacionados, porém as espécies *Briza erecta*, *Briza macrostachya* e *Chascolytrum subaristatum*, bem como *Briza subaristata* var. *interrupta*, não foram discriminadas nos dendrogramas, o que adiciona elementos para justificar a sinonimização desses três táxons sob *Chascolytrum subaristatum*. Os ISSR contribuíram para a resolução de algumas controvérsias taxonômicas no gênero *Chascolytrum*, mas seu uso como um marcador para espécies é limitado no gênero devido ao alto polimorfismo de bandas.

Palavras-chave: *Briza*, marcador molecular, Poaceae, sistemática.

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INTRODUCTION

Chascolytrum Desv. *lato sensu* (Poaceae) is a morphologically diverse and taxonomically controversial grass genus from subtropical and highland areas in South America, some species presenting potential to use as forage, and also there are endangered species. It is represented by perennial species, caespitose or rhizomatous, with extravaginal basal innovations, spikelets many-flowered and panicles open to contracted, erect or pendulous. The spikelets are very variable inside the genus, and this is one of the reasons that made authors propose several different circumscriptions for this alliance of species. These species can be placed into several other genera, like *Briza* L. *lato sensu*, *Poidium* Nees or *Lombardochloa* Roseng. & B. R. Arrill., according to each author. Its evolutionary history was recently analysed (Essi, Longhi-Wagner and Souza-Chies, 2008), and a proposal of a *lato sensu* circumscription was done, excluding only the euroasiatic species (Essi, Longhi-Wagner and Souza-Chies, 2011). Some taxa presents delimitation controversies, forming species complexes not clarified with morphological studies. It is not clear, for instance, whether species like *Briza erecta* Lam. and *Brizamacrostachya* (J. Presl) Steud. should be treated as separate taxa, as done by Longhi-Wagner (1987), or whether they represent only intra-specific variation, and should be synonymised, as proposed by Matthei (1975). The occurrence of several intermediates further complicates this case: morphological links can group these two species with the broadly distributed *Briza subaristata* Lam. (here treated as *Chascolytrum subaristatum* (Lam.) Desv.). There is also no consensus whether varieties distinguishable only by spikelet measures, like *B. subaristata* var. *interrupta* (Hack. ex Stuck.) Roseng., Arrill. & Izag. or based on single discrete characters, like *B. rufa* var. *sparsipilosa* Roseng., Arrill. & Izag., should be accepted, or whether their variation is merely intra-specific polymorphism or environment plasticity. It is often difficult to interpret morphological divergences among closely related taxa. Minor morphological

differences can be a result of environmental variation, species polymorphism or even of evolutionary divergence processes, such as genetic isolation or speciation. The presence or absence of trichomes on certain structures, for instance, may be taxonomically negligible for some groups, or be a stable and reliable morphologic marker for other taxa.

Decisions concerning accepting or not certain features such as taxon markers or like establishing a given taxonomic level for a morphologically distinct group may provide to systematics a certain level of subjectivity. In such cases, the addition of extra approaches can bring light and objectivity to the studies.

Inter Simple Sequence Repeat (ISSR) PCR is a fast, cheap approach to genetic characterization and is widely used for ecological (Wu et al., 2004; Poulin, Weller and Sakai, 2005), population (Esselman et al., 1999; Alexander, Liston and Popovich, 2004; Song et al., 2006; Zhang et al., 2013) or phylogenetic studies (Joshi et al., 2000; Bussell, Waycott and Chappill, 2005), as well as in crop cultivar or native plant fingerprinting (Mattioni et al., 2002; Souza et al., 2005). It has been also applied to explore the hybrid origin of species (Ayres and Strong, 2001) and for systematic purposes (Blair, Panaud and McCouch, 1999; Vanderpoorten, Hedenäs and Jacquemart, 2003; Dinelli, 2004; Vergara et al., 2008).

ISSRs have a few advantages over other molecular markers. Their primers anneal to SSR (Simple Sequence Repeat) that are abundant throughout the eukaryotic genome and evolve rapidly, and hence may reveal a high level of polymorphism (Zietkiewicz, Rafalski and Labuda, 1994; Li and Ge, 2001). In addition, ISSR may produce more reliable and reproducible bands than RAPD (Random Amplified Polymorphic DNA), due to the higher annealing temperature and longer primer sequences (Qian, Ge and Hong, 2001).

The aim of this study was to test whether ISSR can provide markers to help at intra-specific or species-level decisions for taxa belonging to genus *Chascolytrum lato sensu* (with some taxa referred in the text as *Briza*, according to the existence or absence of a combination under *Chascolytrum*),

whenever the study of morphological characters alone is insufficient.

The results obtained by ISSR markers are compared to previous approaches on the genus, and suggestions for applications for ISSR in the group are provided.

MATERIAL AND METHODS

The study was carried out at the Laboratory of Plant Genetics of the Universidade Federal do Rio Grande do Sul, following the procedures described in the *Molecular Protocols*, between the years 2006 and 2007. Plants were collected previously during field trips (from 2003 to 2006), or obtained from herbarium specimens, as described in the *Taxon Sampling*. Data analysis was carried out at the Laboratory of Plant Genetics of the Universidade Federal do Rio Grande do Sul and latter at

the Laboratory of Taxonomy of the Universidade Federal de Santa Maria.

Taxon Sampling

Taxon sampling included representatives of two species complexes, which were analysed separately, according to morphological similarities (Table 1). The first complex (Group 1) included three species (Figures 1 and 2), and the second (Group 2) included one species (Figure 2). Twenty-five accessions were analysed, including representatives of major morphological variation found in each complex. Characters such as spikelet colour and size, lemma width, presence of trichomes, or habitat, were most often considered for sampling. Geographical information was collected, although it was not considered for sampling purposes.

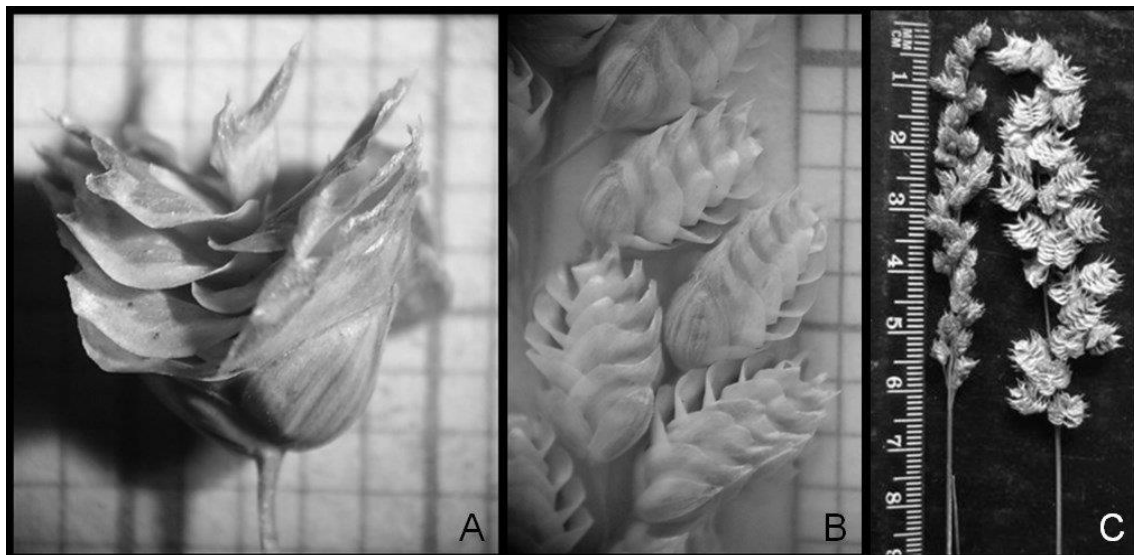


Figure 1: Comparison of specimens showing part of the morphological variability of *Chascolytrum subaristatum*- Size and shape variation in spikelets and panicles. A. “*Briza macrostachya*-like” spikelet (Voucher Information: L. Essi 13, São Pedro do Sul, RS, Brazil, 12/10/2003; ICN herbarium); B. Cylindrical type spikelets (Voucher Information: T. M. Petersen, La Yela, Dep. Empedrado, Prov. Corrientes, Argentina, 29/10/1969; S herbarium); C. Comparison between two panicles, with small spikelets (left) and big spikelets (right) – Location: São Pedro do Sul, RS, Brazil, 2003 (no voucher associated to the picture).Squares [A-B]: 1mm².



Figure 2: Species included in the analysis. A. Lema of *Chascolytrum altimontanum* (Group 1) – Voucher Information: Wood 10768, Oropeza, Chuquisaca, Bolivia, 25/02/1996, LPB herbarium. The arrow shows the complanate trichomes at the base of the lema, typical of the species. Squares: 1mm²; B. Panicle of *Chascolytrum latifolium* (Group 1)– Picture location and date: Urubici, Santa Catarina, Brazil, 07/12/2006; Vouchers Information: Longhi-Wagner *et al.* 10228 – 10230, ICN herbarium; C. Panicle of *Chascolytrum rufum* (Group 2) – Picture location and date: Guarapuava, PR, Brazil, 16/01/2001 (no voucher associated to the picture).

Table 1: Accessions included in this study. All the taxa included are considered belonging to genus *Chascolytrum*; Some taxa were referred as genus *Briza*, when there is not yet a published combination inside *Chascolytrum* [one new combination submitted]. Collector's abbreviations: HI: H. M. Longhi Wagner; Li: L. Essi; W: W. J. R. Wood.

Species [morphological or habitat type]	Collector number	Voucher	Geographical origin of the sample (Country – State – Locality)
Group 1			
<i>C. subaristatum</i> (Lam.) Desv. [<i>Briza erecta</i> Lam.]	HI5056	ICN	Uruguay, Carrasco
<i>C. subaristatum</i> [intermediate <i>C. subaristatum</i> - <i>B. erecta</i>]	Li332	ICN 149.322	Brazil, Santa Catarina, Florianópolis
<i>C. subaristatum</i>	Li7	ICN 132.506	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. subaristatum</i>	Li8	ICN 134.887	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. subaristatum</i>	Li10	ICN 132.509	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. subaristatum</i>	Li11	ICN 132.510	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. subaristatum</i>	Li12	ICN 132.511	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. subaristatum</i>	Li202	ICN 132.542	Brazil, Santa Catarina, Urubici
<i>C. subaristatum</i>	HI5029	ICN	Brazil
<i>C. subaristatum</i>	HI5040	ICN 131.398	Brazil, Rio Grande do Sul, Bagé - Minas de Camaquã
<i>C. subaristatum</i> [<i>B. subaristata</i> var. <i>interrupta</i> (Hack. ex Stuck.) Roseng., Arrill. & Izag.]	Li19	ICN 132.518	Brazil, Rio Grande do Sul, São Pedro do Sul
<i>C. subaristatum</i> [<i>Briza macrostachya</i> (J. Presl) Steud.]	Li13	ICN 132.512	Brazil, Rio Grande do Sul, São Pedro do Sul
<i>C. subaristatum</i> [<i>Briza macrostachya</i>]	Li18	ICN 132.517	Brazil, Rio Grande do Sul, São Pedro

			do Sul
<i>C. subaristatum</i> [<i>Brizamacrostachya</i>]	Li54	ICN 132.553	Brazil, Rio Grande do Sul, Piratini
<i>C. subaristatum</i> [<i>Brizamacrostachya</i>]	Li57	ICN 132.556	Brazil, Rio Grande do Sul, Piratini
<i>C. subaristatum</i> [<i>Brizamacrostachya</i>]	HI5038	ICN 131.396	Brazil, Rio Grande do Sul, Bagé - Minas de Camaquã
<i>Chascolytrum latifolium</i> Essi, Souza-Chies & Longhi-Wagner	Li201	ICN 132.700	Brazil, Santa Catarina, Urubici
<i>Chascolytrum altimontanum</i> Essi, Souza-Chies & Longhi-Wagner	W10768	K, LPB	Bolivia, Chuquisaca, Oropeza
Group 2			
<i>C. rufum</i> J. Presl var. <i>rufum</i> [80 cm tall plant]	Li278	ICN 134.875	Brazil, Rio Grande do Sul, Canguçu
<i>C. rufum</i> var. <i>rufum</i>	Li279	ICN 134.876	Brazil, Rio Grande do Sul, Canguçu
<i>C. rufum</i> var. <i>rufum</i>	Li280	ICN 134.877	Brazil, Rio Grande do Sul, Canguçu
<i>C. rufum</i> var. <i>rufum</i> [broad leaves]	Li372	ICN 132.586	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. rufum</i> var. <i>rufum</i> [broad leaves]	Li373	ICN 132.587	Brazil, Rio Grande do Sul, Porto Alegre
<i>C. rufum</i> [<i>Brizarufa</i> var. <i>sparsipilosa</i> Roseng., Arrill. & Izag., open panicle type]	Li281	ICN 134.878	Brazil, Rio Grande do Sul, Canguçu
<i>C. rufum</i> [<i>Briza rufa</i> var. <i>sparsipilosa</i>]	HI8061	ICN 135.198	Brazil, Rio Grande do Sul, São Francisco de Paula

Molecular Protocols

DNA was extracted from fresh, silica dried or herbarium leaves, following a CTAB method (Doyle and Doyle, 1987) adapted for micro-centrifuge tubes. Total DNA was quantified by agarose gel visualization. Thirteen primers described by Joshi et al. (2000), Martins, Tenreiro and Oliveira, (2003) and Lin et al. (2005) were tested for ISSR amplification, and nine of them were selected (Table 2). PCR reactions were carried out in an Applied Biosystems 2400 thermocycler, in a total volume of 25 µl containing 12 µl sterile Milli-Q purified water, 0.2 µl Taq DNA Polymerase (5U/ul), 2.3 µl MgCl₂ (25mM), 2.5 µl 10× buffer, 1

µl primer 10 pmol, 1 µl of 40 mM dNTP mixture (10mM each dNTP), 1 µl DMSO (2%), and 5 µl DNA (total 30-50 ng). PCR amplifications included 40 cycles of 1 min at 94°C, 45 sec at 50°C and 2 min at 72°C, preceded by a period of 5 min at 92°C and completed by a final extension of 5 min at 72°C, for all the primers, except primer F11, whose annealing temperature was 48°C. The ISSR amplification products were stained by ethidium bromide, run until the complete separation of the ladder (100 and 50 bp, PB-L Produtos Bio-Lógicos, Universidad Nacional de Quilmes), approximately 2h30min, at 100V on 1.8% agarose gel, and visualized by UV.

Table 2: Primers code and sequences included in the analyses.

Primer code:	Primer sequence:	Described by:
P1	(AC) ₈ T	Lin et al., 2005 (as 25)
P2	(GA) ₈ T	Joshi et al., 2000 (as 810)
P3	(CTC) ₄ RC	Poulin, Weller and Sakai, 2005 (as n. 15)
P4	(CT) ₈ G	Joshi et al., 2000 (as 815)
F3	(AG) ₈ YC	Joshi et al., 2000 (as 835)
F4	(GA) ₈ YC	Joshi et al., 2000 (as 841)
F7	(GT) ₈ A	Joshi et al., 2000 (as 819)
F11	(GACA) ₄	Lin et al., 2005 (as 73)
F12	(GTGC) ₄	Martins, Tenreiro and Oliveira, 2003 (as IS06)

Data analyses

Polymorph bands were scored for presence/absence. Analyses were performed using the NTSYS-pc version 2.10 software (Rohlf, 2000). For each of the two groups, the genetic similarity among individuals was calculated using Jaccard's Similarity Coefficient (J), which takes only shared presence into account. The similarity relationships were portrayed by dendrograms built using the clustering method Unweighted Pair Group Method of Arithmetic Average (UPGMA). Bootstrapping analyses, with 2,000 replicates each, were performed by the Winboot software (Yap and Nelson, 1996), to assess the robustness of nodes in the dendrograms, as proposed by Felsenstein (1985).

RESULTS AND DISCUSSION

For Group 1, an average of 12.66 DNA bands was produced using nine primers, 98.24% of which were found to be

polymorphic. For Group 2, the average was 8.6 DNA bands, 85.89% of which were polymorphic. The group with the highest number of monomorphic bands was group 2, with 11 bands (14.11% of the matrix). The primer with the smallest number of bands, considering both complexes, was F7 (total of 7 bands), and the highest number of bands was obtained with the primer P4 (total of 31 bands).

The Jaccard similarity index ranged from 0.2260, between accession li201 and accession w10768, to 0.7442, between accessionHw5038 and accessionHw5056. Low levels of similarity were found in both groups. The absence of a similarity index of 1.0 indicates that no clone or repeated germplasm was included, and all accessions represent plants with distinct fingerprints. The limits for the acceptance of the clusters were established by the similarity average inside the group (see values of the averages for each group in Figures 3 and 4).

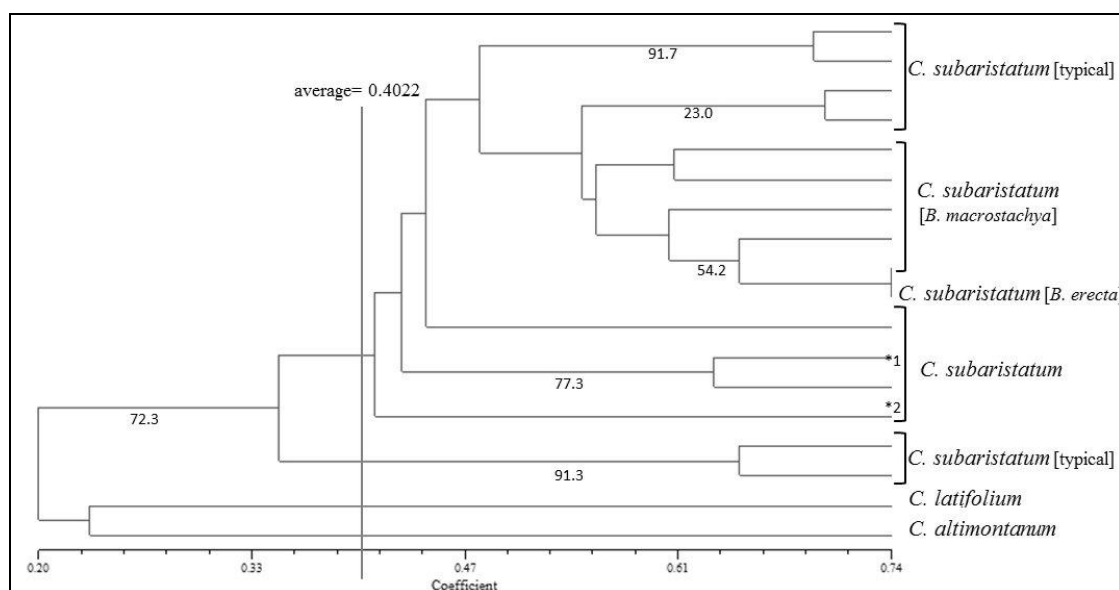


Figure 3: UPGMA dendrogram of individuals from group 1. The cluster is based on the Jaccard similarity index. Vertical line marks the average of similarity indexes. *1= *C. subaristatum* [*B. subaristata* var. *interrupta* (Hack. ex Stuck.) Roseng., Arrill. & Izag.]; *2=*C. subaristatum* [intermediate *C. subaristatum*- *B. erecta*] Numbers below branches correspond to bootstrap values >50.

In Group 1, the two recently described species (accessions li201 and w10768) appeared very distinct from the remaining taxa, being grouped with a very low similarity index. The two are distinct taxa, and grouped together probably due to the absence of other more closely related accessions. All other accessions appeared

clustered. Although the grouping between Cluster A and Cluster B is under the similarity average for the group, it is supported by bootstrap. The high similarity among accessions of *Briza macrostachya* and the accession of *B. erecta* is also supported by bootstrap.

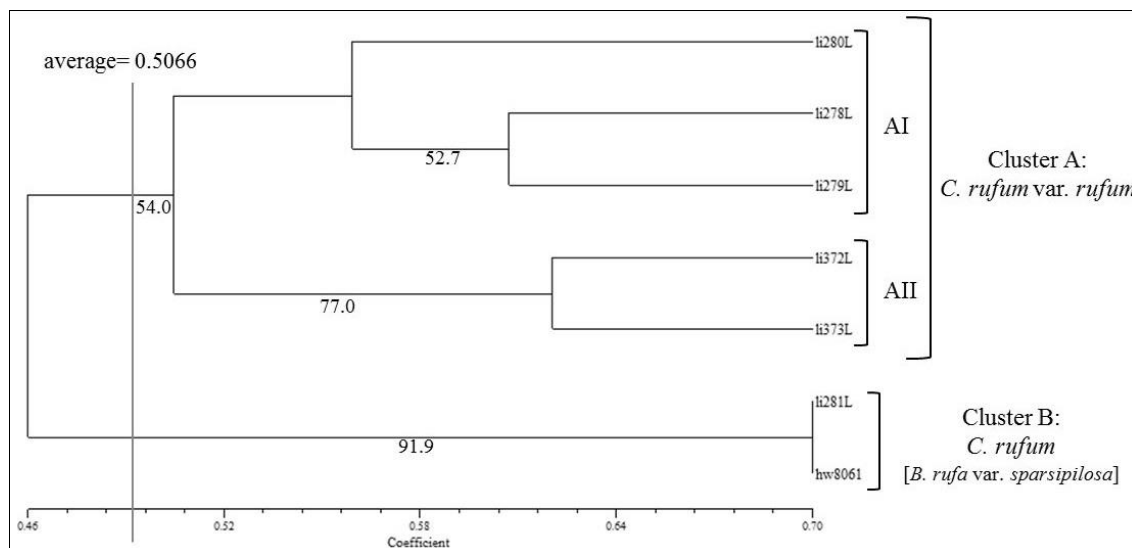


Figure 4: UPGMA dendrogram of individuals from group 2. The cluster is based on the Jaccard similarity index. Vertical line marks the average of similarity indexes. Numbers below branches correspond to bootstrap values >50.

In Group 2, the two varieties could be separated, being variety *sparsipilosa* grouped with the highest similarity level (Cluster B), in spite of its different geographical origins. The main clusters (A and B) represent the two varieties, and the secondary clusters follow a geographical order (AI: Canguçu; AII: Porto Alegre).

Low similarity levels were already expected, due to two main factors: 1) The ISSR are one of the most polymorphic molecular markers; 2) *Chascolytrum* species are autogamous, mainly cleistogamous, so that a low intra-population variability and a high inter-population variability were expected. Although a high level of polymorphism was not a surprise, the use of ISSR as a species marker was not possible: only one band was exclusive of a particular analysed species (however not exclusive when comparing to other *Chascolytrum* species, not included in this paper – data not show), and the similarity indexes were lower than those usually published for species-complexes. Their use as phylogenetic markers for *Chascolytrum* should be avoided, since a minimum monomorphism (20%) across all taxa is required to consider the markers potentially homologous to such studies (Bussell, Waycott and Chappill, 2005). The ISSR helped to solve some critical questions:

1) Concerning the acceptance of two varieties for *Chascolytrum rufum*: ISSR

data supported the recognition of two genetically distinct groups, which correspond to the two varieties sampled. This is also in agreement with other approaches based on flavonoid variation, pollen and satellite chromosomes position (Winge et al., 1984).

2) Concerning the robustness of recently described species: The accessions sampled were absolutely distinct from the remaining morphologically related accessions, thus supporting their acceptance as distinct species, in addition to the morphological findings.

3) Concerning the circumscription for the *Briza macrostachya*- *B. erecta* - *C. subaristatum* complex: The obtained data place *B. erecta* asgenetically very close to *B. macrostachya*, a relation supported by bootstrap. This is in agreement with the opinion of Matthei (1975), who considered these two species synonymous. But the different accessions of *C. subaristatum* are widespread over the clusters, producing the same impact caused by morphological analysis –not only *B. macrostachya* and *B. erecta* should be synonymised, but all three, *B. erecta* and *B. macrostachya* considered an intra-specific polymorphism. This idea may appear not convincing morphologically considering the analysis of the Uruguayan material of *B. erecta*, but it is perfectly acceptable when the Brazilian material is studied. Different spikelet sizes (mature spikelets) are commonly found in the same

plant. And the material usually identified as *B. erecta* is collected mostly in sandy soils. In the case of this complex, a study observing the morphology under different environmental conditions and through different plant generations would be useful to check the influence of the environment on the colour and size of the spikelets, which are the main characters used to distinguish species from this complex. If the three species are accepted as distinct taxonomic entities, it is necessary to consider the hypothesis of hybridization among the species, due to intermediates types. The results obtained for this complex contradict the results of Winge et al. (1984), which allowed the differentiation of the three species, and influenced the circumscription published by Longhi-Wagner (1987). Although their sampling was broader (average of ten individuals per species), it is important to emphasize that some of the markers utilized (morphological, isoesterases) are more affected by environmental conditions than the ISSR markers – which could be the main cause of the distinct phenotypes. Future works with ISSR should include a broader sampling of these three species and greenhouse essays.

CONCLUSIONS

Concluding, ISSR are useful as a tool to support taxonomic decisions in genus *Chascolytrum*, but it is important to keep in mind that ISSR are more effective as markers for polymorphism than for conservative features in this genus.

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