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Diversidade genética em três espécies de *Chascolytrum* Desv. (Poaceae, Pooideae, Poeae) ameaçadas de extinção

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Dedico este trabalho a minha mãe Joana
e aos meus sobrinhos, João Antonio e Gabriel.

“Sou eu, eu mesmo, tal qual resultei de tudo,
Espécie de acessório ou sobressalente próprio,
Arredores irregulares da minha emoção sincera,
Sou eu aqui em mim, sou eu.

Quanto fui, quanto não fui, tudo isso sou.
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Quanto amei ou deixei de amar é a mesma saudade em mim.
(...)

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(...)

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(Álvaro de Campos, heterônimo de Fernando Pessoa)

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SUMÁRIO

RESUMO

A diversidade genética é um dos elementos mais importantes em populações de plantas, principalmente em espécies raras e ameaçadas. A variação genética é essencial para a sobrevivência das populações, uma vez que reduz os efeitos da deriva genética e da endogamia e promove a manutenção do potencial evolutivo. O conhecimento sobre como a diversidade genética está distribuída entre as populações é imprescindível para embasar estratégias de conservação, tendo em vista que a totalidade das populações de uma espécie dificilmente pode ser preservada. Marcadores moleculares de diferentes naturezas podem ser utilizados para estimar a diversidade genética em populações, como os marcadores dominantes, os codominantes e as sequências de DNA. Entre os marcadores dominantes, os do tipo AFLP (*Amplified Fragment Length Polymorphism*) são considerados os mais eficazes e informativos, sendo amplamente utilizados em estudos de genética da conservação de espécies raras e ameaçadas. Além disso, as sequências de DNA plastidial (cpDNA) e nuclear, como os ITS (*Internal Transcribed Spacers*), são as fontes mais utilizadas em estudos filogenéticos e filogeográficos. O uso combinado de diferentes marcadores moleculares aumenta o poder de detecção da variabilidade genética, permitindo inferir sobre o modo preferencial que o fluxo gênico ocorre e evidenciando a história evolutiva das populações. Três espécies do gênero *Chascolytrum* consideradas raras e/ou ameaçadas de extinção foram alvo de um estudo de diversidade genética utilizando pelo menos um dos marcadores moleculares mencionados. Os níveis de diversidade genética e estrutura populacional de *Chascolytrum scabrum* e *C. parodianum* foram estimados a partir do uso de marcadores AFLP, cpDNA (*rpoB-trnC*) e ITS, enquanto os níveis de diversidade genética e estrutura populacional de *C. bulbosum* foram estimados a partir do uso de marcadores AFLP. Altos índices de diversidade genética foram encontrados nas três espécies, independentemente do tamanho da população. Os valores de diferenciação genética entre as populações variaram de acordo com a espécie e, principalmente, com o marcador utilizado. As redes de haplótipos obtidas a partir do marcador plastidial revelaram populações com alta diferenciação geográfica devido a um único haplótipo compartilhado entre duas populações de *C. parodianum*. Os marcadores do tipo AFLP e as sequências de DNA plastidial foram considerados os mais eficientes devido ao suporte estatístico encontrado nas análises, enquanto o uso dos espaçadores ITS em estudos populacionais envolvendo as espécies de *Chascolytrum* deve ser evitado devido à homoplasia e possibilidade de paralogia. Por fim, os

dados obtidos são utilizados para sugerir estratégias para a conservação genética de cada espécie.

Palavras-chave: filogeografia; Gramineae; genética da conservação; Pampa; Mata Atlântica.

ABSTRACT

Genetic diversity in three endangered species of *Chascolytrum* Desv. (Poaceae, Pooideae, Poeae): Genetic diversity is one of the most important elements in plant populations, especially when rare and endangered species are addressed. The genetic variation is essential for the long-term populations survival, since it reduces the genetic drift and inbreeding effects and maintains their evolutionary potential. The knowledge of how genetic diversity is partitioned among populations is crucial to guide conservation strategies, considering that all the populations of a species cannot be effectively preserved. Molecular markers from different nature can be applied to estimate genetic diversity in plant populations, such as dominant markers, co-dominant markers or DNA sequences. Among the dominant markers, the AFLP (Amplified Fragment Length Polymorphism) is considered the most effective and informative markers and it is widely used in genetic conservation for rare and endangered species. Moreover, plastidial (cpDNA) and nuclear DNA sequences, such as the Internal Transcribed Spacers (ITS), are the most used tools for phylogenetic and phylogeographic studies. The combined use of different markers increases the power of detecting genetic variation and allows inferring about the preferential gene flow mode, shedding lights on population's evolutionary history. Genetic diversity of three rare and endangered species of *Chascolytrum* was estimated using at least one of the molecular markers mentioned above. Genetic diversity and population structure for both *C. scabrum* and *C. parodianum* was estimated using AFLP, cpDNA (*rpoB*-*trnC*) and ITS markers, whereas genetic diversity and population structure of *C. bulbosum* was estimated using AFLP markers. High levels of genetic diversity were found for all three species, regardless the population size. The genetic differentiation values among populations vary according to species and, especially, according to molecular marker applied. The haplotype networks based on cpDNA reveal populations with high geographic differentiation, since a single haplotype was found to be shared between two *C. parodianum* populations. The AFLP and cpDNA sequences were considered the most efficient molecular markers due to the high statistical support in data analyses, while the use of ITS spacers in

population studies focusing in *Chascolytrum* species should be discouraged due to high homoplasic nature and possibility of paralogy. Finally, the results are used to suggest prior strategies for genetic conservation of each species.

Key words: phylogeography; Gramineae; conservation genetics; Pampa; Atlantic Forest.

CAPÍTULO I

INTRODUÇÃO GERAL

Campo natural em Pinheiro Machado, RS (Foto: Leonardo Nogueira)

Coração cresce de todo lado. Coração vige feito riacho colominhando por entre serras e varjas, matas e campinas. Coração mistura amores. Tudo cabe.

Guimarães Rosa, in "O Grande Sertão: Veredas"

1 INTRODUÇÃO

1.1 Diversidade genética e o uso de marcadores moleculares na conservação de espécies raras e ameaçadas

A diversidade genética em níveis intraespecíficos pode ser considerada um dos elementos mais importantes em populações naturais de plantas e adquire especial interesse em estudos envolvendo espécies raras e ameaçadas (Schaal *et al.* 1991). A variabilidade genética é essencial para a sobrevivência das populações, uma vez que reduz os efeitos da deriva genética e da endogamia (Buza *et al.* 2000) e promove a manutenção do potencial evolutivo e adaptativo frente a eventos estocásticos e alterações ambientais (Reed & Frankham 2003; Jump *et al.* 2009). Logo, estimativas de índices de diversidade genética são imprescindíveis no momento da elaboração de planos de ação para a conservação de espécies ameaçadas de extinção (Höglund 2009; Cires *et al.* 2013). Além disso, a informação quanto à variabilidade genética dos táxons vem sendo amplamente utilizada como fonte de informação em estudos de caráter evolutivo, na delimitação de espécies, na inferência do modo reprodutivo e estrutura populacional e na estimativa de taxas de migração e fluxo gênico (Avise 2004).

Diversos fatores podem influenciar os níveis de variabilidade genética e estrutura populacional de uma espécie, como o tamanho das populações, a fragmentação, o modo reprodutivo, as taxas de fluxo gênico, a história evolutiva e os padrões de distribuição geográfica (Maguire & Sedgley 1997; Zawko *et al.* 2001; Kim *et al.* 2008). As espécies sob os efeitos da fragmentação geralmente apresentam poucas populações e número reduzido de indivíduos e tendem a exibir baixos índices de diversidade genética e alta diferenciação populacional resultantes da perda de riqueza alélica e isolamento geográfico, tornando-se vulneráveis à extinção (Ellstrand & Elam 1993; Frankham 1995; Honnay & Jacquemyn 2007). Desta forma, populações que se mantêm pequenas ao longo do tempo são mais susceptíveis aos efeitos da deriva genética do que populações grandes (Wright 1938). À medida que se tornam espacialmente isoladas, o fluxo gênico tende a ser restrito, dificultando o reestabelecimento de alelos perdidos e promovendo a redução da variabilidade genética de uma população ao longo das gerações (Ellstrand & Elam 1993).

Contudo, exceções são comuns e espécies consideradas raras, ameaçadas ou de distribuição restrita podem apresentar altos níveis de diversidade genética mesmo quando

comparadas a espécies do mesmo gênero com ampla distribuição geográfica (Gitzendanner & Soltis 2000; Lopez-Pujol *et al.* 2002; Wang *et al.* 2004; Ellis *et al.* 2006). A manutenção de altos níveis de diversidade genética nestes casos pode ser atribuída a diversos fatores, como redução do tamanho populacional e isolamento geográfico recente e altas taxas de fluxo gênico (Zawko *et al.* 2001; Maguire & Sedgley 1997). Neste sentido, altos valores de diversidade genética e fluxo gênico sugerem que efeitos severos de perda de variabilidade (*bottleneck*) ou deriva genética não tenham ocorrido recentemente, mantendo a estabilidade genética mesmo em populações pequenas (Rottenberg & Parker 2003).

Além da variabilidade genética, o conhecimento acerca da distribuição desta dentro e entre populações – estrutura populacional - torna-se importante quando estratégias para a conservação de espécies ameaçadas precisam ser elaboradas e a conservação de todas as populações não é possível (Hamrick & Godt 1996). Desta forma, quando as populações se encontram geneticamente estruturadas, a extinção de uma única população pode reduzir significativamente a diversidade genética total da espécie, enquanto a extinção de uma população de espécies com baixa estruturação resulta em efeitos menos pronunciados a nível específico (Ellis *et al.* 2006). A alta diferenciação genética é promovida principalmente pelo limitado potencial de fluxo gênico entre os indivíduos de populações distintas (Hamrick *et al.* 1991; Ellstrand 1992).

Neste sentido, o fluxo gênico é considerado como um dos principais fatores que promovem a variabilidade genética e influenciam diretamente na estruturação das populações em plantas. Fluxo gênico pode ser definido como a troca de gametas ou indivíduos dentro de uma escala geográfica e, principalmente, pelo movimento de genes via pólen ou dispersão de sementes entre populações (Ellstrand 1992; McDermott & McDonald 1993). À medida que o tamanho da população diminui, a importância do fluxo gênico aumenta, e espera-se que estimativas de migração (*Nm*) de pelo menos um indivíduo por geração seja suficiente para diminuir os efeitos da deriva genética (Wright 1931; Antonovics 1976; Ellstrand & Elam 1993) Por outro lado, populações com elevadas taxas de fluxo gênico podem sofrer "*outbreeding depression*" e tornarem-se susceptíveis a extinção devido à hibridação com populações adaptadas a condições ambientais adversas (Ellstrand 1992).

Diversos marcadores moleculares vêm sendo utilizados para estimar índices de diversidade genética em plantas. Entre os mais utilizados, estão os marcadores dominantes baseados em polimorfismos e "*fingerprinting*", como o "*Amplified Fragment Length Polymorphism*" (AFLP; Vos *et al.* 1995), o "*Random Amplified Polymorphic DNA*" (RAPD)

e o "*Inter-Simple Sequence Repeat*" (ISSR), além dos marcadores microssatélites baseados em polimorfismos de sequências simples ("S*imple Sequence Repeat*"- SSR) e as sequências de DNA (Powell *et al*. 1996; Garcia *et al*. 2004; Luan *et al.* 2006).

Marcadores dominantes apresentam inúmeras vantagens em estudos de genética de populações, como altas taxas de polimorfismo, alta reprodutibilidade e baixo custo, além de não exigirem conhecimento prévio do genoma do organismo a ser estudado (Godwin *et al*. 1997; Cavalli 2003). O uso de marcadores moleculares dominantes para estimar a diversidade genética e sugerir estratégias para a conservação de espécies raras e ameaçadas tem sido amplamente difundido na literatura (Luan *et al.* 2006; Kim *et al.* 2008). Contudo, por serem de herança dominante, oferecem limitada informação por loco quando comparados aos marcadores codominantes, como os microssatélites (Provan *et al.* 2001; Han *et al.* 2009).

A técnica de AFLP está baseada em amplificações de fragmentos digeridos por enzimas de restrição, de tamanhos variáveis, o que resulta em grande poder de detecção de variabilidade genética (acessando altos níveis de polimorfismo) (Vos *et al.* 1995). De fato, marcadores do tipo AFLP são considerados como os mais eficientes e informativos marcadores moleculares dentre os de herança dominante (Russell *et al.* 1997; Garcia *et al.* 2004) e tem sido amplamente utilizados em estudos de genética da conservação (Allphin *et al.* 1998; Cardoso *et al.* 2000; Lucchini 2003; Alexander *et al.* 2004; Li *et al.* 2008; Breinholt *et al.* 2009; Cires *et al.* 2013; Gao *et al.* 2016).

Por outro lado, as sequências de DNA tem sido os marcadores moleculares mais utilizados em estudos filogenéticos e filogeográficos em plantas nas últimas décadas (Birky Jr. 2001; Arthofer *et al*. 2010; Shaw *et al.* 2014). As sequências de DNA plastidial (cpDNA) constituem as melhores ferramentas para inferir estimativas de diversidade genética e relações filogeográficas entre populações de plantas. Em geral, o DNA plastidial apresenta herança uniparental, o que permite determinar relações de ancestralidade e inferir sobre a história evolutiva de uma espécie dentro de um contexto geográfico – a filogreografia (Avise 2000; Avise 2004; Ronikier *et al.* 2008). O genoma plastidial caracteriza-se por apresentar taxas de variabilidade detectáveis a níveis específicos (Ronikier *et al*. 2008; Longo *et al*. 2014), além de baixas taxas de recombinação e mutação e tamanho reduzido, o que o torna uma ótima ferramenta para estudos filogenéticos, filogeográficos e na elucidação de processos históricos e demográficos que moldaram a estrutura populacional atual (Provan *et al.* 2001; Naciri & Gaudel 2007).

O genoma nuclear, em especial a região do DNA ribossomal "*Internal Transcribed Spacer*" (ITS), tem sido amplamente utilizado ao longo de décadas em filogenias moleculares de diversos grupos de organismos e, inclusive, para estimar índices de diversidade genética e relações filogeográficas e evolutivas entre populações (Álvarez & Wendel 2003; Mäder *et al.* 2010). O uso combinado de sequências de DNA plastidial e nuclear traz inúmeras vantagens e possibilita elucidar o modo preferencial do fluxo gênico (via pólen ou semente) e compreender como ocorre a distribuição da variabilidade genética dentro e entre populações (Schaal *et al.* 1998; Ennos *et al*. 1999). Por exemplo, quando o fluxo gênico ocorre preferencialmente via pólen ao invés de semente, as populações exibem estruturação genética mais alta a partir dos marcadores plastidiais em relação aos marcadores nucleares (Petit *et al.* 2005; Pinheiro *et al.* 2011). Porém, o uso dos espaçadores ITS em genética de populações pode se tornar problemático quando não ocorre evolução em concerto completa e diferentes parálogos podem ser detectados, além de outros padrões evolucionários complexos causados pela sua natureza repetitiva (Álvarez & Wendel 2003; Mäder *et al.* 2010).

Estudos que combinam diferentes marcadores moleculares possibilitam diferentes análises de caráter genético, ecológico e geográfico, o que ajuda a elucidar os efeitos que as populações vêm sofrendo devido às ameaças às quais estão sendo expostas. O conhecimento da diversidade genética e da estrutura populacional de espécies ameaçadas é de crucial importância à elaboração de estratégias para sua conservação.

1.2 A família Poaceae e o gênero *Chascolytrum* Desv.

Poaceae (Gramineae) pertence à ordem Poales e constitui uma das maiores famílias de Angiospermas, incluindo cerca de 771 gêneros e 12.074 espécies com distribuição cosmopolita (Soreng *et al.* 2015). No Brasil, o número atual de espécies citadas é de 1486, distribuídas em 498 gêneros (BFG 2015). Para o Rio Grande do Sul, os estudos recentes confirmam a ocorrência 89 gêneros e 450 espécies nativas, sendo 400 espécies ocorrentes em formações abertas e 50 espécies em formações florestais (Boldrini & Longhi-Wagner 2011).

A família apresenta grande importância ecológica e econômica, pela dominância em diversos ecossistemas terrestres e pela sua contribuição direta e indireta na alimentação humana (Boldrini *et al*. 2008). As quatro espécies mais cultivadas em todo o mundo são gramíneas: o trigo (*Triticum aestivum* L.), o arroz (*Oryza sativa* L.), o milho (*Zea mays* L.) e a cana-de-açúcar (*Saccharum officinarum* L.) (Rúgolo de Agrasar & Puglia 2004).

A classificação atual da família reconhece sua divisão em 12 subfamílias (Soreng *et al.* 2015), das quais apenas Puelioideae não ocorre no Brasil (Longhi-Wagner 2012). Dentre as demais subfamílias, Pooideae, que inclui cerca de um terço das espécies de gramíneas (Clayton & Renvoize 1986), destaca-se pelas espécies microtérmicas ou hibernais, que contribuem para a produção de forragem nos campos do Sul do Brasil em períodos críticos, além de incluir os cereais de inverno, como o trigo, a aveia, a cevada e o centeio (Boldrini 2009; Boldrini *et al* 2008).

A sistemática de Pooideae sempre atraiu a atenção de agrostólogos, tanto pela sua grande importância econômica ou por representar uma linhagem de gramíneas C3 muito diversificada em formações abertas temperadas (Davis & Soreng 2007; Boldrini *et al.* 2008; Judd *et al.* 2009). Porém, a classificação desta subfamília tem sido controversa ao longo do tempo, principalmente nas últimas duas décadas, quando as relações filogenéticas entre alguns grupos começaram a ser elucidadas. Algumas linhagens bem sustentadas morfologicamente tiveram seu monofiletismo questionado e a partir de então as relações genéricas dentro de Pooideae passaram a receber especial atenção.

As relações entre as tribos Poeae *s.s.* e Aveneae, por exemplo, têm sido alvo de diversos estudos e suas circunscrições, como tradicionalmente aceitas (Clayton & Renvoize 1986), não têm sido sustentadas nas reconstruções filogenéticas envolvendo representantes destes grupos (Hsiao *et al*. 1995; Grebenstein *et al.* 1998; Soreng & Davis 2000; Döring *et al.* 2007; Quintanar *et al.* 2007; Bouchenak-Khelladi *et al.* 2008; Saarela *et al.* 2010; Soreng *et al.* 2015).

Embora existam caracteres morfológicos marcantes para o reconhecimento destas duas tribos (*sensu* Clayton & Reivoize 1986), o posicionamento atual de alguns gêneros anteriormente reconhecidos em Poeae *s.s.* ou Aveneae tem sugerido uma história evolutiva mais complexa para o grupo, envolvendo múltiplos eventos de hibridação e evolução reticulada (Saarela *et al.* 2010; Soreng *et al.* 2015). Por exemplo, os gêneros *Briza* L. e *Chascolytrum* Desv., tradicionalmente incluídos em Poeae *s.s.*, aparecem indiscutivelmente aliados a gêneros típicos de Aveneae (subtribo Agrostidinae) no clado denomidado grupo "*Chloroplast Aveneae Type"* em diversos estudos (Hsiao *et al*. 1995; Grebenstein *et al.* 1998; Soreng & Davis 2000; Döring *et al.* 2007; Saarela *et al.* 2010; Soreng *et al.* 2015). Desta forma, atualmente a tribo Poeae apresenta uma circunscrição mais ampla, compreendendo gêneros anteriormente incluídos em Poeae *s.s.* e Aveneae (Soreng *et al.* 2015).

Não obstante, os gêneros *Briza* e *Chascolytrum* também apresentam um histórico taxonômico peculiar em comum: tradicionalmente as espécies euroasiáticas de *Briza* foram consideradas relacionadas às espécies sul-americanas de *Chascolytrum* (incluindo *Calotheca* Desv. ex Spreng., *Poidium* Nees e outros gêneros menores) por diversos autores. Diversas circunscrições foram propostas para o grupo, denominado "Complexo *Briza*" (Matthei 1975; Essi *et al.* 2008), ora aceitando gêneros distintos (Parodi 1920; Matthei 1975, Nicora & Rúgolo de Agasar 1981; Bayón 1998), ora aceitando *Briza* em seu sentido amplo, incluindo as espécies sul-americanas (Rosengurt *et al.* 1968; Longhi-Wagner 1987).

A primeira abordagem filogenética envolvendo o "Complexo *Briza*" com base em marcadores moleculares plastidiais (*trnL-trnL-trnF*) e nucleares (ITS e GBBSI) evidenciou a divisão do grupo em dois clados principais (Essi *et al.* 2008), representando duas linhagens evolutivamente distintas: um formado pelas quatro espécies euroasiáticas incluídas em *Briza s.s.* e outro formado pelas 22 espécies sul-americanas. Tais resultados sustentaram o reconhecimento de dois gêneros independentes, o que também pode ser evidenciado através de características citogenéticas (Schifino & Winge 1983) e anatômicas da lâmina foliar (Pelegrin *et al.* 2009). Assim, o gênero *Chascolytrum* passou por uma nova circunscrição de modo a adequar as espécies sul-americanas e o gênero *Briza* passou a incluir apenas as espécies euroasiáticas (Essi *et al.* 2008, 2011).

Em sua circunscrição atual, o gênero *Chascolytrum* inclui 22 espécies nativas na América do Sul, sendo representado no Brasil por 18 espécies e duas variedades, sendo seis espécies e uma variedade consideradas endêmicas (Essi, 2007; BFG 2015). O gênero apresenta maior riqueza específica na Região Sul, sendo Minas Gerais o limite de sua distribuição setentrional no país (BFG 2015), e o Rio Grande do Sul o centro de diversidade, com 16 espécies (Longhi Wagner 1987; Essi 2007). Das espécies ocorrentes no Brasil, cinco estão incluídas no Livro Vermelho da Flora do Brasil (sob o gênero *Briza* ou *Erianthecium* Parodi), sendo que quatro delas ocorrem no Rio Grande do Sul (Filgueiras *et al.* 2013). Estas quatro espécies (*C. brasiliense* (Nees ex Steud.) Essi, Longhi-Wagner & Souza-Chies, *C. bulbosum* (Parodi) Essi, Longhi-Wagner & Souza-Chies, *C. parodianum* (Roseng., Arrill. & Izag.) Matthei e *C. scabrum* (Nees ex Steud.) Matthei) também estão incluídas na Lista de Espécies da Flora Ameaçadas do Rio Grande do Sul, sob diferentes graus de ameaça (Rio Grande do Sul 2014). Vale ressaltar que *C. brasiliense* foi considerada como em perigo (EN) nessa lista, contudo, os registros da espécie para o estado (Cambará do Sul, Itaimbezinho) datam de 1975 (Essi 2007), o que pode sugerir que a espécie tenha uma distribuição muito

restrita ou não ocorra mais no estado. Além disso, algumas espécies apresentam uma discrepância no que se refere aos graus de ameaça que estão incluídas na Lista de Ameaçadas do RS e no Livro Vermelho da Flora do Brasil.

Chascolytrum bulbosum (Figura 1) apresenta espiguetas aristadas e os colmos com os nós basais engrossados, semelhantes a bulbos (Longhi-Wagner 1987). A espécie foi descrita para o Uruguai, sob o nome de *Erianthecium bulbosum* Parodi (Essi 2007), porém sua distribuição no país ainda permanece pouco conhecida devido à falta de coletas e registros em herbários. No Brasil, ocorre exclusivamente no Rio Grande do Sul, na região fisiográfica da Serra do Sudeste do Bioma Pampa (BFG 2015). Ocorre em campos arbustivos com afloramentos graníticos (Essi 2007), vegetando preferencialmente em encostas próximo a baixadas úmidas e locais sombreados. *C. bulbosum* é considerada como criticamente ameaçada de extinção (CR) pelo Livro Vermelho da Flora do Brasil (= *Erianthecium bulbosum*; Martinelli & Moraes 2013) e encontra-se em perigo (EN) pela lista de espécies ameaçadas de extinção do Rio Grande do Sul (2014), sendo o sobrepastejo e a perda de habitat pela conversão dos campos naturais em áreas para a silvicultura as principais causas de ameaça à espécie (Martinelli & Moraes 2013). Até recentemente, a espécie foi considerada rara no estado considerando os poucos registros provenientes dos municípios de Piratini e Pinheiro Machado (Longhi-Wagner 1987). Porém, expedições de coletas mais intensas confirmaram a ocorrência de novos registros em municípios como Lavras do Sul, Bagé, Santana da Boa Vista e Encruzilhada do Sul, ampliando consideravelmente a distribuição geográfica da espécie no Rio Grande do Sul.

Chascolytrum scabrum (Figura 2) é uma espécie restrita ao Rio Grande do Sul, sendo considerada endêmica dos campos de altitude com afloramentos basálticos associados à floresta com Araucária do Bioma Mata Atlântica (Longhi-Wagner 1987; BFG 2015). É caracterizada pela inflorescência contraída, pelas espiguetas com poucos antécios, estes de aspecto rígido e coloração amarelada e alas dos lemas pouco desenvolvidas comparadas a espécie morfologicamente próxima *Chascolytrum lamarckianum* (Nees) Matthei (Longhi-Wagner 1987; Essi 2007). É uma espécie rara e considerada em perigo de extinção (EN) pelo Livro Vermelho da Flora do Brasil (Martinelli & Moraes 2013) e pela lista de espécies ameaçadas de extinção do Rio Grande do Sul (2014), sendo a expansão urbana, o cultivo silvicultural de pinheiros e gramíneas invasoras nas áreas agrícolas e o sobrepastejo as principais ameaças incidentes sobre a espécie (Martinelli & Moraes 2013). Vale ressaltar que algumas populações conhecidas da década de 50 e 70 foram consideradas extintas pela falta

de registros desde então e pela conversão do ambiente em áreas de cultivo. Atualmente, existem registros de apenas quatro locais de ocorrência de *C. scabrum* no Rio Grande do Sul, incluindo dois novos registros em Vacaria e Campestre da Serra, realizados durante as expedições de coletas para este estudo.

Chascolytrum parodianum (Figura 3) é uma espécie restrita ao Bioma Pampa, ocorrendo em topos de morros com afloramentos graníticos, vegetando entre fendas de matacões (Longhi-Wagner 1987). A espécie foi descrita para o Uruguai sob o nome de B*riza parodiana* Roseng., Arrill. & Izag. (Bayón, 1998), porém sua distribuição no país ainda permanece pouco conhecida (Essi 2007). No Rio Grande do Sul, ocorre apenas na região dos municípios de Piratini e Pinheiro Machado e apenas quatro locais de ocorrência da espécie são conhecidos até o momento. De fato, é uma espécie rara e considerada criticamente ameaçada de extinção (CR) pelo Livro Vermelho da Flora do Brasil (Martinelli & Moraes 2013) e pela lista de espécies ameaçadas de extinção do Rio Grande do Sul (2014), sendo a perda de habitat para a silvicultura e o pastejo intensivo as principais ameaças. A espécie é reconhecida principalmente por formar touceiras robustas e apresentar lâminas foliares longas e filiformes (Longhi-Wagner 1987), o que parece ser uma adaptação ao estresse hídrico do ambiente que habita. Neste sentido, cabe dizer que a espécie apresenta uma especificidade de nicho que pode ajudar a explicar sua restrita distribuição geográfica e raridade.

1.3 Objetivos

1.3.1 Objetivo geral

Estimar a diversidade genética e a estrutura populacional de *Chascolytrum scabrum*, *C. parodianum* e *C. bulbosum* através de marcadores moleculares AFLP, sequências de DNA plastidial e nuclear (ITS), contribuindo para a elaboração de planos e estratégias para conservação das espécies através da indicação de populações prioritárias para sua conservação genética e ampliar o conhecimento sobre suas distribuições geográficas.

1.3.2 Objetivos específicos

- Estimar a diversidade genética e estrutura populacional de *Chascolytrm scabrum* e *C. parodianum* através de marcadores moleculares AFLP, cpDNA e ITS;

- Estimar a diversidade genética e estrutura populacional de *C. bulbosum* através de marcadores AFLP;

- Contribuir para a elaboração de planos e estratégias para conservação das espécies ameaçadas de extinção *C. bulbosum, C. parodianum* e *C. scabrum* e indicar populações prioritárias para sua conservação genética;

- Registrar novas populações e ampliar o conhecimento sobre a distribuição geográfica das espécies estudadas.

Figura 1. *Chascolytrum bulbosum.* A. Detalhe da inflorescência, espiguetas em antese; B. Detalhe dos nós basais do colmo engrossados, semelhantes a bulbos. C. Habitat em Pedras Altas, RS; D. Habitat em Encruzilhada do Sul, RS. (Fotos: A, B, e D, Leonardo Nogueira da Silva; C, Liliana Essi)

Figura 2. *Chascolytrum scabrum* A. Detalhe da inflorescência; B. Habitar em Campestre da Serra, RS; C. Habitat em Soledade, RS; D. Habitat em Vacaria, RS. (Fotos: Leonardo Nogueira da Silva).

Figura 3. *Chascolytrum parodianum.* A. Inflorescência jovem; B. Inflorescência madura; C. Detalhe da inflorescência; D. Habitat em Torrinhas, Pinheiro Machado, RS; E. Habitat em Piratini, RS. (Fotos: Leonardo Nogueira da Silva)

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CAPÍTULO II

Genetic diversity and population structure in two rare and endangered species of *Chascolytrum* Desv. (Poaceae, Pooideae, Poeae)

Chascolytrum scabrum, detalhe da inflorescência (Foto: Liliana Essi)

Cresci sob um teto sossegado,
meu sonho era um pequenino sonho meu.
Na ciência dos cuidados fui treinado.
Agora, entre meu ser e o ser alheio
a linha de fronteira se rompeu.

Waly Salomão, "Câmara de ecos"

Genetic diversity and population structure in two rare and endangered species of *Chascolytrum* Desv. (Poaceae, Pooideae, Poeae)

Artigo a ser submetido para Molecular Ecology (A1)

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ABSTRACT

Chascolytrum scabrum and *C. parodianum* are considered rare and endangered species due to several factors, such as small populations facing fragmentation and loss of habitat for silviculture. Furthermore, only four wild populations are known from each species to the present, in Rio Grande do Sul, Brazil. As genetic diversity plays an important role in plant populations, it may be used as to the establishment of management and conservation plans. In order to estimate the genetic diversity and population structure in both species, a genetic survey using three different molecular markers (*rpoB-trnC*, ITS and AFLP) was carried out. The combined use of different molecular markers provides knowledge about evolutionary and demographic process and shed lights on the direction of the gene flow to understand the distribution of genetic diversity and population structure. Remarkable levels of genetic diversity were found for all three molecular markers regardless the population size. However, the population structure in both species varies according to the molecular marker applied. Overall, the plastid marker (*rpoB-trnC*) showed higher population structure compared to the nuclear marker (ITS) suggesting a broad pollen-mediated gene flow in both species, although seed-mediated gene flow seems to be played an important role on population structure in *C. parodianum*. In addition, AFLP and plastid DNA exhibited consistent and well supported results in comparison to nuclear markers. Therefore, ITS region may be not recommended to estimate genetic diversity and population structure in *Chascolytrum* species, since the possibility of paralogy cannot be ruled out. Finally, suggestions for conservation strategies to preserve the genetic resources of this species are outlined.

Keywords: phylogeography; gene flow; AFLP; Pampa; Atlantic Forest.

INTRODUCTION

Rare or endangered species often exhibit low levels of genetic diversity leaded for loss of allelic richness resulting from fragmentation, genetic drift or demographic depression (Ellstrand & Elam 1993; Buza *et al.* 2000; Furches *et al.* 2013). As fragmentation promotes habitat loss and reduces population size, the risk of extinction due to stochastic events become more pronounced (Honnay & Jacquemyn 2007). However, exceptions are common and rare and endangered species can exhibit high levels of genetic diversity even when compared with common and widespread congener species (Gitzendanner & Soltis 2000; Lopez-Pujol *et al.* 2002; Wang *et al.* 2004; Ellis *et al.* 2006). After all, species with restricted ranges and small populations can exhibit strong population structure due to a higher proportion of genetic variation partitioned among populations (Frankham 1995; Avise 2004; Walker & Metcalf 2008). The effects on the increase or decrease of genetic diversity can be associated with several factors such as reduction of population size, fragmentation, breeding system, gene flow, evolutionary history and distribution patterns (Maguire & Sedgley 1997; Zawko *et al.* 2001; Kim *et al.* 2008).

Genetic diversity is an important element in plant populations and the knowledge of genetic structure and variation is fundamental in conservation plans. Thus, molecular markers have become an useful tool to detect and estimate genetic diversity and population structure in rare and endangered species being widely used in plant conservation (Moritz & Faith 1998; Gaudeul *et al.* 2000; Batista & Sosa 2002; Ellis *et all.* 2006; Luan *et al.* 2006; Cires *et al.* 2013; Rodrigues *et al.* 2013). Since the maintenance of genetic variation is essential for conserving rare and endangered species (Avise & Hamrick 1996), studies involving effective markers for detecting genetic variability become required.

Different molecular markers can be applied to access genetic diversity in intraspecific levels, such as Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR), Simple Sequence Repeat (SSR) or DNA sequences (Powell *et al.* 1996; Garcia *et al.* 2004; Luan *et al.* 2006). The AFLP technique (Vos *et al.* 1995) can be considered a higher informative fingerprinting tool for access genetic diversity than any other dominant marker (Russell *et al.* 1997; Garcia *et al.* 2004). Furthermore, AFLP exhibit the advantage of not requiring prior genetic knowledge of the organism studied and can be employed with different molecular markers for increasing the

detection of variability in population genetic studies (Walker & Metcalf 2008; Kitner *et al.* 2012). AFLP has also been used successfully in conservation genetics (Cardoso *et al.* 2000; Lucchini 2003; Li *et al.* 2008; Breinholt *et al.* 2009), even for endemic, rare or endangered species with few and small extant populations (Allphin *et al.* 1998; Schmidt & Jensen 2000; Alexander *et al*. 2004; Cires *et al.* 2013).

Overall, plastid DNA sequences have been the most widely used molecular marker in plant phylogenetic and phylogeographic studies in the last decades (Birky Jr. 2001; Arthofer *et al.* 2010; Shaw *et al.* 2014). The usually uniparental heritage of cpDNA allows its use to infer relationships within several taxonomic categories including intraspecific or population levels (Petit *et al.* 1993). At the intraspecific level, cpDNA may shed light on the processes running within/between populations and also reflect the pattern of gene flow (Schaal *et al.* 2003; Kitner *et al.* 2012). Furthermore, nuclear sequences as the ribosomal Internal Transcriber Spacer (ITS) can also be used to estimate diversity indexes, demographic process and infer breeding system and gene flow (Mäder *et al.* 2010). The combined analysis of nuclear and plastid DNA can highlight the direction of the gene flow and allow understanding the distribution of genetic diversity (Schaal *et al.* 1998; Ennos *et al.* 1999). When gene flow is rather pollen than seed-mediated, plastid markers show higher population genetic structure compared with nuclear markers (Petit *et al.* 2005; Pinheiro *et al.* 2011). The knowledge about pollen and seed contribution in gene flow in population structure can be crucial when *ex situ* conservation or genetic improvement is required, especially for small populations (Ellstrand 1992A).

Regardless the molecular marker applied, several studies addressing genetic diversity and conservation focusing in rare and endangered Brazilian species have been carried in the last decades. In fact, as one of the countries with the highest biodiversity in the world, Brazil has also become a leader in biodiversity conservation (Mittermeier *et al.* 2005). However, most studies focus on species of trees from Atlantic Forest (Cardoso *et al.* 2000; Ramos *et al.* 2007; Ribeiro *et al.* 2011; Mendonça *et al.* 2012) with few studies involving herbaceous species occurring in grasslands or open formations (Cavallari *et al.* 2006). The Rio Grande do Sul is the southernmost state in Brazil and is covered mostly by grasslands (*campos*) derived from two different domains: the northern portion placed in highlands and formed by grasslands interspersed with the *Araucaria angustifolia* (Bertol.) Kuntze forest in Atlantic Forest domain (Dümig *et al.* 2008) and the southern portion placed in Pampa domain (Overbeck *et al.* 2007). Due to some characteristics such as climate and soil, the Southern

Brazilian herbaceous flora is highly diversified, with a great number of restrict, endemic and endangered species (Martinelli & Moraes 2013). Nevertheless, studies accessing genetic diversity and population structure focusing species occurring in this region are still scarce (e.g. Pinheiro *et al.* 2011; Tacuatiá *et al.* 2012; Longo *et al.* 2014).

Chascolytrum Desv. (Pooideae, Poaceae) is a microtermic genus comprising twenty-two species occurring in South America with the biggest diversity in southern Brazilian grasslands (Soreng *et al* 2015; BFG 2015). *Chascolytrum subaristatum* (Lam.) Desv. is a morphologically diverse and widespread species reaching its northern distribution limits in Guatemala. However, species like *C. scabrum* (Nees ex Steud.) Matthei and *C. parodianum* (Roseng., B.R. Arrill. & Izag.) Matthei exhibit restricted range distribution and are considered endangered based on rare occurrence, small population size and habitat disturbance by grazing and silviculture according to the Red Book of Brazilian Flora (Martinelli & Moraes 2013). Molecular phylogenetic studies including *Chascolytrum* based on both nuclear and plastid markers indicate a complex evolutionary history involving hybridization events and adaptive radiation (Essi *et al.* 2008; Saarela *et al.* 2010). Furthermore, preliminary studies using Inter-Simple Sequence Repeat (ISSR) were poorly efficient to sustain taxonomic decisions within the genus (Essi *et al.* 2014). Thus, finding suitable markers to access genetic diversity to investigate the evolutionary history of the group is still necessary.

This study consist in a genetic diversity survey focused on two rare and endangered species of *Chascolytrum* using three different markers: AFLP, plastid and nuclear DNA sequences. Several studies compare the genetic diversity between congener species, but few of them use rare and endangered without a common and widespread species or compare the population structure using different molecular makers. We attempt to answer the following questions: (1) is the level of genetic diversity high or low in populations of *C. scabrum* and *C. parodianum*?; (2) how the populations are structured according to each marker and what may be inferred about the main gene flow system?; (3) which marker is the most efficient in describing the genetic diversity in *Chascolytrum* in a conservation approach?; (4) which populations are priority in genetic conservation of *C. scabrum* and *C. parodianum*?

MATERIAL AND METHODS

Study species, sampling and DNA extraction

Chascolytrum scabrum is restricted to southern Brazil Atlantic Forest highland grasslands (BFG 2015), occurring in areas with basaltic outcrops and roadsides. It is considered rare and Endangered (EN) by the Red Book of Brazilian Flora (Martinelli & Moraes 2013) and the main threats to the species are the urban expansion, *Pinus* L. silviculture and invasive exotic grasses, although field observations show that overgrazing and fire can be other threatening causes. Furthermore, only four collection localities were recorded in herbaria, two of them dating from the 50's and 70's (Longhi-Wagner 1987). Efforts to recollect these populations were made, but due the loss of habitat for soybean crops in the locality that once occurred they were considered extinct. Four populations of *C. scabrum* were included in this study, encompassing two new records for the species in Campestre da Serra and Vacaria, in Rio Grande do Sul state (Table 1).

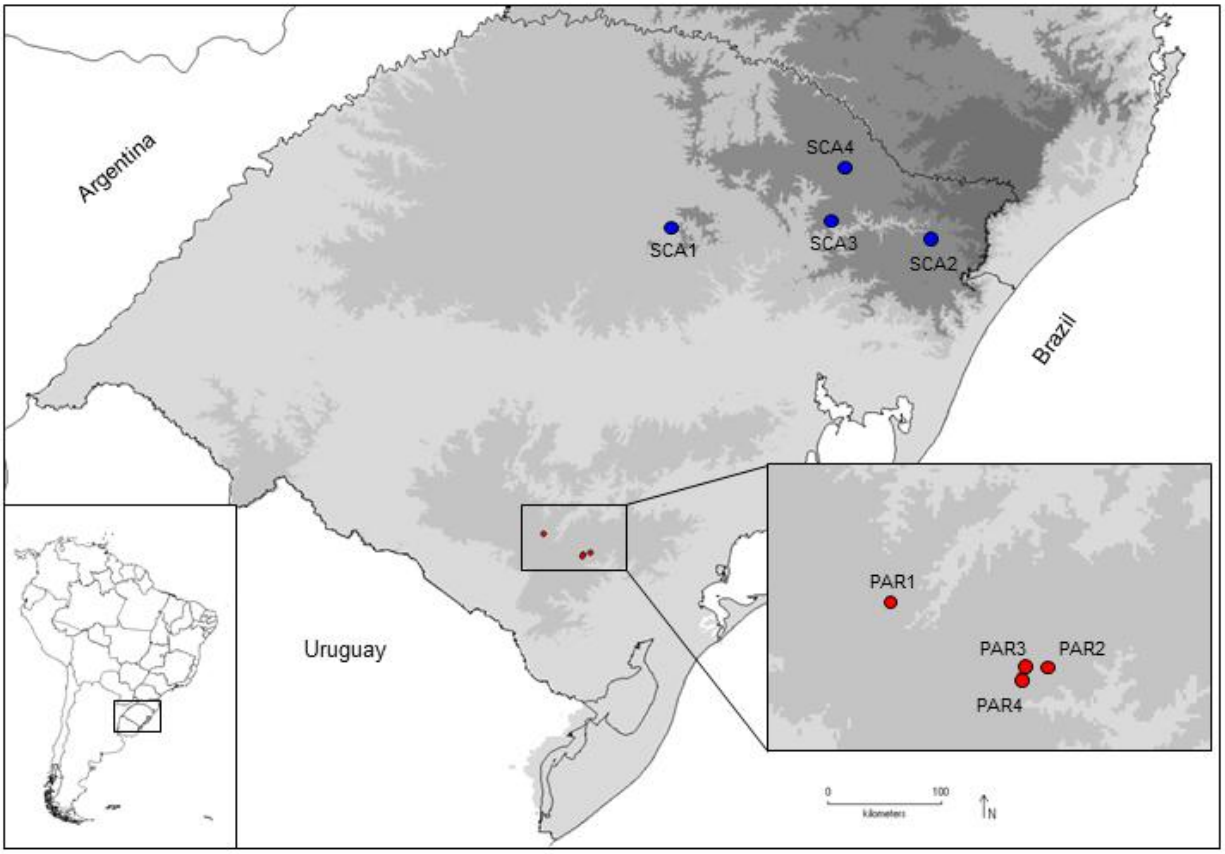

Figure 1. Geographic position of sampled populations of *Chascolytrum scabrum* and *C. parodianum*. Population codes according to Table 1.

Chascolytrum parodianum is restrict to the Pampa domain, occurring in grasslands with granitic outcrops in the southeastern Rio Grande do Sul and Uruguay (BFG 2015). Although it has been described from Uruguay (as *Briza parodiana* Roseng., B.R. Arrill. &

Izag. 1968, Bol. Fac. Agron. Univ. Montevideo 105: 26), its distribution in this country remains unknown. *Chascolytrum parodianum* is considered rare and Critically Endangered (CR), mostly by the *Pinus* silviculture and habitat loss (Red Book of Brazilian Flora; Martinelli & Moraes 2013). Until few years ago, only one collect point was recorded in herbaria from Rio Grande do Sul state, in the municipality of Piratini (Longhi-Wagner 1987). Due to field survey efforts during 2014, three new populations were registered. Field observations show high niche specificity for rock crevices with sandy soil accumulation on granitic hilltops, which can be a limiting factor on its distribution, since the populations are geographically close and have a small number of individuals. Four populations of *C. parodianum* were included in this study (Table 1).

The total sampling according to each marker is available in Table 1. Vouchers from each accession were included in ICN Herbarium of the Universidade Federal do Rio Grande do Sul (UFRGS). The difference in sampling is due to difficulty or lack in amplification or assembly DNA sequence. For DNA extraction, young leaves were collected and dried in silica gel. Total genomic DNA was isolated from approximately 50 mg of dried leaf tissue using the Doyle & Doyle (1987) CTAB method. The integrity and quantity of the DNA were analyzed in 1% agarose gel and quantified in spectrophotometer NanoDrop 2000/2000c (Thermo Scientifics).

Table 1. Sites and sample size of populations of *Chascolytrum scabrum* and *C. parodianum* according to each molecular marker. RS = Rio Grande do Sul state.

						Sampling (number of individuals)		
	Code	**Location**	**Latitude**	**Longitude**	**Altitude (m)**	**AFLP**	***rpo*B-*trn*C**	**ITS**
C. scabrum	SCA1	Brazil, RS, Soledade	28°50'51.60"S	52°26'53.40"W	636	15	17	15
	SCA2	Brazil, RS, Jaquirana	28°54'43.80"S	50°23'54.90"W	832	16	18	15
	SCA3	Brazil, RS, Campestre da Serra	28°46'55.80"S	51°11'55.12"W	732	20	15	19
	SCA4	Brazil, RS, Vacaria	28°21'53.60"S	51°06'03.60"W	863	03	03	02
					Total	54	53	51
C. parodianum	PAR1	Brazil, RS, Pinheiro Machado	31°17'50.00"S	53°29'50.60"W	420	10	10	8
	PAR2	Brazil, RS, Piratini	31°27'00.10"S	53°07'25.90"W	280	03	03	03
	PAR3	Brazil, RS, Piratini	31°27'41.90"S	53°10'44.70"W	387	04	04	04
	PAR4	Brazil, RS, Piratini	31°28'26.20"S	53°11'22.90"W	432	06	06	05
					Total	23	23	20

DNA sequencing

Six DNA regions (ITS, *rpoB-trnC*, *rps16* intron, *trnS-trnG*, *psbA-trnH* and *trnL-trnF*) were amplified in two individuals of each population as a test. The two most variable regions were the nuclear ribosomal DNA internal spacer (ITS) region (Desfeux & Lejeune 1996), including ITS1, ITS2 and the 5.8S gene and the plastidial intergenic spacer *rpoB-trnC* (Shaw *et al.* 2005) and were selected to amplification in all individuals. PCR amplification for ITS region was performed in a 25 µL total volume containing 1 µL of total genomic DNA (approx. 40 ng), 0.5 µM of each primer (forward ITS 92 and reverse ITS 75), 200 µM of dNTP, 1X Taq buffer, 1.5 mM $MgCl_2$ and 1.5 U Taq DNA polymerase. The ITS PCR condition was as follow: an initial step at 94 ºC for 5 min, 40 cycles at 94 ºC for 45 s, 58 ºC for 1 min and 72 ºC for 1.5 min, and a final extension at 72 ºC for 10 minutes. PCR amplification for *rpoB-trnC* region was performed in a 50 µL total volume containing 2 µL of total genomic DNA (approx. 40 ng), 1 µM of each primer, 200 µM of dNTP, 1X Taq buffer, 1.5 mM $MgCl_2$ and 1.5 U Taq DNA polymerase. The *rpoB-trnC* PCR condition was as follow: an initial step at 94 ºC for 1 min, 40 cycles at 94 ºC for 1 min, 53 ºC for 40 s and 72 ºC for 40 s, and a final extension at 72 ºC for 5 minutes. PCR products were purified with 20% polyethylene glycol (PEG) (Dunn & Blattner 1987) and sequenced using an automatic sequencer Genetic Analyser 3500xL (Applied Biosystems). The sequences were assembled with CodonCode Aligner 3.5.3; ambiguous bases were corrected after examination of chromatograms and consensus sequences were edited.

AFLP Protocol

AFLP procedure was performed as described by Vos *et al.* (1995) with modifications according to Baba *et al.* (2015). Approximately 700 ng of total DNA was doubly digested with *Eco*RI (5 U) and *Mse*I (1 U) enzymes in the presence of 2 µL of 10X *Mse*I assay buffer, in a final volume of 20 µL, incubated for 16 h at 37 ºC. The fragments generated were ligated to linker adaptors for *Eco*RI (0.5 µM) and *Mse*I (5 µM) using the T4 DNA ligase enzyme (1 U); 1X T4 DNA ligase buffer; NaCl (0.5 M); BSA (50 ng/µL); and DTT (0.25 mM) in a final volume of 10 µL. The reaction was incubated in a thermocycler at 22º C for 1 h and 70 ºC for 10 min.

The product of restriction/binding reaction was diluted 1:2 ultrapure water. The fragments were amplified with a pair of pre-selective primers containing a selective base. Pre-selective amplification was performed in a final volume of 10 µL, using 3.5 µL of the

GoTaq® kit Green Master Mix (Promega); 0.58 µL of the pre-selective primer (4.75 µM); and 3.0 µL of the dilution of the restriction/binding reaction. The thermocycler program was as follows: 2 min at 72 ºC, followed by 20 cycles of 1 s at 94 ºC, 30 s at 56 ºC, and 2 min at 72 ºC, and finally, 30 min at 60 ºC. The amplified product was diluted 1:10 in ultrapure water.

A 2.5 µL aliquot of the diluted pre-selective product was used for selective amplification, using 0.54 µL of each selective primer, *Mse*I (5 µM) and *Eco*RI (1 µM); and 3.5 µL GoTaq® Green Master Mix (Promega), in a final volume of 10 µL. The selective PCR followed the conditions: initial cycle of 2 min at 94 ºC; 30 s at 65 ºC; and 2 min at 72 ºC; 8 cycles of 1 s at 94 ºC, 30 s at 64 ºC, and 2 min at 72 ºC, decreasing 1 ºC every cycle; 23 cycles of 1 s at 94 ºC, 30 s at 56 ºC, and 2 min at 72 ºC, and finally, 30 min at 60 ºC. Six combinations of *Eco*RI and *Mse*I primers were tested (E-AGC/M-CTC, E-AGC/M-CTG, E-AGC/M-CAG, E-ACT/M-CTC, E-ACT/M-CTG, E-ACT/M-CAG), containing three selective nucleotides, and products were visualized on a 7% polyacrylamide gel. All of the amplifications were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).

Four combinations were selected, based on the number of polymorphic fragments: E-AGC/M-CAG, E-AGC/M-CTC, E-ACT/M-CAG, E-ACT/M-CTC). The *Eco*RI primers for these primer sets were labeled with fluorophores (Table 3) and were subjected to capillary electrophoresis in a 3500 xL automated system (Applied Biosystems). The fragment sizes generated were determined by comparing with 600 LIZ® Size Standard (Applied Biosystems) for standardizing fragments between 100 and 500 bp. The electrophoresis results were combined in a binary matrix using GeneMapper® v.4.1 software (Applied Biosystems).

Table 2. AFLP primers, fluorophores and number of produced fragments for each combination, number and percentage of polymorphic fragments for the *Chascolytrum* species included in this study.

			Number of fragments	
*Eco*RI primer	*Mse*I primer	Fluorophore	*C. scabrum*	*C. parodianum*
AGC	CAG	Hex (green)	164	114
AGC	CTC	Fam (blue)	24	21
ACT	CTC	Hex (green)	104	109
ACT	CAG	Ned (yellow)	204	175
		Total	495	419
		Polymorphic (%)	494 (99.80)	326 (77.80)

Data analysis

DNA sequences

The DNA sequences were aligned using CLUSTAL W (Thompson *et al.* 2001) implemented in MEGA6 (Tamura *et al.* 2013) and manually edited. Due to high levels of homoplasy, the initial portion in *rpoB-trnC* region was excluded in the final alignment and not considered in the analysis (Kelchner 2000). The plastid haplotypes and ITS sequence types were obtained using DnaSP v.5.10.01 software (Librado & Rozas 2009). The information of the number of variable and conserved sites plus nucleotide composition was obtained with MEGA6. The diversity indexes, such as sequence type and haplotype plus nucleotide diversity, were obtained in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010). To test the population differentiation by geographic isolation, pairwise F_{ST} distances between the accessions were calculated using ARLEQUIN and a test of the significance of the genetic and geographical distance correlation (Mantel's test) was calculated using GENALEX 6.5 performed 10.000 permutation rounds (Smouse *et al.* 1986). A plastid DNA haplotype network was constructed by the median joining method (Bandelt *et al.* 1999) using NETWORK v.5 (available at: http://www.fluxux-engineering.com). An analysis of molecular variance (AMOVA) was performed with 10.000 permutations in ARLEQUIN, taking into account two hierarchical approaches: 1) considering all the populations of each species in the same group; and 2) considering two distinct groups obtained previously by the plastid DNA haplotype network of each species. To test the possible clustering between accessions, a Bayesian analysis of genetic population structure was performed in BAPS6 (Corander *et al.* 2004) using the genetic mixture analysis with sequences or linked loci method (Corander & Tang 2007).

AFLP analysis

The AFLP fragments were scored as present (1) or absent (0) in a binary matrix. The diversity indexes, such as percentage of polymorphic loci (P), Shannon's Information Index (*I*), Nei's gene diversity (*H*) (Nei 1973) and gene flow estimates (*Nm*), were obtained using POPGENE v.1.32 (Yeh *et al.* 2000). A Principal Coordinates Analysis (PCoA) was performed based on the Jaccard similarity matrix to visualize the genetic relationships among all individuals analyzed using GENALEX 6.5 (Peakall & Smouse 2006). An analysis of molecular variance (AMOVA) was performed with 10.000 permutations in ARLEQUIN,

taking into account two hierarchical approaches: 1) considering all the populations of each species in the same group; and 2) considering two distinct groups obtained previously by the plastid DNA haplotype network of each species.

The pairwise F_{ST} distances between the accessions were calculated using ARLEQUIN and a test of the significance of the genetic and geographical distance correlation (Mantel's test) was calculated using GENALEX, performed with 10.000 permutation rounds. To verify the possible clustering between the accessions, a Bayesian analysis was performed in STRUCTURE 2.3.4. (Pritchard *et al*. 2000), using the Evano *et al.* (2005) method, with 100.000 Markov Chain Monte Carlo (MCMC) after a 10.000 burn-in repetitions period. For each species a *K* ranging 1 to 7 were tested with 20 independent interactions. The determination of probable *K* clusters was inferred using Structure Harvester (Earl & vonHoldt 2012).

RESULTS

Genetic diversity

The characteristics of the cpDNA haplotypes and ITS sequence types are shown in Table 3. Concerning the *rpoB-trnC* region, 53 individuals of *Chascolytrum scabrum* and 23 individuals of *C. parodianum* were successfully sequenced and produced good sequences. The *rpoB-trnC* final alignment consisted of 1032 bp in *C. scabrum* (GC = 34.5%) and 1099 bp in *C. parodianum* (GC = 21.18%). In this region, five *indels* were found in *C. scabrum* and one *indel* was found in *C. parodianum*. The number of polymorphic sites reached 14 in both species and 14 and 10 haplotypes were found in *C. scabrum* and *C. parodianum* respectively. Gene diversity (*H*) values were similar in both species: 0.8440 (±0.0341) in *C. scabrum* and 0.8419 (±0.0576) in *C. parodianum.* However, *C. scabrum* (0.2490 ± 0.1431) shows higher nucleotide diversity (π) than *C. parodianum* (0.1875 ± 0.1159).

For the ITS region, 51 individuals of *C. scabrum* and 20 individuals of *C. parodianum* were successfully sequenced and produced good sequences. The ITS final alignment consisted of 494 bp in *C. scabrum* (GC = 62.39%) and 504 bp in *C. parodianum* (GC = 62.39%). In this region a single *indel* was found only in *C. scabrum.* The number of polymorphic sites ranged from 4 in *C. parodianum* to 10 in *C. scabrum* and the ITS sequence type ranged from 6 in *C. parodianum* to 19 in *C. scabrum*. Therefore, gene diversity (*H*) values were higher in *C. scabrum* (0.9435 ± 0.0124) than *C. parodianum* (0.8579 ± 0.0363).

Nevertheless, ITS region exhibit low nucleotide diversity (π) for both *C. scabrum* (0.0060 ±0.0035) and *C. parodianum* (0.0028 ± 0.0020).

Table 3. Characteristics of the cpDNA haplotypes and ITS sequence types for the *Chascolytrum* species.

		Sites	Conserved/ variable sites	Indels	Transition/ Transversion	Composition (GC%)
C. scabrum	*rpoB-trnC*	1032	1018 / 14	5	1 / 8	34.50
	ITS	494	484 / 10	2	4 / 5	62.39
C. parodianum	*rpoB-trnC*	1099	1085 / 14	1	5 / 13	21.18
	ITS	504	500 / 4	-	2 / 2	62.39

At the population level, *rpoB-trnC* gene diversity (*H*) ranged from 0 (population SCA4) to 0.8603 (SCA1) in *C. scabrum* and from 0 (PAR2) to 0.8667 (PAR4) in *C. parodianum* (population codes according to Table 1). For ITS region, gene diversity (*H*) ranged from 0 (SCA4) to 0.9524 (SCA1) in *C. scabrum* and from 0.5714 (PAR1) to 1 (PAR2, PAR3) in *C. parodianum*. Haplotype distribution ranged from 1 (SCA4) to 8 (SCA1) in *C. scabrum* and ranged from 1 (PAR2) to 4 (PAR4) in *C. parodianum*. Strikingly, no haplotype was found to be shared in *C. scabrum*, suggesting a phylogeographic differentiation between populations. Haplotype distribution and population indexes of gene diversity (*H*) and nucleotide diversity (π) based on DNA sequences are shown in Table 4. The haplotypes occurring in "SCA1/SCA2 group" are distinguished from haplotypes in "SCA3/SCA4 group" by three-mutation-steps, while remaining haplotypes in *C. scabrum* are distinguished by a single-mutation-step. An exception is haplotype S13, which is distinguished from haplotype S12 by three-mutation-steps. In *C. parodianum*, haplotypes occurring in "PAR1 group" and "PAR2/PAR3/PAR4 group" are distinguished by a single-mutation-step, indicating a phylogeographic population structure. Remarkably, high divergent haplotypes were found within populations. Population PAR1 exhibits a three-mutation-steps haplotype (P3) divergent from P2, and PAR4 exhibits a five-mutation-steps haplotype (P10) divergent from P9. The populations PAR2 and PAR3 (5.45 km apart from each other) shared the P4 haplotype. The haplotype median-joining networks based on plastid marker are shown in Figure 2.

AFLP markers produced 495 fragments in *C. scabrum*, which 494 (99.80%) were polymorphic, and 419 fragments in *C. parodianum*, which 326 (77.80%) were polymorphic, with an average of 215.5 and 148 polymorphic fragments per population, respectively. Nei's gene diversity (*H*) ranged from 0.0640 (SCA4) to 0.1915 (SCA2) in *C. scabrum* and from

0.0938 (PAR2) to 0.1466 (PAR4) in *C. parodianum*. All diversity index based on AFLP markers, including Shannon's Index, are summarized in Table 5.

Table 4: Genetic diversity estimates for *rpo*B-*trn*C and ITS sequences. Population codes according to Table 1. h, haplotype; s, DNA sequence type; *H*, gene diversity; π, nucleotide diversity; In parenthesis are shown the numbers of shared haplotypes or sequence type with other populations.

Populations	***rpo*B-*trn*C**			**ITS**		
	h (shared)	***H***	***π***	**s (shared)**	***H***	***π***
SCA1	8 (0)	0.8603 (±0.0664)	0.0018 (±0.0012)	10 (6)	0.9524 (±0.0344)	0.0063 (±0.0039)
SCA2	2 (0)	0.1111 (±0.0964)	0.0001 (±0.0002)	9 (5)	0.9333 (±0.0397)	0.0052 (±0.0032)
SCA3	3 (0)	0.4571 (±0.1406)	0.0011 (±0.0008)	8 (4)	0.8889 (±0.0376)	0.0042 (±0.0027)
SCA4	1 (0)	0	0	1 (1)	0	0
Species level	14	0.8440 (±0.0341)	0.2490 (±0.1431)	19	0.9435 (±0.0124)	0.0060 (±0.0035)
PAR1	3 (0)	0.3778 (±0.1813)	0.0007 (±0.0006)	2 (0)	0.5714 (±0.0945)	0.0011 (±0.0011)
PAR2	1 (1)	0	0	3 (3)	1.000 (±0.2722)	0.0026 (±0.0027)
PAR3	3 (1)	0.8333 (±0.2224)	0.0011 (±0.0010)	4 (4)	1.000 (±0.1768)	0.0033 (±0.0029)
PAR4	4 (0)	0.8667 (±0.1291)	0.0033 (±0.0022)	3 (3)	0.7000 (±0.2184)	0.0016 (±0.0015)
Species level	10	0.8419 (±0.0576)	0.1875 (±0.1159)	6	0.8579 (±0.0363)	0.0028 (±0.0020)

Table 5. Genetic diversity indexes using AFLP. *P*= polymorphic sites; *H*= Nei's gene diversity; *I*= Shannon's index; *Nm*= gene flow estimates; SD= standard deviation.

	***P* (%)**	***H* (SD)**	***I* (SD)**	***Nm***
SCA1	281 (56.77)	0.1528 (0.1806)	0.2383 (0.2588)	-
SCA2	321 (64.85)	0.1915 (0.1905)	0.2935 (0.2698)	-
SCA3	180 (36.36)	0.1025 (0.1714)	0.1569 (0.2473)	-
SCA4	80 (16.16)	0.0640 (0.1508)	0.0940 (0.2182)	-
Mean	215.5 (43.54)	0.1277	0.1957	-
Species level	494 (99.80)	0.2239 (0.1723)	0.3543 (0.2293)	0.6238
PAR1	185 (44.15)	0.1466 (0.1939)	0.2202 (0.2777)	-
PAR2	96 (22.91)	0.0938 (0.1780)	0.1365 (0.2554)	-
PAR3	140 (33.41)	0.1388 (0.2056)	0.2007 (0.2919)	-
PAR4	171 (40.81)	0.1400 (0.1872)	0.2112 (0.2717)	-
Mean	148 (35.32)	0.1298	0.1922	-
Species level	326 (77.80)	0.1962 (0.1802)	0.3080 (0.2504)	0.9402

Population structure

The genetic divergence (F_{ST}) calculated from each marker was used to estimate the genetic distance among populations (Table 6). Estimates of genetic distance using *rpoB-trnC* ranged from 0.57717 for the most genetically related populations (SCA3 and SCA4), to 0.98044 in the most genetically divergent populations (SCA2 and SCA4) in *C. scabrum*; and ranged from 0.13402 for the most genetically related populations (PAR2 and PAR3) to 0.57845 in the most genetically divergent populations (PAR1 and PAR2) in *C. parodianum*. Most results based on ITS were not statistically supported (Table 6), which can be associated with the sampling or with the highly variable portion of ITS1. However, genetic distance between PAR1 and remaining populations of *C. parodianum* are consistent with the results based on plastid marker. For AFLP data, estimates of genetic distance ranged from 0.12967 (SCA1 and SCA2) to 0.53928 (SCA1 and SCA4) in *C. scabrum*; and ranged from 0.17532 (PAR3 and PAR4) to 0.36231 (PAR1 and PAR2) in *C. parodianum*. Geographic distance can not explain the genetic distance found in *C. parodianum* and *C. scabrum*. Actually, in *C. scabrum* the AFLP's most divergent population (SCA3) is geographically closer to SCA1 and SCA2 than these are from each other. In adittion, the Mantel test did not reveal a significant correlation between the genetic and geographic distances matrices, whereas the P value was not statistically significant from all the markers except from AFLP analysis in *C. parodianum* (r= 0.3817, p= 0.042).

The AMOVA analysis showed contrasting results among species, groups and markers (Table 7). For *C. scabrum*, considerable high population structure was found for ITS and AFLP analysis (F_{ST} = 0.21219 and 0.43945, respectively) when all populations were considered in the same group, but the differentiation was even higher in the *rpoB-trnC* region (F_{ST} = 0.78099). Considering the most genetically related populations as distinct groups ("SCA1/SCA2 group" and "SCA3/SCA4 group"), variation within populations has slightly decreased and the variation among groups ranged from 0.20845 (p=0.33307) in ITS, 0.32995 (p=0.31867) in AFLP, to 0.46727 (p=0.32723) in *rpoB-trnC*. In *C. parodianum* both *rpoB-trnC* and ITS regions showed higher population structure (F_{ST}=0.50348 and 0.40504, respectively) than AFLP (F_{ST} =0.26292). When PAR1 is considered as a distinct group from other populations (PAR2, PAR3 and PAR4), variation within populations decreased in ITS analysis, but showed few differences based on the other markers. Variation among groups was very low considering AFLP data (F_{CT}=0.01528, p=0.49071), but ranged from 0.21095 (p=0.50317) in *rpoB-trnC* to 0.52244 (p=0.24455) in ITS region.

Table 6. Pairwise genetic distance (F_{ST}) and geographic distance (km) between populations of *Chascolytrum scabrum* and *C. parodianum*. In the first square, geographic distances are given above diagonal and genetic distances based on plastid marker below diagonal. In the second square, genetic distances based on AFLP are given above diagonal and genetic distances based on ITS below diagonal. * $P > 0.05$.

					System			
	rpoB-trnC			**Distance (km)**	**ITS**			**AFLP**
C. scabrum	SCA1	SCA2	SCA3	SCA4	SCA1	SCA2	SCA3	SCA4
SCA1	-	199.73	121.99	142.04	-	0.12967	0.47565	0.53928
SCA2	0.67730	-	79.247	91.76	0.01580*	-	0.41316	0.50770
SCA3	0.76881	0.84074	-	47.435	0.28383	0.27976	-	0.53795
SCA4	0.80221	0.98044	0.57717	-	0.18218*	0.02174*	0.28100*	-
C. parodianum	PAR1	PAR2	PAR3	PAR4	PAR1	PAR2	PAR3	PAR4
PAR1	-	39.37	35.27	35.24	-	0.36231	0.26027	0.24920
PAR2	0.57845	-	5.45	6.805	0.56679	-	0.18663	0.30470
PAR3	0.57298	0.13402*	-	1.73	0.51515	0.29577*	-	0.17532
PAR4	0.59398	0.31646*	0.28778	-	0.60997	0.09959*	0.10215*	-

Bayesian analysis was applied to determine the most likely number of clusters (*K*). Simulations performed in STRUCTURE based on AFLP data identified *K*=2 in *C. scabrum* and *K*=5 in *C. parodianum* (Figure 3). In *C. scabrum* the two formed clusters correspond to "SCA1/SCA2 group" and "SCA3/SCA4 group", with the SCA4 population sharing a genetic pool with the first group. The five clusters identified in *C. parodianum* were randomly formed within the populations. Samples in population PAR1 were grouped in two defined clusters and one sample shared a pool from population PAR 3. Meanwhile, populations PAR2 and PAR3 exhibit two distinct pools representing two different clusters, but with admixture between the samples. Lastly, population PAR4 exhibit one distinct pool plus admixture from all other populations. Besides, the gene flow (*Nm*) estimated as 0.6238 individual per generation in *C. scabrum* and 0.9402 in *C. parodianum* suggests a broad gene flow among populations.

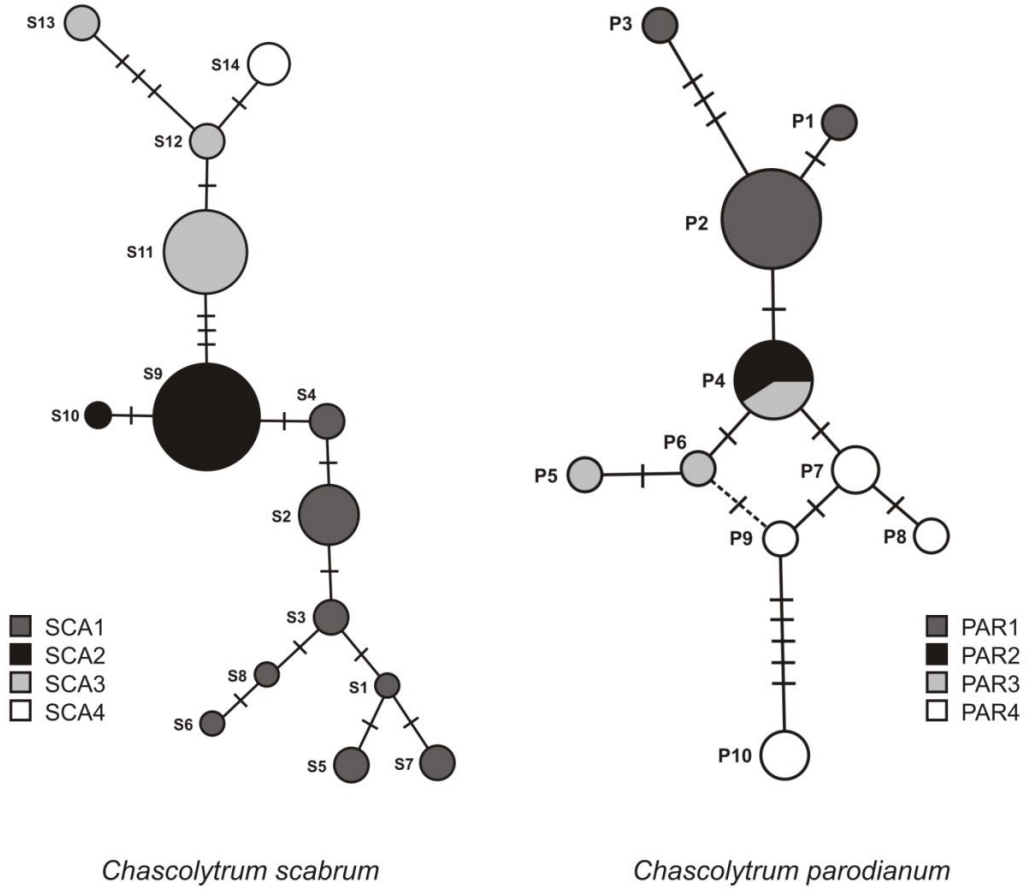

Fig. 2. Haplotype median-joining network based on plastid marker (*rpoB-trnC*) of *Chascolytrum* species. Population codes according to Table 1. The size of each circle is proportional to haplotype frequency across populations. Each line between haplotypes represents a mutational step. The dotted line indicates independent mutation events converging on a shared haplotype. The colors of circles indicate the occurrence of these haplotypes in the four population of each species. See Table 4 for more details.

Table 7. Summary of analysis of molecular variance (AMOVA). d.f., degree of freedom; * $P > 0.05$.

		System					
			***rpo*B-*trn*C**		**ITS**		**AFLP**
	Source of variation (%)	**d.f.**	**% of variation**	**d.f.**	**% of variation**	**d.f.**	**% of variation**
C. scabrum	Among populations	3	78.10	3	21.22	3	43.95
	Within populations	49	21.90	47	78.78	50	56.05
	Total	52	F_{ST}: 0.78099	50	F_{ST}: 0.21219	53	F_{ST}: 0.43945
	Among groups	1	46.73	1	20.84	1	32.99
	Among populations	2	35.03	2	*5.21	2	16.43
	Within populations	49	18.24	47	73.94	50	50.58
	Total	52	F_{CT}: 0.46727	50	F_{CT}: 0.20845*	53	F_{CT}: 0.32995*
C. parodianum	Among populations	3	50.35	3	40.50	3	26.29
	Within populations	19	49.65	16	59.50	19	73.71
	Total	22	F_{ST}: 0.50348	19	F_{ST}: 0.40504	22	F_{ST}: 0.26292
	Among groups	1	21.09	1	52.24	1	1.53
	Among populations	2	*32.33	2	*-1.52	2	25.09
	Within populations	19	46.58	16	49.28	19	73.38
	Total	22	F_{CT}: 0.21095*	19	F_{CT}: 0.52244*	22	F_{CT}: 0.01528*

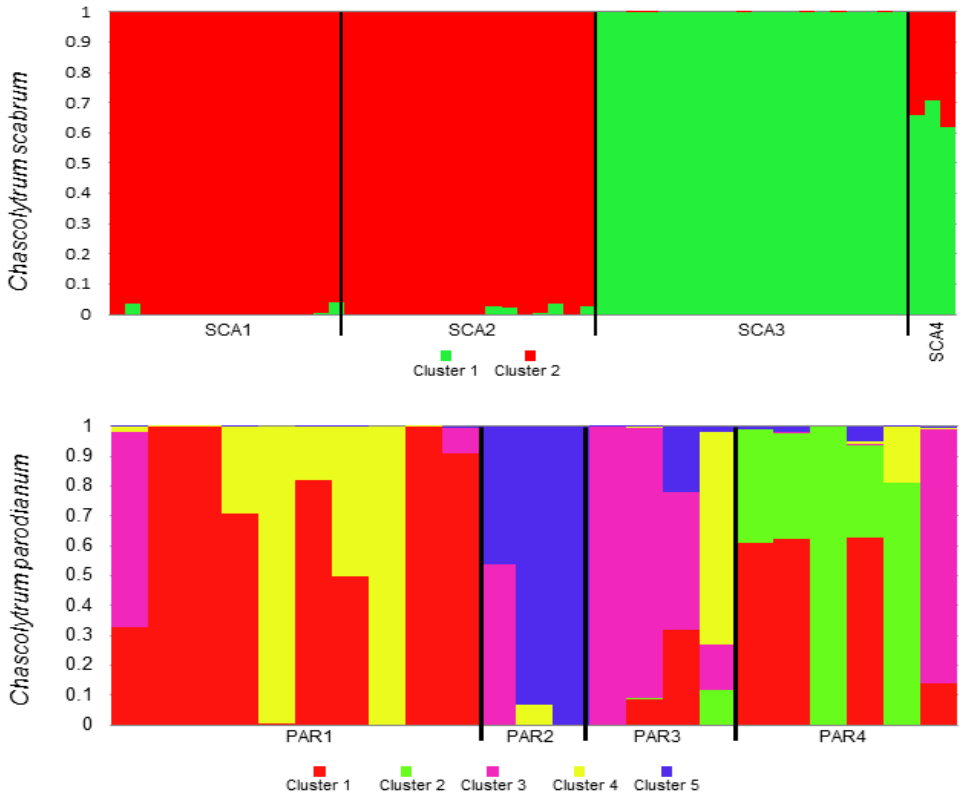

Figure 3. Bayesian analysis of clustering between populations of *Chascolytrum scabrum* and *C. parodianum* obtained in STRUCTURE based on AFLP . Different colors representing different clusters.

The PCoA based on AFLP markers separated the *C. scabrum* samples into two groups (Figure 4). The first group includes all the SCA1 and SCA2 individuals and the second group includes all SCA3 and SCA4 individuals and it is in agreement with the two STRUCTURE clusters. On the other hand, in *C. parodianum* PCoA did not distinguish well-defined populations and the samples were randomly grouped, although the five clusters identified in STRUCTURE can be recognized in the PCoA analysis.

The Bayesian analysis using DNA sequences was performed in BAPS6 and revealed different clustering according to each marker (Figure 5). In *C. scabrum*, *rpoB-trnC* analysis recognized five clusters and ITS analysis recognized six clusters. The *rpoB-trnC* clusters were geographically structured with no admixture between populations, except for populations SCA3 and SCA4 which shared a linked cluster. Unlikely, the ITS clusters were randomly distributed among the populations and exhibit no geographic structure. In *C. parodianum*, *rpoB-trnC* analysis recognized three clusters which one is exclusive from PAR1 and another from PAR4 while the third one is shared between PAR2, PAR3 and PAR4 populations.

Moreover, both ITS and the plastid analysis recovered the separation of PAR1 as an exclusive cluster from the remaining populations. Finally, there is a clear agreement between the BAPS population structure based on *rpoB-trnC* and the haplotype network for both species.

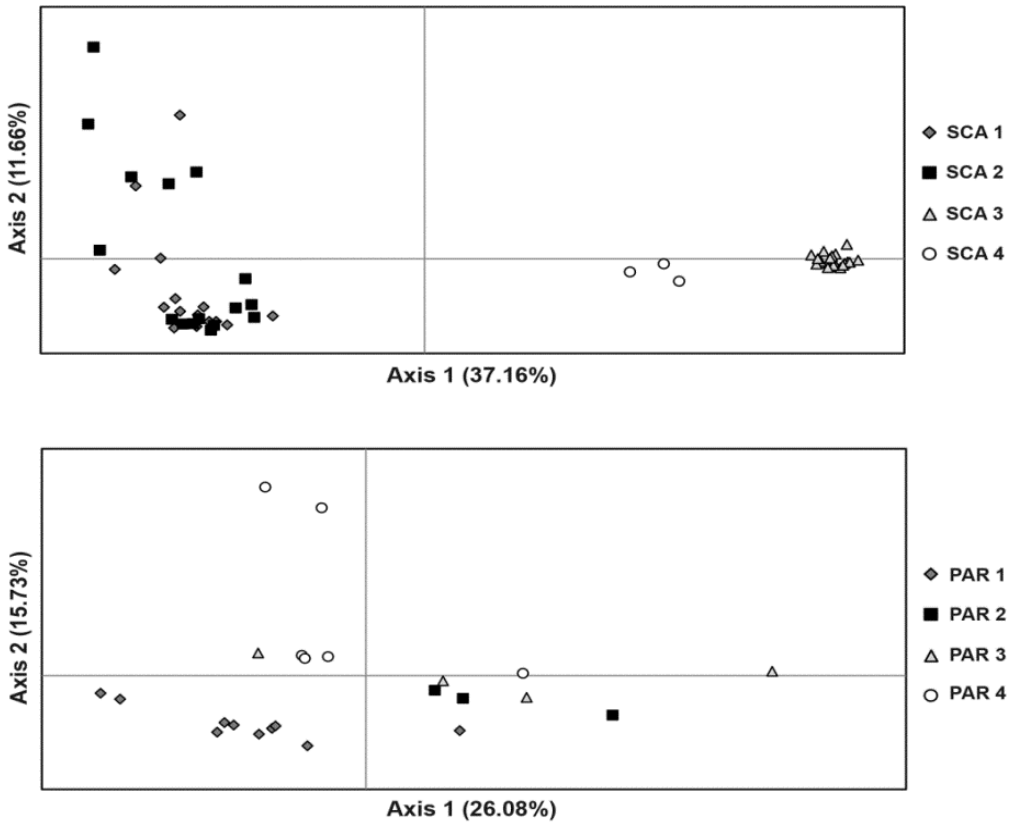

Figure 4. Principal Coordinates Analysis (PCoA) of *Chascolytrum scabrum* and *C. parodianum* based on AFLP. Sum of % axis: *C. scabrum* (56.40 %) and *C. parodianum* (55.46 %).

DISCUSSION

Genetic diversity and population structure in *Chascolytrum scabrum*

Despite its rarity, unexpectedly high levels of genetic diversity were detected in *Chascolytrum scabrum*. The diversity indexes obtained from all molecular markers indicate that SCA1 is the most genetically diversified population (*H* = 0.8603 in *rpoB-trnC*; 0.9524 in ITS; 0.1528 in AFLP). However, population SCA2 exhibits high genetic diversity based on ITS region and AFLP markers (*H* = 0.9333 and 0.1915 respectively), but one of the lowest genetic diversity based on plastid marker (*H*= 0.1111). Indeed, only two haplotypes were

found in this population and one of them was found in a single sampled individual. The most plausible explanation suggests that SCA2 population has been originated with low levels of genetic diversity or have experienced a bottleneck effect. Since the plastid genome is more conserved and accumulates mutations slowly, the highest genetic diversity found in other markers can be linked to recent gene flow mediated by pollen. The ITS region exhibits the highest genetic diversity values than any other marker, except in SCA4 population (H= 0). Several DNA sequence types were found to be shared between populations, suggesting lowest population genetic structure based on the nuclear marker (F_{ST} = 0.21219) compared to the other markers.

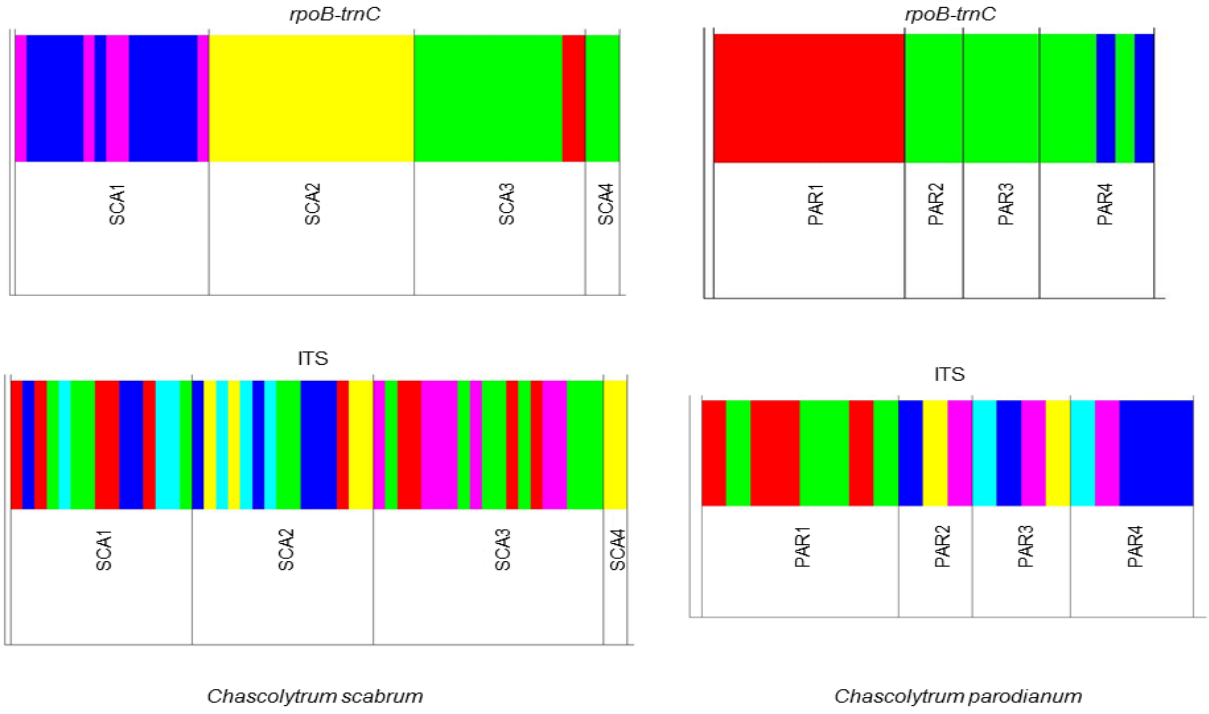

Figure 5. Bayesian Analysis of Population Structure (BAPS) using plastid (*rpoB-trnC*) and nuclear (ITS) markers in *Chascolytrum* species. Population codes according to Table 1. Colors represents different clusters.

However, high population genetic structure was found across the analyzed populations considering plastid (F_{ST} = 0.78099) and AFLP markers (F_{ST} = 0.43945) in comparison with the nuclear marker. The most outstanding pattern recovered by the joint use of different markers in *C. scabrum* was the recognition of SCA3 and SCA4 as a different lineage from the remaining SCA1 and SCA2 populations. This pattern was clearly recovered by the two inferred clusters (K = 2) in the Bayesian analysis using AFLP markers and AMOVA results

revealed a high differentiation among those two groups considering plastid markers (F_{CT} = 0.46727). The remarkable genetic similarity found between the most geographically distant populations (SCA1 and SCA2) can be better explained when a wider past distribution rather than recent long-distance dispersal events is considered (Naciri & Gaudeul 2007). Since the haplotypes found in those populations were closely related but not shared, an isolation event must be occurred a long time ago allowing the accumulation of mutations. It is possible that Holocene expansions in the Brazilian Atlantic Rain Forest from the northeast towards south Brazil led to grasslands fragmentation and may help to explain the population isolation by creating a barrier to gene flow or seed dispersion (Lorscheitter 1997; Dümig *et al.* 2008).

Gene flow can be detected either by the amount of pollen as seeds flow (Batista & Sosa 2002). The highest population structure based on plastid marker (F_{ST}= 0.78099) in comparison with the nuclear marker (F_{ST}= 0.21219) indicates a preferential pollen-mediated gene flow, which may be associated with the limited seed dispersal in some grasses (Peart 1979). The preferential mode of gene flow by pollen can also help to explain the highest genetic diversity found in SCA1, which occurs at the lowest altitude and may be favored by the arrival of pollen through the wind from other populations. On the other hand, the geographic position associated with the lowest genetic diversity found in SCA3 may suggests a founder event derived from a not sampling or extinct population from the north, while SCA4 must have gone through an extreme reduction of population size (Ennos *et al.* 1999). Furthermore, is possible that SCA3 and SCA4 may represent relict populations which gone through severe bottleneck effects leading to the loss of genetic diversity (Naciri & Gaudeul 2007), whereas AFLP markers have revealed a large number of monomorphic loci and plastid DNA showed a reduced number of haplotypes in those populations compared with SCA1. However, the high genetic diversity found in nuclear marker and shared sequence type between the other populations can suggests some gene flow mediated by pollen (Petit *et al.* 2005). Estimate of the effective gene flow (*Nm*) based on AFLP markers was lower than one individual per generation (0.6238) considering all populations. This value must be carefully interpreted since highly divergent populations were considered in analysis and no correlation between geographic distance and genetic differentiation has been found. In addition, *Nm* may also represent historical gene exchange and ancestral polymorphism retention and do not indicate current migration rates (Luan *et al.* 2006).

Genetic diversity and population structure in *Chascolytrum parodianum*

Remarkable genetic diversity was found in populations of *Chascolytrum parodianum*, despite its rarity, population size or geographic distance (see indexes in Table 4 and 5). Populations PAR3 and PAR4 exhibit the highest genetic diversity considering all molecular markers applied. Despite the geographical proximity (1.73 km apart from each other), any haplotype was found to be shared between those two populations, although haplotypes P6 (PAR3) and P9 (PAR4) can be considered related by an independent nucleotide mutation (Figure 2, dotted line). A single haplotype was found in PAR2 ($H = 0$) which are found in PAR3 as well, suggesting a shared origin for both population. Due to habitat disturbance, it is more likely that PAR2 have been originated by seed dispersion from PAR3. Population PAR1 exhibit the highest genetic diversity based on AFLP markers but lowest indexes in plastid or nuclear sequences analysis. Surprisingly, most haplotypes are distinguished by a single mutation-step except haplotypes P3 (PAR1) and P10 (PAR4), which are distinguished respectively by three and five mutation-step from the most related haplotypes. The presence of highly divergent haplotypes suggests a complex population dynamics involving migration and dispersion events with mixture of historically separated populations.

Regardless of the applied marker, population structure analyses have always recognized PAR1 as divergent from the remaining populations. In addition, even DNA sequence types found in PAR1 were exclusive in this population while all DNA sequence types present in remaining populations were found to be shared among them. The fact is that almost all populations have unique genetic traits which reflect the intriguing clustering between the individuals. For instance, the recognition of $K = 5$ in Bayesian analysis by AFLP markers revealed an unexpected pattern in population structure with admixture between all populations but also with a remarkable number of clusters. Besides that, the partitioning of genetic diversity among groups was poorly supported by AMOVA indicating no genetic structure between PAR1 and "PAR2, PAR3, PAR4 group". When all populations were considering in the same group, AMOVA based on AFLP markers revealed that 73.71% of the variance is found within population. In contrast, AMOVA based on plastid and nuclear markers revealed that variance can be found both among either within populations (see Table 7), suggesting a recognition of independent populations with broad gene flow among them.

The estimates of gene flow reached almost one individual per generation ($Nm = 0.9402$) as well expected to minimize the effects of genetic drift in small populations (Ellstrand 1992A). Furthermore, the differentiation among populations were similar in both

plastid and nuclear markers (F_{ST} = 0.50348 and 0.40504, respectively) suggesting mixed gene flow. Thus, seed may contribute as much as pollen in the gene flow, since the populations are geographically close, especially PAR2, PAR3 and PAR4. It is likely that seed dispersal promotes gene exchange among populations as pollen promotes gene exchange within population (Ellstrand 1992B). After all, the long-distance seed dispersion can also play a significant role in population dynamics due to the presence of highly divergent haplotypes which may be arising from other genetically distinct populations (Ellis *et al.* 2006). As *C. parodianum* exhibit niche specificity, seed establishment may be hampered by limited suitable habitats. Therefore, populations may be structured rather by habitat colonization by individuals from genetically distinct populations. This pattern of migration may also suggest the recognition of subpopulations within a metapopulation model (Tero *et al.* 2003), but this hypothesis must be tested in further analyses. Although the genetic differentiation (F_{ST}) have increased with geographic distance, no correlation in Mantel's test was significantly supported, except for AFLP analysis (r = 0.3817, P = 0.042).

The use of molecular markers

The DNA sequences are the most reliable source of genetic information being largely applied in phylogenetic, phylogeographic and population studies (Álvarez & Wendel 2003; Shaw *et al.* 2014). As evolving following an evolutionary model, DNA sequences exhibit features that may reflect the evolutionary and demographic history of populations. On the other hand, dominant markers such RAPD, ISSR and AFLP have been widely used in population genetics to infer genetic diversity, population structure, gene flow and mating system (Zhivotovsky 1999). As do not require prior knowledge of the studied plant genome, dominant markers are easily reproducible and low cost tools to be implemented in conservation genetic studies. However, finding suitable DNA markers that provide an amount of genetic information at intraspecific levels require prior laboratory work. In this study, three different markers were applied in a genetic survey in two *Chascolytrum* species: plastid DNA sequences (*rpoB-trnC*), nuclear DNA sequences (ITS) and the dominant marker AFLP.

Despite that ITS seems to be a highly phylogenetic informative marker, its use in intrapopulational studies in *Chascolytrum* is still limited to infer genetic relationships due to homoplasy, mainly in ITS1 region. This held the possibility of paralogy due to incomplete concerted evolution since heterogeneity seems to be a common feature in plant species (Ruggiero & Procaccini 2004; Mäder *et al.* 2010). However, the high genetic diversity found

in ITS region from both *C. scabrum* and *C. parodianum* can be useful to highlight the preferential system of gene flow between the analyzed populations. Although most of the population genetic structure patterns revealed by the plastid marker were reproduced by AFLP markers, the AFLP technique must be used carefully. As a dominant marker, AFLP accesses the entire genome, specially the nuclear (Ley & Hardy 2013), without an evolutionary model and may not reflect historical or demographic processes that shaped the genetic structure in analyzed populations as well as plastid DNA sequences. However, AFLP must be considered as a highly informative marker for access genetic diversity in *Chascolytrum* and could be applied in genetic conservation as an economically viable alternative to DNA sequences (Lucchini 2003). Finally, the use of plastid DNA sequences should be rather stimulated once reflects historical and demographic process that shaped population structure in a phylogeographic approach. Within *Chascolytrum*, we suggest the combined analysis of cpDNA and AFLP markers for accurate conservation decisions, but AFLP can be applied by itself for a rapid detection of genetic diversity and population structure in population genetic studies.

Implications for conservation

Genetic diversity has been widely used to distinguish and indicate priority populations of endangered species since the maintenance of genetic variation is one of the major goals in plant conservation (Ribeiro *et al*. 2011). Thus, the use of different molecular markers can increase the power of detecting genetic variability plus shed lights on historical and demographic process, which must be taken into account when developing management plans and conservation are required. The criteria for the selection of priority populations must include both the uniqueness and its diversity level of its allelic composition (Petit *et al.* 1998). In the case of *C. scabrum* and *C. parodianum*, the combined analysis of AFLP and DNA sequences has provided enough information for making conservation decisions, since AFLP presented high levels of polymorphism and DNA sequences have elucidated the population evolutionary history. Thus, actions to conserve both species should be employed immediately.

All extant populations of *C. scabrum* exhibit their own genetic signatures, which is related with several factors such demography, geographic position and evolutionary history. Despite populations SCA1 and SCA2 were included in the same cluster inferred by AFLP markers, the haplotype constitution plus geographic position makes both populations as priority for genetic conservation. The same applies to the SCA3 population, which exhibit

highly divergent genetic features that may represent a relict population. Finally, it seems difficult includes population SCA4 in an *in situ* conservation plan due to reduction of population size by intensive grazing. For this population, we suggest an *ex situ* conservation trhough seed germination, which can be used as a source of individuals to increase genetic diversity and haplotype richness in population SCA3.

The interesting pattern of genetic structure and clustering found in *C. parodianum* has suggested a complex population dynamics. Taking into account the priority conservation, we suggest that at least PAR1, PAR3 and PAR4 must be recognized as independent populations, since high levels of genetic diversity and haplotypes with geographic differentiation has been found. The small population size makes the development of conservation plans urgent whereas any population is included in any protected area (Loyola *et al.* 2014). Due to high habitat specificity, the *ex situ* conservation may be limited by difficulties in seed germination or maintenance of individuals in a greenhouse. Therefore, we encouraged *in situ* conservation by the establishing of conservation units or conservation management.

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CAPÍTULO III

Genetic diversity and population structure of the endangered *Chascolytrum bulbosum* (Poaceae, Pooideae, Poeae) in Southern Brazil accessed by AFLP markers

Chascolytrum bulbosum em antese (Foto: Leonardo Nogueira da Silva)

Apesar das ruínas e da morte,
Onde sempre acabou cada ilusão,
A força dos meus sonhos é tão forte,
Que de tudo renasce a exaltação
E nunca as minhas mãos ficam vazias.

Sophia de Mello Breyner Andresen, in 'Antologia Poética'

Genetic diversity and population structure of the endangered *Chascolytrum bulbosum* (Poaceae, Pooideae, Poeae) in Southern Brazil accessed by AFLP markers

Artigo a ser submetido para Biochemical Systematics and Ecology (B1)

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ABSTRACT

Chascolytrum bulbosum is a perennial grass restricted to shrubby fields with granite outcrops from southern South America and it is considered endangered due to increasing habitat loss and overgrazing. Recent field collections verify a wider distribution than previously recorded. After all, no population genetic study and conservation management plan for the species has been conducted until now. AFLP markers were used to investigate the genetic diversity and infer the population structure of 13 natural populations across *C. bulbosum* distribution in Rio Grande do Sul, Brazil. Three selective primer combinations of AFLP produced a total of 651 fragments, which 98.20% were polymorphic. At the species level, genetic diversity were considered moderated (H = 0.149486) compared to previously studies focusing endangered species. The genetic differentiation and population structure were considered moderate to high (G_{ST} = 0.2275; F_{ST} = 0.34054) and no correlation between genetic and geographic distance was found (r = 0.0564, P = 0.05). The major analyses recovered the separation of all individuals sampled into two groups, although AMOVA indicates that only 13.20% of the variation relies among them. High variation within population was found (65.95%). The estimates of gene flow (*Nm*) by migration were high in all analyses: 1.689 among populations, 2.059 among populations in group A, 1.932 among populations in group B and 3.490 among groups. The results suggest that no recent severe bottleneck may be occurred in populations analyzed in this study. As gene flow occurs broadly through the geographical range of the species in Rio Grande do Sul, we suggest the stimulation of *in situ* rather than *ex situ* conservation, and priority populations to genetic conservation of *C. bulbosum* are indicate.

Keywords: plant conservation; genetic conservation; gene flow; population genetics.

INTRODUCTION

Genetic diversity at intraspecific levels is considered one of the most important elements in plant populations especially when rare and endangered species are addressed (Schaal *et al.* 1991). The genetic variation is crucial for the long-term survival of population facing fragmentation because it may reduces the effects of genetic drift or inbreeding depression (Buza *et al.* 2000). Fragmented populations often exhibit high degrees of genetic differentiation and population structure and may be more susceptible to stochastic and extinction events (Frankham 1995; Hensen & Oberprieler 2005; Rodrigues *et al.* 2013). However, high genetic diversity and low population structure can often be found in endangered species suggesting a broad gene flow between populations (Breinholt *et al.* 2009; Cires *et al.* 2013). In addition, the lack of genetic structure due to high gene flow can also be detrimental since populations suffering "outbreeding depression" are also apt to extinction due to hybridization (Ellstrand 1992). After all, the importance of gene flow increases as population size decreases and gene flow between 0.5 and one individual per generation is considered sufficient to minimize the effects of genetic drift (Wright 1931; Antonovics 1976; Ellstrand & Elam 1993).

A large number of different molecular markers has been used in population genetic studies, especially dominant markers based on banding patterns (Luan *et al.* 2006; Kim *et al.* 2008; Kitner *et al.* 2012). Amplified fragment length polymorphism (AFLP; Vos *et al.* 1995) is considered a higher informative fingerprint tool than any other commonly dominant marker (Russell *et al.* 1997; Garcia *et al.* 2004) and has been widely used for resolving genetic issues among individuals, populations and species (Zhang *et al.* 2001; Subudhi *et al.* 2005; Lambertini *et al.* 2006; McKinnon *et al.* 2008; Erol *et al.* 2011; Gao *et al.* 2016). Moreover, AFLP technique has also been well applied to estimate genetic diversity and infer population structure in endangered species as a guide for conservation plans (Cardoso *et al.* 2000; Gauduel *et al.* 2000; Luchini 2003; Breinholt *et al.* 2009; Cires *et al.* 2013).

Chascolytrum bulbosum (Parodi) L. Essi, Longhi-Wagner & Souza-Chies is a perennial and microtermic grass restricted to shrubby fields with granite outcrops of southern South America, including the southern portion of Rio Grande do Sul state (Brazil) and Uruguay, in Pampa Domain (Longhi-Wagner 1987). The species is easily recognized within the genus by awned spikelets, pilose and flat leaf blades and the thickened basal nodes, similar to bulbs (Longhi-Wagner 1987). In Brazil, *C. bulbosum* has been included in some

Red Lists, such as the Red List of Rio Grande do Sul (EN; Rio Grande do Sul 2014) and the Red Book of Brazilian Flora (CR; Martinelli & Moraes 2013, under the name *Erianthecium bulbosum* Parodi), and the major threats to the species include habitat loss and overgrazing. For a long time, the species was considered rare and supposedly found only in the region of Piratini and Pinheiro Machado counties, in Rio Grande do Sul state. Collection efforts made in the last two years allow to record new localities of occurrence which expanded significantly the geographical range of the species. Nevertheless, no population genetic study and conservation management plan for the species has been conducted until now.

Since the maintenance of genetic variation is essential for conserving endangered species (Avise & Hamrick 1996), this study applied AFLP markers to estimate both genetic diversity and population structure of natural populations of *Chascolytrum bulbosum* occurring in Rio Grande do Sul state in Southern Brazil. We attempt to answer the following questions: (1) what is the level of genetic diversity within and among populations of *C. bulbosum* based on AFLP markers?; (2) how is the genetic diversity distributed within and between populations? and finally (3) what is the estimation of gene flow and how it affects the population genetic structure? In addition to genetic diversity and population structure, the results are used to discuss and propose conservation and management strategies for the species.

MATERIAL AND METHODS

Plant material and DNA isolation

Young leaves from 179 individuals from 13 geographically distinct locations were collected and dried in silica gel. The locations of the populations are shown in Figure 1. The number of samples per population varied from four to 21 (Table 1). Total genomic DNA was isolated from approximately 50 mg of dried leaf tissue using the Doyle & Doyle (1987) CTAB method. The integrity and quantity were analyzed in 1% agarose gel and quantified in spectrophotometer NanoDrop 2000/2000c (Thermo Scientifics).

AFLP protocol

AFLP procedure was performed as described by Vos *et al.* (1995) with few modifications following Baba *et al.* (2015). Approximately 700 ng of total DNA was doubly digested with *Eco*RI (5 U) and *Mse*I (1 U) enzymes in the presence of 2 μL of 10X *Mse*I

assay buffer, in a final volume of 20 µL, and incubated for 16 h at 37 ºC. The fragments generated were ligated to linker adaptors for *Eco*RI (0.5 µM) and *Mse*I (5 µM) using the T4 DNA ligase enzyme (1 U); 1X T4 DNA ligase buffer; NaCl (0.5 M); BSA (50 ng/µL); and DTT (0.25 mM) in a final volume of 10 µL. The reaction was incubated in a thermocycler at 22º C for 1 h and 70 ºC for 10 min.

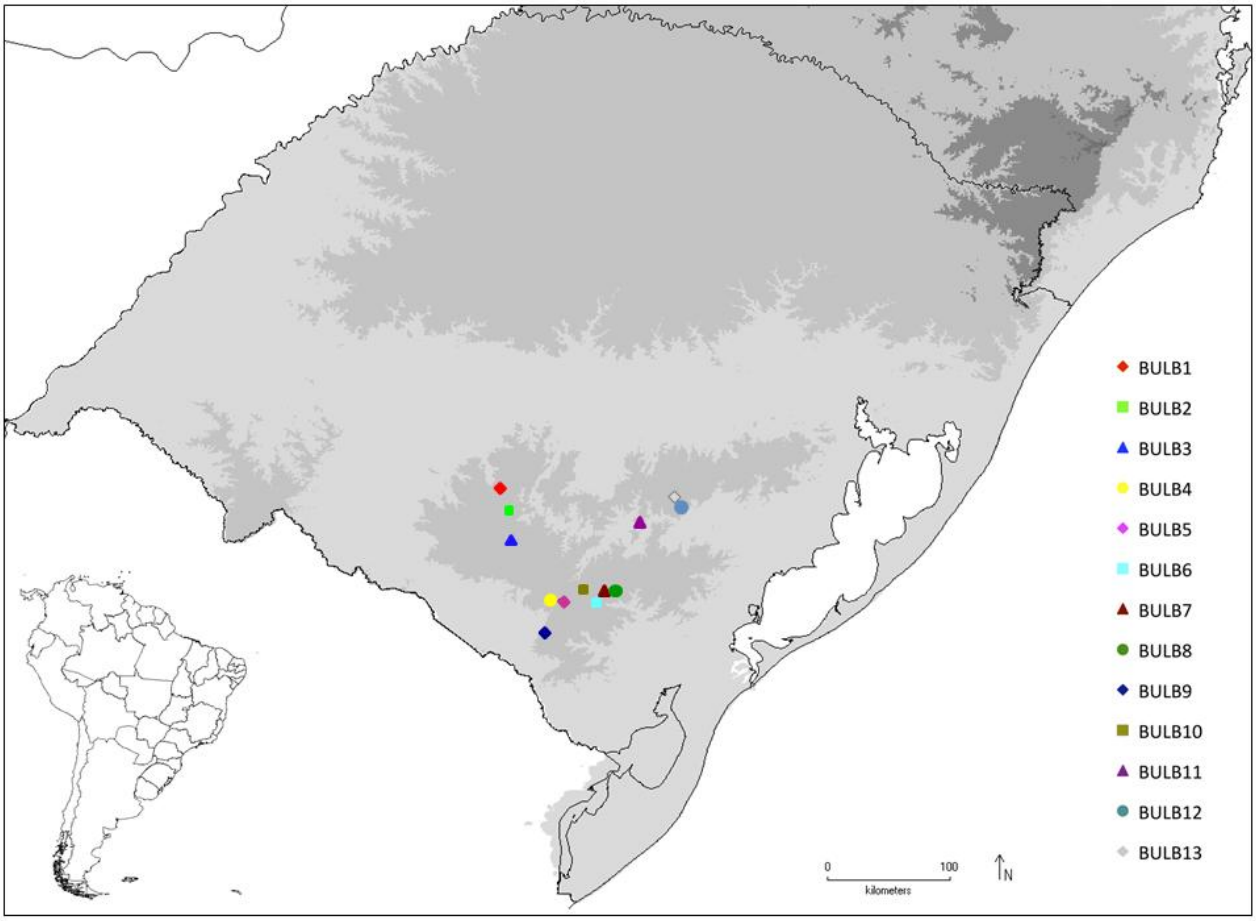

Figure 1. Geographic position of the 13 sampled populations of *Chascolytrum bulbosum* included in analysis. Population codes according to Table 1.

The product of restriction/binding reaction was diluted 1:2 in ultrapure water. The fragments were amplified with a pair of pre-selective primers containing a selective base. Pre-selective amplification was performed in a final volume of 10 µL, using 3.5 µL of the GoTaq® kit Green Master Mix (Promega); 0.58 µL of the pre-selective primer (4.75 µM); and 3.0 µL of the dilution of the restriction/binding reaction. The thermocycler program was as follows: 2 min at 72 ºC, followed by 20 cycles of 1 s at 94 ºC, 30 s at 56 ºC, and 2 min at 72 ºC, and finally, 30 min at 60 ºC. The amplified product was diluted 1:10 in ultrapure water.

Table 1: Sites, population codes and sample size of *Chascolytrum bulbosum*. RS, Rio Grande do Sul state; ICN, Herbarium of Universidade Federal do Rio Grande do Sul (UFRGS); N, number of individuals.

Population	Location (Brazil, RS)	Voucher (ICN)	Latitude (S)	Longitude (W)	Elevation (m)	N
BULB1	Lavras do Sul	*L.N.Silva 656*	31º27'52.6"	53º10'48.4"	320	8
BULB2	Lavras do Sul	*L.N.Silva 676*	30º50'53.4"	53º53'13.4"	325	14
BULB3	Bagé	*L.N.Silva 680*	31º04'38.8"	53º53'.22.5"	290	12
BULB4	Candiota	*L.N.Silva 689*	31º31'07.9"	53º34'33.1"	276	9
BULB5	Pinheiro Machado	*L.N.Silva 684*	31º31'04.8"	53º30'43.1"	375	18
BULB6	Pinheiro Machado	*L.N.Silva 669*	31º31'18.8"	53º14'10.3"	346	15
BULB7	Piratini	*L.N.Silva 660*	31º27'52.2"	53º10'48.4"	403	9
BULB8	Piratini	*L.N.Silva 670*	31º27'00.1"	53º07'25.9"	278	21
BULB9	Pedras Altas	*L.N.Silva 692*	30º56'41.5"	52º56'03.4"	379	18
BULB10	Pinheiro Machado	*D.B.Lucas S.N.*	31º26'06.7"	53º21'09.8"	409	4
BULB11	Santana da Boa Vista	*L.N.Silva 694*	30º56'41.5"	52º56'03.4"	186	14
BULB12	Encruzilhada do Sul	*L.N.Silva 690*	30º47'50.6"	52º37"39.6"	176	19
BULB13	Encruzilhada do Sul	*L.N.Silva 691*	30º45'54.6"	52º39'27.9"	335	18

A 2.5 µL aliquot of the diluted pre-selective product was used for selective amplification, using 0.54 µL of each selective primer, *Mse*I (5 µM) and *Eco*RI (1 µM); and 3.5 µL GoTaq® Green Master Mix (Promega), in a final volume of 10 µL. The selective PCR followed the conditions: initial cycle of 2 min at 94 ºC; 30 s at 65 ºC; and 2 min at 72 ºC; 8 cycles of 1 s at 94 ºC, 30 s at 64 ºC, and 2 min at 72 ºC, decreasing 1 ºC every cycle; 23 cycles of 1 s at 94 ºC, 30 s at 56 ºC, and 2 min at 72 ºC, and finally, 30 min at 60 ºC. All amplifications were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).

Three primers combinations were used for selective amplifications (Table 2). The *Eco*RI primers were labeled with fluorophores and were subjected to capillary electrophoresis in a 3500 xL automated system (Applied Biosystems). The fragment sizes generated were determined by comparing with 600 LIZ® Size Standard (Applied Biosystems) for standardizing fragments between 100 and 500 bp. The electrophoresis results were combined in a binary matrix using GeneMapper® v.4.1 software (Applied Biosystems).

Data analysis

The AFLP fragments were scored as present (1) or absent (0) in a binary matrix. The number and percentage of polymorphic loci (P) and Nei's gene diversity (*H*) (Nei 1973) were computed using both POPGENE v.1.32 (Yeh *et al.* 2000) and AFLP-SURV 1.0 (Vekemans

2002). A Principal Coordinates Analysis (PCoA) was performed based on the Jaccard similarity matrix to visualize the genetic relationships among all individuals analyzed using GENALEX 6.5 (Peakall & Smouse 2006). To verify the partitioning of genetic variation among groups and populations, an analysis of molecular variance (AMOVA) was performed under 10,000 permutations using ARLEQUIN.

Table 2. AFLP primers, fluorophores and number of produced fragments for the *Chascolytrum bulbosum* samples included in this study.

*Eco*RI primer	*Mse*I primer	Fluorophore	Fragments
AGC	CTC	Fam (blue)	67
ACT	CTC	Hex (green)	235
ACT	CAG	Ned (yellow)	349
		Total	651
		Polymorphic (%)	639 (98.2%)

The pairwise F_{ST} distances between the accessions were calculated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010) and a test of the significance of the genetic and geographical distance correlation (Mantel's test) was calculated using GENALEX, performed with 10.000 permutation rounds. The pairwise F_{ST} distance matrix was used with MEGA6 (Tamura *et al*. 2013) to produce both Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Neighbor-Joining (NJ) dendrograms. To verify the possible clustering of the accessions, a Bayesian analysis was performed in STRUCTURE 2.3.4. (Pritchard *et al*. 2000), using the Evano *et al.* (2005) method, with 100,000 Markov Chain Monte Carlo (MCMC) after a 10,000 burn-in repetitions period. The most likely number of clusters (K) was estimated under the admixture model and correlated allele frequencies, with no prior information on population origin. *K* values ranging from 1 to 16 were tested, with 20 independent interactions. The determination of probable *K* clusters was inferred using Structure Harvester (Earl & vonHoldt 2012). The population genetic differentiation G_{ST} was computed using AFLP-SURV 1.0 and gene flow among populations, within and among groups were estimated indirectly from the equation $Nm = 0.5(1-G_{ST})/G_{ST}$ (McDermott & McDonald 1993).

RESULTS

Genetic diversity and population structure

AFLP analysis of 179 individuals using three pairs of primers produced a total of 651 fragments of which 639 (98.20%) were polymorphic (Table 2). The number of polymorphic fragments ranged from 129 (19.82%) in BULB1 (population code according to Table 1) to 333 (51.15%) in BULB6 with an average of 217.85 (33.46%) polymorphic fragments per population (Table 3). Overall, genetic diversity (*H*) reached 0.149486 at the species level with an average of 0.104406 at the population level. Among the 13 populations analyzed, population BULB7 had the highest genetic diversity (*H* = 0.179724) while population BULB5 had the lowest genetic diversity (*H* = 0.065249). The genetic diversity values found in all populations are shown in Table 3.

Genetic differentiation (F_{ST}) and geographic distance between all pairs of populations are listed in Table 4. The result of Mantel's test revealed that no correlation was found between genetic and geographic distance (r = 0.0564; *P* = 0.05). Indeed, it is remarkable as geographically close populations have highest genetic differentiation than geographically distant populations. For instance, populations BULB4 and BULB5 (5.973 km apart from each other) exhibit a differentiation index of 0.33891, while population BULB3 and BULB4 (57.494 km apart from each other) exhibit a differentiation index of 0.10908. After all, the lowest value of genetic differentiation was found between populations BULB3 and BULB4 (see above) and the highest value of genetic differentiation was found between populations BULB5 and BULB11 (F_{ST} = 0.56294; 128.813 km apart from each other). In addition, population BULB11 exhibits the highest values of genetic differentiation between any populations regardless the geographic distance (F_{ST} = from 0.32861 to 0.56294).

The PCoA analysis separated almost all the individuals sampled into two major groups (Group A and Group B; Figure 2), although the first three axes explained, respectively, only 16.74%, 11.93% and 7.04% of the total variation found. Furthermore, in agreement with the PCoA the Bayesian inference of clustering performed by STRUCTURE revealed the most likely *K* = 2 (Figure 3), with remarkable genetic admixture between populations. Indeed, when a hierarchical AMOVA (Table 5) was carried out taking into account those two genetically groups, only 13.20% of the variation was partitioned among the groups (F_{CT} = 0.13199; *P* < 0.01) while 25.50% and 61.30% of the variation was found among populations within groups and within populations, respectively. Considering all populations at the same

hierarchical level, the genetic differentiation among populations increased to 34.05% (F_{ST} = 0.34054; $P < 0.01$), but the variation within population remained close to the hierarchical analysis (65.95%). Both UPGMA and NJ dendrograms recovered the same clustering between populations with few differences in topology of the groups (Figure 4). Besides, either BULB1 or BULB11 appeared as the most divergent populations in both analyses.

Table 3. Diversity indexes and genetic structure of 13 populations of *Chascolytrum bulbosum* based on AFLP markers using POPGENE. *P*, polymorphic fragments; *H*, Nei's gene diversity; N, population size. Population codes according to Table 1.

Population	**Group**	***P* (%)**	***H***	**N**
BULB1	A	129 (19.82)	0.074007 (±0.040973)	8
BULB2	A	216 (33.18)	0.107004 (±0.055145)	14
BULB3	A	206 (31.64)	0.100079 (±0.052360)	12
BULB4	A	214 (32.87)	0.115975 (±0.062729)	9
BULB5	A	149 (22.89)	0.065249 (±0.033227)	18
BULB6	A	333 (51.15)	0.111331 (±0.057029)	15
BULB7	A	319 (49.00)	0.179724 (±0.096884)	9
BULB8	A	178 (27.34)	0.073689 (±0.037114)	21
BULB9	B	172 (26.42)	0.086062 (±0.043646)	18
BULB10	B	134 (20.58)	0.109575 (±0.072163)	4
BULB11	B	171 (26.27)	0.074020 (±0.038323)	14
BULB12	B	283 (43.47)	0.130901 (±0.065869)	19
BULB13	B	328 (50.38)	0.129665 (±0.065472)	18
	Average	217.85 (33.46)	0.104406	-
	Species level	639 (98.20)	0.149486 (±0.071411)	179

Estimation of gene flow (*Nm*) from G_{ST} was carried out considering the migration rates between all populations, between populations within the same group and between the two groups (Table 6). The *Nm* between all populations was 1.689 individual per generation, while *Nm* between populations within Group A was 2.059, between populations within Group B was 1.932 and between groups were 3.490 individuals per generation, suggesting high gene flow in *Chascolytrum bulbosum*.

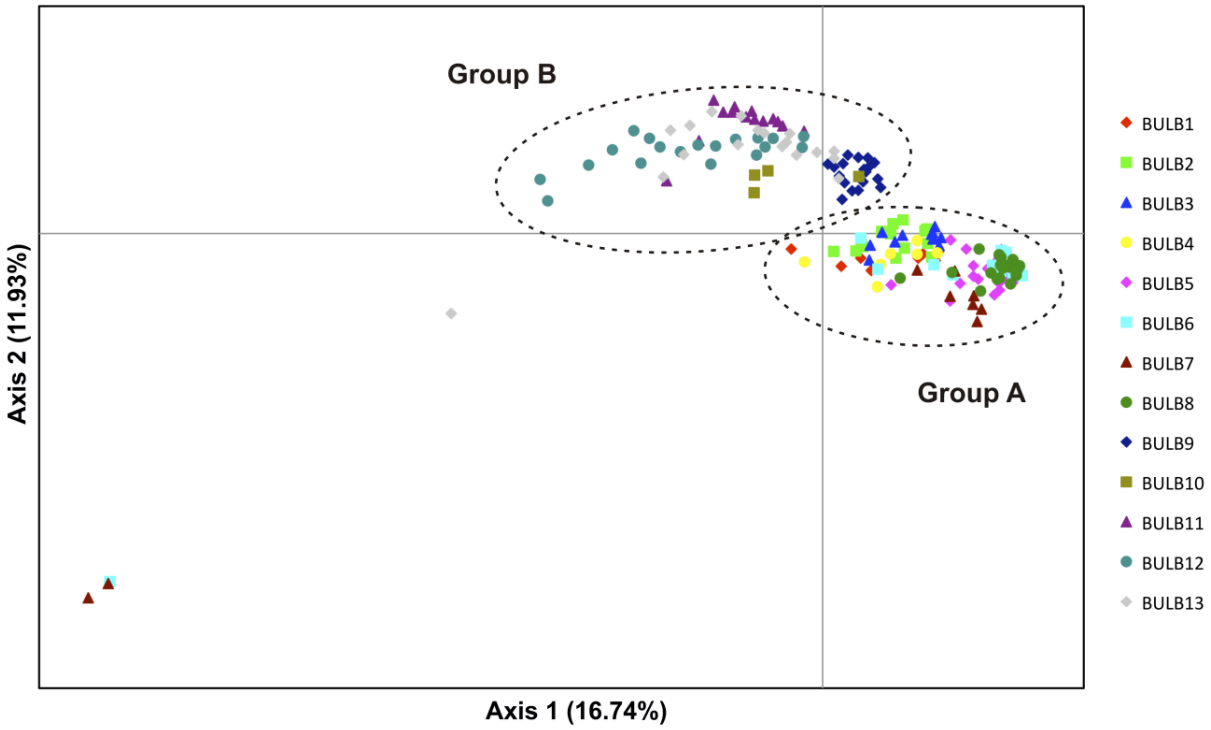

Figure 2. Principal Coordinates Analysis (PCoA) of *Chascolytrum bulbosum* based on AFLP. Population codes according to Table 1.

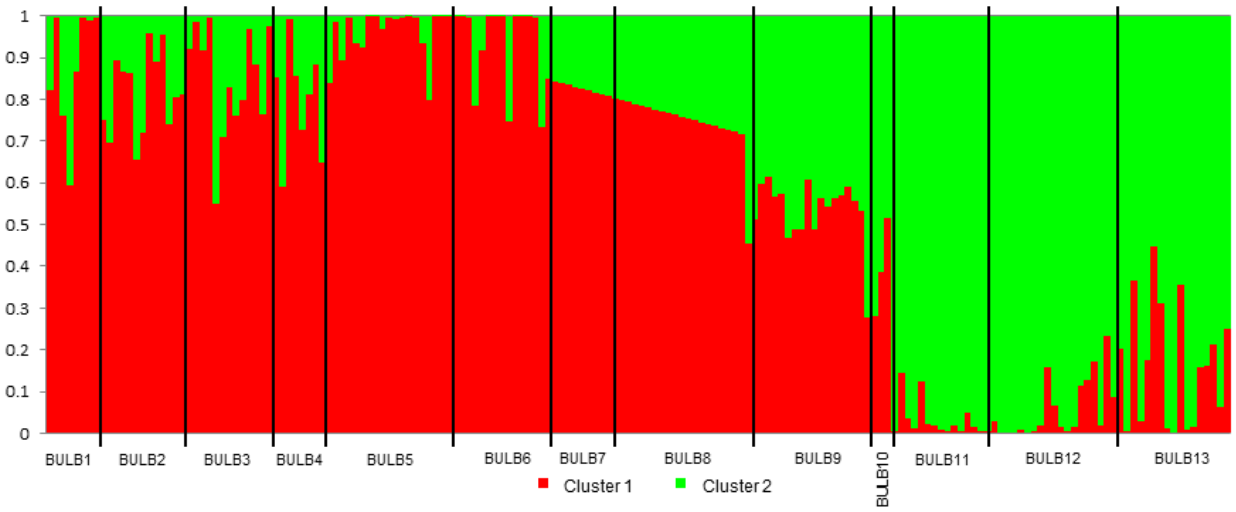

Figure 3. Bayesian analysis of clustering between populations of *C. bulbosum* obtained in STRUCTURE based on AFLP. Different colors representing different clusters. Population codes according to Table 1.

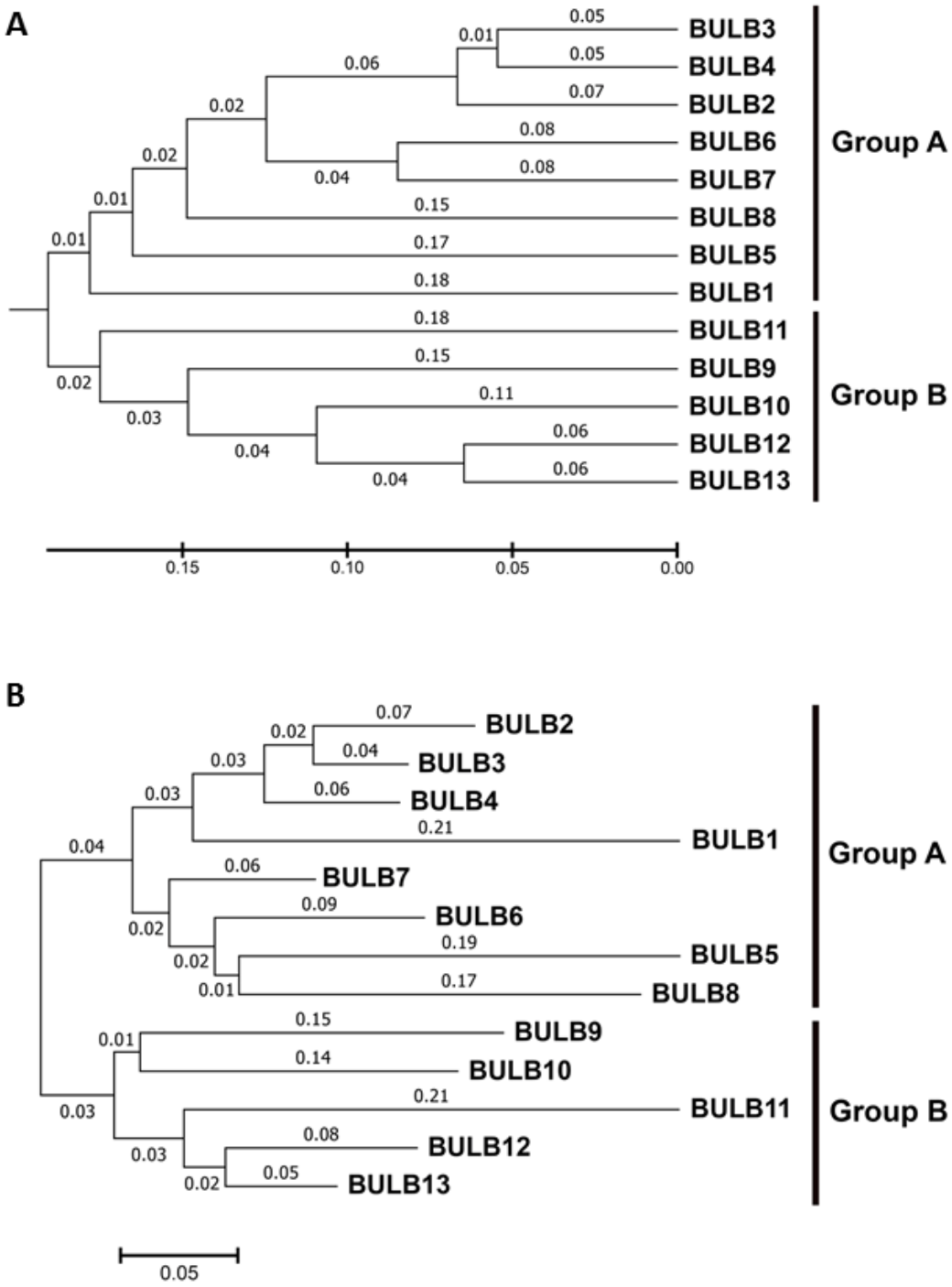

Figure 4. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram (A) and Neighbor-Joining (NJ) dendrogram (B) between 13 populations of *C. bulbosum* based on AFLP markers. Population codes according to Table 1.

Table 4. Pairwise geographical distance (in km) between populations (values above diagonal) and F_{ST} pairwise (values below diagonal) between 13 populations of *C. bulbosum* based on analysis of 651 AFLP fragments.

	BULB1	BULB2	BULB3	BULB4	BULB5	BULB6	BULB7	BULB8	BULB9	BULB10	BULB11	BULB12	BULB13
BULB1	-	18.427	42.829	98.697	101.028	115.084	113.580	116.053	123.699	100.843	102.549	128.813	125.621
BULB2	0.31868	-	25.464	80.298	82.601	97.135	96.008	98.757	105.559	82.782	91.556	120.545	117.833
BULB3	0.29485	0.10914	-	57.494	60.726	79.289	79.952	83.709	81.176	64.732	92.207	124.417	122.535
BULB4	0.30235	0.15719	0.10908	-	5.973	32.134	37.949	43.449	27.920	23.118	88.331	120.828	121.086
BULB5	0.49178	0.37159	0.35626	0.33891	-	26.164	32.047	37.561	29.397	17.760	84.226	116.340	116.755
BULB6	0.33926	0.28889	0.26028	0.25362	0.29556	-	8.268	13.233	45.033	14.576	70.292	99.247	100.513
BULB7	0.28196	0.28143	0.22770	0.18235	0.25923	0.16961	-	5.535	53.139	16.632	62.376	91.001	92.304
BULB8	0.46511	0.31389	0.30040	0.32391	0.35915	0.26281	0.28530	-	58.264	21.694	59.096	86.714	88.195
BULB9	0.48613	0.37388	0.37577	0.38083	0.42841	0.35329	0.32112	0.39907	-	44.520	112.035	143.019	143.855
BULB10	0.50292	0.35610	0.36281	0.30730	0.48113	0.31553	0.21543	0.44731	0.29053	-	67.541	99.042	99.645
BULB11	0.55968	0.41707	0.30479	0.43725	0.56294	0.45976	0.44322	0.52881	0.41059	0.40237	-	33.635	33.096
BULB12	0.36379	0.33211	0.24255	0.31794	0.42619	0.36339	0.32503	0.43412	0.34644	0.23967	0.32861	-	4.630
BULB13	0.35383	0.26566	0.42961	0.25077	0.37871	0.29539	0.27913	0.38069	0.25314	0.19772	0.25866	0.12925	-

Table 5. Analysis of Molecular Variance (AMOVA) of 13 populations of *Chascolytrum bulbosum* based on AFLP markers. d.f., degree of freedom. Number of permutations: 10000. All results are supported with $P < 0.01$.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index
Among populations	12	3182.353	17.04305 Va	34.05	F_{ST} = 0.34054
Within population	166	5478.731	33.00440 Vb	65.95	
Total	178	8661.084	50.04745		
Among groups	1	793.643	7.10683 Va	13.20	F_{CT} = 0.13199
Among populations	11	2388.710	13.73054 Vb	25.50	F_{ST} = 0.38701
Within populations	166	5478.731	33.00440 Vc	61.30	
Total	178	8661.084	53.84178		

Table 6. Genetic diversity and population structure calculated in AFLP-SURV based on AFLP markers. *H*, Nei's gene diversity; G_{ST}, coefficient of genetic differentiation among populations; *Nm*, gene flow.

	H (SD)	G_{ST}	*Nm*
Among all populations	0.1158 (±0.0069)	0.2275	1.689
Among populations in group A	0.1170 (±0.0090)	0.1954	2.059
Among populations in group B	0.1131 (±0.0109)	0.2056	1.932
Among groups	0.1278 (±0.0012)	0.1253	3.490

DISCUSSION

Genetic diversity and population structure in Chascolytrum bulbosum

A large number of polymorphic and homologous AFLP fragments was detected in *C. bulbosum*, providing a highly informative measure of genetic diversity within and among populations. No significant numbers of private fragments were found in the analysis, suggesting that the variation and divergence among populations must be attributed to difference in fragments frequency rather than allele fixation (Luan *et al*. 2006). In addition, an analysis of correlation indicates that genetic diversity is not significantly influenced by the population size (r = -0.2591), since small populations can exhibits high values of gene diversity (*H*) (see Table 3).

According to the characteristics of *C. bulbosum* populations (i.e. small size, fragmentation and overgrazing), low levels of genetic diversity and high population structure were expected, especially between distant populations. However, the genetic diversity values found in this study were similar or slightly higher compared with several studies based on AFLP markers focusing endangered species (Gauduel *et al.* 2000; Rottenberg & Parker 2003; Barnaud & Houliston 2010; Cires *et al.* 2013). Furthermore, a recent genetic survey involving two rare and endangered species of *Chascolytrum* Desv. exhibits similar values to *C. bulbosum* and proposes that genetic diversity within the genus can be better explained by the pattern of gene flow and evolutionary history rather than rarity, geographic distance or population size (da Silva *et al.* 2016, in prep.).

The highest amount of genetic variation within populations in both hierarchical analyses (65.95% and 61.30%, Table 5) suggests outcrossing reproduction (Hamrick & Godt 1996). Despite all analyses indicate the separation of the individuals sampled in two major groups (i.e PCoA, Figure 2; K = 2, Figure 3; and UPGMA and NJ dendrograms, Figure 4), AMOVA results revealed that only 13.20% (F_{CT} = 0.13199) of genetic variation relies between them. However, when all sampling was considered in the same hierarchical group, differentiation between populations has increased (F_{ST} = 0.34054) indicating that populations within a same group can exhibit higher genetic differentiation than populations from different groups (see Table 4 for pairwise F_{ST}) and no correlation between genetic and geographic distance was revealed by Mantel's test (r = 0.0564; P = 0.05). Furthermore, differentiation measures across all populations was higher (G_{ST} = 0.2275) than differentiation among groups (G_{ST} = 0.1253), indicating a high gene flow between the two groups (Nm = 3.490 individuals

per generation; Table 6). Indeed, gene flow value among groups was remarkably higher than gene flow within groups, suggesting high rates of migration between all the geographic localities.

Finally, the maintenance of high genetic diversity in endangered species can be attributed to several factors, such as recent fragmentation and isolation, large population size or recurrent gene flow (Zawko *et al.* 2001). Although population genetic theory predicts that larger populations tend to maintain higher genetic diversity (Ellstrand & Elam 1993), remarkable genetic diversity was found in small populations of *C. bulbosum* (i.e. BULB4, BULB7, BULB10; Table 3). The moderate values of genetic diversity plus high values of gene flow (*Nm*) found in our study suggest that probably no bottleneck or genetic drift has been occurred recently, since genetic diversity has been maintained even in small populations (Rottenberg & Parker 2003). However, *Nm* values tend to represent historical genetic exchange and are not indicative of current migration (Luan *et al.* 2006). Thus, the high *Nm* values found in *C. bulbosum* may indicate a previous intermingling or recent extinction of intermediary populations (Luan *et al.* 2006).

Implications for conservation

The utility of genetic diversity measures inferred by AFLP in biological conservation is widely documented in literature. The maintenance of genetic diversity is one of the most important elements in population genetics and may be used as a guide in conservation plans (Gaudeul *et al.* 2000; Ellis *et all.* 2006; Luan *et al.* 2006; Cires *et al.* 2013; Rodrigues *et al.* 2013). Therefore, the criteria for the selection of priority populations must include both the uniqueness and the diversity level of their allelic composition (Petit *et al*. 1998). After all, most endangered taxa are not driven to extinction before genetic factors affect them adversely (Spielman *et al.* 2004). In the case of *Chascolytrum bulbosum*, a set of factors must be taken into consideration: (1) it is evident that the inclusion under threatened categories in actual red lists was based mainly on lack of field collections; (2) as predicted previously (Martinelli & Moraes 2013), overgrazing is a feature which may reduce the population size and eliminate the species from the habitat; and (3) the values of genetic diversity and low population structure indicated a broad gene flow even between distant populations. In conclusion, since no severe loss of genetic diversity was detected in the present study, most efforts must be addressed to ensure the populations viability through *in situ* conservation and grazing control. Taking into account both genetic features (i.e. number of polymorphic fragments, genetic

diversity and population clustering) and geographic position, we encourage the priority conservation of the populations BULB2, BULB6, BULB7, BULB9, BULB12 and BULB13 in order to guarantee the genetic conservation of *C. bulbosum* in Southern Brazil.

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CAPÍTULO IV

CONSIDERAÇÕES FINAIS

Campo com afloramentos graníticos em Pedras Altas, RS (Foto: Liliana Essi)

Ultimatum

"Eu da raça dos descobridores
Desprezo o que seja menos
Que descobrir um novo mundo

Proclamo isso bem alto
Braços erguidos
Fitando o Atlântico
E saudando abstratamente o infinito."

Álvaro de Campos, 1917

4 CONSIDERAÇÕES FINAIS

A presente dissertação apresentou contribuições significativas para o conhecimento da variabilidade genética presentes nas populações naturais de *Chascolytrum bulbosum, C. parodianum* e *C. scabrum* no Rio Grande do Sul. Os esforços de coleta realizados durante dois períodos de florescimento (setembro a dezembro de 2014 e 2015) possibilitaram o registro de novas populações das três espécies, ampliando o conhecimento sobre a distribuição geográfica destas. Um total de 260 indivíduos, distribuídos em 21 populações das três espécies, foram coletados em sílica-gel para análise molecular.

Ao contrário do esperado, altos índices de diversidade genética foram detectados entre as populações das espécies estudadas, independentemente do tamanho da população. No caso das espécies *C. scabrum* e *C. parodianum*, três marcadores moleculares foram implementados para as análises de diversidade genética e estrutura populacional: AFLP, cpDNA (*rpoB-trnC*) e ITS. Os resultados obtidos são discutidos no capítulo II e sugerem que os marcadores de sequências de DNA plastidial e AFLP são os mais indicados para estudos populacionais e de conservação, como já referido na literatura (Garcia *et al.* 2004; Ribeiro *et al.* 2011; Cires *et al.* 2013). Por outro lado, os espaçadores ITS mostraram-se altamente variáveis e homoplásicos e a possibilidade de paralogia não pode ser descartada devido à presença de sequências muito divergentes nos alinhamentos preliminares, que não foram consideradas nas análises finais. Mesmo assim, a análise comparativa de fragmentos dos genomas nuclear e plastidial possibilitou inferir sobre o modo preferencial do fluxo gênico, permitindo compreender melhor como as populações estão estruturadas atualmente a partir de sua história evolutiva e demográfica. As redes de haplótipos obtidas a partir do marcador plastidial indicam populações com alta diferenciação geográfica para ambas as espécies. Com exceção de um haplótipo compartilhado entre duas populações de *C. parodianum*, os demais haplótipos ocorrem exclusivamente em uma única população. Neste sentido, a alta estruturação genética encontrada nas populações de *C. scabrum* e *C. parodianum* pode ser um fator preocupante, uma vez que a perda de uma única população pode reduzir drasticamente a variabilidade genética no nível específico (Ellis *et al.* 2006). Não obstante, nenhuma das populações de *C. scabrum* e *C. parodianum* incluídas nas análises deste estudo encontram-se dentro de unidades de conservação ou áreas de manejo conservativo e uso sustentável (Loyola *et al.*

2014). Desta forma, a elaboração de estratégias para a conservação do patrimônio genético e proteção das populações existentes de *C. scabrum* e *C. parodianum* torna-se urgente.

Em relação a *C. bulbosum*, marcadores AFLP foram utilizados para estimar os índices de diversidade e estrutura populacional. Até recentemente, a espécie era considerada rara no Rio Grande do Sul, fato que deve ter justificado sua inclusão em listas de espécies ameaçadas de extinção (Martinelli & Moraes 2013; Rio Grande do Sul 2014). Contudo, expedições de coletas intensivas em toda a extensão da região fisiográfica da Serra do Sudeste permitiram o registro de 13 populações naturais da espécie até o momento. Neste sentido, *C. bulbosum* parece ser uma espécie muito mais comum do que se imaginava, sendo bem distribuída dentro dos limites dos campos arbustivos com afloramentos graníticos no Rio Grande do Sul. Estima-se que mais populações devem ocorrer em locais de difícil acesso e campos naturais, uma vez que o pastejo parece ser a principal ameaça à perpetuação da espécie (Marinelli & Moraes 2013). Os resultados obtidos e discutidos no capítulo III revelaram altos níveis de diversidade genética, moderada estruturação populacional (G_{ST} = 0.2275; F_{ST} = 0.34054) e altas taxas de fluxo gênico, sugerindo que a espécie não tenha passado por efeitos severos de deriva genética e perda de diversidade genética (*bottleneck*) recentemente. Contudo, a fragmentação dos habitats frente à expansão de áreas para cultivos de espécies florestais exóticas pode representar uma barreira ao fluxo gênico futuramente. Sendo assim, ações que protejam e monitorem as populações naturais de *C. bulbosum* são necessárias para a conservação da espécie.

Os resultados mais importantes obtidos neste estudo indicam que mesmo espécies raras e ameaçadas podem exibir altos níveis de diversidade genética. A manutenção de índices em populações enfrentando declínio demográfico pode ser associada a diversos fatores, mas principalmente aos padrões de fluxo gênico (Ellstrand 1992; Ellstrand & Elam 1993), o que se aplica bem ao caso das espécies de *Chascolytrum* foco deste estudo. Além disso, análises filogenéticas anteriores envolvendo o gênero sugerem uma história evolutiva bastante complexa para o grupo, envolvendo eventos de hibridação e radiação adaptativa (Essi *et al.* 2008; Saarela *et al.* 2010), o que pode estar ocorrendo também em níveis infraespecíficos e populacionais. De fato, o uso combinado de marcadores de sequências de DNA plastidial e nuclear confirmam uma complexa dinâmica populacional em *C. scabrum* e *C. parodianum*, sugerindo que eventos de dispersão e mistura de populações historicamente separadas tenham moldado a distribuição da diversidade genética encontrada atualmente nas populações estudadas destas espécies.

As perspectivas futuras para este trabalho incluem coletas mais intensivas para o registro de novas populações, principalmente no Uruguai, onde a distribuição de *C. parodianum* e *C. bulbosum* ainda é pouco conhecida. A inclusão de novas populações deve trazer novas informações importantes acerca da história evolutiva destas espécies, permitindo um melhor entendimento de sua estruturação e variabilidade genética, dentro de um contexto geográfico (filogeografia; Avise 2000). Além disso, o uso de marcadores de sequências de DNA plastidial nas populações de *C. bulbosum* deve ser estimulado, pois deve trazer novas evidências sobre os padrões filogeográficos da espécie, complementando os dados já obtidos no capítulo III através do uso dos marcadores AFLP. Por fim, sugere-se um monitoramento contínuo das populações estudadas, incluindo novas estimativas de diversidade genética para averiguar os possíveis efeitos da fragmentação sobre estas ao longo do tempo.

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