UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

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COLLETIA PARADOXA (SPRENG.) ESCALANTE (RHAMNACEAE) IDENTIFICAÇÃO DE FLAVONOIDES, DIVERSIDADE GENÉTICA E CONSERVAÇÃO

Santa Maria, RS
2019

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Doutor em Ciências Farmacêuticas**.

Orientadora: Dr.ª Marli Matiko Anraku de Campos

Coorientadora: Dr.ª Liliana Essi

Santa Maria, RS
2019

Bastos-Záchia, Nelci Rolim
Colletia paradoxa (spreng.) Escalante (Rhamnaceae) identificação de flavonoides, diversidade genética e conservação / Nelci Rolim Bastos-Záchia.- 2019.
105 p.; 30 cm

Orientadora: Marli Matiko Anraku de Campos
Coorientadora: Liliana Essi
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós Graduação em Ciências Farmacêuticas, RS, 2019

1. Antioxidante 2. Marcadores moleculares 3. Flavonoides 4. Taxonomia 5. Quina I. Matiko Anraku de Campos, Marli II. Essi, Liliana III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 09 de Janeiro de 2019:

Marli Matiko Anraku de Campos, Dr.ª (UFSM)
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Raquel Lüdtke, Dr.ª (UFPEL) - PARECER

DEDICATÓRIA

À Deus.

À minha família,
por ser o alicerce da minha vida.

De tudo ficaram três coisas:
a certeza de que estava sempre começando,
a certeza de que preciso continuar
e a certeza de que seria interrompido antes de terminar.
Fazer da interrupção um caminho novo,
fazer da queda um passo de dança,
do medo, uma escada,
do sonho uma ponte,
da procura, um encontro.

Fernando Pessoa

AGRADECIMENTOS

Agradeço a Deus por estar vivendo esse momento! Entre tantos desafios que Ele já me proporcionou, e me guiou até o final, este foi especialmente desafiador!

E, talvez por isso, na mesma proporção, foi enriquecedor!

Agradeço a Deus por todo o aprendizado, por todas as pessoas que conheci e tantos amigos que descobri... Por isso, quero manifestar minha gratidão a essas pessoas que se fizeram parceiras nesta conquista, em especial:

Às minhas orientadoras,

À Profa. Margareth Linde Athayde (*in memorian*) que apesar do pouco tempo que estivemos juntas ficou marcada em minha vida, pelo carinho, acolhimento, nunca duvidando que eu pudesse cumprir esse desafio. Conseguimos!

Profa. Dr.ª Marli Matiko Anraku de Campos, pela amizade, por não me deixar desistir quando o desânimo tomou conta e por ter aceito assumir como minha orientadora, mesmo já tendo sua cota de orientados completa.

Profa.Dr.ª Liliana Essi pela orientação de toda a parte relativa à genética de populações, taxonomia, técnicas de laboratório em sistemática filogenética, conhecimento bibliográfico, com sua grande experiência, dinamismo e sabedoria. Obrigada pela amizade, confiança e exemplo de profissional.

Ao meu esposo, Renato Aquino Záchia, responsável pelo meu primeiro contato com a Profa. Margareth e por iniciar todo esse processo de aprendizado. Tua presença em todos os momentos da minha vida foram fundamentais, obrigada pelo estímulo, apoio e confiança. Te amo.

Ao meu filho Gabriel, por todas as músicas suaves que selecionava para me ajudar a relaxar e estudar e que fazia muito efeito! E ainda por toda a ajuda e participação nas atividades do lar.Te amo filho.

A minha filha Júlia, por todas as horas de nosso convívio que cedeu para o meu trabalho, além de toda compreensão e ajuda doméstica. Te amo filhinha.

Ao meu pai Wilson José de Bastos e a minha mãe Guiomar Rolim Bastos, por serem o meu exemplo de fé, coragem e perseverança. Obrigada por estarem sempre ao meu lado. Amo vocês.

Ao meu irmão Nilton e a minha irmã Neusa, pelo companheirismo, confiança e apoio, sempre me ajudando em todo o tipo de desafio.

Aos meus sobrinhos e sobrinhas, Leonardo, Lucas, Marília, Vitória, Victor, Laura e Leozinho, por terem sempre uma palavra de estímulo e de carinho e acreditarem em mim.

Aos professores da UFSM, em especial Prof. Antônio Carlos Ferreira da Silva, João Marcelo dos Santos Oliveira, Fernando Teixeira Nicoloso, Luiz Augusto Salles das Neves, Élgion Loreto, Felipe Ricachenevsky e às professoras Solange Tedesco, Daniela Guimarães Simão, Luciane Tabaldi, Lenira Sepel, pelo apoio nos laboratórios sempre que precisei.

À coordenação e ao Colegiado do PPG – Ciências Farmacêuticas pelo apoio durante a execução do trabalho.

Aos professores e técnicos diretamente ligados ao PPG – Ciências Farmacêuticas – CCS, pelos ensinamentos, apoio logístico, compreensão e profissionalismo.

Aos laboratoristas, técnicos e aos colegas de bancada de todos os laboratórios onde desenvolvemos as análises, em especial Benardete Panno e Tânia Viana do herbário, Carmine, Maria e Raquel dos laboratórios de botânica, Mariana, Roberta, Fallon, Ritiel e Aline do CCS.

À Débora, por ter sido fundamental em todo esse processo, ajudando-me em cada momento do meu dia, obrigada amiga.

À minha grande família, tios, tias, primos e primas que mesmo à distância confiam e torcem sempre por mim.

Ao meu filho do coração, Jean, por estar sempre pronto pra entrar em cena, toda vez que eu precisasse, e à Sílvia por todo o estímulo.

Aos meus amigos queridos Fallon e Francisco, por caminharem juntinho comigo, sempre que precisei. Novos filhos do coração que ganhei!

Aos meus compadres Maria Salete, Telmo e Bruna pelo incentivo e confiança no meu trabalho.

À Thamyres que com pouco tempo na família, já conquistou seu lugar, obrigada pelo estímulo.

Ao colega Igor Wassiljew Moia pelo apoio e estímulo nesta caminhada.

Às minhas filhas do coração, Larissa e Stefani, por todas as diferentes maneiras que vocês me ajudam estando por perto.

À vó Solange, Rosélia e Geovana, Suane e Isabelle, Ellen e Júlia, por todas as vezes que assumiram a minha Júlia, para que eu pudesse ir a campo, foi importante poder sair tranquila.

À Márcia, pelas tantas conversas na tentativa de me fazer encontrar a segurança, perdida em algum lugar!

À Lúcia Sevegnani e à Nice, por acreditarem em mim, sei o quanto estariam orgulhosas se estivessem aqui!

Enfim, seria impossível citar a todos os que de alguma maneira me ajudaram a conseguir cumprir esse desafio, pois somos feitos de relações; e foram muitas pessoas que fizeram

comigo essa caminhada. Então, à todos aqueles, que por descuido da minha memória e não por falta de merecimento, não foram citados, meu mais profundo agradecimento.

Indivíduos de *Colletia paradoxa* no ambiente natural (Pinheiro Machado, RS). Foto de Liliana Essi.

RESUMO

COLLETIA PARADOXA (SPRENG.) ESCALANTE (RHAMNACEAE): IDENTIFICAÇÃO DE FLAVONOIDES, DIVERSIDADE GENÉTICA E CONSERVAÇÃO

AUTOR: Nelci Rolim Bastos-Záchia
ORIENTADORA: Marli Matiko Anraku de Campos
COORIENTADORA: Liliana Essi

Colletia Comm. ex Juss. é um gênero da família Rhamnaceae, que conta cinco espécies exclusivas da América do Sul, com duas delas chegando até o Brasil. A espécie *C. paradoxa* apresenta um grande polimorfismo em suas formas, podendo ser identificados três morfotipos, *C. paradoxa* morfotipo "paradoxa", *C. paradoxa* morfotipo "exserta", *C. paradoxa* morfotipo "intermediário", resultando em questionamentos sobre os limites desta espécie. Sabe-se que *C. paradoxa* ocorre em pequenas populações fragmentadas e que o alto grau de degradação e fragmentação dos ambientes naturais pode ocasionar, através da redução de habitats específicos, o isolamento das populações, diminuindo o fluxo gênico entre elas e consequentemente perdendo a variabilidade genética, que pode assim, iniciar um processo de extinção da espécie. É uma espécie medicinal, sendo as raízes e cascas dos ramos utilizadas para baixar a febre e está na lista das ameaçadas de extinção no RS e no Brasil. O objetivo deste estudo é identificar os flavonoides existentes no extrato bruto das folhas e ramos de *C. paradoxa* e seus diferentes morfotipos, investigar a ação antimicobacteriana e a atividade antioxidante destes extratos, contribuir para um melhor conhecimento da espécie *C. paradoxa* e sua delimitação taxonômica e estimar a diversidade genética de populações de *C. paradoxa* por meio de marcadores moleculares, considerando sua condição de espécie ameaçada. A capacidade de eliminação dos radicais livres foi determinada pelo método DPPH, a atividade antimicobacteriana pelo método de microdiluição em caldo e o conteúdo fenólico quantificado pelo método espectrofotométrico e por cromatografia líquida de alta eficiência (HPLC). A diversidade genética foi estimada através de marcadores ISSR e a delimitação da espécie foi realizada através de análise morfológica associada a marcadores ISSR. Concluiu-se que *C. paradoxa* possui potencial antioxidante natural, além de apresentar atividade antimicobacteriana classificada como moderada a significativa. A similaridade nos resultados fitoquímicos dos indivíduos dos três morfotipos, a análise morfológica e de ISSR corroboram com a ideia de que sejam uma única espécie, *C. paradoxa*, entretanto incentiva-se a continuidade das pesquisas no intuito de aprofundar o conhecimento sobre a biologia, potencialidades e preservação da espécie. Com relação à diversidade genética da espécie, conclui-se que a mesma apresenta diversidade moderada, o que somados aos riscos ambientais identificados nas populações naturais reforça que a espécie apresenta-se ameaçada de extinção no estado do Rio Grande do Sul.

Palavras chave: Antioxidantes. Antimicobacteriano. Marcadores moleculares. Caracterização Polifenois. Quina. Taxonomia.

ABSTRACT

COLLETIA PARADOXA (SPRENG.) ESCALANTE (RHAMNACEAE) IDENTIFICATION OF FLAVONOIDS, GENETIC DIVERSITY AND CONSERVATION

AUTHOR: Nelci Rolim Bastos-Záchia
ADVISOR: Marli Matiko Anraku de Campos
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Colletia Comm. ex Juss. is a genus of the family Rhamnaceae, which counts five unique species of South America, with two of them arriving until Brazil. *C. paradoxa* morphotype "paradoxa", *C. paradoxa* morphotype "exserta", *C. paradoxa* morphotype "intermediate", resulting in questions about the limits of this species. It is known that *C. paradoxa* occurs in small fragmented populations and that the high degree of degradation and fragmentation of the natural environments can cause, through the reduction of specific habitats, the isolation of the populations, reducing the gene flow between them and consequently losing the genetic variability, which can thus initiate a process of extinction of the species. It is a medicinal species having the roots and bark of the branches used to lower the fever and is on the list of endangered species in RS and Brazil. The objective of this study is to identify the flavonoids present in the crude extract of the leaves and branches of *C. paradoxa* and its different morphotypes, to investigate the antimicrobial action and the antioxidant activity of these extracts, contribute to a better knowledge of the species *C. paradoxa* and its taxonomic delimitation and to estimate the genetic diversity of populations of *C. paradoxa* of molecular markers, considering their condition as an endangered species. The capacity of elimination of free radicals was determined by the DPPH method, the antimycobacterial activity by the broth microdilution method and the phenolic content quantified by the spectrophotometric method and by high performance liquid chromatography (HPLC). Genetic diversity was estimated through ISSR markers and species delimitation was performed through morphological analysis associated with ISSR markers. It was concluded that *C. paradoxa* has a natural antioxidant potential, in addition to presenting an antimycobacterial activity classified as moderate to significant. The similarity in the phytochemical results of the individuals of the three morphotypes, the morphological analysis and the ISSR corroborate with the idea that they are a single species, *C. paradoxa*, however, it is encouraged the continuity of the researches in order to deepen the knowledge about the biology, potentialities and preservation of the species. With respect to the genetic diversity of the species, it is concluded that it presents moderate diversity, which added to the environmental risks identified in the natural populations reinforces that the species is threatened with extinction in the state of Rio Grande do Sul.

Key words: Antioxidants. Antimicobacterial. Molecular markers. Characterization Polyphenols. Quina. Taxonomy.

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LISTA DE ABREVIATURAS, SIGLAS, ACRÔNIMOS E SÍMBOLOS

Abs_{blank}	*Absorbance of the crude extracts without addition of* DPPH
$Abs_{control}$	(*Absorbance of DPPH solution in ethanol*)
Abs_{sample}	*Absorbance of the crude extracts*
AFLP	(*Amplified Fragment Length Polymorphism*): Polimorfismo de comprimento de fragmentos amplificados.
AMOVA	*Molecular Variance Analyses*
CD	(*Central Depression*): Depressão Central
CGEN	(*Management Council for Genetic Heritage*)Conselho de Gestão do Patrimônio Genético
CTAB	*Cetil Trimethylammonium bromide*
DAD	*Detector Diode Array*
DNA	(*desoxyribonucleic acid*): Ácido desoxiribonucleico
dNTP	(*deoxynucleotide*): Deoxinucleotídeos
DMSO	(*dimethyl sulfoxide*): Sulfóxido de dimetilo ou Dimetilsulfóxido
DPPH	2,2-diphenyl-1-picrylhydrazyl
F	Herbário do Field Museum - Chicago
FAS-II	*Enzyme Fatty Acid Synthase*
FLOR	Herbário da Universidade Federal de Santa Catarina - Florianópolis
GAE	*Equivalent of Gallic Acid*
GST	*Coefficient of gene differentiation*
HAS	Herbário Alarich Schultz – Fundação Zoobotânica do Rio Grande do Sul
H	*Nei's gene diversity*
HDCF	Herbário do Departamento de Ciências Florestais - UFSM
HPLC	*High Performance Líquid Chromatography*
HUCS	Herbário da Universidade de Caxias do Sul.
HUEFS	Acrônimo do Herbário da Universidade Estadual de Feira de Santana
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IC_{50}	*Concentration of the extracts necessary to eliminated 50% of free radicals of DPPH present in the medium*
ICN	Herbário Instituto de Ciências Naturais – Departamento de Botânica UFRGS
IUCN	International Union for Conservation of Nature
I	*Shannon's Information index*

ICMBIO	Instituto Chico Mendes
ISSR	*Inter-simple sequence repeats*: Entre sequências simples (microssatélites) repetidas
K	Herbário Kew - Londres
LD	Limite de Detecção
LQ	Limite de Quantificação
MEL	Herbarium de Melbourne - Austrália
MIC	*Minimum Inhibitory Concentration*
ng	*nanograms:* nanogramas
Na	*Observed number of alleles*: Número de alelos observados
Ne	*Effective number of alleles*: Número efetivo de alelos
NY	Acrônimo do Herbário New York Botanical Garden
PACA	Herbarium Porto Alegre Colégio Anchieta – UNISINOS, São Leopoldo
pb	*base pairs*: pares de bases
PCoA	*Principal Coordinates Analyses*: Análises das Coordenadas principais
PPB	*Percentage of Polymorphic Band*: Percentual de Bandas Polimórficas
PCR	*Polymerase Chain Reaction*: Reação em cadeia da polimerase
RAPD	*Random Amplified Polymorphic* DNA
RB	Sequência de número de coletas de Rolim Bastos
RFLP	*Restriction Fragment Length Polymorphism*
SISBIO	Sistema de Atendimento à Distância do ICMBIO
SMDB	*Herbarium of the Department of Biology of* UFSM
SPG	*Southern Plateau Grasslands*: Campos de Cima da Serra
SS	*Sul- Rio-Grandense Shield*: Escudo-Sul-Riograndense (Serra do Sudeste)
SSR	*Simple Sequence Repeats*: Sequências simples repetidas
TA	*Annealing Temperature*
TP	*Total Polyphenols*
UFSM	Federal University of Santa Maria
UPGMA	*Unweighted Pair Group Method with Arithmetic Mean Average*
W	Herbarium de Viena - Austria
λ-	Lambda
α -	Alfa
μ -	Micro
Δ –	Delta

APRESENTAÇÃO

Esta tese foi organizada de modo a divulgar seus resultados sob a forma de artigos científicos que serão apresentados a seguir. Os artigos estão formatados de acordo com as revistas onde serão publicados e estão exibidos nessa sequência:

MANUSCRITO 1 - Phytochemical Analyses and Evaluation of Antioxidant and Antimycobacterial Activity of *Colletia paradoxa* from Brazil.

Constitui-se num estudo fitoquímico da espécie *C. paradoxa* com o objetivo de investigar a composição fitoquímica dos extratos brutos de seus diferentes morfotipos e avaliar a atividade antioxidante e antimicobacteriana, das amostras das diferentes regiões fisiográficas.

MANUSCRITO 2 - Circumscription of the species *Colletia paradoxa* - *Colletia exserta* (Colletieae, Rhamnaceae): morphological and molecular evidence.

Aqui analisamos as variações morfológicas dos indivíduos nas diferentes populações e morfotipos, buscando compreender quais implicações isto tem para a taxonomia do gênero, através de uma abordagem morfológica e dados moleculares auxiliares.

MANUSCRITO 3 - Genetic Variability and Population Structure of *Colletia paradoxa*: a medicinal species threatened with extinction.

Conduz uma análise e discussão sobre a diversidade genética de populações naturais da espécie *C. paradoxa* no Rio Grande do Sul e sul de Santa Catarina.

Na sequência, serão comentados aspectos gerais dos resultados, as conclusões e fornecidas as referências bibliográficas relativas às seções introdução e considerações finais.

SUMÁRIO

1 INTRODUÇÃO

1.1 RHAMNACEAE

Rhamnaceae é uma família de angiospermas que pertence a ordem Rosales (APG IV, 2016), é formada por cerca de 52 gêneros e 900 espécies (STEVENS, 2001) organizadas em doze tribos. Com distribuição cosmopolita, tem cerca de 28 gêneros e 170 espécies ocorrendo na região neotropical (LIMA, 2006).

No Brasil, ocorrem 14 gêneros e 47 espécies distribuídas em sete tribos (LIMA, 2015). São encontradas em quase todos os ecossistemas brasileiros, habitando preferencialmente locais ensolarados, como bordas de matas e tendo como centro de maior diversidade a Mata Atlântica e a Amazônia (GIULIETTI et al, 2005, 2014). No Rio Grande do Sul a família é representada por nove gêneros e 13 espécies (LIMA, 2015).

As Rhamnaceae podem ser árvores, arbustos, subarbustos, ervas e lianas. Caracterizam-se pelas pétalas cuculadas, frequentemente unguiculadas, alternas às sépalas, estames opostos às pétalas e a presença de disco nectarífero forrando o receptáculo, concrescido com o ovário ou não. São polinizadas por insetos, principalmente por abelhas (melitofilia) e dispersadas por zoocoria, barocoria ou anemocoria (LIMA, 2013). Entre alguns indivíduos da família podemos observar características xeromórficas como folhas reduzidas ou ausentes, aglomeração de folhas, encurtamento dos eixos do ramo e a presença de espinhos (RICHARDSON et al, 2000) como no caso do gênero *Colletia*.

As Rhamnaceae têm pouco valor econômico, sendo que os seus representantes mais notáveis são o joazeiro-do-norte (*Ziziphus joazeiro* Mart.), a coronilha (*Scutia buxifolia* Reiss.) e a uva-do-japão (*Hovenia dulcis* Thunb.). Porém, fora do Brasil há um grande interesse em seu uso como ornamentais, em especial os gêneros *Ceanothus*, *Colletia*, *Pomaderris*, *Rhamnus* e *Phylica* (JUDD et al, 2009).

Algumas espécies são de interesse alimentício pois possuem frutos comestíveis, como é o caso do joazeiro ou laranjeira-do-vaqueiro (*Zizyphus joazeiro* Mart.), árvore típica da Caatinga nordestina que é inteiramente aproveitada pela população, para o tratamento de diversos males (problemas dermatológicos, do sistema respiratório, sistema digestório, cicatrizante e até como dentifrício) entre outros usos. A uva japonesa (*Hovenia dulcis* Thunb.), espécie asiática muito bem adaptada no Brasil, fornece pseudofrutos que são os pedúnculos florais entumecidos, carnosos e muito doces e suculentos (SOUZA, LORENZI, 2008).

Entre as espécies de uso medicinal, podemos citar *Rhamnus purshiana* DC, cáscara sagrada, usada como laxativa (LÔBO, 2012), *Ampelozizyphus amazonicus* Ducke, infusão da raspa da raiz usada na região amazônica para a cura de resfriado e da malária (LIMA, 2006) e *Rhamnidium elaeocarpum* Reissek, espécie conhecida como "cafezinho" ou "cafezinho-do-cerrado", usadas para acalmar a coceira das gengivas em crianças, no início da dentição e também para tratar dores de estômago (LIMA, 2011).

Rhamnaceae apresenta espécies com grande potencial de produtos biologicamente ativos, tendo sido investigado e confirmado em algumas espécies como *Rhamnus sphaerosperma* var. *pubescens* (Reissek) M.C. Johnston, como antioxidante, antimicrobiano, hemolítico (MOREIRA, 2013); *Scutia buxifolia* Reissek, com atividade cardiotônica, diurética e hipotensora, (BOLIGON et al, 2009a); *Condalia buxifolia* Reissek, alcaloide ciclopeptidico antibacterial, condaline-A, (MOREL et al, 2002); *Discaria americana* Gillies & Hooker, como ansiolítica, antinociceptiva, (SILVA et al, 2012). O extrato metanólico das folhas de *Rhamnidium elaeocarpum* contém quantidades significativas de flavonoides, alcaloides, saponinas e taninos, sugerindo que a presença dessas substâncias possam justificar a ação anti-úlcera demonstrada por esse extrato, já que os compostos apresentam alta atividade antioxidante (SILVA et al, 2015). Silva et al (2010) também confirmam as atividades antioxidante e antimicrobiana de extratos de cascas e folhas de *Ziziphus joazeiro* Mart.

1.2 O GÊNERO *COLLETIA* Comm. ex Juss.

Colletia é um gênero exclusivo da América do Sul, onde ocorrem cinco espécies, *C. spinosissima* Gmel., *C. ulicina* Gill. & Hook., *C. hystrix* Clos., *C. paradoxa* (Spreng.) Esc. e ***C. spartioides*** Colla (LOEFGREN, 1917; SUESSENGUTH, 1953; JOHNSTON, FREITAS SOARES, 1972; TORTOSA, 1989, LIMA, GIULLIETTI, 2014). Duas destas espécies chegam até o Brasil: *C. paradoxa* que também ocorre no Uruguai e Argentina, e *C. spinosissima* ocorrendo no Equador, Peru, Bolívia, Argentina, Uruguai, Chile e Brasil (MARZOCCA, MARTHI, 1951, TORTOSA, 1989). Sua maior diversidade de espécies está ao sul do paralelo 30º sul, e sua distribuição está associada aos Andes, na América do Sul (AAGESEN et al, 2005).

Colletia paradoxa é encontrada nos estados do Rio Grande do Sul, Santa Catarina e Paraná, e *C. spinosissima* ocorre no Rio Grande do Sul e em Santa Catarina (BASTOS-ZÁCHIA, MORAES, 1999; LIMA, GIULLIETTI, 2014). No Rio Grande do Sul, *C. paradoxa*, ocorre na floresta da encosta da Serra do Sudeste e eventualmente no sul da Planície Costeira (SOBRAL et al, 2006). Também é encontrada nas florestas nebulares e pequenos capões de

matas ciliares, na região dos Campos de Cima da Serra. Na mesma região, encontra-se *C. spinosissima*, em pequenas formações campestres próximo ao cânion ou nas escarpas.

Os indivíduos de *C. paradoxa* são arbustos, ou pequenas árvores de até 5m de altura, caracterizando-se por apresentarem ramos espinescentes, cobertos por fina pilosidade acinzentada ou então glabros. Seu aspecto xeromórfico é ressaltado com a presença de folhas muito reduzidas, caducas, ramos curtos e espinescentes. Apesar de abundante floração produzem poucos frutos viáveis (D'AMBROGIO, MEDAN, 1993). Durante a floração, as flores brancas exalam perfume adocicado que lembra a baunilha (observação pessoal).

Colletia paradoxa morfotipo "paradoxa", detalhe do ramo com frutos. Foto de Liliana Essi.

1.2.1 A variabilidade morfológica em *Colletia paradoxa*

Os indivíduos de *C. paradoxa* apresentam uma grande variabilidade morfológica em seus ramos espinescentes. Devido a isso, parte destes indivíduos eram identificados como outra espécie, *C. exserta*, considerada como espécie distinta de *C. paradoxa* (REISSEK, 1861; JOHNSTON, FREITAS SOARES, 1972). Entretanto, Tortosa (1989) considerou que a diferença na forma e largura dos espinhos era apenas uma variação devido a fatores ambientais, o que foi seguido por outros pesquisadores (BASTOS-ZÁCHIA, MORAES, 1999; RICHARDSON et al, 2000; RICHARDSON et al, 2004; AAGESEN et al, 2005; ZULOAGA, 2008), que aceitaram a proposta de sinonimização daquela espécie.

Lima & Giullietti (2014), revisaram este conceito reestabelecendo *C. exserta* como uma espécie distinta de *C. paradoxa*. Para isso argumentaram que a diferença está principalmente nos ramos estreitos, delicados, ascendentes e curvados de *C. exserta*, além de flores tubulosas; enquanto que *C. paradoxa* apresenta ramos largos, grossos, patentes (inclusive em ramos jovens) e flores urceoladas.

A grande variação morfológica existente em *C. paradoxa* faz com que os extremos dessa variabilidade sejam notados como taxa diferentes, entretanto devemos considerar a existência de uma continuidade de caracteres intermediários entre eles. Por isso, neste estudo, trataremos esses morfotipos como *C. paradoxa* morfotipo "paradoxa", *C. paradoxa* morfotipo "exserta" e *C. paradoxa* morfotipo "intermediário". No manuscrito dois desta tese trataremos mais especificamente sobre essa grande variabilidade observada, com intenção de discutir a larga plasticidade existente dentro da espécie *C. paradoxa.*

D'Ambrogio e Medan (1993) ao realizar um estudo sobre o comportamento reprodutivo de *C. paradoxa* concluíram que essa espécie tem reprodução exclusivamente sexuada e xenógama, sendo polinizada por himenópteros (*Apis melifera*) e alguns dípteros (Syrphidae). Foi observado durante o mesmo estudo (1993), que *C. paradoxa* apresenta como estratégia, uma floração com uma fase pós floral prolongada (4 a 8 meses), disponibilizando a flor neste período, que passa por diferentes estações do ano. Isso permite que uma maior diversidade de insetos possa visitar as flores, já que elas apresentam uma estrutura floral pouco especializada, permitindo o acesso desses visitantes e aumentando as chances de ocorrer a polinização. Ainda segundo D'Ambrogio e Medan, (1993) apenas uma flor em cada quatro delas forma fruto, e destes, apenas 6,1% amadurecerão. O baixo nível de frutificação os leva a crer, que o pólen não seja transportado pelo vento.

Colletia paradoxa, no Rio Grande do Sul, ocupa ambientes variados podendo ser encontrada sobre diferentes substratos, como solos intemperizados com origem em rochas metamórficas ou graníticas, pedregosos, arenosos ou com um maior percentual de matéria orgânica, rasos ou até mesmo profundos mas sempre associados à umidade em diferentes graus. Foi encontrada, até o momento, em apenas quatro núcleos populacionais muito distantes entre si: Depressão Central (Porto Alegre, São Pedro do Sul), Serra do Sudeste (Piratini, Pinheiro Machado, Canguçu), Litoral (Chuí, Mostardas/Tavares) e Campos de Cima da Serra (São Francisco de Paula, Cambará-do-Sul, Jaquirana). É uma espécie de distribuição descontínua e inexpressiva, também em Santa Catarina, (JOHNSTON & FREITAS SOARES,1972, [sub *C. exserta*]) formando populações esparsas em ambientes muito diferenciados. Ocorrem na borda de pequenas matas campestres ou de galeria e eventualmente em campo aberto, formando pequenos aglomerados. Também foi encontrada à beira de estrada pavimentada ou secundária, isoladas ou em pequenos grupos.

Considerando suas características morfológicas e ecológicas, sua distribuição geográfica, seu potencial medicinal, e seu status como espécie ameaçada, e sabendo-se que essa espécie até o momento foi pouco estudada, percebe-se a necessidade de gerar conhecimento sobre a espécie em diversas áreas de interesse. Santos & Marchiori (2009) alertam para a necessidade de estudos multidisciplinares, como forma de resolver as questões duvidosas na família.

Segundo Ramawat et al (2009) as pesquisas modernas sobre plantas medicinais devem incorporar um enfoque multidisciplinar que possa combinar técnicas botânicas, fitoquímicas, biológicas e moleculares.

Da mesma forma, Richardson et al. (2000) consideram que nestes casos de questionamentos taxonômicos, principalmente, deve-se fazer uso do conhecimento de múltiplas áreas, em especial de anatomia, morfologia e química.

Neste estudo, procuramos investigar através da análise fitoquímica, morfológica e molecular diferentes populações de *C. paradoxa* e contribuir para um melhor conhecimento dessa espécie nativa, fornecendo dados que favoreçam um manejo eficiente visando o uso sustentável através de estratégias de conservação.

Colletia paradoxa morfotipo “paradoxa”, detalhe do ramo. Foto de Liliana Essi.

1.2.2 Estado de conservação da espécie

Colletia paradoxa foi classificada como ameaçada de extinção na categoria vulnerável (VU A3c) em 2012, no processo de reavaliação das listas da fauna e da flora do Rio Grande do Sul, organizado pela Comissão de Reavaliação e pelos Coordenadores de cada grupo taxonômico. Durante a avaliação dos critérios para a classificação da espécie *C. paradoxa*, observou-se a escassez de informações a respeito da real situação das populações das espécies nativas, principalmente aquelas menos conhecidas.

A distribuição de *C. paradoxa* é fomentadora da discussão sobre o seu estado de conservação, pois existem poucos locais de ocorrência onde essa espécie já tenha sido encontrada. Como os diferentes morfotipos ocorrem em regiões que sofrem grande pressão antropogênica, sua principal ameaça é a redução ou destruição de hábitats (Lima et al, 2014), que são causados pela da urbanização, o corte e queimada da vegetação, entre outros fatores. Por ocuparem ambientes nas florestas do sul da Mata Atlântica, através de Porta de Torres e na

encosta da Serra do Sudeste, áreas bastante ameaçadas pela agricultura extensiva e invasão de espécies exóticas e madeireiras, suas áreas de ocupação foram estimadas em 36 km² para *C. paradoxa stricto sensu* e 44 km² para *C. exserta*, colocando-os na categoria "Em Perigo" (EN) na Lista Vermelha de Espécies de Flora Ameaçadas do Brasil (MARTINELLI et al, 2013). Como são núcleos bem isolados, sugerem uma fragmentação de hábitats que poderá ter consequências sobre a manutenção da variabilidade genética da espécie, podendo assim influenciar diretamente na sua conservação. Pode ser considerado também um fator de risco, o costume da população de usá-la como planta medicinal, extraindo-lhe cascas e raízes.

1.2.3 **Etnobotânica e fitoquímica**

No Brasil, *Colletia paradoxa* é um arbusto espinhoso, conhecido popularmente no meio rural como quina, podendo ser reconhecida também pelos seguintes nomes: quina-do-rio-grande (Corrêa, 1974), quina-de-porto-alegre, quina-cruzeiro, quina-coroa-de-cristo, curro, curá-manoel, cruzeiro, espinho-de-cruz (JOHNSTON & FREITAS SOARES, 1972; TORTOSA, 1995), assim como na Argentina é chamada de espina-de-la-cruz, espina-cruz, curru-mamoel, curro, cura-mamuel, crucero, quina (ESCALANTE, 1946; MARZOCCA & MARTHI, 1951; TORTOSA, 1989,1995) e no Uruguai, espina-de-cruz (ARECHAVALETA, 1900). Apesar das variadas combinações de nome para *C. paradoxa*, quina é o nome mais comumente usado, além de ser também, um nome popular atribuído à outras espécies.

Há referência ao uso de *C. paradoxa*, assim como a outras espécies do gênero, com diversas finalidades que datam desde a segunda metade do século XIX (TORTOSA, 1989). Isso nos permite dizer que o conhecimento de seus usos populares são bem difundidos, enquanto que na área da fitoquímica há ainda muito o que fazer. Sob o ponto de vista da análise fitoquímica temos ainda poucos estudos realizados com *C. paradoxa*, no entanto, já está comprovado seu potencial biológico. Este foi mais um motivo que nos estimulou a realizar esta pesquisa, aliado a isso a importante constatação de que a espécie em estudo é utilizada como planta medicinal. Conhecida por suas propriedades tônica e febrífuga, é também referida segundo Tortosa (1989) como purgante, além de outros usos. Seus ramos e raízes são ricos em saponinas e por isso eram muito utilizados para lavar roupas de lã, além do aproveitamento de sua madeira e lenha de ótima qualidade.

A espécie é utilizada na Argentina, Europa e Estados Unidos como ornamental devido ao seu aspecto peculiar (MARZOCCA, MARTHI, 1951; TORTOSA, 1989). No Brasil, ainda é pouco conhecida no meio urbano, porém também lhe é atribuído o uso de suas cascas e /ou

raízes, como eficiente medicamento contra a febre. Este tipo de uso, da matéria prima, já traz em si o problema da ameaça a sua conservação, que é a prática de utilizar as cascas e raízes, causando a supressão dos exemplares utilizados. Isso poderia causar impactos na população acessada pelos coletores. Somente esta questão, já demandaria estudos de biologia da reprodução e ecologia de populações com sugestões de trabalho bastante interessantes, que podem resultar em propostas de manejo que permitiriam a exploração sustentável. Além disso, sua presença em áreas de atividade agropecuárias, produzem danos ao gado causando prejuízos. Esse fato faz com que haja uma ação antrópica no sentido de remover os indivíduos da área, seja através da foice e facão ou pela prática do fogo, sendo necessária uma orientação de manejo sustentável. Na Argentina, costumava-se contratar pessoas que trabalhavam especificamente com a função de remover os chamados "currales" formados pelas populações de *C. paradoxa*, e por isso, foram exterminadas extensas áreas (TORTOSA, 1989). No Brasil, não se tem registro destas ações, a não ser pela prática de "limpar o campo" através do uso do facão ou do fogo, principalmente nas regiões onde se desenvolvem atividades agropecuárias e do seu uso como lenha.

População de *Colletia paradoxa*, na localidade Pai João do município de Mostardas, RS. Foto R. Záchia.

1.2.4 Atividades Antioxidante e Antimicobacteriana

Um dos objetivos dessa tese é investigar o potencial de ação antioxidante e de atividade antimicobacteriana apresentado pelos extratos brutos da espécie *C. paradoxa* e seus diferentes morfotipos. As micobacterioses, doenças causadas pelas micobacterias não tuberculosas (MNT), são responsáveis pelo aumento do surto de infecções cirúrgicas que acometem cada vez mais pacientes pós-cirurgia, em estado de baixa imunidade. A rápida identificação dos organismos é importante para que o diagnóstico e a escolha do tipo de tratamento, seja eficiente no controle da doença (WILDNER et al, 2011). Assim, cada vez mais aumenta o interesse em pesquisas que investiguem novas fontes de fitoterápicos. Nesse sentido muitas pesquisas têm sido realizadas, utilizando-se micobactérias de crescimento rápido e não patogênicas, para investigar a atividade bacteriana de extratos e compostos derivados de plantas (KUETE et al, 2008; BROWN et al, 2007). No primeiro manuscrito dessa tese, avaliamos a atividade antimicobacteriana do extrato bruto de *C. paradoxa*. Além dessa, a outra atividade biológica avaliada foi a ação antioxidante.

Apresentamos aqui, uma breve descrição da importância desses ensaios antimicobacterianos para as ciências farmacêuticas. O gênero *Mycobacterium* está relacionado à doenças importantes sendo, *Mycobacterium leprae* agente etiológico da hanseníase e *M. tuberculis*, o agente causador da tuberculose (MACEDO et al, 2009). O gênero *Mycobacterium* apresenta 175 espécies e 13 subespécies (EUZEBY, 2018).

Esse estudo foi realizado utilizando espécies *M. abscessus*, *M. fortuitum* e *M. massiliense*. *Mycobacterium abscessus* é o principal agente etiológico associado à doença nodular bronquiectásica, enquanto *M. fortuitum*, apresenta-se clinicamente na forma de doenças cutâneas, ósseas ou de tecidos moles (ANTUNES et al, 2012). *Mycobacterium massiliense* tem sido apontado como agente etiológico predominante na maioria das cidades brasileiras, sendo responsável por grande parte das infecções causadas por micobactérias de crescimento rápido (MCR), incrementada pelo aumento da ocorrência do HIV (WILDNER et al, 2011). Todas elas pertencem ao grupo IV (Micobactérias de Crescimento Rápido – MCR). São espécies patógenas oportunistas que normalmente estão relacionadas a infecções pós-operatórias de vários tipos (ANVISA, 2009). Nessa tese, foi realizado um ensaio para avaliar a ação antimicobacteriana, do extrato bruto de *C. paradoxa* e seus diferentes morfotipos, em *M. abscessus*, *M. fortuitum e M. massiliense*.

Nosso organismo produz normalmente radicais livres como resultado da atividade metabólica normal. O termo "radical livre" refere-se a um átomo ou molécula altamente reativo, que contêm número ímpar de elétrons em sua última camada eletrônica. Este "não emparelhamento de elétrons" da última camada, é que confere alta reatividade a esses átomos

ou moléculas (COTINGUIBA et al, 2013; FERREIRA, MATSUBARA, 1997; RIBEIRO et al, 2005).

Estes radicais livres reagem com substâncias oxidáveis existentes no meio, além do DNA, RNA e proteínas porém, nessa busca pela estabilidade química, eles causam reações em cadeia que determinam alterações de diversos tipos em vários componentes celulares (LIMA, BEZERRA, 2012). No nosso corpo, o excesso dessas moléculas resulta no "estresse oxidativo", que é o desequilíbrio entre o número de moléculas oxidantes e a quantidade de antioxidantes disponíveis no nosso organismo para combatê-las, causando danos celulares (BIANCHI, ANTUNES, 1999). Em relação a isso, nosso corpo produz defesas antioxidantes que agem no sentido de manter o equilíbrio das reações metabólicas, monitorando a formação e a remoção das substâncias radicalares aí existentes (BIANCHI, ANTUNES, 1999; FERREIRA, MATSUBARA, 1997; LIMA, BEZERRA, 2012). O efeito prejudicial dos radicais livres ocorre quando eles estão presentes no organismo em quantidades muito maiores do que a capacidade de defesa antioxidante, do organismo, de neutralizá-los. Um antioxidante é qualquer substância capaz de retardar ou prevenir a oxidação de proteínas e lipídios celulares ou mesmo alterações no DNA, estando presente em pequenas concentrações, quando comparado ao agente oxidante (SILVA et al, 2010).

Os flavonoides pertencem a uma classe de compostos naturais vegetais, que têm sido reconhecidos pelas suas importantes atividades biológicas, como ações antialérgicas, antivirais, anti-inflamatórias e vasodilatadoras, mas especialmente pela capacidade de inibir ou reduzir a formação de radicais livres e quelar metais (SIMÕES, 2004).

Apesar dessa atividade biológica já ter sido confirmada na família e reconhecida em outros gêneros, ainda não havia sido investigada para essa espécie. O detalhamento do procedimento e seus resultados são apresentados no manuscrito 1.

1.2.5 **Marcadores moleculares para caracterização genética**

Os marcadores morfológicos são utilizados como descritores para identificar variações dentro de uma mesma espécie, relacionados com a forma ou padrão fenotípico de uma determinada característica. Porém, quando não é possível fazer a identificação de uma planta através da análise morfológica, pode-se recorrer aos marcadores moleculares, através do processo de obtenção de um padrão de bandas específicas para determinada espécie. Esse

conjunto de bandas exclusivo e que caracteriza a espécie chamamos de *fingerprinting*, que equivale a uma impressão digital (GUIMARÃES, 2009; SOUZA-CHIES et al, 2014).

Padrões de banda podem ser utilizados, também, para avaliar a variedade genética existente em populações naturais. Várias técnicas de biologia molecular podem ser utilizadas para esse fim, mas as mais comumente usadas são AFLP (*Amplified Fragment Length Polymorphism*), microssatélites (SSR), RFLP (*Restriction Fragment Length Polymorphism*), RAPD (*Random Amplified Polymorphic DNA*) e ISSR (*Inter Simple Sequence Repeat*) que é a técnica utilizada no presente estudo.

Os marcadores *Inter Simple Sequence Repeat* (ISSR) utilizam um único *primer* desenhado com base nas sequências repetidas dos microssatélites na extremidade 5', podendo possuir alguns nucleotídeos extras na extremidade 3' (*primers ancorados*). Assim, os primers anelam-se dentro das repetições e amplificam as regiões genômicas entre os SSRs, cujo tamanho dos fragmentos são limitados pela própria técnica da PCR (GUIMARÃES et al, 2009). Há dois tipos de marcadores, quanto à revelação dos fragmentos Os marcadores ISSR, assim como ocorre nas técnicas AFLP e RAPD, são consideradas dominantes. Isso significa que alelos de um mesmo loco, são revelados pela presença ou ausência de uma banda que resulta da amplificação de um fragmento de determinado tamanho, mas que não é possível saber se o loco amplificado está em homozigose ou heterozigose. Já os marcadores SSRs e RFLP são ditos co-dominantes, pois permitem a identificação de heterozigotos, revelando a presença de dois alelos e são particularmente úteis em programas de melhoramento.

Os marcadores do tipo ISSRs, são derivados de primers não específicos. De forma geral, são amplamente usados em estudos de diversidade genética, devido ao seu alto polimorfismo e baixo custo. Têm a vantagem de não ser necessário ter informação prévia sobre o genoma, principalmente quando se trata de plantas nativas, além de serem de fácil aplicação a várias espécies vegetais (GUIMARÃES et al, 2009). A forma de análise é semelhante à técnica de RAPD, porém a repetibilidade dos padrões de banda do ISSR é bastante superior àquela. Atualmente são bastante utilizados no estudo de populações de plantas ameaçadas de extinção (CAO et al, 2006; QI et al, 2015, TIAGO et al, 2018).

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar a morfologia, a composição fitoquímica, a atividade antioxidante e antimicobacteriana e a diversidade genética de populações de *Colletia paradoxa*, contribuindo com informações para a avaliação do status de *C. paradoxa* na Lista Vermelha das Espécies Ameaçadas, através dos critérios IUCN.

2.2 OBJETIVOS ESPECÍFICOS

- Determinar o teor de compostos fenólicos nos extratos brutos da espécie *C. paradoxa* e seus morfotipos de diferentes regiões fisiográficas, e caracterizá-los através de Cromatografia Líquida de Alta Eficiência (HPLC);
- Avaliar a atividade antimicobacteriana dos extratos brutos de *C. paradoxa* e seus morfotipos, frente às bactérias *Mycobacterium abscessus*, *Mycobacterium fortuitum* e *Mycobacterium massiliense*;
- Avaliar a atividade antioxidante dos extratos brutos de *C. paradoxa* e seus morfotipos;
- Identificar os compostos bioativos nos extratos brutos;
- Relacionar os resultados obtidos nas análises moleculares das amostras de *C. paradoxa* com as características morfológicas apresentadas pela espécie e seus diferentes morfotipos;
- Analisar a diversidade genética entre e dentro de populações de *C. paradoxa*;
- Avaliar o uso de marcadores ISSR para analisar fragmentos de DNA de *C. paradoxa*.

3 PUBLICAÇÕES CIENTÍFICAS

3.1 MANUSCRITO 1[1]

Phytochemical Analyses and Evaluation of Antioxidant and Antimycobacterial Activity of *Colletia paradoxa* from Brazil

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ABSTRACT

Colletia paradoxa (Spreng.) Esc. (Rhamnaceae) is a medicinal plant, threatened with extinction in Brazil, presenting great morphological variability. Our objective is to investigate the phytochemical components, antioxidant capacity and antimycobacterial activity of different morphotypes of *C. paradoxa* in different environments. For this, the crude extract of the leaves and branches of the individuals sampled was used. The elimination capacity of the free radicals was determined by the DPPH method, the antimycobacterial activity by the broth microdilution method and the phenolic content by the spectrophotometric method using the Folin-Ciocalteu reagent and by HPLC. The extracts of *C. paradoxa* and its morphotypes showed significant amounts of phenolic compounds, including quercetin, quercitrin and rutin, besides considerable antioxidant potential and moderate antimycobacterial activity. The results indicate considerable free radical capture capacity, being potential sources of natural antioxidant and antimycobacterial activity. No connection was detected between the phytochemical composition and different morphotypes of *C. paradoxa.*

Key Words: Antimicobacteriana; Flavonoids; Morphotypes; Quina; Rhamnaceae.

[1] Este manuscrito foi aceito para publicação no periódico *Natural Product Research*, o qual está formatado de acordo com as normas exigidas pela citada revista.

1. Introduction

Phenolic compounds from the secondary metabolism of plants are good natural antioxidants and potential bacteriostatic agents, attracting more and more people's attention. The antioxidant potential compounds are much higher than that of all other antioxidants known in the diet, since they are reductive schedules that along with other reducers such as vitamin C, vitamin E and carotenoids, protect our body from oxidative stress (Scalbert and Williamson, 2000). However, medicinal plants are of great importance in public health, especially in developing countries. Besides that, they are object of interest to the researchers (Mossi et al, 2009).

In general, the Rhamnaceae family presents species with great potential of biologically active products. This biological potential has been investigated and confirmed in some species as *Rhamnus sphaerosperma* var. *pubescens* (Reissek) M.C. Johnston - antioxidant, antimicrobial, hemolytic (Moreira et al, 2013), *Scutia buxifolia* Reissek - cardiotonic, diuretic and hypotensive activity (Boligon et al, 2009a), *Condalia buxifolia* Reissek - antibacterial cyclopeptide alkaloid, identified as condaline-A (Morel et al, 2002) and *Discaria americana* Gillies & Hooker - anxiolytic and antinociceptive (Silva et al, 2012), among others.

Phytochemical studies on members of the Rhamnaceae family, including *C. paradoxa*, describe the presence of triterpenoids and steroids in their composition, mainly in the aerial parts of the plant. Furthermore, the antimicrobial activity of the *C. paradoxa* triterpenic isolates against *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Salmonella setubal*, *Escherichia coli* and *Pseudomonas aeruginosa* has been reported (Giacomelli et al, 2006).

Maldaner (2005) studied the secondary metabolites and the antimicrobial activities of two Rhamnaceae, being they *Scutia buxifolia* Reissek. and *Condalia buxifolia* Reissek. In *Condalia buxifolia* identified four metabolites: condalin A, β-sitosterol, lupeol and taraxerol. In *Scutia buxifolia* isolated two metabolites, ursolic acid and β-sitosterol, and identified three cyclopeptide alkaloids, they are, scutiania B, scutianina C and scutianina D. In the studies of antimicrobial acticity through bioautography of fractions and isolated metabolites, both species showed well defined zones of inhibition against Gram positive and Gram negative bacteria.

Giacomelli (2005) carried out a phytochemical study of three species belonging to the family Rhamnaceae: *Discaria americana*, *Colletia paradoxa* and *Gouania ulmifolia*. In this study, it was able to isolate and identify, from the aerial parts of *C. paradoxa*, a new metabolite, named seco-3,4-germanicone, along the known compounds ursolic acid, lupenol, betulinic acid, ceanotic acid, taraxerol, taraxerone, seco-3,4-taraxerone, germanicol, germanicone, β-sitosterol and β-sitosterol-glycosylated. Beyond that, it was isolated, from the root bark, ziziberenalic acid together with the compounds of betulinic acid, ceanotic acid, ursolic acid, β-sitosterol and β-sitosterol-glycosylated.

According to Boligon et al (2009b), the fractions and the crude extract of the leaves and stem bark of *S. buxifolia* present DPPH capture activity, besides being efficient inhibitors in the production of TBARS. The authors relate this activity to the presence of quercetin, quercitrin, isoquercitrin and rutin in their extracts.

Nishijima et al (2010) report important anti-inflammatory and antinociceptive activities of the methanolic extract of *Rhamnidium elaeocarpum* Reissek, when studying the effects of extracts and fractions of plant species on the total neutralization capacity against hemorrhage caused by *Bothrops jararaca*.

The genus *Colletia* presents a great vegetative polymorphism. It belongs to the Colletieae tribe that has as a center of dispersion of its species the south of South America (Aagesen et al, 2005). In Rio Grande do Sul State, Brazil, occupies a very diversified environment, where two to three species occur, *C. spinosissima*, Gmel., *C. paradoxa* (Spreng.) Esc. and *C. exserta* Klotzsch ex Reiss (Bastos-Záchia and Moraes 1999; Lima and Giullietti, 2014). *C. paradoxa* and *C. exserta* have a wide and fragmented occurrence, and its circumpscription remains controversial. Before the work of Lima and Giulietti (2014), some authors (Tortosa, 1989; D'Ambrogio and Medan, 1993; Bastos-Záchia and Moraes, 1999) considered that *C. exserta* would be synonymous with *C. paradoxa*, circumscription here adopted. The Brazilian Red Book of Endangered Species (Martinelli and Moraes, 2013) considered these taxa as distinct species, both endangered. Either treated as a single species or two different species (here treated as morphotypes of a single one), these plants are considered as medicinal by popular knowledge. There is not any study demonstrating if these different morphotypes bear phytochemical differences. Therefore, it is considered necessary a study that contributes to a better knowledge of the phytochemical composition in different environments and morphotypes. Thus, the objective of this study is to investigate the phytochemical

components, antioxidant capacity and antimycobacterial activity of different morphotypes of *C. paradoxa* in different environments.

2. Material and methods

2.1 The material studied

The material to be studied consists of samples of three morphotypes of *Colletia paradoxa*: morphotype "paradoxa" (individuals with morphology corresponding to *C. paradoxa stricto sensu*), morphotype "exserta" (individuals with morphology corresponding to *C. exserta*) and morphotype "intermediate" (individual with morphology resembling both species). Morphotype "paradoxa" includes shrubs or small trees with spinescent branches, shows broad, thick, straight, patent branches (even in young plants) and urceolate flowers. Morphotype "exserta" is distinguished form the former by the presence of narrow, delicate, curved and ascending branches and tubular flowers. Morphotype "intermediate" includes one individual with intermediate characteristics to previous described morphotypes, that is, branches ascending and sometimes broad, patent and delicate, and still with varying flowers as to form. In addition, the plants were sampled at different points of collection. Details about individuals and vouchers are provided in Table I.

Table I – List of different *C. paradoxa* morphotypes studied, with location, coordinates and *voucher* number.

Access code	**Morphological type**	**Location**	**Collection date**	**Physiographic region**	**Coordinates**	***Voucher***
RB 501	Morphotype "paradoxa"	Piratini, RS	30/Apr./2016	SS	S 31° 21' 5,61" W 053° 03' 23,57"	SMDB 16.913
RB 507	Morphotype "exserta"	Porto Alegre, RS	15/Apr./2016	CD	S 30º 03' 6.07'' W 51º 10' 37.95''	SMDB 16.918
RB 508	Morphotype "intermediate"	São Pedro do Sul, RS	17/Apr./2016	CD	S 29º 37' 14'' W 54º 10' 44''	SMDB 16.914
RB 512	Morphotype "paradoxa"	Pinheiro Machado, RS	30/Apr./2016	SS	S 31°33'13,38" W 053°24'42,39"	SMDB 16.915
RB 515	Morphotype "exserta"	Cambará do Sul, RS	10/Apr./2016	SPG	S 29° 1' 00.16" W 50° 08' 30.94"	SMDB 16.912

RB: collection number of N. Rolim Bastos; Physiographic regions according Fortes (1959) SS: Sul-Rio-Grandense Shield; CD: Central Depression; SPG: Southern Plateau Grasslands; SMDB: Herbarium of the Department of Biology of UFSM.

2.2 Collection of samples

The branches and leaves of different individuals of *C. paradoxa* were collected for analyses in five municipalities of three physiographic regions of Rio Grande do Sul, representing also different morphotypes (Table I), being two samples of the morphotype "paradoxa", two of the morphotype "exserta" and one of the morphotype "intermediary", because no other population of this morphotype was found until that date. After the collection were conditioned in newspapers and dried in greenhouse. The collections were always duly authorized by the responsible bodies (IBAMA - SISBIO) and the Voucher material of the research is deposited in the SMDB Herbarium of the Federal University of Santa Maria (Table I). The identification of the material was performed by Nelci Rolim Bastos-Záchia (SMDB). Analyses of types and types pictures was also performed, besides consulting specialized bibliography.

2.3 Phytochemical analyses

2.3.1 Extract preparation

Each plant sample was dehydrated in an oven at 40° C until completely dry and then crushed. The extracts were obtained by hydroalcoholic (EtOH: H_2O, 7: 3, v/v) maceration of the material (15 g/L) in a closed vessel. The macerate was submitted to manual daily shaking, for a period of seven days, for the total extraction of the existing compounds in the sample. At the end of this period the contents were filtered in cotton, repeating this protocol three times. After the hydroalcoholic extracts were submitted to the rotary evaporator to eliminate the ethanol, and later they were taken to dry in the rotary evaporator, to obtain the crude extract, without application of vacuum.

2.3.2. *Reagents and equipments*

All chemical reagents were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid and catechin were purchased from merck (Darmstadt, Germany). Quercitrin, quercetin, canferol, luteolin and rutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). the quantification and identification of the phenolics compounds were performed on the Shimadzu Prominence Auto System Liquid chromatograph equipped with alternative pumps (Shimadzu LC-20AT) connected to a degasser (20A5 DGU) (CBM 20A), a diode arrangement detector (SPD-M20A) and software (LC solution SP1 1.22). Quantification of polyphenols and DPPH test were performed on a Shimadzu-UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan).

2.3.3. *Total Phenolics Content*

The total polyphenols were evaluated by colorimetric method, using the reagent *Folin-Ciocalteu* 2N and spectrophotometer reading at 730 nm. The crude extract was prepared in the concentration of 0,150 mg/mL, being diluted in ethanol in triplicate. The total polyphenol content was calculated by a calibration curve using gallic acid as reference (Chandra et al, 2004).

2.3.4. *Characterization by HPLC - Chromatographic conditions*

Chromatographic analyses were performed in reverse phase under gradient conditions using column Phenomenex C_{18} (4,6mm x 150mm) charged with particles with a diameter of 5,0 µm. The mobile phase used was water containing 2% acetic acid (A) and methanol (B) and the composition gradient was 5% (B) during 2 minutes, 25% (B) until 10 minutes, 40, 50, 60, 70 e 80% (B) every 10 minutes, following the method described by Barbosa Filho et al (2014) with modifications. The crude extracts were analyzed at a concentration of 15 mg/mL.

The flow used was 0.6 mL/min and the injection volume was 50 µL. The wavelength was 271 nm for gallic acid, 280 nm for catechin, 327 nm for caffeic acid, and 365 nm for quercetin, quercitrin, luteolin, canferol and rutin. Samples and the mobile phase were filtered through a 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Reference solutions were prepared in the mobile phase for HPLC at concentrations of 0.050-250 mg/mL. Chromatographic peaks were confirmed by comparing their retention time with those of the reference standards and by the DAD spectra (200 to 600 nm). The calibration curve for gallic acid was: Y=12609x + 1135,3 (r = 0,9998); catechin: Y = 11972x + 1258.1 (r = 0.9997), caffeic acid: Y = 11864x + 1305.9 (r = 0.9999); quercetin: Y = 12648x + 1352.7 (r = 0.9996); quercetin: Y = 13573x + 1369.4 (r = 0.9999); Y = 11965x + 1287.6 (r = 0.9997), rutin: Y = 11874x + 1196.8 (r = 0.9999) and luteolin: Y = 12845x + 1359.7 (r = 0.9999). All chromatographic operations were performed at room temperature and in triplicate. The limit of detection (LD) and the limit of quantification (LQ) were calculated based on the standard deviation and slope of the responses of three independent analyses curves, as defined by ICH (2005). LD and LQ were calculated as 3.3 and 10 σ/S, respectively, where **σ** is the standard deviation of the response and S is the slope of the calibration curve.

2.3.5. *Statistic of the results of the phytochemical analyses*

The tests were performed in triplicate and the data were submitted to a variance analyses (ANOVA) and the means were compared by the Tukey test with a statistical significance ($p < 0.05$) considered significant, using the software package Statistical Analyses System (SAS) 9.0 STAT.

2.4 Biological analyses

2.4.1. *Evaluation of antioxidant activity*

The evaluation of the antioxidant activity was performed using the spectrophotometric method with 2, 2-diphenyl, 1-picrylhydrazyl (DPPH) as reagent, according to Boligon et al. (2009b). This reaction occurs because of the ability antioxidant compounds have to capture the DPPH free radicals. The crude extracts of *C. paradoxa* were used in concentrations of 250; 125; 62.5; 31.25; 15.62 e 7.81 µg/mL in ethanol (2.5 mL). To 2.5 mL of each sample was added 1 mL of 0.3 mM DPPH solution in ethanol. After 30 minutes, the absorbance readings were taken at 518 nm in a spectrophotometer (Shimadzu - UV-1201). A solution of DPPH (1mL, 0.3 mM) in ethanol (2.5 mL) was used as the negative control and ascorbic acid was used as standard (positive control) at the same sample concentrations. Ethanol was used to clear the spectrophotometer, with blank solutions for each sample (without addition of DPPH), to minimize interference of sample components in the reading. The assay was performed in triplicate and the calculation of the percentage of antioxidant activity followed the equation:

$$\% \text{ inibition} = 100 - \left[\left(\frac{Abs_{sample} - Ab_{blank}}{Abs_{control}}\right) \times 100\right]$$

Where, $\boldsymbol{Abs_{sample}}$ is the absorbance of the crude extracts, $\boldsymbol{Abs_{blank}}$ is the absorbance of the crude extracts without addition of DPPH and $\boldsymbol{Abs_{control}}$ is the absorbance of the DPPH solution in ethanol. The percent inhibition of the DPPH radical was calculated and a plot of percent inhibition *versus* the crude extract concentration was constructed. The free radical capacity was measured by spectrophotometric analyses, where the inhibitory concentration (IC_{50}), which is the concentration of the extract necessary to eliminate 50% of free radicals of DPPH present in the medium, was also determined.

2.4.2. *Evaluation of antimycobacterial activity*

2.4.2.1 Microrganisms

To carry out this study, were used the strains of *Mycobacterium abscessus* (ATCC 19977), *Mycobacterium fortuitum* (ATCC 6841) and *Mycobacterium massiliense* (ATCC 48898). The colonies were isolated in Löwesten-Jensen solid media (HiMedia Laboratories)

and then cultured in Middlebrook 7H9 broth medium (Difco Laboratories) containing 0.2% (vol/vol) glycerol and 10% (vol/vol) OADC (oleic acid-albumin-dextrose-catalase).

2.4.2.2 *Susceptibility tests*

The minimum inhibitory concentration values were determined by broth microdilution method (CLSI, 2011). The assay was performed on 96-well sterile U-bottomed microplates containing 12 columns and 8 lines, identified from A to H. Initially, 0.1 ml of Mueller-Hinton broth (MHB-Merck, Darmstadt, Germany) was transferred onto line A of columns 1 to 8. In column 1, 0.1 ml of the extract to be tested were individually inoculated and 0.1 ml transferred after homogenization to the bore of column 2, repeating the procedure to column 8, thereby obtaining concentrations in a range of 5.0 mg/mL to 0.039 mg/mL.

The bacterial suspensions were prepared in 1.0 x 10^8 CFU/mL physiological saline solution equivalent to the McFarland 0.5 scale and then inoculated in a volume of 100 µL into the column holes 1 through 8, leaving a final concentration of 10^4 CFU/mL. The holes in columns 9, 10, 11 and 12 were intended for the control tests of the experiment. Column 9 was reserved for the sterility control, adding 100 µL of Mueller-Hinton broth and 100 µL of extract. Columns 10 and 11 were reserved for the negative control of the inhibitory activity of the ethanol and DMSO diluent, by adding 100 µL of 10% ethanol solution and 100 µL of 2% DMSO solution in 100 µL of Mueller-Hinton broth and 5 µL of bacterial inocula. The orifice of column 12, reserved for positive control or bacterial viability, was prepared with 100 µL of Mueller-Hinton broth and 100 µL of bacterial inocula.

The microplates were incubated at 30° C for 72 hours, in aerobic condition, and then 20 µl of 1% aqueous solution of 2, 3, 5-triphenyltetrazolium chloride (TTC-Merck, Darmstadt, Germany) was added. These microplates were reincubated for three hours at 30° C, after which time the results were read. The formation of red staining in the orifices was interpreted as absence of antibacterial activity, whereas the non-formation of red staining was considered as presence of antibacterial activity. Each test was performed in triplicate (Ayres et al, 2008).

3. Results and Discussion

3.1 Chromatographic analyses

The chromatographic profiles of the crude ethanolic extracts of the aerial parts of the different *Colletia paradoxa* individuals were compared using as parameters the retention time and the molecular absorption spectrum obtained through the diode arrangement detector. The signals that showed similarity to the monitored parameters for the samples were numbered and are identified in Figure 1.

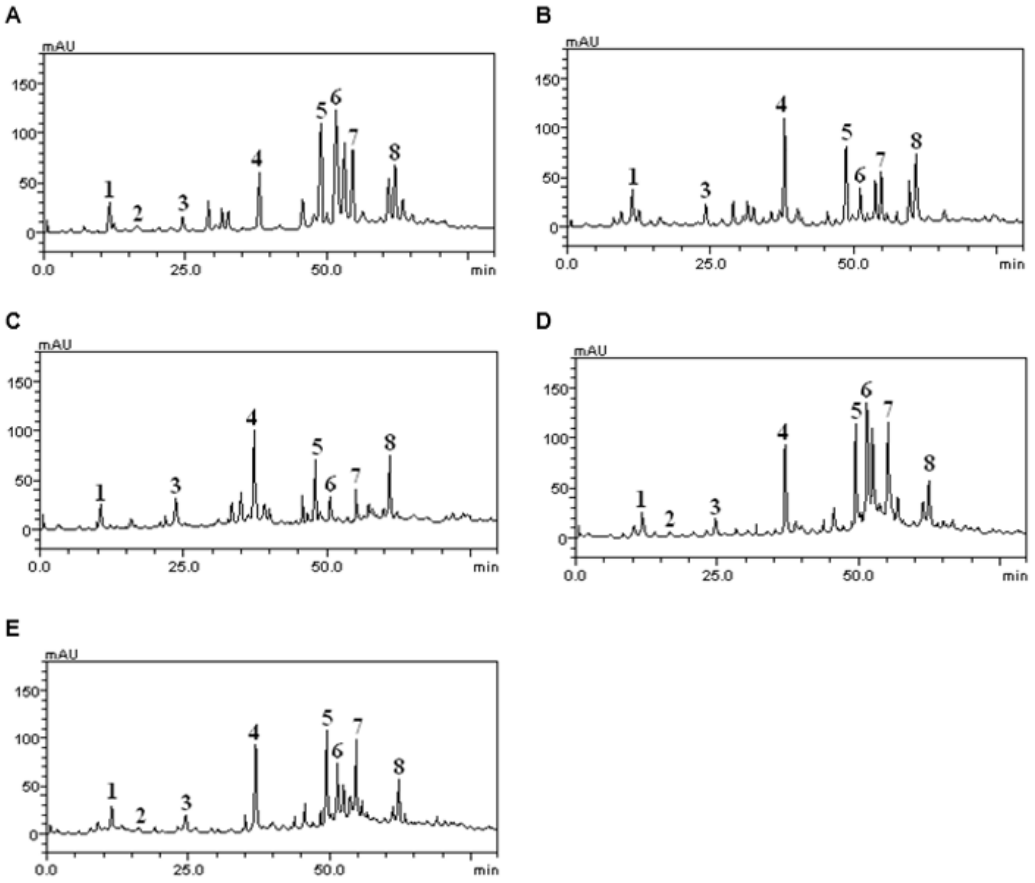

FIGURE 1 - Chromatographic profile representative of the crude extract of *Colletia paradoxa* morphotype "paradoxa" (A: RB 501, B: RB 512); morphotype "exserta" (C: RB 507, E: RB 515) e morphotype "intermediate" (D: RB 508). UV detection was at 327nm. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), quercetin (peak 6), canferol (peak 7) and luteolin (peak 8).

The crude extracts of the samples of the different populations of *C. paradoxa* were analyzed by HPLC and the result of quantification of the phenolic compounds there is presented in Table II. Chromatograms obtained for the extracts revealed the presence of the following compounds: gallic acid (t_R = 10,57 min; pico 1), catechin (t_R = 16,09 min; pico 2), caffeic acid

(t_R = 24,81 min; pico 3), rutin (t_R = 38,15; pico 4), quercitrin (t_R = 48,03 min; pico 5), quercetin (t_R = 51,87 min; pico 6), canferol (t_R = 54,62 min; pico 7) and luteolin (t_R = 63,45 min; pico 8) according to Figure 1 e Table II.

Table II. Phenolic compounds present in the crude extract of different morphotypes of *Colletia paradoxa*, through HPLC-DAD.

Compounds	***RB 501*** ***CP*** **mg/g**	***RB 512*** ***CP*** **mg/g**	***RB 508*** ***CI*** **mg/g**	***RB 507*** ***CE*** **mg/g**	***RB 515*** ***CE*** **mg/g**	**LOD** **µg/mL**	**LOQ** **µg/mL**
Gallic acid	2.35±0.01 a	2.48±0.03 a	1.17± 0.03 a	1.83±0.01 a	1.06±0.02 a	0.025	0.083
Catechin	0.26±0.02 b	-	0.35 ± 0.01 b	-	0.39±0.03 b	0.009	0.027
Caffeic acid	0.87±0.01 c	1.09±0.01 b	0.92 ± 0.01 c	2.62±0.04 b	0.84±0.01 c	0.028	0.095
Rutin	4.95±0.01 d	10.24±0.02 c	8.76±0.02 d	8.41±0.01 c	8.15±0.01 d	0.017	0.053
Quercitrin	10.18±0.03 e	7.26±0.03 d	9.83±0.04 e	5.03±0.03 d	9.63±0.04 e	0.008	0.024
Quercetin	12.56±0.02 f	2.45±0.01 a	12.15±0.01 f	1.79±0.02 a	5.29±0.01 f	0.030	0.099
Canferol	7.05±0.01 g	4.17±0.01 e	9.87±0.01 e	2.58±0.01 b	8.12±0.01 d	0.011	0.037
Luteolin	4.83±0.01 d	7.20±0.04 d	5.01±0.03 g	5.09±0.01 d	5.18±0.01 f	0.016	0.051

Results are expressed as mean of three determinations ± SD (standard deviation). The averages followed by different letters differ by the Tukey test at p <0.05. LOD (limit of detection), LOQ (limit of quantification), CP (*C. paradoxa* morphotype "paradoxa"), CE (*C. paradoxa* morphotype "exserta") and CI (*C. paradoxa* morphotype "intermediate").

Analysing the chromatograms of the samples (Figure 1) it is observed that only catechin could not be detected in all extracts, however is present for the three morphotypes (individuals RB 501, RB 515, and RB 508). The other signals are present in all samples. It was observed that in the extracts of the morphotype "paradoxa" RB 501 presented quercetin (12.56 mg/g) followed by quercitrin (10.18 mg/g) and canferol (7.05 mg/g) , while RB 512 had the highest concentration with rutin (10.24 mg/g) followed by quercitrin (7.26 mg/g) and luteolin (7.20

mg/g). As for extracts from the exserta morphotype, RB 507 was found to have rutin (8.41 mg/g), followed by luteolin (5.09 mg/g) and quercitrin (5.03 mg/g) as its (9.63 mg/g) followed by rutin (8.15 mg/g) and canferol (8.12 mg/g) as major values. On the other hand, the intermediate morphotype RB 508 presented a high concentration of quercetin (12.15 mg/g) followed by canferol (9.87 mg/g), quercitrin (9.83 mg/g). All the compounds present in the extracts, in greater or lesser concentration, have antioxidant action.

Among the flavonoids found, quercitrin was, on average, the one of greater quantity of compounds in the *Colletia paradoxa* extracts, followed by quercetin, rutin, canferol, luteolin, gallic acid, caffeic acid and catechin. Several studies have demonstrated that these compounds have antioxidant properties (Silva et al, 2010; Silva et al, 2015; Merino et al, 2015), confirming the results found in the antioxidant capacity assay of *C. paradoxa* extracts.

The identified tannins were gallic acid and catechin. These compounds can be found in fruits, seeds, bark and leaves of plants and are rich in phenolic groups. They are chemically classified as condensates or hydrolyzable and have pharmacological properties that confer on them antibacterial, anti-inflammatory, antiparasitic and gastroprotective activities (Mello and Santos, 2001).

The antioxidant activity has been observed in several studies on the Rhamnaceae family, but this is the first time that this activity has been checked for *C. paradoxa.* Concerning the composition variation observed between the extracts, we can affirm that there is no significant differentiation between them that could be characterized as in the different morphotypes.

3.1.1 *Total phenolic content and antioxidant capacity*

The antioxidant capacity of the extracts was evaluated by analyzing the ability to sequester the free radicals stable DPPH in the analyzed medium (Figure 2). The IC_{50} values (concentration of the extract capable of sequestering 50% of the DPPH radicals in solution) are shown in Table III, as well as the content of total polyphenols (TP) obtained by the Folin-Ciocalteu method expressed in milligrams equivalent of gallic acid (GAE) per gram of dry plant.

Table III. Total polyphenols and antioxidant capacity of the crude extracts of the morphotypes of *C. paradoxa* samples.

Crude extracts of different samples	**TP ± SD (mg GAE/g)**	**IC_{50} ± SD (µg/mL)**
RB 501 *C. paradoxa* morphotype "paradoxa"	318,26 ± 0,11	94,83 ± 16,93
RB 512 *C. paradoxa* morphotype "paradoxa"	169,56 ± 0,64	515,02 ± 23,60
RB 508 *C. paradoxa* morphotype "intermediate"	169,48 ± 0,38	507,89 ± 29,28
RB 507 *C. paradoxa* morphotype "exserta"	150,99 ± 0,46	211,65 ± 22,70
RB 515 *C. paradoxa* morphotype "exserta"	123,46 ± 1,17	217,53 ± 10,77

Results are expressed as mean of three determinations ± SD (standard deviation). TP = Total polyphenols, GAE/g = equivalent of gallic acid per gram.

The extract with the highest amount of total polyphenols was obtained from the RB 501 access, with 318.26 mg GAE/g and IC_{50} 94.83 µg/mL. Boligon et al. (2012b) found similar values (323.47 mg GAE/g in the butanolic fraction and 141.09 mg GAE/g) in the crude extract of the leaves of *Scutia buxifolia* Reiss. The extracts RB 508 and RB 512 had the second highest amount of polyphenols (169.48 mg GAE / g and 169.56 mg GAE / g, respectively), although they presented higher values of IC50. The other accessions also presented considerable amounts of total polyphenols, but with significantly lower IC_{50} values, with RB 515 having the lowest value of total polyphenols (123.46 mg GAE/g). Although there is a directly proportional relationship between the amount of total polyphenols and the capacity to capture free radicals through the DPPH method (Mustafa et al., 2010; Janovik et al., 2011; Brum et al., 2013), some studies suggest that this does not always occur (Kahkonen et al., 1999). In the extracts of *C. paradoxa* RB 508 and RB 512, although they present the second highest amount of total polyphenols, showing the lowest antioxidant activity (higher IC_{50} values). According to Brum et al. (2013), this can occur due to several reasons such as the presence of different active compounds in the plant that can cause a differentiation in the antioxidant action, synergistic effects of different compounds in each extract. The experimental conditions that can vary and the methods used to perform the reaction and also the fact that the samples come from different physiographic regions. In addition, there are compounds that react rapidly with DPPH, while others have a slower rate reaction. Alviano et al (2008) evaluated the antioxidant activity of

Ziziphus joazeiro, which presented very good results (IC_{50} = 821.4 ± 35.3 µg/mL). The leaf extract (IC_{50} = 461.88 µg/mL) and the extract of the peels (IC_{50} = 1743) were evaluated using the same method, using ethanolic extracts (leaves and peels) of *Z. joazeiro*, 5 µg/mL). Our extracts presented even more promising results, with IC_{50} ranging from 515.02 ± 23.60 µg / mL to 94.83 ± 16.93 µg/mL, confirming the antioxidant potential for the Rhamnaceae family. However, it was not possible to observe a relationship between the different morphotypes and the total number of polyphenols through this assay, since the amount of polyphenols varied significantly even within the same morphotypes.

With regard to the antioxidant capacity of the extracts, it was observed that all the extracts presented antioxidant capacity, but inferior to the control (ascorbic acid). The value of factor ***F*** obtained in the ANOVA calculation was 7.118, being much larger than the ***critical F*** of 2.533, thus indicating a significant difference of the antioxidant action of the extracts in relation to the standard (ascorbic acid). The value of ***p*** = 0.000171 (<0.05) confirms this significance. Using the Tukey test, it was observed that only the RB 501 sample showed no significant difference in relation to the standard, presenting the highest antioxidative potential among the samples evaluated. It was verified that the two accessions of morphotype "exserta" (RB 507 and RB 515) presented similar antioxidant activity, whereas the accessions of morphotype *"*paradoxa*"* presented quite different antioxidant activity, representing the largest (RB 501) and smaller (RB 512) measurements, the latter coinciding with RB 508.

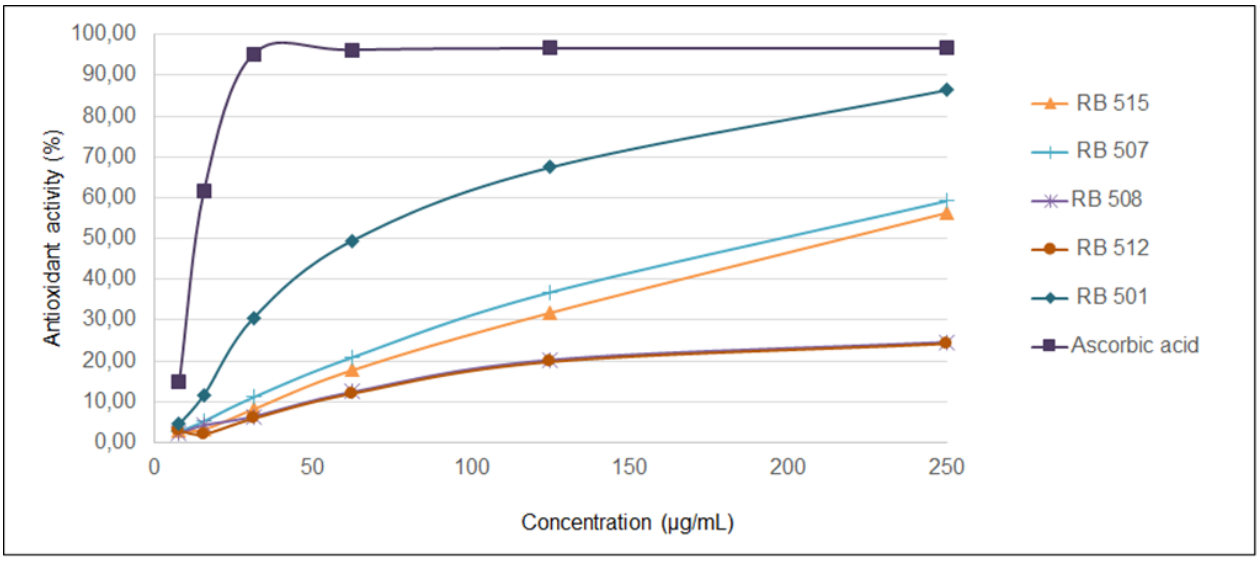

FIGURE 2 – Antioxidant capacity of the crude extracts of the morphotypes of *C. paradoxa* and ascorbic acid (positive standard) by the DPPH method.

3.2. *Determination of Minimum Inhibitory Concentration (MIC)*

MIC values were obtained between 78.12 and 1250 µg/mL (Table IV). The lowest MIC for *Mycobacterium abscessus* was 312.5 µg/mL, for the extract of RB 501, and the highest was 1250 µg/mL, for the extract of RB 508. For *M. fortuitum*, the lowest MIC was 312.5 µg/mL, for the extracts of RB 515 and RB 501, and the highest MIC was obtained for the extracts of RB 507 and RB 508, 1250 µg/mL. For *M. massilense*, the lowest MIC value, 78.12 µg/mL, was obtained with the morphotype "exserta" extract RB 515, while the highest was obtained for another access of the morphotype RB 507, with MIC 625 µg/ml (Table IV).

Table IV. Determination of the Minimum Inhibitory Concentration (MIC) of the crude extracts of the different morphotypes of *C. paradoxa*.

EXTRACT	*M.abscessus* (µg/mL)	*M. fortuitum* (µg/mL)	*M. massiliense* (µg/mL)
RB 515 morphotype "exserta"	625	312,5	78,12
RB 507 morphotype "exserta"	625	1250	625
RB 508 morphotype "intermediate"	1250	1250	312,5
RB 512 morphotype "paradoxa"	625	625	312,5
RB 501 morphotype "paradoxa"	312,5	312,5	312,5
Clarithromycin	16*	1	32*

*Resistance index. RB: colletion number of N. Rolim Bastos.

Giacomelli (2005) demonstrated that the hexane, dichloromethane and ethyl acetate extracts from the aerial parts of *C. paradoxa* presented significant antimicrobial activity for *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Klebsiella pneumoniae, Salmonella setubal* and *Escherichia coli*, with values of MIC ranging from 6.2 to 50 mg.

Quercitrin and quercetin were considered the major components in the variations of *C. paradoxa* investigated. Similar phytochemical profile was also observed in the Boligon et al analyses (2012b), which evaluated the chemical composition of the crude extract and different fractions of *Scutia buxifolia* (Rhamnaceae). In addition, it evaluated the antimicrobial activity against *Mycobacterium smegmatis, M. tuberculosis* and *M. avium* verifying that the flavonoids

present in the plant are the chemical components involved to a greater degree for the observed antimycobacterial activity (Boligon et al, 2012a).

It is suggested that the potential action of flavonoids against components of the genus *Mycobacterium* may be involved in their ability to inhibit enzymes involved in the biosynthesis of fatty acids, mainly mycolic acids, a major component of the mycobacterial cell wall. Considering the chemical structure of flavonoids, it is suggested that their lipophilic character allows these compounds to penetrate with less difficulty in the mycobacterial cell, since the extremely lipophilic character of the cell wall of the mycobacteria is a prominent factor when studying the resistance presented by these microorganisms, giving them physical resistance to antimicrobial agents and biocides. In addition, the enzyme fatty acid synthase II (FAS-II) has no action in the presence of the oxygen present in the cyclic structure that is part of the chemical structure of these flavonoids (Boligon et al, 2012a; Brown et al, 2007).

According to the classification proposed by Kuete (2010), the activity of crude extracts is considered significant if MIC values are lower than 100 μg/mL, moderate between 100-625 μg/mL or low when MIC> 625 μg/mL. From this, it can be observed that, in general, the crude extracts obtained from the leaves and branches of *C. paradoxa* showed moderate inhibitory activity against the tested microorganisms (Table IV). The extract RB 515 morphotype "exserta" presented a significant value for *M. massiliense* (78.12 μg / mL), but also presented moderate value for *M. fortuitum* (312.5 μg / mL) and low value for *M. abscessus* (625 μg / mL). Thus, it can be considered that the best result was from the RB 501 morphotype "paradoxa" extract, which showed moderate values for the three *Mycobacterium* species. Thus, further trials evolving the investigation of the antimicrobial activity of the extracts should be performed, *in vitro* and *in vivo*¸ to determine their potential use as an anti-infective agent. Despite this, there are not enough differences in MIC values capable of characterizing and separating the species studied.

Finally, we encourage the continuity of the studies to evaluate phytotherapeutic and toxicological action of *C. paradoxa*, to determine the safety of its use and the feasibility of its application in the pharmaceutical area and other areas.

4. Conclusions

It was verified that extracts of *C. paradoxa* morphotype “paradoxa” (RB 501 and RB 512), morphotype “exserta” (RB 507 and RB 515) and morphotype “intermediate” (RB 508), have significant amounts of phenolic compounds and consequently, a considerable antioxidant potential, probably due to the presence of quercitrin, rutin, quercetin, canferol, luteolin, gallic acid, caffeic acid and catechin that were identified in extracts by HPLC.

These results suggest that *C. paradoxa*, because it presents this amount of compounds, can be considered as potential sources of natural antioxidants. Also, due to the presence mainly of quercitrin and quercitin, moderate extractive activity was observed in inhibition of the growth of *Mycobacterium abscessus*, *M. fortuitum* and *M. massiliense*, suggesting a potential antimicobacterial activity of the evaluated extracts, mainly in the RB 501 morphotype "paradoxa" extract.

Conflicts of interest

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

The authors would like to thank PhD Aline Auguste Boligon for collaborating in the analyses of the extracts of *Colletia paradoxa*.

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3.2 MANUSCRITO 2[2]

Circumscription of *Colletia paradoxa* (Colletieae, Rhamnaceae): morphological and molecular evidence

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Abstract

Often the circumscription of a taxon based only on morphology can become a challenge due to the great morphological variability presented by certain plant groups. This happens with *Colletia paradoxa* and its possible synonym, *C. exserta.* Some researchers consider them as a single species while others prefer to keep both valid. Thus, we sought to evaluate the delimitation of *Colletia paradoxa*, concerning its variation (morphotypes) through the morphological analyses of the individuals collected in the field and those in herbarium collections, combined with the molecular analyses. Twenty-four samples were included in the molecular approach, collected in different municipalities of Rio Grande do Sul and one from Santa Catarina State, representing the typical morphological types of *C. paradoxa* and morphotypes corresponding to *C. exserta* concept, as well as individuals with intermediate morphology. Four ISSR primers were selected, which produced 64 amplified DNA fragments for 24 individuals, all polymorphic. From these results, a binary matrix of presence and absence was produced, which was converted into a Nexus file for analyses in the PAUP* 4.0 software. Genetic similarity dendrograms were generated with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean average) and *Neighbor-joining* methods. ISSR dendrograms resulted in genetic clusters mixing morphotypes. Finally, we observed that the morphological state of characters, such as size and shape of the thorns, hairiness of the branches and shape of the floral tube, overlap in both species, resulting in a wide diversity of shapes and sizes in the different populations and even in the same individual. These observations corroborate the results of the molecular analyses in such a way that the combination of these data suggests that the synonimization of *C. exserta* and *C. paradoxa* should be accepted, with *C. paradoxa* circumscription in a broader sense.

Key words: Molecular markers, Polymorphism, Quina, Genetic similarity, Taxonomy.

[2] Este manuscrito foi submetido ao periódico *PHYTOTAXA*, o qual está formatado de acordo com as normas exigidas pela citada revista.

Introduction

Rhamnaceae is a family with about 52 genera and 900 species (Stevens, 2001) of cosmopolitan distribution, occupying the tropical, subtropical and temperate areas. They exhibit a wide variety of habits from trees, shrubs, climbing sub-bushes and herbs. Rhamnaceae is a monophyletic family and is very close to Dirachmaceae and Barbeyaceae (Richardson *et al*, 2000a).

Colletia is an exclusive genus of South America, where five species occur, *C. spinosissima* Gmelin (1791: 408), *C. ulicina* Gillies *et* Hooker (1829: 155), *C. hystrix* Clos (1846: 32), *C. paradoxa* (Sprengel 1825: 219) Escalante (1946: 219, *C. spartioides* Bertero ex Colla (1834: 52) (Loefgren, 1917, Suessenguth, 1953, Johnston & Freitas Soares, 1972, Tortosa, 1989, Lima & Giullietti, 2014). Two of these species occur in Brazil, *C. paradoxa* e *C. spinosissima*.

Colletia paradoxa occurs in Southeastern Brazil, Uruguay and Argentina (Tortosa, 1989). They are isolated shrubs, agglomerates, or trees up to 5m high and are characterized by a large number of spinescent branches, with deciduous leaves, small flowers, usually without petals, with nectariferous disk coating the receptacle, the edge free and revolute on the inner lower third of the floral tube. They occupy a diversity of environments and can be found in the countryside, roadside, on the edge of riparian forests or in the interior of small fields (Sobral et al, 2006). Living on shallow, rocky, well-drained soils or on deep soils, on the banks of rivers, streams, or on weathered soils near streams.

Colletia paradoxa is known as a medicinal plant (popularly known as "quina-cruzeiro" or "espinho-de-cruz" in Brazil) used in rural areas as purgative, antispasmodic, and antithermal due to its tonic and febrile properties. It is mentioned the use of its wood as firewood of excellent quality and in the woodwork industry; its branches and roots are rich in saponins (Tortosa, 1989, Marzocca & Marthi, 1951). In addition, because of their unique appearance, *Colletia* species generally present great potential for use as hedges (Marzocca & Marthi, 1951) and as ornamental (grown in Europe, United States and Argentina) (Tortosa, 1989). *Colletia paradoxa*, as well as other species of the genus, is able to fix the atmospheric nitrogen in the symbiotic nodules of the roots, through actinomycetes of the genus *Frankia*. Because of this, these plants

can grow on soils of low nutrient condition (D'Ambrogio & Medan, 1993, Tortosa, 1988, Medan & Tortosa, 1976).

The concept of Lima & Giullietti (2014) on the circumscription of *C. paradoxa* and *C. exserta* (which they accept as a separate species) is not adopted by all taxonomists. Some authors have suggested that this is a synonym for *C. paradoxa* (Tortosa, 1989., 2000; Richardson et al, 2000b, 2004; Aagesen et al, 2005; Zuloaga, 2008). Collections with intermediate characteristics are common to the typical patterns of these taxa, and considering that both names appear in lists of endangered species (Martinelli & Moraes, 2013, FZB-RS, 2014), it is necessary to define the most appropriate circumscription for this pair of species, helping to define their geographical distribution and, consequently, conservation policies.

Morphological characters, as well as biochemical and cytological characters, are used as descriptors to identify variations within the same species (Rossi *et al,* 2014), regarding the shape or phenotypic pattern of a particular characteristic. However, because they are influenced by environmental conditions, they become limited as a marker. Thus, when it is not possible to identify a plant through morphological evaluation, one can resort to DNA sequence analyses. However, for species that have more recently diverged, traditionally used DNA sequences will not always present adequate phylogenetic signal. In these situations, more polymorphic molecular markers are sought (Rossi *et al,* 2014). In addition, it is possible to use several techniques such as biochemical and molecular, based on the analyses of polymorphism of enzymes and DNA fragments through the process of obtaining a specific band pattern for a given species or in the identification of cultivars. This unique set of bands that characterizes the species is called fingerprinting, and is equivalent to a fingerprint (Souza-Chies *et al,* 2014).

Band standards can also be used to assess the genetic variability in natural populations or to access evolutionary history (Rossi *et al,* 2014, Rivas *et al,* 2013, Brandão *et al,* 2011). Several molecular biology techniques can be used for this purpose, but the most commonly used are AFLP (Amplified fragment length polymorphism), microsatellites, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter simple Sequence Repeat). However, some of these techniques have limitations such as low reproducibility, high cost and need of prior knowledge of the sequences for the production of specific primers (Reddy *et al,* 2002, Souza-Chies *et al,* 2014).

In general, markers such as ISSRs, which are derived from nonspecific primers, are widely used in studies of genetic diversity due to their high polymorphism, low cost, abundance

in the genome, with the advantages of not needing prior information about the genome, especially when it comes to native plants. They are relatively easy to apply to studies with various plant species (Pharmawati *et al*, 2004, Souza-Chies *et al*, 2014, Chagas *et al*, 2015), and may be useful also combined with additional evidences of other nature, as other molecular data or morphological data. The combined analyses is widely used, in which the morphological characteristics obtained in previous research, analysed together with the molecular data, give a consistent support to the results (Richardson *et al*, 2000a; Richardson *et al*, 2000b, Kellerman *et al*, 2005, Aagesen *et al*, 2005, Kellerman & Udovicic, 2008).

A morphological study was carried out, complemented with analyses of ISSR markers, in order to evaluate two circumscriptions classically presented in previous works: *C. paradoxa lato sensu* (including *C. exserta*) or *C. paradoxa stricto sensu*, taking *C. exserta* as another species. In order to simplify the text, since there are intermediate morphologies included, all specimens are referred as *C. paradoxa*. The distinction between the potentially different taxa is done using the term "morphotype". Specimens which fit in the *C. exserta* concept are referred as morphotype "exserta", *C. paradoxa stricto sensu* is referred as morphotype "paradoxa" and specimens with intermediate morphology between these two taxa are referred ad morphotype "intermediate". Sampling considered mainly morphology, and diversity of physiographic regions in Southern Brazil.

Thus, the objective of this study is to evaluate the delimitation of *Colletia paradoxa*, concerning its variation (morphotypes) through the morphological analyses of the individuals collected in the field and those in herbarium collections, combined with the molecular analyses.

Material and Methods

Study taxon

For morphological analyses and molecular analyses, specimens of *Colletia paradoxa* with diverse morphology were sampled*:* morphotype "paradoxa" (corresponding to *C. paradoxa stricto sensu),* morphotype "exserta" (corresponding to *C. paradoxa lato sensu)* and morphotype "intermediate" (corresponding to specimens partially similar to both taxa).

Sampling and molecular biology protocols

The material used in this study was obtained through field collections in nine localities of seven municipalities of the states of Rio Grande do Sul and one of Santa Catarina, belonging to different physiographic regions (Figure 1), in a total of 24 individuals. Leaves or epidermis and cortex of young branches (when there was scarcity of leaves), were used. They were stored in silica gel immediately after collection. For each location, a voucher was stored in the SMDB herbarium (Universidade Federal de Santa Maria - UFSM).

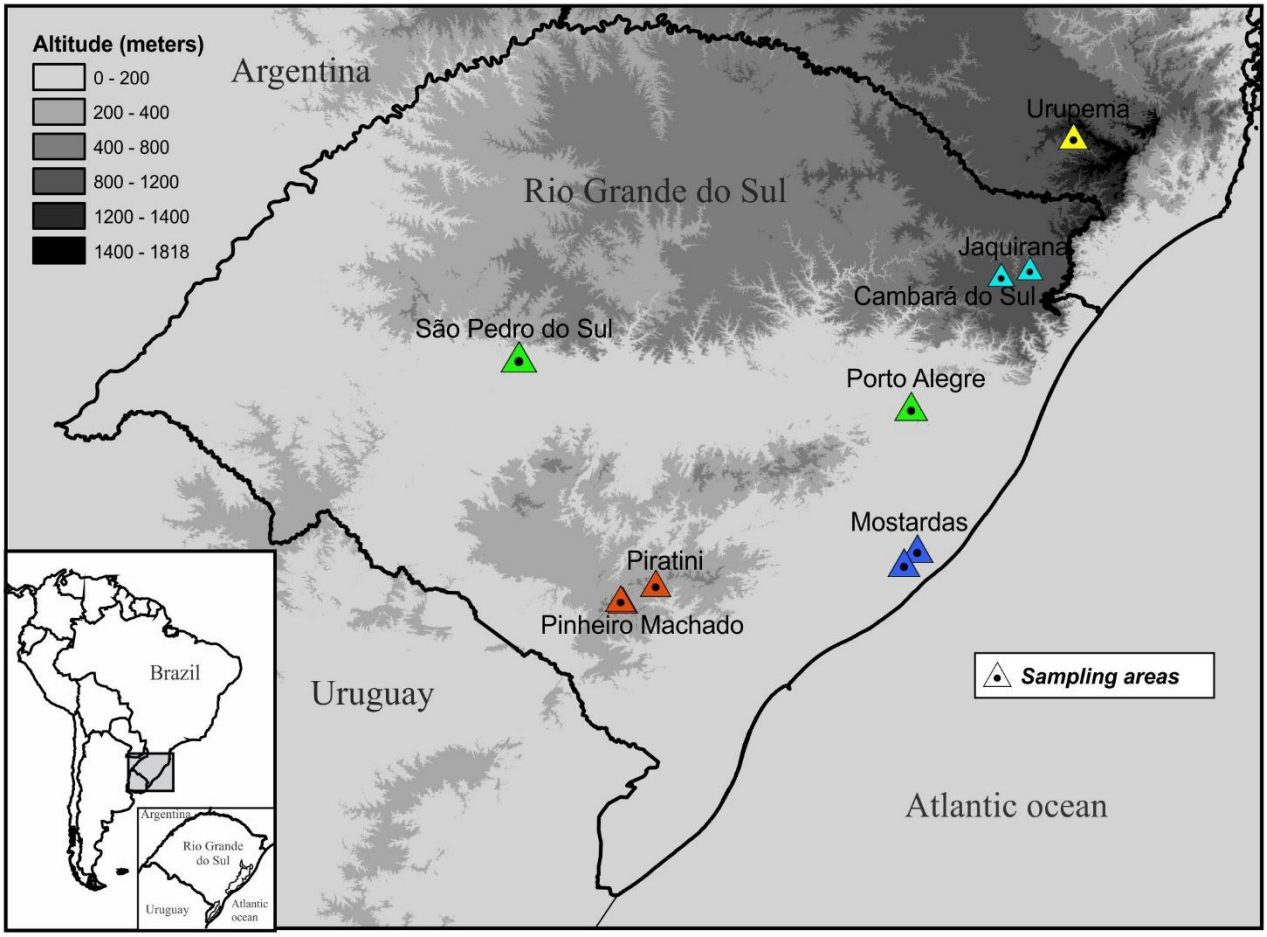


FIGURE 1. Localities of collection of *C. paradoxa* and and its morphotypes in the states of Rio Grande do Sul and Santa Catarina, Brazil.

The collections were authorized by IBAMA - SISBIO/CGEN. The collection sites and Vouchers are presented in Table 1.

After drying on silica, the leaves or branches were weighed (30 mg-50 mg) and macerated in liquid nitrogen until powder state, from which the DNA was extracted. Total DNA extraction followed the protocol adapted from Doyle & Doyle (1987) adapted for microcentrifuge tubes. Samples were evaluated and quantified by 0.8% agarose gel electrophoresis, stained with Safer

Dye or GelRed and compared to the standard of known amounts of Phage λ DNA (25, 50, 100, 200, 482 ng). After the assay, aliquots were prepared with standard concentrations (20-25 ng of DNA). The samples were stored at -20 °C. We used the ISSR markers for the evaluation of genetic similarity.

TABLE 1. Accession code, investigated accessions, morphological type of the species in the field, municipality and physiographic region where they were collected, geographic location and Voucher number.

Accession code	**Investigated accession**	**Morphological type of *C. paradoxa***	**Locality**	**Physiographic Region**	**Coordinates**	**Voucher**
	ISSR					
RB 501	-	morphotype "paradoxa"	Piratini, RS	SS	S 31° 21' 5,61 W053° 03'23,57"	SMDB 16913
RB 506	506	morphotype "paradoxa"	Porto Alegre, RS	CD	S 30º 03' 6.07'' W 051º 10' 37.95''	SMDB 16925
RB 507	-	morphotype "exserta"	Porto Alegre, RS	CD	S 30º 03' 6.07'' W 051º 10' 37.95''	SMDB 16918
RB 508	508A, 508B, 508C, 508D, 508E, 508F, 508G	morphotype "intermediate"	São Pedro do Sul, RS	CD	S 29º 37' 14'' W 054º 10' 44''	SMDB 16914
RB 512	512A, 512C	morphotype "paradoxa"	Pinheiro Machado, RS	SS	S 31°33'13,38" W 053°24'42,39"	SMDB 16915
RB 513	513A, 513B, 513C, 513D, 513E	morphotype "paradoxa"	Pinheiro Machado, RS	SS	S 31°33'37,29" W 053° 24' 6,48"	SMDB 16916
Li 977	-	morphotype "paradoxa"	Pinheiro Machado, RS	SS	S 31° 36' 00,3" W 053° 24' 20,7"	SMDB 16927
RB 514	514A, 514B, 514C	morphotype "intermediate"	Jaquirana, RS	SPG	S 29° 01' 4,17" W 050° 15'31,73"	SMDB 16 917
RB 515	515C, 515D, 515E	morphotype "exserta"	Cambará do Sul, RS	SPG	S 29° 1' 00.16" W 50° 08' 30.94"	SMDB 16918
Li 934	934A, 934C, 934F	morphotype "intermediate"	Urupema, SC	SC	S 27°57'18,45" W 49°52'38,86"	SMDB 16926

RB: acronym and collection number of N. Rolim Bastos; Li: acronym and collection number of Liliana Essi; (-) = Amostras de DNA não amplificadas. Physiographic regions according to Fortes (1956), SS: South-Rio-Grandense Shield; CD: Central Depression; SPG: Southern Plateau Grasslands; SC: Urupema, Santa Catarina State. SMDB: Herbarium of the UFSM Botanic Garden.

Analyses of genetic similarity with ISSR

The 24 samples were obtained from different municipalities of Rio Grande do Sul and Santa Catarina, representing different morphotypes, as explained previously (Table 1). The amplification of the ISSRs were performed by PCR reactions in a final volume of 25 μL [containing 20-25 ng of total DNA, 0.25 μL Taq DNA Polymerase (5U/μL), 2.3 μL $MgCl_2$ (25 mM) 5 μL of 10 × buffer, 1 μL primer 10 pmol, 1 μL of 40 mM dNTPs mixture (each dNTP at 10 mM), 1 μL DMSO (2%), and sterile ultrapure water]. The amplification was performed in a Minicycler thermocycler in 40 cycles of 1 min at 94 °C, 45 sec at 50 °C and 2 min at 72 °C, preceded by a 5 min cycle at 92 °C and completed with a final extension cycle of 5 min at 72 °C. For the amplification of the fragments, 15 primers were tested, of which four were selected since they presented an efficient reprodutibility. They are: P3, F3, P4 and O4 (Table 2).

PCR products were separated on 1.5% agarose gel and stained with Safer Dye or GelRed. The electrophoresis was carried out for 2 hours and 30 minutes at about 60 mA or 73 V and 4W. The result was visualized in UV transilluminator and photographed with digital camera, for later analyses of the patterns and assembly of the binary matrix. The size of the amplified fragments was estimated by comparison to the molecular marker of DNA Ladder 100 pb (Ludwig Biotec Brazil).

In an Excel spreadsheet, a unified matrix was produced with the patterns of the four primers selected for the 24 DNA samples, which was converted into a Nexus file for analyses in the PAUP* 4.0 software (SWOFFORD, 2003). Dendrograms of genetic similarity were generated with the UPGMA (Unweighted Pair-Group Method with Arithmetic average) and *Neighbor-joining* methods, all characters with the same weight, using midpoint posterior rooting.

Analyses of morphological descriptors

The plants collected for the molecular analyses and those samples that were already incorporated in the collections of the herbaria HDCF, HUCS and SMDB were measured. In

addition, we considered the measurements obtained in material of the herbaria PACA, ICN, HAS and FLOR (Table 4 appendix) resulting from analyses of the material described in the article of Bastos-Záchia and Moraes (1999). The following morphological characteristics were observed: leaf size, color of branches and garment, characteristics of the spinous branches, thickness, length and width (at the base) of the spines, shape of the calicine tube. We also analysed the photos of the *Colletia* types of the herbaria W, F, K, MEL, as well as the information system of basic data of scientific collections, Species Link http://www.splink.org.br/index?lang=pt and Tropicos Database https://www.tropicos.org/Name/27501050, for comparison of the observations made in the field material.

Results

Analyses of genetic similarity with ISSR

The four initiators used amplified 64 DNA fragments, all of which were polymorphic. The highest number of bands was generated by primer P3 (21 bands), and the lowest number of bands was generated by the primer F3 (Table 2). The amplified fragments ranged from 250 bp to 2000 bp and had a mean number of 16 loci per primer.

TABLE 2. Primers used, their sequences, references, number of bands and maximum, average and minimum size of bands per primer.

Primer code	Sequence	Reference	Number of bands	Size of the bands (pb) Max. (Medium) Min.
P3	$(CTC)_4$ RC	Poulin, Weller & Sakai, 2005 (*as* n.15)	21	2000 (786) 250
P4	$(CT)_8G$	Joshi *et al*, 2000 (*as* 815)	18	2000 (1236) 500
F3	$(AG)_8YC$	Joshi *et al*, 2000 (*as* 835)	10	1200 (640) 400
O4	$(AC)_8C$	Lee *et al*, 2003 (*as* UBC 826)	15	1.000 (663) 300

From a unified matrix with the four primers, in a total of 64 characters and 24 terminals we obtained two dendrograms, one generated by UPGMA (Figure 2) and another by Neighbor Joining (Figure 3). The dendrogram generated by UPGMA recovered two main clusters (Cluster 1 and Cluster 2).

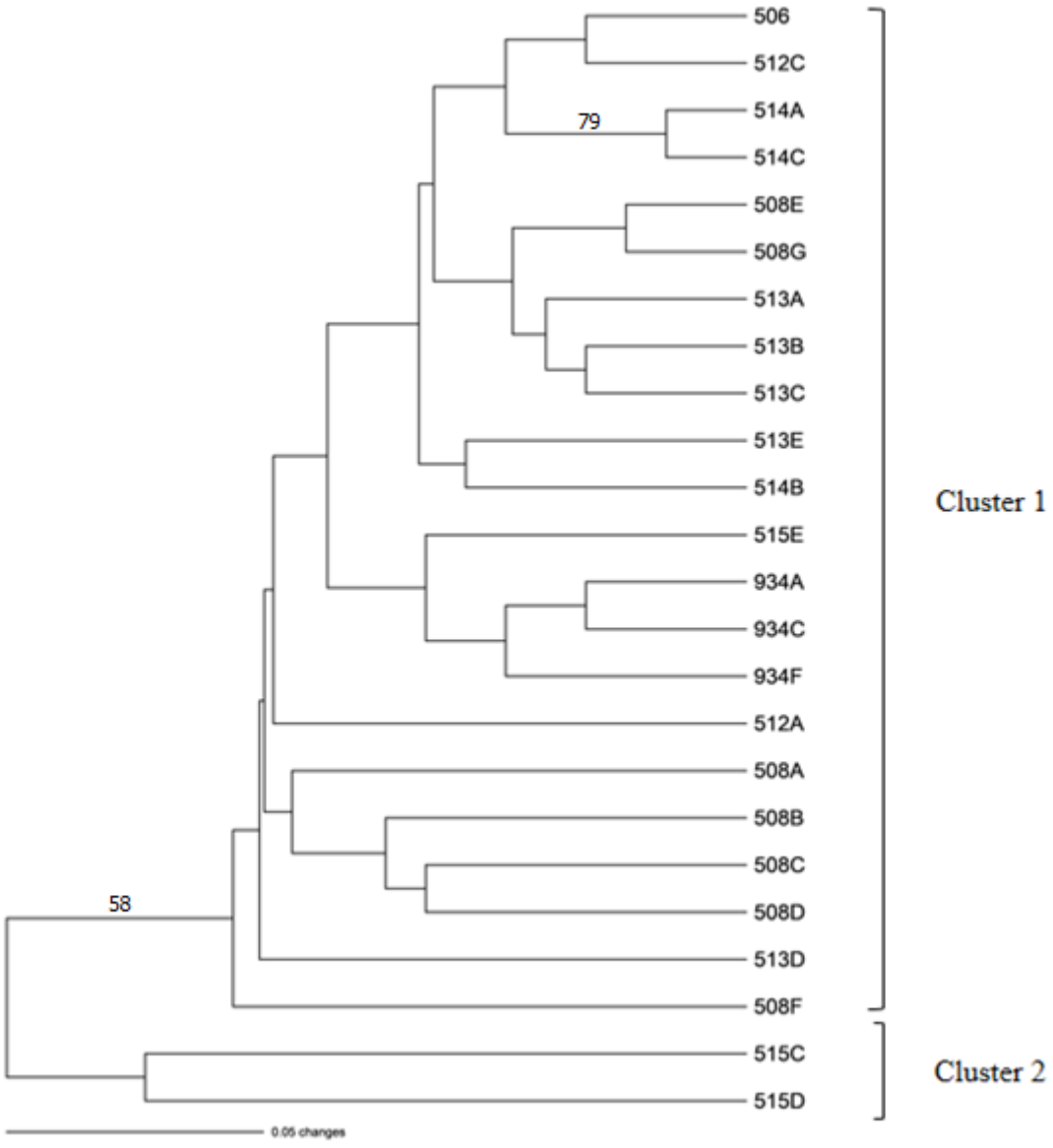


FIGURE 2. Dendrogram generated by the UPGMA method from a 64-character ISSR data matrix, highlighting two main Clusters. Numbers above branches correspond to bootstrap values > 50.

Cluster 2, formed by two individuals of the population from Cambará do Sul (515C and 515D), represents the morphotype "exserta", being the most differentiated cluster of the dendrogram and thus forming a separate cluster. However, an individual from the same locality (515E) appears in another distant group (Cluster 1), together with a morphotype "intermediate" (934A, 934C and 934F) from Urupema, Santa Catarina. The second major group (Cluster 1) is formed by all the other individuals, where one representative of São Pedro do Sul (508F, "intermediate"), and another from Pinheiro Machado (513D, "paradoxa") presented the second highest value of genetic differentiation, followed by the individual 512A that also formed a separate cluster in the dendrogram. However, the other individuals of the Cluster 1 represent different sites and morphotypes varied between *"*paradoxa*"* and *"*exserta*"*, not being evident neither grouping by geographic distance nor by morphology.

The dendrogram generated by Neighbor Joining showed six main clusters (Figure 3). When we evaluated the genetic relationships among the 24 individuals sampled, using the genetic distance grouping method Neighbor Joining, we could observe that the dendrogram has a division into two main groups, one formed by clusters 1, 2, 3, 4, and 5, and another further, here numbered as Cluster 6. Cluster 6 groups individuals from Cambará do Sul (515C, 515D, and 515E), all from the same locality and who have fewer representatives with intermediate morphological characteristics. In the other set of clusters (1 to 5), we observe the grouping of individuals from Urupema, Santa Catarina in Cluster 5. Despite the grouping in geographic terms, Cluster 5 has individuals with intermediate characteristics. The other individuals analyzed are distributed between clusters 1 to 4. Cluster 4 has an individual from Pinheiro Machado together with another one from Jaquirana, both presenting "paradoxa" and "intermediate" morphotypes. Cluster 3 was attended by almost all the representatives of São Pedro do Sul, which are considered individuals of intermediate characteristics, since they mix characteristics of the two morphotypes. Cluster 2 brings together individuals from the same locality, Pinheiro Machado, with great morphological variability, presenting characteristics of the two morphotypes and a representative of São Pedro do Sul (morphotype "intermediate"). Cluster 1 has individuals of *C. paradoxa* morphotype "paradoxa" of Pinheiro Machado, together with individuals from the population of Jaquirana, "intermediate" morphotypes.

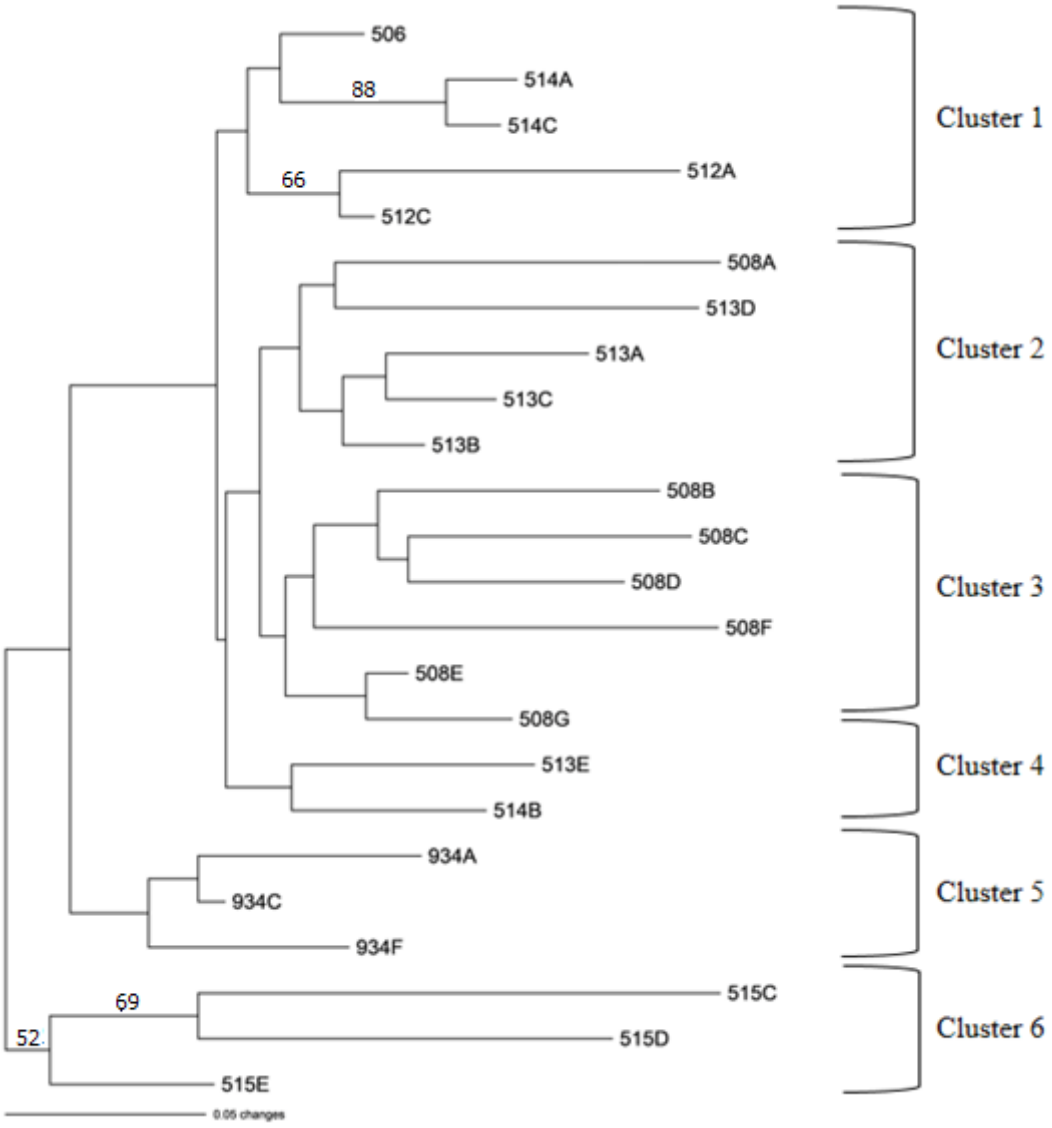


FIGURE 3. Neighbor Joining Dendrogram, generated from a 64-character ISSR matrix, emphasizing six main clusters. Numbers above branches correspond to bootstrap values > 50.

Morphological analyses

Photos of specimens from the *C. paradoxa* and *C. exserta* collection were examined, and more than 50 specimens were analyzed in detail, which are described on Table 3.

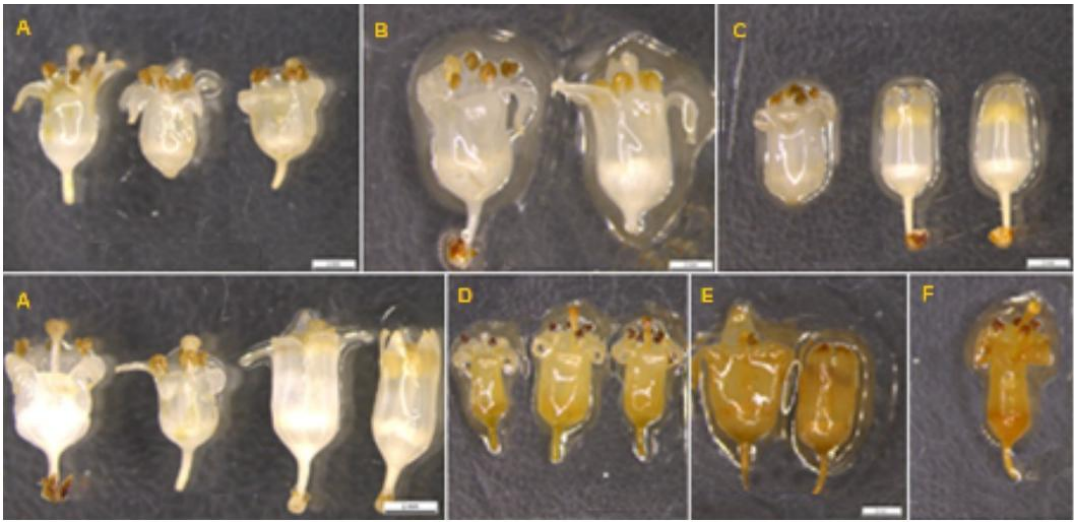
TABLE 3. Comparison between morphological characteristics observed for each species. Vouchers are shown in parentheses.

OBSERVED CHARACTER	***C. paradoxa* morphotype "paradoxa"**	***C. paradoxa* morphotype "exserta"**	***C. paradoxa* morphotype "intermediate"**
Measurement of leaves	(2,5) 5,0-7,5 (9,0) mm length	(4,0) 9,0-14,0 mm length	4,0-10,0 mm length
Color of the branches	Green-gray	Olive green	Variable, olive to greyish
Characteristics of the branches	Patent thorns, broad triangular, thick, dense grayish pubescence (Fig. 6 left)	Thorns thin, some narrow triangular, others acicular, delicate, ascending, olive color (Fig. 6 right)	Thorns thin, some narrow triangular, others acicular, ascending, olive green, delicate. Thorns patent, narrow triangular, strong, dense grayish pubescence (Fig. 7) Branches with great morphological variation and intermediate forms. Wide and opposite spines although thin in thickness, pubescent or glabrous
Thorn Thickness	Thick, ca. 2.0 mm, wide triangular	Thin, delicate, less than or equal to 1,0 mm thick	Thin in thickness, 1.0-1.5 mm in thickness and wide triangular, other fine, delicate, upward
Length of spines	(0,7) cm 2,5-4,5 (5,5) cm	0,8-6,0 cm	0,8-5,0 cm
Width of base of the spines	4,0-17,0 mm	1,0-8,0 mm	0,7-12,0 mm
Indumento	Smooth grayish pubescence (Fig. 6 left)	Branches without pubescence (Fig. 6 right)	Branches glabros, even in those similar to *C. paradoxa* morfotipo "paradoxa" or slight pubescence to almost glabrous (Fig. 5)
Form of the calicine tube	Urceolate to tubules (Fig. 4A)	Tubules to campanulas Tubules, small (Fig. 4E)	Tubules to urceolate or campanulate Button tubing and dilated at disk height (Fig. 4)

***C. paradoxa* morphotype "paradoxa"**: **SMDB** 16922 (Fig. 5), 16923, 16927A, **HUCS** 32820, 36039.

***C. paradoxa* morphotype "exserta"**: **SMDB** 16912, 16918, **HUCS** 38652, 13378, 19904, 15559, 19904, 39875, 13378.

***C. paradoxa* morphotype "intermediária"**: **SMDB** 6136, 2510, 10197, 16913, 16915, 16915A, 16916, 16917, 16926B, 16926C, 16926G, 16926H, 16926i, **HUCS** 1228, 7937, 15559, 39875, 1337.



. **FIGURE 4.** Detail of the morphological variability of the calicine tube in different individuals of *Colletia*. **A.** SMDB 16921 **B.** SMDB 16916 **C.** SMDB 16913 **D.** HUCS 39875 **E.** SMDB 16915 **F.** HUCS 13378. Fotos produzidas no Laboratório de Anatomia Vegetal, do Prof. João Marcelo dos S. Oliveira.

***Colletia paradoxa* morphotype "paradoxa"** is characterized by spinescent branches usually covered by dense and tenuous grayish hairiness (sometimes lacking). Its thorns are thick, strong, patent and lax. However, they may have secondary branches that sprout at the base and / or upper branches, exhibiting great morphological variability, including narrow, delicate or strong spines and even acicular, patent or ascending, glabrous or hairy spines. The flowers also present variability in the shape of the calyx tube that can vary from urceolate to tubulose (Fig. 4). Examples of these characteristics are the SMDB 16913 exsicate (Fig. 5).

***Colletia paradoxa* morphotype "exserta"** is characterized by olive green, glabrous, ascending, delicate and lax branches as in the HUCS 39875. From these branches can appear sprouts similar to the mother plant as well as showing branches with thorns patent, triangular, thicker and with slight pubescence, as observed in the SMDB 16917 specimen of the Jaquirana city. Also in the specimen SMDB 16912 and HUCS 15559 these characteristics of the morphotype "exserta" appear interspersed with eventual pubescence of the branches and flowers ranging from 3.0 to 5.0 mm in length and tubular calicine tube to urceolate. The variation in flower size is important because it shows that there is no discontinuity in this characteristic, between the morphotypes "paradoxa" and "exserta".

***Colletia paradoxa* morfotipo "intermediário"** are individuals that present characters either the morphotype "exserta" or the morphotype "paradoxa" or even a combination of these characteristics. Thus, we can observe in a single individual, branches with narrow, delicate, glabrous, ascending or not, while others are patent, wide triangular, covered by fine gray hairiness and flowers larger than those of the "exserta" morphotype. There are also features mixed as strong, narrow spines with grayish pubescence or broad thorns, patent, delicate and glabrous or pubescent as we can see in the examples SMDB 16915 and 16926; HUCS 1228 and 7937. The pilosity of the branches should be observed with caution because this characteristic is very variable, being able to present some pubescent branches and others completely glabrous, in the same individual (Tortosa, 1989). This characteristic morphological variability presented by the individuals collected in Pinheiro Machado city (SS) was also observed in the populations of Mostardas city (CP) where, despite of different regions, individuals with characteristics of the three morphotypes occur.

FIGURE 5. *Colletia paradoxa* on environment, Piratini, SMDB 16913 (RB 501), RS.

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FIGURE 6. Overview of the variability morphologic of *Colletia paradoxa*, SMDB 16922, to the left. SMDB 16920, on the right. Photos by Renato Záchia.

The question about *C. exserta*, whether or not it is synonymous with *C. paradoxa*, was first debated by Tortosa (1989), who discussed and sustained *C. exserta* synonimization in favor of *C. paradoxa*, considering that specimens with spines narrow and arched – one of which Reissek (1861) used to describe *C. exserta* – was a simple morphological variation, possibly due to environmental factors.

The differentiation proposed by Lima and Giulietti (2014), it can be stated that the observations made in the field, through examination of the herbarium material consulted, and type photographs do not confirm the characteristics proposed by the authors, for the maintenance of the species *C. exserta*,

due to the great morphological variability presented by individuals from different regions of Rio Grande do Sul and southern Santa Catarina, which were collected and analysed by us (Fig. 7). Thus, at the end of the analyses, we did not observe discontinuity in the characteristics that justified the maintenance of both species.

We respect the position of other authors regarding the validity of *C. exserta* species and suggest new research, perhaps using other types of molecular markers. However, after analyzing all these samples and comparing them with the results of the molecular analyses, we consider that the arguments used to maintain the validity of the *C. exserta* species become so fragile that they become insufficient to justify, characterizing clearly and undoubtedly, the existence of this species.

Thus, the revised description of *Colletia paradoxa*, taken in its broad sense, follows.

FIGURE 7: Overview of *Colletia paradoxa* in vegetative stage, evidencing differences in the morphology of the branches (SMDB 16919). A. Patent branches; B. Patent and thin branches; C. Thin branches. Photos by Renato Záchia.

Colletia paradoxa (Sprengel) Escalante

M.G. Escalante, *Boletín de la Sociedad Argentina de Botánica* 1 (3): 219. 1946.

Basonym: *Condalia paradoxa* Spreng. Syst. Veg. 1: 825. 1825. Isotype photograph: Montevidéu (Uruguai), 06.II.1962, Sello (MEL!, W!).

Colletia cruciata Gillies *et* Hooker Bot. Misc. 1: 152, tab. 43. 1829 (1830). Photograph: Ad. Maldonado (Uruguai), 28.IV.1820, Gillies *s.n*. (K!)

Colletia bictonensis Lindl. J. Hort. Soc. London 5: 31, 1850.

Colletia exserta Klotzsch ex Reissek in Martius, Fl. Bras. 11(1): 100. 1861.

Photograph: Brasilia (Brasil), *s.d.*, Sello 4268 (5849 F!)

Shrubs may reach arboreal size, spinescent, apparently subphylactic, olive-green sometimes grayish, pubescence variable from pubertal to almost glabrous. **Branches** spinescent cylindrical or compressed at the base, pubescent or glabrous, ending at a sharp apex, pungent with dark color, lax thorns, decussate, narrow to triangular, flat, forming an angle of 35º to 90º with the branch from which they depart, from 0.7-5.5 (6.0) cm long and (2.0) 4.0-10.0 (15.0) mm wide. **Leaves** reduced, opposing or subopposite, decussed, stipulated, deciduous, petiolate, in the axillary of spines, elliptic, narrow elliptic or ovate when well developed, whole or with toothed margin, trinerved or peninerved, with (2.5) 5.0-8.0 (11.0) mm of length and acute or emarginated apex, always mucronate, with attenuated base. Stipules 2, small triangular, hairy, persistent. Two sprouts in each leaf axilla, the superior ones originate thorns or spiniform twigs, the inferior ones, flower brachiblasts. Apical buds of brachiblasts eventually originating macroblasts. **Flowers** solitary or fasciculate, axilla of thorns, tetrameric or pentameric, perfect, actinomorphic, glabrous, greenish white. Urceolate or tubular calicine tube, (3.0) 3.8-4.0 (7.0) mm long and 2.0-3.5 (4.0) mm wide, circumcised near the base, deciduous after anthesis. Lacinia 4-5, triangular, reflex, with pronounced median nerve on the adaxial side, (1.0) 1.5-2.5 (3.0) mm long, flower pedicel with 1.5-2.0 (3.0) mm in length. Devoid of petals. **Stamens** 4-5, intrusive, exserted, inserted into the throat of the floral tube, between the teeth of the chalice. Bithecous, sintecas, intrusive, 0.5-0.7 mm long, with a loci, horseshoe-shaped anthers. **Nectariferous disk** covering the bottom of the tube, annular on the lower third of the internal floral tube, with free edges, revolute, fleshy. **Gynoecium** 3 (2-4) carpelar. **Ovary** semi-inferior, conical-globose, glabrous, often trilocular, 3-carpelar, with a commotion in the basal part. Solitary, erect, anatropous ovules. **Estiylus** cylindrical, 2.7-3.7 mm long, included, reaching the throat of the tube or exsert, sometimes, divided into the distal

portion. Trilobate stigma. **Fruit** dry, tricoco, with elastic dehiscence, 4.5-5.0 (6.0) mm long. **Seed** triquetric, flattened, with endosperm, unilocular, 2.0 to 3.5 mm long.

Analysed specimens:—BRAZIL. Rio Grande do Sul: **Bagé**, Casa de Pedra, 01 July 1991, *J. Gianchin & I. Fernandes 909* (PACA); Estrada Bagé-Pinheiro Machado**,** 10 July 1974**,** *A.M. Girardi & B. Irgang s.n.* (ICN 26736); **Bom Jesus**, 16 January 1942, *B. Rambo s.n.* (PACA 9030); Águas Brancas Farm, near Rio dos Touros, 03 February 1991, *J. Larocca s.n.* (PACA 70802); Cilho's Farm, on edge of forest, 23 November 2002, *R. Wasum 1636* (HUCS), Boqueirão's Farm, on the edge of forest, 13 February 1998, *Rossato & Wasum s.n.* (HUCS 13378), Rio Pelotas, 22 February 1952, *A. Sehnem 5837* (PACA, HUCS); **Cambará do Sul,** Road near Jaquirana 04 January 1978, *M. Fleig 889* (ICN); Arvoredo's Farm, 06 May 1984, *S. Miotto 943* (ICN); At the roadside, 19 March 2000, *R. Wasum 527* (HUCS), Canyon Fortaleza, Aparados da Serra, 08 October 2003, *J.N.C. Marchiori & L.P. Deble s.n.* (HDCF 5717); Capão Penso, Mr. Portássio Tittoni's Farm, 10 April 2016, *N.R. Bastos-Záchia e R. Záchia, 500* (SMDB); **Canguçu**, Alto da Tuna, 05 June 1986, *A.Alvarez Filho s.n.* (SMDB 2510); 15 km from Canguçu, side of the road to Piratini, 11 October 1972, *J.C. Lindman s.n. et al.* (ICN 2667); 500m off the limit of the road to Piratini – Canguçu, 09 April 1991, *N.R. Bastos 97 et al.* (PACA); Canguçu, rocky outcrop at roadside, 24 November 2006, *J.N.C. Marchiori s.n.* (HDCF 5711); Canguçu, rocky outcrop at roadside, 24 November 2006, *J.N.C. Marchiori & S.R. Santos s.n.* (HDCF 5713); **Caxias do Sul**, 44 km W from Lageado Grande, 08 February 1994, *Eggli Labhart & Hillmann 2527* (PACA); **Jaquirana,** 20 February 1952, *B. Rambo s.n.* (PACA 52017); On the edge of the ciliary forest, 27 February 2013, *M. Grizzon 192* (HUCS); Cascata dos Venâncio**,** 09 April 2016, *N.R. Bastos-Záchia & R. Záchia 514* (SMDB); **Mostardas**, Balneário Mostardense, Trilha das Dunas, 22 April 2016, *N.R. Bastos-Záchia & R. Záchia 517* (SMDB); Balneário Mostardense, Pai João locality, 22 April 2016, *N.R. Bastos-Záchia & R. Záchia 518* (SMDB); Lagoa Barros-Velho, about a sambaqui, 09 January 2008, *R. Wasum 4376* (HUCS); **Pedras Altas**, Pinheiro Machado park, 20 July 1985, *J.N.C. Marchiori s.n.* (HDCF 1786); **Pinheiro Machado**, BR 293, 3 Km after entrance of the city, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 512* (SMDB); BR 293, 1.4 km of the entrance to Pinheiro Machado, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 513* (SMDB); BR 293, Km 97, from Piratini towards Pinheiro Machado, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 520* (SMDB); BR 293, 500 m from the Km 97 plate, from Piratini towards Pinheiro Machado, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 521* (SMDB); BR 293, close to Km 98, Piratini towards Pinheiro Machado, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 522* (SMDB); earth road in RS 608, between Pedras Altas and Piratini, 16 October 2015, *L. Essi, 977* (SMDB); **Piratini,** 09 August 1960, *J.K. Amaral 02* (PEL);

100 m from the limit between Piratini and Canguçu, 09 April 1991, *N.R. Bastos & R. Wasum 96* (PACA); Venda de Lata**,** Mr. Pedico's Farm, 30 April 2016, *N.R. Bastos-Záchia & R. Záchia 501* (SMDB); **Porto Alegre**, Morro da Polícia, 03 June 1980, *O. Bruno 2546* (HAS); Morro da Polícia, 19 October 1933, *B. Rambo s.n.* (SMDB); Morro da Polícia, 26 August 1939, *Ir. Augusto s.n.* (ICN 18707); Morro da Polícia, 12 January 1991, *N.R. Bastos & P. Vargas 192* (PACA); Morro da Polícia, 19 May 1933, *B. Rambo s.n.* (PACA 391); Morro da Polícia, June 1952, *B. Rambo s.n.* (PACA 52796); Morro da Polícia, 03 June 1980, *L. Aguiar & L. Martau 470* (HAS); Morro da Polícia, August 1960, *A.R. Schultz 2375* (ICN); Morro da Polícia, May 2009, *M. Grings & V. Camejo 768* (HUCS); Zoobotanical foundation of Rio Grande do Sul, 15 April 2016, *N.R. Bastos-Záchia 506* (SMDB); Zoobotanical foundation of Rio Grande do Sul, 15 April 2016, *N.R. Bastos-Záchia 507* (SMDB); **São Francisco de Paula**, February 1948, *B. Rambo.* (PACA 36361); On edge of forest, 10 October 2011, *C.A. Marchett 106* (HUCS), Passo da Ilha, 23 February 1980, *L.A.B. Ferreira s.n.* (ICN 47046); **São José dos Ausentes**, close to Silveira water stream, bush at the roadside, 27 October 2006, fr., *J.N.C. Marchiori s.n.* (HDCF 5712); **São Pedro do Sul**, Cerro Itaquatiá, 17 April 2016, *N.R. Bastos-Záchia 508* (SMDB); **Vacaria,** Ronda's Farm, 10 Octuber 1947, *B. Rambo s.n.* (PACA 35012). Santa Catarina: **Araranguá**, Morro dos Conventos, 15 November 1971, *J.C. Lindman & M.L. Porto s.n.* (ICN 9128); **São Joaquim**, Eco Tourism Coffee Farm, 11 October 1992, *N.R. Bastos s.n.* (PACA 72161); São Joaquim, Pericó District, 05 December 2003, sterile with buds, *L.P. Deble & A.S. Oliveira s.n.* (HDCF 5718); **São José dos Ausentes**, near Silveira water stream, bushes with Araucária, side of the road, 27 October 2006, *J.N.C. Marchiori & S.R. Santos s.n.* (HDCF 5719); **Tainhas**, 20 August 1960, *L.R.M. Baptista s.n.* (ICN 2376); **Urubici**, Vacas Gordas, 07 April 1991, *G. Hatschbach & Barbosa 55320* (HUCS); **Urupema**, Cedrinho, side of the road, at the entrance of Pousada Recanto das Araucárias, 03 May 2015, *L. Essi 934, V.B. Fortes, E.S. Muller, et al.* (SMDB). URUGUAY – Santa Teresa Park, 05 March 1973, *B. Irgang s.n.* (ICN 21661).

Discussion

The great morphological variability already mentioned by Tortosa (1989), and that motivated this study, was confirmed in *C. paradoxa* specimens observed in the field (Fig. 6).

The individuals found in the region of Piratini and Pinheiro Machado are characterized by grayish branches, with broad, thick, lax and patent thorns. We often find shoots, especially in the upper branches, maintaining the same aspect of the adult plant. However, it is very common to find budding branches in the lower part of these plants (or sometimes in the upper branches) that are very different

from the mother plant, presenting narrow, thin, olive-green, ascending, sometimes acicular and leafy thorns, evidencing a great morphological variability of this species, even in the same individual. These observations were also confirmed by Santos (2008). The specimens from São Pedro do Sul present branches with thick but narrow and ascending thorns, considered in this study as examples of morphotype "intermediate".

The comparison between morphotypes evidenced an overlap of the morphological characteristics, as shown in Table 3. It is noteworthy the occurrence of characteristics described for *C. paradoxa* and *C. exserta* in branches of the same individual, as it can be observed in the specimen SMDB 2510, that shows broad, with tenuous gray hairiness, patent, thick branches; and presents flowers with campanulate or urceolate calicine tube, as well as delicate, narrow and ascending branches. In the same way, we can observe this variation in a specimen (NY 533381) collected by L.B. Smith, in São Joaquim, SC, which presents narrow and delicate spines but sometimes ascending, sometimes patents. Another specimen that showed such variability was collected in São Joaquim (HUEFS 208518), with thorns in varying slope, from patents to upwards, of little thickness, and varying in the greyish aspect of the branches.

Conclusion

The ISSR markers generated polymorphic state characters as expected, allowing the recognition of small genetic subgroups. These groups, however, only reaffirm the great diversity already observed at morphological level. Therefore, the molecular data do not distinguish the species *C. paradoxa* and *C. exserta*, in the same way that the morphological analyses was not able to distinguish such taxa.

The comparison between *C. paradoxa stricto sensu* and *C. exserta* (*sensu* Lima and Giulietti, 2014) and intermediate forms showed a continuity of the morphological characteristics, as presented in this article. Morphological characters such as the size and shape of the thorns, hairiness of the branches and shape of the floral tube are characteristics that overlap in both species, characterizing a wide diversity of shapes and sizes in different populations and even in the same individual.

These observations corroborate the results of the molecular analyses through of Neighbor joining method (Fig. 3) in a way that the combination of these data suggests the synonimization of *C. exserta* under *C. paradoxa*. Molecular data do not refute this.

Therefore, *C. exserta* is considered as synonymous with *C. paradoxa*, since we did not observe discontinuity in the morphological and molecular characteristics that justify the maintenance of the two species separately.

Acknowledgements

The authors thank Prof. Dr. Jean Carlos Budke for the elaboration of the map, to Prof. Dr. João Marcelo Santos de Oliveira for the availability of the laboratory and the guidelines in the elaboration of the photos with magnifying glass and to Francisco Rossatto Neto for the adjustment of the image and also to the curators of the herbaria consulted and to the colleagues Larissa Schumacher, Vanise Uberti, Bruno Moscato Soares and Jessica Mena Barreto de Freitas by collaborating in the bench activities.

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APPENDIX

TABLE 4. List of consulted herbaria.

Herbarium Acronym	Herbarium Name
F	Field Museum of Natural History, U.S.A. Illinois. Chicago.
FLOR	Herbarium Flor Federal University of Santa Catarina, Florianópolis SC
HAS	Herbário Alarich R.H. Schultz, Museu de Ciências Naturais da FZB RS
HDCF	Herbário do Departamento de Ciências Florestais, UFSM, RS
HUCS	Herbário da Universidade de Caxias do Sul, UCS, Caxias do Sul RS
ICN	Herbário ICN do Instituto de Biociências, UFRGS, Porto Alegre,RS
K	Royal Botanic Gardens, U.K. England, Kew
MEL	Royal Botanic Gardens Victoria, Australia, Victoria, Melbourne
PACA	Herbarium Anchieta, IAP-UNISINOS, São Leopoldo, RS
SMDB	Herbário do Departamento de Biologia, UFSM, Santa Maria, RS
W	Herbarium, Department of Botany, Naturhistorisches Museum Wien.

3.3 MANUSCRITO 3

Genetic Diversity and Population Structure of *Colletia paradoxa* (Rhamnaceae) a medicinal species threatened with extinction in Brazil

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Abstract

In order to obtain a better knowledge about the endangered species *C. paradoxa* (Rhamnaceae), which appears in fragmented populations, a study was carried out with six populations from different physiographic regions of Rio Grande do Sul and Santa Catarina states, Brazil. The objective of this study is to investigate the real conditions of genetic diversity in populations, through the use of ISSR molecular marker, and to evaluate the distribution of genetic diversity among and within populations, in order to plan conservation and management strategies. Five selected primers produced 112 DNA fragments in 46 samples from individuals that were evaluated. The result of the analyses showed 100% polymorphism. The Nei's genetic diversity index (H) was 0.3083. The Shannon's information index (I) was 0.4880. The Bayesian inference of clustering performed by Structure revealed the most likely K= 4 to *C. paradoxa*. The analysis of molecular variance among all populations showed that the greatest

genetic diversity occurs within the populations (82%), while among them there is less differentiation (18%), although in some populations good levels of genetic migration occur (Nm = 1.0465) indicate that genetic changes among populations of *C. paradoxa* are still occurring.

Keywords: Genetic conservation. Genetic diversity. ISSR markers. Morphotypes. Quina.

1. Introduction

Colletia paradoxa (Sprengel) Escalante (Rhamnaceae), is a South American, species with occurrence, in southern Brazil. The individuals of *C. paradoxa* are shrubs or rarely small trees, with xeromorphic characteristics, and can be found in a great diversity of environments. It is a selective, hygrophilic and heliophyte species (Johnston & Freitas Soares, 1972). It occurs in Rio Grande do Sul and Santa Catarina, being able to reach Paraná state (Brazil). In spite of its use as an ornamental plant in other countries, in Brazil it is known only in the rural region where it was widely used as a medicinal plant, recognized as purgative, antispasmodic and antipyretic due to its tonic and febrile properties. The populations of *C. paradoxa* identified up to now in Rio Grande do Sul are fragmented and composed of a number of isolated or agglomerated individuals, and in Santa Catarina also it presents a wide dispersion, but it is non-expressive, discontinuous and irregular (Johnston & Freitas Soares, 1972). It is considered endangered in Brazil (Martinelli & Moraes, 2013), and there is not any study about the genetic diversity of natural population so far. According to Souza-Chies et al. (2014), there is still a large gap in knowledge about phylogenetic relationships and levels of genetic diversity in the broad sense for native species, despite the great diversity of Brazilian Flora. Both genetic diversity and gene flow reveal the percentage of polymorphism between different populations and individuals providing information about the evolutionary and adaptive relationships of species to adverse or threatened environments, helping to understand the reason and process of the threat mechanism (Chai et al, 2014, Yang et al, 2016).

In this study we used the molecular marker of the ISSR fingerprinting type, because they are semi-arbitrary, amplified by PCR in the presence of a complementary oligonucleotide for a given microsatellite, of wide distribution along the genome and without the need for prior knowledge of the genome (Souza-Chies et al. 2014, Reddy et al, 2002; Rossi et al, 2014), mainly because it is a native species not yet characterized genetically.

The objective of this study is to verify the patterns of genetic diversity between and within populations, of *C. paradoxa* and how it affects the structure of these populations, through the use of

ISSR markers. We aim to contribute to the knowledge of the real conditions of the populations so that conservation programs can be implemented for this species.

2. Material and Methods

2.1. Plant material

Forty-six samples of *Colletia paradoxa* were collected from seven municipalities in Rio Grande do Sul and one municipality in Santa Catarina State, Brazil (Fig. 1). The natural populations of Rio Grande do Sul are fragmented and belong to different physiographic regions (Fortes, 1956). CD: Central Depression; SS: South-Rio-Grandense Shield; SPG: Southern Plateau Grasslands; CP: Coastal Plain. The location and size of the populations is given in Table 1. These are populations that vary greatly in size, so that the number of representatives sampled in each collection site varied from one to nine. To avoid collecting clones, a minimum distance of ten meters between sampled individuals was observed. Specimens were grouped according to the physiographic region, threated as five *a priori* populations.

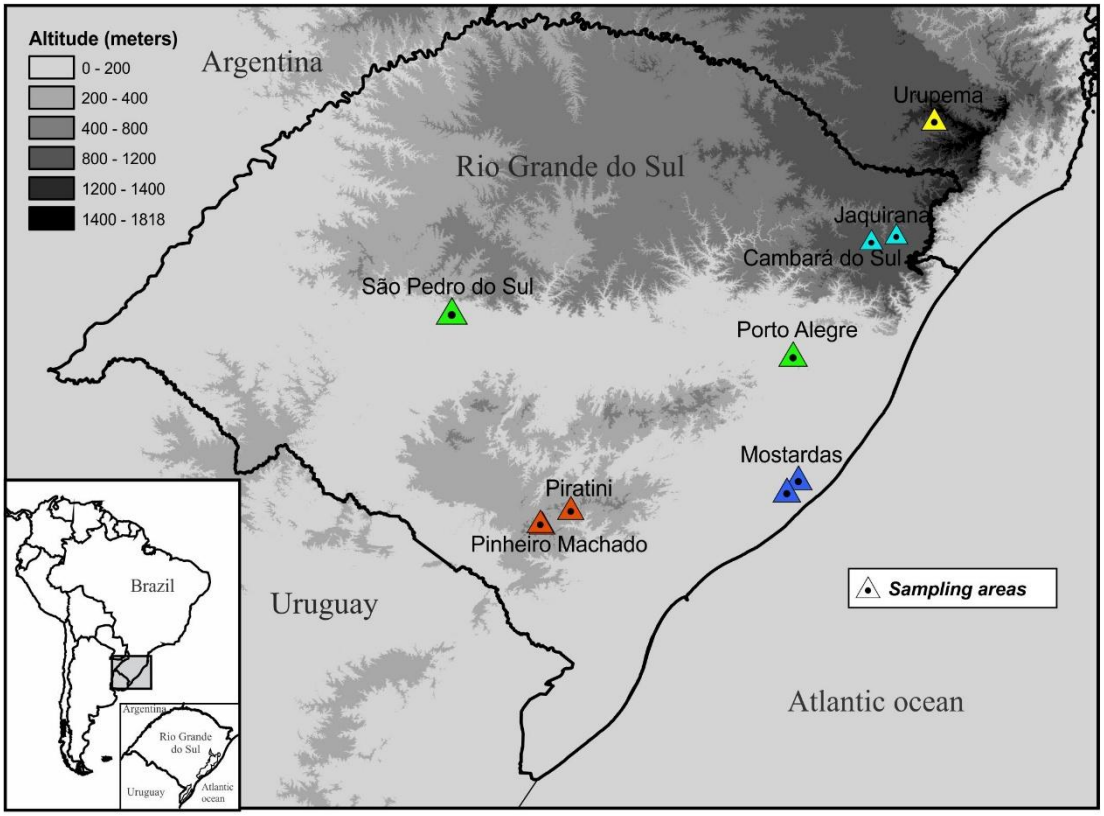

Figure 1 - A map showing the sampling sites of *C. paradoxa*. Different collors represent distinct physiographic regions. Green: CD- Central Depression, Red: SS- South-Rio-Grandense Shield, Light Blue: SPG- Southern Plateau Grasslands, Dark Blue: CP- Coastal Plain; Yellow: Urupema, Santa Catarina State.

Table 1 - Locations and sampling number of natural populations of *C.paradoxa*, and its corresponding Voucher.

Access	Pop. Code	No. of samples	Latitude (N)	Longitude (E)	Region	City/State	Voucher
506	1	1	30°03’6,1’’S	051°10’37,9’’W	CD	Porto Alegre, RS	SMDB 16925
507	1	1	30°03’6,07’’S	051°10’37,95’’W	CD	Porto Alegre, RS	SMDB 16918
508	1	8	29°37’14’’S	054°10’44’’W	CD	São Pedro do Sul, RS	SMDB 16914
501	2	1	31°21'5,6”S	053°03’23,7”W	SS	Piratini, RS	SMDB 16913
512	2	3	31°33’13,4”S	053°24’42,4”W	SS	Pinheiro Machado, RS	SMDB 16915
513	2	5	31°33’37,3”S	053°24’6,5”W	SS	Pinheiro Machado, RS	SMDB 16916
521	2	1	31°36’50,5”S	053°17’09,4”W	SS	Pinheiro Machado, RS	SMDB 16922
977	2	2	31°36’00,3”S	053°24’20,7”W	SS	Pinheiro Machado, RS	SMDB 16927
514	3	4	29°01’4,17”S	050°15’31,73”W	SPG	Jaquirana, RS	SMDB 16917
500	3	1	29°01’00,2”S	050°08’30,9”W	SPG	Cambará do Sul, RS	SMDB 16912
515	3	5	29°01’00,1”S	050°08’24,4”W	SPG	Cambará do Sul, RS	SMDB 16912
934	4	9	27°57’18,4”S	049°52’38,9”W	SC	Urupema, SC	SMDB 16926
517	5	2	31°06’39,5”S	050°52’09,7”W	CP	Mostardas, RS	SMDB 16919
518	5	3	31°03’21,2”S	050°48’49,8”W	CP	Mostardas, RS	SMDB 16920

CD- Central Depression; SS- South-Rio-Grandense Shield; SPG- Southern Plateau Grasslands; CP- Coastal Plain; SC - Urupema, Santa Catarina State.

2.2. DNA extraction

Samples were obtained from leaves, or epidermis of young shoots when there was a shortage of leaves, which were stored on silica gel immediately after collection. A voucher was deposited at the SMDB herbarium (Federal University of Santa Maria), for each sampled locality. Genomic DNA was extracted from 30-50mg of samples by the adapted CTAB method (Doyle & Doyle, 1987). Total DNA

was quantified by 0.8% agarose gel electrophoresis and standardized aliquots (20-25ng of DNA) were prepared, which were stored at -20ºC until the moment of use.

2.3. PCR reaction conditions and ISSR analyses

Fifteen primers were tested, of which five (P1, P3, P4, F3 and O4) were shown to be efficient producing clear and polymorphic bands which were used in the ISSR analyses of this study (Table 2). For the amplification of the ISSRs, PCR reactions were performed in final 25µl volumes, containing 20-25ng of total DNA, 0.25µl Taq DNA Polymerase (5U / µl), 2.3µl $MgCl_2$ (25mM), 5 µl of 10 × buffer, 1 µl primer 10 pmol, 1 µl of 40 mM dNTPs mixture (each 10 mM dNTP), 1 µl DMSO (2%), and sterile ultrapure water. The amplification was performed in a Minicycler thermocycler in 40 cycles of 1 minute at 94° C, 45 seconds at 50° C and 2 minutes at 72° C, preceded by a 5 minutes cycle at 92° C and completed with a final extension cycle of 5 minutes at 72° C. The annealing temperature was 50° C for all selected primers. PCR products were separated on 1.5% agarose gel, stained with Safer Dye or GelRed. The electrophoresis ran for two hours and 30 minutes at about 60 mA or 73 V and 4W. The result was visualized in UV transilluminator and photographed with digital machine, for later analyses of the standards and assembly of the binary matrix. The size of the amplified fragments was estimated by comparison to the molecular marker of DNA Ladder 100pb (Ludwig Biotec Brazil).

2.4. Analyses of molecular data

The amplified fragments of ISSR were encoded in a binary matrix as present (1) or absent (0), considering only well defined bands as valid. From this matrix, data matrices were generated for GenAlEx 6.5 programs (Peakall & Smouse, 2012) and STRUCTURE (Pritchard et al, 2000).

Genetic diversity analyses of populations PCoA and Molecular Variance Analyses (AMOVA) were calculated through the GenAlEx 6.5 Software (Peakall & Smouse, 2012), using matrices of each primer separately, without inclusion of the missing data. Each matrix contained five (P4, P3) or four populations (primers F3, O4 and P1). In addition, analysis with STRUCTURE and POPGENE were performed using unified matrix with data from the five selected primers (P1, P3, P4, F3 and O4), including missing data for populations with low number of amplified individuals (eg Population 5, from Mostardas) and the seven collection sites that make up the five populations previously considered.

The software POPGENE 1.31 (Yeh et al, 1999) was used to calculate the statistics of the genetic diversity parameters of each population considering that they are in Hardy-Weinberg equilibrium, were

percentage of polymorphic loci (%), effective number of alleles (Ne), number of alleles (Na), Nei's genetic diversity index (H) (Nei, 1973), Shannon's information index (I), genetic differentiation coefficient (Gst), based on the cophenetic matrices.

The study of the population structure of *C. paradoxa lato sensu* was carried out through a Bayesian cluster analyses with the purpose of estimating the number of existing populations through the Structure v. 2.3.4. (Pritchard et al, 2000). The analyses was performed using the Evano et al. (2005) method, with 100.000 Markov Chain Monte Carlo (MCMC) simulations and 10.000 burn-in repetition period. The choice of the *K* value was performed using the Structure Harvester software (Earl & von Holdt, 2012) using the logarithms of the data probabilities in order to infer the best *K* through the *DeltaK* statistic (Evanno et al, 2005). The *K* values were tested from 1 to 16 with 20 independent interactions. An analysis of molecular variance (AMOVA) was performed to verify the distribution of genetic variation between and within populations. In addition, it was calculated population genetic differentiation and between clusters (G_{ST}) and gene flow among populations, which was estimated from the $Nm = 0.5 (1- G_{ST}) / G_{ST}$ equation (McDermott & McDonald, 1993), according applied in POPGENE v. 1.31. The Mantel test (Mantel, 1967) was implemented by the MXCOMP routine in the NTSYS-pc program, version 2.2K, with 1,000 random permutations.

3. Results

3.1. *Genetic diversity of Colletia paradoxa*

The five selected primers produced for 46 individuals of *C. paradoxa* a total of 112 bands, all polymorphic. The amplified fragments varied from 250 to 2.100 bp, with average of 851pb and the number of bands amplified per primer ranged from 15 (F3) to 32 (P1) with a mean of 21.8 bands per primer (Table 2).

The genetic parameters of *C. paradoxa* revealed very different genetic diversity results between the six sample populations (Table 3). The number of polymorphic fragments ranged from 49 to 104 with a mean of 84.6 polymorphic fragments per population, with a total of 100% polymorphism. The Shannon's information index (I) varied from 0.2961(±0.3601), for CP, to 0.6342 (±0.2944), for SPG, with a mean of 0.4880 (±0.3139) for all populations. Nei's genetic diversity index (H) ranged from 0.1907 (±0.2286), for PC to 0.3875 (±0.1799), for SPG, with a mean of 0,3083 (±0.2010), for all populations. The population SPG (3) presented the highest diversity index and the highest percentage of polymorphism (92.86%). High (H) values indicate that there is 19% to 38.7% of heterozygosis

within populations. The highest number of alleles observed (Na) in these populations was 2.3750 (±0.6170) per population (3-SPG), while the highest number of effective alleles (Ne) was 1.7819 (±0.4927) per population. The population that presented the highest number of effective alleles (Ne) was population 2 (SS) followed by population 3 (SPG) with 1.7787 (±0.5280), while the lowest values (Ne) occurred in populations 5 (CP) and 4 (SC), 1.3686 (±0.4873) and 1.5044 (±0.4759), respectively (Table 3).

Table 2 - Primers selected, their sequences and references, number and size of the polymorphic bands. The annealing temperature (TA) used for all primers was 50°C.

Primer	**Sequence**	**Reference**	**Number of bands**	**Size of the bands (pb)** **Max. (Medium) Min.**
P1	ACACACACACACACACT	Lin *et al*, 2005 (*as* 25)	32	2.100 (867) 300
P3	CTCCTCCTCCTCRC	Poulin, Weller & Sakai, 2005 (*as* n.15)	30	2.080 (877) 250
P4	CTCTCTCTCTCTCTCTG	Joshi *et al*, 2000 (*as* 815)	16	1.700 (1150) 500
F3	AGAGAGAGAGAGAGAGYC	Joshi *et al*, 2000 (*as* 835)	15	1.800 (710) 300
O4	ACACACACACACACACC	Lee *et al*, 2003 (*as* UBC 826)	16	1.000 (653) 300

Table 3 - Statistical variation of measures of genetic diversity of populations of *C. paradoxa*.

Population	Size	Na	Ne	H	I	Nº Polymorphic bands	Polymorphic percentage (%)
Pop 1 – CD	10	2.0893 ±0.5624	1.5489 ±0.4177	0.3071 ±0.1808	0.4881 ±0.2616	99	88%
Pop 2 - SS	12	2.1429 ±0.6826	1.7819 ±0.4927	0.3855 ±0.2031	0.5955 ±0.3219	93	83%
Pop 3- SPG	10	2.3750 ±0.6170	1.7787 ±0.5280	0.3875 ±0.1799	0.6342 ±0.2944	104	93%
Pop 4 - SC	9	1.8661 ±0.6778	1.5044 ±0.4759	0.2709 ±0.2129	0.4262 ±0.3315	78	70%
Pop 5 - CP	5	1.5536 ±0.6954	1.3686 ±0.4873	0.1907 ±0.2286	0.2961 ±0.3601	49	44%
Average	**9.2**	**2.0054 ±0.6470**	**1.5965 ±0.4803**	**0.3083 ±0.2010**	**0.4880 ±0.3139**	**84.6**	**75%**

(Na) observed alleles; (Ne) number of effective alleles; (H) Nei's gene diversity index; (I) Shannon's information index.

3.*2 Genetic relationships among Colletia paradoxa populations*

The coefficient of gene differentiation across the entire population was 0.3233 ($G_{ST=}$ 0.3233), meaning that there is a 32% divergence between populations, ie there is a low genetic differentiation between them.

The analyses of the molecular variance (AMOVA) obtained by the different primers indicated that in the five primers tested, an average of 76.8% of the total genetic variability occurred within the populations and only 23.2% occurred between populations (Table 4). Considering only the primers that amplified fragments in the five populations, the percentage of genetic variability among populations was even lower, with values of 18% between them and 82% of variability within populations.This means that intrapopulation genetic differentiation is much greater than interpopulation differentiation (Fig. 2). These results are in agreement with several other authors who have worked with allogamous species and also found intrapopulational genetic diversity greater than interpopulational (Freitas et al, 2005; Rossi et al, 2014; Qi et al, 2015; Silva et al, 2016; Souza et al, 2008).

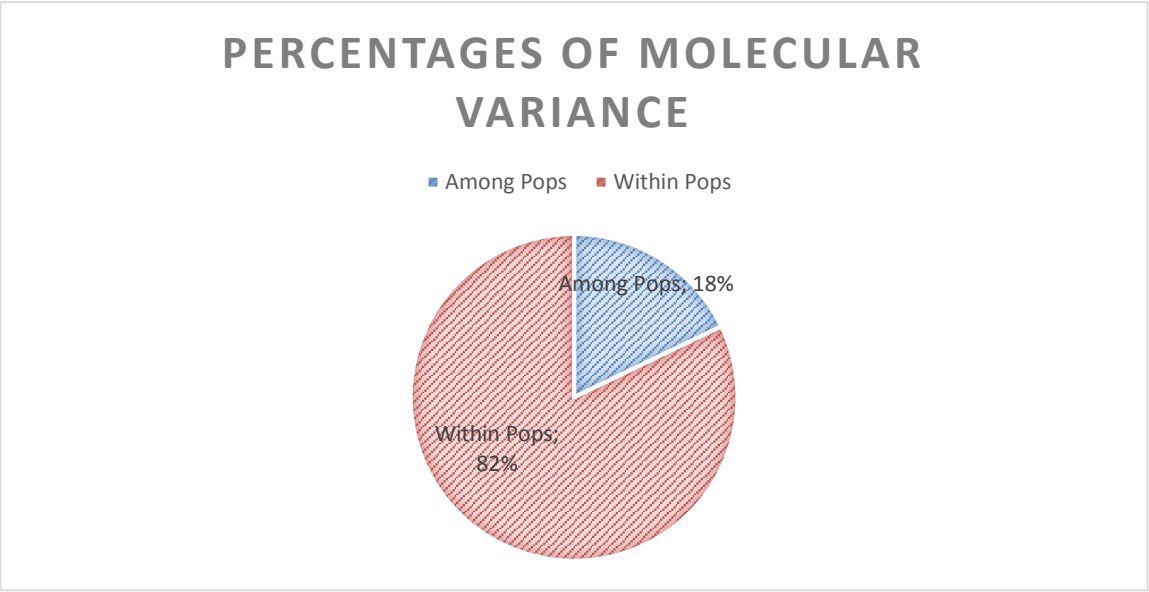

Figure 2 - Analyses of the molecular variance in the five populations, with primers P3 e P4.

Table 4. Molecular variation analyses for all primers used (AMOVA).

Primer	AMOVA Among Pop	AMOVA Within Pop
F3	19%	81%
P4	43%	57%
O4	19%	81%
P1	7%	93%
P3	28%	72%
Average	**23%**	**77%**

The Mantel test reveals if genetic distance and geographic distance are related. For this test, the population dendrogram was constructed with the UPGMA method using the Nei's genetic distance values (Nei, 1973). The Nei's genetic distance and the geographical distance between the five populations are listed in Table 5.

Table 5 - Nei's unbiased measures of genetic identity and genetic distance between *Colletia paradoxa* populations.

	CD	SS	SPG	SC	CP
CD	****	**0.9247**	**0.9209**	**0.8423**	**0.4820**
SS	**0.0783**	****	**0.9057**	**0.8473**	**0.6983**
SPG	**0.0824**	**0.0990**	****	**0.9400**	**0.4778**
SC	**0.1717**	**0.1657**	**0.0618**	****	**0.4324**
CP	**0.7299**	**0.3591**	**0.7385**	**0.8384**	****

Nei's genetic distance (below diagonal) and genetic identity (above diagonal).

The Mantel Test revealead that there is no correlation between genetic distance and geographic-distance (R = -0.03584, P <0.5774), as also showed in the Scalterplot of the software Alleles in Space (Figure 5). In the y-axis is represented the genetic distance and in the x-axis the geographic distance. It can be observed along the graph that the variations appear similar and independent of the geographic distance, demonstrating a homogeneity of the data, that is, the geographic distance does not really seem to be related to the genetic distance, which can be observed in the generated scatterplot by Allelic Agregation Index Analyses. The small differentiation observed is likely to be more at random than due to geographical distances between populations.

The G_{ST} value obtained by the Mantel Test was 0.3233 (G_{ST} values ranging from zero to 0.05 = low, 0.05 to 0.15 = mean, and 0.15 to 0.25 = relatively high - H. Zhao et al, 2016). In addition, the average percentage of polymorphic bands in the five populations of *C. paradoxa* was 84.6%, which

together with the Nei's genetic diversity index (H) of 0.3083 and the Shannon's polymorphism information index (I) was 0.4880 the populations have moderate to high rates of genetic diversity. According to Qi et al. (2015) and Silva et al. (2016), species that have a wide geographic distribution generally exhibit high levels of genetic diversity. This fact may justify the good level of genetic diversity for the populations of *C. paradoxa*, indicated through the observed parameters, if we consider that although they present themselves in dispersed and distant nuclei of one another, their populations occur in a wide geographic distribution. Fan et al. (2009), when studying the genetic differentiation in the populations of *Shiraia bambusicola* also founded similar results. Qi et al. (2015) obtained similar results when studying the populations of *Shiraia bambusicola* (PPB= 99.6%, H= 0.3314, I= 0.4996) from East China through the use of ISSR markers, obtaining values similar to ours (PPB= 84.6%, H= 0.3083, I= 0.4880). A probable explanation would be that the ancestors of these populations were already established in a more homogeneous way in their geographic distribution, exhibiting a diversified gene pool that was maintained despite population reduction (Qi et al, 2015). In addition, another factor that we should also consider is the fact that these populations are from alternating (preferably cross-pollinated) and xenogamous plants (D'Ambrogio & Medan, 1993), which would partly explain these levels of genetic diversity.

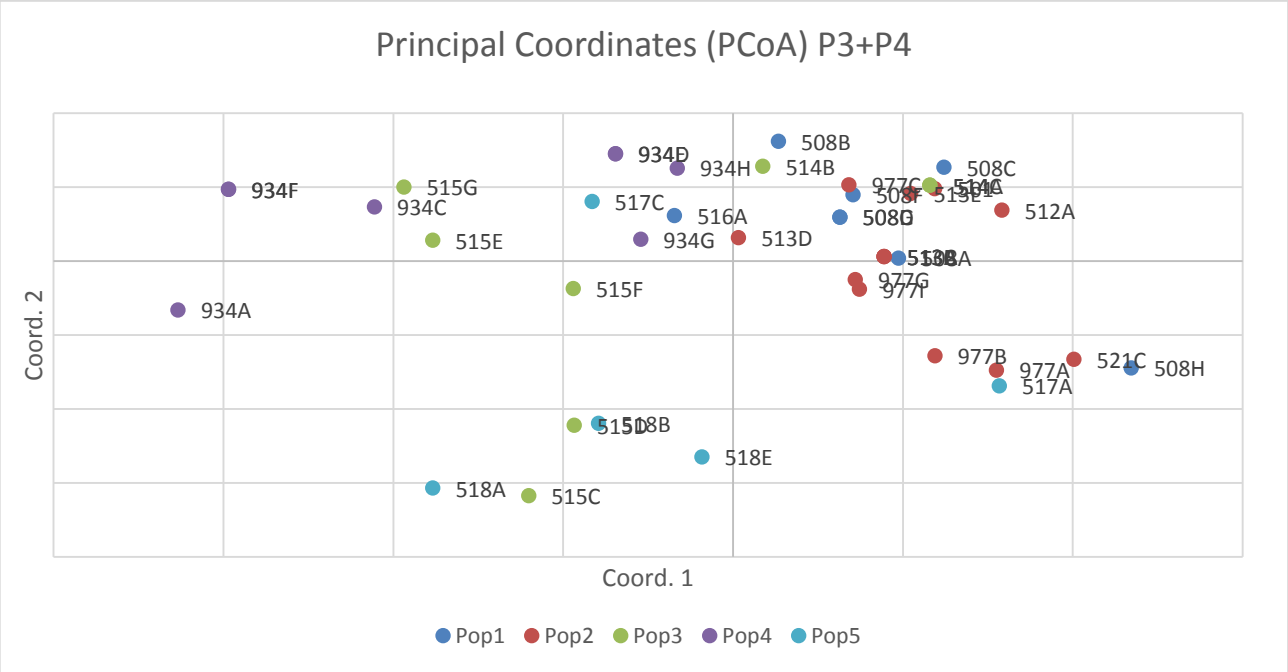

Figure 3 – Analysis of Principal Coordinates (PCoA) in populations of *C. paradoxa* in Rio Grande do Sul and southern Santa Catarina (Urupema). Note that there is no evident population structure. The colors identify the

populations. Pop1: CD - Central Depression, Pop2: SS - South-Rio-Grandense Shield, Pop3: SPG - Southern Plateau Grasslands, Pop4: SC - Urupema, Santa Catarina State, Pop5: CP - Coastal Plain.

Through the analysis of the main coordinates we were able to observe the spatial distribution of the populations and the relation of the individuals between and within them. The population 2 is observed occupying the second and fourth quadrant, demonstrating a certain homogeneity in the population. Also identified in the extreme left of the main axis are some individuals from the isolated population 4 (Santa Catarina), suggesting that they present the greatest differences in relation to the population of the Sul-Riograndense Shield. However, the presence of individuals of this same population in the intermediate portion of the x-axis, next to the representatives of the other populations, indicates that also in the population 4 (SC), individuals occur similar to the other populations, that is, the presence of individuals with characteristic intermediaries. In the y-axis, although the differential gradient is smaller than in the x-axis, the grouping consisting of individuals from all populations occurs. However, some individuals from populations 3 and 5 (Costal Plain) also appear at the lower end of the and axis and indicate some difference with individuals at the upper end. The coastal population, which appears to be well delimited, appears with an individual characterization in population 2 and population 4 (fourth and first quadrants, respectively). It was concluded that the result of this analysis, corroborates the indices observed in the other parameters obtained, such as the value of the number of migrants, calculated from the genetic differentiation coefficient among all populations (G_{ST}), which generated a Nm = 1.0465, considered relatively reasonable (> 1).

3.3. Analyses of genetic differentiation

The Bayesian inference of clustering performed by Structure revealed the most likely K = 4 (Figures 4), with remarkable genetic admixture between populations. This admixture is in agreement with the results of AMOVA, which revealed that most of the molecular variance is within populations. Comparing the barplot obtained with the five groups corresponding to physiographic regions, it is possible to observe that populations 1 and 2 of Rio Grande do Sul are genetically similar, and clearly distinct from population 4, from Urupema, Santa Catarina. Populations 3 (Jaquirana and Cambará do Sul, South-Rio-Grandense Shield) and 5 (Mostardas, Rio Grande do Sul Coast) are also distinct. In

population 3, it is possible to recognize similarities with all other populations. It seems that this population connects genetically the other regions.

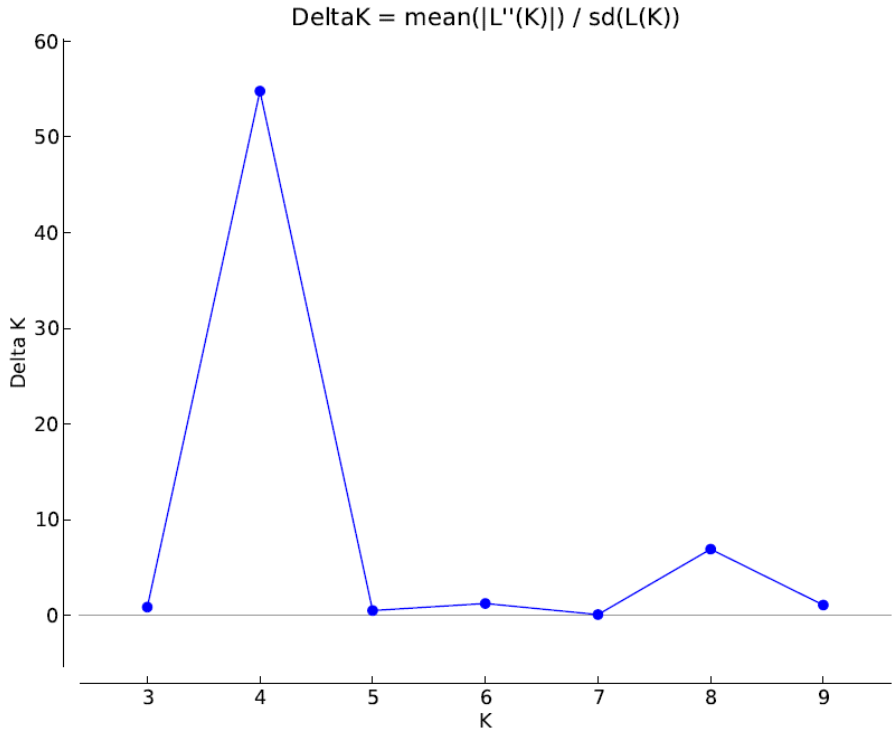

Figure 4 - Estimation of the best *K* by the *DeltaK* statistic, inferred with the Structure program.

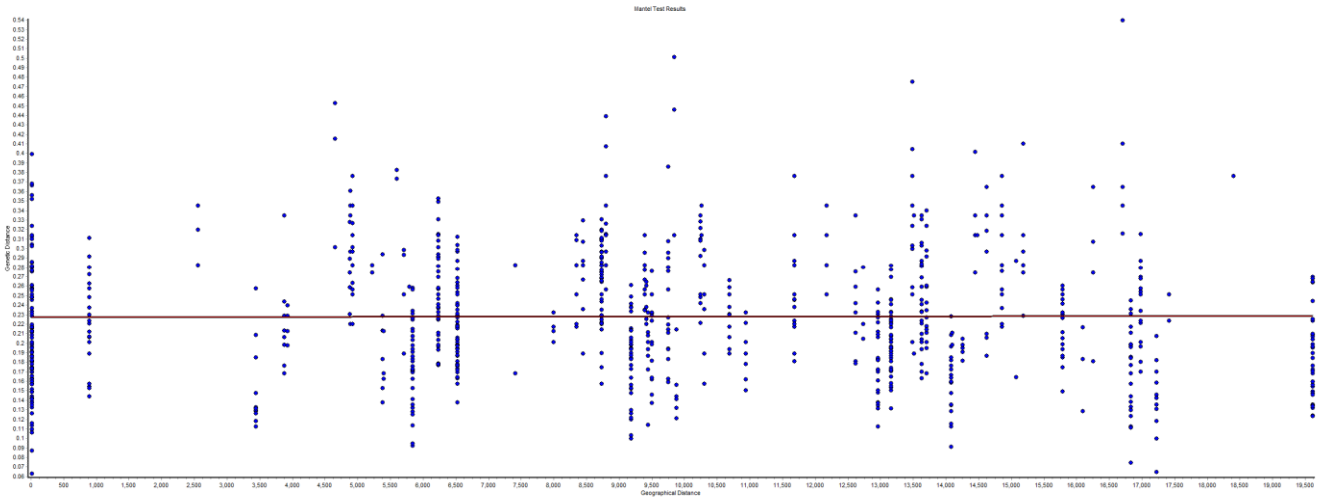

Figure 5 - Scatterplot obtained with software Alleles in Space, Allelic Agregation Index Analyses (AAIA). There is no correlation between genetic distance and geographic-distance.

The five populations of *C. paradoxa* previously defined for this study, presented individuals with characteristics that included the three morphotypes (*C. paradoxa* morphotype "paradoxa", *C. paradoxa* morphotype "exserta" and *C. paradoxa* morphotype "intermediate"), considered at the beginning of this research, and which represent the great morphological variability already confirmed for *C. paradoxa* lato sensu species. The five populations proposed *a priori*, were thus considered, taking into account, mainly, the different regions occupied by the species, than by the morphological characteristics presented by it. At the end of the analysis, it is possible to state: the populations that obtained the highest values of Nei's genetic diversity (H) in this study were population 3 (SPG: Jaquirana e Cambará do Sul) with H = 0.3875 (± 0.1799), followed closely by population 2 (SS: Piratini e Pinheiro Machado) with H = 0.3855 (± 0.2031). Population 1 (CD: Porto Alegre e São Pedro do Sul) H = 0.3071 (± 0.1808), followed for population 4 (SC: Urupema) with H = 0.2709 (± 0.2129) and population 5 (CP: Mostardas) H = 0.1907 (± 0.2286). In the same order the values for the Shannon's information were obtained, to population 3 (SPG) with I = 0.6342 (± 0.2944), followed closely by population 2 (SS) with I = 0.5955 (± 0.3219) were observed, Population 1 (CD) I =0.4881 (± 0.2616), followed for Population 4 (SC) I = 0.4262 (±0.3315) and Population 5 (CP) I = 0.2961 (± 0.3601).

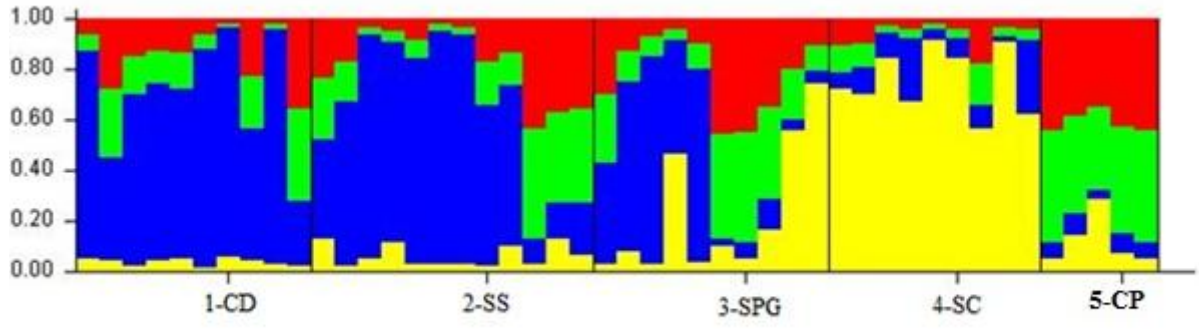

Figure 6 - Bayesian analyses of genetic clustering between populations of *C. paradoxa* obtained in STRUCTURE software for K = 4. The numbers (1-5) correspond to the populations previously defined.

DISCUSSION

Soil conditions and the close distances between the populations have significant importance in the diversity analyses (Li, 2009). Also, the variability within and between populations can be determined by three factors: gene flow, genetic drift and habitat reduction (Li, 2009). Reducing

population size, whether by deforestation or other biotic or abiotic factors, can lead to fragmentation of populations. The breeding system may also influence genetic diversity, and cross-breeding species usually have a higher genetic variation than autogamous species (Hamrick & Godt, 1989, 1996; Rossi et al, 2014) consequently, they present high genetic diversity within the populations and low diversity among the populations, when in relation to the autogamous plants.

The fragmentation observed in field seems to have not significant impact to genetic diversity of the species so far. This diversity found may be a result of ancestral polymorphism, representing a time when these populations were connected. The presence of admixture between genetic groups and the absence of correlation between geographic and genetic distance corroborate this hypothesis.

They have a moderate to hight genetic variability within the populations and high levels of genetic diversity, in all analysed parameters. In addition, all fragments obtained through the use of ISSR genetic markers were polymorphic and the high value obtained for the gene flow indicated that the gene migration among the populations of *C. paradoxa* are still occurring.

Conclusion

In view of the results obtained in the analysis and discussed here. We can conclude that:

- There is gene flow occurring among the populations studied of *C. paradoxa* through migrants;
- Molecular variance within populations is much greater than genetic diversity among populations;
- There is no correlation between geographic distance and genetic distance,
- ISSR molecular markers were effective polymorphic markers capable of identifying genetic diversity in *C. paradoxa* populations.

Acknowledgements

We thank Prof. Dr. Renato Aquino Záchia and Msc. Fallon dos Santos Siqueira for the suggestions and reading of the manuscript. We would also like to thank the lab leaders and staff of

the Departments of Biology and Biochemistry and Molecular Biology for support in laboratories whenever required.

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4 CONSIDERAÇÕES FINAIS

A busca por uma vida mais saudável e equilibrada faz com que cada vez mais pessoas questionem suas relações com o meio ambiente buscando alternativas para evitar, controlar e/ou curar doenças por meio de fontes naturais. A demanda pelo consumo de produtos naturais e o interesse pela ação de compostos antioxidantes tem se intensificado nos últimos anos, principalmente devido à preocupação com a prevenção contra o envelhecimento. Do mesmo modo, doenças degenerativas, relacionadas aos danos causados ao organismo pelo excesso de radicais livres, como câncer, doenças cardiovasculares, complicações do *Diabetes mellitus* e disfunções cerebrais, como Alzheimer e Parkinson (FERREIRA, MATSUBARA, 1997; VELLOSA et al, 2007) têm sido cada vez mais frequentes. Além disso, devido à disseminação da resistência microbiana e ao reduzido arsenal de medicamentos disponíveis para o tratamento de infecções bacterianas, surge a preocupação em obter-se novos medicamentos. As plantas têm sido intensivamente estudadas, porque são prováveis fontes de matéria prima para a obtenção de novos antibióticos entre outros produtos de interesse farmacêutico, podendo ser uma fonte alternativa, que têm sido usada na medicina tradicional para esse fim (MENDES et al, 2011).

O produto resultante do metabolismo primário das plantas é responsável pelo atendimento de suas funções vitais, enquanto os compostos resultantes do metabolismo secundário podem ter efeitos medicinais ou tóxicos, além de exercer funções protetivas contra os predadores e atuar na atração de agentes polinizadores (SIMÕES, 2004). Compostos fenólicos resultantes do metabolismo secundário de plantas, são bons antioxidantes naturais e potenciais agentes bacteriostáticos, atraindo crescente atenção dos pesquisadores. O potencial antioxidante destes compostos é muito superior ao de todos os outros antioxidantes conhecidos na dieta. São agentes redutores que, juntamente com outros, como vitamina C, vitamina E e carotenóides, protegem nossos corpos do estresse oxidativo (SCALBERT, WILLIAMSON, 2000). Portanto, as plantas medicinais desempenham um papel muito importante na saúde pública, especialmente nos países em desenvolvimento, além de despertarem o interesse dos pesquisadores (MOSSI et al, 2004, VELLOSA et al, 2007).

Estudos fitoquímicos em membros da família Rhamnaceae, incluindo *Colletia paradoxa*, descrevem a presença de triterpenóides e esteróides em sua composição, principalmente nas partes aéreas da planta. Além disso, a atividade antimicrobiana dos isolados triterpênicos de *C. paradoxa*

contra *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Klebsiella pneumoniae, Salmonella setubal, Escherichia coli* e *Pseudomonas aeruginosa* foi comprovada por Giacomelli et al, (2006).

Este estudo teve como objetivo fazer uma análise multidisciplinar de uma espécie de uso medicinal, *Colletia paradoxa*, da família Rhamnaceae, avaliando ao mesmo tempo aspectos farmacológicos, taxonômicos e moleculares, obtendo um amplo espectro informativo de uma única espécie. Através da análise do extrato bruto, foram obtidas as informações sobre sua ação antioxidante, ação antimicobacteriana e identificação dos flavonóides presentes. Quanto à capacidade antioxidante, pode-se observar que o maior valor de IC_{50} foi obtido pelo extrato de *C. paradoxa* morfotipo “paradoxa” (RB 501) e o segundo maior valor pela amostra RB 507 que é um representante do morfotipo "exserta". Essas duas amostras estão em populações de diferentes municípios, Piratini (SS) e Porto Alegre (DC) respectivamente. O outro extrato do morfotipo "paradoxa" apresentou o menor valor de IC_{50}, sugerindo não haver relação dos resultados em quanto aos diferentes morfotipos. Em relação às diferentes regiões de coleta de amostras, os resultados também foram variados, mostrando que as regiões Serra do Sudeste (SS) e Depressão Central (DC) apresentaram os maiores e também os menores valores de IC_{50}, não mostrando relação com a região onde foram coletadas.

Na análise para a determinação da Concentração Inibitória Mínima dos extratos observou-se que um valor significativo de CIM foi obtido com o extrato do morfotipo "exserta" (RB 515, de Cambará do Sul) para cepas de *M. massiliense*. Enquanto o extrato de amostra RB 501 (Piratini), representando o morfotipo "paradoxa", apresentou o segundo melhor valor de CIM para as cepas das três espécies de *Mycobacterium*. Apesar da distância e da peculiaridade geográfica entre os locais de coleta das duas amostras, isso não pareceu interferir nos resultados obtidos.

Ao analisar o cromatograma para identificar os flavonóides nos diferentes extratos, observou-se que, com exceção da catequina que não foi detectada nos extratos RB 512 e RB 507, todos os outros compostos detectados no cromatógrafo estavam presentes nos extratos das amostras estudadas. Considerando a quantidade de compostos fenólicos presentes em cada amostra, verificou-se que as maiores concentrações são qualitativamente coincidentes em todas as amostras. Quercitrina, quercetina, rutina, canferol e luteolina são as que apresentam maior quantidade, aparentemente não relacionadas às diferenças morfotípicas ou às regiões onde foram coletadas. No entanto, como este estudo não monitorou as diferenças ambientais de cada local de coleta, não podemos avaliar a relação desses resultados com o ambiente onde as amostras foram coletadas.

Na análise taxonômica, pode-se confirmar que toda a variabilidade morfológica observada está contida em uma única espécie, *C. paradoxa*. Os resultados da análise molecular contribuem com várias informações sobre as populações, como diversidade genética, fluxo gênico e correlação entre distância genética e distância geográfica. Entretanto, tanto na análise da ação antimicobacteriana quanto na ação antioxidante e nos resultados da identificação dos flavonóides não houve relação com as diferenças morfológicas apresentadas por cada morfotipo. Esses resultados instigam novas pesquisas sobre um maior número de amostras e análises de diferentes frações dos extratos analisados. Por isso temos a responsabilidade de preservar esta espécie que já demonstrou seu grande potencial biológico e que está ameaçada de extinção.

Apesar da nossa rica biodiversidade, é possível ver que um número impressionante de espécies está sendo extinto e nem sempre são conhecidas. Infelizmente, os programas de pesquisa e conservação estão se desenvolvendo em um ritmo mais lento do que o ritmo acelerado da degradação ambiental. Esse mecanismo ameaça os ecossistemas e a biodiversidade.

A conservação de uma espécie ameaçada como *C. paradoxa* depende de uma série de fatores que devem ser articulados para formar uma ponte entre a pesquisa e as ações correspondentes. A permanência do fluxo gênico entre populações distantes, a existência de variabilidade genética dentro e entre populações, bem como a atenção à existência de bancos de sementes são itens fundamentais a serem observados para conservação. Esses mecanismos microevolutivos devem ser bem compreendidos, considerando restrições ecológicas e genéticas, estratégias adaptativas, integração de métodos ecológicos e genéticos, na compreensão da dinâmica populacional (MARTINS, 1987).

Como as populações desta espécie formam núcleos muito distantes entre si; diante das demandas previamente explicitadas, é necessário mapear essas áreas, buscar populações intermediárias entre elas e propor ações de conservação que aumentem a proteção dos habitats.

Colletia paradoxa apresenta uma distribuição ampla e em mosaico, portanto, seria necessário manter intactos todos os fragmentos, a fim de garantir a considerável variabilidade genética das populações estudadas, conhecida através dos resultados do presente trabalho.

5 CONCLUSÃO

Essa tese de doutorado buscou, através da integração de conhecimento de diversas áreas, fornecer subsídios que colaborem na conservação mais efetiva desta espécie ameaçada de extinção, sem deixar de lado sua grande potencialidade em pesquisa e utilização dentro das ciências farmacêuticas.

Assim, ao final deste estudo foi possível:

- confirmar considerável quantidade de importantes compostos fenólicos responsáveis pelas atividades biológicas desta espécie;
- confirmar o potencial dos extratos de *C. paradoxa* como fonte de ação antioxidante natural;
- confirmar o potencial dos extratos de *C. paradoxa* como fonte de atividade antimicobacteriana, avaliados sobre as espécies *Mycobacterium abscessus, M. fortuitum* e *M. massiliense*;
- conhecer melhor a espécie *C. paradoxa* e sua delimitação taxonômica, tornando as identificações mais seguras;
- confirmar que nenhum morfotipo estudado pode ser entendido como uma espécie distinta de *C. paradoxa*;
- constatar a existência de fluxo gênico ocorrendo entre as populações de *C. paradoxa*;
- constatar que a diversidade genética dentro das populações é muito maior do que a diversidade genética entre as populações;
- constatar que não há correlação entre distância geográfica e distância genética das populações estudadas;
- confirmar que os marcadores moleculares ISSR foram muito eficientes e polimórficos, capazes de identificar uma considerável diversidade genética em populações de *C. paradoxa*.

Por fim, incentivamos a continuidade dos estudos:

- para avaliar a ação fitoterápica e toxicológica de *C. paradoxa*, com a finalidade de determinar a segurança de seu uso e a viabilidade de sua aplicação na área farmacêutica e em outras áreas;

- para avaliar o uso de marcadores codominantes para obter maiores informações sobre o fluxo gênico, heterozigozidade e as relações entre as populações, para que seja monitorada a variabilidade genética desta espécie.

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