UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

Tese de Doutorado

ESTUDOS EVOLUTIVOS EM *VERBENOXYLUM REITZII* (MOLDENKE) TRONC. (VERBENACEAE)

VERÔNICA AYDOS THODE

Porto Alegre, 13 de maio de 2013.

ESTUDOS EVOLUTIVOS EM *VERBENOXYLUM REITZII* (MOLDENKE) TRONC. (VERBENACEAE)

VERÔNICA AYDOS THODE

Tese de Doutorado apresentado ao Programa de Pós-Graduação em Botânica, do Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para obtenção do título Doutor em Ciências com ênfase em Botânica.

Orientadora: Profa. Dra. Loreta Brandão de Freitas

Comissão Examinadora
Profa. Dra. Clarisse Palma-Silva
Prof. Dr. João Renato Stehmann
Profa. Dra. Tatiana Teixeira de Souza Chies

Porto Alegre, 13 de maio de 2013.

"O HOMEM, FILHO DESTA TERRA, QUE LHE FORNECE O PÃO DE CADA DIA E OS SÍMBOLOS DE SUA VIDA ESPIRITUAL, SENTE UM RESPEITO INATO PERANTE A FISIONOMIA DESTA SUA MÃE E PÁTRIA. ENQUANTO O ESPAÇO É SUFICIENTE E A DENSIDADE DEMOGRÁFICA PEQUENA, NÃO SE TORNAM MUITO CONSCIENTES TAIS SENTIMENTOS, MAS, NO MOMENTO EM QUE AS NECESSIDADES BRUTAIS DA VIDA FORÇAM A INTERFERIR SEMPRE MAIS NA EXPRESSÃO NATURAL DO AMBIENTE, DESPERTA A DOR PERANTE A DESTRUIÇÃO DE SUAS FEIÇÕES NATURAIS E O DESEJO DE AS CONSERVAR, SENÃO NO CONJUNTO, AO MENOS EM ALGUNS LUGARES E NOS TRAÇOS MAIS CARACTERÍSTICOS."

BALDUÍNO RAMBO

(A FISIONOMIA DO RIO GRANDE DO SUL, 1942)

AGRADECIMENTOS

Agradeço, com grande satisfação, a todas as pessoas que auxiliaram na realização deste trabalho e Instituições que possibilitaram sua execução. A todos que contribuíram nestes anos para o meu crescimento pessoal e profissional, que estiveram ao meu lado e compartilharam momentos importantes durante esta construção.

A minha família, pai, irmã e especialmente a minha mãe, que sempre me apoiaram, incentivaram e estiveram ao meu lado em todos os momentos. Aos meus queridos amigos que me deram ouvidos, momentos de descontração, alegrias e força.

A minha orientadora Loreta Brandão de Freitas por possibilitar e viabilizar que este trabalho tenha sido executado, pela confiança, autonomia e por ter me auxiliado a amadurecer como pesquisadora.

Aos colegas de laboratório Caroline Turchetto, Ana Lúcia Segatto, Maikel Reck, Lauís Brisolara Corrêa, Geraldo Mädder, Gustavo Arias, Ana Luiza Cazé, Michel Barros, Priscilla M. Zamberlan, Jefferson Fregonezi, pelo companheirismo, ensinamentos e experiências adquiridas juntos. Agradeço especialmente à Alice Backes, que auxiliou com grande empenho na construção do artigo que compõe o capítulo 2 desta tese e pela amizade que foi fruto de um trabalho em equipe.

As minhas amigas, professoras e inspiradoras, Dra. Lilian Auler Mentz e Dra. Márcia Vignoli por terem incentivado o meu amor pela Botânica.

Ao Dr. Richard G. Olmstead por possibilitar que eu realizasse um estágio em seu laboratório na University of Washington (Seattle, USA) que foi muito enriquecedor,

tanto científica quanto pessoalmente. Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa sanduíche concedida.

Às Dras. Nataly O'Leary e Maria Múlgura do Instituto de Botánica Darwinion (San Isídro, Argentina) e Fátima Salimena (Universidade Federal de Juíz de Fora) por compartilharem comigo todo o seu conhecimento e amor pelas verbenáceas.

Aos membros da minha banca de qualificação pelas sugestões enriquecedoras ao artigo que compõe o capítulo 1 desta tese, Dra. Lilian Auler Mentz, Dra. Andréia Carina Turchetto Zolet e Dr. Nelson J. R. Fagundes.

Ao Instituto de Biociências da UFRGS, ao Departamento de Botânica e professores do PPG-Botânica pela oportunidade de cursar o doutorado. Ao PPG-Genética da UFRGS que possibilitou minha participação em disciplinas e cursos concedidos pelo Programa e ao secretário administrativo do mesmo, Sr. Elmo Cardoso.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de doutorado concedida e ao CNPq e Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) que viabilizaram a execução deste trabalho.

SUMÁRIO

RESUMO

Verbenoxylum (Verbenaceae – Lamiales) é um gênero monotípico de hábito arbóreo restrito ao limite sul da Mata Atlântica. Apesar da presença de um estudo filogenético sobre a classificação da família Verbenaceae empregando marcadores moleculares, o posicionamento de *Verbenoxylum reitzii* permanece incerto. Através de estudos filogenéticos moleculares é possível investigar o relacionamento entre os organismos e processos evolutivos que possam ter tido papel importante na diferenciação e distribuição dos mesmos. Análises de diversidade genética com marcadores moleculares que tenham histórias evolutivas distintas podem promover um entendimento mais profundo de eventos históricos e recentes que possam ter influenciado na distribuição da variabilidade genética atual. Estudos genéticos populacionais e de modelagem de nicho ecológico (ENM; combina distribuição geográfica e variáveis ambientais) em espécies vegetais da Mata Atlântica podem auxiliar a desvendar a influência das mudanças climáticas geradas pelos ciclos glaciais/interglaciais na composição desta floresta. Este trabalho teve por objetivo contribuir para o entendimento da história evolutiva de *V. reitzii* através de análises filogenéticas e de diversidade genética populacional. Foram realizados quatro trabalhos que correspondem aos capítulos desta tese. No primeiro capítulo, foi inferido o posicionamento de *V. reitzii* em Verbenaceae com base nos marcadores moleculares *trnL-trnF* e *ndhF*. Os resulatdos mostraram que esta espécie está incluída na tribo Duranteae, sendo grupo-irmão de *Recordia* Moldenke (gênero monotípico endêmico da Bolívia). Foi proposta uma nova combinação para *V. reitzii* que foi incluída em *Recordia*. No segundo capítulo, foram desenvolvidos 11 marcadores variáveis do tipo SSRs para *V. reitzii* e foi testada a transferabilidade destes nos gêneros relacionados *Recordia* e *Duranta*. Dez *loci* de SSRs amplificaram em *Recordia* e nenhum em *Duranta*. No terceiro capítulo, foram utilizados dez *loci* de SSRs (desenvolvidos no capítulo 2) junto com regiões do cpDNA para caracterizar a variabilidade intraespecífica de *V. reitzii*. Além disto, foram realizadas análises de ENMs e testes de hipóteses de divergência e conservatismo de nicho para *V. reitzii* e *R. boliviana*. Os resultados apontaram uma diversidade genética baixa para *V. reitzii*, que

pode ser associada com a sua distribuição restrita, pequeno tamanho populacional e efeito fundador. As ENMs indicaram que a distribuição atual de *Verbenoxylum* e *Recordia* deve ter sido influenciada por mudanças climáticas do passado e os testes de divergência e conservatismo de nicho mostraram a importânica de variáveis ambientais na divergência e distribuição das duas espécies. Por fim, no quarto capítulo, foram analisadas as relações evolutivas dentro da tribo Duranteae, a qual *Verbenoxylum* pertence, utilizando marcadores do cpDNA e nucleares de sequência.

ABSTRACT

Verbenoxylum (Verbenaceae – Lamiales) is a monotypic tree genus restricted to the southern limit of the Brazilian Atlantic Forest. Despite of the presence of a phylogenetic study on the classification of the Verbenaceae family using molecular markers, the placement of *Verbenoxylum reitzii* remains uncertain. Using molecular phylogenetic studies it is possible to investigate the relationship between organisms and the evolutionary processes that might have had an important role on their differentiation and distribution. Genetic diversity analyses with molecular markers that present distinct evolutionary histories may provide a deeper understanding of historical and recent events that might have influenced on the present distribution of genetic variability. Population genetic studies and ecological niche modeling (ENM; combines geographic distribution and environmental variables) for plant species from the Atlantic forest might help to reveal the influence of environmental changes generated as consequences of the glacial/interglacial cycles in the composition of this forest. The aim of the present project was to contribute for the understanding of *V. reitzii* evolutionary history through phylogenetic and population genetic diversity analyses. Four studies were developed which correspond to the chapters of the present thesis. In the first chapter, the position of *V. reitzii* within the Verbenaceae was inferred based on the molecular markers *trnL-trnF* e *ndhF*. The results showed that this species is included in the tribe Duranteae, sister to *Recordia* Moldenke (monotypic genus endemic to Bolivia). A new combination for *V. reitzii* was proposed, including the species in *Recordia*. In the second chapter, 11 variable SSRs markers were developed for *V. reitzii* and cross-amplification was tested in the related genera *Recordia* and *Duranta*. Ten loci amplified in *Recordia* and all failed in *Duranta.* In the third chapter, ten SSR loci (developed in chapter 2) were used along with cpDNA regions to evaluate the intraspecific variability in *V. reitzii*. Besides, ENMs and tests of hypotheses of niche divergence and conservatism were performed for *V. reitzii* and *R. boliviana*. The results indicated that *V. reitzii* has a low genetic diversity, which may be related to its narrow distribution, small population size, and founder effect. The ENMs pointed that the present distribution of *Verbenoxylum* and *Recordia* may have been influenced by past

climatic changes and the tests of niche divergence and conservatism showed the importance of environmental variables on the diversification and distribution of the two species. Lastly, in the fourth chapter, evolutionary relationships within Duranteae, the tribe where *Verbenoxylum* belongs, were analyzed using plastidial and nuclear markers.

INTRODUÇÃO GERAL

INTRODUÇÃO GERAL

VERBENACEAE, *VERBENOXYLUM* E *VERBENOXYLUM REITZII*

A família Verbenaceae J. St.-Hil. (Lamiales) possui cerca de 34 gêneros e 1200 espécies que ocorrem principalmente nas Américas, com apenas dois gêneros que possuem representantes exclusivos do Velho Mundo: *Chascanum* E. Mey., encontrado em Madagascar, Arábia e Índia, e *Coelocarpum* Balf. f., que ocorre em Madagascar, Somália e Socotra (Atkins 2004). No Brasil ocorrem 17 gêneros com cerca de 310 espécies (Salimena *et al*. 2012).

Verbenaceae, como família, foi reconhecida em 1805 por Saint-Hilaire. Diversos autores propuseram classificações para Verbenaceae (Chamisso 1832; Schauer 1847; Bentham & Hooker 1876; Briquet 1895; Junell 1934; Troncoso 1974; Sanders 2001; Atkins 2004; Marx *et al*. 2010). A família já possuiu aproximadamente 100 gêneros e 2600 espécies (Cronquist 1981), porém, com base em evidências morfológicas e moleculares, vários gêneros passaram para Lamiaceae ou para outras famílias (Cantino 1992; Wagstaff & Olmstead 1997; Wagstaff *et al.* 1998). A classificação proposta por Marx e colaboradores (2010) foi baseada em dados moleculares e amostrou todos os gêneros, com a exceção de *Verbenoxylum* Tronc. Esta filogenia molecular apresentou muitas diferenças com relação aos sistemas de classificação de Verbenaceae anteriormente publicados.

Tradicionalmente, o gênero *Verbenoxylum* era incluído na tribo Citharexyleae, a qual foi estabelecida por Briquet em 1895, compreendendo quatro gêneros: *Citharexylum* L., *Coleocarpum* Balf. f., *Duranta* L. e *Rhaphitamnus* Miers. Mais tarde, foram incluídos os gêneros *Baillonia* Bocq., *Recordia* Moldenke, *Rehdera* Moldenke e *Verbenoxylum* Tronc

(Sanders 2001). Atkins (2004) considerou sete destes gêneros como pertencentes à tribo e atribuiu posição incerta ao gênero *Coelocarpum*. Na filogenia molecular da família (Marx *et al*. 2010), Citharexyleae ficou representada por *Baillonia*, *Citharexylum* e *Rehdera* (*Verbenoxylum* não foi amostrado).

Verbenoxylum é um gênero monotípico de hábito arbóreo. A espécie *Verbenoxylum reitzii* (Moldenke) Tronc. foi encontrada até o momento apenas no Brasil, nos estados do Rio Grande do Sul e Santa Catarina, restrita ao limite sul da Mata Atlântica, de 10 a 550 metros de altitude, não alcançando os campos no platô da Serra Geral (Troncoso 1974; Reitz *et al*. 1978, 1983; Sobral *et al*. 2006). Esta espécie está incluída na Lista de Espécies Ameaçadas do Estado do Rio Grande do Sul (SEMA 2003) e suas informações ecológicas, biológicas e genéticas são escassas ou mesmo inexistentes (Troncoso 1971; von Poser *et al.* 1997; Bueno & Leonhardt 2011).

Verbenoxylum reitzii foi descrita inicialmente por Moldenke (1949) como *Citharexylum* e mais tarde foi segregada deste gênero por Troncoso (1971) por possuir flores com a corola infundibuliforme, estames com filetes longos e tecas notavelmente divergentes na base, dispondo-se quase em plano horizontal na antese, por engrossamento do conetivo e frutos que se dividem em dois mericarpos alongados quando maduros. As espécies de *Citharexylum,* por sua vez, possuem corola subcilíndrica, estames com filetes curtos e tecas paralelas na antese, sem engrossamento na base do conetivo e frutos que não se dividem quando maduros (Troncoso, 1971, 1974) (Figura 1).

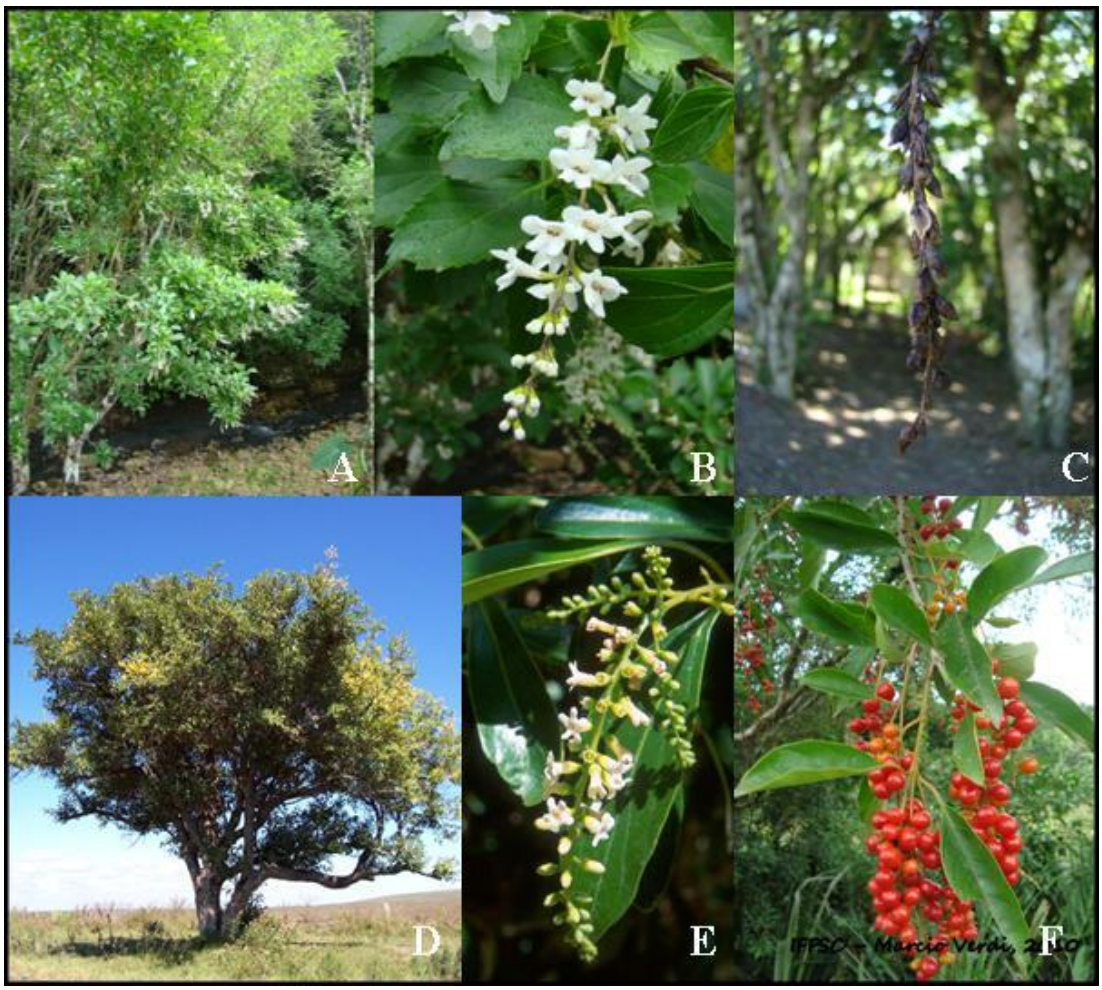


Figura 1. Representantes de Verbenaceae. Em A. hábito, B. Inflorescência, e C. frutos maduros de *Verbenoxylum reitzii* (Moldenke) Tronc.; em D. hábito de *Citharexylum montevidense* (Spreng.) Moldenke; em E. inflorescência de *Citharexylum spinosum* L.; e em F. frutos maduros de *Citharexylum myrianthum* Cham.

FILOGENIA MOLECULAR

A reconstrução filogenética consiste em estimar as relações de ancestralidade e descendência para um determinado grupo de organismos (Miyaky *et al*. 2001). Em sistemática molecular de plantas, genes do DNA plastidial e nuclear são amplamente utilizados na busca por caracteres filogeneticamente informativos para o esclarecimento de relações evolutivas entre *taxa*.

Uma vez conhecida a filogenia molecular de um determinado grupo, esta pode ser utilizada para estudar o posicionamento filogenético dos organismos e aprimorar sistemas de classificação tradicionais. É possível também analisar diversos aspectos evolutivos como evolução de estado ancestral de caracteres morfológicos e análises biogeográficas, como a reconstrução de área ancestral e de cenários de dispersão e vicariância. Dependendo da existência de possíveis pontos de calibração na filogenia molecular inferida, como registros fósseis bem identificados para o grupo, é possível datar a filogenia e realizar diversos estudos como a estimativa de tempo de divergência entre espécies.

Estudos de diversidade genética de plantas na Mata Atlântica

Estudos de diversidade genética procuram analisar como a variabilidade genética está distribuída nas populações ao longo da área de ocorrência de uma espécie. A distribuição da variabilidade genética pode ser afetada por diversos fatores como o tamanho de área de ocorrência da espécie, sua capacidade de dispersão e a presença de barreiras geográficas (Wright 1978). Neste tipo de análise é necessário encontrar marcadores moleculares que contenham variação no nível intraespecífico e permitam explorar a estrutura genética de uma espécie abrangendo toda a sua distribuição geográfica. Os mais comuns em estudos de diversidade genética de plantas atualmente são os marcadores microssatélites (SSRs), espaçadores internos transcritos do rDNA nuclear (ITS) e espaçadores intergênicos do DNA plastidial, como por exemplo, *trnT-trnF*, *trnL-trnF*, *psbA-trnH*, *psbC/trnS3* (Collevatti *et al*. 2003; Lorenz-Lemke *et al*. 2005, 2010; Palma-Silva *et al*. 2007, 2009; Ramos *et al*. 2007, 2009). Através de análises de estruturação populacional é possível observar a existência de fluxo gênico histórico e

recente (Beebee & Rowe 2008) e identificar as populações de uma espécie como unidades geneticamente distintas (Slatkin 1994).

Os microssatélites ou SSRs (*simple sequence repeats*) são marcadores moleculares que apresentam alto grau de polimorfismo e por isso são muito utilizados em estudos genéticos de populações. Os mesmos consistem de motivos formados por um a seis nucleotídeos repetidos diversas vezes (Kelkar *et al.* 2010). Estes marcadores são multi-alélicos, codominantes (permite identificar indivíduos homozigotos e heterozigotos em cada loco), distribuídos por todo o genoma e amplificados por PCR (*Polymerase Chain Reaction*). Como consequência de suas elevadas taxas de mutação, os SSRs são bastante polimórficos: as variações se manifestam como diferenças numéricas na repetição dos motivos. Para desenvolver estudos populacionais com este tipo de marcador é necessário desenvolver *primers* específicos que podem também ser testados e utilizados em espécies proximamente relacionadas.

A riqueza de espécies representa a medida mais amplamente utilizada para a biodiversidade, entretanto, através de estudos genéticos intraespecíficos também é possível identificar linhagens evolutivas. Estudos genéticos intraespecíficos podem ser usados como uma ferramenta para a criação de Unidades de Manejo (*Management Units* – MUs) e o estabelecimento de linhagens distintas geograficamente e geneticamente como as Unidades Evolutivamente Significativas (*Evolutionary Significant Units* – ESUs) (Moritz 1994; Rull 2011). O estabelecimento e utilização prática destes conceitos na proteção de espécies envolve a determinação da estrutura genética das populações e seu relacionamento evolutivo (Moritz & Faith 1998). Análises de processos evolutivos nas espécies com distribuição restrita, endêmicas e raras podem ser cruciais na tentativa de conservar populações que sejam geneticamente distintas e na tomada de medidas de

preservação, uma vez que raridade também é um indicativo de prioridade para a conservação (Moritz & Faith 1998).

Estudos genéticos populacionais para as espécies vegetais na Mata Atlântica vêm sendo conduzidos em número crescente nos últimos anos (Margis *et al*. 2002; Conte *et al*. 2003; Lorenz-Lemke *et al*. 2005, 2010; Ribeiro *et al*. 2005; Alcantara *et al*. 2006; Palma-Silva *et al*. 2007, 2009; Ramos *et al*. 2007, 2009; Carnaval & Moritz 2008; Collevatti *et al*. 2012), sendo o terceiro bioma/ecorregião da América do Sul mais incluído em estudos filogeográficos, perdendo apenas para a Amazônia e os Andes (Turchetto *et al*. 2013).

A Mata Atlântica ocorre no Brasil e em regiões do Paraguai e da Argentina. No Brasil, extende-se desde o Rio Grande do Sul até o Piauí (30^{o} S a 3^{o}S) ao longo da costa leste, desde o nível do mar até ca. 2700 m de altitude (SOS Mata Atlântica/INPE 2008; Metzger 2009; Stehmann *et al*. 2009). Esta ecorregião é considerada um *hotspot* da biodiversidade, contendo um enorme número de espécies endêmicas de plantas vasculares, porém grande parte de seu habitat original foi devastado (Conservation International 2010). Foram estimadas cerca de 15.487 espécies de plantas (incluindo Angiospermas, Gimnospermas, Samambaias e Licófitas), sendo 9.164 endêmicas (Lista de Espécies da Flora do Brasil 2013). Restam hoje apenas ca. 7-11 % da sua estrutura original distribuída em fragmentos isolados (Ranta *et al*. 1998; SOS Mata Atlântica/INPE 2008; Ribeiro *et al*. 2009; Vieira *et al*. 2009).

A Mata Atlântica é formada pelas florestas Ombrófila Densa, Ombrófila Mista (mata de araucárias), Estacional Semidecidual e Estacional Decidual e pelos ecossistemas associados, como manguezais, restingas, brejos interioranos, campos de altitude e ilhas costeiras e oceânicas (SOS Mata Atlântica/INPE 2008).

Conexões passadas ligando a Mata Atlântica e a Floresta Amazônica, separadas principalmente pelo Cerrado brasileiro, constituindo possíveis rotas migratórias para plantas foram propostas por Rizzini (1963) e Andrade-Lima (1964). Extensas similaridades florísticas entre o sudeste da Mata Atlântica e o leste da Floresta Amazônica sugeriram a existência de uma rota denominada de "ponte sudeste-noroeste" (Bigarella *et al*. 1975). Esta rota poderia ter existido como um corredor florestal atravessando o Brasil central ou como fragmentos em série que permitiam migração por *island-hopping* (Oliveira-Filho & Ratter 1995). Outras áreas de contato entre estas duas florestas foram descritas no nordeste do Brasil, ao longo de matas de galeria do Cerrado e no sul através da bacia do Rio Paraná (Por 1992; Costa 2003).

Análises baseadas em dados palaeoclimáticos e genética de populações na Mata Atlântica sugeriram a presença de três refúgios históricos que permitiram a sobrevivência de espécies desta floresta durante períodos climáticos menos favoráveis: o refúgio Bahia que compreende uma grande área entre os rios São Francisco e rio Doce; o refúgio Pernambuco que corresponde a uma área menor ao norte do rio São Francisco, e o refúgio rio Doce que abrange áreas ao sul do rio Doce (Carnaval & Moritz 2008). Este mesmo estudo sugeriu que a área da floresta progressivamente migrou na direção sul, corroborando o registro fóssil (Ledru *et al*. 2005). No entanto, este padrão não é regra e os refúgios propostos por Carnaval & Moritz (2008) podem ser resultado não apenas da barreira física composta pelos refúgios, mas sim de diversos fatores em conjunto (Martins 2011).

Desde o descobrimento do Brasil, a Mata Atlântica vem sendo reduzida, pela exploração de seus recursos naturais, pela destruição da floresta para dar espaço a plantações de monoculturas e pela fundação e expansão de grandes cidades localizadas

ao longo do litoral brasileiro (Conservation International 2010). Esta área tem passado por intensa interferência antrópica e as espécies nativas vêm sendo substituídas por monoculturas de soja, arroz, *Eucaliptus*, *Pinus*, *Acacia*, entre outras espécies exóticas ou de interesse comercial. Foram retirados da Mata Atlântica, por métodos extrativistas rudimentares e predatórios, diversas espécies de fontes de madeira, palmito, plantas ornamentais, aves, peixes e pequenos animais. Com a destruição do habitat e a caça predatória, espécies animais e vegetais foram sendo deslocados ou eliminados destas matas (Leite & Klein 1990).

O estudo da Mata Atlântica e suas formações é muito importante para compreender a dinâmica da vegetação, considerando mudanças ambientais em escala local e global, e conhecer as causas do grande número de endemismos e da alta diversidade desta região (Leite 2002; Quadros & Pillar 2002). Muitas espécies ainda não são conhecidas pela ciência (Lewinsohn & Prado 2005), e com a degradação e perda de biodiversidade em escala exponencial, muitas poderão ser extintas sem nunca terem sido estudadas.

Estudos de diversidade genética de espécies que ocorrem na Mata Atlântica podem auxiliar a compreender os processos evolutivos que influenciam na diferenciação e distribuição das populações.

MODELAGEM DE NICHO ECOLÓGICO

O nicho ecológico se refere ao conjunto de condições bióticas e abióticas necessárias para a sobrevivência e reprodução (Hutchinson 1957; Brown & Lomolino 1998). A modelagem de nicho ecológico combina dados sobre a ocorrência das espécies com sistemas de informação geográfica (GIS) baseados em variáveis ambientais (Warren

2008). Esta abordagem vem sendo utilizada para inferir padrões de distribuição dos organismos no passado, descrever a distribuição presente e prever alterações futuras, combinando dados da distribuição atual com variáveis climáticas e ecológicas. Tais estudos permitem também analisar a evolução de nicho ecológico e testar hipóteses de divergência ou conservatismo de nicho (Wiens & Graham 2005; Warren *et al*. 2008; McCormack *et al*. 2010). Estes estudos podem ainda, auxiliar a elucidar questões evolutivas e as consequências de mudanças climáticas na distribuição de organismos e na formação de biomas (Behling & Lichte 1997; Behling & Negrelle 2001; Behling *et al*. 2002; Carnaval & Moritz 2008). Este tipo de abordagem pode servir como uma importante ferramenta para estabelecer estratégias de conservação de espécies e dos locais onde ocorrem.

CONSERVATISMO OU DIVERGÊNCIA DE NICHO

O conservatismo de nicho propõe que espécies relacionadas tendem a ocupar nichos ecológicos semelhantes. O conservatismo de nicho está relacionado com baixa adequabilidade e incapacidade de sobreviver em condições ecológicas distintas (Harvey & Pagel 1991; Wiens & Graham 2005; Warren 2008). Por outro lado, a divergência de nicho propõe a evolução de linhagens para novas condições ambientais, resultando em expansão na distribuição e aumento da tolerância de nicho (*niche breadth*) (Wiens & Donoghue 2004).

Ferramentas baseadas em GIS e modelagens de nicho ecológico podem ser utilizadas para quantificar conservatismo e divergência de nicho entre *taxa* proximamente relacionados (Wiens & Donoghue 2004; Warren *et al.* 2008; McCormack *et al*. 2010). Estas análises podem auxiliar a elucidar o papel da distribuição geográfica e de

atributos ecológicos na diversificação dos organismos (Wiens & Graham 2005; Warren *et al*. 2008; McCormack *et al*. 2010; Wellenreuther *et al*. 2012; Wooten & Gibbs 2012; Hung *et al*. 2013) e a desvendar fatores que possam ter contribuído na formação de comunidades vegetais mais complexas (Antonelli & Sanmartín 2011).

OBJETIVOS

O objetivo geral deste trabalho foi contribuir para o entendimento da história evolutiva de *Verbenoxylum reitzii* através de análises filogenéticas e de diversidade genética. Como objetivos específicos, teve-se:

1. Determinar o posicionamento filogenético de *V. reitzii*, a fim de compreender seu relacionamento dentro da família Verbenaceae.
2. Avaliar o padrão de evolução dos caracteres morfológicos utilizados tradicionalmente para separar grupos em Verbenaceae.
3. Desenvolver uma biblioteca de microssatélites para *V. reitzii* e caracterizar marcadores variáveis para serem utilizados em análises de diversidade genética da espécie.
4. Caracterizar a variabilidade intraespecífica de *V. reitzii* através de sequências de DNA e marcadores do tipo microssatélites.
5. Modelar o nicho ecológico de *V. reitzii* no presente e passado e testar hipóteses de divergência e conservatismo de nicho em comparação com um grupo irmão.
6. Compreender as relações evolutivas dentro da tribo Duranteae utilizando marcadores plastidiais e nucleares e investigar padrões de distribuição geográfica neste grupo.

JUSTIFICATIVA

Estudos evolutivos em plantas endêmicas da Mata Atlântica são de grande importância para gerar informações que auxiliem na compreensão de padrões complexos que possam ter influenciado na formação desta ecorregião. Até o momento, não existiam estudos moleculares para *Verbenoxylum* e sua relação com os demais membros da família Verbenaceae permanecia incerta. *Verbenoxylum reitzii* está na lista das espécies da flora ameaçadas de extinção do Rio Grande do Sul (SEMA 2002) e ocorre em um ambiente altamente degradado e sujeito à ação antrópica constante.

Devido ao estado de degradação em que a Mata Atlântica se encontra, o estudo de espécies que ocorrem nessa região é de elevada importância (Carnaval e Moritz 2008). Sendo assim, existe a necessidade de melhor compreender o posicionamento filiogenético de *Verbenoxylum reitzii*, a variabilidade genética e o entendimento da dinâmica populacional desta espécie. Estudos como a modelagem de nicho ecológico e testes de conservatismo e divergência de nicho também podem contribuir para um melhor entendimento da história evolutiva deste táxon e do ambiente onde ocorre.

A análise filogenética da tribo Duranteae é necessária para esclarecer as relações evolutivas dentro da tribo. Sequências gênicas têm provado ser uma ferramenta eficiente para inferir relações de parentesco entre organismos (Albach *et al*. 2001). Genes do DNA plastidial e nuclear vêm sendo estudados na busca de caracteres filogeneticamente informativos para estabelecer relações entre os grupos.

CAPÍTULO 1

PHYLOGENETIC POSITION OF THE MONOTYPIC GENUS *VERBENOXYLUM* (VERBENACEAE) AND NEW COMBINATION UNDER *RECORDIA*

PHYLOGENETIC POSITION OF THE MONOTYPIC GENUS *VERBENOXYLUM* (VERBENACEAE) AND NEW COMBINATION UNDER *RECORDIA*

Verônica A. Thode, [1,4] Nataly O'Leary, [2] Richard G. Olmstead, [3] and Loreta B. Freitas[1,4]

[1] Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Bento Gonçalves 9500, Porto Alegre, Brazil

[2] Instituto de Botánica Darwinion, Labardén 200, San Isidro, Argentina

[3] Department of Biology and Burke Museum, University of Washington, Seattle, Washington 98195, U. S. A.

[4] Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil

Artigo aceito para publicação na revista *Systematic Botany*

Abstract—In spite of the recent studies on the phylogeny of Verbenaceae, the position of the monotypic *Verbenoxylum,* endemic to the Atlantic rainforest in southeastern Brazil, remains unsolved. Molecular data were here analyzed to infer the phylogenetic placement of this genus; furthermore morphological data was studied in order to examine traits that support relationships among taxa. Sequences of the plastid regions of *ndhF* gene and *trnL–trnF* intergenic spacer were analyzed to conduct phylogenetic studies with maximum parsimony, maximum likelihood, and Bayesian inference. Morphological traits that had been traditionally used to distinguish tribes within Verbenaceae, as well as those employed to characterize *Verbenoxylum,* were examined. *Verbenoxylum* is nested within the tribe Duranteae, sister to *Recordia*, a monotypic genus endemic to Bolivia, a placement never reported before. The morphological traits analyzed prove not to be useful to distinguish tribes but are important at lower taxonomic levels. Based on the sister relationship and morphological similarities between the genera *Verbenoxylum* and *Recordia*, we propose the inclusion of *Verbenoxylum reitzii* into *Recordia*, forming the new combination ***Recordia reitzii***.

Keywords—Bolivia, Brazil, character evolution, molecular phylogeny, *ndhF*, *trnL–trnF*.

Verbenaceae (Lamiales) comprises about 35 genera and 800 species of herbs, shrubs, trees, and lianas that can occur in a broad variety of habitats. The genera are distributed mostly in the New World, with only *Chascanum* E. Mey. and *Coelocarpum* Balf. f. exclusive to Africa and the Indian Ocean Rim, respectively (Atkins 2004; Marx et al. 2010). Most of the classification systems proposed for Verbenaceae were based on morphological characters (Schauer 1847; Briquet 1895; Junell 1934; Troncoso 1974; Sanders 2001; Atkins 2004), whereas Marx et al. (2010) proposed a classification based on molecular markers (Table 1).

Verbenoxylum Tronc., as currently circumscribed, has only one species, *Verbenoxylum reitzii* (Moldenke) Tronc., endemic to southeastern Brazil. It occurs in the southern limit of the Atlantic rainforest in the Brazilian states of Rio Grande do Sul and Santa Catarina (Troncoso 1974; Reitz et al. 1978, 1983; Sobral et al. 2006) from 10 to 550 meters above sea level (Figs. 1A, 2). *V. reitzii* is considered vulnerable to extinction in the List of threatened species of Rio Grande do Sul Brazilian state (SEMA 2003), and according to the classification of rarity proposed by Rabinowitz (1981), which is based on geographical distribution, habitat distribution, and local population size, this species would belong to form 7, the most restricted form of rarity (Caiafa and Martins 2010). There are few biological studies on this species (Troncoso 1971; von Poser et al. 1997; Bueno and Leonhardt 2011).

Verbenoxylum was first described by Moldenke (1949) under *Citharexylum* L. However, Troncoso (1971), based on flower characters (corolla tube, thecal orientation, style length, and stigma) and fruit type, segregated it from *Citharexylum* under the new genus *Verbenoxylum*. This genus has been traditionally placed within the tribe Citharexyleae Briquet (Troncoso 1974; Sanders 2001; Atkins 2004) because of

its previous relation to *Citharexylum*. Troncoso (1974) included *Verbenoxylum* in Citharexyleae, which also comprised the genera *Baillonia* Bocq., *Citharexylum*, *Duranta* L., and *Rhaphithamnus* Miers. Sanders (2001) expanded this tribe including genera *Coelocarpum*, *Rehdera* Moldenke, *Rhaphithamnus*, and *Recordia* Moldenke. This concept of Citharexyleae was later followed by Atkins (2004) with the exception of *Coelocarpum*, which was not assigned to any tribe. However, based on molecular evidence Marx et al. (2010) identified a clade they recognized as Citharexyleae, comprising *Citharexylum*, *Baillonia*, and *Rehdera*. Since *Verbenoxylum* was the only genus of Verbenaceae not represented in the molecular phylogeny of Verbenaceae (Marx et al. 2010), its phylogenetic placement remains uncertain.

We present here a phylogenetic analysis using the plastid regions *ndhF* and *trnL–trnF* for all Verbenaceae genera, and examine twelve morphological traits in an evolutionary context to answer the following questions: 1) What is the phylogenetic position of *V. reitzii* within Verbenaceae? 2) Does it belong to the tribe Citharexyleae as proposed in previous taxonomic treatments for the family? 3) Are the morphological characters used in traditional classifications, here evaluated, useful to delimit tribes or genera within Verbenaceae?

MATERIALS AND METHODS

Taxon and Gene Sampling— The plastid regions *ndhF* gene, *trnL* intron, and *trnL–trnF* intergenic spacer were sequenced for field collected *V. reitzii* and combined with sequences from a previous molecular phylogeny of Verbenaceae (Marx et al. 2010). Sampling in the species-rich tribes Lantaneae and Verbeneae was reduced in relation to Marx et al. (2010). All Verbenaceae genera were included in this analysis,

among a total of 79 species. The outgroup was composed of 18 species representing other families in Lamiales. Voucher information and GenBank accession numbers can be found in Appendix 1.

DNA Extraction, Amplification, and Sequencing—Total genomic DNA was extracted from silica-gel dried tissue using a modified Doyle and Doyle (1987) CTAB protocol. Amplification and sequencing were performed using protocols described in Olmstead et al. (2008, 2009) with primers listed in Table 2. The PCR products were purified by precipitation from a 20% polyethylene glycol solution and washed in 70% ethanol (Dunn and Blattner 1987).

Phylogenetic Analyses—Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Corp., Ann Arbor, Michigan, U. S. A.) and manually aligned using Se-Al 2.0a11 (Rambaut 2002) or with MAFFT v.6 (http://mafft.cbrc.jp/alignment/server/) followed by manual adjustments. Nucleotide composition and variable sites were estimated using Mega 5 (Tamura et al. 2011).

Gaps from the *ndhF* and *trnL–trnF* sequences were coded as binary characters (Graham et al. 2000; Simmons and Ochoterena 2000) using GapCoder (Young and Healy 2003) and combined into a single dataset with the plastid regions in all analyses.

Maximum parsimony (MP) analyses were conducted in PAUP* 4.0b10 (Swofford 2002). Heuristic searches were performed with 1,000 replicates of random sequence additions, maximum of 10 trees saved per replicate, and tree-bisection-reconnection (TBR) branch swapping. Characters were unordered and equally weighted. Statistical support was estimated using 1,000 bootstrap (BSP) replicates (Felsenstein 1985) with three random addition replicates.

For maximum likelihood (ML) analyses and Bayesian inference (BI), the evolutionary models were selected using jModelTest 0.1.1 (Guidon and Gascuel 2003; Posada 2008) with Akaike Information Criterion (AIC). A TVM + G model was determined to best-fit both *ndhF* and *trnL–trnF*. Because the same model was selected and they are both noncoding plastid regions, they were concatenated. The gaps were treated as binary characters in the analyses. Maximum likelihood analyses were conducted using GARLI 2.0 (Zwickl 2006) with two independent search replicates and 1,000 bootstrap (BSL) replicates. The consensus tree was constructed in PAUP* 4.0b10 (Swofford 2002).

The BI analyses were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with 10 million generations sampled every 100 generations in two independent runs, each with four simultaneous Markov chains initiated with a random tree. The convergence between runs was checked with Tracer 1.5 (Rambaut and Drummond 2009). The first 25% of the sampled trees were discarded as burn-in and the remaining were used to build a consensus tree.

Morphological Ancestral State Reconstruction—We analyzed twelve morphological characters (Table 3) that were important in the taxonomy of the family or were used to distinguish taxa from *Verbenoxylum* (Schauer 1847; Briquet 1895; Junell 1934; Troncoso 1971, 1974; Sanders 2001; Atkins 2004) (Table 1). The character states were mapped into the Bayesian consensus tree obtained in the molecular analyses, using parsimony in Mesquite 2.75 (Maddison and Maddison 2011), according to the literature and herbaria specimens. The tree was collapsed to tips that represent the Verbenaceae genera and with outgroup taxa removed.

RESULTS

Phylogenetic Analyses—The gene region *ndhF* presented sequence lengths from 2,071 to 2,140 bp, with the aligned sequence including 1,164 conserved, 940 variable, 660 parsimony informative characters, a consistency index (CI) of 0.51, and a retention index (RI) of 0.75. Sequence lengths of *trnL–trnF* ranged from 891 bp to 1,232 bp, with the aligned sequence including 605 conserved, 447 variable, 276 parsimony informative characters, a CI of 0.64, and a RI of 0.8. The total length of the combined data unambiguously aligned was 3,372 bp, being 1,769 conserved, 1,387 variable, and 936 parsimony informative sites and 209 gaps that were scored as binary characters in the analyses. The combined dataset presented a CI of 0.56 and a RI of 0.78.

The MP, ML, and BI trees have similar topologies and are consistent with respect to relationships among genera within Verbenaceae. The analyses using the two plastid regions confirm the results previously obtained by Marx et al. (2010) for the phylogenetic relationships within Verbenaceae. *Verbenoxylum* forms a well-supported clade (BSP = 100, BSL = 100, PP = 1.00) with genus *Recordia*, within the tribe Duranteae Bentham, with these two genera sister to *Duranta* (BSP = 81, BSL = 90, PP = 0.92) (Fig. 3). The rest of the tribe forms a second clade comprising *Bouchea* Cham., *Chascanum*, and *Stachytarpheta* Vahl (BSP = 100, BSL = 100, PP = 1.00). Genera from the tribe Duranteae as circumscribed here are illustrated in Fig. 1A-F. Matrices and final tree files can be accessed on TreeBASE (study number S13802).

Morphological Analyses—We mapped character states for twelve discrete morphological traits for all Verbenaceae genera (Fig. 4A–L). All characters are shown to be homoplastic among tribes within the family, but can be important to distinguish

genera within a tribe. Characters are described in Table 3, and ancestral state reconstructions along with their utility for distinguishing genera in Duranteae are summarized in Table 4. Some characters have ambiguous reconstructions (more than one state can be the basal condition) for the common ancestor (for the family or tribe) due to the lack of information for a taxon or because the states are equally parsimonious.

The tribe Duranteae is composed of two clades, one formed by *Duranta*, *Recordia*, and *Verbenoxylum* (D + R + V) and the other by *Bouchea*, *Chascanum*, and *Stachytarpheta* (B + C + S). Many morphological differences were found between these two clades, contributing to the lack of a single morphological synapomorphy that could distinguish this tribe from the rest (Fig. 4A–L).

Within Duranteae, the D + R + V clade shares one synapomorphic trait: presence of linear floral bracts (Fig. 4H). Pedicellate flowers (Fig. 4I) are found in all members of the D + R + V clade, constituting a plesiomorphic trait for this clade, however it helps to distinguish the latter from the B + C + S clade, where sessile flowers constitute a synapomorphy.

Duranta has two synapomorphic traits: fleshy fruits (Fig. 4B) and four carpels (Fig. 4C). The presence of a short style (Fig. 4E) differentiates *Duranta* from the rest of Duranteae. However, this trait is ambiguously reconstructed for the ancestor of the tribe, thus it is not possible to distinguish this as the synapomorphic or the plesiomorphic condition for *Duranta*.

The *Recordia* + *Verbenoxylum* clade has four synapomorphic traits: they are trees (Fig. 4A), have long stamen filaments (Fig. 4J), divergent thecae (Figs. 4K, 5O), and exerted anthers (Figs. 4L, 5N). A bicarpellate ovary (Fig. 4C) is also shared by the

Recordia + *Verbenoxylum* clade, but this character is ambiguously reconstructed, so the state for the ancestor of Duranteae is unknown.

The B + C + S clade is supported by two synapomorphic traits: herbs or suffrutescent shrubs (Fig. 4A) with sessile flowers (Fig. 4I). Presence of one carpel (Fig. 4C) also characterizes this clade but it is not possible to distinguish which state of this trait is derived or plesiomorphic due to the ambiguous reconstruction for the ancestor of Duranteae. The *Bouchea* + *Chascanum* clade shares two synapomorphies: an oblique stigma (Fig. 4D) and absence of a staminode (Fig. 4G). Genus *Stachytarpheta* has two synapomorphic traits: two fertile stamens (Fig. 4F), and vertical thecal orientation (Fig. 4K).

DISCUSSION

Phylogenetic Position of Verbenoxylum Within Verbenaceae—Our results strongly support the placement of *V. reitzii* within the tribe Duranteae, as sister to *Recordia boliviana* Moldenke (Fig. 3). These two species are sister to *Duranta*, composing a clade sister to the rest of the tribe, represented by genera *Bouchea*, *Chascanum*, and *Stachytarpheta*. Marx et al. (2010) showed that Citharexyleae of earlier classifications (Troncoso 1974; Sanders 2001; Atkins 2004) was not monophyletic, which left open the question of which clade *Verbenoxylum* belonged to. Troncoso (1971) mentioned affinities between *Verbenoxylum* and *Recordia*, such as similar habit and similar flowers. However, a placement near *Citharexylum*, rather than *Duranta*, was implied in those classifications. The tribe Duranteae was established by Bentham in 1839, composed of four genera: *Citharexylum*, *Duranta*, *Petrea* L., and *Rhaphithamnus*. In the molecular phylogeny of Verbenacaeae proposed by Marx et al.

(2010), the tribe is circumscribed to include *Bouchea*, *Chascanum, Stachytarpheta*, *Duranta*, and *Recordia*. Our phylogenetic analyses suggest that *V. reitzii* also belongs to this tribe (Appendix S1, see online Supplemental Data). This expands the composition of Duranteae and resolves the placement of *Verbenoxylum* to tribe.

Morphological Characters Within Duranteae—With a well-resolved phylogeny for the family it is possible to interpret the evolution of morphological traits and to evaluate the characters used traditionally to distinguish tribes or genera. According to our results, none of the twelve characters here studied are informative for distinguishing tribes within the family but they can be important to distinguish genera or suprageneric groups within a tribe. These traits probably have multiple origins within Verbenaceae (Fig. 4A-L). Marx et al. (2010) mentioned that none of the traditional treatments for Verbenaceae matches their molecular phylogeny, suggesting that homoplasy is frequent in the characters used in those classifications. A synapomorphic trait to support Duranteae, as here circumscribed, was not identified. However, the morphological characters here studied can be important to define groups within this tribe (Fig. 4A-L; Table 4).

Recordia and *Verbenoxylum* (Duranteae), and *Citharexylum* and *Rehdera* (Citharexyleae) are the only genera in Verbenaceae with species that are trees. Within Duranteae, this character is a synapomorphy for the *Recordia* + *Verbenoxylum* clade, having evolved from ancestors that were shrubs or small trees. On the other hand, in the ancestor of the B + C + S clade there was a shift to herbs or suffrutescent shrubs (Fig. 4A).

Dry fruits are present in the majority of Verbenaceae genera. However, *Duranta* has fleshy fruits, and constitutes the only genus in Duranteae without dry

fruit. Sanders (2001) associated *Duranta* with *Citharexylum* by the presence of fleshy fruits. Nevertheless, our study shows that fleshy fruits have evolved independently in both genera. Fruit type was one of the morphological differences noticed by Troncoso (1971) to distinguish *Verbenoxylum* from *Citharexylum*, being dry in the first (Fig. 5E-F) and fleshy in the latter (Fig. 4B).

In Verbenaceae, plants can have one, two, or four carpels. In Duranteae, the B + C + S clade is characterized by one carpel, *Recordia + Verbenoxylum* clade has two carpels (Fig. 5H), and *Duranta* is the only genus in the family with four carpels. A shift from two to four carpels is the most probable explanation for the carpel condition in *Duranta*. This means that the ancestor of the D + R + V clade probably had two carpels and this might constitute a synapomorphy for this clade. However, the ancestral carpel condition in Duranteae remains uncertain in this analysis (Fig. 4C).

A capitate stigma is plesiomorphic in Verbenaceae and is the state shared by most Duranteae (Fig. 5I). However, an oblique stigma, which also is found independently in several other tribes, is a synapomorphy for the *Bouchea* + *Chascanum* clade (Fig. 4D).

Style length is ambiguously reconstructed for the ancestor of Duranteae, nevertheless the presence of a short style (less than three times the ovary length) in *Duranta* separates this genus from the rest of the tribe, which have long styles (more than three times the ovary length). Style length was another trait mentioned by Troncoso (1971) to segregate *Verbenoxylum*, with a long style, from *Citharexylum*, with short styles. However, in extraordinary cases, species of *Citharexylum* from Central America can have long styles (Gibson 1970). This character has been used traditionally

to distinguish the genus *Glandularia* J.F. Gmel. from *Verbena* L. (Botta 1993; O'Leary et al. 2010) (Fig. 4E).

Most Verbenaceae have four fertile stamens (Fig. 5O), only the monotypic genus *Hierobotana* Briq., from the tribe Verbeneae, and *Stachytarpheta* have two fertile stamens, thus distinguishing *Stachytarpheta* within Duranteae (Fig. 4F).

Presence of a staminode is less frequent within Verbenaceae than absence. Within Duranteae, presence of staminode is a plesiomorphic state found in *Stachytarpheta*, *Recordia* (Fig. 5O), *Verbenoxylum*, and certain species of *Duranta*. This last genus has species with and without staminodes, as well as taxa with five fertile stamens, showing that this trait is variable within *Duranta*. Absence of a staminode is a synapomorphy for the *Bouchea* + *Chascanum* clade (Fig. 4G).

The shape of the floral bracts in Verbenaceae is quite variable and often is used to distinguish species within a genus (e.g., Atkins 2005; Thode and Mentz 2010). However, linear floral bracts constitute a synapomorphy for the D + R + V clade. The linear floral bracts found in the *Recordia* + *Verbenoxylum* clade (Fig. 5D, L) are usually difficult to observe in herbarium material because are early deciduous. The genera from the B + C + S clade has lanceolate floral bracts (Fig. 4H).

Within Duranteae, the presence of a floral pedicel (longer than 2 mm) is a plesiomorphic state for the D + R + V clade, and sessile flowers is a synapomorphy for the B + C + S clade (Fig. 4I).

Anthers subtended by a long filament (more than 2 mm long) are found in a few taxa within Verbenaceae, with sessile or subsessile anthers occurring most frequently in the family. In Duranteae, presence of long filaments constitutes a synapomorphy for the *Recordia* + *Verbenoxylum* clade (Fig. 5O). *Duranta* species have

subsessile filaments; however there is one species, *Duranta serratifolia* (Griseb.) Kuntze, which has long filaments. Troncoso (1971) used this character to distinguish *Verbenoxylum* from *Citharexylum*, with sessile or subsessile anthers in the latter (Fig. 4J).

The most common thecal orientation in Verbenaceae is parallel. Divergent thecae are present in *Rhaphithamnus*, *Coelocarpum*, and within Duranteae is a synapomorphy for the *Recordia* + *Verbenoxylum* clade (Fig. 5O). Vertical thecae are a synapomorphy for *Stachytarpheta*. The rest of the tribe have parallel thecae. Troncoso (1971) used this trait to segregate *Verbenoxylum*, with divergent thecae, from *Citharexylum*, which has parallel thecae (Fig. 4K).

In Verbenaceae anthers are most frequently included in the corolla tube. Nevertheless, exerted anthers appear independently several times in all Verbenaceae tribes, except in the tribe Citharexyleae. In Duranteae, exerted anthers state is a synapomorphy for the *Recordia* + *Verbenoxylum* clade (Fig. 5N), thus this character is useful to differentiate these taxa from the rest of the tribe (Fig. 4L).

The twelve characters studied here showed to be informative to distinguish taxa within Duranteae (Table 4). The *Recordia* + *Verbenoxylum* clade is morphologically distinct from the rest of the genera in the tribe (Figs. 1A-F, 4A-L). *Verbenoxylum* and *Recordia* differ from the other genera by five traits: both are trees, have a bicarpellate ovary, flowers that have anthers with long filaments, with divergent, and exerted thecae. The floral traits of the *Recordia* + *Verbenoxylum* clade seem to show a distinct reproductive strategy comparing with the rest of the tribe, possibly associated with different pollinators, but further biological studies on both taxa are necessary. The differences between the two genera lie in floral bract, calyx, and leaf pubescence and

margins, with *Verbenoxylum* being almost glabrous and having serrated leaves, and *Recordia* being hirsute and having entire leaf margins (Fig. 5A-F). Both are narrow endemics and monotypic; *Verbenoxylum* is distributed in the subtropical Atlantic rainforest in Rio Grande do Sul and Santa Catarina, Brazil. *Recordia* is endemic to Bolivia, common in the low mountains west of Santa Cruz de la Sierra, on the highway between Samaipata and Cochabamba (M. Nee, pers. comm.), occurring between 500 and 1,850 meters above sea level in dry subtropical semi-deciduous forest (Fig. 2).

New Combination—Our molecular and morphological analyses strongly support the combination of *Verbenoxylum reitzii* and *Recordia boliviana* as a single genus, despite the large distributional gap (Figs. 2–5). Our results show that the molecular and morphological differences do not justify different generic rank. We propose a new combination for *Verbenoxylum reitzii*, transferring it to the genus *Recordia*, and reducing *Verbenoxylum* to synonymy. Consequently, *Recordia* is now circumscribed to include two species, *Recordia reitzii* endemic to Brazil and *Recordia boliviana* endemic to Bolivia.

TAXONOMIC TREATMENT

Recordia reitzii (Moldenke) Thode & O'Leary, comb. nov. *Verbenoxylum reitzii* (Moldenke) Tronc. Darwiniana 16: 626. 1971. *Citharexylum reitzii* Moldenke, Phytologia 3: 59. 1949. —TYPE: BRAZIL. Santa Catarina: Arar (Araranguá), Rodeio da Areia, 12 Nov 1943, *R. Reitz c175* (holotype: NY!; isotypes: NY!, RB!, SI!).

KEY TO THE SPECIES OF *RECORDIA*

1. Plants mostly glabrous, calyx scarcely pubescent, leaf margins serrated, endemic to Brazil ..... *R. reitzii*

2. Plants mostly puberulous, calyx hirsute, leaf margins entire, endemic to Bolivia ..... *R. boliviana*

ACKNOWLEDGEMENTS. This study was supported by a doctoral fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior to VAT, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul, by the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220080100177/09) to NO'L, by the U.S. National Science Foundation grants DEB 0542493, DEB 0710026, and DEB 1020369 to RGO. We thank Priscilla Zamberlan and Jéferson Fregonezi for help with molecular data and analyses, Erin Tripp and Luzmila Arroyo for permission to use images, and the anonymous reviewers and the associate editor Andrew Hipp for comments on the manuscript.

LITERATURE CITED

Atkins, S. 2004. Verbenaceae. Pp. 449–468 in *The families and genera of vascular plants* vol. 7, ed. J. W. Kadereit. Berlin: Springer-Verlag.

Atkins, S. 2005. The genus *Stachytarpheta* (Verbenaceae) in Brazil. *Kew Bulletin* 60: 161–272.

Bentham, G. 1839. Enumeration of plants collected by Mr. Schomburgk in British Guiana. *Annals & Magazine of Natural History* 2: 441–451.

Botta, S. M. 1993. Notas en el género *Glandularia* (Verbenaceae-Verbenoideae) III. Estudio taxonómico de las especies patagónicas. *Parodiana* 8: 9–36.

Briquet, J. 1895. Verbenaceae. Pp. 132–182 in *Die naturlichen Pflanzenfamilien* Tiel 4/3a, ed. A. Engler, and K. Prantl. Leipzig: Engelmann.

Bueno, O. L. and C. Leonhardt. 2011. Distribuição e potencial paisagístico dos gêneros *Citharexylum* L. e *Verbenoxylum* Tronc. no Rio Grande do Sul, Brasil. *Iheringia. Série Botânica* 66: 47–60.

Caiafa, A. N. and F. R. Martins. 2010. Forms of rarity of tree species in the southern Brazilian Atlantic rainforest. *Biodiversity and Conservation* 19: 2597–2618.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh tissue. *Phytochemical Bulletin* 19: 11–15.

Dunn, I. S. and F. R. Blattner. 1987. Charons 36 to 40: multi–enzyme, high capacity, recombination deficient replacement vectors with polylinkers and polystuffers. *Nucleic Acids Research* 15: 2677–2698.

Felsenstein, J. 1985. **Confidence limits on phylogenies: an approach using the bootstrap.** *Evolution* **39:** 783–791.

Gibson, D. N. 1970. Verbenaceae. Flora of Guatemala. *Fieldiana Botany* 24: 167–236.

Graham, S. W., P. A. Reeves, A. C. E. Burns, and R. G. Olmstead. 2000. Microstructural changes in noncoding chloroplast DNA: interpretation, evolution, and utility of indels and inversions in basal angiosperm phylogenetic inference. *International Journal of Plant Science* 161 (6 Suppl.): S83–S96.

Guidon, S. and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696–704.

Holmgren, P. K., N. H. Holmgren, and L. C. Barnett, eds. 1990. *Index Herbariorum 1: The herbaria of the world.* Ed. 8. *Regnum Vegetabile*. New York: New York Botanical Garden.

Junell, S. 1934. Zur Gynaceummorphologie und Systematik der Verbenaceen und Labiaten. *Symbolae Botanicae Upsaliensis* 4:1–219.

Maddison, W. P. and D. R. Maddison. 2011. Mesquite: a modular system for evolutionary analysis, version 2.75. [computer program]. Available at http://mesquiteproject.org.

Marx, H. E., N. O'leary, Y. Yuan, P. Lu-Iirving, D. C. Tank, M. E. Múlgura, and R. G. Olmstead. 2010. A molecular phylogeny and classification of Verbenaceae. *American Journal of Botany* 97: 1–17.

Moldenke, H. N. 1949. Notes on new and noteworthy plants VII. *Phytologia* 3: 59.

O'Leary, N., M. E. Múlgura de Romero, and O. Morrone. 2010. Revisión taxonómica de las especies del género *Verbena* L. (Verbenaceae) II: serie Verbena. *Annals of the Missouri Botanical Garden* 97: 369–428.

Olmstead, R. G. and J. A. Sweere. 1994. Combining data in phylogenetic systematics - an empirical approach using three molecular data sets in the Solanaceae. *Systematic Biology* 43: 467–481.

Olmstead, R. G., L. Bohs, H. Abdel Migid, E. Santiago-Valentin, V. F. Garcia, and S. M. Collier. 2008. A molecular phylogeny of the Solanaceae. *Taxon* 57: 1159-1181.

Olmstead, R. G., M. L. Zjhra, L. G. Lohmann, S. O. Grose, and A. J. Eckert. 2009. A molecular phylogeny and classification of Bignoniaceae. *American Journal of Botany* 96: 1731–1743.

Posada, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.

Rabinowitz, D. 1981. Seven forms of rarity. Pp. 205–217 in *The biological aspects of rare plant conservation*, ed. H. Synge. *Chichester: John Wiley and Sons.*

Rambaut, A. 2002. Se-Al: Sequence alignment editor v2.0a11. [computer program]. Available at http://evolve.zoo.ox.ac.uk/.

Rambaut, A. and A. J. Drummond. 2009. Tracer v1.5 [computer program]. Available at http://tree.bio.ed.ac.uk/software/tracer/.

Reitz, R., R. M. Klein, and A. Reis. 1978. Projeto Madeira de Santa Catarina. *Sellowia* 28–30: 1-320.

Reitz, R., R. M. Klein, and A. Reis. 1983. Projeto Madeira do Rio Grande do Sul. *Sellowia* 34–35: 1-525.

Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Sanders, R. W. 2001. The genera of Verbenaceae in the southeastern United States. *Harvard Papers in Botany* 5: 303–358.

Schauer, J. C. 1847. Verbenaceae. Pp. 522–700 in *Prodromus Systematis Naturalis Regni Vegetabilis* 11, ed. A. P. De Candolle. Paris: Fortin, Masson et Sociorum.

SEMA. 2003. Lista final das espécies da flora ameaçadas – RS, Decreto Estadual n° 42.099, Available at www.fzb.rs.gov.br/downloads/flora_ameacada.pdf.

Simmons, M. P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.

Sobral, M., J. A. Jarenkow, P. Brack, B. Irgang, J. Larocca, and R. S. Rodrigues. 2006. Flora arbórea e arborescente do Rio Grande do Sul, Brasil. São Carlos, Brazil: RiMA/Novo Ambiente.

Swofford, D. L. 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods), version 4b10. Sunderland: Sinauer Associates.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.

Thode, V. A. and L. A. Mentz. 2010. O gênero *Glandularia* J. F. Gmel. (Verbenaceae) no Rio Grande do Sul, Brasil. *Acta Botanica Brasilica* 24: 529–557.

Troncoso, N. S. 1971. *Verbenoxylum*, nuevo género de Verbenáceas arbóreas de Brasil austral. *Darwiniana* 16: 622–626.

Troncoso, N. S. 1974. Los géneros de Verbenáceas de Sudamérica extratropical. *Darwiniana* 18: 295–412.

von Poser, G. L., M. E. Toffoli, M. Sobral, and A. T. Henriques. 1997. Iridoid glucosides substitution patterns in Verbenaceae and their taxonomic implication. *Plant Systematics and Evolution* 205: 265–287.

Young, N. and J. Healy. 2003. **GapCoder automates the use of indel characters in phylogenetic analysis.** *BMC Bioinformatics* **4:** 6.

Zwickl, D. J. 2006. *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion.* Austin: University of Texas, U. S. A.

TABELAS

CAPÍTULO 1

TABLE 1. Classification systems for Verbenaceae s.s. and characters that they used.

Authors	**Schauer (1847)**	**Briquet (1895)**	**Junell (1934)**	**Troncoso (1974)**	**Sanders (2001)**	**Atkins (2004)**	**Marx et al. (2010)**
Infrafamilial division	5 subtribes	5 subfamilies	6 tribes	7 tribes	4 tribes	6 tribes	8 tribes
	Casseliinae	Citharexyleae	Casselieae	Casselieae	Citharexyleae	Casselieae	Casselieae
	Durantinae	Euverbeneae	Citharexyleae	Citharexyleae	Lantaneae	Citharexyleae	Citharexyleae
	Lantaninae	Lantaneae	Lantaneae	Lantaneae	Petreeae	Lantaneae	Duranteae
	Petreinae	Monochileae	Petreeae	Parodiantheae	Verbeneae	Petreeae	Lantaneae
	Verbeninae	Petreeae	Priveae	Petreeae		Priveae	Neospartaneae
		Priveae	Verbeneae	Priveae		Verbeneae	Petreeae
				Verbeneae			Priveae
							Verbeneae
Characters	Calyx and corolla				Calyx and corolla	Inflorescence	
	Inflorescence	Inflorescence	Gynoecium	Gynoecium	Inflorescence	Anther and connective	Seven DNA markers
	Fruit	Gynoecium		Fruit	Anther and connective	Staminode	
					Staminode	Style	
					Style	Carpel number	
					Carpel number	Fruit	
					Fruit		

TABLE 2. Polymerase chain reaction (PCR) and sequencing (seq.) primers for the two plastid regions, primer sequence, and source.

Gene region and primer		Primer sequence (5'-3')	Source
ndhF	1	ATGGAACAKACATATSAATATGC (PCR and seq.)	Olmstead and
gene	536	TTGTAACTAATCGTG TAGGGGA (seq.)	Sweere (1994)
	536R	TCCCCTACACGATTAGTTACAA (seq.)	
	972	GTCTCAATTGGGTTATATGATG (PCR and seq.)	
	972R	CATCATATAACCCAATTG AGAC (seq.)	
	1318	GGATTAACYGCATTTTATATGTTTCG (seq.)	
	1318R	CGAAACATATAAAATGCRGTTAATCC (PCR and seq.)	
	1603	CCTYATGAATCGGACAATACTATGC (seq.)	
	1603R	GCATAGTATTGTCCGATTCATRAGG (seq.)	
	2110R	CCCCCTAYATATTTGATACCTTCTCC (PCR and seq.)	
trnL–trnF	*trnLF*_c	CGAATCGGTAGACGCTACG (PCR and seq.)	Taberlet et al.
region	*trnL/F*_d	GGGGATAGAGGGACTTGAAC (seq.)	(1991)
	*trnLF*_e	GGTTCAAGTCCCTCTATCCC (seq.)	
	*trnLF*_f	TTTGAACTGGTGACACGAG (PCR and seq.)	

TABLE 3. Characters and corresponding states used in this study for the morphological ancestral state reconstruction.

1. Habit: Tree (0); shrub or small tree (1); herb or suffrutescent shrub (2). **2. Fruit:** Dry (0); fleshy (1). **3. Carpel number:** One (0); two (1); four (2). **4. Stigma:** Capitate (0); bilobed (1); oblique (2). **5. Style length:** Long = more than three times the ovary length (0); short = less than three times (1). **6. Fertile stamens:** Two (0); four or more (1). **7. Staminode:** Absence (0); presence (1). **8. Floral bracts:** Linear (0); lanceolate (1). **9. Pedicel:** Absence = sessile or subsessile (0); presence = longer than 2 mm (1). **10. Stamen filament:** Absence or subsessil = less than 2 mm (0); presence = more than 2 mm (1). **11. Thecal orientation:** Divergent (0); vertical (1); parallel (2). **12. Anther exertion:** Absence/included (0); presence/exerted (1).

TABLE 4. Results for the ancestral state reconstruction using parsimony analyzed with Mesquite. B = *Bouchea*; C = *Chascanum*; D = *Duranta*; R = *Recordia*; S = *Stachytarpheta*; V = *Verbenoxylum*.

Character	Number of steps	Ancestral state for Verbenaceae	Ancestral state for Duranteae	State for *Recordia* + *Verbenoxylum*	Taxa distinguished within Duranteae
1	10	Shrub or small tree	Shrub or small tree	Tree	(B + C + S)/D/(R + V)
2	8	Ambiguous	Dry	Dry	D
3	7	Ambiguous	Ambiguous	Two	(B + C + S)/D/(R + V)
4	10	Capitate	Capitate	Capitate	(B + C)
5	7	Ambiguous	Ambiguous	Long	D
6	2	Four (or +)	Four (or +)	Four (or +)	S
7	6	Presence	Presence	Presence	(B + C)
8	7	Lanceolate	Lanceolate	Linear	(B + C + S)/(D + R + V)
9	8	Presence	Presence	Presence	(B + C + S)/(D + R + V)
10	8	Absence or subsessile	Absence or subsessile	Presence	(R + V)
11	4	Parallel	Parallel	Divergent	(B + C + D)/S/(R + V)
12	12	Absence (included)	Absence (included)	Presence (exerted)	(R + V)

APÊNDICES

CAPÍTULO 1

APPENDIX 1. List of specimens sampled: Taxon, place of collection, vouchers, and GenBank accession numbers (*ndhF*, *trnLF*). Sequences not available are indicated by —. Abbreviations of herbaria follow Holmgren et al. (1990).

Ingroup —***Acantholippia salsoloides*** Griseb., Argentina: Salta, *R. Olmstead 2007-23* (WTU), HM216682, HM216586. ***Aloysia catamarcensis*** Moldenke, Argentina: La Rioja, *R. Olmstead 2007-82* (WTU), HM216683, HM216587. ***Aloysia gratissima*** (Gillies & Hook.) Tronc., Cultivated, *K. -J. Kim 12803* (TEX), AF130154, HM216592. ***Baillonia amabilis*** Bocq. ex Baill., Bolivia: El Poston-Chiqueta, *M. Cardenas 4522* (US), HM216691, HM216595. ***Bouchea dissecta*** S. Watson, Mexico: Municipio de Nogales, *A. L. Reina G., T. R. Van Devender, P. Merlin 2004-951* (TEX), HM216692, HM216596. ***Bouchea fluminensis*** (Vell.) Moldenke, Cultivated*, H. Rimpler 1141* (FB), HM216693, HM216597. ***Bouchea linifolia*** A. Gray, U. S. A.: Texas, *B. L. Turner 20-423* (TEX), HM216694, HM216598. ***Burroughsia appendiculata*** (B.L.Rob. & Greenm.) Moldenke, Mexico: Coahuila, *J. Henrickson 14273* (LL), HM216695, HM216600. ***Casselia confertiflora*** (Moldenke) Moldenke, Brazil: Goiás, *R. C. Mendonça et al. 2859* (US), HM216697, HM216602. ***Casselia glaziovii* var. *serrata*** Moldenke, Brazil: Minas Gerais, *M. A Silva et al. 3630* (US), HM216698, HM216603. ***Casselia integrifolia*** Nees & Mart., Brazil: Espírito Santo*, J. R. Pirani et al. 3449* (US), HM216699, HM216604. ***Chascanum humbertii*** Moldenke, Madagascar, *Miller and Randrianasola 6127* (MO), HM216700, HM216605. ***Chascanum laetum*** Walp., Ethiopia: Wollo, *J. DeWilde 6923* (MO), HM216701, HM216606. ***Citharexylum argutedentatum*** Moldenke, Peru: Cusco: Urubamba, *R. Olmstead 2009-32* (WTU), –, HM216607. ***Citharexylum argutedentatum*** Moldenke, Peru: Cusco: Calca, *R. Olmstead 2009-36* (WTU), HM216702, HM216608. ***Citharexylum berlandieri*** B. L. Rob., Cultivated: Fairchild Tropical Gardens 78169B, *J. Francisco-Ortega* (FTG), HM216703, HM216609. ***Citharexylum fruticosum*** L., Cultivated: Kew 000-69-14013, no voucher, HM216704, –. ***Citharexylum fruticosum*** L., Cuba: Pinar del Rio, *R. Olmstead 96-113* (WTU), –, HM216610. ***Citharexylum herrerae*** Mansf. 1, Peru: Apurimac: Abancay, *R. Olmstead 2009-11* (WTU), HM216705, HM216611. ***Citharexylum herrerae*** Mansf. 2, Peru: Apurimac: Abancay, *R. Olmstead 2009-21* (WTU), HM216706, HM216612. ***Citharexylum ilicifolium*** Kunth, Peru: Cusco: Urubamba, *R. Olmstead 2009-31* (WTU), HM216707, HM216613. ***Citharexylum ligustrinum*** van Houtte, Cultivated: Kew 000-69.51235, no voucher, HM216708, HM216614. ***Citharexylum mocinnoi*** D. Don, Nicaragua: Jinoteca, *S. Grose 151* (HULE), HM216709,

HM216615. ***Citharexylum montevidense*** (Spreng.) Moldenke, Argentina: Buenos Aires, *R. Olmstead 2004-102* (WTU), HM216710, HM216616. ***Coelocarpum madagascariense*** Scott-Elliot 1, Madagascar, *Schatz 2977* (MO), HM216712, HM216618. ***Coelocarpum madagascariense*** Scott-Elliot 2, Madagascar, *Phillipson and Milijaona 3569* (MO), HM216711, HM216617. ***Coelocarpum swinglei*** Moldenke, Madagascar, *Phillopson et al. 3443* (MO), HM216713, HM216619. ***Diostea juncea*** Miers 1, Cultivated: RBG Edinburgh 19300262, no voucher, HM216715, HM216621. ***Diostea juncea*** Miers 2, Cultivated: RBG Kew 1969-35347, no voucher, HM216714, HM216620**.** ***Dipyrena glaberrima*** (Gillies & Hook.) Hook., Argentina: Mendoza, *R. Olmstead 2004-179* (WTU), HM216716, HM216622. ***Duranta erecta*** L., Cultivated: Jardin Botanica Nacional, Havana, Cuba, *R. Olmstead 1996-100* (WTU), HM216717, HM216623. ***Duranta fletcheriana*** Moldenke, Cuba: Topes de Collantes, *R. Olmstead 1996-71* (WTU), HM216718, HM216624**.** ***Duranta serratifolia*** (Griseb.) Kuntze, Argentina: Salta*, R. Olmstead 2007-009* (WTU), HM216719, HM216625. ***Duranta sprucei*** Briq, Cultivated: Waimea Bot. Gard. 75S356, *R. Olmstead 1992-221* (WTU), HM216720, HM216626. ***Duranta triacantha*** Juss., Peru: Apurimac, *R. Olmstead 2009-20* (WTU), HM216721, HM216627. ***Glandularia aurantiaca*** (Speg.) Botta, Argentina: Mendoza, *R. Olmstead 2004-196* (WTU), HM216722, EF571554. ***Glandularia bipinnatifida*** (Nutt.) Nutt., U. S. A.: Colorado, *R. Olmstead 92-133* (WTU), HM216723, —. ***Glandularia bipinnatifida*** (Nutt.) Nutt., U. S. A.: Texas, *Y. -W. Yuan 2005-12* (WTU), —, EF571535. ***Glandularia tenera*** (Spreng.) Cabrera, Cultivated: Waimea Bot. Gard. 74P1415, *R. Olmstead 92-222* (WTU), HM216728, —. ***Glandularia tenera*** (Spreng.) Cabrera, Argentina: Mendoza, *R. Olmstead 2004-148* (WTU), —, EF571556. ***Hierobotana inflata*** Briq., Ecuador: Pichineha, *E. Asplund 17069* (US), HM216729, HM216628. ***Junellia crithmifolia*** (Gillies & Hook.) N. O'Leary & P. Peralta, Argentina: Mendoza, *R. Olmstead 2004-169* (WTU), HM216730, EF571558. ***Junellia seriphioides*** (Gillies & Hook.) Moldenke, Argentina: Mendoza, *R. Olmstead 2004-147* (WTU), HM216732, EF571561. ***Lampaya castellani*** Moldenke, Argentina: Jujuy, *R. Olmstead 2007-063* (WTU), HM216736, HM216630. ***Lampaya hieronymi*** Schum. ex Moldenke 1, Argentina: Catamarca, *E. Marilienez Carretero 2092* (MERL), HM216737, HM216631. ***Lampaya hieronymi*** Schum. ex Moldenke 2, Argentina: Catamarca, *F. Biurrun et al. 4960* (SI), HM216738, HM216632. ***Lantana canescens*** Kunth, Argentina: Salta, *R. Olmstead 2007-006* (WTU), HM216740, HM216634. ***Lantana trifolia*** L., Cultivated: Jardin Botanica Nacional Havana, Cuba, *R. Olmstead 1996-98* (WTU), HM216745, HM216639. ***Lippia integrifolia*** Hieron., Argentina: Catamarca, *R. Olmstead 2007-78* (WTU), HM216749, HM216643.

Mulguraea asparagoides (Gillies & Hook.) O'Leary & Peralta, Argentina: Mendoza, *R. Olmstead 2004-192* (WTU), HM216756, EF571567. ***Mulguraea scoparia*** (Gillies & Hook.) O'Leary & Peralta, Argentina: Mendoza, *R. Olmstead 2004-178* (WTU), HM216758, EF571566. ***Nashia inaguensis*** Millsp., Cultivated: Fairchild Tropical Gardens 8655, no voucher, HM216759, HM216650. ***Neosparton aphyllum*** (Gillies & Hook.) Kuntze, Argentina: Mendoza, *R. Olmstead 2004-193* (WTU), HM216760, HM216651. ***Neosparton ephedroides*** Griseb., Argentina: Catamarca, *R. Olmstead 2007-077* (WTU), HM216761, HM216652. ***Parodianthus ilicifolius*** (Moldenke) Troncoso, Argentina: San Luis, *R. Olmstead 2004-181* (WTU), HM216762, HM216653. ***Petrea kohautiana*** C. Presl, Ecuador, *J. L. Clark 6554* (US), HM216763, HM216654. ***Petrea volubilis*** L. 1, Cultivated: RBG Kew 000.73.17818, no voucher, AY919283, HM216655. ***Petrea volubilis*** L. 2, Cultivated: RBG Kew 326.75.03134, no voucher, FJ887872, FJ870052. ***Phyla cuneifolia*** (Torr.) Greene, U. S. A.: Colorado, *R. Olmstead 1992-134* (WTU), HM216765, HM216657. ***Pitraea cuneato-ovata*** (Cav.) Caro 1, Argentina: Mendoza, *R. Olmstead 2004-186* (WTU), HM216768, HM216661. ***Pitraea cuneato-ovata*** (Cav.) Caro 2, Argentina: Catamarca, *R. Olmstead 2007-81* (WTU), HM216769, HM216662. ***Priva cordifolia*** Druce, South Africa: Natal, *W. Vos 391* (NU), HM216770, HM216663. ***Priva lappulacea*** (L.) Pers., Cuba: Villa Clara, *R. Olmstead 1996-86* (WTU), HM216771, HM216664. ***Recordia boliviana*** Moldenke, Bolivia: Santa Cruz, *M. Nee 24092* (TEX), HM216772, HM216665. ***Rehdera penninervia*** Standl. & Moldenke, Guatemala: El Peten, *C. L. Lundell and E. Contreras 19938* (TEX), HM216773, —. ***Rehdera penninervia*** Standl. & Moldenke, Guatemala: El Peten, *M. Pena-Chocarro and N. Bonilla 1378* (MO), —, HM216666. ***Rehdera trinervis*** (Blake) Moldenke, Mexico: Compeche, *E. Martinez S., D. Alvarez M., S. Ramirez A. 31706* (TEX), HM216774, HM216667. ***Rhaphithamnus spinosus*** (Juss.) Moldenke, Cultivated: RBG Kew 128-83.01596, no voucher, L36409, FJ870056. ***Rhaphithamnus venustus*** B. L. Rob., Chile: Juan Fernandez Islands, *T. F. Stuessy 11855* (OS), HM216775, HM216668. ***Stachytarpheta cayennensis*** (Rich.) Vahl, Argentina: Corrientes, *R. Olmstead 2004-113* (WTU), HM216776, HM216669. ***Stachytarpheta dichotoma*** (Ruiz & Pav.) Vahl, U. S. A.: Hawaii, *R. Olmstead 951* (WTU), L36414, HM216670. ***Stachytarpheta frantzii*** Polak., Cultivated: Fairchild Tropical Gardens 2001-0533B, no voucher, HM216777, HM216671. ***Stachytarpheta jamaicensis*** (L.) Vahl, Cuba: Topes de Collantes, *R. Olmstead 1996-68* (WTU), HM216778, HM216672. ***Stachytarpheta mutabilis*** (Jacq.) Vahl, Cultivated: Waimea Bot. Gard. 75C1444, *R. Olmstead 1992-207* (WTU), HM216779, HM216673. ***Tamonea boxiana*** (Moldenke) R. A. Howard, U. S. A.: Puerto Rico, *R. Olmstead*

2003-12 (WTU), HM216780, HM216674. ***Tamonea curassavica*** (L.) Pers., Cultivated: Germany, *H. Rimpler 1917* (FB), HM216781, HM216675. ***Verbena carnea*** Medik, U. S. A.: Florida, *Zomlefer 693* (WTU), HM216783, HM216676. ***Verbena intermedia*** Gillies & Hook., Argentina: Entre Rios, *R. Olmstead 2004-106* (WTU), HM216785, EF571522. ***Verbena montevidensis*** Spreng., Argentina: Corrientes, *R. Olmstead 2004-112* (WTU), HM216788, EF571521. ***Verbena officinalis*** L., Cultivated, *R. Olmstead 98-55* (WTU), HM216789, EF571525. ***Verbenoxylum reitzii*** (Mold.) Tronc., Brazil: Rio Grande do Sul, *V. Thode 162* (ICN), KC466433, KC466432. ***Xeroaloysia ovatifolia*** (Moldenke) Troncoso, Argentina: San Luis, *R. Olmstead 2004-184* (WTU), HM216792, HM216678. ***Xolocotzia asperifolia*** Miranda 1, Mexico: Chiapas, *D. Neill 5477* (MO), HM216793, HM216680. ***Xolocotzia asperifolia*** Miranda 2, Nicaragua: Matagalpa, *W. D. Stevens 22332* (MO), HM216794, HM216679.

Outgroup –*Antirrhinum majus* L., Cultivated, no voucher, L36413, –. ***Antirrhinum majus*** L., Cultivated, *Erixon and Bremer 10* (UPS), –, AJ430929. ***Barleria prionitis*** L., Cultivated: Uppsala Bot. Gard. 1977-3036 (UPS), U12653, –. ***Barleria prionitis*** L., *R. Scotland s.n.*, –, AF063118. ***Bignonia capreolata*** L., Cultivated: RBG Kew 1980-3846, no voucher, J887855/DQ222566, FJ870021. ***Buddleja araucana*** Phil., Argentina: Neuquen, *R. Olmstead 2007-94* (WTU), –, HM216599. ***Buddleja davidii*** Franch., Cultivated, *R. Olmstead 88-007* (WTU), L36394, –. ***Callicarpa dichotoma*** Raeusch , Cultivated: Beal Bot. Gard., *R. Olmstead* 88-012 (WTU), L36395, AF363665. ***Digitalis purpurea*** L., Cultivated, *K. -J. Kim 13943* (YNUH), AF130150, –. ***Digitalis purpurea*** L., Cultivated, *E. Freeman s.n.*, –, AF034871. ***Eccremocarpus scaber*** Ruiz & Pav., Cultivated: RBG Kew 1988-132, *M. W. Chase 2999* (K), AF102630, FJ870030. ***Elytraria crenata*** Vahl., *R. Scotland s.n*., U12657, –. ***Elytraria imbricata*** (Vahl.) Pers., U. S. A.: Arizona, *McDade and Jenkins 1155* (ARIZ), –, AF061819. ***Jacaranda mimosifolia*** D. Don, Brazil, *L. Lohmann 369* (MO), EF105012, EF105070. ***Lamium purpureum*** L., U. S. A.: Ohio, *Wagstaff 88-031* (BHO), U78694, AF363664. ***Martynia annua*** L., U. S. A.: Arizona, *P. Jenkins 97-149* (ARIZ), HM216755, HM216649. ***Myoporum mauritianum*** A. DC., Cultivated: RBG Kew 1984-4220, no voucher, L36403, AJ299257. ***Nematanthus hirsutus*** (Mart.) Wiehler, Cultivated, no voucher, L36404, –. ***Nematanthus strigillosus*** (Mart.) H. E. Moore, Cultivated: USBRG, *J. Skog 7751* (US), –, AY047148. ***Nyctanthes arbor-tristis*** L., Cultvated: RBG Kew 099-86.00993, no voucher, U78708, –. ***Nyctanthes arbor-tristis*** L., *K. Dahlstrand s.n.* (GB), –, AF231863. ***Paulownia tomentosa*** (Thunb.) Steud., Cultivated, *R. Olmstead 88-008* (WTU), L36406, –. ***Paulownia tomentosa*** (Thunb.) Steud., Cultivated*, Erixon and Bremer 22* (UPS), –, AJ430926. ***Schlegelia***

parviflora (Oerst.) Monach., Venezuela (Cultivated at MO), *Gentry 14221* (MO), L36410, AJ608570. ***Scrophularia californica*** Cham. & Schldtl., U. S. A.: California, *C. W. dePamphilis s.n.* (PAC), L36411, –. ***Scrophularia californica*** Cham. & Schldtl., *E. Freeman s.n.*, –, AF118802. ***Sesamum indicum*** L., Cultivated, no voucher, L36413, –. ***Sesamum indicum*** L., Cultivated, *P. Jenkins 97-141* (ARIZ), –, AF067067.

APPENDIX 2. Vouchers corresponding to the localities plotted on the map (Fig. 2).

Recordia boliviana. BOLIVA. Santa Cruz: Florida, 06 Nov 2005, *D. Villarroel 182* (MO); Florida, 12 Dec 2007, *D. Villarroel 1660* (MO); Vallegrande, 28 Nov 2011, *G. A. Parada , Y. Inturias & M. Betancur 3846* (MO); Vallegrande, s.d*., M. Nee 38482* (MO); the plain around Santa Cruz that lies within the Flora de la Región del Parque Nacional Amboró, s.d., *M. Nee 38027* (MO); within the Flora de la Región del Parque Nacional Amboró, but above the 700 m contour, s.d., *M. Nee 47855* (MO).

Verbenoxylum reitzii. BRAZIL. Santa Catarina: Criciúma, 19 Jan 2010, *V. Thode 314* (ICN); Orleans, 12 Nov 2009, *V. Thode 291* (ICN); Praia Grande, 06 Nov 2009, *V. Thode 278* (ICN); Timbé do Sul, 07 Dec 2009, *V. Thode 284* (ICN). Rio Grande do Sul: Osório, 05 Nov 2009, *V. Thode 269* (ICN); Três Forquilhas, 06 Nov 2009, *V. Thode 282* (ICN).

FIGURAS

CAPÍTULO 1

FIG. 1. Members of the tribe Duranteae (all scales = 1 cm). A. *Verbenoxylum reitzii*. B. *Recordia boliviana*. C. *Duranta vestita* Cham.. D. *Bouchea fluminensis* (Vell.) Moldenke. E. *Chascanum* sp.. F. *Stachytarpheta reticulata* Mart. ex Schauer. A, C, D, and F. Verônica Thode; B. Luzmila Arroyo, Museo Noel Kempff; E. Erin Tripp, Rancho Santa Ana Botanic Garden.

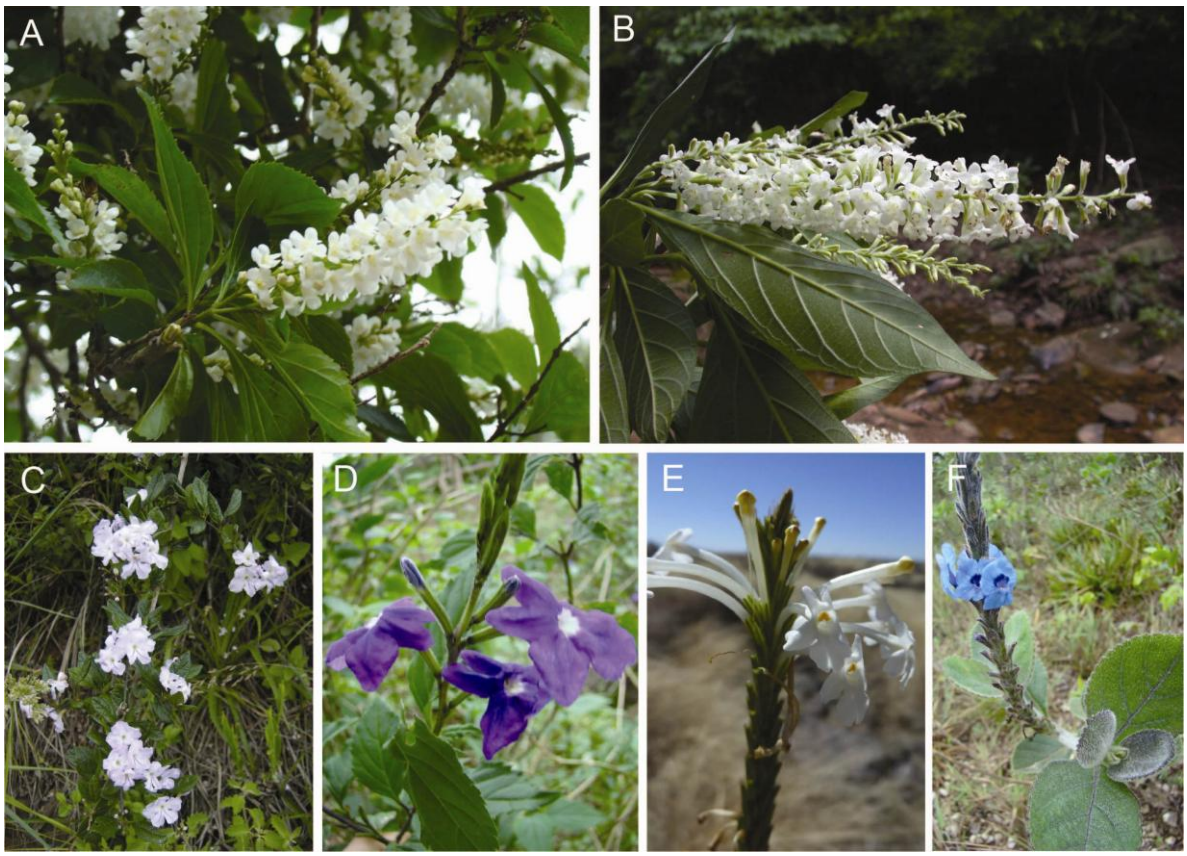


FIG. 2. Map of South America showing known localities of distribution of *Verbenoxylum reitzii* (black squares) and *Recordia boliviana* (white squares). Voucher information can be found in Appendix 2.

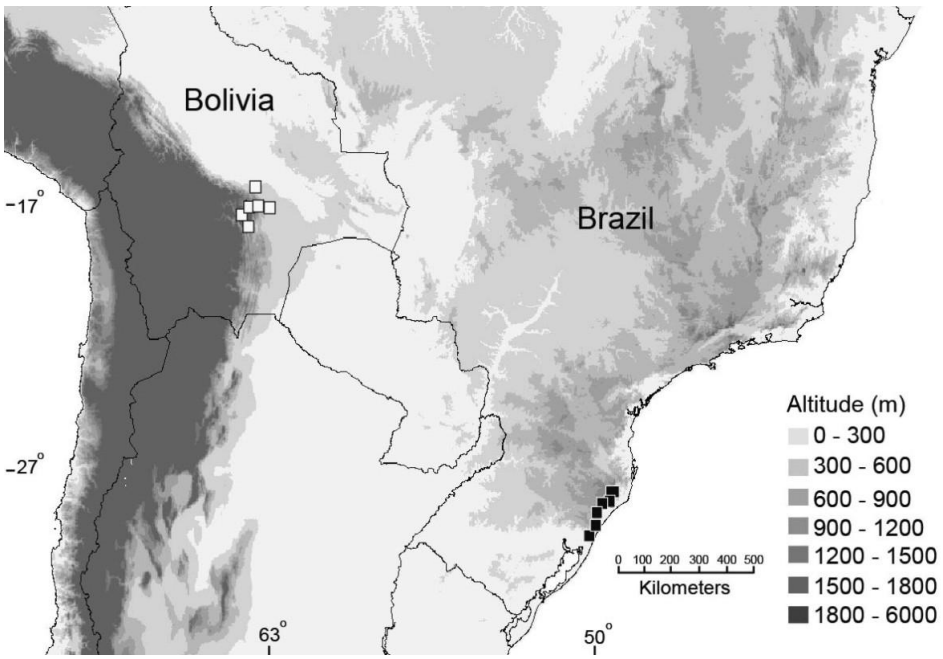


FIG. 3. Bayesian consensus tree topology based on combined data from the plastid markers *ndhF* and *trnL–trnF* inferred with MrBayes. Branches with MP/ML bootstrap support (BSP/BSL) and BI posterior probability (PP). Asterisk indicates maximum support and "–" clade not obtained in the tree. Letters on branches indicate Verbenaceae tribes: L. Lantaneae. V. Verbeneae. N. Neospartoneae. Pi. Priveae. Ci. Citharexyleae. Ca. Casselieae. Pe. Petreeae. Out. outgroups. Cross indicates genera not assigned to tribe.

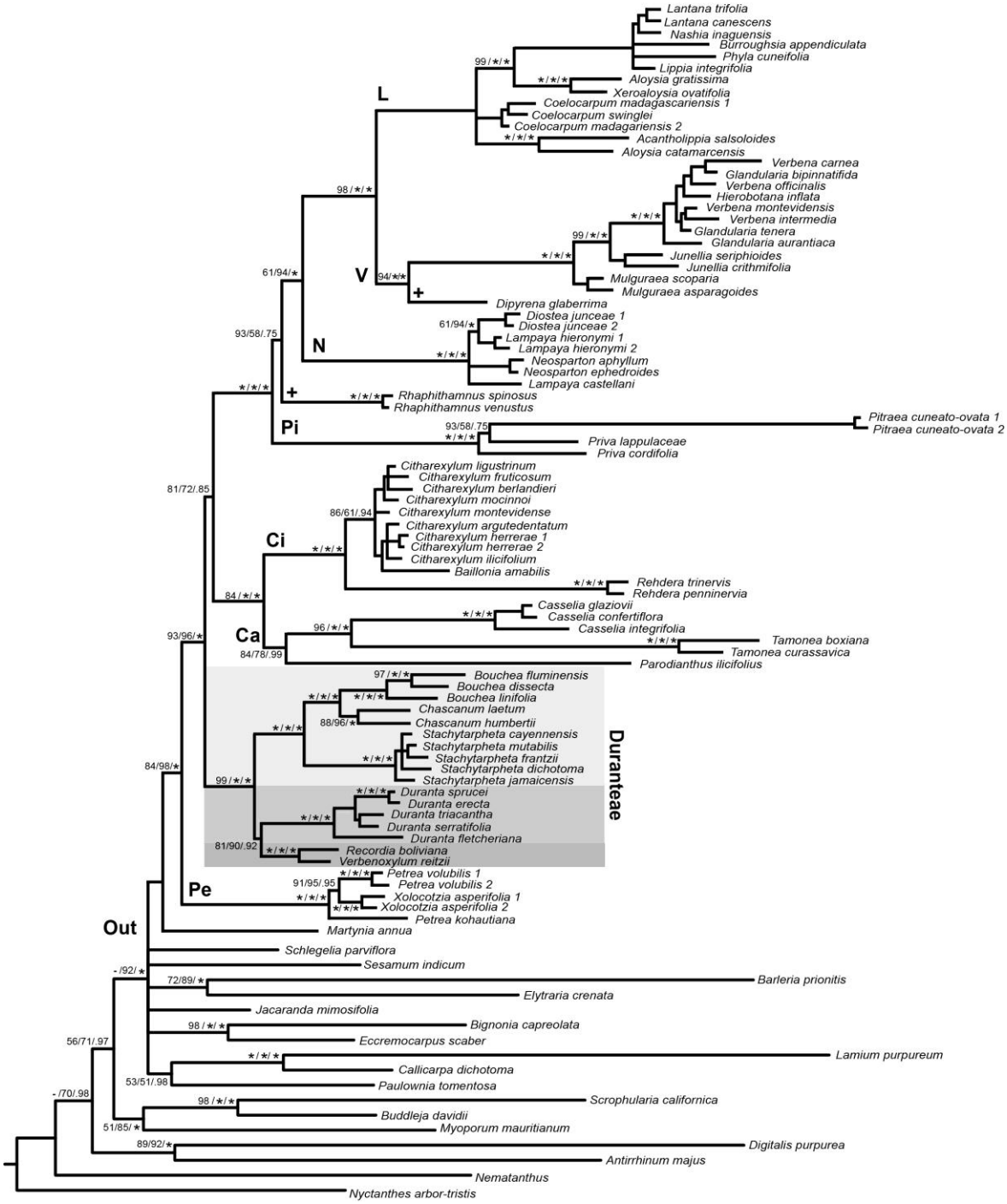
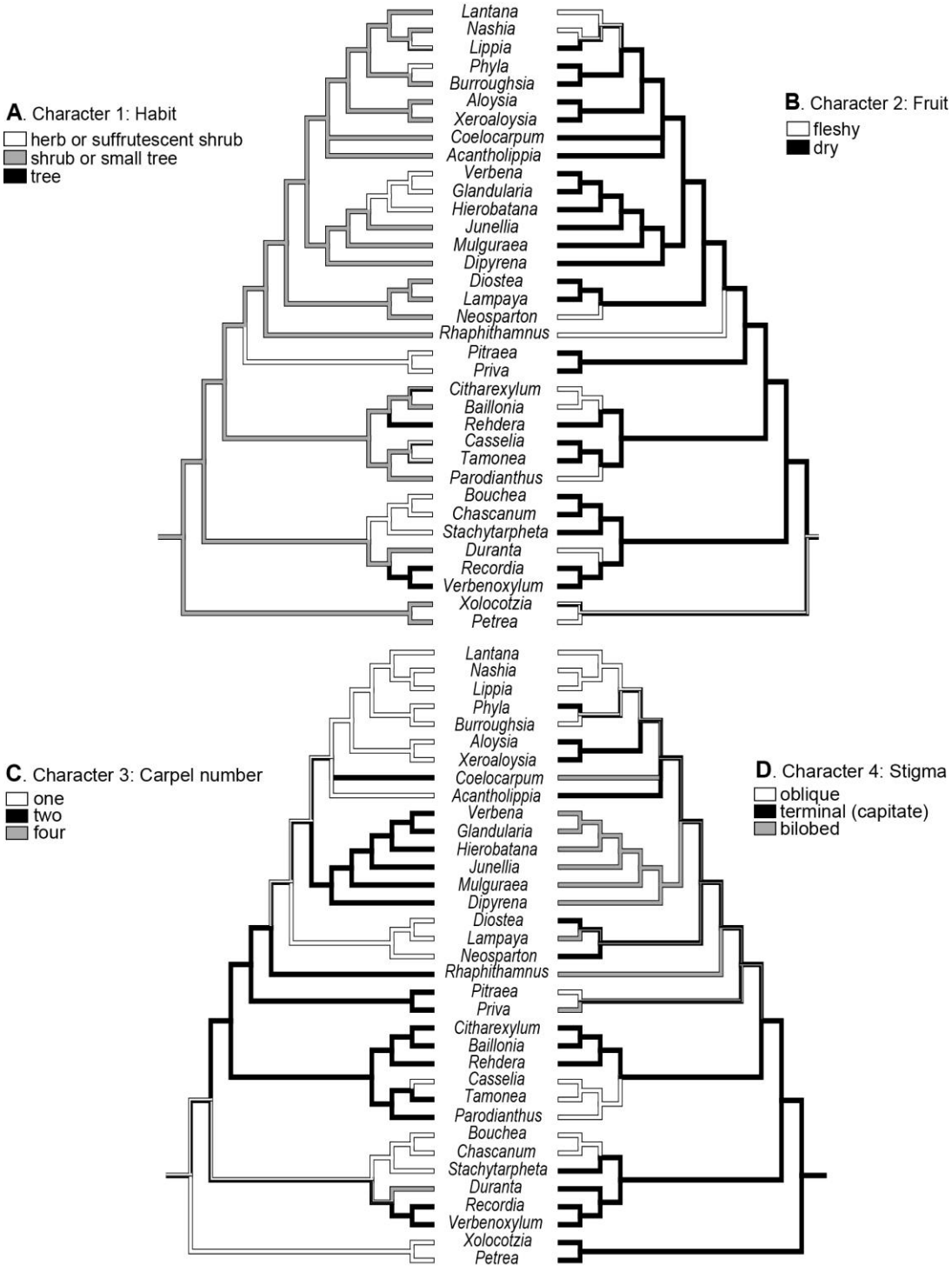
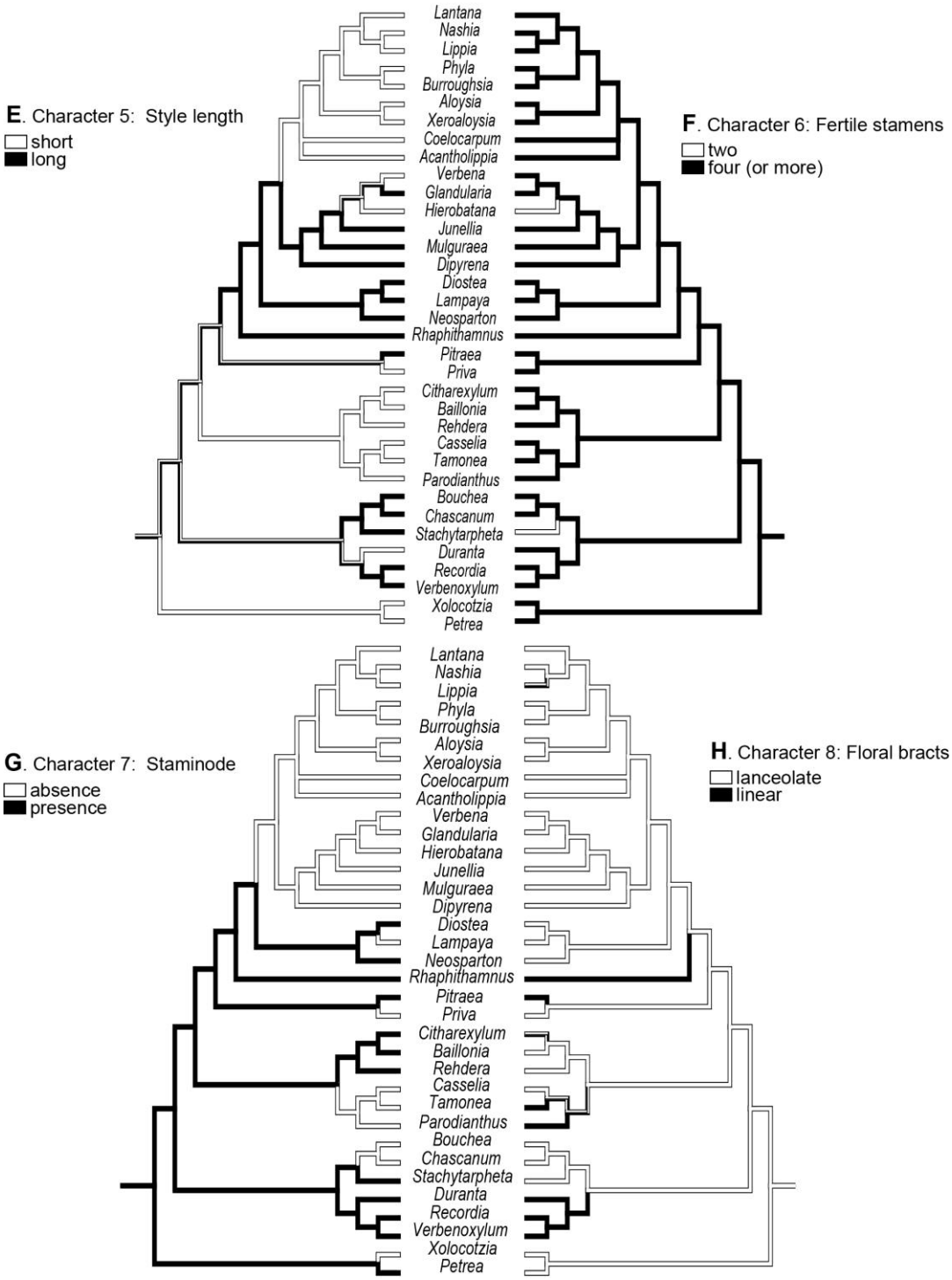


FIG. 4. A-D. Ancestral state reconstruction mapped on the Bayesian consensus tree with terminal collapsed to tips that represent the Verbenaceae genera, using parsimony in Mesquite of A. Habit. B. Fruit. C. Carpel number. D. Stigma.


A. Character 1: Habit
herb or suffrutescent shrub
shrub or small tree
tree
B. Character 2: Fruit
fleshy
dry
C. Character 3: Carpel number
one
two
four
D. Character 4: Stigma
oblique
terminal (capitate)
bilobed
Lantana
Nashia
Lippia
Phyla
Burroughsia
Aloysia
Xeroaloysia
Coelocarpum
Acantholippia
Verbena
Glandularia
Hierobatana
Junellia
Mulguraea
Dipyrena
Diostea
Lampaya
Neosparton
Rhaphithamnus
Pitraea
Priva
Citharexylum
Baillonia
Rehdera
Casselia
Tamonea
Parodianthus
Bouchea
Chascanum
Stachytarpheta
Duranta
Recordia
Verbenoxylum
Xolocotzia
Petrea

CONT. FIG. 4. E-H. E. Style length. F. Fertile stamens. G. Staminode. H. Floral bracts.



CONT. FIG. 4. I-L. I. Pedicel. J. Stamen filament. K. Thecal orientation. L. Anther exertion.

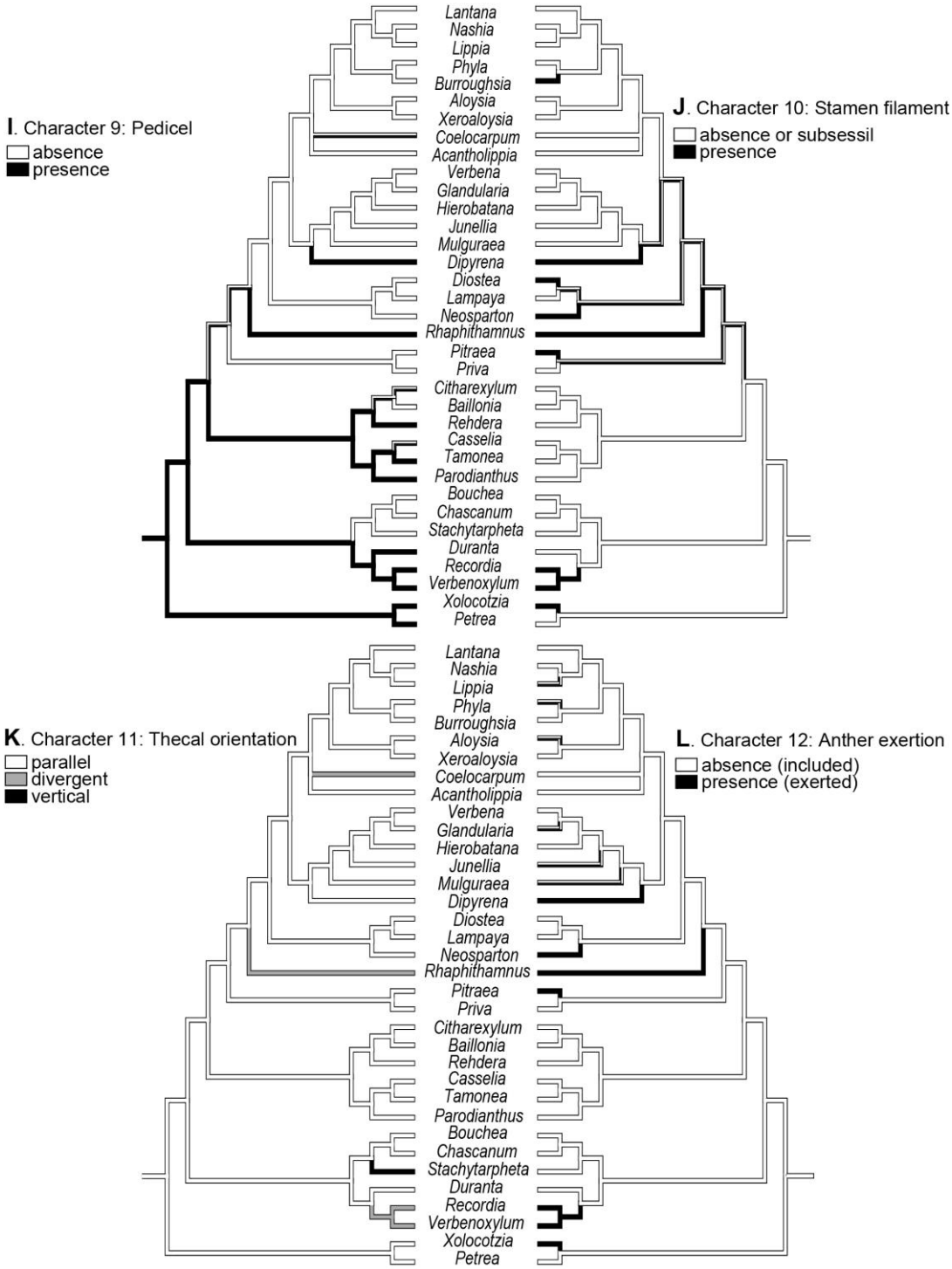
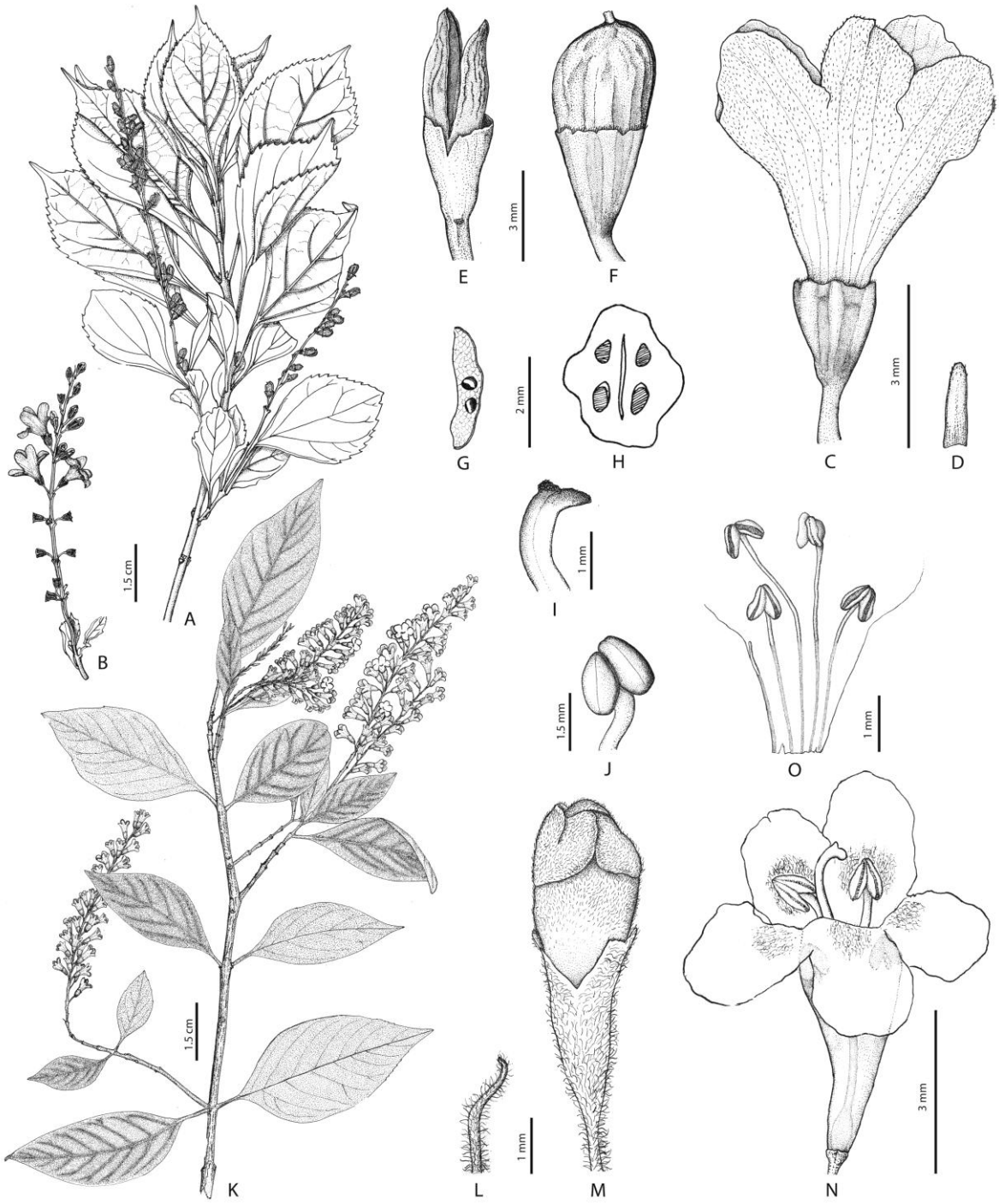


FIG. 5. *Verbenoxylum reitzii.* A. Plant branch in fruit. B. Florescence with flowers. C. Flower. D. Floral bract. E. Mature fruit with calyx. F. Fruit with calyx. G. Mericarp, cross section. H. Ovary, cross section. I. Stigma. J. Anther. *Recordia boliviana.* K. Plant branch. L. Floral bract. M. Flower in blossom. N. Flower with exerted anthers. O. Open corolla with androecium (Modified from Tronsoco 1974).



CAPÍTULO 2

DEVELOPMENT OF MICROSATELLITE FOR *VERBENOXYLUM REITZII* (VERBENACEAE), A TREE ENDEMIC TO THE BRAZILIAN ATLANTIC RAINFOREST

DEVELOPMENT OF MICROSATELLITE FOR *VERBENOXYLUM REITZII* (VERBENACEAE), A TREE ENDEMIC TO THE BRAZILIAN ATLANTIC RAINFOREST[1]

Verônica A. Thode[2,3], Alice Backes[3], Geraldo Mader[3], Raquel Kriedt[3], Sandro L. Bonatto[4], and Loreta B. Freitas[2,3]

[2]Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Bento Gonçalves 9500, Porto Alegre, Brazil

[3]Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil

[4]Genomic and Molecular Biology Laboratory, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, Porto Alegre, Brazil.

Artigo aceito para publicação na revista *Applications in Plant Sciences* (*American Journal of Botany*)

ABSTRACT

Premise of the study: Microsatellite markers were developed for *Verbenoxylum reitzii* (Verbenaceae), a tree endemic to the Brazilian Atlantic Rainforest, to investigate their usefulness in population genetic studies. The loci were tested for cross-amplification in the related genera *Recordia* and *Duranta*.

Methods and Results: Eleven polymorphic microsatellite markers were isolated from an enriched library of *V. reitzii* and characterized. The primers were tested on 60 individuals from three populations of this species. The number of alleles per locus ranged from two to 11, and the observed and expected heterozygosities varied from 0.0 to 1.0 and from 0.088 to 0.758, respectively. Ten loci successfully amplified in *Recordia boliviana* and all failed in *Duranta vestita*.

Conclusions: Our results suggest the usefulness of the microsatellite loci developed here to access genetic variability for phylogeographic and population genetics studies in *V. reitzii*, which are important for the conservation of this rare species.

Key words: Cross-amplification; microsatellite; *Recordia*; Verbenaceae; *Verbenoxylum*.

INTRODUCTION

Verbenoxylum Tronc. (Verbenaceae) is a monotypic genus with a restricted distribution, endemic to the Atlantic Rainforest in the Brazilian states of Rio Grande do Sul and Santa Catarina. *Verbenoxylum reitzii* (Moldenke) Tronc. is a tree vulnerable to extinction in the List of threatened species of Rio Grande do Sul Brazilian state (SEMA, 2003). It is distributed in remaining preserved fragments and riverine areas of the forest, occurring only in the lowland subtropical seasonal deciduous forest, below 550 meters elevation (Troncoso, 1974). We developed and characterized 11 microsatellite loci for *V. reitzii* and tested for cross-amplification in the related and also monotypic genus *Recordia* Moldenke, a Bolivian endemic, and in a representative of the genus *Duranta* L. endemic to southeastern Brazil and Argentina (Troncoso, 1974; Sobral et al., 2006; Thode et al., in press). This is the first time that microsatellite loci are developed for *V. reitzii*. The new markers presented will be used in further genetic diversity studies, which are important to the conservation of this rare species and to preserve the Brazilian Atlantic Rainforest.

METHODS AND RESULTS

Total genomic DNA was extracted from silica gel-dried tissue from one individual using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). An enriched library methodology was used to isolate simple sequence repeats (SSRs) (Beheregaray et al., 2004). The total DNA was digested with the restriction enzyme *Rsa*I and the fragments were linked to two oligo adapters and amplified using polymerase chain reaction (PCR) in a thermocycler (Applied Biosystems, Foster City, California, USA) with an initial denaturation at 95°C for 4 min, followed by 20 cycles of

94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. The products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), enriched in three motifs - $(dAT)_8$, $(dGA)_8$, $(dGAA)_8$ - , and selectively captured using streptavidin magnetic particles (Invitrogen, Carlsbad, California, USA). The selected DNA fragments were amplified by PCR with an initial denaturation at 95°C for 1 min, followed by 25 cycles of 94°C for 40 s, 60°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 5 min. The resulting fragments were cloned into a pGEM-T vector (Promega, Madison, Wisconsin, USA), inserted into competent XL1-Blue *E. coli*, and incubated. A total of 100 randomly chosen clones were PCR-amplified with an initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. The products were purified and sequenced on a MegaBACE™ 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, PA, USA). Twenty clones presented SSRs and 14 were adequate for primer design using Primer3 4.0.0 (Rozen and Skaletsky, 2000) with primer sizes between 18 and 25 base pairs, GC contents ranging from 48% to 60%, and annealing temperatures varying from 55 to 65°C.

The resulting markers were tested in three populations of *V. reitzii* in southeastern Brazil: Rio Grande do Sul, Osório (29°52'54''S, -50°17'15''W; *N*=20; voucher: ICN 166749), Santa Catarina, Praia Grande (29°10'58''S, -49°59'43''W; *N*=20; voucher: ICN 166755), and Santa Catarina, Orleans (28°22'16''S, -49°14'34''W; *N*=20; voucher: ICN 166763). The vouchers are deposited in the ICN Herbarium (Universidade Federal do Rio Grande do Sul, Brazil). The DNA from 60 individuals was extracted using a CTAB protocol (Doyle and Doyle, 1987). PCR was performed in 15 µL reaction containing 10-30 ng/µL of template genomic DNA, 200 µM each dNTP (Invitrogen,

Carlsbad, California, USA), 2.0 pmol each of fluorescently labeled M13(-21) primer and reverse primer, 0.4 pmol of forward primer with a 5'-M13(-21) tail, 2.0 mM $MgCl_2$ (Invitrogen), 0.5 U of *Taq* Platinum DNA polymerase, and 1× *Taq* Platinum reaction buffer (Invitrogen), with the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 53-65°C for 30 s, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The forward primers were FAM-, NED-, or HEX-labeled. The products were analyzed on MegaBACE™ 1000 automated sequencer using ET-ROX 550 size ladder (GE Healthcare). Alleles were verified using GENETIC PROFILER 2.0 (GE Healthcare).

Thirteen of the 14 primers amplified successfully; of these, two were monomorphic and 11 were polymorphic. The primer sequences, repeat motif, fragment size range (bp), and the respective annealing temperatures of 13 microsatellite loci that were successfully amplified are shown in Table 1. The number of alleles per locus (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e), and Hardy–Weinberg equilibrium (HWE) were analyzed with ARLEQUIN version 3.5 (Excoffier and Lischer, 2010) (Table 2), and linkage disequilibrium using Bonferroni correction was tested with FSTAT version 1.2 (Goudet, 1995).

All the individuals presented one or two alleles (consistent with a diploid condition) with the expected sizes. In population Osório, the number of alleles per locus varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.088 to 0.672, respectively (Table 2). In population Praia Grande, the number of alleles per locus varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 0.6 and from 0.097 to 0.73, respectively (Table 2). In population Orleans, the loci Vx07 and Vx14 were monomorphic and the number of alleles per locus for the

polymorphic loci varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.3 to 0.758, respectively (Table 2). Considering all the populations together, the total number of alleles per locus ranged from two (Vx06 and Vx14) to 11 (Vx04), and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.088 to 0.758, respectively (Table 2). The loci Vx05 and Vx06 showed significant deviation from HWE ($p < 0.005$) in all the three populations, Vx10 and Vx13 in the populations Osório and Praia Grande, and Vx02 and Vx04 showed significant deviation in the population Orleans (Table 2). No linkage disequilibrium was detected for any loci ($P > 0.004$). Cross-amplification of the 11 microsatellite loci was tested in two individuals of *Recordia boliviana* Moldenke, and two of *Duranta vestita* Cham. All markers, except Vx14, amplified successfully in *R. boliviana* with the same PCR conditions, and all failed in *D. vestita*.

CONCLUSIONS

The 11 microsatellite loci presented here revealed polymorphism in *V. reitzii* populations, providing a powerful tool for future population genetics and phylogeographic studies. The success in the cross-amplification of most of these markers in *R. boliviana* suggests that they can be used for the same finality in this species. These loci may help to provide valuable genetic information to build conservation strategies for these rare trees.

Acknowledgments: This study was funded by a doctoral fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior to V.A.T., by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Fundação de Amparo à Pesquisa do

Estado do Rio Grande do Sul. The authors thank P. Zamberlan and A. L. Segatto for technical support.

LITERATURE CITED

BEHEREGARAY, L. B., L. M. MÖLLER, T. S. SCHWARTZ, N. L. CHAO, AND G. CACCONE. 2004. Microsatellite markers for the cardinal tetra *Paracheirodon axelrodi*, a commercially important fish from central Amazonia. *Molecular Ecology Notes* 4: 330–332.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh tissue. *Phytochemical Bulletin* 19: 11–15.

EXCOFFIER, L.G.L., AND H.E.L. LISCHER. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.

GOUDET, J. 1995. FSTAT ver 1.2: A computer program to calculate F-Statistics. *Journal of Heredity* 86: 485–486.

ROZEN, S., AND H. J. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Krawetz and S. Misener [eds.], Bioinformatics methods and protocols: Methods in molecular biology, 365–386. Humana Press, Totowa, New Jersey, USA.

SEMA. 2003. Lista final das espécies da flora ameaçadas – RS, Decreto Estadual n° 42.099, Website http://www.fzb.rs.gov.br/downloads/flora_ameacada.pdf [accessed 15 December 2012].

SOBRAL, M., J. A. JARENKOW, P. BRACK, B. IRGANG, J. LAROCCA, AND R. S. RODRIGUES. 2006. Flora arbórea e arborescente do Rio Grande do Sul, Brasil. RiMA/Novo Ambiente, São Carlos, Brazil.

THODE, V. A., O'LEARY N., OLMSTEAD R. G., AND FREITAS L. B. 2013. Phylogenetic position of the monotypic genus *Verbenoxylum* (Verbenaceae) and new combination under *Recordia*. *Systematic Botany*: in press.

TRONCOSO, N. S. 1974. Los Géneros de Verbenáceas de Sudamérica extratropical. *Darwiniana* 18: 295–412.

TABELAS

CAPÍTULO 2

Table 1. Characteristics of the 13 microsatellite loci that were successfully amplified in *Verbenoxylum reitzii*. For each marker are shown the forward and reverse primer sequences, repeat motif, fragment size range (bp), annealing temperature (*Ta*), and GenBank accession number.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession No.
Vx01	F: TCACTTGATATGTTGCCACTTT	$(CT)_7$	253-269	64	KC417432
	R: TGAATGCGCTTATGGCTATC				
Vx02	F: CACTTGTCGGGAGGAATGTC	$(CT)_{10}$	253-277	64	KC417433
	R: TGAGAACGCAAAACTGGATG				
Vx03	F: TAACAGGCAATGACGGATCG	$(CT)_9$	191-195	62	KC417434
	R: CCCACAAACACCCGAAAG				
Vx04	F: CTGTGATTGATTGGGCAGGAAG	$(GA)_8(GAAA)_1(GA)_8$	164-194	62	KC417435
	R: TTTTTGGCCCGATGGAAGTTA				
Vx05	F: TCACATTAAATAGATGGTCAGACG	$(TG)_7(AG)_8$	153-163	62	KC417436
	R: GGTGAAACCCTATCCAATTCCAGGC				
Vx06	F: CTGGGGCTAAAGTTGGTCAC	$(CT)_9$	189-217	62	KC417437
	R: TTCGCCATTTCACATCGTC				
Vx07	F: CGCAAGATTCCCAATTTCTG	$(CT)_9$	174-188	64	KC417438
	R: TCGATTTCCACCTCGTGTG				
Vx08	F: GGGGAACTTGGATGAGGAAG	$(CTTT)_4(CTT)_5$	151	64	KC544260
	R: AATCTCTCCAGCACCCACTG				
Vx09	F: TCGGAGGTTCCATATCCTTC	$(TTC)_{10}$	219-234	65	KC417439
	R: GAGTTTGTCATCAGCGACTCC				
Vx10	F: GACCTTGTGCGAAAATGAGC	$(GAA)_{14}$	252-276	65	KC417440
	R: GTGATCTCCCTTCGCTTCC				
Vx12	F: GGCTATTTCGTCATTAGGCATC	$(GA)_7$	247	64	KC544261
	R: ATTCGGTAACTCACACCTGCTG				
Vx13	F: CACAAACATGCTACGCTTGAC	$(GA)_{18}(GGGA)_1(GA)_3$	263-271	53	KC417441
	R: GAGGTCCTTCACTCGTCTCTTAC				
Vx14	F: GCAAGACACATGCCGTTTTACC	$(GAA)_8$	254-257	65	KC417442
	R: CTGGGCTCCTTCTCTTAACG				

Table 2. Genetic diversity of the three populations of *Verbenoxylum reitzii*. Number of alleles (*A*), observed heterozygosity (*H*o), and expected heterozygosity (*H*e) are given for each microsatellite marker and population.

Locus	Osório			Praia Grande			Orleans			Total
	A	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*
Vx01	5	0.285	0.265	4	0.250	0.427	4	0.200	0.352	8
Vx02	4	0.411	0.613	4	0.600	0.541	5	0.523	0.629	7
Vx03	3	0.304	0.478	2	0.600	0.492	2	0.318	0.510	3
Vx04	7	0.608	0.672	7	0.222	0.730*	7	0.400	0.758*	11
Vx05	2	1.000	0.512*	5	0.555	0.622*	2	1.000	0.521*	5
Vx06	2	0.000	0.459*	2	0.133	0.496	2	0.000	0.507*	2
Vx07	2	0.090	0.088	4	0.411	0.572*	1	M	M	4
Vx09	2	0.318	0.273	4	0.400	0.352	3	0.217	0.300	4
Vx10	4	0.333	0.556*	6	0.578	0.603	4	0.523	0.602	7
Vx13	3	0.000	0.553*	3	0.150	0.524*	3	0.000	0.317*	4
Vx14	2	0.043	0.124	2	0.000	0.097	1	M	M	2

Notes: * = deviation from Hardy-Weinberg equilibrium ($p < 0.005$); M = monomorphic.

CAPÍTULO 3

GENETIC DIVERSITY AND ECOLOGICAL NICHE MODELING OF THE RESTRICTED DISTRIBUTED *RECORDIA REITZII* (VERBENACEAE) FROM SOUTHERN BRAZILIAN ATLANTIC FOREST

GENETIC DIVERSITY AND ECOLOGICAL NICHE MODELING OF THE RESTRICTED DISTRIBUTED *RECORDIA REITZII* (VERBENACEAE) FROM SOUTHERN BRAZILIAN ATLANTIC FOREST

VERÔNICA A. THODE,[1,2] GUSTAVO ADOLFO SILVA ARIAS,[1] CAROLINE TURCHETTO,[1] ANA LÚCIA ANVERSA SEGATTO,[1] ANA LUÍZA RAMOS CAZÉ,[1] GERALDO MADER,[1] MAIKEL RECK-KORTMANN, [1] SANDRO LUIS BONATTO[3] and LORETA BRANDÃO DE FREITAS[1,2*]

[1]Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil, [2]Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Bento Gonçalves 9500, Porto Alegre, Brazil, [3]Genomic and Molecular Biology Laboratory, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, RS, Brazil, [*] Corresponding author: E-mail: loreta.freitas@ufrgs.br

Artigo em preparação para ser submetido à revista *Botanical Journal of the Linnaean Society*

The Atlantic forest is well known for its great amount of diversity and endemism. The intensive deforestation of this region, caused mainly by the human impact, brings serious consequences to this complex area. Genetic diversity studies of restricted distributed species may bring important insights on the formation of the Atlantic forest, on the assembly of its biodiversity, and may also provide valuable information to understand diversification processes in this endangered region. Here, we studied the genetic diversity of *Recordia reitzii*, a tree restricted to the Brazilian Atlantic forest, using three plastidial (cp) DNA regions (*trnS-trnG*, *psbA-trnH*, and *rpl32-trnL*) and ten microsatellite (SSR) loci. To assess the historical processes that may have influenced distribution of the extant *R. reitzii* populations, the present potential distribution of *R. reitzii* and *Recordia boliviana*, its close related species restricted to semi-deciduous dry forests in Bolivia, were modeled and projected onto past models (Last Glacial Maximum (LGM) and Last Interglacial (LIG). Using the ecological niche models (ENM), niche divergence was quantified between *Recordia* species. According to our cpDNA data, we found that *R. reitzii* has low genetic diversity, which may be a result of its narrow distribution, small population size, and founder effect. In spite of the species' limited range, the cpDNA and SSR data showed that a north-south pattern of diversity distribution might be observed. Our data also indicated well-structured populations, suggesting that gene flow and seed dispersal are probably limited. The ENMs showed a wider palaeodistribution during the LIG and a retraction at the LGM, for both species. The niche divergence and conservatism tests indicated that ecological factors might have influenced in the diversification of the *Recordia* species. Our results are consistent with the limited distribution and rarity of *R. reitzii*.

ADDITIONAL KEYWORDS: cpDNA – microsatellite – niche divergence – niche conservatism

INTRODUCTION

The Atlantic forest is widely known for its great number of organisms and endemism, as well as for its diversity loss (Mori *et al*. 1981; Terborgh 1992; Morellato & Haddad 2000; Myers *et al*. 2000; Perret *et al*. 2006; Ribeiro *et al*. 2009; Stehmann *et al*. 2009). This forest extends from north to south Brazil (3°S to 30°S), along the east cost, from the sea level up to 2700 m elevation (Metzger 2009; Stehmann *et al*. 2009). Nowadays, the Atlantic forest comprises only 7-11% of its original structure and it is restricted to isolated fragments, which leads to the reduction of the biodiversity, especially of the endemic species (*Ranta et al*. 1998; Vieira *et al*. 2009; Ribeiro *et al*. 2009; SOS Mata Atlântica/INPE 2008). In despite of the great loss of diversity, many species still unknown (Lewinsohn & Prado 2005, Sobral & Stehmann 2009) and more studies are necessary to investigate the evolutionary processes that may have influenced on the assembly of this biome (Perret *et al*. 2006). The study of the Atlantic forest and its formation are of great importance to comprehend the driving factors of its diversity and high number of endemics, moreover, to understand the consequences of the past, present, and future environmental changes on this region and on its extant biodiversity (Behling & Lichte 1997; Behling & Negrelle 2001; Behling *et al*. 2002; Carnaval & Moritz 2008).

Population genetics analyses on plants that occur in the Atlantic forest and the influence of climatic changes and glacial/interglacial cycles on the distribution, genetic diversity, and population structure of species have been investigated (Reis *et al*. 2000; Conte *et al*. 2003; Ribeiro *et al*. 2005; Alcantara *et al*. 2006; Palma-Silva *et al*. 2009; Ramos *et al.* 2009; Lorenz-Lemke *et al*. 2010; Pinheiro *et al* 2011; Collevatti *et al*. 2012a, Turchetto-Zolet *et al*. 2012), however, little is known about the genetic

diversity of restricted distributed species and about the factors that might have influenced in their present isolation. Small-scale studies are important to evaluate larger events and to elucidate the diversification processes in a biome as a whole (Silva *et al.* 2012). Genetic diversity studies with chloroplast DNA (cpDNA) (maternally inherited) and nuclear (biparentally inherited) markers may provide a deeper understanding of ancient and recent events that have influenced the current distribution of genetic variability (Ennos 1994, Collevatti *et al.* 2003, Palma-Silva *et al*. 2009).

Many forest organisms had a broader distribution during interglacial periods, due to the warmer and more humid climatic conditions and had their ranges reduced during the glacial periods, which were dryer and with colder temperatures, staying confined to suitable refugia (Behling & Lichte 1997, Bennett & Provan 2008, Lorenz-Lemke *et al*. 2010). During the Quaternary, changes in distribution and persistence in microrefugia, small areas with adequate features, were more frequent than complete extinctions in the Neotropics (Vegas-Vilarrubia *et al*. 2011; Turchetto-Zolet *et al*. 2013). Lineages of trees and shrubs went through different histories after ice ages, thus, leading to distinct levels of genetic variation between and within populations (Lascoux *et al*. 2004).

Studies based on paleoclimatic data and population genetics on the Brazilian forest predicted the presence of three historical forest refugia along the Atlantic coast: a large area between the rivers São Francisco and Doce (Bahia), a smaller region north of the São Francisco river (Pernambuco), and another encompassing areas south of the Doce river (Rio Doce) (Ledru *et al*. 2005; Carnaval & Moritz 2008). This latter also suggested that the forest range has progressively migrated south, in agreement with

the pollen record. However, the refugia may be different across species, thus, the forest refugial model proposed by Carnaval & Moritz (2008) cannot be used as a pattern for a large set of organisms (Porto *et al*. 2012).

Ecological niche modeling (ENM) combines data from the species occurrence with geographic information systems (GIS) environmental layers (Warren 2008). This approach has been used to understand past, present, and future patterns of distribution and also to study niche evolution (Wiens & Graham 2005; Warren *et al*. 2008; McCormack *et al*. 2010). Ecological niche is the set of biotic and abiotic conditions in which a species is able to survive, reproduce, and maintain viable populations (Hutchinson 1957; Brown & Lomolino 1998). Studying the niche's differences and similarities among closely related species may bring insights into the role of the geographical distribution and ecological features on the diversification of these organisms (Wiens & Graham 2005; Warren *et al*. 2008; McCormack *et al*. 2010; Wellenreuther *et al*. 2012; Wooten & Gibbs 2012; Hung *et al*. 2013). These analyses may bring light into important evolutionary issues and serve as powerful tools for the conservation of this biome and to protect its species.

Niche conservatism is manifested by the tendency of related species to occupy environmental niches that are similar and the inability of the organisms to survive under different conditions (low suitability) (Harvey & Pagel 1991; Wiens & Graham 2005; Warren 2008). On the other hand, niche divergence refers to the evolution for new conditions and expansion of niche breadth, increasing the distribution of a clade (Wiens & Donoghue 2004). GIS-based tools can be used to quantify niche conservatism and divergence between closely related taxa (Wiens & Donoghue 2004; Warren *et al.* 2008; McCormack *et al*. 2010). Studying the biotic interchange would help understand

the historical assembly of biomes and select biological corridors for future conservation (Antonelli & Sanmartín 2011).

Recordia reitzii (Moldenke) Thode & O'Leary (Verbenaceae) is a tree restricted to the southern limit of the Brazilian Atlantic rainforest. Its geographical distribution comprises a small area along the coast, with ca. only 200 km of extension in the Brazilian states of Rio Grande do Sul and Santa Catarina, from 10 to 550 m elevation, not reaching the higher areas of the southern Brazilian plateau (Serra Geral), which can have in this area up to ca. 1,000 m elevation (Troncoso 1974; Reitz *et al*. 1978, 1983; Sobral *et al*. 2006; Behling & Pillar 2007; Thode *et al*. in press) (Fig. 1). *Recordia reitzii* occurs mostly in remaining preserved fragments and riverine areas of the forest (Thode *et al*. in press) and is included in the List of threatened species of Rio Grande do Sul Brazilian state (SEMA 2003). This species is considered endemic to the Atlantic forest (Salimena *et al*. 2009; Salimena *et al*. 2012). Until recently, this species was the only representative of the genus *Verbenoxylum* Tronc. and based on genetic and morphological data, it was inferred to be sister to *R. boliviana* (Thode *et al*. in press). *Verbenoxylum reitzii* (Moldenke) Tronc. was transfered to *Recordia* and *Verbenoxyum* reduced to synonymy. *Recordia boliviana* is a tree with a narrow distribution in the semi-deciduous dry forests of Bolivia, common in the low mountains west of Santa Cruz de la Sierra, between Samaipata and Cochabamba (M. Nee, pers. comm.), occurring between 500 and 1,850 m elevation. These two species share several morphological traits and compose a genus with a large distributional gap (Thode *et al*. in press). *Recordia reitzii* and *R. boliviana* are trees, different from the most of Verbenaceae. Within the 34 genera of the family, only *Citharexylum* L. and *Rehdera* Moldenke, also share this habit (Thode *et al*. in press).

Using three chloroplast regions and ten microsatellite loci, we aimed to investigate the genetic diversity and structure in *R. reitzii* populations. In addition, niche modeling the LGM and LIG and tests of niche divergence and conservatism for *R. reitzii* and *R. boliviana* were performed to investigate the effects of the past climate changes in the processes that may have played a role in the present narrow distribution of *R. reitzii*.

MATERIAL AND METHODS

POPULATION SAMPLING

One hundred and thirty five individuals from 15 populations of *R. reitzii* were genotyped for 10 microsatellite loci, and three chloroplast regions were sequenced for a representative subset of 81 individuals of these. The localities were sampled throughout the species distribution in southeastern Brazil (Fig. 1, Table S1). Besides three localities (OSO, PGR, and ORL) it was not possible collect many individuals due to the rarity of this taxon and anthropic habitat disturbs. Moreover, no individuals were found farther north than ORL (28°22'16.7"S, 49°14'34.3"W) or farther south than OSO (29° 52' 54.8"S, 50° 17' 15.0"W).

DNA AMPLIFICATION, CHLOROPLAST DNA SEQUENCING, AND MICROSATELLITE ANALYSES

Genomic DNA was extracted using the CTAB protocol Roy *et al*. (1992) from field-collected silica-dried leaves. Three cpDNA intergenic spacers *psbA-trnH* (Sang *et al*. 1997), *trnS-trnG* (Hamilton 1999), and *rpl32-trnL* (Shaw *et al*. 2007) were amplified by polymerase chain reaction (PCR) in a thermocycler (Model Veriti, Applied Biosystems, Foster City, California, USA). PCR was performed in 25 µL reaction containing 5 ng/µL

of template genomic DNA, 200 µM each dNTP (Invitrogen, Carlsbad, California, USA), 0.2 µM of each primer, 2.0 mM $MgCl_2$, 0.5 U of *Taq* Platinum DNA polymerase (Invitrogen), and 1× *Taq* Platinum reaction buffer (Invitrogen), with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min (50° for the *rpl32-trnL*), and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. PCR products were purified according Dunn and Blattner (1987) and sequenced on MegaBACE™ 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA) using the DYEnamic ET Terminator Sequencing Premix Kit (GE Healthcare). The sequences were assembled using Chromas 2.4 (Technelysium, Helensvale, Australia) and manually aligned and edited in Mega 5 (Tamura *et al*. 2011). Inversions and insertion/deletion events (indels) were coded as a single evolutionary event. The three cpDNA regions were concatenated for all analyses.

PCR for the 10 microsatellite loci was performed in 15 µL reaction containing 10-30 ng/µL of template genomic DNA, 200 µM each dNTP (Invitrogen, Carlsbad, California, USA), 0.2 µM each of fluorescently labeled M13(-21) primer and reverse primer, 0.04 µM of forward primer with a 5'-M13(-21) tail, 2.0 mM $MgCl_2$, 0.5 U of *Taq* Platinum DNA polymerase (Invitrogen), and 1× *Taq* Platinum reaction buffer (Invitrogen), with an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 53-65°C for 30 s, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The forward primers were FAM-, NED-, or HEX-labeled. The products were analyzed on MegaBACE™ 1000 automated sequencer using ET-ROX 550 size ladder (GE Healthcare). Alleles were verified using Genetic Profiler 2.0 (GE Healthcare).

DATA ANALYSES OF CPDNA SEQUENCES

Haplotype (*h*) and nucleotide (π) diversities (Nei 1987), and analyses of molecular variance (AMOVA, Excoffier *et al*. 1992) were calculated with Arlequin v. 3.5.1.2 (Excoffier & Lischer 2010), the latter was performed with significance tests using 10000 permutations. A median-joining network (Bandelt *et al.* 1999) of the haplotypes was estimated in the program Network 4.1.0.9 (available at www.fluxux-engineering.com). Spacial analyses of molecular variance (SAMOVA, Dupanloup *et al*. 2002) were conducted in SAMOVA v. 1.0 to assess the geographical structure of the chloroplast haplotypes. Mantel tests and Spatial Autocorrelation Analyses (SAA) to compare genetic and geographic distances was performed among populations in Alleles in Space v. 1.0 (AIS, Miller 2005) using 1000 permutations. The neutrality tests Tajima's *D* (Tajima 1989) and Fu's *F*s (Fu 1997) were carried out in Arlequin v. 3.5.1.2 to verify the neutral equilibrium model.

DATA ANALYSES OF SSR LOCI

Genetic diversity indices for each population (*A* = observed number of alleles, *AR* = allelic richness, *H*o = observed heterozygosity, *H*e = expected heterozygosity) were calculated in Arlequin and Fstat v. 2.9.3.2 (Goudet 2002). Significant departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium with Bonferroni correction was tested with Arlequin and Fstat, respectively. To identify evidence of possible null alleles we used the software Micro-Checker 2.2.3 (Van Oosterhout *et al*. 2004). Analyses of Molecular Variance (AMOVA) were performed in Arlequin to verify the degree of genetic differentiation among populations and among individuals within populations. The pairwise population genetic differentiation was calculated using *F*st

(incorporates the infinite alleles model) and *R*st (designed for SSR data, incorporates a stepwise mutation model) (Slatkin 1995). The significance was tested using 10000 permutations. The Bayesian clustering method was implemented in Structure v. 2.3.3 (Pritchard *et al.* 2000) to investigate population genetic structure. This approach allows to elucidate an optimal number of genetic clusters (*K*) based on individuals and does not assume a priori membership to a population. The analyses were performed with 10 runs for each *K* (ranging between 1 and 20) with burn-in of 250000 Markov chain Monte Carlo (MCMC) periods using 1000000 MCMC replicates, allowing admixture, with no prior population information, and correlated allele frequencies (Pritchard *et al.* 2000; Falush *et al.* 2003). To infer the best *K* value we used the ΔK method (Evanno *et al*. 2005), which favors the model with the greatest second-order rate of change in lnPr(X|K). The individual ancestry coefficients for the Structure runs were calculated using Clumpp 1.1.2 (Jakobsson & Rosenberg 2007) with the average pairwise similarity of individual assignments across runs using the Greedy method and with statistics *G'*. The result from Clumpp was plotted using Distruct 1.1 (Rosenberg 2004). The role of gene flow through pollen versus through seed was calculated using the following formula from Ennos (1994):

$$\text{Pollen flow / seed flow} = \frac{[(1 / \Phi_{SC(B)} - 1) - 2(1 / \Phi_{SC(M)} - 1)]}{(1 / \Phi_{SC(M)} - 1)}$$

where $\Phi_{SC(B)}$ and $\Phi_{SC(M)}$ are levels of molecular variance calculated among populations in Arlequin from biparentally (nuclear SSRs) and maternally (cpDNA) inherited markers, respectively.

ECOLOGICAL NICHE MODELING

Occurrence points for *R. reitzii* and *R. boliviana* (Table S2) were obtained from herbaria records, resulting in only 21 and 12, respectively, congruent with the restricted distribution of both. Only records with GPS coordinates and detailed locality were used. All localities were plotted onto a map of South America, encompassing southern Brazil and Bolivia with DIVA-GIS v. 7.1.7.2 (Hijmans *et al*. 2004) to investigate the potential ranges of *R. reitzii* and *R. boliviana* during the present and to project it onto the last glacial maximum (LMG; 21 kyr BP) and last interglacial (LIG; 120-140 kyr BP) paleoclimatic conditions. The environmental data for both locations were obtained from the 19 climatic variables from WorldClim (Hijmans *et al*. 2005) with a resolution of 30 arc seconds (~1 Km^2). The grid layers were cut to the area that include both *Recordia* species and then exported to ASCII format with the software DIVA-GIS v. 7.1.7.2 (Hijmans *et al*. 2004). To avoid highly correlated variables in our dataset, Pearson correlation coefficients were calculated in ENMTools 1.3 (Warren *et al.* 2008) for all pairs of the 19 climatic variables. The pairs of variables with $R > 0.75$ were identified and the ones with the lower percent importance to the model in a preliminary run were discarded (Peterson 2007; Nakazato *et al.* 2010) (Table S3). Ten climatic variables were excluded for analyses due to high correlations and nine were used in the ENMs (Table S4). The maximum entropy algorithm in Maxent v. 3.3 (Phillips *et al.* 2006) was used to generate niche models based on the species presence data. Prediction models were run for 10 iterations using 10% random records to test for each run, and the subsample option for each replicated run type. The quality of the models was evaluated by the area under the curve (AUC) scores, which ranges from 0.5 and 1, where values above 0.7 are acceptable (Pearce & Ferrier 2000).

Besides allopatric distribution of the two species of *Recordia*, we tested whether these taxa conserved or diverged their bioclimatic preferences after their evolutionary split. We used two similarity metrics to calculate the niche overlap of *R. reitzii* and *R. boliviana* by comparing the obtained estimates of habitat suitability from the ENMs. We calculated, with ENMtools, the Schoener's *D* and Hellinger's *I* metrics, which range from 0 (niche models have no overlap) to 1 (niche models are identical, equally suitable for both species) (Schoener 1968; van der Vaart 1998; Warren *et al*. 2008). The statistical significance of the niche overlap values was assessed by the identity test implemented in ENMTools.

The identity test was executed to verify if the habitat suitability scores, obtained from the ENMs for *R. reitzii* and *R. boliviana*, exhibit statistically significant differences (Warren *et al*. 2008). The identity test was performed with 100 replicates to obtain a pseudoreplicated null distribution. The observed values of niche overlap (Schoener's *D* and Hellinger's *I*) were compared to the null distribution generated. The null hypothesis of this test is rejected when the estimated value for *D* and/or *I* is significantly different from the null distribution.

The background test implemented in ENMTools was used to determine whether species' niches were more distinct than expected based on the environmental background differences. Null models were taken into account to test weather observed differences were due to spatial autocorrelation caused by geographic distance (Warren *et al.* 2008; McCormack *et al*. 2010). The test compares the suitability scores of one species with the geographical background of the other, and vice versa. Based on 100 overlap values resulted from the comparison of the ENM of

one species to ENMs created from random points plotted within the geographical range of the other (Warren *et al.* 2008; McCormack *et al*. 2010). The number of background points equals to the sample size of the taxon from whose range the random points were plotted. Two null distributions are generated (one for each taxon). Evidence for niche divergence or conservatism requires two conditions respectively: niche characteristics are different/similar between species and these differences are greater/smaller than a null distribution of the niche similarity indices between one species and random points within the other species' range.

We conducted a Principal Components Analysis (PCA) to explore differences in the environmental space with the raw data in Mystat (Students version of Systat, Stepp & Leitner 1989). We used the environmental data from the nine climatic variables used in the ENMs (Table S4) for the species' occurrence points and 1000 random points plotted in a minimum convex polygon drawn within the taxa geographic ranges with DIVA-GIS. The nine variables were reduced with PCA of the correlation matrix. The most important axes that explained a potion of the overall variance (>1%) and had a clear biological interpretation based on loading scores of each variable were retained. We tested niche divergence and conservatism against a null model of background divergence on each of the axes by comparing the observed difference in mean niche values on a given principal component to the difference in mean background values. The significance was assessed with 1000 Jackknife replicates of the mean background values.

RESULTS

cpDNA DATA

Amplified sequences of the cpDNA intergenic spacers *psbA-trnH* (354 base pairs - bp), *trnS-trnG* (682 bp), and *rpl32-trnL* (993 bp) were concatenated for all analyses showing a total length of 2029 bp. The overall alignment presented one inversion of 17 bp and seven indels that were coded as one evolutionary step each. We also found six single base substitutions (4 transitions and 2 transversions) and five haplotypes for the 81 individuals from 15 populations of *R. reitzii*. The overall haplotype (*h*) and nucleotide (π) diversities were 0.429 (standard deviation (SD) = 0.060) and 0.0015 (SD = 0.0009), respectively. The haplotype H1 was the most frequent, present in all populations but not in the northern CRI and ORL, which shared haplotype H5. The southernmost populations OSO and CAR were the only to present more than one haplotype (OSO presented H1, H3, and H4 and CAR H1 and H2) (Fig. 1). The analyses of molecular variance (AMOVA) showed a high differentiation among populations (Fst = 0.957, $P < 0.001$). The SAMOVA with the cpDNA data indicated two population groups (Fct = 0.975, $P < 0.0001$), with one group comprising CRI and ORL (the two populations that share haplotype H5) and the other group comprising the rest of the populations. Mantel tests (r = 0.173, $P < 0.001$) and the SAA (0.0015, $P < 0.00001$) (Fig. S1) presented a significant association between genetic and geographic distances. The neutrality tests Tajima's D and Fu's Fs were positive but not significant (0.574, P = 0.743, and 5.255, P = 0.949, respectively) indicating that the populations remained demographically stable.

SSR DATA

Individual population analyses showed low to high levels of genetic variability (Table 1), with the lowest mean number of alleles (*A*) for populations CAR and MAQ (1.7) and the highest mean for PGR (4.1). The mean allelic richness (AR) ranged from 1.5 (CAR) to 2.1 (PGR). The mean observed heterozygosity (*H*o) varied between 0.178 (ORL) and 0.410 (S84) and the mean expected heterozygosity (*H*e) ranged from 0.243 (CAR) to 0.536 (PGR). Evidence for linkage disequilibrium was not detected and significant departures from HWE were observed in different loci and in distinct populations (Table 1). Because of the reduced sample size of the population CRI for the SSR dataset, it was removed from the genetic variability analyses. The overall SSR data verified using Micro-Checker suggested no evidence of genotyping errors due to large allelic dropout, but indicated that null alleles might be present for Vx04, Vx06, and Vx13, and genotyping errors resulting from stuttering for Vx04 and Vx13. However, these latter, were double-checked with Genetic Profiler and all allele peaks were clear and well defined. The same analyses performed per population suggested the presence of null alleles in five loci in different populations: Vx01 (PGR and ORL), Vx04 (PGR and ORL), Vx06 (PGR, ORL, and SID), Vx10 (SID), and Vx13 (PGR, ORL, SID, and S86). The number of private alleles per population ranged from 0 (CAR, TCA, MAZ, TSU, and SID) to 6 (PGR). Populations MAQ and ORL also showed a high number of private alleles with 5 each. The mean frequency of private alleles was 0.150. The AMOVA for SSR data revealed a low differentiation among populations and high differentiation within populations (*F*st 0.262, $P < 0.001$; *R*st = 0.193, $P < 0.001$). The Bayesian cluster analyses identified the best group structure with $K = 4$ clusters (Fig. 2). Using the results from the AMOVA among populations for the cpDNA (*F*st = 0.957) and SSR data

(*F*st 0.262), the ratio of pollen flow to seed flow (Ennos 1994) resulted ca. 60, indicating that the role of gene flow through pollen in *R. reitzii* is ca. 60 times more efficient than through seed.

ECOLOGICAL NICHE MODELING

As indicated by high area under the curve (AUC) average values for the replicate runs of the Maxent distribution model (*R. reitzii* = 0.962, SD = 0.090 and *R. boliviana* = 0.994, SD = 0.004), both predictions had a good quality (AUC > 0.7 = acceptable; Pearce & Ferrier 2000). Maxent analyses pointed the variable "Temperature Annual Range" as the greater contribution for *R. reitzii* model (43.7%) and "Precipitation of Driest Month" for *R. boliviana* (52.3%) (Table S4). The ENMs under current climatic conditions predicted suitable localities for both species matching mostly their known observed distributions. Nevertheless, other small suitable localities were indicated out of the present ranges of the species, but no records are known for those areas (Fig. 3A, B). The potential geographical ranges at LGM of *R. reitzii* (Fig. 3C) and *R. boliviana* (Fig. 3D) showed to be somewhat wider than the present, however, the predictions for the LIG presented the widest ranges of suitable localities for both species (Fig. 3E, F).

The model of LGM distribution for *R. reitzii* showed that this species could have mainly occurred in the same restricted area along the southern Brazilian coast at that time, nevertheless, presenting more suitable localities (from its current distribution towards the coast) due to the presence of more land available as a result of the lower sea levels at the LGM (Fig. 3C). The ENM at the LGM for *R. boliviana* (Fig. 3D) presented more suitable localities than its current model (Fig. 3B), including part of the area predicted for *R. reitzii* at the LGM along the Brazilian coast (Fig. 3D).

The predicted distribution at the LIG for *R. reitzii* suggested that its range could have been significantly wider during this period. This ENM presented strong prediction for several areas forming a diagonal encompassing southern Brazil, Paraguay, and Bolivia, including areas within the current distribution of *R. boliviana* (Fig. 3E). The ENM at the LIG for *R. boliviana* (Fig. 3F) indicated a similar diagonal pattern, nevertheless, with weaker predictions and not reaching the suitable distribution of *R. reitzii* as at the LGM (Fig. 3D). Strong predictions were pointed for *R. boliviana* for localities along the west coast, surpassing the Andes, disjunct from its present range and out of the known distribution for the species (Fig. 3D, F). The altitude layer plotted on the LIG models (Fig. 3E, F) corresponds to the present elevation model and was included by means of geographic reference. Nevertheless, the current altitude pattern for the area under study, including the Andes, was probably similar to that at the LIG (Gregory-Wodzicki 2000).

TESTS FOR NICHE CONSERVATISM AND DIVERGENCE

The niche identity test indicated that the niche overlap indices obtained for *R. reitzii* and *R. boliviana* were significantly different from the pseudoreplicated data sets (Schoener's $D = 0.058$; Hellinger's $I = 0.214$; $p < 0.000$) (Fig. 4A, B).

The background randomization tests did not indicate either significant evidence for niche divergence or conservatism. The observed niche overlap value, Schoener's *D*, was not significantly different than the null distribution (Fig. 4C).

The complementary test for niche conservatism and divergence based on PCA of the raw bioclimatic data showed three axes that explain 87.6% of the total variation and availed themselves to biological interpretation (Table 2). Variables associated with

these axes had high loads and only the first component (PC1) showed high longitude/latitude correlation (Table 2). The low overlap indices (*D* and *I*) obtained may also be attributed to the differences between background environmental conditions, owed to the high spatial autocorrelation of the bioclimatic variables (Table 2).

DISCUSSION

cpDNA and SSR data

The cpDNA data showed that the *R. reitzii* populations showed lower haplotype and nucleotide diversities compared to other Neotropical trees (Ramos *et al.* 2009; Novaes *et al.* 2010; Garcia *et al.* 2011; Collevatti *et al.* 2012b), which might be related to the sample size, rarity, and restricted distribution of *R. reitzii* (only ca. 200 km of extension north-south along the Brazilian southeastern coast). The high differentiation among populations indicated by the AMOVA, the low intrapopulational genetic diversity, and the presence of fixed haplotypes may be a result of a founder effect and small population sizes. The two population groups indicated by the SAMOVA refer to one group formed by the two northern populations that share haplotype H5 (CRI and ORL) and a second group comprising the rest of the populations that share haplotype H1. The southernmost populations CAR and OSO are the only that present more than one haplotype besides H1: H2, exclusive of CAR, and H3 and H4, exclusive of OSO. The Mantel tests and the SAA (Fig. S1) also presented a significant association between genetic and geographic distances that indicates a north-south pattern of diversity in the Atlantic forest, which have been previously observed for other plant groups in this biome (Lorenz-Lemke *et al*. 2005; Palma-Silva *et al*. 2009; Turchetto-Zolet *et al*. 2012).

Different from the cpDNA (*F*st = 0.957), the SSR data pointed a higher differentiation within populations (*F*st = 0.261; *R*st = 0.193). The pollination and seed dispersal systems of *R. reitzii* are unknown, nevertheless, the *F*st values from the analyses of molecular variance may indicate that gene flow through pollen is ca. 60 times more efficient than through seeds (Ennos 1994; Ennos *et al.* 1999; Palma-Silva *et al*. 2009). The group structure with four clusters (Fig. 2) may indicate a north-south pattern of diversity (as observed in the cpDNA results) with closer populations being more similar to each other than distant populations. Besides the lack of information about the pollination systems, it is possible that some gene flow might be present between the *R. reitzii* populations (Fig. 2). However, the cpDNA and SSR data indicated well-structured populations, suggesting that gene flow and seed dispersal should be limited. Our data show, at least, three different genetic units (south, central, and north) influenced by the distance between populations.

SPECIES DISTRIBUTION MODELING

The ENMs suggested that the current range of the species of *Recordia* might be a relict of ancestors with a wider distribution during the LIG, due to the presence of suitable conditions for forest organisms to expand at that time (warmer and more humid) (Behling 2002; Carnaval & Moritz 2008; Lorenz-Lemke *et al*. 2010) (Fig. 3E, F). In the cooler and dryer LGM, the ancestral populations probably retracted and persisted disjunct and locally restricted to locations with suitable ecological conditions that allowed the survival of the two know extant species (Fig. 3C, D). The Atlantic forest is mainly separated from other forests by a 'dry diagonal' of seasonally dry forests, which are open vegetation and savanna-like areas, that includes the biomes of

Caatinga, Cerrado, and Chaco (Vanzolini 1963; Collevatti *et al*. 2012a). The changes in distribution and persistence in refugia with adequate features were more frequent than complete extinctions (Vegas-Vilarrubia *et al*. 2011; Turchetto-Zolet *et al*. 2013). During past climatic conditions, plant lineages from different vegetation formations (such as the Amazon and dry forests) may have had connections with the Atlantic forest by migration routes, such as forest corridors (e.g. gallery forests along rivers) or a series of patches (which allowed migration through island-hopping) (Rizzini 1963; Andrade-Lima 1964; Bigarella *et al*. 1975; Por 1992; Oliveira-Filho & Ratter 1995; Costa 2003).

TESTS FOR NICHE CONSERVATISM AND DIVERGENCE

Recordia reitzii occur restricted to the southernmost portion of the Atlantic forest, between the Serra Geral and the Atlantic Ocean, as a possible relict of a wider distributed species. This isolation may have been influenced by past habitat shifts that influenced on the forests expansions and retractions (Behling 2002; Carnaval & Moritz 2008; Lorenz-Lemke *et al*. 2010).

The background test performed with the reduced bioclimatic space obtained with the PCA showed a weak niche divergence accounted for variables reduced in the first principal component (PC1) (Table 2; Fig. S2). However, the PC1 showed strong geographic autocorrelation, indicating that the variables related might be diminishing the divergence pattern of the other variables in the ENMs niche overlap analysis realized with ENMTools.

The second principal component (PC2) indicated that variables related with extreme climatic conditions (especially the temperature of the coldest month and dry

seasons) might be the influencing factors of a strong signal of bioclimatic divergence between the *Recordia* species (Table 2). This result may be related with the wider bioclimatic space that *R. reitzii* holds in the PC2 (Fig. S2). This pattern could be explained by possible processes of expansion of niche breath of *R. reitzii*, which occupies more extreme weather conditions of the southern Atlantic Brazilian coast, compared with the more tamponed conditions close to the Andes mountains.

Our results suggest that late Pleistocene fluctuations and ecological factors might have played an important role on the diversification events of the *Recordia* species (Moritz *et al*. 2000). Finally, to make further inferences on the patterns of diversification in *Recordia*, dated phylogenies and analyses of divergence time estimates are necessary for the Verbenaceae, however, no fossil records are known for the family to calibrate these studies.

CONSERVATION IMPLICATIONS

More in depth biological studies, such as pollination and dispersal systems, are required to help to comprehend what factors influence the low genetic diversity presented in *R. reitzii* populations. Analyses of intraspecific genetic diversity are important to identify lineages that are geographically and genetically distinct by means of conservation (Moritz 1994; Moritz & Faith 1998; Moritz *et al*. 2000). The Atlantic forest and its species are under threat from habitat destruction and degradation. The conservation of areas that harbor endemic species is very important to preserve the genetic diversity (Moritz *et al*. 2000).

The climate change and the evolutionary history of the organisms seem to be connected, thus, the study of past climate changes are extremely important to obtain

information for future situations and biodiversity conservation (Rull 2011). Climatic changes may affect species range as they track suitable environments (Hewitt 1996) and may influence on distribution and rates of diversification. In the evolutionary past, corridors for the migration of the organisms were available, allowing the movement of species during climate changes (Donoghue 2008). However, human habitat fragmentation might limit organisms' dispersal in present and future climate changes and may influence the assembly of regional species pool (Donoghue 2008).

Information generated on this study on the genetic variability and climate change responses of *R. reitzii* might be useful to generate conservation strategies for this endangered species.

ACKNOWLEDGEMENTS

This study was funded by a doctoral fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior to VAT, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul.

REFERENCES

Alcantara S, Semir J, Solferini VN. 2006. Low genetic structure in an epiphytic Orchidaceae (*Oncidium hookeri*) in the Atlantic Rainforest of South-eastern Brazil. *Annals of Botany* **98:** 1207–1213.

Andrade-Lima D. 1964. Contribuição à dinâmica da flora do Brasil. *Arquivos do Instituto Ciências da Terra* **2:** 15–20.

Antonelli A, Sanmartín I. 2011. Why are there so many plant species in the Neotropics? *Taxon* **60:** 403–414.

Bandelt H-J, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16:** 37–48.

Behling H, Lichte M. 1997. Evidence of dry and cold climatic conditions at glacial times in tropical SE Brazil. *Quaternary Research* **48:** 348–358.

Behling H, Negrelle RRB. 2001. Tropical Rain Forest and climate dynamics of the Atlantic lowland, Southern Brazil, during the Late Quaternary. *Quaternary Research* **56:** 383–389.

Behling H, Arz HW, Patzold J, Wefer G. 2002. Late Quaternary vegetational and climate dynamics in southeastern Brazil, inferences from marine cores GeoB 3229-2 and GeoB 3202-1. *Palaeogeography, Palaeoclimatology, Palaeoecology* **179:** 227–243.

Behling H, Pillar V. 2007. Late Quaternary vegetation, biodiversity and fire dynamics on the southern Brazilian highland and their implication for conservation and management of modern Araucaria forest and grassland ecosystems. *Philosophical Transactions of the Royal Society B* **362:** 243–251.

Bigarella JJ, Andrade-Lima D, Riehs PJ. 1975. Considerações a respeito das mudanças paleoambientais na distribuição de algumas espécies vegetais e animais no Brasil. *Anais da Academia Brasileira de Ciências* **47:** 411–464.

Brown JH, Lomolino MV. 1998. Biogeography. Sunderland: Sinauer Associates.

Carnaval A, Moritz C. 2008. Historical climate modelling predicts patterns of current

biodiversity in the Brazilian Atlantic forest. *Journal of Biogeography* **35:** 1187–1201.

Collevatti RG, Terrible LC, Lima-Ribeiro MS, Nabout JC, Oliveira G, Rangel TF, Rabelo SG, Diniz-Filho JA. 2012a. A coupled phylogeographical and species distribution modelling approach recovers the demographical history of a Neotropical seasonally dry forest tree species. *Molecular Ecology* **21**: 5845–5863.

Collevatti RG, de Castro TG, de Souza Lima J, de Campos TM. 2012b. Phylogeography of *Tibouchina papyrus* (Pohl) Toledo (Melastomataceae), an endangered tree species from rocky savannas, suggests bidirectional expansion due to climate cooling in the Pleistocene. *Ecology and Evolution* **2:** 1024–1035.

Conte R, Nodari RO, Vencovsky R, Reis MS. 2003. Genetic Diversity and recruitment of the tropical palm *Euterpe edulis* Mart., in a natural population from the Brazilian Atlantic Forest. *Heredity* **91**: 401–406.

Costa LP. 2003. The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography* **30**: 71–86.

Dunn IS, Blattner FR. 1987. Sharons 36 to 40: Multi-enzyme, high capacity, recombination deficient replacement vectors with polylinkers and polystuffers. Nuc. Ac. Res. 15: 2677-2698.

Donoghue MJ. 2008. A phylogenetic perspective on the distribution of plant diversity. *Proceedings of the National Academy of Sciences* **105:** 11549–11555.

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* **11:** 2571-2581.

Ennos RA. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* **72**: 250–259.

Ennos RA, Sinclair WT, Hu X-S, Langdon A. 1999. Using organelle markers to elucidade the history, ecology, and evolution of plant populations. In: Hollingsworth PM, Bateman RM, Gornall RJ, eds. *Molecular Systematics and Plant Evolution*. London: Taylor & Francis.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564–567.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. *Genetics* **131**: 479–491.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of cluster of individuals using the software structure: a simulation study. *Molecular Ecology* **14:** 2611–2620.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* **164**: 1567–1587.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915–925.

Garcia MG, Silva RS, Carniello MA, Veldman JW, Rossi AAB, de Oliveira LO. 2011. Molecular evidence of cryptic speciation, historical range expansion, and recent intraspecific hybridization in the Neotropical seasonal forest tree *Cedrela fissilis* (Meliaceae). *Molecular Phylogenetics and Evolution* **61:** 639–649.

Goudet J. 2002. FSTAT: a computer program to calculate F-statistics (Version 2.9.3.2.). *Journal of Heredity* **86:** 485–486.

Gregory-Wodzicki KM. 2000. Uplift history of the Central and Northern Andes: A review. *Geological Society of America Bulletin* **112**: 1091–1105.

Hamilton MB. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**: 521–523.

Harvey PH, Pagel M. 1991. The Comparative Method in Evolutionary Biology. Oxford: Oxford University Press.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**: 247–276.

Hijmans R, Cameron S, Parra J, Jones P, Jarvis A. 2005. Very high resolution

interpolated climate surfaces for global land areas. *International Journal of Climatology* **25**: 1965–1978.

Hijmans RJ, Guarino L, Bussink C, Mathur P, Cruz M, Barrentes I, Rojas E, 2004. DIVA-GIS. v.7.1.7. A geographic information system for the analysis of species distribution data. Available at: http://www.diva-gis.org (accessed 1 February 2013).

Hung C -M, Drovetski SV, Zink RM. 2013. Recent allopatric divergence and niche evolution in a widespread Palearctic bird, the common rosefinch (*Carpodacus erythrinus*). *Molecular Phylogenetics and Evolution* **66**: 103–111.

Hutchinson GE. 1957. A Treatise on Limnology. Vol. 1. New York: John Wiley & Sons.

Jakobsson M, Rosenberg NA. 2007. Clumpp: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatic **23**: 1801–1806.

Lascoux M, Palmé AE, Cheddadi R, Latta RG. 2004. Impact of Ice Ages on the genetic structure of trees and shrubs. *Philosophical Transactions of the Royal Society of London. Series B, Biological* **359**: 197–207.

Ledru MP, Rousseau DD, Cruz FW, Riccomini C, Karmann I, Martin L. 2005. Paleoclimate changes during the last 100.000 years from a record in the Brazilian Atlantic rainforest region and interhemispheric comparison. *Quaternary Research* **64**: 444–450.

Lewinsohn TM, Prado PI. 2005. How many species are there in Brazil? *Conservation Biology* **19**: 619–624.

Lorenz-Lemke AP, Muschner VC, Bonatto SL, Cervi AC, Salzano FM, Freitas LB. 2005. Phylogeographic inferences concerning evolution of brazilian *Passiflora aciínia* and *P. elegans* (Passifloraceae) based on ITS (nrDNA) variation. *Annals of Botany* **95:** 799–806.

Lorenz-Lemke AP, Togni PD, Mäder G, Kriedt RA, Stehmann JR, Salzano FM, Bonatto SL, Freitas LB. 2010. Diversification of plant species in a subtropical region of eastern South American highlands: a phylogeographic perspective on native

Petunia (Solanaceae). *Molecular Ecology* **19**: 5240–5251.

McCormack JE, Zellmer AJ, Knowles LL. 2010. Does niche divergence accompany allopatric divergence in Aphelocoma jays as predicted under ecological speciation? insights from tests with niche models. *Evolution* **64**: 231–1244.

Metzger JP, Martensen AC, Dixo M, Bernacci LC, Ribeiro MC, Teixeira, AMG, Pardini R. 2009. Time-lag in biological responses to landscape changes in a highly dynamic Atlantic forest region. *Biological Conservation* **142**: 1166–1177.

Miller MP. 2005. Alleles in Space: computer software for the joint analysis of interindividual spatial and genetic information. *Journal of Heredity* **96**: 722–724.

Morellato LM, Haddad CFB. 2000. Introduction: The Brazilian Atlantic forest. *Biotropica* **32**: 786–792.

Mori SA, Boom BM, Prance GT. 1981. Distribution patterns and conservation of eastern Brazilian coastal forest tree species. *Brittonia* **33**: 233–245.

Moritz C. 1994. Defining "Evolutionary Significant Units" for conservation. *Trends in Ecology and Evolution* **9**: 373–375.

Moritz C, Faith DP. 1998. Comparative phylogeography and the identification of genetically divergent areas for conservation. *Molecular Ecology* **7**: 419–429.

Moritz C, Patton JL, Schneider CJ, Smith TB. 2000. Diversification of Rainforest Faunas: An Integrated Molecular Approach. *Annual Review of Ecology and Systematics* **31**: 533–563.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.

Nakazato T, Warren DL, Moyle LC. 2010. Ecological and geographic modes of species divergence in wild tomatoes. *American Journal of Botany* **97**: 680–693.

Nei M. 1987. Molecular Evolutionary Genetics. New York: Columbia University Press.

Novaes RML, de Lemos Filho JP, Ribeiro RA, Lovato MB. 2010. Phylogeography of *Plathymenia reticulata* (Leguminosae) reveals patterns of recent range expansion towards northeastern Brazil and southern Cerrados in Eastern Tropical South

America. *Molecular Ecology* **19:** 985–998.

Oliveira-Filho AT, Ratter JA. 1995. A study of the origin of central Brazilian forests by the analysis of plant species distribution patterns. *Edinburgh Journal of Botany* **52**: 141–194.

Palma-Silva C, Lexer C, Paggi GM, Barabará T, Bered F, Bodanese-Zanettini MH. 2009. Range-wide patterns of nuclear and chloroplast DNA diversity in *Vriesea gigantea* (Bromeliaceae), a Neotropical forest species. *Heredity* **103**: 503–512.

Pearce J, Ferrier S. 2000. Evaluating the predictive performance of habitat models developed using logistic regression. *Ecological Modelling* **133**: 225–245.

Perret M, Chautems A, Spichiger R. 2006. Dispersal-vicariance analyses in the tribe Sinningieae (Gesneriaceae): a clue to understanding biogeographical history of the Brazilian Atlantic forest. *Annals of the Missouri Botanical Garden* **93**: 340–358.

Peterson AT. 2007. Why not WhyWhere: the need for more complex models of simpler environmental spaces. *Ecological Modelling* **203**: 527–530.

Phillips S, Anderson R, Schapire R. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* **190**: 231–259.

Pinheiro F, Barros F de, Palma-Silva C, Fay MF, Lexer C, Cozzolino S. 2011. Phylogeography and genetic differentiation along the distributional range of the orchid *Epidendrum fulgens*: a Neotropical coastal species not restricted to glacial refugia. *Journal of Biogeography* **38**: 1923–1935.

Por FD. 1992. Sooretama: the Atlantic rain forest of Brazil. The Hague: SPB Academic Publishing.

Porto TJ, Carnaval AC, Rocha PLB da. 2012. Evaluating forest refugial models using species distribution models, model filling and inclusion: a case study with 14 Brazilian species. *Diversity and Distributions* **19**: 330–340.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structureusing multilocus genotype data. *Genetics* **155**: 945–959.

Ramos ACS, Lemos-Filho JP, Lovato MB. 2009. Phylogeographical structure of the neotropical forest tree *Hymenaea courbaril* (Leguminosae: Caesalpinioideae) and

its relationship with the vicariant *Hymenaea stigonocarpa* from Cerrado. *Journal of Heredity* **100**: 206–216.

Ranta P, Blom T, Niemelä J, Joensuu E, Siitonen M. 1998. The fragmented Atlantic rain forest of Brazil: size, shape and distribution of forest fragments. *Biodiversity and Conservation* ***7*****:** 385–403.

Reis MS, Fantini AC, Nodari RO, Reis, A, Guerra MP, Montovani A. 2000. Management and conservation of natural populations in Atlantic Rain Forest: the case study of palm heart (*Euterpe edulis* Martius). *Biotropica* **32**: 894–902.

Reitz R, Klein RM, Reis A. 1978. Projeto Madeira de Santa Catarina. *Sellowia* **30:** 1–320.

Reitz R, Klein RM, Reis A. 1983. Projeto Madeira do Rio Grande do Sul. *Sellowia* **34–35:** 1–525.

Ribeiro RA, Ramos ACS, Lemos Filho JP, Lovato MB. 2005. Genetic variation in remnant populations of *Dalbergia nigra* (Papilionoideae), an endangered tree from the Brazilian Atlantic Forest. *Annals of Botany* **95:** 1171–1177.

Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM. 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation* **142**: 1141–1153.

Rizzini CT. 1963. Nota prévia sobre a divisão fitogeográfica do Brasil. *Revista Brasileira de Geografia* **25**: 1–64.

Rosenberg NA. 2004. Distruct: a program for the graphical display of population structure. *Molecular Ecology Notes* **4**: 137–138.

Roy A, Frascaria N, Mackay J, Bousquet J. 1992. Segregating random amplified polymorphic DNAs (RAPDS) in *Betula alleghaniensis*. *Theoretical and Applied Genetics* **85**: 173–180.

Rull V. 2011. Neotropical biodiversity: timing and potential drivers. *Trends in Ecology & Evolution* **26**: 508**–**513.

Salimena FRG, Múlgura de Romero ME, O' Leary N, Peralta P, Thode VA. 2009. Verbenaceae. In: Stehmann JR, Forzza RC, Salino A, Sobral M, Costa DP, Kamino

LHY, eds. *Plantas da Floresta Atlântica*. Rio de Janeiro: Jardim Botânico do Rio de Janeiro.

Salimena FRG, Thode V, Mulgura M , França F, Silva TRS. 2012. Verbenaceae. In: *Lista de Espécies da Flora do Brasil*. Jardim Botânico do Rio de Janeiro. Available at: *http://floradobrasil.jbrj.gov.br/2012/FB015217*) (accessed 1 February 2013).

Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* **84**: 1120–1136.

Schoener TW. 1968. Anolis lizards of Bimini: resource partitioning in a complex fauna. *Ecology* **49**: 704–726.

SEMA. 2003. Lista final das espécies da flora ameaçadas – RS, Decreto Estadual n° 42.099. Available at: http://www.fzb.rs.gov.br/downloads/flora_ameacada.pdf [accessed 15 December 2012].

Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetics studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* **94**: 275–288.

Silva SM, Moraes-Barros N, Ribas CC, Ferrand N, Morgante JS. 2012. Divide to conquer: a complex pattern of biodiversity depicted by vertebrate components in the Brazilian Atlantic Forest. *Biological Journal of the Linnean Society* **107**: 39**–**55.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**: 457–462.

Sobral M, Jarenkow JA, Brack P, Irgang B, Larocca J, Rodrigues RS. 2006. Flora arbórea e arborescente do Rio Grande do Sul, Brasil. São Carlos: RiMA/Novo Ambiente.

Sobral M, Stehmann JR. 2009. An analysis of new angiosperm species discoveries in Brazil (1990 - 2006). *Taxon* **58**: 227–232.

SOS Mata Atlântica, Instituto Nacional de Pesquisas Espaciais (INPE). 2008. Atlas dos remanescentes florestais da Mata Atlântica, período de 2000 a 2005. Available at: http://www.sosmatatlantica.org.br (accessed 1 February 2013).

Stehmann JR, Forzza RC, Salino A, Sobral M, Costa DP, Kamino LHY. 2009. Plantas da Floresta Atlântica. Rio de Janeiro: Jardim Botânico do Rio de Janeiro.

Stepp SL, Leitner DW. 1989. Software review: Mystat™: A personal version of systat (statistical). *Nonprofit Management Leadership* **28:** 33–34.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585–595.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28:** 2731–2739.

Terborgh J. 1992. Maintenance of the diversity in tropical forests. *Biotropic* **24**: 283–292.

Thode VA, O'Leary N, Olmstead RG, Freitas LB. 2013. Phylogenetic position of the monotypic genus *Verbenoxylum* (Verbenaceae) and new combination under *Recordia*. *Systematic Botany*: In press.

Troncoso NS. 1974. Los Géneros de Verbenáceas de Sudamérica extratropical. *Darwiniana* **18:** 295–412**.**

Turchetto-Zolet AC, Pinheiro F, Salgueiro F, Palma-Silva F. 2013. Phylogeographical patterns shed light on evolutionary process in South America. *Molecular Ecology* **22**: 1193–1213.

Van der Vaart AW. 1998. Asymptotic statistics. Cambridge: Cambridge Univerity Press.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes **4**: 535–538.

Vanzolini PE. 1963. Problemas faunísticos do cerrado. In: Simpósio sobre o Cerrado, ed. Ferri MG. São Paulo: University of São Paulo Press.

Vegas-Vilarrubia T, Rull V, Montoya E, Safont E. 2011. Quaternary palaeoecology and nature conservation: a general review with examples from the neotropics. *Quaternary Science Reviews* **30**: 2361–2388.

Vieira MV, Olifiers N, Delciellos AC, Antunes VZ, Bernardo LR, Grelle C.V, Cerqueira R. 2009. Land use vs. fragment size and isolation as determinants of small mammal composition and richness in Atlantic Forest remnants. *Biological Conservation* **142**: 1191–1200.

Warren DL, Glor RE, Turelli M. 2008. Environmental niche equivalency versus conservatism: quantitative approaches to niche evolution. *Evolution* **62**: 2868–2883.

Wellenreuther M, Larson KW, Svensson EI. 2012. Climatic niche divergence or conservatism? Environmental niches and range limits in ecologically similar damselflies. *Ecology* **93**: 1353–1366.

Wiens JJ, Donoghue MJ. 2004. Historical biogeography, ecology and species richness. *Trends in Ecology and Evolution* **19**: 639–644.

Wiens JJ, Graham CH. 2005. Niche conservatism: integrating revolution, ecology, and conservation biology. *Annual Review of Ecology and Systematics* **36:** 519–539.

Wooten JA, Gibbs HL. 2012. Niche divergence and lineage diversification among closely related *Sistrurus* rattlesnakes. *Journal of Evolutionary Biology* **25:** 317–328.

TABELAS

CAPÍTULO 3

Table 1. Measures of genetic diversity for the ten microsatellite loci for 14 populations of *R. reitzii*.

		OSO (n = 23)						CAR (n = 3)						MAQ (n = 5)						TFO (n = 6)						TCA (n = 6)						MAZ (n = 6)						PGR (n = 20)					
Locus	Size range (bp)	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e
Vx01	253-269	21	5	0	1.557	0.286	0.266	2	1	0	1.000	M	M	3	1	0	1.000	M	M	6	4	0	2.869	0.000*	0.788	6	2	0	1.919	0.500	0.530	6	1	0	1.000	M	M	16	4	0	1.904	0.250	0.427
Vx02	253-277	17	4	0	2.303	0.412	0.613	3	3	0	2.404	1.000	0.733	5	3	1	2.462	0.600	0.644	6	5	0	2.331	0.667	0.576	5	5	0	2.595	0.600	0.667	6	4	0	2.982	1.000	0.818	20	4	0	2.103	0.600	0.541
Vx03	191-195	23	3	1	1.883	0.304	0.478	3	1	0	1.000	M	M	5	1	0	1.000	M	M	6	2	0	1.333	0.167	0.167	6	2	0	1.333	0.167	0.167	6	2	0	1.857	0.667	0.485	20	2	0	1.864	0.600	0.492
Vx04	164-194	23	7	0	2.519	0.609	0.478	3	3	0	2.149	0.333	0.600	5	3	0	3.062	0.600	0.822	5	3	0	2.595	0.200	0.733	6	2	0	1.919	0.500	0.530	6	3	0	2.533	0.50	0.712	18	7	1	2.746	0.222*	0.730
Vx05	153-163	20	2	0	1.894	1.000*	0.513	3	1	0	1.000	M	M	5	1	2	2.462	0.600	0.644	5	2	0	1.400	0.200	0.200	6	2	0	1.939	0.333	0.545	5	2	0	1.924	0.00	0.533	18	5	1	2.397	0.555*	0.622
Vx06	189-217	15	2	0	1.816	0.000*	0.513	2	1	0	1.000	M	M	5	1	0	1.667	0.000	0.356	4	1	2	1.000	M	M	6	2	0	1.576	0.333	0.303	6	2	0	1.745	0.167	0.409	15	2	0	1.870	0.133	0.497
Vx07	174-188	22	2	0	1.175	0.091	0.089	3	1	0	1.000	M	M	3	1	0	1.000	M	M	3	4	0	3.200	0.333	0.867	5	2	0	1.400	0.200	0.200	6	1	0	1.000	M	M	17	4	1	2.219	0.412*	0.572
Vx09	219-234	22	2	0	1.513	0.318	0.089	2	2	0	1.786	0.500	0.500	5	2	0	1.000	M	M	6	1	0	1.000	M	M	6	3	0	1.909	0.333	0.439	6	1	0	1.000	M	M	20	4	1	1.732	0.400	0.353
Vx10	252-276	21	4	0	2.212	0.333*	0.556	3	3	0	2.149	0.667	0.600	5	3	1	2.462	0.800	0.644	5	4	0	2.710	0.600	0.733	6	3	0	1.667	0.333	0.318	6	4	0	2.402	0.500	0.636	19	6	1	2.353	0.579	0.603
Vx13	263-271	22	3	0	2.043	0.000*	0.554	2	1	0	1.000	M	M	4	1	1	1.786	0.000	0.429	5	3	0	2.305	0.400	0.644	6	2	0	1.919	0.167	0.530	6	2	0	1.576	0.000	0.303	20	3	1	2.072	0.150*	0.524
Mean/total	–	3.4	1	1.892	0.202	0.41	–	1.7	0	1.449	0.250	0.243	–	1.7	5	1.790	0.260	0.354	–	2.9	2	2.074	0.257	0.471	–	2.5	0	1.818	0.347	0.423	–	2.2	0	1.802	0.283	0.390	–	4.1	6	2.126	0.256	0.536

	TSU (n = 5)						SID (n = 8)						S86 (n = 7)						S84 (n = 6)						TRE (n = 8)						LMU (n = 7)						ORL (n = 23)					
Locus	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e	N	A	PA	AR	H_o	H_e
Vx01	4	1	0	1.000	M	M	2	1	0	1.000	M	M	4	2	1	1.786	0.000	0.429	6	2	1	1.745	0.167	0.409	3	2	0	1.933	0.000	0.533	6	1	0	1.000	M	M	20	4	2	1.732	0.200	0.353
Vx02	5	4	0	2.102	0.600	0.533	8	3	0	1.668	0.375	0.342	7	2	1	1.286	0.143	0.143	5	3	0	2.305	1.000	0.644	8	3	0	1.700	0.375	0.342	7	2	0	1.286	0.143	0.143	21	5	1	2.373	0.524	0.630
Vx03	5	2	0	1.790	0.600	0.467	8	2	0	1.431	0.250	0.233	7	2	0	1.286	0.143	0.143	6	2	0	1.939	0.333	0.545	8	2	0	1.876	0.500	0.500	7	2	0	1.789	0.571	0.440	22	2	0	1.891	0.318	0.511
Vx04	5	1	0	1.000	M	M	8	3	0	1.937	0.125	0.492	5	2	0	1.667	0.000	0.356	4	3	0	2.414	0.250	0.679	6	4	1	2.929	0.000*	0.800	3	2	0	1.933	0.000	0.533	20	7	2	2.807	0.400*	0.759
Vx05	5	1	0	1.000	M	M	8	1	0	1.000	M	M	7	1	0	1.000	M	M	6	1	0	1.000	M	M	8	2	0	1.450	0.000	0.233	7	1	0	1.000	M	M	13	2	0	1.907	1.000*	0.522
Vx06	4	2	0	1.727	0.5	0.429	6	2	0	1.312	0.167	0.167	4	2	0	1.786	0.500	0.429	5	3	0	2.229	0.400	0.600	6	2	0	1.919	0.167	0.530	4	3	0	2.557	0.000	0.714	20	2	0	1.886	0.000*	0.508
Vx07	2	2	0	1.786	0.5	0.500	1	1	0	1.000	M	M	7	3	0	2.510	0.286	0.703	4	2	0	1.929	0.250	0.536	5	1	0	1.000	M	M	5	1	0	1.000	M	M	21	1	0	1.000	M	M
Vx09	5	1	0	1.000	M	M	8	3	0	1.668	0.375	0.342	7	3	0	1.955	0.571	0.473	6	2	0	1.919	0.500	0.530	8	4	0	2.346	0.625	0.642	7	3	1	1.791	0.429	0.385	23	3	0	1.582	0.217	0.300
Vx10	5	3	0	2.234	0.600	0.622	8	5	0	2.823	0.750	0.792	7	3	0	2.145	0.714	0.582	5	4	0	2.610	0.800	0.711	8	4	0	2.445	0.500	0.650	7	3	0	2.475	0.857	0.692	21	4	0	2.325	0.524	0.603
Vx13	5	2	0	1.624	0	0.356	8	3	0	2.121	0.000*	0.567	7	4	1	2.501	0.286	0.659	5	2	0	1.667	0.400	0.356	8	3	0	2.497	0*	0.700	7	4	1	2.347	0.143*	0.626	22	3	0	1.631	0.000*	0.317
Mean/total	–	1.9	0	1.526	0.280	0.291	–	2.4	0	1.596	0.204	0.293	–	2.4	3	1.792	0.264	0.392	–	2.4	1	1.976	0.410	0.501	–	2.7	1	2.010	0.217	0.493	–	2.2	2	1.718	0.200	0.353	–	3.3	5	1.913	0.178	0.450

N = sample size, A = observed number of alleles, PA = private alleles, AR = allelic richness, Ho = observed heterozigosity, He = expected heterozigozity, * = deviation from Hardy-Weinberg equilibrium ($p < 0.005$), M = monomorphic.

Table 2. Test of niche divergence vs. conservatism for *Recordia reitzii* and *R. boliviana*.

	Niche axes		
Paiwise comparison	PC1	PC2	PC3
Rr x Rb*	2.01	0.12	-0.45
Null distribution	(1.91; 1.95)	(-0.07; 0.08)	(0.04; 0.20)
Niche**	Divergence	Divergence	Conservatism
Expalined (%)	46.4	26.7	14.5
Variable loading 1	Precipitation of Driest Month (0.968)	Min. Temperature of Coldest Month (0.830)	Temperature Annual Range (0.935)
Variable loading 2	Temperature Seasonality (0.929)	Mean Temperature of Driest Quarter (0.762)	Mean Temperature of Wettest Quarter (0.485)
Variable loading 3	Isothermality (-0.926)	Mean Temperature of Wettest Quarter (0.715)	-
Variable loading 4	Annual Precipitation (0.891)	Precipitation of Wettest Month (0.617)	-
Interpretation	Seasonality	Temperature and humidity extremes	Temperature Seasonality
Longitude correlation	0.96	0.02	0.08
Latitude correlation	-0.97	0.02	-0.02

* observed differences between *R. reitzii* and *R. boliviana*, ** if the null distribution is smaller than the observed niche value, then the niche is considered divergent; if larger, considered conserved.

FIGURAS

CAPÍTULO 3

Figure 1. Map of Brazil and detail of part of Rio Grande do Sul and Santa Catarina Brazilian states showing the distribution of cpDNA haplotypes (A) and a median-joining network (B) for the populations. Circle size is proportional to sample size, and colors represent the haplotypes. Darker areas in A represent higher altitude.

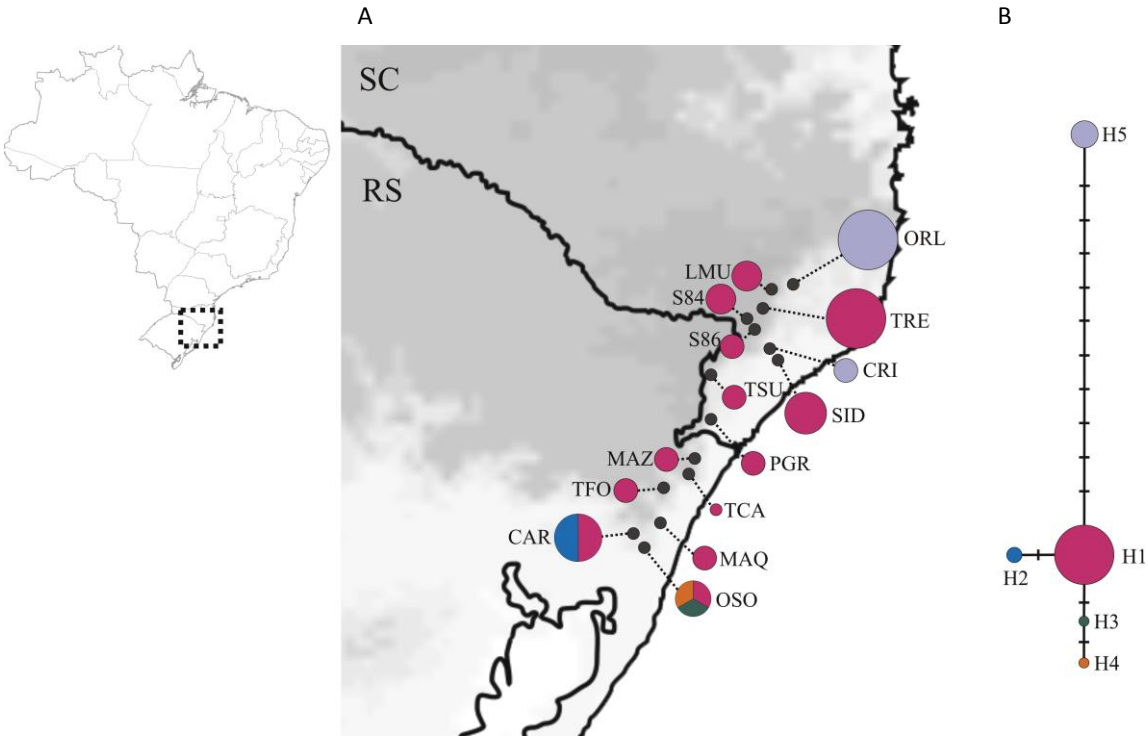


Figure 2. Bayesian structure analysis of *R. reitzii*. Each vertical line represents an individual portioned into the number of inferred clusters (*K* = 4) represented by the colors and the black vertical lines delimit populations.

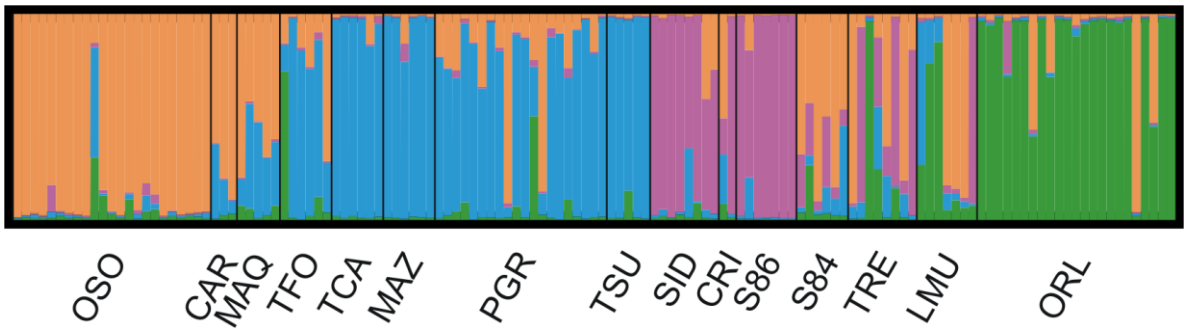
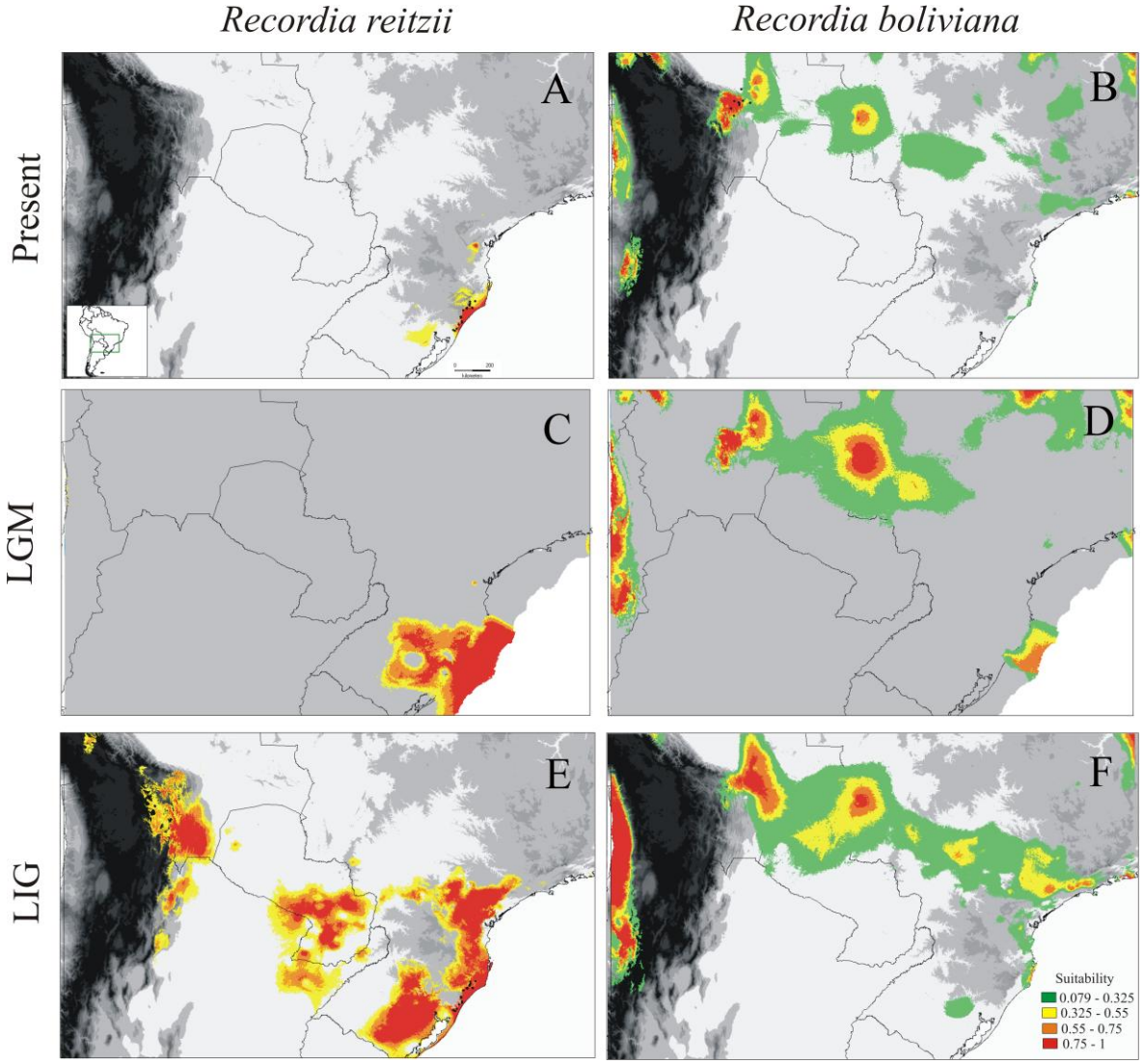
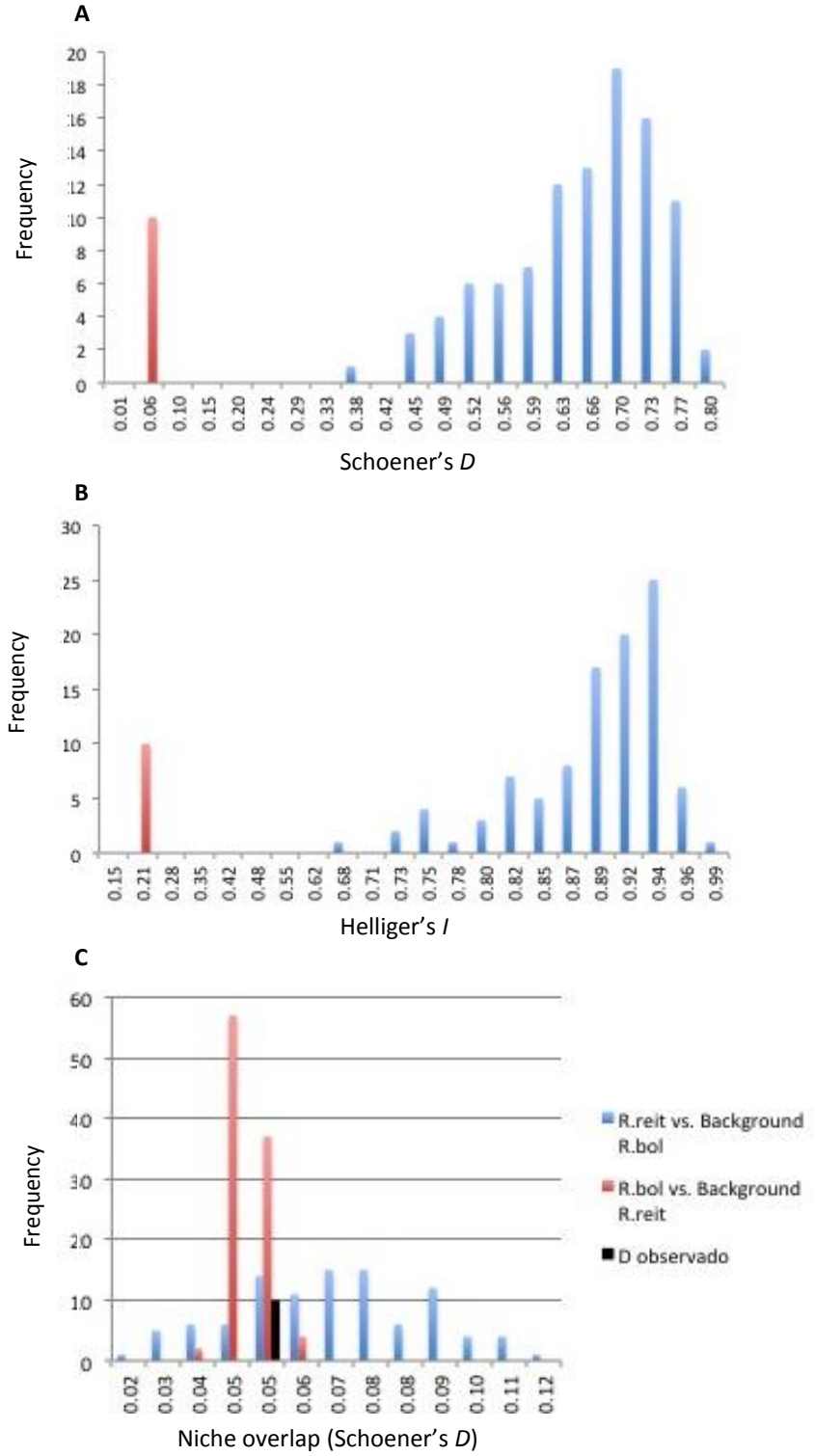


Figure 3. The predicted distribution models of *Recordia reitzii* and *R. boliviana* for the present (A, B), the Last Glacial Maximum (C, D), and the Last Interglacial (E, F) calculated with Maxent. Black dots represent point localities on which the models were based.



Treshold = Minimum training presence (*R. reitzii* = 0.325 and *R. boliviana* = 0.079).

Figure 4. Niche overlap values (red bars) for Schoener's *D* (A) and Helliger's *I* (B) compared to null distributions generated with the identity test. The similarity scores (*D* and *I*) are lower than the null hypotheses of niche equivalency, indicating that the bioclimatic niches are more different than expected by chance. Background test for Schoener's *D* (C). The observed niche overlap Schoener's *D* value (black bar), was not significantly different than the background null distribution, not indicating either significant evidence for niche divergence or conservatism.



APÊNDICES

CAPÍTULO 3

Figure S1. Spatial Autocorrelation Analysis results.

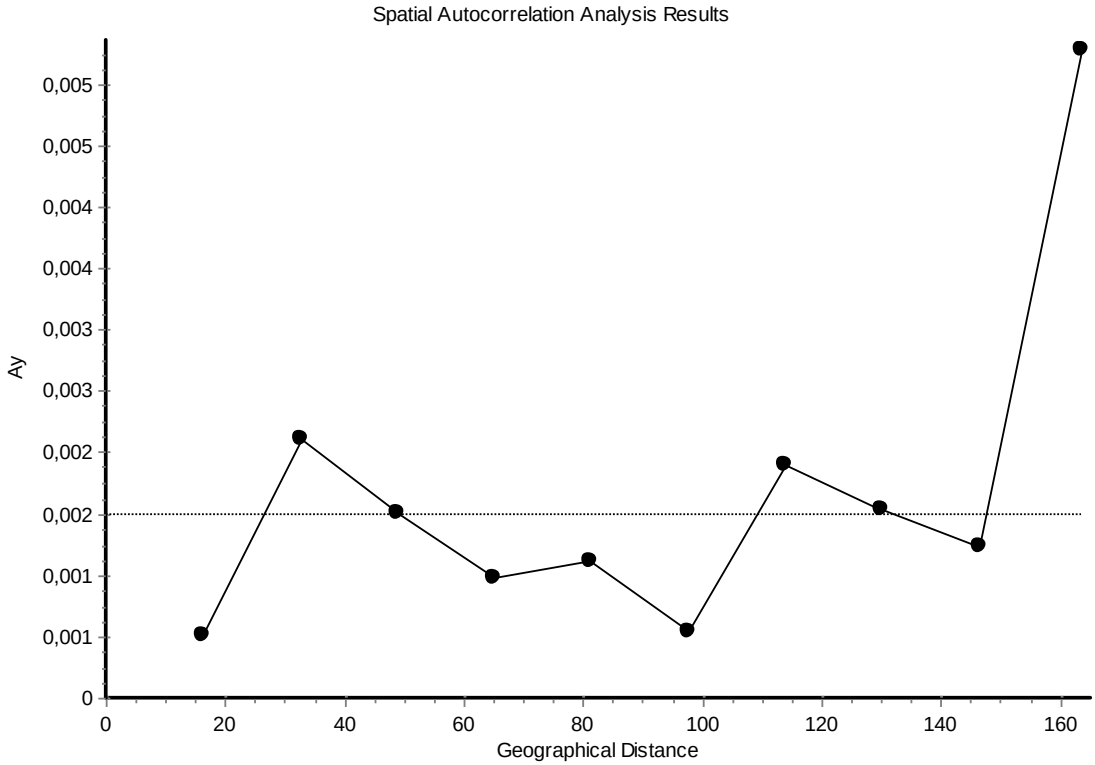


Figure S2. Scatter plot for the two main axes obtained from Principal Components Analysis with the environmental data from the nine climatic variables used in the ENMs for the *Recordia reitzii* (red points) and *R. boliviana* (black points) occurrence points.

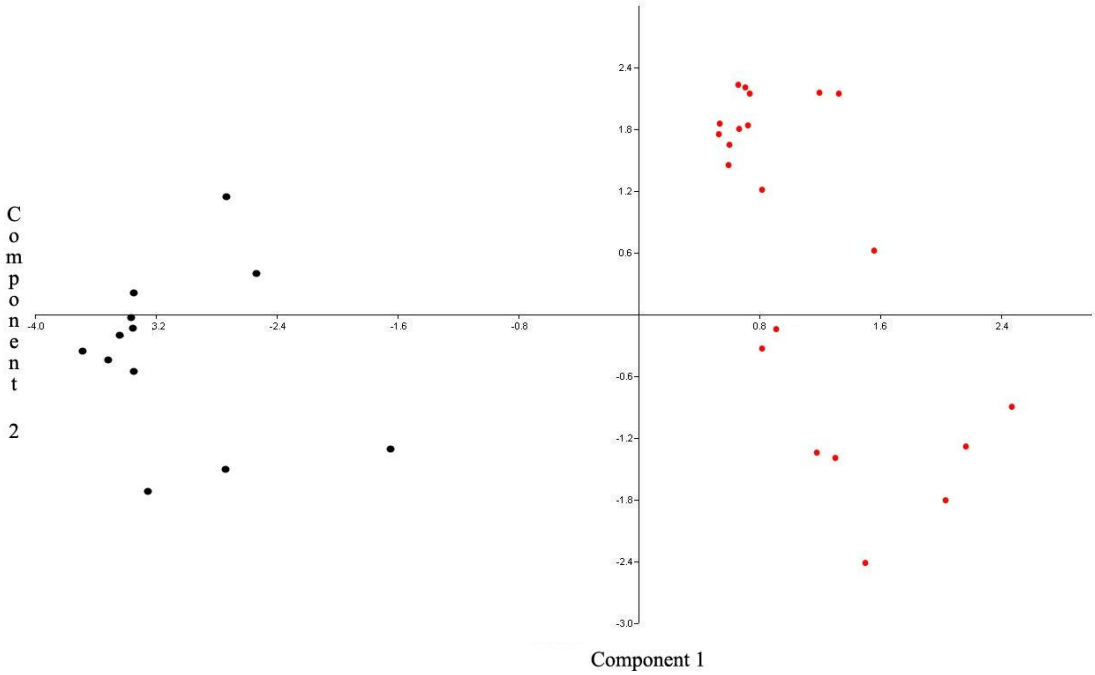


Table S1. Sampling locations of the 15 populations of *R. reitzii*.

Code	Population Locality	Voucher	Latitude	Longitude	Atitude (m.a.s.l.)
OSO	RS, Osório	V. Thode 269, ICN 166749	S 29 52 54.8	W 50 17 15.0	180
CAR	RS, Caraá	V. Thode 266, ICN 166748	S 29 50 07.1	W 50 21 29.1	142
MAQ	RS, Maquiné	V. Thode 275, ICN 166751	S 29 45 57.2	W 50 11 55.0	19
TFO	RS, Três Forquilhas	V. Thode 282, ICN 166751	S 29 32 55.8	W 50 04 43.3	30
TCA	RS, Três Cachoeiras	V. Thode 280, ICN 166754	S 29 27 38.6	W 49 56 10.0	11
MAZ	RS, Morro Azul	V. Thode 272, ICN 166750	S 29 24 18.5	W 49 55 51.1	29
PGR	SC, Praia Grande	V. Thode 281, ICN 166755	S 29 10 58.4	W 49 59 43.0	572
TSU	SC, Timbé do Sul	V. Thode 284, ICN 166757	S 28 49 12.3	W 49 52 12.3	148
SID	SC, Siderópolis	V. Thode 315, ICN 166765	S 28 43 10.1	W 49 22 12.6	28
CRI	SC, Criciúma	V. Thode 314, ICN 166764	S 30 42 25.3	W 49 24 30.6	32
S86	SC, Santo Antônio	V. Thode 286, ICN 166759	S 28 35 17.1	W 49 31 24.5	130
S84	SC, Santo Antônio	V. Thode 285, ICN 166758	S 28 34 03.1	W 49 32 36.4	162
TRE	SC, Trveiso	V. Thode 317, ICN 166766	S 28 29 34.6	W 49 31 15.2	403
LMU	SC, Lauro Muller	V. Thode 405	S 28 23 16.5	W 49 30 13.6	536
ORL	SC, Orleans	V. Thode 291, ICN 166763	S 28 22 16.7	W 49 14 34.3	103

Table S2. Geographic coordinates of *R. reitzii* (Rr) and *R. boliviana* (Rb) occurrence records used in Ecological Niche Modeling.

Species	Longitude	Latitude
Rr	-50.36	-29.84
Rr	-50.29	-29.88
Rr	-50.20	-29.77
Rr	-50.08	-29.55
Rr	-50.00	-29.18
Rr	-49.98	-29.17
Rr	-49.97	-29.20
Rr	-49.94	-29.46
Rr	-49.93	-29.41
Rr	-49.87	-28.82
Rr	-49.68	-28.71
Rr	-49.66	-28.75
Rr	-49.66	-28.70
Rr	-49.54	-28.57
Rr	-49.54	-28.58
Rr	-49.52	-28.59
Rr	-49.52	-28.49
Rr	-49.50	-28.39
Rr	-49.41	-28.71
Rr	-49.37	-28.72
Rr	-49.24	-28.37
Rb	-64.11	-18.49
Rb	-63.96	-18.87
Rb	-63.95	-18.11
Rb	-63.72	-18.19
Rb	-63.69	-18.24
Rb	-63.68	-18.24
Rb	-63.67	-18.27
Rb	-63.67	-18.31
Rb	-63.67	-18.26
Rb	-63.60	-17.50
Rb	-63.50	-18.10
Rb	-63.12	-18.20

Table S3. Correlation matrix for all pairs of the 19 climatic variables from WorldClim (Hijmans *et al.* 2005) calculated in ENMTools (Warren *et al*. 2008) with Pearson correlation coefficients

	BIO1	BIO2	BIO3	BIO4	BIO5	BIO6	BIO7	BIO8	BIO9	BIO10	BIO11	BIO12	BIO13	BIO14	BIO15	BIO16	BIO17	BIO18	BIO19
BIO1	0.00	-0.54	-0.05	-0.18	0.92	0.96	-0.36	0.94	0.92	0.96	0.98	0.58	0.59	0.15	-0.38	0.61	0.18	0.57	0.23
BIO2	0.00	0.00	0.16	0.26	-0.37	-0.71	0.68	-0.40	-0.64	-0.50	-0.58	-0.67	-0.49	-0.55	0.72	-0.52	-0.55	-0.53	-0.59
BIO3	0.00	0.00	0.00	-0.89	-0.36	0.07	-0.60	-0.20	0.13	-0.31	0.14	0.05	0.34	-0.35	0.43	0.32	-0.34	0.13	-0.27
BIO4	0.00	0.00	0.00	0.00	0.20	-0.36	0.87	0.04	-0.40	0.10	-0.39	-0.38	-0.58	0.08	-0.11	-0.57	0.07	-0.38	-0.01
BIO5	0.00	0.00	0.00	0.00	0.00	0.80	0.02	0.94	0.76	0.99	0.82	0.41	0.36	0.15	-0.39	0.38	0.17	0.39	0.19
BIO6	0.00	0.00	0.00	0.00	0.00	0.00	-0.58	0.84	0.96	0.87	0.98	0.68	0.66	0.25	-0.48	0.68	0.28	0.63	0.34
BIO7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.12	-0.58	-0.12	-0.54	-0.58	-0.62	-0.22	0.27	-0.62	-0.24	-0.52	-0.32
BIO8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.77	0.96	0.87	0.43	0.45	0.05	-0.30	0.47	0.07	0.49	0.10
BIO9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.82	0.96	0.63	0.63	0.22	-0.42	0.65	0.24	0.56	0.33
BIO10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.88	0.49	0.44	0.19	-0.44	0.46	0.21	0.48	0.25
BIO11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64	0.68	0.14	-0.36	0.70	0.17	0.62	0.24
BIO12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.87	0.68	-0.60	0.88	0.70	0.85	0.73
BIO13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	-0.23	1.00	0.29	0.87	0.34
BIO14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.77	0.29	0.99	0.40	0.97
BIO15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.25	-0.78	-0.35	-0.79
BIO16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.88	0.35
BIO17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.42	0.98
BIO18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44
BIO19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S4. The nine climate models used for Ecological Niche Modeling of *Recordia reitzii* and *R. boliviana.*

Variable	Percent contribution		Permutation importance	
	R. reitzii	***R. boliviana***	***R. reitzii***	***R. boliviana***
BIO3 = Isothermality (BIO2/BIO7) (* 100)	9.00	7.70	1.10	22.6
BIO4 = Temperature Seasonality (standard deviation *100)	1.80	7.40	1.00	14.6
BIO6 = Min Temperature of Coldest Month	0.00	1.00	0.00	2.10
BIO7 = Temperature Annual Range (BIO5-BIO6)	43.7	13.3	56.2	0.00
BIO8 = Mean Temperature of Wettest Quarter	12.5	0.40	22.5	0.40
BIO9 = Mean Temperature of Driest Quarter	5.50	2.90	11.4	0.30
BIO12 = Annual Precipitation	13.6	12.4	7.30	11.9
BIO13 = Precipitation of Wettest Month	10.9	2.60	0.40	0.60
BIO14 = Precipitation of Driest Month	3.00	52.3	0.00	47.5

CAPÍTULO 4

ESTUDO DAS RELAÇÕES EVOLUTIVAS NA TRIBO DURANTEAE UTILIZANDO MARCADORES PLASTIDIAIS E *LOCI* NUCLEARES DE PPR (*PENTATRICOPEPTIDE REPEAT*)

ESTUDO DAS RELAÇÕES EVOLUTIVAS NA TRIBO DURANTEAE UTILIZANDO MARCADORES PLASTIDIAIS E *LOCI* NUCLEARES DE PPR (*PENTATRICOPEPTIDE REPEAT*)

Verônica A. Thode, [1] Richard G. Olmstead, [2] and Loreta B. Freitas[1,3]

[1] Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Bento Gonçalves 9500, Porto Alegre, Brazil

[2] Department of Biology and Burke Museum, University of Washington, Seattle, Washington 98195, U. S. A.

[3] Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil

A ser submetido para publicação na revista *Biological Journal of the Linnean Society*

INTRODUÇÃO

A tribo Duranteae (Verbenaceae – Lamiales) foi estabelecida por Bentham em 1839, compreendendo quatro gêneros: *Citharexylum* L., *Duranta* L., *Rhaphithamnus* Miers e *Petrea* L. Outros sistemas de classificação foram propostos para apresentando diferentes divisões intrafamiliares Verbenaceae (Schauer 1847; Bentham & Hooker 1876; Briquet 1895; Junell 1934; Troncoso 1974; Sanders 2001; Atkins 2004; Marx *et al*. 2010). A classificação proposta por Marx e colaboradores (2010) foi baseada em marcadores do DNA plastidial (cpDNA) e amostrou todos os gêneros, com a exceção de *Verbenoxylum* Tronc. Este estudo apresentou muitas diferenças com relação aos sistemas de classificação anteriormente publicados, influenciando também na composição de Duranteae. Neste mesmo, a tribo foi amostrada por cinco gêneros, representados por 16 espécies (três espécies de *Bouchea* Cham., duas de *Chascanum* E. Mey., cinco de *Duranta*, cinco de *Stachytarpheta* Vahl e a única conhecida para *Recordia* Moldenke até o momento). Mais tarde, também com base em marcadores do cpDNA, o gênero *Verbenoxylum* foi incluído nesta tribo, sendo grupo-irmão de *Recordia*. *Verbenoxylum* foi transferido para *Recordia* que passou a possuir duas espécies disjuntas, *Recordia reitzii* (Moldenke) Thode & O'Leary, restrita ao extremo sul da Mata Atlântica brasileira e *Recordia boliviana* Moldenke, endêmica de florestas na Bolívia (Thode *et al.*, *in press*).

O número de espécies e a distribuição geográfica dos representantes de Duranteae podem ser visualizados na tabela 1. Esta tribo possui um gênero exclusivo da América do Sul, *Recordia*, que apresenta uma lacuna distribucional separando as suas duas espécies; gêneros com representantes nas Américas do Sul, Central e do Norte (*Bouchea*, *Duranta* e *Stachytarpheta*) e um gênero com espécies exclusivas do

Velho Mundo (*Chascanum*). Dos 33 gêneros de Verbenaceae, além de *Chascanum*, apenas *Coelocarpum* Balf. f. (tribo Lantaneae) não ocorre nas Américas (Atkins 2004; Thode *et al*., *in press*). Estes distintos padrões de distribuição presentes em Duranteae tornam esta tribo particularmente interessante para análises filogenéticas, biogeográficas e evolutivas que busquem um melhor entendimento dos mecanismos associados à diversificação e distribuição de espécies.

PPR (*pentatricopeptide repeat*) é um potencial marcador nuclear por possuir uma combinação única de três características: possui um grande número de *loci* com ortólogos estabelecidos, não possui íntrons e possui evolução rápida. Os genes *PPR* possuem muitas vantagens como marcadores filogenéticos, pois geram alinhamentos confiáveis e esforço mínimo para gerar sequências (Yuan *et al*. 2009). As proteínas *PPR* são caracterizadas por 2–26 repetições em *tandem* de um motivo altamente degenerado de 35 aminoácidos (Small & Peeters 2000; Lurin *et al*. 2004). Estudos de proteínas *PPR* indicam que elas funcionam como ligantes de RNA sequência-específicos em muitos processos pós-transcricionais, incluindo processamento, edição e estabilidade do RNA e tradução (Delannoy *et al*. 2007; Schmitz-Linneweber & Small 2008). Técnicas para a utilização de *loci* de *PPR* em Verbenaceae foram desenvolvidas como uma fonte de marcadores nucleares adequados para estudos filogenéticos (Yuan *et al*. 2009). Alguns estudos envolvendo estas regiões nucleares tem tido sucesso na determinação das relações filogenéticas entre espécies de plantas em diferentes níveis de relacionamento evolutivo e taxonômico (Yuan *et al*. 2010; Lu-Irving & Olmstead 2013).

Para melhor compreender as relações evolutivas dentro da tribo Duranteae e utilizar a filogenia gerada como base para futuras análises biogeográficas foram

estudados marcadores dos genomas nucleares (três *loci* de *PPR*) e plastidiais (*ndhF* e *trnL-trnF*) em análises filogenéticas de máxima verossimilhança e Bayesiana.

MATERIAL E MÉTODOS

MATERIAL VEGETAL

Para desenvolver o estudo filogenético de Duranteae foram amostrados os cinco gêneros da tribo, representados por 45 espécies que foram selecionadas para representar a distribuição geográfica na tribo (cinco espécies de *Bouchea*, quatro de *Chascanum*, onze de *Duranta*, 23 de *Stachytarpheta* e as duas de *Recordia*). As amostras foram coletadas em campo ou provenientes de exsicatas de herbário. Como grupos externos foram utilizadas 13 espécies pertencentes a outras tribos de Verbenaceae. A tabela S1 mostra os *taxa* amostrados neste estudo e países onde foram coletados.

EXTRAÇÃO DE DNA

O DNA genômico foi extraído de folhas jovens coletadas e armazenadas em sílica gel ou de material herborizado utilizando a técnica de Roy *et al.* (1992), eluído em água ultra pura e armazenado a -20 °C. A qualidade do DNA extraído foi verificada através de eletroforese horizontal em gel de agarose 1%, corado com GelRed™ (Biotium) e visualizado em transiluminador de luz ultravioleta. Os produtos foram quantificados em espectrofotômetro NanoDrop ND-1000 (NanoDrop Technologies).

AMPLIFICAÇÃO E SEQUENCIAMENTO

Para as análises filogenéticas foram amplificados o gene que codifica a subunidade 5 da enzima NADH desidrogenase (*ndhF*) e o espaçador entre os genes que codificam os RNAs transportadores de leucina e fenilalanina (*trnL-trnF*) do genoma plastidial incluíndo o íntron *trnL*, além de sequências parciais da proteína *PPR* (*pentatricopeptide repeat*) (AT1G09680, AT3G09060 e AT5G39980) (Yuan *et al*. 2009, 2010). As amplificações foram realizadas através da técnica de PCR (*Polimerase Chain Reaction*) em termocicladores automáticos (MJ Res. Inc., Eppendorf e Applied Biosystems), foram verificadas através de eletroforese horizontal em gel de agarose 1%, coradas com GelRed^{TM} (Biotium) e visualizadas em transiluminador de luz ultravioleta. As sequências dos *primers* de amplificação e sequenciamento e suas referências originais encontram-se na tabela S2. A purificação dos produtos de PCR foi feita com polietilenoglicol 20% seguindo protocolo de Dunn & Blattner (1987) antes do sequenciamento. As reações de sequenciamento das regiões do genoma nuclear (AT1G09680, AT3G09060 e AT5G39980) foram purificadas com Sephadex G-50 de acordo com protocolo descrito pelo fabricante. As reações de sequenciamento das regiões plastidiais *ndhF* e *trnLF* foram purificadas com acetato de amônio e etanol absoluto. O sequenciamento dos fragmentos de DNA foi realizado em sequenciador automático ABI 3730 (Applied Biosystems) usando o kit comercial BigDye v.3.1 (Applied Biosystems), de acordo com as indicações do fabricante. Todos os fragmentos foram sequenciados em ambas as direções com os *primers* indicados na tabela S2.

Análise das sequências de DNA

As sequências foram visualizadas no programa Sequencher 4.5 (Gene Codes Corporation) e alinhadas manualmente no programa SeAl v2.0a11 (Rambaut 2002) ou com auxílio do programa MAFFT v.6 (http://mafft.cbrc.jp/alignment/server/) seguido de correções manuais quando necessário. Os sítios variáveis e a composição nucleotídica foram estimados no programa MEGA 5.0 (Tamura *et al*. 2011).

Análises Filogenéticas

As sequências dos dois marcadores plastidiais (*trnL-trnF* e *ndhF*) foram concatenadas e analisadas como um conjunto de dados único. Os alinhamentos dos *loci* nucleares (AT1G09680, AT3G09060 e AT5G39980) foram tratados separadamente. As reconstruções filogenéticas foram realizadas individualmente para cada conjunto de dados e com os dados de todos os *loci* (plastidiais e nucleares) concatenados em uma supermatriz. Foram realizadas análises filogenéticas através dos métodos de máxima verossimilhança e inferência Bayesiana com os modelos evolutivos selecionados no jModelTest 0.1.1 (Guidon & Gascuel 2003; Posada 2008) com *Akaike Information Criterion* (AIC) O modelo GTR + I + G foi selecionado e aplicado em todas as análises.

Análises de máxima verossimilhança foram conduzidas utilizando o GARLI 2.0 (Zwickl 2006) com duas réplicas independentes de busca e 1000 réplicas de *bootstrap*. A árvore consenso foi construída no PAUP* 4.0b10 (Swofford 2002).

As análises de inferência Bayesiana foram realizadas no MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) com 10 milhões de gerações amostradas a cada 100 em duas corridas independentes, com quatro cadeias de Markov cada, iniciadas com

uma árvore aleatória. A convergência entre as corridas foi verificada no Tracer 1.5 (Rambaut & Drummond 2009).

RESULTADOS, DISCUSSÃO E PERSPECTIVAS

As análises filogenéticas utilizando três *loci* de *PPR* do genoma nuclear (AT1G09680, AT3G09060 e AT5G39980) e duas regiões do cpDNA (*trnL-trnF* e *ndhF*) apresentaram os relacionamentos entre os gêneros da tribo Duranteae coerentes com filogenias anteriores realizadas apenas com marcadores plastidias (Marx *et al*. 2010), porém, com maior representatividade específica. Todas as espécies amostradas ficaram posicionadas dentro dos seus respectivos gêneros. A Figura 1 mostra os resultados da análise filogenética realizada com a supermatriz com todos os conjuntos de dados concatenados.

Depois de alinhada e concatenada, a supermatriz com todas as sequências consistiu de 6581 pares de base (pb). A quantidade de posições alinhadas para cada marcador foi: *trnL-trnF* = 1031 pb, *ndhF* = 2116 pb, AT1G09680 = 1211 pb, AT3G09060 = 994 pb e AT5G39980 = 1229 pb.

Com uma maior representatividade de espécies em relação ao trabalho anteriormente publicado (Marx *et al.* 2010), poderão ser realizadas análises biogeográficas em Duranteae. Esta tribo apresenta gêneros com diferentes padrões de distribuição que podem ser investigados evolutivamente para elucidar processos históricos como a distribuição disjunta em um mesmo continente, migração de espécies entre as Américas e a ocorrência de linhagens proximamente relacionadas em continentes diferentes. Até o momento, nenhum fóssil foi identificado para a família Verbenaceae, o que impossibilita a datação das filogenias moleculares geradas e

estimativas de tempo de divergência das linhagens para compreender os processos de diversificação. Entretanto, muitas análises evolutivas podem ser realizadas com base em filogenias moleculares.

Estudos de reconstrução de área ancestral serão realizados utilizando os métodos de parcimônia no programa Mesquite 2.75 (Maddison & Maddison 2011) onde as localidades serão plotadas, a partir de uma matriz de presença e ausência, na árvore Bayesiana consenso gerada. Uma análise de máxima verossimilhança será implementada no programa LaGrange (Ree & Smith 2008) para inferir a evolução da amplitude geográfica na filogenia gerada, modelando a dispersão de linhagens e extinção local em um conjunto de áreas como eventos estocásticos em tempo contínuo (Ree *et al*. 2005; Ree & Smith 2008). Esta abordagem irá incorporar informações de divergências de linhagens e conexões disponíveis entre as áreas ou rotas de dispersão (Ree *et al*. 2005; Ree & Smith 2008). Cenários de dispersão e vicariância serão testados com análise Bayesiana no RASP (Yu *et al*. 2011).

REFERÊNCIAS BIBLIOGRÁFICAS

Atkins S. 2004. Verbenaceae. Pp. 449–468 *in* Kadereit, J. (ed)., *The Families and Genera of Vascular Plants*. VII. Flowering Plants: Dicotyledons: Lamiales (except Acanthaceae including Avicenniaceae). Springer, Berlin.

Bentham G. 1839. Enumeration of plants collected by Mr. Schomburgk in British Guiana. *Annals & Magazine of Natural History* 2: 441–451.

Bentham G & Hooker JD (1876) *Genera Plantarum* 2:1131-1160.

Briquet J. 1895. Verbenaceae. Pp. 132–182 in *Die naturlichen Pflanzenfamilien* Tiel 4/3a, ed. A. Engler, and K. Prantl. Leipzig: Engelmann.

Delannoy E, Stanley WA, Bond CS & Small ID. 2007. Pentatricopeptide repeat (*PPR*) proteins as sequence-specificity factors in post-transcriptional processes in organelles. *Biochem. Soc. T.* 35: 1643-1647.

Dunn IS & Blattner FR. 1987. Sharons 36 to 40: Multi-enzyme, high capacity, recombination deficient replacement vectors with polylinkers and polystuffers. *Nuc. Ac. Res*. 15: 2677-2698.

Junell S. 1934. Zur Gynaceummorphologie und Systematik der Verbenaceen und Labiaten. *Symbolae Botanicae Upsaliensis* 4: 1–219.

Lurin C, Andres C, Aubourg S, Bellaoui M, Bitton F, Bruyere C, Caboche M,Debast C, Gualberto J, Hoffmann B, *et al*. 2004. Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 16: 2089–2103.

Lu-Irving P & Olmstead RG. 2013. Investigating the evolution of Lantaneae (Verbenaceae) using multiple loci. *Botanical Journal of the Linnean Society* 171: 103–119.

Marx HE, O'leary N, Yuan Y, Lu-Iirving P, Tank DC, Múlgura ME & Olmstead RG. 2010. A molecular phylogeny and classification of Verbenaceae. *American Journal of Botany* 97: 1–17.

Maddison WP & DR Maddison. 2011. Mesquite: a modular system for evolutionary analysis, version 2.75. [computer program]. Available at http://mesquiteproject.org.

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.Rambaut A. 2002. Se-Al: Sequence Alignment Editor, v2.0a11. Available at http://evolve.zoo.ox.ac.uk/.

Rambaut A & Drummond AJ. 2009. Tracer v1.5 [computer program]. Available at http://tree.bio.ed.ac.uk/software/tracer/.

Ree RH, Moore BR, Webb CO & Donoghue MJ. 2005. A likelihood framework for inferring the evolution of geographic range on phylogenetic trees. *Evolution* 59: 2299–2311

Ree, RH and S.A. Smith. 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology* 57: 4–14.

Ronquist F & Huelsenbeck JP. 2003. *MrBayes 3*: phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Roy A, Frascaria N, MacKay J & Bousquet J. 1992. Segregating random amplified polymorphic DNAs (RAPDs) in *Betula alleghaniensis*. *Theoretical and Applied Genetics* 85: 173–180.

Sanders RW. 2001. The genera of Verbenaceae in the southeastern United States. *Harvard Papers in Botany* 5: 303–358.

Schauer JC. 1847. Verbenaceae. Pp. 522–700 in *Prodromus Systematis Naturalis Regni Vegetabilis* 11, ed. A. P. De Candolle. Paris: Fortin, Masson et Sociorum.

Schmitz-Linneweber C & Small I. 2008. Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends in Plant Sciences* 13: 663-670.

Small I & Peeters N. 2000. The *PPR* motif – a TPR-related motif prevalent in plant organellar proteins. *Trends in Biochemichal Sciences* 25: 46–47.

Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4b10. Sunderland, MA, USA: Sinauer Associates, Inc.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, & Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.

Thode VA, O'Leary N, Olmstead RG & Freitas LB. 2013. Phylogenetic position of the monotypic genus *Verbenoxylum* (Verbenaceae) and new combination under *Recordia*. *Systematic Botany*: in press.

Troncoso NS. 1974. Los generos de Verbenacea de Sudamerica extratropical. *Darwiniana* 18: 295–412 .

Yu Y, Harris AJ & He XJ. 2011. RASP (Reconstruct Ancestral State in Phylogenies), version 1.1. Disponível em http://mnh.scu.edu.cn/soft/blog/RASP (acessado em 01 de janeiro de 2013).

Yuan Y, Liu C, Marx HE & Olmstead RG. 2009. The pentatricopeptide repeat (*PPR*) gene family, a tremendous resource for plant phylogenetic studies. *New Phytologist* 182: 272–283.

Yuan Y, Liu C, Marx HE & Olmstead RG. 2010. An empirical demonstration of using pentatricopeptide repeat (*PPR*) genes as plant phylogenetic tools: Phylogeny of Verbenaceae and the *Verbena* complex. *Molecular Phylogenetics and Evolution* 54: 23–35.

Zwickl DJ. 2006. *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion.* Austin: University of Texas, U. S. A.

TABELAS

CAPÍTULO 4

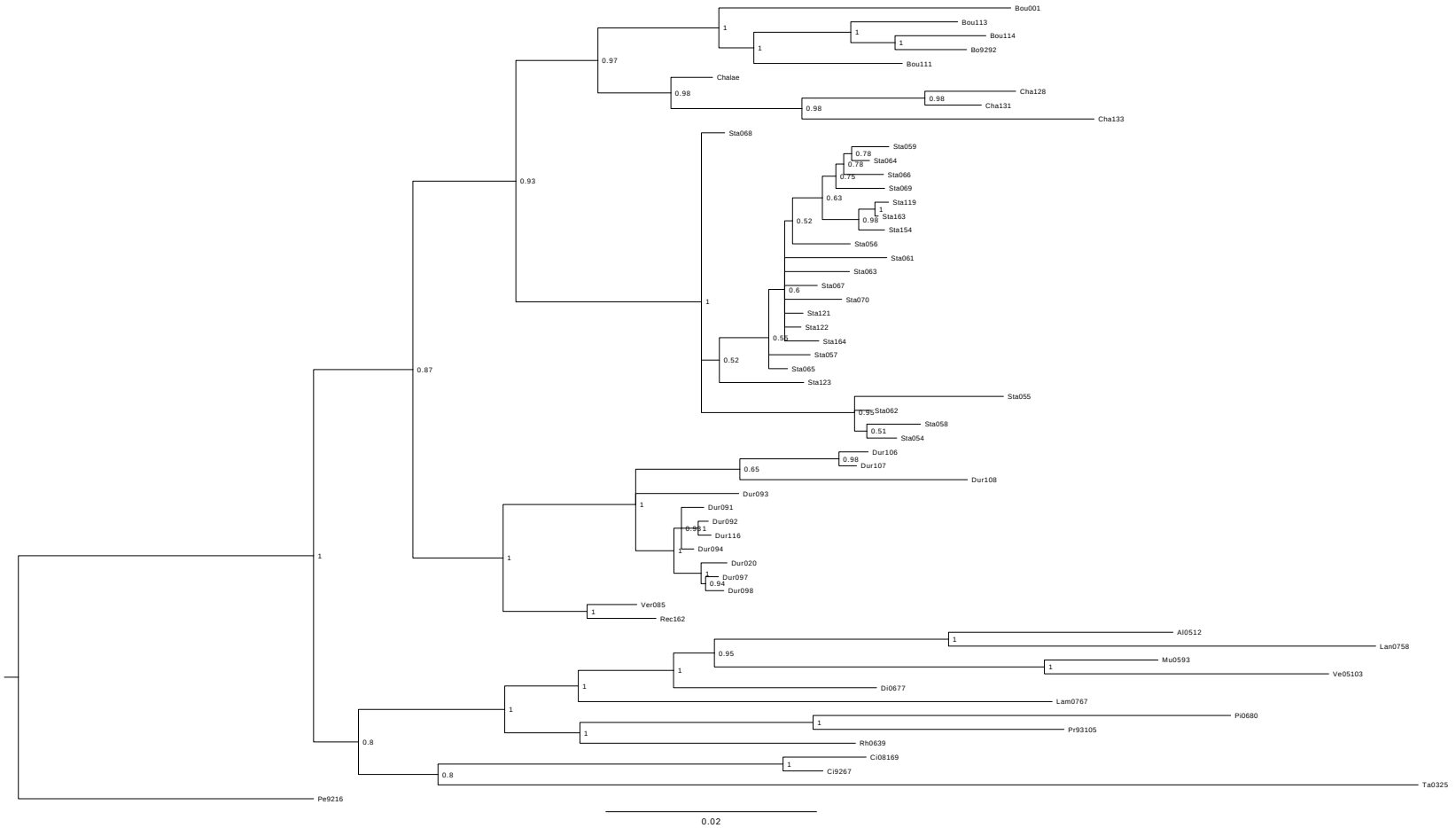
Tabela1. Representantes da tribo Duranteae, número de espécies por gênero, distribuição e referência.

Gênero	Número de sp.	Distribuição	Referência
Bouchea	9	Estados Unidos até Argentina	Marx *et al.* 2010
Chascanum	ca. 27	África, Madagascar, Península Arábica e Índia	Marx *et al*. 2010
Stachytarpheta	ca. 130	América Tropical	Atkins 2005
Duranta	17 to 34	América do Sul e Caribe	Troncoso 1974
Recordia	2	Bolívia e sul do Brasil	Thode *et al. in press*

FIGURAS

CAPÍTULO 4

FIGURA 1. Árvore consenso Bayesiana inferida a partir de todas as sequências combinadas (*trnL-trnF*, *ndhF*, AT1G09680, AT3G09060 e AT5G39980 = 6581 posições alinhadas), para 45 espécies de Duranteae e 13 espécies de grupo externo. Os valores nos ramos indicam a probabilidade posterior. Os códigos para os nomes estão na Tabela S1.



APÊNDICES

CAPÍTULO 4

Table S1. Espécies amostradas nas análises filogenéticas de Duranteae com marcadores do cpDNA (*trnL-trnF* e *ndhF*) e sequências parciais da proteína *PPR* (AT1G09680, AT3G09060 e AT5G39980) e países onde foram coletados.

Código	Espécies	País coletado
Bo9292	*Bouchea fluminensis var. pilosa*	Argentina
Bou001	*Bouchea ingrestis*	Brasil
Bou111	*Bouchea linifolia*	Estados Unidos
Bou113	*Bouchea dissecta*	México
Bou114	*Bouchea boliviana*	Bolivia
Cha128	*Chascanum garipense*	Namibia
Cha131	*Chascanum hildebrandtii*	Kenya
Cha133	*Chascanum sessilifolium*	Diredawa
Chalae	*Chascanum laetum*	Etiópia
Dur020	*Duranta vestita*	Brasil
Dur093	*Duranta fletcheriana*	Cuba
Dur091	*Duranta serratifolia*	Argentina
Dur092	*Duranta rupestris*	Peru
Dur094	*Duranta triacantha*	Peru
Dur097	*Duranta sprucei*	Peru
Dur098	*Duranta erecta*	Cuba
Dur106	*Duranta mandonii*	Ecuador
Dur107	*Duranta mandonii*	Peru
Dur108	*Duranta erida*	República Dominicana
Dur115	*Duranta sp.*	Peru
Sta054	*Stachytarpheta glabra*	Brasil
Sta055	*Stachytarpheta harleyi*	Brasil
Sta056	*Stachytarpheta gesnerioides 1*	Cultivada
Sta057	*Stachytarpheta glabra*	Brasil
Sta058	*Stachytarpheta mexiae*	Cultivada
Sta059	*Stachytarpheta reticulata*	Brasil
Sta061	*Stachytarpheta sp. 1*	Brasil
Sta062	*Stachytarpheta sp. 2*	Brasil
Sta063	*Stachytarpheta sp. 3*	Brasil
Sta064	*Stachytarpheta sp. 4*	Brasil
Sta065	*Stachytarpheta sp. 5*	Brasil
Sta066	*Stachytarpheta sp. 6*	Brasil
Sta067	*Stachytarpheta sp. 7*	Brasil
Sta068	*Stachytarpheta sp. 8*	Brasil
Sta069	*Stachytarpheta sp. 9*	Brasil
Sta070	*Stachytarpheta sp. 10*	Brasil
Sta119	*Stachytarpheta sp. 11*	Peru
Sta121	*Stachytarpheta jamaicensis*	Cuba
Sta122	*Stachytarpheta mutabilis*	Peru
Sta123	*Stachytarpheta crassifolia*	Brasil
Sta154	*Stachytarpheta dichotoma*	Estados Unidos, Hawaii
Sta164	*Stachytarpheta frantzii*	Cultivada
Sta163	*Stachytarpheta cayennensis*	Argentina
Rec162	*Recordia boliviana*	Bolivia
Ver085	*Verbenoxylum reitzii*	Brasil
Pe9216	*Petrea volubilis*	
Ci9267	*Citharexylum ligustrinum*	
C08169	*Citharexylum montevidense*	
Ve05103	*Verbena bonariensis*	
Rh0639	*Rhaphithamnus venustus*	
Pi0680	*Pitraea cuneatoovata*	
Pr93105	*Priva cordifolia*	Outgroup
Al0512	*Aloysia virgata*	
Lan0758	*Lantana canescens*	
Lam0767	*Lampaya castellani*	
Ta0325	*Tamonea boxiana*	
Mu0593	*Junellia asparagoides*	
Di0677	*Dipyrena glaberrima*	

Tabela S2: *Primers* utilizados neste estudo para *Polimerase Chain Reactions* e sequenciamento.

Marcador	Condições de Amplificação			"Primer"	Sequência dos "primers" (5' -3')	Referência
		94	2m	1F	ATG GAA CAK ACA TAT SAA TAT GC (PCR e seq.)	
		94	1m	536F	TTG TAA CTA ATC GTG TAG GGG A (seq.)	
ndhF 1F-1318R	35x	52	1m	536R	TCC CCT ACA CGA TTA GTT ACA A (seq.)	Olmstead e Sweere (1994)
		72	3m	972F	GTCTCAATTGGGTTATATGATG (seq.)	
		72	10m	972R	CAT CAT ATA ACC CAA TTG AGA C (seq.)	
				1318R	CGAAACATATAAAATGCRGTTAATCC (PCR e seq.)	
		94	2m	972F	GTCTCAATTGGGTTATATGATG (PCR e seq.)	
		94	1m	1318F	GGA TTA ACY GCA TTT TAT ATG TTT CG (seq.)	
ndhF 972F-2110R	35x	50	1m	1603F	CCT YAT GAA TCG GAC AAT ACT ATG C (seq.)	Olmstead e Sweere (1994)
		72	3m	1603R	GCA TAG TAT TGT CCG ATT CAT RAG G (seq.)	
		72	10m	2110R	CCC CCT AYA TAT TTG ATA CCT TCT CC (PCR e seq.)	
		80	5m	c	CGA ATC GGT AGA CGC TAC G (PCR e seq.)	
		94	1m	d	GGG GAT AGA GGG ACT TGA AC (seq.)	
trnL-trnF	30x	52	1m	e	GGT TCA AGT CCC TCT ATC CC (seq.)	Taberlet *et al.* (1991)
e trnL íntron		72	2m	f	TTT GAA CTG GTG ACA CGA G (PCR e seq.)	
		72	5m			
				180F	ACC RCC CTW TCT CAA GCC ATC CAA A (PCR e seq.)	Yuan *et al.* (2010)
				320F	TCT TCT CTT TCT TCA CAT GGC T (PCR e seq.)	
				336F	CTT ATT GCA CTA TGA TCC A (PCR e seq.)	V. Thode, dados não publicados
				850F	GTT AGT TTC AAT ACT TTG ATG AA (seq.)	
PPR AT1G09680				850R	TTC ATC AAA GTA TTG AAA CTA AC (seq.)	
				1300F	TTG TAA GGA RGG AGA TYT GGA (seq.)	Yuan *et al.* (2010)
				1300R	TCC ARA TCT CCY TCC TTA CAA (seq.)	
				1590R	TAA CCG TTC ATA AGC ACA TTG TA (seq.)	
				1760R	TAR TCA AGA ACA AGC CCT TTC GCA C (seq.)	
				930F	AGT GCT YTG ATT CAT GGG TTG TG (PCR e seq.)	Yuan *et al.* (2010)
				1000F	TCA CCT GAT GCT GTT GTA TRT (PCR e seq.)	
		94	2m	1000F2	GTC ACC TGA TGC TGT TGT ATA TA (PCR e seq.)	V. Thode, dados não publicados
		94	30s	1310F	GAG GTG TTC TAG ATG CTT TTG C (seq.)	
PPR AT3G09060	35x	48	30s	1310R	GCA AAA GCA TCT AGA ACA CCT C (seq.)	Yuan *et al.* (2010)
		72	3m	1760F	ATG CAT AAT ATT TTG ATT CAT GG (seq.)	
		72	10m	1760 R	CCA TGA ATC AAA ATA TTA TGC AT (seq.)	
				1966R	GCA AGA ACA AAG ACC TTT AAG AG (PCR e seq.)	V. Thode, dados não publicados
				2000R	GAT ATT CTA TTG CAA GAA CA (PCR e seq.)	Yuan *et al.* (2010)
				2080R	ACA GCT CKR ACA AGT ATR TTC CA (PCR e seq.)	
				550F	CAC GGR CTG TTC GAC GAA ATG CG (PCR e seq.)	Yuan *et al.* (2010)
				594F	CGCATTTTGGGAAAGAGGGTCTC (PCR e seq.)	V. Thode, dados não publicados
				650F	TCT TGG CTT CAG AAG ATG GA (seq.)	Yuan *et al.* (2010)
				949F	GTG TTT GCA GAA ATG AGG GAG AT (seq.)	V. Thode, dados não publicados
PPR AT5G39980				949R	ATC TCC CTC ATT TCT GCA AAC AC (seq.)	V. Thode, dados não publicados
				1030F	TGT AAT ATA ATG ATA GAT GTK TA (seq.)	
				1030R	GCC CAT AMA CAT CKA TCA TTA T (seq.)	
				1370F	GGG AAG YTR GAT AGA GCA GC (seq.)	Yuan *et al.* (2010)
				1620R	AAG ACC GTT ATR TCC TTG ACC TC (seq.)	
				1890R	AGA CTC AGC ATC TGR AAA TGA AC (PCR e seq.)	

CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

Os principais objetivos deste trabalho foram realizar estudos evolutivos com *Verbenoxylum reitzii* através de análises filogenéticas, diversidade genética**,** modelagem de nicho ecológico e testes de hipótese de conservatismo de nicho para contribuir com um conjunto de informações que possam servir como ferramentas para inferir padrões evolutivos da espécie e gerar medidas de conservação para a mesma.

No capítulo 1, observou-se que os estudos filogenéticos moleculares são de grande importância para resolver questões taxonômicas. Através da utilização de marcadores moleculares foi possível inferir o posicionamento de *Verbenoxylum* em Verbenaceae, posicionando-o na tribo Duranteae, como grupo-irmão do também monotípico *Recordia*, cuja distribuição é restrita à Bolívia. Com auxílio de dados morfológicos diagnósticos foi possível verificar que as espécies destes dois gêneros são muito semelhantes, sugerindo que ambas poderiam compor um único gênero. Troncoso (1971), quando descreveu *Verbenoxylum*, mencionou sua similaridade com *Recordia* mas, talvez pela grande lacuna de distribuição entre as espécies e o pouco material de ambas disponível em herbários, tal semelhança foi descartada. No entanto, *Verbenoxylum* e *Recordia* já haviam sido posicionados na mesmo tribo (Citharexyleae) em sistemas de classificação anteriores (Sanders 2001; Atkins 2004). Porém, um estudo baseado em dados moleculares de Marx e colaboradores (2010) apresentou composições das tribos de Verbenaceae diferentes das classificações tradicionais, além de não ter incluído *V. reitzii*. Através dos resultados obtidos no capítulo 1, *V. reitzii* foi transferida para o gênero *Recordia* (descrito por Moldenke em 1934), reduzindo *Verbenoxylum* a sinônimo deste. Os estudos de reconstrução de

estado de caráter ancestral mostraram que os caracteres utilizados tradicionalmente em classificações de Verbenaceae evoluíram independentemente e não são capazes de caracterizar as tribos propostas pela filogenia molecular de Marx e colaboradores (2010).

No capítulo 2, marcadores do tipo microssatélite foram desenvolvidos e caracterizados para *V. reitzii* e foi testada sua transferabilidade para *Recordia boliviana* e *Duranta vestita* (o gênero *Recordia* foi inferido como grupo-irmão de *Verbenoxylum* e *Duranta* foi estabelecido como o grupo-irmão de *Verbenoxylum* + *Recordia* no capítulo 1). O sucesso na amplificação da maior parte dos *loci* desenvolvidos para *V. reitzii* em amostras de *R. boliviana* sugere uma grande proximidade filogenética entre as espécies, apesar da distância geográfica que as separa. Estes mesmos *loci* não foram capazes de amplificar as amostras de *D. vestita*, o que reforça tal conclusão. Além de permitirem o estudo da diversidade genética em *V. reitzii*, estes *loci* de SSR poderão ser utilizados em estudos de diversidade genética de *R. boliviana* permitindo futuras análises comparativas entre as duas espécies.

No capítulo 3, tratamos de determinar a distribuição da variabilidade genética do que passamos a nomear como *Recordia reitzii* (*Verbenoxylum reitzii* foi transferido para o gênero *Recordia* no capítulo 1). Este estudo associado à modelagem de nicho ecológico e testes de hipótese de conservatismo x divergência de nicho trouxe informações importantes sobre a história evolutiva desta espécie em particular, contribuindo para o entendimento do mecanismo de distribuição e diferenciação deste pequeno gênero de Verbenaceae. Estas análises foram realizadas com o objetivo de investigar a dinâmica populacional em *R. reitzii* e estudar os efeitos de mudanças

climáticas na atual distribuição das duas espécies de *Recordia*. Os dados de cpDNA apresentados neste capítulo sugerem que *R. reitzii* possui uma diversidade baixa, a qual pode ser associada a sua distribuição restrita, pequeno tamanho populacional e efeito fundador. Apesar da baixa diversidade, as análises de cpDNA e SSRs mostram um padrão norte-sul de distribuição de diversidade. Os resultados gerados nestas análises podem auxiliar na tomada de medidas de conservação para a *R. reitzii*, uma vez que estes identificaram grupos geneticamente distintos que são importantes para a preservação da diversidade genética da espécie. Estudos biológicos e ecológicos em *R. reitzii* são necessários para uma melhor compreensão dos processos evolutivos que possam estar influenciando na diversidade de suas populaçõoes e que possam ser associados com os resultados encontrados. Os ENMs indicam que a atual distribuição das espécies de *Recordia* deve ter sido influenciada por mudanças climáticas pretéritas e oscilações de ciclos glaciais/interglaciais. Os modelos sugerem que durante o LIG (períodos mais quentes e húmidos) a distribuição era mais ampla e durante o LMG (perídos mais frios e secos) as populações ancestrais de *Recordia* provavelmente sofreram uma retração na distribuição. Durante estes períodos de condições menos favoráveis para organismos florestais, estes podem ter permanecido em refúgios.

Os dados obtidos durante o estágio sanduíche no laboratório do Dr. Richard Olmstead (University of Washington, Seattle, Estados Unidos) estão descritos no capítulo 4. Estes resultados ainda necessitam de muitas outras análises antes de serem considerados prontos para publicação. Eles comporão um artigo a ser submetido ao periódico *Biological Journal of the Linnaean Society*. No capítulo 4, marcadores do genoma plastidial e nuclear foram utilizados para analisar as relações evolutivas

dentro da tribo Duranteae (Verbenaceae) e gerar filogenias moleculares que possam ser utilizadas em outras análises. Foram amostrados os cinco gêneros da tribo, representados por 45 espécies. Foi gerado um alinhamento total de 6581 pares de base (três *loci* de *PPR* do genoma nuclear: AT1G09680, AT3G09060 e AT5G39980 e duas regiões do cpDNA: *trnL-trnF* e *ndhF*). A tribo Duranteae apresenta gêneros com diferentes padrões de distribuição que podem ser investigados evolutivamente, com base nas filogenias moleculares geradas, para elucidar processos biogeográficos que possam ter influenciado na diversificação de linhagens e formação de biomas. Como por exemplo: análises de reconstrução de área ancestral, abordagens que incorporam informações de divergência de linhagens e conexões disponíveis entre as áreas ou rotas de dispersão e testes de cenários de dispersão e vicariância podem ser realizados. Em conjunto, os resultados que compõem os capítulos desta tese sugerem que estudos evolutivos em organismos raros e de distribuição restrita podem trazer informações importantes para elucidar processos amplos que possam servir como ferramenta para gerar medidas de conservação de *Recordia reitzii* e da Mata Atlântica.

REFERÊNCIAS BIBLIOGRÁFICAS

REFERÊNCIAS BIBLIOGRÁFICAS

Alcantara S, Semir J, Solferini VN. 2006. Low genetic structure in an epiphytic Orchidaceae (*Oncidium hookeri*) in the Atlantic Rainforest of South-eastern Brazil. *Annals of Botany* **98:** 1207–1213.

Andrade-Lima D. 1964. Contribuição à dinâmica da flora do Brasil. *Arquivos do Instituto Ciências da Terra* **2:** 15–20.

Antonelli A, Sanmartín I. 2011. Why are there so many plant species in the Neotropics? *Taxon* **60:** 403–414.

Beebee TJC, Rowe G. 2008. An introduction to molecular ecology. New York: Oxford University Press.

Behling H, Lichte M. 1997. Evidence of dry and cold climatic conditions at glacial times in tropical SE Brazil. *Quaternary Research* **48:** 348–358.

Behling H, Negrelle RRB. 2001. Tropical Rain Forest and climate dynamics of the Atlantic lowland, Southern Brazil, during the Late Quaternary. *Quaternary Research* **56:** 383–389.

Behling H, Arz HW, Patzold J, Wefer G. 2002. Late Quaternary vegetational and climate dynamics in southeastern Brazil, inferences from marine cores GeoB 3229-2 and GeoB 3202-1. *Palaeogeography, Palaeoclimatology, Palaeoecology* **179:** 227–243.

Bigarella JJ, Andrade-Lima D, Riehs PJ. 1975. Considerações a respeito das mudanças paleoambientais na distribuição de algumas espécies vegetais e animais no Brasil. *Anais da Academia Brasileira de Ciências* **47:** 411–464.

Brown JH, Lomolino MV. 1998. Biogeography. Sunderland: Sinauer Associates.

Carnaval A, Moritz C. 2008. Historical climate modelling predicts patterns of current biodiversity in the Brazilian Atlantic forest. *Journal of Biogeography* **35:** 1187–1201.

Collevatti RG, de Castro TG, de Souza Lima J, de Campos TM. 2012. Phylogeography of *Tibouchina papyrus* (Pohl) Toledo (Melastomataceae), an endangered tree species from rocky savannas, suggests bidirectional expansion due to climate cooling in the Pleistocene. *Ecology and Evolution* **2:** 1024–1035.

Conte R, Nodari RO, Vencovsky R, Reis MS. 2003. Genetic Diversity and recruitment of the tropical palm *Euterpe edulis* Mart., in a natural population from the Brazilian Atlantic Forest. *Heredity* **91**: 401–406.

Costa LP. 2003. The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography* **30**: 71–86.

Harvey PH, Pagel M. 1991. The Comparative Method in Evolutionary Biology. Oxford: Oxford University Press.

Hung CM, Drovetski SV, Zink RM. 2013. Recent allopatric divergence and niche evolution in a widespread Palearctic bird, the common rosefinch (*Carpodacus erythrinus*). *Molecular Phylogenetics and Evolution* **66**: 103–111.

Hutchinson GE. 1957. A Treatise on Limnology. Vol. 1. New York: John Wiley & Sons.

Ledru MP, Rousseau DD, Cruz FW, Riccomini C, Karmann I, Martin L. 2005. Paleoclimate changes during the last 100.000 years from a record in the Brazilian Atlantic rainforest region and interhemispheric comparison. *Quaternary Research* **64**: 444–450.

Lewinsohn TM, Prado PI. 2005. How many species are there in Brazil? *Conservation Biology* **19**: 619–624.

Lista de Espécies da Flora do Brasil 2013. Available at: http://floradobrasil.jbrj.gov.br/ (accessed 15 April 2013).

Lorenz-Lemke AP, Togni PD, Mäder G, Kriedt RA, Stehmann JR, Salzano FM, Bonatto SL, Freitas LB. 2010. Diversification of plant species in a subtropical region of eastern South American highlands: a phylogeographic perspective on native *Petunia* (Solanaceae). *Molecular Ecology* **19**: 5240–5251.

Martins FDM. 2011. Historical biogeography of the Brazilian Atlantic forest and the

Carnaval-Moritz model of Pleistocene refugia: what do phylogeographical studies tell us? *Biological Journal of the Linnean Society* **104**: 499–509.

Marx HE, O'leary N, Yuan Y, Lu-Irving P, Tank DC, Múlgura ME, Olmstead RG. 2010. A molecular phylogeny and classification of Verbenaceae. *American Journal of Botany* **97**: 1–17.

McCormack JE, Zellmer AJ, Knowles LL. 2010. Does niche divergence accompany allopatric divergence in Aphelocoma jays as predicted under ecological speciation? insights from tests with niche models. *Evolution* **64**: 231–1244.

Moldenke. HN. 1934. *Phytologia* **1**: 99. 1934.

Moritz C. 1994. Defining "Evolutionary Significant Units" for conservation. *Trends in Ecology and Evolution* **9**: 373–375.

Moritz C, Faith DP. 1998. Comparative phylogeography and the identification of genetically divergent areas for conservation. *Molecular Ecology* **7**: 419–429.

Oliveira-Filho AT, Ratter JA. 1995. A study of the origin of central Brazilian forests by the analysis of plant species distribution patterns. *Edinburgh Journal of Botany* **52**: 141–194.

Palma-Silva C, Lexer C, Paggi GM, Barabará T, Bered F, Bodanese-Zanettini MH. 2009. Range-wide patterns of nuclear and chloroplast DNA diversity in *Vriesea gigantean* (Bromeliaceae), a Neotropical forest species. *Heredity* **103**: 503–512.

Por FD. 1992. Sooretama: the Atlantic rain forest of Brazil. The Hague: SPB Academic Publishing.

Ramos ACS, Lemos - Filho JP, Lovato MB. 2009. Phylogeographical Structure of the Neotropical Forest Tree *Hymenaea courbaril* (Leguminosae: Caesalpinioideae) and Its Relationship with the Vicariant Hymenaea stigonocarpa from Cerrado. *Journal of Heredity* **100**: 206–216.

Ranta P, Blom T, Niemelä J, Joensuu E, Siitonen M. 1998. The fragmented Atlantic rain forest of Brazil: size, shape and distribution of forest fragments. *Biodiversity and Conservation* ***7*:** 385–403.

Reitz R, Klein RM, Reis A. 1978. Projeto Madeira de Santa Catarina. *Sellowia* **30:** 1–320.

Reitz R, Klein RM, Reis A. 1983. Projeto Madeira do Rio Grande do Sul. *Sellowia* **34–35:** 1–525.

Ribeiro RA, Ramos ACS, Lemos Filho JP, Lovato MB. 2005. Genetic variation in remnant populations of *Dalbergia nigra* (Papilionoideae), an endangered tree from the Brazilian Atlantic Forest. *Annals of Botany* **95:** 1171–1177.

Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM. 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation* **142**: 1141–1153.

Rizzini CT. 1963. Nota prévia sobre a divisão fitogeográfica do Brasil. *Revista Brasileira de Geografia* **25**: 1–64.

Rull V. 2011. Neotropical biodiversity: timing and potential drivers. *Trends in Ecology & Evolution* **26**: 508**–**513.

Salimena FRG, Thode V, Mulgura M , França F, Silva TRS. 2012. Verbenaceae. In: *Lista de Espécies da Flora do Brasil*. Jardim Botânico do Rio de Janeiro. Available at: *http://floradobrasil.jbrj.gov.br/2012/FB015217*) (accessed 1 February 2013).

SEMA. 2003. Lista final das espécies da flora ameaçadas – RS, Decreto Estadual n° 42.099. Available at: http://www.fzb.rs.gov.br/downloads/flora_ameacada.pdf (accessed 15 December 2012).

SOS Mata Atlântica, Instituto Nacional de Pesquisas Espaciais (INPE). 2008. Atlas dos remanescentes florestais da Mata Atlântica, período de 2000 a 2005. Available at: http://www.sosmatatlantica.org.br (accessed 1 February 2013).

Stehmann JR, Forzza RC, Salino A, Sobral M, Costa DP, Kamino LHY. 2009. Plantas da Floresta Atlântica. Rio de Janeiro: Jardim Botânico do Rio de Janeiro.

Thode VA, O'Leary N, Olmstead RG, Freitas LB. 2013. Phylogenetic position of the monotypic genus *Verbenoxylum* (Verbenaceae) and new combination under *Recordia*. *Systematic Botany*: In press.

Turchetto-Zolet AC, Pinheiro F, Salgueiro F, Palma-Silva F. 2013. Phylogeographical

patterns shed light on evolutionary process in South America. *Molecular Ecology* **22**: 1193–1213.

Vieira MV, Olifiers N, Delciellos AC, Antunes VZ, Bernardo LR, Grelle C.V, Cerqueira R. 2009. Land use vs. fragment size and isolation as determinants of small mammal composition and richness in Atlantic Forest remnants. *Biological Conservation* **142**: 1191–1200.

Warren DL, Glor RE, Turelli M. 2008. Environmental niche equivalency versus conservatism: quantitative approaches to niche evolution. *Evolution* **62**: 2868–2883.

Wellenreuther M, Larson KW, Svensson EI. 2012. Climatic niche divergence or conservatism? Environmental niches and range limits in ecologically similar damselflies. *Ecology* **93**: 1353–1366.

Wiens JJ, Graham CH. 2005. Niche conservatism: integrating revolution, ecology, and conservation biology. *Annual Review of Ecology and Systematics* **36:** 519–539.

Wooten JA, Gibbs HL. 2012. Niche divergence and lineage diversification among closely related *Sistrurus* rattlesnakes. *Journal of Evolutionary Biology* 25: 317–328.

Wright S. 1978. Evolution and the genetics of populations. Chicago: The University of Chicago Press.