ÉRICA MANGARAVITE

A COMPLEXA HISTÓRIA EVOLUTIVA DE *Cedrela fissilis* (MELIACEAE) DA MATA ATLÂNTICA BRASILEIRA DURANTE AS MUDANÇAS CLIMÁTICAS DO QUATERNÁRIO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para a obtenção do título de *Doctor Scientiae.*

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Marcia Flores da Silva Ferreira

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Luiz Orlando de Oliveira
(Orientador)

Aos meus pais, José Carlos e Maria Julia,

Aos irmãos, Igor e Vítor,

Aos sobrinhos, Maria Luisa e Luiz Gustavo

Ao namorado, Wendel

Dedico!

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BIOGRAFIA

ÉRICA MANGARAVITE, filha de José Carlos Soares Mangaravite e Maria Julia de Medeiros Mangaravite, nasceu na cidade de Cachoeiro de Itapemirim, Espírito Santo (ES), em 13 de outubro de 1987.

Concluiu o Ensino Fundamental em 2001, na Escola Estadual de Ensino Fundamental e Médio "Waldemiro Hemerly" em Rio Novo do Sul-ES e o ensino médio em 2004, na Escola de Ensino Médio "Guimarães Rosa", em Cachoeiro de Itapemirim-ES.

Graduou-se na modalidade bacharelado no Curso de Ciências Biológicas em fevereiro de 2011, pela Universidade Federal do Espírito Santo, em Alegre, Espírito Santo.

Obteve o título de mestre em setembro de 2012, no Programa de Pós-graduação em Genética e Melhoramento, área de concentração Genética Vegetal, na Universidade Federal de Viçosa, em Viçosa, Minas Gerais.

Em Outubro de 2012 iniciou o Doutorado no Programa de Pós-graduação em Genética e Melhoramento, área de concentração Genética Vegetal, na Universidade Federal de Viçosa, em Viçosa, Minas Gerais.

No período de abril de 2015 à março de 2016 realizou parte do doutorado pela *Universität Leipzig*, em Leipzig, na Alemanha. Também morou em Frankfurt am Main quando esteve alocada no *Biodiversität und Klima Forschungszentrum* (BiK-F).

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RESUMO

MANGARAVITE, Érica. D.Sc., Universidade Federal de Viçosa, setembro de 2016. **A complexa história evolutiva de *Cedrela fissilis* (meliaceae) da Mata Atlântica brasileira durante as mudanças climáticas do quaternário.** Orientador: Luiz Orlando de Oliveira.

A floresta Atlântica brasileira, uma das regiões de maior biodiversidade no mundo, está entre as cinco áreas mais importantes, *hotspot* de biodiversidade, possuindo mais de 8.000 espécies endêmicas. Entretanto, ela é uma das florestas mais ameaçadas do mundo. Estudos vêm sendo conduzidos com o objetivo de tentar entender as razões que levaram a este grande acúmulo de biodiversidade. Nós estudamos o modelo de distribuição de espécies, diversidade genética e filogeografia em populações de *Cedrela fissilis* da floresta Atlântica, com o intuito de responder se populações estariam em refúgios secos ou em florestas úmidas durante o último máximo glacial (LGM). Nossos resultados mostraram suporte para ambas as hipóteses, sugerindo assim que provavelmente uma combinação de processos atuaram no espaço e no tempo formando a diversidade que existe atualmente. Além disso, Estudos moleculares necessitam de DNA de qualidade e, em plantas, as folhas não estão sempre disponíveis. Dessa forma, nós testamos uma completa metodologia de extração de tecido do câmbio em diferentes espécies (*Anadenanthera peregrina* var. *peregrina*, *Cedrela fissilis*, *Ceiba speciosa* e *Dimorphandra wilsonii*) a fim de obter um DNA adequado para amplificação. O protocolo utilizado aqui, desde a coleta até a extração, foi efetivo para a obtenção de produtos de PCR para sequenciamento e genotipagem.

ABSTRACT

MANGARAVITE, Érica. D.Sc., Universidade Federal de Viçosa, September, 2016. **The complex history of *Cedrela fissilis* (Meliaceae) from Brazilian Atlantic forest during Quaternary climate changes.** Adviser: Luiz Orlando de Oliveira.

The Brazilian Atlantic forest, one of the most biodiverse regions in the world, is among the five most important areas, biodiversity hotspot, harbouring more than 8,000 endemic species. However, it exhibits the most threatened forests in the world. Studies have been conducted in order to understand the reasons that led to this high accumulation of biodiversity. We evaluated species distribution model, genetic diversity and phylogeography of populations of *Cedrela fissilis* from Atlantic range, in order to answer whether populations were in dry refugia or in moist forests during the last glacial maximum (LGM). Our results showed support for both hypotheses, suggesting that likely a mixture of processes have acted through space and time. In addition to this study, molecular studies require DNA isolated, and in plants the leaves are not always available. Thus, we also tested a complete methodology of DNA extraction from cambium tissue in different species (*Anadenanthera peregrina* var. *Peregrina*, *Cedrela fissilis*, *Ceiba speciosa* and *Dimorphandra wilsonii*) in order to obtain a suitable DNA for amplification. The protocol used here, showed from the collected steps until extraction, was effective for obtaining PCR products for sequencing and genotyping.

INTRODUÇÃO GERAL

A floresta Atlântica brasileira (Mata Atlântica) é uma das regiões de maior biodiversidade no mundo. Esta floresta está entre as cinco áreas mais importantes, *hotspot* de biodiversidade, possuindo mais de 8.000 espécies endêmicas (Myers et al., 2000) (Figura 1). Entretanto, ela possui as florestas mais ameaçadas do mundo, com cerca de 11,4 - 16% da área total de suas florestas ainda remanescentes (Tabarelli et al., 2005; Ribeiro et al., 2009). Estudos vêm sendo conduzidos com o objetivo de tentar entender as razões que levaram a este grande acúmulo de biodiversidade e sugerem que o isolamento em refúgios florestais seja um mecanismo importante de promoção da diversificação nas florestas Atlânticas brasileiras (Carnaval e Moritz, 2008; Carnaval et al., 2009; Amaral et al., 2013; Álvarez-Presas et al., 2014).

Figura 1. Distribuição geográfica de um dos 10 biomas mais ameaçados no mundo: a Mata Atlântica, com predominância de ocorrência no Brasil. Fonte: http://www.cepf.net/where_we_work/regions/south_america/atlantic_forest/Pages/default.aspx

Refúgios são grandes fragmentos florestais que permitem que as espécies persistam durante ocasiões de grandes alterações climáticas, como um ciclo glacial ou interglacial, por exemplo (Ashcroft, 2010; Stewart et al., 2010). Essas regiões são muito importantes para conservação, pois preservam a variação genética que pode recolonizar áreas adjacentes, apresentando grande influência na trajetória sucessional e na capacidade dos ecossistemas serem re-estabelecidos. Essa recolonização é relatada tanto em animais (Chester e Robson, 2011; Parkos et al., 2011; Robinson et al., 2013), quanto em plantas (Dimitrov et al., 2012; Zhao et al., 2013).

Regiões de maior estabilidade climática podem ser refúgios (Carnaval e Moritz, 2008; Carnaval et al., 2009); áreas de altitude elevada, por exemplo, podem agir como um refúgio histórico (Dimitrov et al., 2012; Devitt et al., 2013) e apresentam grande relevância em estudos ecológicos e evolutivos (Körner, 2007). Essas regiões de refúgio, entretanto, não estão protegidas nos últimos anos. Regiões de altitude elevada encontram-se amplamente ameaçadas no mundo (Doumenge et al., 1993; Aldrich et al., 1997; Bruijnzeel et al., 2010; Pompeu et al., 2014), ocupando uma área estimada de 215.000 km², o que corresponde apenas a 1,4% de todas as florestas topicais e 6,6% de todas as florestas montanas tropicais (Scatena et al., 2010). Dentre as principais ameaças Bruijnzeel e Hamilton (2000) destacaram: a conversão para pastagens e para culturas cultivadas, o corte de lenha, colheita de produtos não madeireiros e de caça; introdução de espécies exóticas; qualidade do ar e mudanças climáticas, fogo, entre outras.

Florestas montanas

Visto que as florestas de altitude apresentam diversas denominações, no Simpósio Internacional de Floresta Montana Tropical (*Tropical Mountain Cloud Forest* – TMCF), realizado em San Juan, Puerto Rico, 1993, foram escolhidos alguns termos para padronizar as suas designações, dentre eles: floresta montana, floresta nebular, floresta de altitude, mata de neblina, entre outros (Hamilton et al., 1995). Florestas montanas são definidas como um tipo de floresta ombrófila encontradas em áreas montanhosas tropicais, nas quais a interação das condições climáticas com a vegetação levam à formação de nuvens e névoas (Hamilton et al., 1995). Essa definição reconhece a influência dos níveis de temperatura e umidade no zoneamento dessas florestas

(Scatena et al., 2010). Além disso, elas são compostas de ecossistemas florestais de florística distinta, com endemismo frequentemente elevado (Hamilton et al., 1995). A elevada riqueza e diversidade em florestas montanas devem-se principalmente à história climática e geológica associada à evolução; à dispersão das espécies no tempo; e às perturbações ambientais sobre a adaptação das espécies (Martinelli, 2007). Em 2004, ocorreu em Waimea, Hawai, o Simpósio "Ciência para Conservação e Manejo de Floresta Montana Tropical". Assim como em 1993, este simpósio também reuniu novas iniciativas e colaborações, que resultaram em uma agenda com planos para conservação destas florestas (Bubb et al., 2004).

As TMCF ocorrem em escala global dentro de uma ampla variação de altitude. Entre 1.200-1.500 m são denominadas de baixo-montana (*Lower Montane Cloud Forest* – LMCF), apresentando a altura do dossel médio de 35 m, com árvores emergentes até 45 m, a temperatura é cerca de 18ºC e a cobertura de briófitas nos troncos de 25-50%. A transição da LMCF para alto-montana (*Upper Montane Cloud Forest* - UMCF) é regida principalmente pelo nível de condensação de nuvens persistentes, pois nas LMCF a incidência de névoa é insignificante, enquanto nas UMCF a névoa é moderada. Nas UMCF, altitude entre 2.000-3.000 m, observa-se uma redução no dossel, para 2-20 m, e a cobertura de musgos fica em torno de 70-80%. Em grandes montanhas equatoriais, a transição de UMCF para a floresta subalpine (*Subalpine Cloud Forest* - SCF) é observada geralmente em altitudes entre 2.800 e 3.200 m. As SCF são encontradas apenas nas montanhas de maior elevação, chegam a 3.900 m de altitude, sendo a maioria na América Latina e na Nova Guiné. Além disso, essas florestas não são caracterizadas apenas pela suas baixas estaturas (até 9 m) e temperaturas (<10ºC), mas também pelas folhas ainda menores, e ausência de epífitas (Bruijnzeel e Hamilton, 2000; Bruijnzeel e Scatena, 2011).

No continente sul-americano a feição orográfica mais importante da borda do atlântico é formada pelas montanhas das Serras do Mar e da Mantiqueira (Almeida e Carneiro, 1998) (Figura 2), as quais influenciam fortemente o relevo e as condições climáticas das regiões Sul e Sudeste do Brasil. A Serra da Mantiqueira está localizada na Região Sudeste, encontrada entre os biomas Mata Atlântica e Cerrado, e se estende pelos estados de São Paulo, Minas Gerais, Espírito Santo e Rio de Janeiro. Sua maior porção está no estado de Minas Gerais e a menor no Espírito Santo, na Serra do Caparaó (Pelissari e Romaniuc Neto, 2013). As florestas montanas da Serra da Mantiqueira ocorrem tipicamente acima de 1.100 m (Pompeu et al., 2014). Trabalhos de fitofisionomia realizado nessas florestas têm mostrado formação diferenciada

(Bertoncello et al., 2011), elevada diversidade e riqueza de espécies arbóreas (Pompeu et al., 2014); e presença de espécies endêmicas, com distribuição restrita (Meireles et al., 2014).

Figura 2. Florestas montanas na América do Sul. Na região Atlântica a principal feição orográfica está localizada nas Serras do Mar e da Mantiqueira (Fonte: *Tropical Mountain Cloud Forest Initiative*).

Uma das Unidades de Conservação situadas na Serra da Mantiqueira é o Parque Nacional do Itatiaia (PNI) e abrange parte dos estados do Rio de Janeiro e Minas Gerais. O seu relevo é caracterizado por montanhas e elevações rochosas, com altitude variando de 600 a 2.791 m, no seu ponto culminante, o Pico das Agulhas Negras. A área do PNI abrange nascentes de 12 importantes bacias hidrográficas regionais (PARNA Itatiaia, 2014). As montanhas do Itatiaia estão entre as mais altas elevações da Serra da Mantiqueira (Almeida e Carneiro, 1998). Mais ao norte do PNI,

em uma ramificação da Serra da Mantiqueira, encontra-se outra Unidade de Conservação, o Parque Nacional do Caparaó (PNC), com áreas florestais de formação secundária e altitude variando de 800 a 2892 metros, no seu ponto culminante, o Pico da Bandeira, que é a terceira maior montanha do Brasil. Esta Unidade de Conservação está localizada entre os estados de Minas Gerais e Espírito Santo, na Serra do Caparaó que está interligada com as Serras do Brigadeiro e do Pai Inácio em Minas Gerais (PARNA Caparaó, 2014). Na Serra do Brigadeiro encontra-se o Parque Estadual da Serra do Brigadeiro (PESB), que ocupa o extremo norte da Serra da Mantiqueira, no estado de Minas Gerais. É uma Unidade de Conservação predominantemente de Mata Atlântica. A Serra do Brigadeiro, situada mais a oeste da Serra do Caparaó, possui inúmeras nascentes, que contribuem de maneira significativa para a formação de duas importantes bacias hidrográficas do Estado: a do Rio Doce e a do Paraíba do Sul (IEF, 2015). O PESB apresenta relevo montanhoso com altitude média de 1.424 m, variando de 945 m no fundo dos vales mais baixos, a 1.985 m no Pico do Soares (Silva et al., 2007). O PESB apresenta alta similaridade florística em relação a outras florestas montanas de mesma tipologia (Soares et al., 2006). Outra região de elevada altitude, situada mais a leste da Serra do Caparaó, está situada no município de Venda Nova do Imigrante, região serrana do Estado do Espírito Santo. O relevo do município tem como predominância o tipo montanhoso e escarposo e a sede está a uma altitude de 730 m (Silva et al., 2011).

Na Serra do Mar, o relevo é formado por um conjunto de escarpas festonadas na costa do Atlântico, com cerca de 1.000 km de extensão, que se estende do Rio de Janeiro (Serra dos Órgãos) ao norte de Santa Catarina (Almeida e Carneiro, 1998) no bioma da Mata Atlântica. Semelhante à Serra da Mantiqueira, a Serra do Mar também apresenta florestas montanas com fitofisionomias diferenciadas (Tabarelli e Mantovani, 1998; Koehler et al., 2002; Mocochinski e Scheer, 2008; Bertoncello et al., 2011; Scheer et al., 2011). No limite norte da Serra do Mar, há o Parque Nacional da Serra dos Órgãos (PNSO) que é caracterizado por uma topografia acidentada e por grandes desníveis, possuindo altitudes que variam entre 80 a 2263 m, onde se encontra seu ponto culminante, a Pedra do Sino. O parque protege os mananciais que drenam para as duas principais bacias hidrográficas fluminenses, a do Paraíba do Sul e a da Baía de Guanabara (PARNA Serra dos Órgãos, 2014).

A espécie *Cedrela fissilis*

Popularmente conhecida como ‘cedro branco’ e ‘cedro rosa’, a espécie *Cedrela fissilis* é monoica, possuindo flores masculinas e femininas, sendo as flores masculinas com ovário bem desenvolvido, mas não funcional; e as flores femininas possuindo estames bem desenvolvidos, mas não funcionais; além disso, há abertura em diferentes momentos das flores masculinas e femininas (Pennington, 2010). As árvores são submetidas, portanto, a polinização cruzada (alógamas), sendo normalmente mediada por mariposas e abelhas. O cedro produz muitas sementes aladas, cuja dispersão é feita pelo vento e favorecida pelo porte das árvores que formam grandes copas e atingem até 45 m de altura e 1,5 m de diâmetro, facilitando a liberação das sementes do alto do dossel da floresta (Carvalho, 1994; Pennington, 2010).

Cedrela fissilis é uma das 17 espécies do gênero *Cedrela* que pertence a família Meliaceae. O gênero é monofilético e compreende árvores decíduas, com a maioria delas distribuídas de forma restrita em florestas sazonais nas Américas Central e do Sul (Muellner et al., 2010; Pennington e Muellner, 2010) (Figura 3). *Cedrela fissilis*, no entanto, é uma espécie de ampla distribuição que ocorre em florestas estacionais, florestas de galeria e em ecótones com o Cerrado (Carvalho, 1994; Muellner et al., 2010; Pennington, 2010). Considerando o grupo sucessional, *C. fissilis* é uma espécie que se comporta como secundária inicial ou secundária tardia, ocorrendo tanto na floresta primária, principalmente nas bordas da mata ou clareiras, quanto na floresta secundária (Carvalho, 1994). A espécie se encontra classificada na categoria “ameaçada” da Lista Vermelha de espécies ameaçadas de extinção da *International Union for Conservation of Nature* (IUCN, 2016); e, segundo o Livro Vermelho da Flora do Brasil, ela se encontra na categoria “vulnerável” (Martinelli e Moraes, 2013). Isso está relacionado ao alto valor da espécie no setor madeireiro, devido à boa qualidade da madeira e à grande perda do seu habitat.

Garcia et al. (2011) exploraram as relações filogenéticas e filogeográficas do gênero *Cedrela* utilizando sequências da região completa dos espaçadores internos transcritos (ITS) dos genes nucleares de RNA ribossomal 18S-26S e de três regiões cloroplastídicas (espaçadores trnT-trnL; espaçadores trnS-trnG; e genes psbB, psbT e psbN). Foi constatado que *C. fissilis* não forma um grupo monofilético e compreende sequências encontradas em indivíduos identificados como sendo *Cedrela odorata* e *Cedrela balansae*. A espécie *C. odorata* é formada por diferentes entidades genéticas

não bem delimitadas (Muellner et al., 2009), e a *C. odorata* presente neste grupo monofilético de *C. fissilis* tem ocorrência exclusiva no Brasil. Garcia et al. (2011) verificaram também que a espécie *C. fissilis* é formada por duas linhagens genealógicas distintas; a localização de cada uma destas linhagens é separada pelo Cerrado, sendo uma localizada a oeste e outra a leste deste domínio (Figura 4). Essas linhagens foram confirmadas utilizando 10 locos de marcadores microssatélites (Mangaravite et al., 2016). Em ambos os trabalhos as amostras foram coletadas em boa parte do Brasil e na planície oriental da Bolívia, constatando-se que as linhagens evoluíram em isolamento geográfico, isto é, em alopatria. A divergência entre as linhagens ocorreu no início do Plioceno e Mioceno tardio (Garcia et al., 2011) e o encontro delas mais recentemente na região central do Brasil, chegando ao nordeste (Mangaravite et al., 2016).

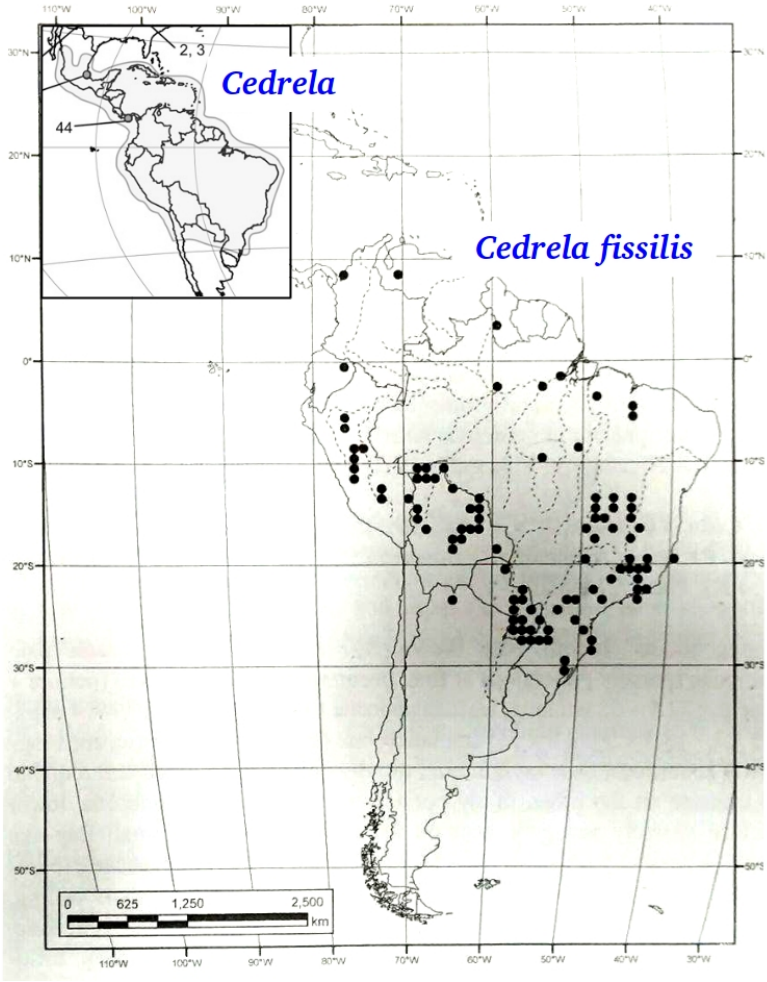

Figura 3. A distribuição moderna (novo mundo) do gênero *Cedrela* (topo da figura) (Fonte: Muellner et al., 2010, modificado). Distribuição da espécie *Cedrela fissilis* (Fonte: Pennington and Muellner, 2010).

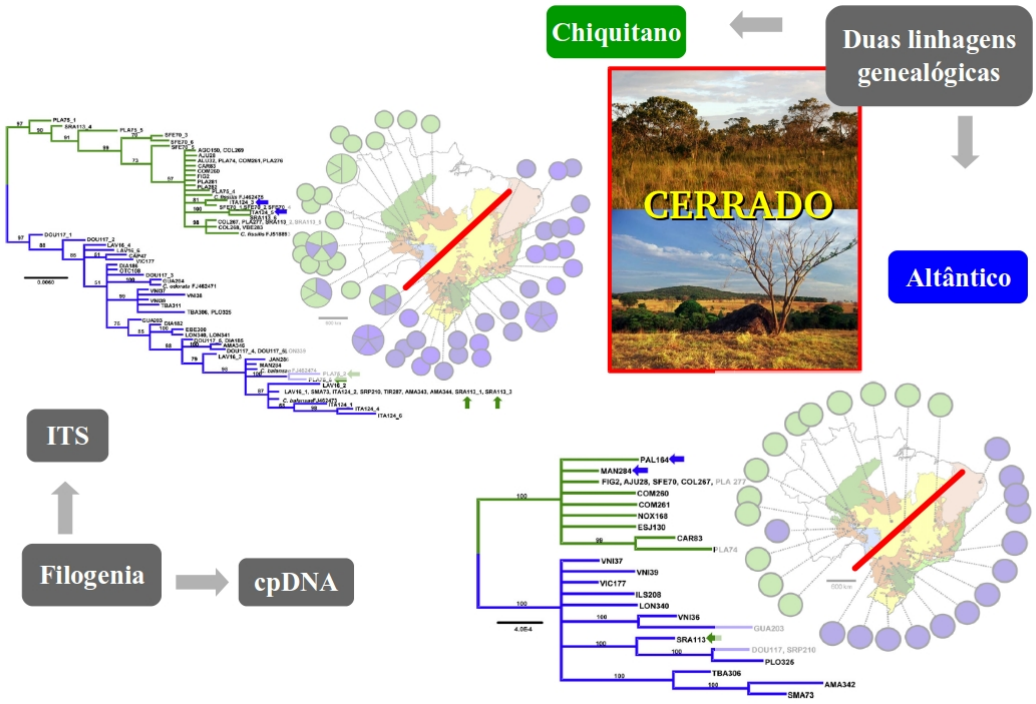

Figura 4. Representação esquemática das duas linhagens genealógicas situadas à leste (linhagem do Atlântico, em azul) e à oeste (linhagem do Chiquitano, em verde) do Cerrado, obtidas a partir de filogenia utilizando sequências de ITS (figura à esquerda) e de cpDNA (figura à direita) (Fonte: Garcia et al., 2011, modificada).

Dentro da linhagem do Atlântico, a espécie *C. fissilis* apresentou uma maior diferenciação genética de uma população localizada na Serra do Caparaó, em uma variação de altitude de 647 a 1.638 m acima do nível do mar, quando comparada com populações de baixa elevação (Mangaravite et al., 2016). Porém, não se conhece as causas dessa variação genética, tampouco como ocorre a diversidade em outras populações de *C. fissilis* de florestas montanas do Atlântico brasileiro. Estudos utilizando diferentes marcadores genéticos (microsatélites, AFLP, regiões nucleares e/ou cloroplastídicas) vêm sendo realizados para esclarecer a diversidade genética e a distribuição de populações em regiões de altitude. Utilizando essas ferramentas moleculares, foi demonstrado que diferentes regiões de elevada altitude apresentam fluxo gênico frequente e grande estruturação genética nas espécies *Castanopsis eyrei* (Shi et al., 2011); *Saintpaulia* sp. (Dimitrov et al., 2012); *Myrtus nivellei* (Migliore et al., 2013).

Ferramentas moleculares e modelagem climática

A filogeografia molecular trata dos princípios e processos que governam as distribuições geográficas das linhagens genealógicas, especialmente aquelas dentro e entre espécies muito próximas entre si, fornecendo informações sobre diversidade genética não somente na dimensão espacial, mas também na dimensão temporal (Avise, 2000). Ferramentas de filogeografia molecular têm sido mais frequentemente utilizadas em estudos de ecologia, conservação genética e biogeografia (e.g., Oliveira et al., 2010; Garcia et al., 2011; Leite et al., 2016; Melo et al., 2016). Com o surgimento da teoria coalescente (Hudson, 1983; Kingman, 1982a,b; Tajima, 1983), as análises de genética populacional mudaram de foco, não sendo mais de interesse predizer a frequência alélica de gerações futuras (Ewens, 1979), mas sim utilizando de amostra de alelos para modelar as genealogias de genes para trás no tempo sob praticamente qualquer história demográfica complexa, a fim de estimar os parâmetros filogeográficas como tamanhos populacionais, tempos de divergência, e as taxas de migração (Wakeley, 2008). Inicialmente, marcadores plastidiais foram promovidos como marcador molecular de escolha para estudos de filogeografia molecular devido à sua falta de recombinação, a neutralidade putativa, e tamanho efetivo da população pequena (Hickerson, et al., 2010).

Posteriormente, fez-se necessário a avaliação de populações com o uso de marcadores genético multilocos, e por isso os microssatélites tornaram-se ferramentas importantes, sendo amplamente utilizadas para estudos de filogeografia molecular. Microssatélites (Litt e Luty, 1989) são pequenas sequências, de um a seis pares de base, repetidas em tandem, que também são chamadas de SSR (*Simple Sequence Repeats*, Jacob et al., 1991) ou STR (*Short Tandem Repeats*, Edwards et al., 1991). Apresentam distribuição randômica e dispersa ($10^4 – 10^5$) no genoma (Tautz, 1993); são bastante abundantes em plantas (Delseny et al., 1983; Tautz; Renz, 1984), são multi-alélicos, com alto grau de polimorfismo de comprimento (Zane et al., 2002); são codominantes, a maioria seletivamente neutros (Charlesworth et al., 1994) e apresentam alta taxa de mutação (Schlötterer, 2000). Com isso, estes marcadores são altamente informativos e muito eficientes (ver Kalia et al. (2011) para detalhamentos deste tipo de marcador).

Associados às informações genéticas, dados paleoclimáticos vêm sendo utilizados para esclarecer a variação genética e a distribuição. Modelos de distribuição de espécies (SDMs, *Species Distribution Models*) combinam observações de ocorrência de espécies com estimativas ambientais, auxiliando na obtenção de novas informações

ecológicas e evolutivas (Elith e Leathwick, 2009). Os SDMs são aplicados em estudos de dinâmica de nichos (Pearman et al., 2007). O conservadorismo de nicho, tendência de um nicho de espécies manter-se inalterado ao longo do tempo (Pearman et al., 2007), ajuda a explicar a diversidade em padrões espaciais da distribuição das espécies (Wiens e Graham, 2005; Pearman et al., 2007; Antonelli e Sanmartín, 2011). Os SDMs contribuem para a detecção de mudanças de nicho que ocorrem em uma espécie ao longo do tempo ou entre populações separadas no espaço geográfico (Pearman et al., 2007). Para o gênero *Cedrela*, sabe-se que ele apresenta complexa dinâmica de nicho, sofrendo ações tanto do conservadorismo quanto da evolução de nicho em escalas temporais e climáticas (Koecke et al., 2013).

Refúgio seco *vs.* Florestas úmidas

Mudanças climáticas têm moldado a distribuição geográfica das espécies e podem afetar sua diversidade genética (Stewart et al, 2010;. Homburg et al, 2013; Ramírez-Barahona e Eguiarte, 2013). Flutuações climáticas podem levar tanto ao declínio da diversidade genética e, então à extinção de espécies ou, à diversificação dentro das espécies e potencialmente especiação (Hewitt, 2000). As temperaturas globais diminuíram ao longo do Cenozóico (há 65 milhões de anos;. Zachos et al, 2008), com oscilações aumentando em frequência e amplitude desde o Quaternário (há 2,4 milhões de anos; Hewitt, 2000) (Figura 5). O último máximo glacial (*Last Glacial Maximum,* LGM, há aproximadamente 21.000 anos) foi a maior manifestação da mudança climática natural que permanece relativamente bem preservada no registro geológico (Mix et al., 2001). Durante o LGM, lençóis de gelo atingiram a sua máxima extensão, tornando este período um tema importante para os estudos paleoclimáticos já que era dramaticamente diferente do clima de hoje (Mix et al., 2001), e também diferente do clima antes. Assim, LGM, provavelmente, teve um grande impacto sobre a distribuição geográfica e/ou diversidade genética das espécies (Silva et al., 2012; Ramírez-Barahona e Eguiarte, 2013).

Na América do Sul, os primeiros estudos sugeriram condições frias e secas na região amazônica durante o LGM, levando a fragmentos florestais que serviram de refúgio, isolados em vegetação abertas não-florestais (Haffer, 1969, Hammen e Hooghiemstra, 2000). No sul da floresta atlântica brasileira (FAB), os dados palinológicos mostram que pastagens dominava a paisagem durante o LGM onde existem ecossistemas florestais, indicando também um clima mais frio e seco do que

hoje (Behling e Lichte, 1997, Behling e Negrelle de 2001, Behling, 2002). Além disso, Carnaval e Moritz (2008) usaram modelos paleoclimáticos e previram refúgios florestais histórico no FAB. Eles descobriram que as áreas climaticamente mais estáveis estavam combinadas a centros atuais de endemismo em um subconjunto de cinco espécies de vertebrados. Além disso, estudos com rãs (Carnaval et al., 2009) e aves (Amaral et al, 2013) mostraram a possibilidade de uma área de refúgio seco no FAB que explicaria a alta biodiversidade desta área.

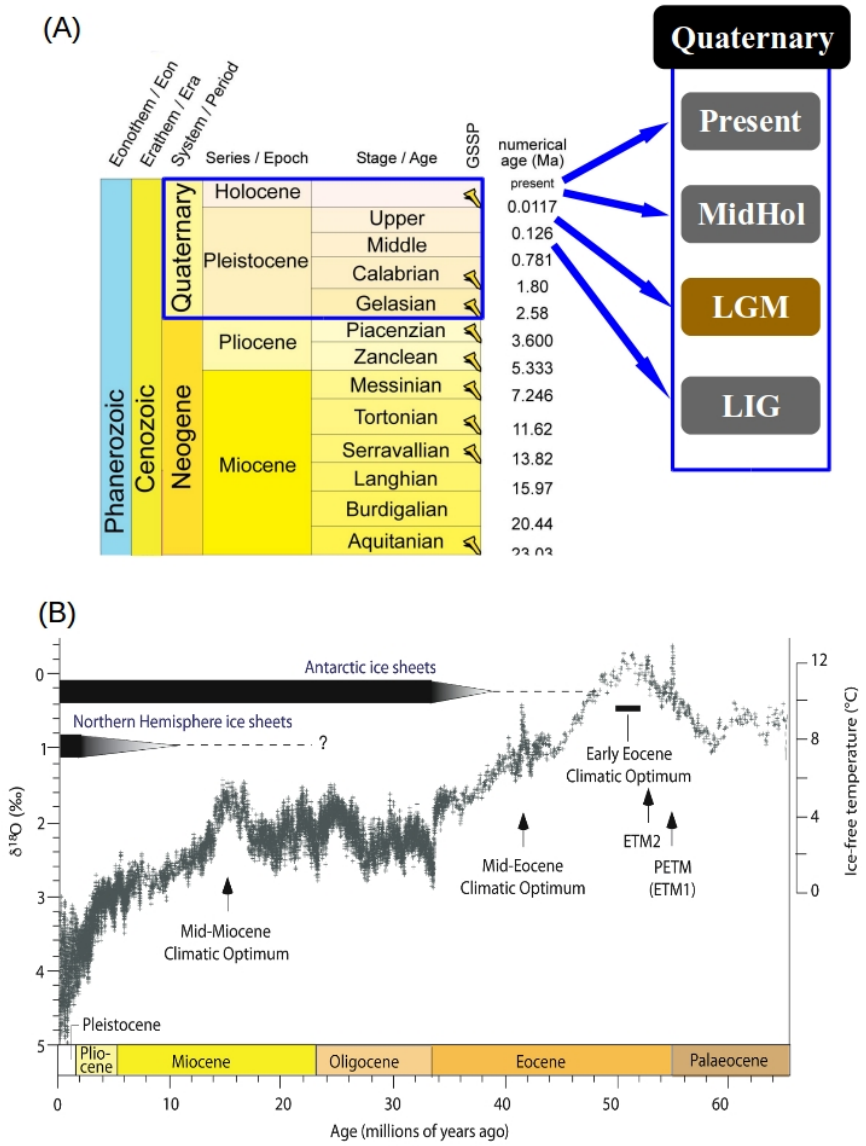

Figura 5. Quadro de cronoestatigrafia mostrando os períodos e as últimas épocas da Era Cenozoica (A). Os retângulos em azul delimitam o período do Quaternário e os estágios mais avaliados (Presente, MidHol, LGM e LIG, veja texto para descrição) (Fonte: www.stratigraphy.org 2014). Em (B) um gráfico de clima para o período do Cenozoico (65 milihões de anos antes do presente) é mostrado. Nele é possível observar que as temperaturas globais diminuíram ao longo do tempo e que nos últimos 2 milhões de anos as oscilações aumentaram em frequência e amplitude (Fonte: Zachos et al., 2008).

Ao contrário da hipótese refúgio seco (*Dry Refugia Hypotheses*, DRH), alguns dados de pólen não indicam uma substituição de floresta por vegetação seca na Amazônia, o que sugere que a cobertura florestal pode ter permanecido estável e não fragmentada (Colinvaux et al, 2000;. Rull, 2008). Leite et al. (2016) observaram utilizando cinco espécies de pequenos mamíferos, que uma expansão de condições climáticas adequadas na plataforma continental emergiu durante o LGM e sugeriu que refúgios florestais desempenharam apenas um papel menor, se algum, neste *hotspot* de biodiversidade durante os períodos glaciais. Além disso, um estudo sobre sapos endêmicos da FAB sugeriu a persistência de habitats florestais no sul (Thomé et al., 2010) em vez de uma colonização de refúgio do norte após a LGM (Carnaval e Moritz, 2008). Em adição a este cenário alternativo, as montanhas exibem um papel importante para manter a umidade. As características das florestas montanas influenciam diretamente a disponibilidade de umidade por uma entrada constante de grandes quantidades de água como precipitação horizontal (Foster, 2001). Isto sugere que, durante o LGM, montanhas poderiam ter sido um habitat úmido estável, em que as espécies teriam sobrevivido e linhagens foram geradas ou persistiram por mudanças de altitude (Hewitt, 2000).

Ramírez-Barahona e Eguiarte (2013) revisaram os registros paleoecológicas da região Neotropical e mostraram a existência de dois cenários de umidade durante o LGM para a floresta nebular: o DRH e as hipóteses de florestas húmidas (*Moist Forest Hypotheses*, MFH) (Figura 6). Eles destacaram como ambos os cenários podem levar a padrões distintos na diversidade genética atual. Sob o DRH, áreas de distribuição geográficas contraídas podem levar à fragmentação durante os ciclos glaciais. Como consequência, há uma perda da diversidade genética durante a contração. Posteriormente, populações com pequenos tamanhos populacionais efetivos voltam a colonizar áreas e expandir-se deixando uma marcada estrutura genética. Além disso, as populações podem mostrar diferenciação genética se elas originam-se de diferentes linhagens situadas em refúgio (Ramírez-Barahona e Eguiarte, 2013). Em contraste, sob a MFH, as populações se moveriam para médias ou baixas altitudes, onde estariam conectadas. Depois disso, ao voltar para as altitudes originais, apresentariam então pouca ou nenhuma expansão demográfica. A principal consequência desta ligação será o aumento da diversidade genética resultante da heterogeneidade e estruturação genética difusa das populações (ver Ramírez-Barahona e Eguiarte, 2013, para mais detalhes).

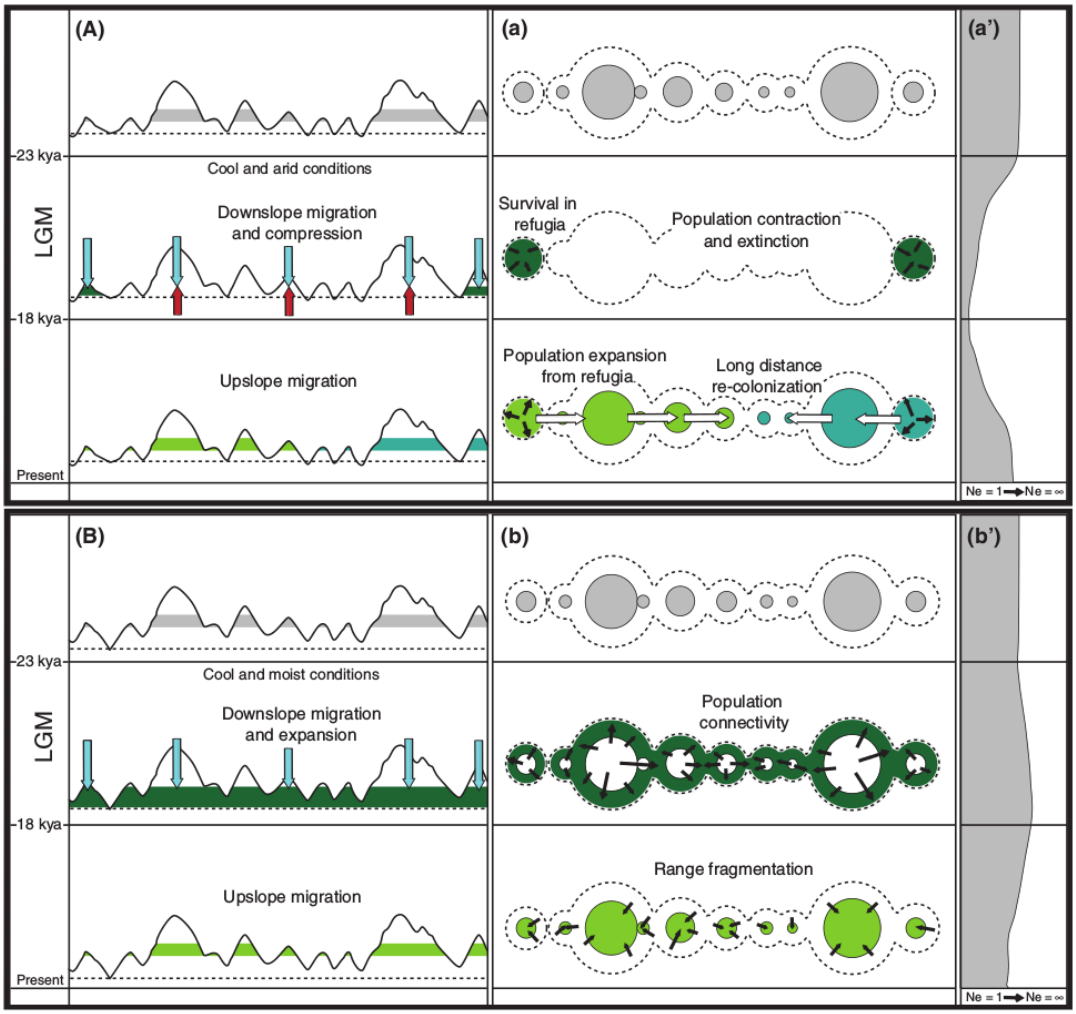

Figura 6. Modelo de distribuição e dinâmica demográfica de florestas montanas, para o LGM. Em (A) modelo de refúgio seco (DRH) e em (B) modelo de florestas húmidas (MFH). A figura é mostrada em corte transveral (A e B) e em vista aérea (a e b) das florestas montanas (Fonte: Ramírez-Barahona e Eguiarte, 2013).

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CAPÍTULO 1

The complex history of *Cedrela fissilis* (Meliaceae) from Brazilian Atlantic forest during Quaternary climate changes

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Abstract

In South America, the most important orographic feature of the Atlantic coast is formed by the mountains of the Serra do Mar and Serra da Mantiqueira that strongly influence the relief and climatic conditions in the South and Southeast of Brazil. Those mountains harbour part of the Brazilian Atlantic Forest (BAF) which is the fourth global biodiversity hotspots. Climatic changes shape the geographic distribution of species and may affect their genetic diversity. The Last Glacial Maximum (LGM) was the largest manifestation of natural climate change that remains well preserved in the geological record and reached their maximum extent of ice sheets, making this period an important topic for paleoclimatic studies. In South America there is a debate on glacial aridity, then not clear whether cloud forests were contracted or not into forest refugia during the LGM. Thus, to test this we used species distribution modeling, and evaluate the genetic diversity and phylogeography of populations of *Cedrela fissilis*. Our conclusions are that different genetic patterns of highland population and an intermediate level of genetic structure reflected a mixture of events acting over the Quaternary. Long-term dispersion and vicariance may have been the most important process that influenced the populations contraction and expansion during the LGM. Finally, the usage of plant species as biological model revels an interesting and complex pattern of diversification, and bring new insights of evolutionary in BAF.

Introduction

Mountain regions around the world are exceptionally rich centres of biodiversity and endemism (Kier et al., 2009). In South America, the most important orographic feature of the Atlantic coast is formed by the mountains of the Serra do Mar and Serra da Mantiqueira (Almeida and Carneiro, 1998) that strongly influence the relief and climatic conditions in the South and Southeast of Brazil. Studies carried out in these highlands have shown floristic similarities between them (Bertoncello et al, 2011; Meireles and Shepherd, 2015); high richness of tree species (Pompeu et al, 2014), and the presence of endemic species with restricted distributions (Meireles et al., 2014). Those mountains harbour part of the Brazilian Atlantic Forest (BAF) (Oliveira-Filho and Fontes, 2000). The BAF is the fourth global biodiversity hotspots due to its exceptional high number of endemic species (almost 8,600, among plant and vertebrate species) and to its high level of habitat loss (Myers et al., 2000). It is one of the most threatened regions in the world, with only about 11.4 - 16% of the total forest area remaining (Tabarelli et al, 2005; Ribeiro et al., 2009).

Climatic changes shape the geographic distribution of species and may affect their genetic diversity (Stewart et al, 2010; Homburg et al., 2013; Ramírez-Barahona and Eguiarte, 2013). Over time, the climate fluctuations will lead either to a decline of genetic diversity and ultimately species extinction or to diversification within species and potentially speciation (Hewitt, 2000). Global temperatures decreased throughout the Cenozoic (65 million of years ago; Zachos et al., 2008), with oscillations increasing in frequency and amplitude since the Quaternary (2.4 million of years ago, Hewitt, 2000). The Last Glacial Maximum (LGM, about 21,000 years ago) was the largest manifestation of natural climate change that remains relatively well preserved in the geological record (Mix et al., 2001). During the LGM, ice sheets reached their maximum extent, making this period an important topic for paleoclimatic studies since it was dramatically different from the climate of today (Mix et al., 2001), and also different from the climate before. Thus, the LGM will likely have had a major impact on the geographic distribution and/or genetic diversity of species (Silva et al., 2012; Ramírez-Barahona and Eguiarte, 2013).

In South America, early studies suggested cold and dry conditions in the Amazon region during the LGM, leading to forest fragments that served as refugia, isolated by open non-forest vegetation (Haffer, 1969, Hammen and Hooghiemstra,

2000). In the south of the BAF, palynological data show that grasslands dominated the landscape during the LGM where forest ecosystems exist today, also indicating a colder and drier climate than today (Behling and Lichte, 1997, Behling and Negrelle, 2001, Behling, 2002). In addition, Carnaval and Moritz (2008) used palaeoclimatic models and predicted historical forest refugia in the BAF. They found that climatically more stable areas matched the current centres of endemism in a subset of five vertebrate species. Further, studies with frogs (Carnaval et al., 2009) and birds species (Amaral et al, 2013) have shown the possibility of a dry refuge area in the BAF that would explain the high biodiversity of this area.

Contrary to the dry refuge hypothesis (DRH), some pollen data do not indicate a replacement of forest by grassland vegetation in Amazonia, suggesting that forest cover may have remained stable and not fragmented (Colinvaux et al., 2000; Rull, 2008). Leite et al. (2016) observed an expansion of suitable climatic conditions onto the emerged continental shelf during the LGM for five species of small mammals, and suggested that forest refuges played only a minor role, if any, in this biodiversity hotspot during glacial periods. Also, a study on toads endemic to the BAF suggested the persistence of forested habitats in the south (Thomé et al., 2010) instead of a colonization from northern refugia after the LGM (Carnaval and Moritz, 2008). In addition to this alternative scenario, the mountains exhibit an important role to keep the humidity. The cloud forests are characterized by persistent cloud immersion, which directly influences the moisture availability by a constant input of large amounts of water as horizontal precipitation (Foster, 2001). This suggests that, during the LGM, mountains could have been a stable moist habitat, in which species may have survived and lineages have been generated or persisted by altitudinal shifts (Hewitt, 2000).

Ramírez-Barahona and Eguiarte (2013) reviewed paleoecological records from the Neotropics and showed the existence of two humidity scenarios during the LGM for the cloud forest: the DRH and the moist forest hypotheses (MFH). They highlighted how both scenarios should lead to distinct patterns in genetic diversity today. Under the DRH, geographic ranges contract and become fragmented during the glacial cycles. As a consequence, there is a loss of genetic diversity during the contraction. Afterwards, the populations will re-colonize areas and expand from small effective population sizes leaving a marked genetic structure. Additionally, populations may show genetic differentiation if they come from different refuge lineages (Ramírez-Barahona and Eguiarte, 2013). In contrast, under the MFH, the populations will move downslope to mid or low elevations, where they remain connected. Afterward, they move upslope and

experience range fragmentation and little to no demographic expansion. The main consequence of this connection will be the increase of genetic diversity resulting from spatial heterogeneity and diffuse genetic structuring of populations (see Ramírez-Barahona and Eguiarte, 2013, for more details).

Given the debate on glacial aridity, it remains unsubstantiated whether cloud forests were contracted or not into forest refugia during the LGM (Ramírez-Barahona and Eguiarte, 2013). In the BAF's mountains, paleoecological data and phylogeography studies have shown agreement with the DRH (Behling, 2002), while others have shown disagreement with it (Thomé et al., 2010; Amaro et al., 2012). Also, there is a lack of studies that discuss biota evolution using plant species as biological models in South America (Turchetto-Zolet et al, 2013). Thus, to test if the Brazilian Atlantic cloud forests were in DRH or in MFH during the LGM, we have used species distribution modeling, and evaluated the genetic diversity and phylogeography of populations of *Cedrela fissilis* Vell. (Meliaceae). Commonly called in Brazil as "cedro branco" or "cedro rosa" (Carvalho, 1994), *C. fissilis* is a deciduous tree that belongs to an arboreal genus with 17 hardwood species (Pennington and Muellner, 2010). It is valued for the high quality of its wood, and it is listed in the IUCN Red List of endangered species (IUCN, 2016). *Cedrela fissilis* Vell. (Meliaceae) is widely distributed occurring throughout most of the areas where the genus is found such as seasonal forests, ecotones that are adjacent to seasonal forests and to Cerrado, the Brazilian Savanna; and gallery forests (Carvalho, 1994; Pennington et al., 1981, Pennington and Muellner, 2010). Garcia et al. (2011) explored phylogenetic relationships and the phylogeography of *Cedrela* in Brazil and the eastern part of Bolivia. They found that *C. fissilis* consists of two distinct genealogical lineages: one located in the Chiquitano range and the other one in the Atlantic range separated by the Cerrado. The divergence between lineages might have occurred during the Late Miocene to Early Pliocene (Garcia et al., 2011). These patterns were confirmed using ten microsatellite markers by Mangaravite et al. (2016). Also, the specimens that belong to the Atlantic range occur in a wide range of altitude, from 20m to more than 1600m above sea level (Mangaravite et al., 2016). The trees have trunks up to 1.5 m in diameter and form large canopies that can reach up to 45 m high. They undergo cross-pollination mediated by moths and bees, and produce wind-dispersed seeds (Carvalho, 1994; Pennington and Muellner, 2010).

Thus, we will address the following questions: (1) Are the populations from highland genetically similar between them? Do they display a marked or a diffuse genetic structure? (2) Which evolution process most explain the genetic diversity,

structure and gene genealogies of populations of *C. fissilis* from BAF? (3) Did the populations of *C. fissilis* contract or expand in BAF during the LGM? (4) How do these results contribute to explain the diversity of the BAF?

Material and methods

Sampling strategy

We evaluated 410 specimens from 50 localities of *C. fissilis* throughout the Atlantic range. For the population genetic analysis, we genotyped 344 specimens from 21 populations, while for the phylogeographic analysis we investigated 148 specimens from all 50 localities, comprising both new collections and published data (Garcia et al., 2011; Mangaravite et al., 2016). The population codes, localities, coordinates, number of specimen and the references are shown in Table 1 and Table 2. The populations with the terrestrial ecoregions (Olson, et al., 2001) and the elevation in the region are shown in Figure 1. The populations MPA, PEU, VNI, CAP, PSB, PSO and PNI occur in elevations higher than 600 m and they were used hereafter as highland populations.

Table 1. Populations of *C. fissilis* and genetic diversity over 11 microsatellite loci. Codes: *n*, number of samples evaluated; A_{priv}, number of private alleles; *A/locus*, mean number of alleles per locus; H_E, expected heterozygosity; H_O, observed heterozygosity; and *F*, inbreeding coefficient.

Locality (population code)	*n*	A_{priv}	A_{priv}/n	*A/locus*	H_E	H_O	*F*	Reference
Tobias Barreto and Poço Verde, SE (SER)	10	0	0	6.09	0.70	0.61	0.13	Mangaravite et al. (2016)
Januária, MG (JAN)	9	1	0.11	6.20	0.82	0.77	0.09	Mangaravite et al. (2016)
Mata do Passarinho, Bandeira, MG (MPA)	8*							Present study
Uberlândia, MG (UBE)	19	7	0.37	11.64	0.86	0.75	0.14	Present study
Diamantina, MG (DIA)	13	3	0.23	10.18	0.86	0.73	0.16	Mangaravite et al. (2016)
Conceição da Barra, ES (CON)	**3***							Present study
Sooretama, ES (SOO)	**7***							Present study
Rio Doce State Park, MG (PRD)	3*							Present study
Uaimií State Park, MG (PEU)	17	4	0.24	11.18	0.84	0.75	0.11	Present study
Venda Nova do Imigrande, ES (VNI)	37	4	0.11	12.64	0.80	0.68	0.15	Present study
Caparaó Nacional Park, MG, (CAP)	35	8	0.23	13.55	0.82	0.73	0.11	Mangaravite et al. (2016)
Serra do Brigadeiro State Park, MG (PSB)	10	2	0.20	8.64	0.83	0.64	0.24	Present study
Aguapei river mouth, SP (FR2)	5*							Present study
Itaporã, MS (ITA)	9	3	0.33	7.55	0.83	0.72	0.14	Mangaravite et al. (2016)
Serra dos Órgãos Nacional Park, RJ (PSO)	25	9	0.36	11.91	0.87	0.74	0.16	Present study
Itatiaia Nacional Park, RJ (PNI)	61	8	0.13	16.27	0.86	0.71	0.17	Present study
Três Barras do Paraná, PR (TBA)	13	3	0.23	9.64	0.88	0.79	0.10	Mangaravite et al. (2016)
Palotina, PR (PLO)	28	6	0.21	13.27	0.86	0.72	0.16	Mangaravite et al. (2016)
Campinhos, PR (CAM)	7*							Mangaravite et al. (2016)
Blumenau, SC (BLU)	18	3	0.17	11.09	0.82	0.72	0.11	Mangaravite et al. (2016)
Santa Maria, MS (SMA)	7*							Mangaravite et al. (2016)
Overall sum	304**	61**	0.20					
Overall mean	21.71**	4.35**		10.70**	0.83**	0.72**	0.14**	

Note: *, populations with n < 9. **, values which do not include data from populations with n<9.

Table 2. Populations of *Cedrela fissilis* sampled for analyses of phylogeography; their Latitude and Longitude; their number of samples (*n*), and the genebank accesion number of the samples evaluated in previous study.

Locality (population code)	Latitude/ Longitude	*n*	GenBank accession
Qeixada, CE (CEA)	-4.968º/-39.017º	3	Present study
Guarabira, PB (GUA)	-6.8340º/-35.490º	1	JF922136.1
Poço Verde, SE (POV)	-10.711º/-38.185º	3	Present study
Tobias Barreto, SE (TOB)	-11.173º/-38.013º	3	Present study
Manga, MG (MAN)	-14.791º/-43.939º	1	JF922134.1
Januária, MG (JAN)	-15.189º/-44.206º	1	Present study
Mata do Passarinho, Bandeira, MG (MPA)	-15.817º/-40.544º	2	Present study
Catalão, GO (CAT)	-18.196º/-47.956º	1	Present study
Uberlândia, MG (UBE)	-18.979º/-48.037º	2	Present study
Diamantina, MG (DIA)	-18.409º/-43.490º	5	Present study
Flona do Rio Preto, Conceição da Barra, ES (CON)	-18.359º/-39.849º	1	Present study
Sooretama, ES (SOO)	-19.052º/-40.156º	3	Present study
Rio Doce State Park, MG (PRD)	-19.713º/-42.732º	2	Present study
Ouroeste, SP (OUR)	-20.011º/-50.363º	1	Present study
Uaimií State Park, MG (PEU)	-20.249º/-43.571º	1	Present study
Venda Nova do Imigrande, ES (VNI)	-20.312º/-41.133º	11	JF922130.1; JF922131.1, JF922132.1 and Present study
Ouro Preto, MG (ORP)	-20.389º/-43.507º	1	Present study
Ouro Branco, MG (ORB)	-20.505º/-43.713º	2	Present study
Caparaó Nacional Park, MG, (CAP)	-20.529º/-41.920º	5	Present study
Serra do Brigadeiro State Park, MG (PSB)	-20.684º/-42.445º	8	Present study
Viçosa, MG (VIC)	-20.773ª/-42.874º	1	JF922135.1
São José do Rio Preto, SP (SRP)	-20.785º/-49.320º	1	Present study
Cedral, SP (CED)	-20.907º/-49.284º	1	Present study
Tiradentes, MG (TIR)	-21.106º/-44.179º	1	Present study
Aguapei river mouth, SP (FR2)	-21.124º/-51.741º	2	Present study

Ijac, MG (IJA)	-21.163º/-44.923º	3	Present study
Lavras, MG (LAV)	-21.250º/-44.989º	3	Present study
Cecalpina, MS (CEC)	-21.257º/-51.973º	1	Present study
Itápolis, SP (IPO)	-21.582º/-48.830º	2	JF922137.1, JF922138.1
Itaporã, MS (ITA)	-22.002º/-54.714º	11	Present study
Trajano de Morais, RJ (TRA)	-22.087º/-42.062º	3	Present study
Dourados, MS (DOU)	-22.199º/-54.817º	2	JF922144.1 and present study
Serra dos Órgãos Nacional Park, RJ (PSO)	-22.438º/-43.137º	8	Present study
Itatiaia Nacional Park, RJ (PNI)	-22.447º/-44.611º	5	Present study
Amaporã, PR (AMA)	-23.086º/-52.796º	1	JF922139.1
Londrina, PR (LON)	-23.162º/-51.165º	1	JF922140.1
Itu, SP (ITU)	-23.266º/-47.299º	1	Present study
BR – Umuarama, PR (BRP)	-23.652º/-53.370º	5	Present study
Engenheiro Beltrão, PR (EBE)	-23.805º/-52.262º	5	Present study
Campo Mourão, PR (CMO)	-24.038º/-52.409º	4	Present study
Três Barras do Paraná, PR (TBA)	-24.309º/-53.127º	1	JF922142.1
Palotina, PR (PLO)	-24.309º/-53.909º	1	JF922141.1
Porto Camargo – Icaraíma, PR (PCA)	-24.401º/-53.632º	4	Present study
São Pedro do Iguaçu, PR (SPI)	-24.933º/-53.848º	4	Present study
Campinhos, PR (CAM)	-25.035º/-49.090º	3	Present study
Blumenau, SC (BLU)	-26.918º/-49.066º	7	Present study
Concórdia, SC (COC)	-27.239º/-52.011º	4	Present study
Otacílio Costa, SC (OTC)	-27.477º/-50.120º	5	Present study
Pinhal da Serra, RS (PIS)	-27.874º/-51.063º	1	Present study
Santa Maria, RS (SMA)	-29.717º/-53.724º	1	JF922143.1
	Total	148	

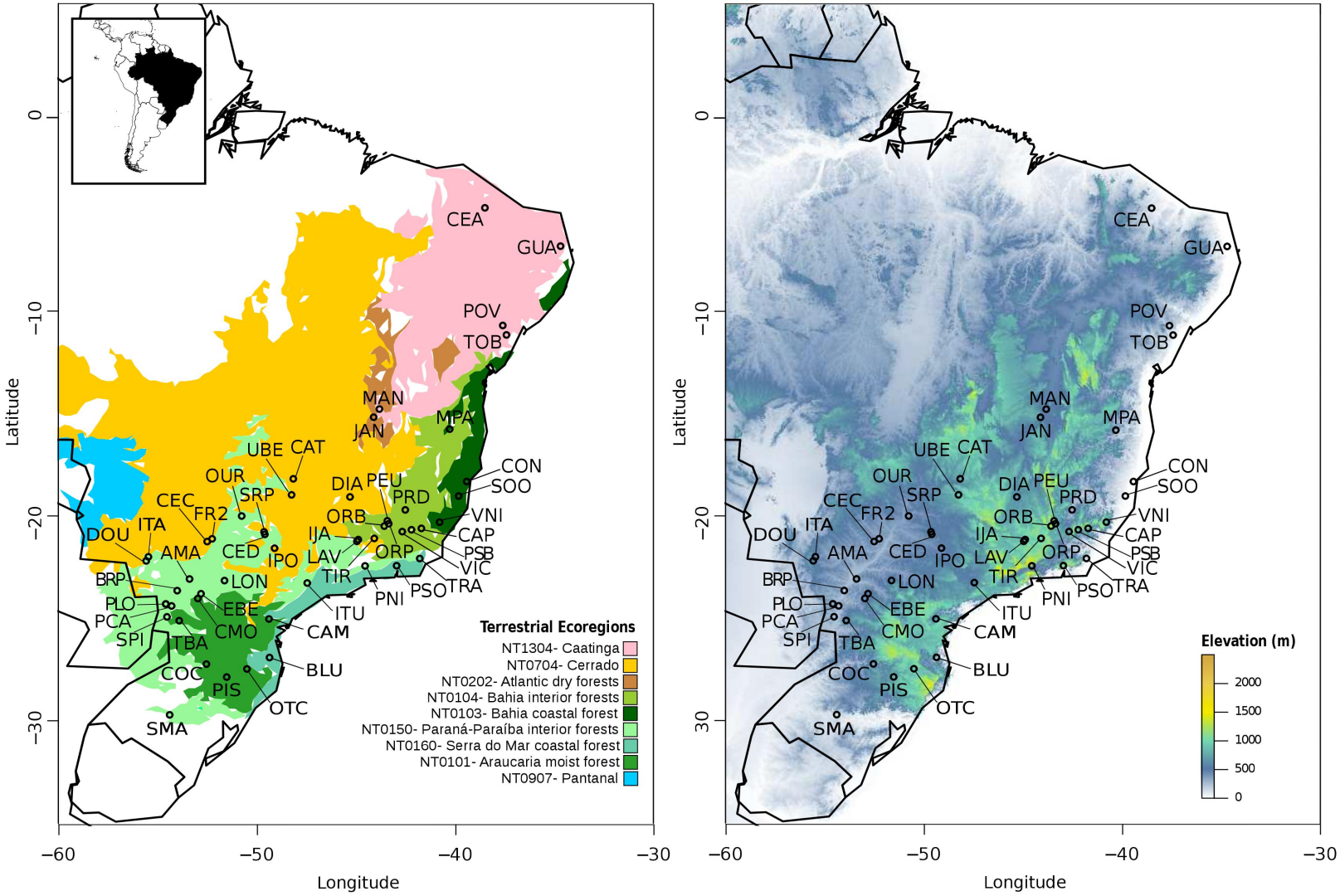

Figure 1. Map with the locations of sampled populations of *C. fissilis* in eastern South America with the terrestrial ecoregions (left) and the elevation (right) associated with them. (Refer to Table 2 for population codes).

Specimens and habitat distribution modelling

Georeferenced occurrence localities of *C. fissilis* were gathered in the field and from published data (Garcia et al., 2011, Mangaravite et al., 2016). In total, we used 50 occurrences to model habitat suitability. To alleviate the impact of spatial autocorrelation, we only included records with a minimum pairwise distance of 10 km. The models were built using the maximum entropy algorithm as implemented in MAXENT (Phillips et al., 2006). In particular, we used a 10-fold cross validation approach with 80% of the occurrences for training and 20% for testing. To assess model performance, we used the area under the ROC (receiver operating characteristic) curve (AUC, Hanley and McNeil, 1982). The AUC determines the model's predictive ability compared to a random prediction. In addition, models were validated by assessing the sensitivity, specificity, and accuracy values averaged across the replicates. To estimate habitat suitability, we used bioclimatic variables from WorldClim (Hijmans et al., 2005) at 2.5-min resolution as explanatory variables. Among the 19 bioclimatic variables (Table S1), we selected those with a Pearson-correlation pairwises of less than 0.71 (Table S2) and as well as high contribution and permutation importance in the models (Table S3). We retained a subset of seven predictors (bio02, Mean Diurnal Range; bio04, Temperature Seasonality; bio05, Max Temperature of Warmest Month; bio08, Mean Temperature of Wettest Quarter; bio16, Precipitation of Wettest Quarter; bio18, Precipitation of Warmest Quarter; and bio19, Precipitation of Coldest Quarter).

For modelling we used the *group discrimination* technique, that requires the absence data (background); and has been more reliable than the *profile* technique (Mateo et al., 2010), which requires presence-only data. However, confirming the absence is convolute, subjective and expensive. Mateo et al. (2010) showed that the 'target-group absences', instead of use random pseudo-absences, is more appropriate for the model, but they suggest the former method in case sufficient data are available. Thus, since we have not a large amount of records to improve our model fits and also no target-group, it is needed to find an appropriate way to calculate the pseudo-absence data (PA). We generated twice the number of presences as pseudo-absences for improved model reliability using the *three-step* (TS) selection procedure as described in Iturbide et al. (2015). The first step of the TS defines the environmentally unsuitable areas using a presence-only profiling algorithm; the second one calculates different maximum distance thresholds to each presence location; and the last step selects the optimum background extent and the corresponding fitted model from all possible

pseudo-absence configurations generated in step 2 (for more details see Iturbide et al., 2015). In addition, we included a 10 km of buffer around the collection localities to avoid grid cells with both presence and pseudo-absence data (Hirzel et al., 2001; Chefaoui and Lobo, 2008; Mateo et al., 2010; Iturbide et al., 2015).

The present-day model predictions were projected onto three past climatic layers: the Middle Holocene (MidHol) period about 6,000 years ago, the last glacial maximum (LGM) period about 21,000 years ago, and the last interglacial (LIG) period 120,000 to 140,000 years ago. For the MidHol and LGM we created an average of three circulation models: CCSM4 (Community Climate System Model of National Center for Atmospheric Research, USA); MIROC-ESM (Earth System Model of Japan Agency for Marine-Earth Science and Technology, Atmosphere and Ocean Research Institute (The University of Tokyo), and National Institute for Environmental Studies); and MPI-ESM-P (Earth System Model of Max Planck Institute for Meteorology, Germany). For LIG we employed the data from Otto-Bliesner et al. (2008). All bioclimatic layers are available in the WorldClim website (www.worldclim.org/).

We calculated the fragmentation of all four predictions in R software version 3.3.1 (http://www.r-project.org/), using the package 'SDMTools' (VanDerWal et al., 2015). Firstly, we converted the habitat suitability into binary data according to different cutoffs. These cutoffs were the suitability threshold in which the suitability was considered as 1 (if higher than the cutoff) or 0 (if lower than the cutoff). For the first cutoff (equal to 0.4), habitat suitability values higher than 0.4 were considered as 1, and values smaller than 0.4 were considered as 0. The same procedure was performed for the following cutoffs (0.5, 0.6, 0.7, 0.8). Since some layers exhibited no values higher than 0.9, the last cutoff evaluated was 0.8. Therefore, we measured, for every cutoff, the ratio of the patch perimeter (m) to area (m^2) using the 'PatchStat' function and we plotted the values in a chart.

DNA extraction, microsatellite genotyping and gene sequencing

Leaf samples were dried using silica gel and kept at room temperature until the DNA was extracted. Total genomic DNA was extracted according to Cota-Sánchez et al. (2006) with modifications (Riahi et al., 2010).

Genotyping was carried out using 11 microsatellite loci: eight markers (Ced2, Ced18, Ced41, Ced44, Ced54, Ced65, Ced95, and Ced131) developed by Hernandez et al. (2008) for *Cedrela odorata*, and three markers (CF26 and CF66) developed by

Gandara et al. (2015) for *C. fissilis* (Table 3). Primer pair CF66 amplified two loci — CF66A and CF66B, respectively (Gandara et al., 2015); distinct ranges of allele sizes (113–167 bp for CF66A; 199–253 bp for CF66B) allowed us to clearly differentiate the genotypes of each of the two loci (Table 3). The polymerase chain reaction (PCR) was performed in two multiplexes – when we amplify more the one marker at the same PCR – M1 (Ced54-Ced41) and M2 (Ced2-Ced65), then two other primers were amplified separately (Ced95 and Ced131) and combined with the M1 and to M2, respectively, to be genotyped with them to reduce costs. Also, two primers were amplified separately, but combined later for genotyping – pseudo-multiplex M3 (Ced18-CF26). Finally, the the last markers (Ced44 and CF66) had the amplification and genotyping carried out separately. We used 12 μL volumes with 10 ng of DNA, 1 × buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, and 1% Triton X-100), 0.2 μM of each primer (forward and reverse), 2.5 mM of $MgCl_2$, 0.25 mg/mL of Bovine Serum Albumin (BSA) (Invitrogen, São Paulo, Brazil), 0.2 mM of dNTPs, and 0.5 U of Taq DNA Polymerase (Phoneutria Biotecnologia, Belo Horizonte, Brazil).

The amplifications were carried out on a Vapo Protect Eppendorf Thermal Cycler (Eppendorf, São Paulo, Brazil). For the difficult amplification (as happened with the primer Ced95) we conducted the PCR with 1X of the special buffer IVB (Phoneutria Biotecnologia, Belo Horizonte, Brazil). The amplification program (Gandara, 2009) consisted of a preheating step of 96°C for two minutes, 30 cycles of 94°C for one minute, 55°C for one minute, and 72°C for one minute; at the end of the 30 cycles there was an extension step of 72°C for seven minutes. Each forward primer was labeled with either 6-FAM, HEX (MWG-Biotech, Ebersberg, Germany), or NED (Applied Biosystems, São Paulo, Brazil) fluorescence (Table 3). Some of the fragments were separated on a 96 capillary sequencer ABI PRISM 3130 × 1 DNA Analyzer (Applied Biosystems, São Paulo, Brazil), and PCR products were sized relative to a molecular size marker (ROX 500—Applied Biosystems, São Paulo, Brazil). Part of the samples were genotyped by Macrogen Inc., South Korea (www.macrogen.com/) and the PCR products were sized relative to a molecular size marker (ROX 400). The fragments were scored using GeneMapper version 4.0 (Applied Biosystems, São Paulo, Brazil). For the 11 loci, the average percentage of missing data was 12.87% (ranging from 4.70%, for Ced41, to 24.7%, for Ced95).

Table 3. Description of 11 microsatellite loci. Code: N_A, number of alleles per locus, bp, basepairs.

Locus	Primer Sequence (5'-3')	Array	Fluorescence	N_A	Allele Size (bp)	Missing data	Null Allele Mean Frequency	Reference
Ced2	F: TTTGCTTTGAGAAACCTTGT R: AACTTTCGAATTGGTTAAGG	$(GA)_{20}$	6-FAM	22	131-241	6.4%	0.0365	Hernández, et al. (2008)
Ced18	F: CAAAGACCAAGATTTGATGC R: ACTATGGGTGGCACAACTAC	$(GA)_{23}$	HEX	24	115-161	10.5%	0.0349	Hernández, et al. (2008)
Ced41	F: TCATTCTTGGATCCTGCTAT R: GTGGGAAAGATTGTGAAGAA	$(TC)_{18}$	HEX	28	68-186	4.7%	-0.0140	Hernández, et al. (2008)
Ced44	F: ACTCCATTAACTGCCATGAA R: ATTTTCATTCCCTTTTAGCC	$(TG)_{14}(AG)_{17}$	6-FAM	33	154-238	14.2%	-0.0098	Hernández, et al. (2008)
Ced54	F: GATCTCACCCACTTGAAAAA R: GCTCATATTTGAGAGGCATT	$(GA)_{15}(AG)_6G(GA)_5$	6-FAM	18	100-218	12.8%	0.0095	Hernández, et al. (2008)
Ced65	F: GAGTGAGAAGAAGAATCGTGATAGC R: GAGGTTCGATCAGGTCTTGG	$(GA)_7(CA)_{14}$	HEX	20	121-199	16.0%	0.1655	Hernández, et al. (2008)
Ced95	F: ATTTTCATTCCCTTTTAGCC R: TTATCATCTCCCTCACTCCA	(CT)17(AC)13	NED	31	115-253	24.7%	0.0160	Hernández, et al. (2008)
Ced131	F: CTCGTAATAATCCCATTCCA R: GGAGATATTTTTGGGGTTTT	$(CT)_{16}$	NED	29	66-172	15.4%	0.1002	Hernández, et al. (2008)
CF26	F: CCAAATTCCAGAGGAGAG R: GTTCTGCTTCATCGAAGG	$(GA)_9$	6-FAM	25	119-191	9.90%	0.1823	Gandara et al. (2015)
CF66A	F: CAGCAGTTCTGAAACAGTAA R: ATTCAGCAACTTGAGAGC	$(GA)_{19}$	6-FAM	28	113-167	11.9%	0.0089	Gandara et al. (2015)
CF66B	F: CAGCAGTTCTGAAACAGTAA R: ATTCAGCAACTTGAGAGC	$(GA)_{19}$	6-FAM	25	191-253	15.1%	0.0974	Gandara et al. (2015)

The trnT–trnL spacer from the chloroplast genome was amplified using standard PCR protocols (Muellner et al., 2009; Oliveira et al., 2010). The spacer was amplified in two independent reactions. The first reaction was performed using the primers A (5'-CATTACAAATGCGATGCTTCT-3') and B (5'-TCTACCGATTTCGCCATATC-3') which amplify the intergenic spacer between trnT (UGU) and the 5' exon of trnL (UAA) (Taberlet et al., 1991). The second reaction was performed with primers C (5'-CGAAATCGGTAGACGCTACG-3') and D (5'-GGGGATAGAGGGACTTGAAC-3') and amplified the intron of trnL (UAA) (Taberlet et al., 1991). The PCRs were carried out with 25 μL final volume, with about 100 ng of DNA, 1 X of the special buffer IVB (Phoneutria Biotecnologia, Belo Horizonte, Brazil), 0.5 μM of each primer (forward and reverse), 2 mM of $MgCl_2$, 17,5 μg/mL of Bovine Serum Albumin (BSA) (Invitrogen, São Paulo, Brazil), 0.2 mM of dNTPs, and 1.25 U of GoTaq DNA Polymerase (Promega, Belo Horizonte, Brazil). The amplifications were carried out on a Vapo Protect Eppendorf Thermal Cycler (Eppendorf, São Paulo, Brazil) following the amplification program with a preheating step of 94°C for two minutes, 36 cycles of 94°C for one minute, 58°C for one minute, and 72°C for one minute; in the end the extension step was at 72°C for five minutes. All the PCR products were cleaned using 0.75 U of both enzyme EXO (Exonuclease I, Affimetrix, São Paulo, Brazil) and SAP (Shrimp Alkaline Phosphatase, Affimetrix, São Paulo, Brazil) in the proportion of 15μL of PCR to 3μL of the enzymes (diluted in 50 mM Tris, pH 8.0). Each reaction was incubated at 37°C for 45 minutes to degrade remaining primers and nucleotides, followed by one step at 80ºC for 20 minutes to inactivate the ExoSAP. Sequencing was performed by Macrogen Inc., South Korea, using the same primers as in the PCR amplifications. Sequences were edited and aligned in the program Sequencher 4.8 (Gene Codes) using the default settings. In addition, the ReAligner (Anson and Myers, 1997) option was used to optimize the gap placement. Gaps longer than three sites in the alignments were coded as insertion/deletion (indel), and different sizes of indels were coded as different evolutionary events, following previous methodologies (e.g. Garcia et al., 2011; Zelener et al., 2016). We did not use indels that were bordered by mononucleotide repeats (e.g. polyA) as a source of information in subsequent statistical analyses to avoid effects that can arise from experimental errors or evolutionary lability associated with this type of indels (Mast et al., 2001).

Data analyses

For the microsatellite data, the populations with small sample sizes (n < 9) (as populations MPA, CON, SOO, PRD, FR2, CAM and SMA) were included only in analysis that do not require a priori identification of populations (as in Structure — see below). Initially, we estimated the null allele frequencies for each marker with the program Micro-Checker, version 2.2.3 (Van Oosterhout et al., 2004). Further, we used the software FSTAT, version 2.9.3.2 (Goudet, 2002) to calculate the statistical significance of deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci in all populations. In order to describe the populations we ran the GDA software (Lewis and Zaykin, 2002) and presented, for each population, the number of specimens (*n*), number of alleles per locus (*A/locus*), number of private alleles (A_{PRIV}), number of private alleles per specimens (A_{PRIV}/n), expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (F_{IS}). Concerning the diversity among populations, we estimated two indexes: F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995; Goodman, 1997). The FSTAT software (Goudet, 2002) estimated both F_{ST} and R_{ST} over all samples. To test the significance of each estimate, we used 1000 permutations, with 99% confidence intervals. The F_{ST} index assumes the infinite alleles model (IAM), while the R_{ST} is based on allele size (stepwise mutation model, SMM). Estimates of R_{ST} may be useful when mutations contribute substantially to allelic differences among populations, resulting in greater insights into the patterns of relationships among populations than provided by F_{ST} (Holsinger and Weir, 2009). We also computed the pairwise *Fst* of populations using ARLEQUIN (Excoffier et al., 2005). The *p*-value of the test is the proportion of permutations leading to a F_{ST} value larger or equal to the observed one (Manual of ARLEQUIN), we used 1000 permutations, with 95% confidence intervals. In Arlequin, we selected 'Slantkin's distance' and instead of 'Number of different alleles (F_{ST})' we activated the option 'Sum of squared size difference (R_{ST})', which allowed us to use more information for each locus.

For the next genetic structure analysis we included all 21 populations. The Bayesian clustering approach of software Structure version 2.3.4 (Pritchard et al., 2000; Hubisz et al., 2009) inferred the number of genetic groups of *C. fissilis* using the Monte Carlo Markov Chain (MCMC) approach. We set runs with a burn-in period of 250,000 steps followed by 750,000 steps, with 20 independent replications. The Structure assumes a model in which there are *K* genetic groups, each of which is characterized by

a set of allele frequencies at each locus. Individuals in the sample are assigned (probabilistically) to one or more genetic groups, if their genotypes indicate that they are admixture (Pritchard et al., 2010). As Evanno et al. (2005) suggested, we set the *K* from 1 to 24, which is from 1 to the number of sampling sites (21) plus 3. To find the best *K*, we used the *ΔK* method (Evanno et al., 2005), as implemented in Structure Harvester (Earl and VonHoldt, 2011). We converged the data of the 20 interactions in the best *K* with the software Clumpp (Jakobsson and Rosenberg, 2007), then we graphically displayed the results using the software Distruct (Rosenberg, 2004). For each population, we summed the membership coefficients for all samples to obtain a diagram depicting the relative contribution of each lineage to the contemporary gene pool of that population.

In order to assess the diversity of the sequences obtained, the program DnaSP 5:10 (Rozas et al., 2009) determined the haplotypes in the data set. Gene genealogies based on coalescent theory were inferred using a Median Joining haplotype network (Bandelt et al., 1999) as implemented in NETWORK 4.5.1.6 (www.fluxus-technology.com).

Results

Habitat distribution modelling

Climate-based distribution models of *C. fissilis*, built with current conditions (AUC = 0.808), exhibited the best distance threshold of 860 km. The models indicated the presence of fragmented high-suitable areas in the south of Brazil, as well as in fewer areas through Atlantic coast. Present habitat suitability was projected into the past and the paleomodels differed substantially over the past 140,000 years (Figure 2). High habitat suitability during the LIG, deeper in the past, was predicted in a large area southward to Brazil (number 1 in Figure 2A) and in two smaller areas in the Paraná basin (number 2 in Figure 2A) and Amazon basin (number 3 in Figure 2A). Lower suitability was found in the Northeast during the LIG (Figure 2A). Later, at the LGM, the highest suitability was displaced north and westward to the former projection (the * in Figure 2B). Also, high suitability onto the continental shelf was observed, specially at the Abrolhos Bank, and on the coast of the north-east (Figure 2B). At the MidHol, similarly to the present habitat distribution modelling, high suitability regions

were scattered south and eastward to Brazil, and also a small area in the north-east (Figure 2C and 2D). These four time period exhibited differences concerning fragmentation (Figure 3). Regardless the cutoffs (0.4, 0.5, 0.6, 0.7 and 0.8), the chart shows the highest habitat fragmentation in the present, followed by MidHol, LGM, and the LIG. Thus, the fragmentation increased towards the present day.

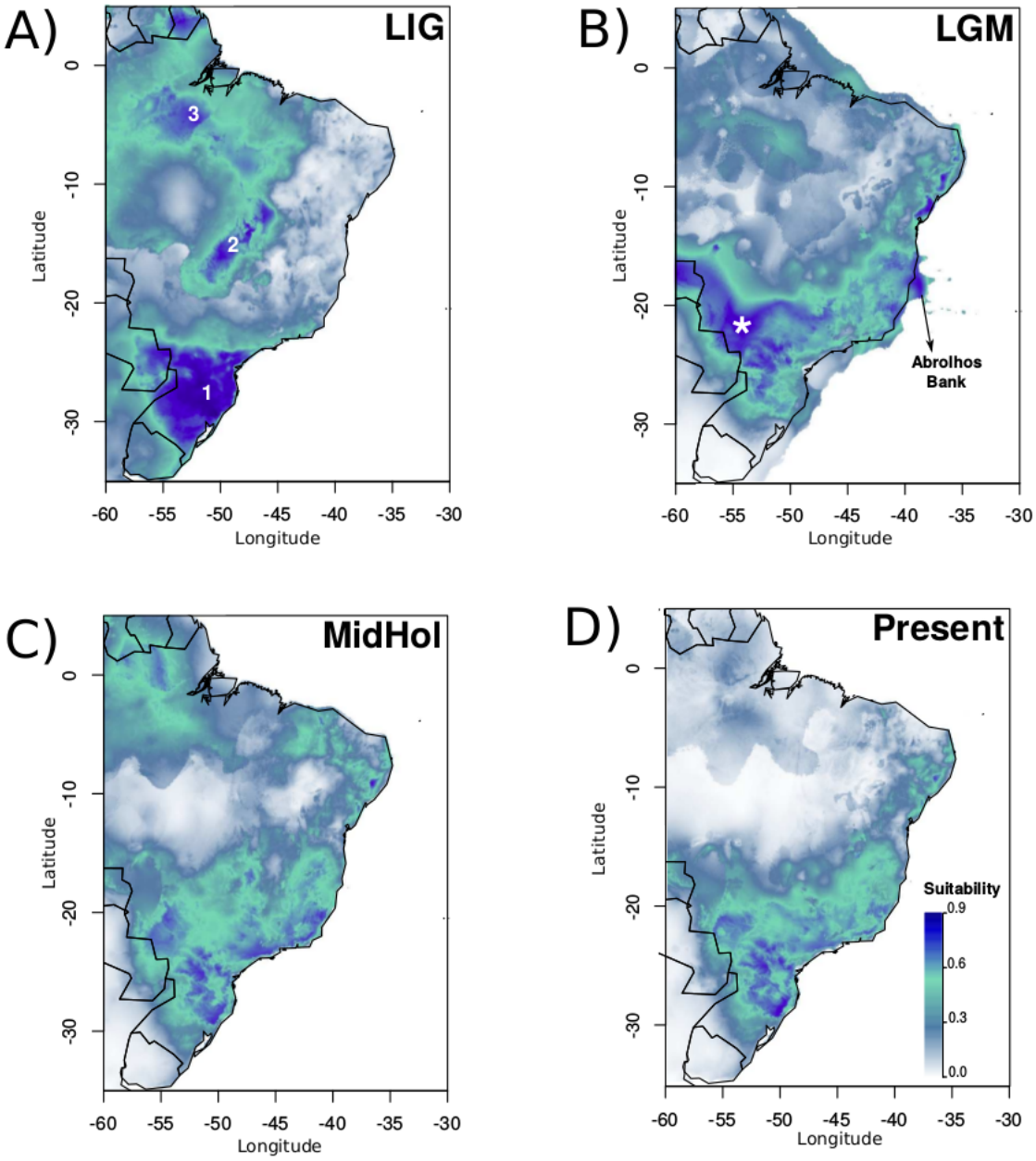

Figure 2. Habitat suitability of *C. fissilis* populations through time in eastern South America. The habitat suitability was projected to (A) the last interglacial (LIG, 120,000 to 140,000 y B.P); (B) the last glacial maximum (LGM, 22,000 y B.P.), including the exposed continental shelf; (C) Middle Holocene (MidHol, 6,000 y B.P.); and (D) the present. In (A) the numbers 1, 2, and 3, exhibited the higher suitability at the LIG projections. In (B) the * represent the higher suitability at the LGM. All pictures follow the suitability colour scale showed in (D).

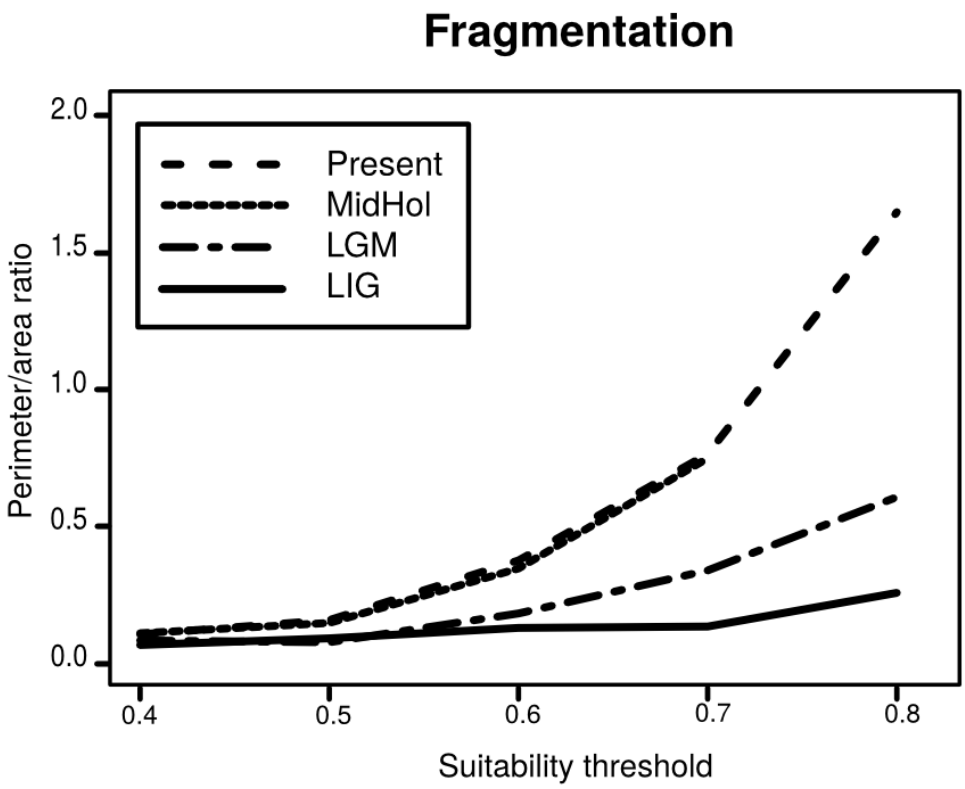

Figure 3. Fragmentation chart presented for each time (Present, MidHol, LGM, and LIG) considering different suitability thresholds.

Genetic diversity and structure

We identified 283 alleles across the 11 microsatellite loci; the number of alleles per locus ranged from 18 (Ced54) to 33 (Ced44). None of the 11 loci exhibited high null allele frequency (Table 3). Also, the 11 loci were at Hardy-Weinberg equilibrium, and linkage equilibrium. Fourteen populations (with population size ≥ 9) exhibited 61 private alleles (A_{priv}), ranging from 0 (SER) to 9 (PSO). When we divided the A_{priv} by number of sample we pick up the A_{priv} regardless the different population size, the major A_{priv}/n occurred in the populations UBE (A_{priv}/n = 0.37) and PSO (A_{priv}/n = 0.36). The mean expected heterozygosity was H_E = 0.83 ranged from H_E = 0.7 (SER) to H_E = 0.88 (TBA), while the observed heterozygosity was H_O = 0.72, ranging from H_O = 0.61 (SER) to H_O = 0.79 (TBA). The H_E was slightly higher than the H_O, indicating some degree of inbreeding in all populations. The mean inbreeding coefficient was F = 0.14, ranging from F = 0.11 (CAP) to F = 0.24 (PSB) (Table 1). We detected a significant but low F_{ST} overall 14 populations (F_{ST} = 0.058, P<0.01). Similarly the R_{ST} was significant and low (R_{ST} = 0.0826, P<0.01). Since the R_{ST} value was higher than F_{ST}, the observed differentiation among the populations received a strong contribution of the SMM. Thus, the pairwise genetic diversity was calculated with the matrix of Slatkin linearized F_{ST}. For the pairwise R_{ST} (Table 4), we obtained 65 significant values (P<0.05) out of 91 pairwise values. The genetically closest populations was UBE x ITA (R_{ST} = 0.019) and the genetic farthest populations SER x VNI (R_{ST} = 0.225). The population SER exhibited the higher pairwise values when compared to other populations.

Table 4. Matrix of Slatkin linearized pairwise F_{ST} of 14 populations of *C. fissilis*. All pairwise values are significant (P>0.05)

	SER	JAN	UBE	DIA	PEU	VNI	CAP	PSB	ITA	PSO	PNI	TBA	PLO	BLU
SER	-													
JAN	0.049	-												
UBE	0.184	ns	-											
DIA	0.168	ns	ns	-										
PEU	0.197	ns	0.025	0.041	-									
VNI	0.225	ns	0.097	0.097	0.073	-								
CAP	0.175	ns	0.070	0.052	0.072	ns	-							
PSB	0.172	ns	0.036	0.065	0.043	0.103	0.072	-						
ITA	0.155	0.030	0.019	0.036	0.058	0.079	0.040	0.030	-					
PSO	0.119	ns	ns	ns	ns	ns	ns	ns	ns	-				
PNI	0.178	ns	0.030	0.025	0.047	0.070	0.039	0.038	0.024	ns	-			
TBA	0.156	ns	0.037	0.022	0.050	0.077	0.043	0.040	ns	ns	0.021	-		
PLO	0.154	0.021	0.026	0.023	0.042	0.084	0.036	0.044	0.024	ns	ns	ns	-	
BLU	0.172	ns	0.095	0.081	0.086	0.097	0.068	0.075	0.066	ns	0.054	0.064	0.042	-

Code: ns non significant values.

The genetic structure of the 21 populations (now including those populations $n < 9$) exhibited a pattern according to where they occur geographically and to every terrestrial ecoregion they belong (Figure 4A). They were assigned into five genetic groups ($K = 5$, Figure 4B). Populations with a majority of genetic group 1 (PSO, PNI, CAM and BLU) were mainly distributed in the coast of the southeast, then naming it as 'Serra do Mar' (depicted in dark blue). There was one exception (CON), which can be found further north in close proximity to populations from other genetic groups (SOO and PRD). The distribution of this group was regardless the elevation, because the populations CAM, BLU and CON belong to low elevations, while the populations PSO and PNI are from highlands. Populations which the majority genetic group is the 'Central-West' (number 2, depicted in lighter blue) are widely spread in the central of Brazil, occurring either in lowland (ITA, FR2, UBE, DIA and PRD) or in highland (PSB and PEU). Three populations from lowland (PLO, TBA and SMA) exhibited major assignment in the 'South' genetic group (number 3, depicted in yellow). The highland population PNI is also associated with group 3. The 'Serra do Caparaó' group (number 4, depicted in green) occurred specially in highland population, with a high proportion (in CAP and VNI), but also and less expressed in the populations PRD and MPA. The later population was the highest admixture population. The last genetic group, the 'Northeast' (number 5, depicted in lightest blue), occurred basically into three lowland populations (JAN, SER and SOO) and the population SOO is furthest from the other two populations.

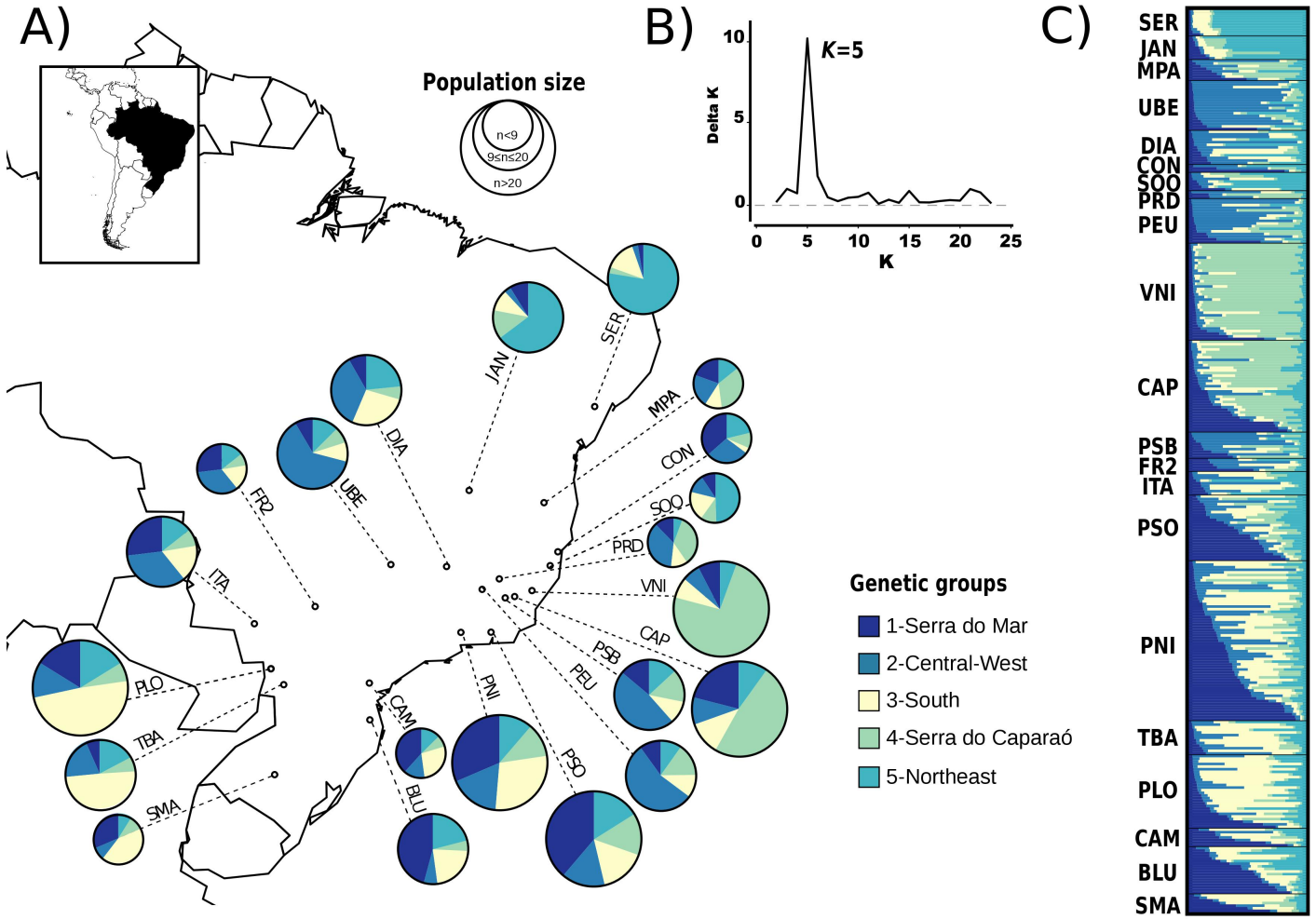

Figure 4. Clustering analyses and the geographic distribution of the five genetic groups of *C. fissilis*. (A) The geographical occurrence of the populations and their lineage assignment. The pie charts represent the mean of each membership coefficient for all samples from that population. (B) The best K (K=5) was calculated according to the ΔK method (Evanno et al., 2005). (C) Plot of the clustering analysis in STRUCTURE, showing the five lineages content per sample. Each horizontal bar represents a sample and the different five colors is a membership coefficient of each sample that represent the fraction of its genome that has ancestry in that lineage. (Refer to Table 2 for population codes).

Phylogeography

The alignment with 1370 bp revealed the presence of 20 polymorphic sites were characterized by indels or substitutions of bases in 148 individuals. We observed 19 substitutions, 5 transitions and 15 transversions. The remaining polymorphic site showed indel with 7 base pair. The analysis of 148 specimens *Cedrela fissilis* revealed 16 haplotypes (Figure 5).

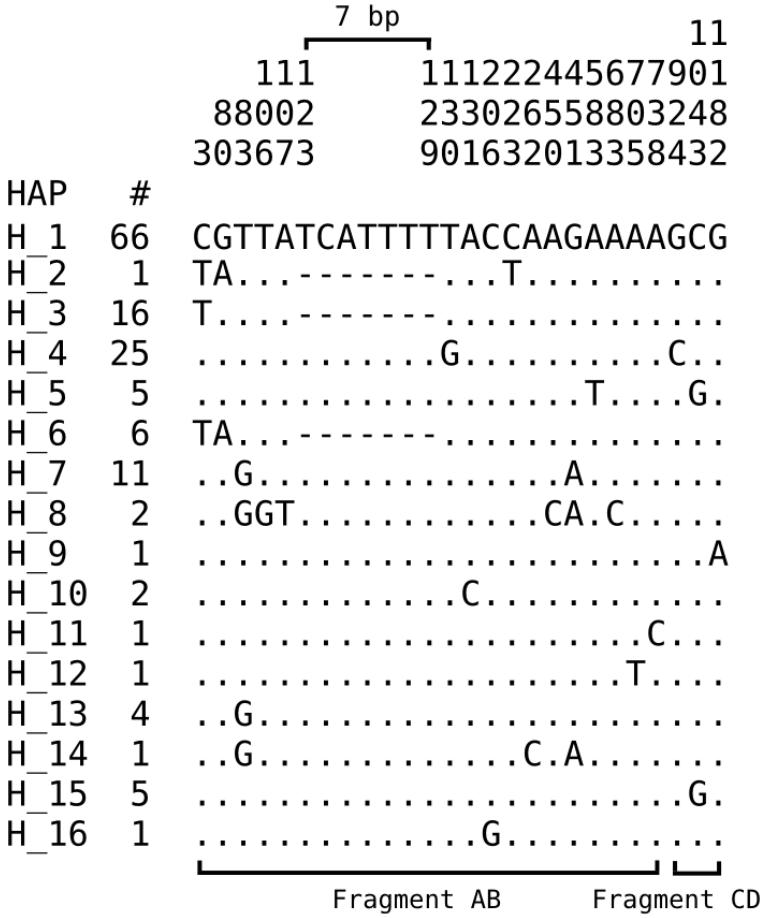

Figure 5. Out of 1370 sites, 21 were polymorphic in the two cpDNA fragments (Fragment AB and Fragment CD) of the 16 haplotypes (HAP from H_1 to H_16) from 148 specimens of *C. fissilis*. Dots indicate similarity to haplotype 1 and hyphens indicate gaps. Numbers on top indicate the nucleotide position with haplotype 1, acting as the reference sequence during alignment. The # indicates the numbers of occurrences of the haplotypes.

The haplotype network shows seven genealogical lineages (hereafter haplogroup) indicated by different colours (Figure 6). The central haplogroup (H1, represented in blue) was the most frequent. This haplogroup presented 66 specimens from 22 populations wide scatter in the Atlantic range. The haplogroups represented in yellow (H4) and in orange (H2, H3 and H6) were two different haplogroup that exhibited an overlapped occurrence, that is mostly south-westward, apart from the population SOO, which is from the east coast, but also contains a sample that is from haplogroup H4. The haplogroups represented in pink (H7 and H14) and in purple (H5) are also two haplogroups with geographic overlap, in this case in the north-east of Brazil. The haplogroup H5 is connected to the central one by haplotypes from population VNI (H16). While the pink haplogroup is connected to the central one by haplotypes from population PSB (H13). VNI and PSB are closely geographically. The haplogroup represented in green is the farthest one to the central haplogroup, distant by six mutation steps. The green haplogroup evidence a connexion between the west and the east populations through the north-east. Five populations are very close geographically (in the west area) although each one belongs to a different haplogroups (CEC, FR2, OUR, CED, and SRP). Similarly to the genetic structure of the populations, the haplogroups were not associated to the different elevations of the populations.

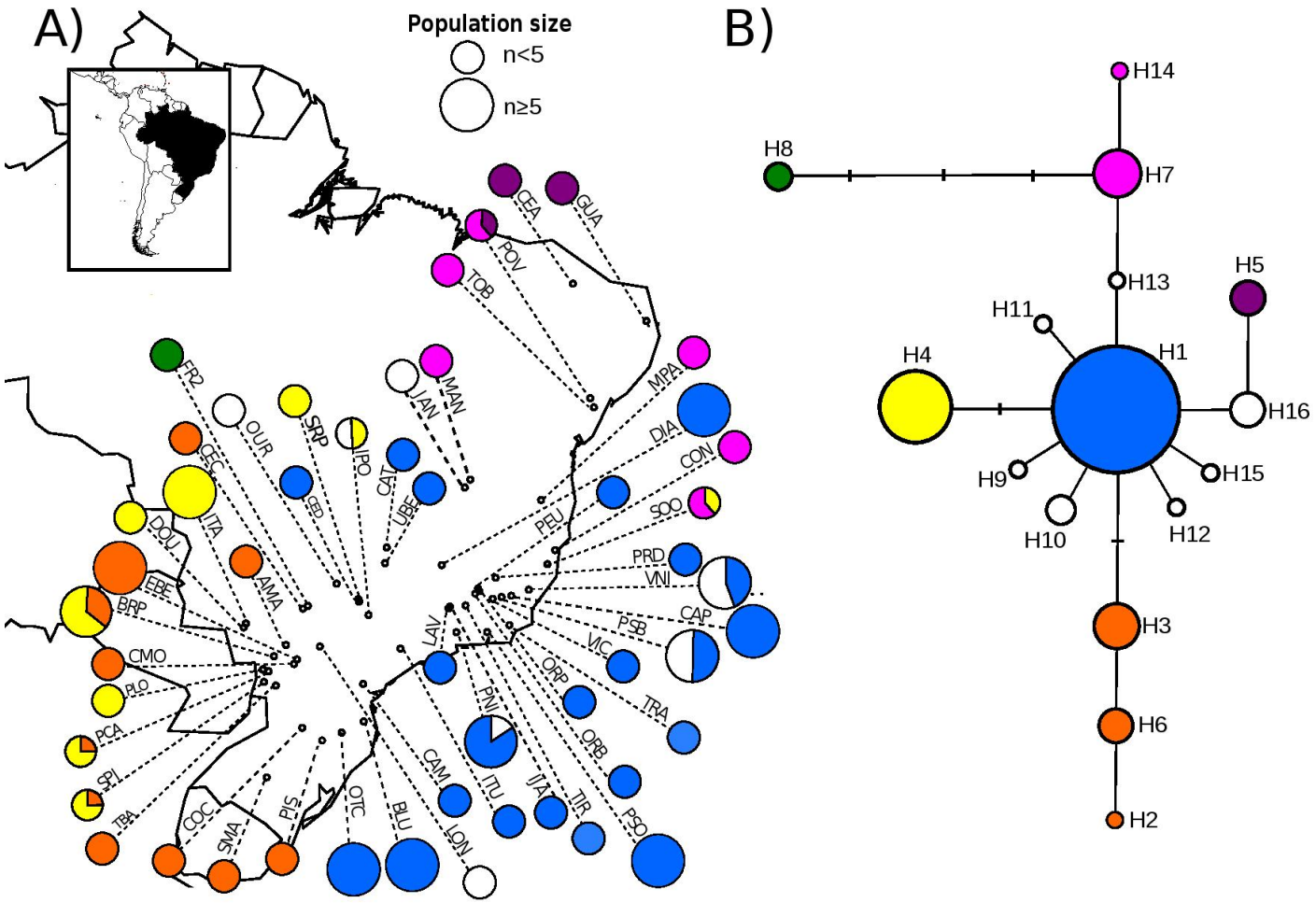

Figure 6. Median-joining network and the geographic distribution of the 16 haplotypes of *C. fissilis*. (A) The geographical occurrence of the populations and their lineage assignment. Each pie circle represents one population and the colors depict the haplogroup which the population belongs to. Refer to Table 2 for population codes. (B) Median-joining network obtained with trnT-trnL haplotypes. The network shows the relationships among different genealogical lineages of *C. fissilis*. Each circle represents one haplogroup (coded from H1 to H16). Their sizes are proportional to the relative frequencies and the colors depict the haplogroup. The number of mutation steps are indicated with small bars just in case more than one step occurs.

Discussion

In this study, we have used species distribution modelling and two molecular markers (microsatellite and cpDNA) to assess the complex evolutionary patterns in the Brazilian Atlantic forest (BAF). We evaluated the tree species *Cedrela fissilis* (Meliaceae) regarding their wide distribution, elevation range and large ecological plasticity. Our results support both the dry refugia hypothesis and the moist forest hypothesis, suggesting that most likely a mixture of processes have acted through space and time.

The molecular data were largely congruent (between microsatellite and cpDNA) and minor differences may be due to the higher mutation rates and levels of polymorphism of the microsatellite (Schlötterer, 2000; Zane et al., 2002). The fact that most of the populations which occurred in the same ecoregion were consistently genetically clustered suggests a correspondence between phylogeographic history of *C. fissilis* and the BAF. However, it should be noted that some highland populations did not show high genetic similarities, even though they were geographic close or ocurred in the same terrestrial ecoregion (e.g., CAP and PSB). This pattern appears to be in contrast with other tropical highland regions (Dimitrov et al, 2012; Migliori et al., 20013; Shi et al., 2011). We suggest that different highland populations were genetically different due to the following non-mutually exclusive processes: (1) dispersal toward highlands from genetically distinct areas; or (2) differentiation by genetic drift.

The level of admixture among the populations reflects recent gene flow likely by dispersion within the whole Atlantic range. Indeed, the long-term dispersion has been the most important process that shaped the current distribution of *C. fissilis* (Garcia et al., 2011; Mangaravite et al., 2016). At the north-east of the Brazilian Atlantic coast, the low habitat suitability during the LIG contrary to the high suitability in the present is likely another indication that long-term dispersion might have occurred during the Quaternary climatic changes. The north-east populations (the genetic group number 5 and the pink and purple lineages), however, showed little admixture and were genetically more divergent than the other populations. Similar patterns have also been shown for frog species in the 'Pernambuco refugium' (Carnaval et al., 2009) and fire-eye antbirds in the north-east (Maldonado-Coelho, 2012). The north-east seems to be a mixture between distinct lineages that arrived by two dispersion routes. One may have

occurred from central and another from south of Brazil. Spichiger et al. (2004) have called them 'Peri-Amazonian' and 'Parana Atlantic' main gradient, respectively. These two independent biogeographical routes were also reported previously (Maldonado-Coelho, 2012), confirming that those dispersion were important for the genetic structure of organisms within the BAF. We also consider that the 'Parana Atlantic' route may play an important role for the northern population diversity, as the network analysis that indicated two independent connections between the north-east and the Atlantic coast. Similar patterns in the distinct northern lineage of an endemic passerine bird were reported, but explained by a recent bottleneck effect (D'Horta et al., 2011). For *C. fissilis*, bottlenecks are unlikely to have occurred due to the homozygote excess observed here and in Mangaravite et al. (2016). We propose that populations in the north-east are different because they exist in a meeting point of different lineages where: (1) the specific genes exist due to genetic drift; or (2) the hitchhiking effect of the neutral genes evaluated herein with the genes under strong selection, due to the climatic extreme environment of Caatinga biome, compared to rainforest.

Other evidence of long-term dispersion is the presence of a highly diverse area (different genealogical lineages) in the Paraná basin. This diversity is likely a relict from a joint distribution range during the LGM between central and south of Brazil. This region is known to have been wetter than today (Spichiger et al., 2004; Varela and Fariña, 2016) and climatically stable (Carnaval and Moritz, 2008). These features suggest, then, a possible presence of refugia. Similarly, populations in the Atlantic coast (CON, SOO and PRD) were highly admixed and also occur in a climatic stable area (Carnaval and Moritz, 2008). They seem to be another relict of a meeting area between dispersal from the south of Brazil and from the continental shelf of the LGM (where high habitat suitability was predicted). However, instead of presence of refugia, the latter evidence support the theory that the continental shelf has accommodated the expansion of the BAF, keeping more connections and gene flow, playing an important role for biodiversity (Leite et al., 2016).

In contrast to the long-term dispersion, the phylogeographic history of *C. fissilis* shows a remarkable geographic fragmentation increasing from the LIG to the present-day distribution, leading to allopatric differentiation, isolation and genetic inbreeding. Thus, we cannot exclude that range fragmentation process – vicariance – have also ocurred over time in populations of *C. fissilis* from BAF. Correspondingly, ecological vicariance (Cabanne et al., 2008) and isolation (Martins et al., 2009) have

explained spatial divergence in the BAF. This evidence agrees to contraction in the LGM of dry refugia hypotheses. When regional climate became unsuitable for species, their ranges contracted onto places – refugia – of limited spatial extent that provided suitable environments and allowed them to persist in these sites until climatic conditions improved (Keppel et al., 2012). Despite that, the projections for the LGM seems to be less contracted than the present-day, with higher connection over the range, in expansion and in agreement to the moist forest hypotheses.

Although some authors suggested the presence of dry refugia in the BAF (Cabanne et al., 2008; Carnaval et al., 2009; Martins et al., 2009; D'Horta et al., 2011; Maldonado-Coelho, 2012; Amaral et al., 2013), a number of studies have refuted this hypotheses over the past years (e.g., Thomé et al., 2010, 2014; Álvarez-Presas et al., 2011, 2014; Amaro et al., 2012; Batalha-Filho et al., 2012; Cabanne et al., 2013; Leite et al., 2016). In spite of our results did not exclude the presence of refugia in the LGM, the colonization at the BAF from refugia of the north-east may not be the sole process responsible for the high genetic diversity. Instead, complementary processes may have influenced this biome through space and time. We propose that several regions had their distribution shifted due to the drier and colder climate that are reported by palynological data from most of the BAF (Behling e Lichte, 1997, Behling e Negrelle de 2001, Behling, 2002). Later, populations returned to their original place, southward in Brazil, after ameliorate the climatic conditions. This return, however, proceeded with increasing fragmentation until the present-day. Although the fragmentation may not affect genetic diversity (Cabanne et al., 2016), the present-day distribution suggests the presence of several micro-refugias throughout the BAF, playing a role in the survival and evolution after the glaciations (Keppel et al., 2012).

This study is the first which reports the LGM as more fragmented relatively to the LIG, and as more connected, with expansion, relatively to the present. This resulted in an intermediate level of genetic structure detected today, between admixture and strong structure. The BAF is an environmentally complex biome, thus the climate changes of Quaternary may have had a major (e.g., Carnaval et al., 2009) or a minor impact (e.g., Álvarez-Presas et al., 2011, 2014) on the genetic diversity and the phylogeography of species. Indeed, climatic variability impacted the northern and the southern forests differently (Carnaval et al., 2014) and reflected distinct demographic scenarios (D'Horta et al., 2011). In this sense, we do not refute the influence of tectonics in BAF geographic variation before the the Quaternary (Amaro et al., 2012),

and neither exclude the possibility of other events driving the genetic diversity (Thomé et al., 2014, Leite et al., 2016).

In summary, the different genetic patterns of highland population and the intermediate level of genetic structure reflect a mixture of events acting over the Quaternary. Long-term dispersion and vicariance may have been the most important process that influenced the populations contraction and expansion during the LGM. Finally, the usage of plant species as biological example reveals a complex pattern of diversification, and brings new insights into the evolutionary history of the Brazilian Atlantic forest.

Supplementary tables

Table S1. Percentage contribution and permutation importance in the models for all 19 variables. The subset of seven predictors are in bold.

Code	Variable	Percent contribution	Permutation importance
bio4	**Temperature Seasonality (standard deviation *100)**	27.9	9
bio11	Mean Temperature of Coldest Quarter	18.1	8.4
bio9	Mean Temperature of Driest Quarter	17.1	2.4
bio5	**Max Temperature of Warmest Month**	13.3	2.4
bio3	Isothermality (BIO2/BIO7) (*100)	7.7	35
bio8	**Mean Temperature of Wettest Quarter**	3.3	1.1
bio10	Mean Temperature of Warmest Quarter	2.3	0.1
bio2	**Mean Diurnal Range (Mean of monthly (max temp - min temp)**	2.2	7.3
bio16	**Precipitation of Wettest Quarter**	2.1	8.3
bio18	**Precipitation of Warmest Quarter**	1.7	5.7
bio19	**Precipitation of Coldest Quarter**	1.5	1.8
bio14	Precipitation of Driest Month	1.5	3
bio12	Annual Precipitation	0.7	0.5
bio13	Precipitation of Wettest Month	0.3	14.2
bio17	Precipitation of Driest Quarter	0.2	0.7
bio15	Precipitation Seasonality (Coefficient of Variation)	0	0.2
bio6	Min Temperature of Coldest Month	0	0
bio7	Temperature Annual Range (BIO5-BIO6)	0	0
bio1	Annual Mean Temperature	0	0

Table S2. Pearson correlation pairwises among the 19 bioclimatic variables. The pairwise correlation of the retained subset of seven predictors are in bold. For the name of every variable see Table S1.

	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19
bio1	1.00	0.15	0.80	0.81	0.75	0.92	0.66	0.83	0.90	0.88	0.98	0.32	0.56	0.44	0.57	0.56	0.42	0.45	0.32
bio2		1.00	0.24	**0.15**	**0.24**	0.50	0.73	**0.03**	0.31	0.15	0.20	0.24	0.15	0.40	0.22	**0.15**	0.40	**0.39**	**0.57**
bio3			1.00	0.92	0.33	0.86	0.82	0.56	0.82	0.49	0.87	0.34	0.63	0.39	0.64	0.62	0.37	0.43	0.43
bio4				1.00	**0.37**	0.83	0.77	**0.53**	0.85	0.44	0.91	0.41	0.69	0.48	0.67	**0.69**	0.46	**0.35**	**0.33**
bio5					1.00	0.53	0.06	**0.71**	0.60	0.87	0.66	0.19	0.30	0.35	0.33	**0.31**	0.35	**0.35**	**0.11**
bio6						1.00	0.88	0.69	0.92	0.77	0.94	0.39	0.59	0.25	0.46	0.58	0.25	0.58	0.52
bio7							1.00	0.40	0.75	0.41	0.73	0.35	0.52	0.07	0.35	0.50	0.08	0.48	0.56
bio8								1.00	0.58	0.83	0.75	0.14	0.35	0.45	0.48	**0.35**	0.43	**0.24**	**0.13**
bio9									1.00	0.72	0.93	0.39	0.60	0.31	0.49	0.59	0.29	0.53	0.44
bio10										1.00	0.77	0.20	0.33	0.25	0.31	0.33	0.24	0.46	0.29
bio11											1.00	0.38	0.64	0.46	0.62	0.64	0.44	0.46	0.35
bio12												1.00	0.85	0.30	0.14	0.88	0.35	0.01	0.67
bio13													1.00	0.12	0.34	0.99	0.01	0.01	0.56
bio14														1.00	0.86	0.12	0.99	0.15	0.39
bio15															1.00	0.32	0.87	0.26	0.19
bio16																1.00	0.07	**0.05**	**0.56**
bio17																	1.00	0.16	0.42
bio18																		1.00	**0.32**
bio19																			1.00

Table S3. Percentage contribution and permutation importance in the models for the retained subset of seven predictors.

Variable	Percent contribution	Permutation importance
Temperature Seasonality (bio4)	49.8	40.5
Max Temperature of Warmest Month (bio5)	35.2	16.3
Mean Diurnal Range (bio2)	6.0	9.2
Mean Temperature of Wettest Quarter (bio8)	5.2	17.7
Precipitation of Warmest Quarter (bio18)	1.6	1.5
Precipitation of Wettest Quarter (bio16)	1.5	13.7
Precipitation of Coldest Quarter (bio19)	0.6	1.2

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CAPÍTULO 2

A robust methodology for obtaining genomic DNA from cambium tissue for molecular studies on Neotropical tree species

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Research in Population Genetics, Phylogeny and Phylogeography with trees species have a great importance because they help to elucidate genetic, evolutionary and geographic distribution patterns. For these studies, the DNA sequence comparisons are used, but before it is required to use DNA isolation methods. As long as in plants you can not always get the leaves, this paper presents an efficient methodology for extracting DNA from trunk, which is suitable for further molecular analysis.

Abstract

The Neotropical forests consist of a mosaic of phylogeographical and interaction patterns. The deciphering of molecular clues hidden on the genomic DNA is beginning to reveal their complex evolutionary history. Herein we describe a robust methodology for extracting genomic DNA from cambium tissues, a resource can be sampled from seasonal forest species throughout the year. We explain the protocol fully to show how tissue samples should be collected, processed, and stored during field expeditions, and how high-quality genomic DNA can be extracted after getting back to the laboratory. There is no requirement for either liquid Nitrogen or commercial kit for DNA extraction. We applied our protocol in a wide range of conditions using four widespread tree species commonly found on seasonal forest (*Anadenanthera peregrina* var. *peregrina*, *Cedrela fissilis*, *Ceiba speciosa* and *Dimorphandra wilsonii*). Our protocol was effective in obtaining PCR-grade genomic DNA that was suitable for both DNA sequencing (both nuclear and chloroplastic regions) and genome-wide microsatellite genotyping.

Key words: PCR Amplification; DNA Isolation; ITS; SSR; *trn*L intron; Woody tissues.

Introduction

Nowadays there is an intense focus on understanding how biodiversity is distributed around the world. Molecular data has been particularly useful to unravel hidden aspect of the evolutionary history of the flora in America (e.g., Soltis *et al.* 2006; Hoorn *et al.* 2010; Shafer *et al.* 2010; Hughes *et al.* 2013; Turchetto-Zolet *et al.* 2013), Africa (e.g., Lorenzen *et al.* 2012), Europe (e.g., Taberlet *et al.* 1998; Feliner 2011), and in Oceania (e.g., Byrne 2008; Wallis and Trewick 2009). In the past years, the number of works related to biodiversity has increased and most of them have employed tools from phylogeny, phylogeography, and population genetic studies (e.g., Avise 2009; Flynn *et al.* 2011; Losos *et al.* 2013; Tucker and Cadotte 2013; Morlon 2014). Therefore, these studies, performed with some group or even for a single species, have described the genetic diversity, detected new evolutionary patterns and/or suggested some conservation advices. The Neotropical forests of South America have called attention of many investigators because of its complex mosaic of phylogeographical and interaction patterns (Antonelli and Sanmartín 2011; Turchetto-Zolet *et al.* 2013). In particular, studies have addressed the evolutionary patterns of seasonal forests that are spread in disjunct distribution but related floristically among them (Bigarella 1975; Ab'Saber 1977). These blocks of forest hold species unrelated to species from surrounding areas (Prado 2000; Pennington *et al.* 2006). Some plant species have been used to clarify these patterns by approaching their genetic diversity such as *Anadenanthera peregrina* (L.) Speg. var. *peregrina* (Leguminoseae-Mimosoideae) (Silveira *et al.*, unpublished results); *Cedrela fissilis* Vell. (Meliaceae) (Garcia *et al.* 2011; Mangaravite *et al.* 2016); and *Ceiba speciosa* (A. St.-Hil.) Ravenna (Malvaceae) (Collevatti, Lima-ribeiro *et al.* 2013; Collevatti, Terribile, *et al.* 2013). Additionally, the Brazilian Savanna, called Cerrado, is also an important Biome and some plant species have had their genetic accessed such as *Dimorphandra wilsonii* Rizzini (Leguminoseae-Caesalpinioideae) (Souza and Lovato 2010; Fernandes and Rego 2014; Vinson *et al.* 2015).

Accessing molecular information from DNA requires either DNA sequencing or DNA genotyping and there are many studies combine both source of information to obtain robust inferences. Assembly molecular databases are carried out through the polymerase chain reaction (PCR) as one of the first steps during data acquisition. It is now clear that PCR requires genomic DNA of high purity, that is, the genomic DNA

should be free of polysaccharides and polyphenols, because these compounds interfere with the DNA polymerase enzymatic activity and may render PCR useless when present in the solution (Mannerlöf and Tenning 1997). There are several methods of DNA extraction described. In addition to traditional methods (Dellaporta *et al.* 1983; Doyle and Doyle 1987; Doyle 1990); there are those that were modified for a specific taxon (e.g., Lodhi *et al.* 1994; Cruz *et al.* 1997); to reduce the quantity of polysaccharide and/or polyphenol components in plants (e.g., Cruz *et al.* 1997; Sánchez-Hernández and Gaytán-Oyarzún 2006); or for herbarium samples (e.g., Rogers and Bendich 1985; Cota-Sánchez *et al.* 2006; Riahi *et al.* 2010). However, most of these methods were designed to have leaves as the primary source of genomic DNA.

In the field, however, there are many instances in which leaves are not immediately available as a source of genomic DNA. Firstly, tropical trees from seasonal dry forest can reach very high canopy that hamper or inhibit the leaves accessible. For the species mentioned before, *A. peregrina* var. *peregrina* reachs about 10 m (Altschul 1964; Queiroz 2009); *C. speciosa* reachs up to 20 m (Gibbs and Semir 2003); *D. wilsonii*, reachs about 30-m tall (Vinson *et al.* 2015); and *C. fissilis* reachs up to 45 meters high (Carvalho 1994; Pennington and Muellner 2010). Secondly, plants that occur in the seasoanl dry forest release usually all their leaves in the driest period of the year (Veloso *et al.* 1991; Oliveira-Filho and Fontes 2000). Therefore, when the specimens are leafless, the sample collection is impaired.

Another key point is the long time required for DNA extraction. To overcome this drawback, commercial kits can be used to extract DNA quickly from leaves. Although these kits might save time, they become expensive given the high number of samples used when population genetic studies are carried out. The use of a protocol that targets a distinct organ of the plant, such as cambium tissues, can be an alternative to circumvent those difficulties. In addition, the use of alternative sources of genomic DNA would reduce the chances to take foreign DNA along with the target DNA, given that leaf tissue contains imperceptible guests (like micro-organisms and small insects). There are few studies that intend to obtain DNA from cambium tissue in tree species. However, most of them adopted some commercial kit to help the extraction method (Asif and Cannon 2005; Colpaert *et al.* 2005; Rachmayanti *et al.* 2006; Tibbits *et al.* 2006; Novaes *et al.* 2009). Moreover, these methods are time-consuming and require many steps, which included long incubation and precipitation steps (Asif and Cannon 2005; Colpaert *et al.* 2005; Novaes *et al.* 2009; Lanes *et al.* 2013), or the need of liquid

nitrogen on the first steps (Asif and Cannon 2005; Novaes *et al.* 2009; Lanes *et al.* 2013).

Taking into account the drawbacks aforementioned, we aim to develop a robust methodology for extracting genomic DNA from cambium tissues. We describe herein a protocol with a complete description, from sampling in the field to the DNA extraction steps in the laboratory. We tested the protocol on genomic DNA obtained from four Neotropical tree species. We assessed both the time after field sampling and the different storage conditions and their interactions. Our protocol avoids both liquid nitrogen and any commercial kit of DNA extraction. Our aim was to provide a simpler protocol, as inexpensive and fast as possible, but powerful enough to provide sufficient amounts of high purity genomic DNA. We proved the effectiveness of our protocol using PCR for genes sequencing (cpDNA and rDNA) and microsatellite genotyping (SSR markers), two distinct tools widely used in phylogeny, phylogeography, and population genetic studies.

Material e methods

Field sampling and preservation strategies

We sampled cambium tissues from ten specimens: three specimens of *Anadenanthera peregrin*a var. *peregrina* (ANA1, ANA2 and ANA3), three specimens of *Cedrela fissilis* (CED1, CED2 and CED3), three specimens of *Ceiba speciosa* (CEI1, CEI2 and CEI3), and one specimen of *Dimorphandra wilsonii* (DIM1) (Figure 1). To sample the cambium tissue, we used a puncher (diameter, 1 cm; thickness, 0.2 cm; length, 16 cm). We hammered the puncher into the tree trunk until it reached the wood fibrous layer, probably the secondary xylem. Subsequently, we removed the piece of cambium from the trunk for storage (see below). Before punching another trunk, we cleaned it with a piece of paper, and then sterilised by washing the puncher using a solution of 100% ethanol. To avoid the growth of micro-organisms, we filled the puncture with expanding foam (Cascola).

To explore the best conditions for tissue conservation, we collected three samples from each tree specimen. The first sample was kept in Dithiothrietol transport buffer, hereafter referred to as "DTT" treatment. The second sample was kept in ascorbic acid transport buffer, hereafter referred to as "AA" treatment. Both DTT and AA treatment were kept in 15 mL Falcon tubes that were wrapped in aluminium foil to avoid degradation of buffer constituents when exposed to light. The third sample was kept in an air-sealed plastic bag containing about 50 g of silica gel beads, hereafter referred to as "SIL" (Figure 1). Both the DTT and AA buffers were intended to maintain the integrity of the genomic DNA while sampling in the field; they were prepared following Colpaert *et al.* (2005) with modifications. They consisted of 2/3 of absolute ethanol and 1/3 of 1X CTAB buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB); 100 mM Tris-HCl, pH 7.5; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 4% (w/v) polyvinylpyrrolidone (PVP)]. Diffetencially to Colpaert et al. (2005), we did not use the antioxidant agent at the same time and also did not add the β-mercaptoethanol, instead we tested two different buffers (DTT and AA). In the DTT, we added 3 mM dithiothrietol; while in the AA, we added 0.3% (w/v) of ascorbic acid. Samples kept in Falcon tubes were kept at 4º C at the laboratory, while samples in air-sealed bags remained at room temperature (about 25º C), in order to better preserve them.

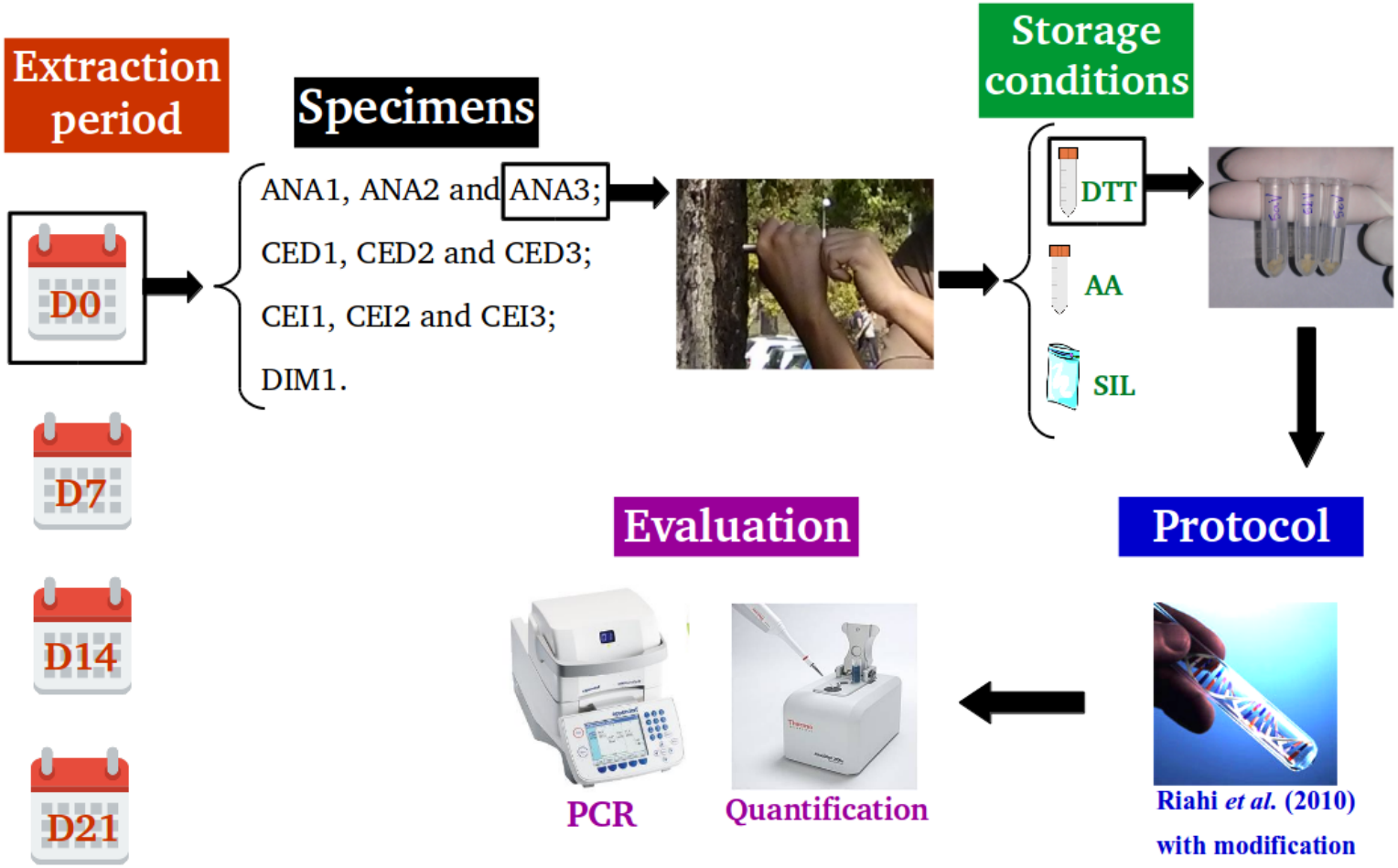

Figure 1. Framework of the method. We considered four extraction periods (D0, D7, D14 and D21). Ten different specimens (ANA1, ANA2, ANA3, CED1, CED2, CED3, CEI1, CEI2, CEI3, and DIM1) of four species were collected by hammering the puncher into the trunk tree. For every tree, we collected three samples and storage them into three conditions (DTT, AA and SIL). For every storage condition we used three replicates for the extraction protocol. Finally, the DNA extracted were submitted to evaluation with PCR and quantification.

Sample preparation

A key factor during genotyping is PCR-grade genomic DNA. Thus, we set up experiments intended to investigate whether storage conditions would decrease the overall quality of the genomic DNA over time. Extraction of genomic DNA from DTT and AA treatments was carried out at four periods: "D0" (when the extraction was carried out at the same day the samples were taken); "D7", "D14" and "D21" (when the extraction was performed after 7, 14 and 21 days after the samples were taken, respectively) (Figure 1). DNA extraction from SIL was performed after 14 and 21 days only, as we had to wait until the tissues were dehydrated, as the color-changing silica beads displayed.

Prior to DNA extraction, the buffer-stored samples (DTT and AA) were washed with distilled water to remove any buffer residues, sliced into small pieces, and dried with toilet paper. The silica-dried samples (SIL) were removed from the plastic bags and sliced. Sliced samples (approximately 40 to 70 mg) were placed into 2.0 mL microcentrifuge tubes together with two 3.2-mm-chrome-steel beads per tube. Every bead after its usage was washed with water and neutral detergent and later kept for 5 minutes in HCl 4M. Next, the samples were homogenised using a Bead Beater system (BioSpec). For extracting the genomic DNA, we developed our protocol based on previously published procedure Cota-Sánchez *et al.* (2006) as modified by Riahi *et al.* (2010). Our choice of protocol for DNA extraction exhibited the following advantages: it has been useful to extract genomic DNA from high-phenolic-compounds samples; it was a very fast procedure; it did not use any commercial kit; and it has been shown to be efficient in isolating genomic DNA from cambium tissues.

Protocol for extraction of genomic DNA

We performed the following 8-step protocol:

1. Preheat the CTAB buffer (2% CTAB, 100 mM Tris-HCl, pH 7.5, 1.4 M NaCl, 20 mM EDTA, pH 8.0; 4% PVP; adding 1% β-mercaptoethanol immediately prior to

use) to 65°C.

2. Add 800 µL CTAB buffer to the 2.0-mL microcentrifuge tubes containing the samples and the steel beads.

3. Next, use a Bead Beater (BioSpec) to pulverise the samples. The silica-dried samples for 3 min at 2,500 oscillations per minute and the buffer-stored samples for 5 min at 3,000 oscillations per minute. In case the grinding is not complete, repeat this step once more.

4. Incubate grinded samples in a water bath at 65°C for 15 min with occasional swirling.

5. Cool the samples by keeping tubes on ice for about 2 min. Add 750 µL CIA solution (chloroform:isoamyl alcohol; 24:1) to each tube and invert 50 times. Centrifuge for 15 min at 10,000 rpm (Eppendorf centrifuge 5424). Transfer the supernatant (about 600 µL) to a new 1.5-mL microcentrifuge tube and repeat CIA step.

6. Transfer the supernatant (about 400-500 µL) to a new 1.5-mL microcentrifuge tube and add 0.7 vol (about 350 µL) ice-cold isopropanol; invert gently 10 times. Centrifuge for 15 min at 10,000 rpm. Discard supernatant without disturbing the pellet;

7. Wash the pellet by adding 1 mL 70% ethanol. Centrifuge for 10 min at 10,000 rpm and pour off ethanol; repeat this step, but adding 1 mL absolute ethanol. Dry pellets at room temperature (about 1 hour). Do not let them over-dry.

8. Resuspend each pellet in 30 µL TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8,0), containing RNAse A (10 mg/mL). Incubate DNA sample at 37°C for 30 min. and keep it at 4°C until subsequent use.

Efficiency of isolation of genomic DNA

The presence of aromatic rings in both purine and pyrimidines bases moieties allow for the DNA to have peak absorbance of UV light at 260 nm (A_{260}); thus

concentration of DNA can be estimated based on A_{260}. Contaminant proteins and phenolic compounds have peak absorbance of UV light at 280 nm (A_{280}), while other organic contaminants absorb UV light at 230 nm (A_{230}). Thus, the ratios A_{260}/A_{280} and A_{260}/A_{230} can be taken as indicators of DNA purity (Maniatis *et al.* 1982). A NanoDrop Spectrophotometer (Thermo Scientifics) provided the absorbance at two wavelengths (260 and 280 nm) and the ratios A_{260}/A_{280} and A_{260}/A_{230} and calculated the DNA concentration (expressed as ng/μL). The ratio A_{260}/A_{280} greater than 1.8 represent a high purity of the DNA, and the ratio A_{260}/A_{230} might be higher than the ratio A_{260}/A_{280}, as mentioned in the T042 Technical Bulletin of the NanoDrop Spectrophotometer (Thermo Scientifics), this value is commonly in the range of 2.0-2.2.

Analyses of variance (ANOVA) determined the effect of extraction period (D0, D7, D14, and D21), storage condition (DTT and AA), and their interactions on the final concentration of DNA. A second ANOVA evaluated the effect of extraction period (D14 and D21), storage condition (DTT, AA and SIL), and their interactions on DNA yield. Both ANOVAs had a completely randomised design, with three replications each. Furthermore, the Tukey's test was used to evaluate the differences between the treatments and the interactions. We performed the analyses in the R software (http://www.r-project.org/) using the ExpDes Package (Ferreira *et al.* 2013).

The samples were visually inspected for degradation using DNA electrophoresis. We ran 2 μL of each sample on 1% (w/v) agarose gels with 1x TBE buffer (w/v 10.8% tris base, 5.5% boric acid and 0.83% EDTA) at constant voltage (120V) for approximately 40 min. The gel was stained with 0.02% ethidium bromide (w/v) and the image recorded under UV fluorescence at an L-Pix Image system (Loccus Biotecnology). To aid visual inspection of the genomic DNA after electrophoresis, we loaded the agarose gel with a 1 kb DNA ladder (Promega) alongside the samples.

Competence of the genomic DNA as PCR template

We tested whether the genomic DNA was suitable as template for PCR. We performed amplifications using either specific gene regions and genome-wide, microsatellite markers (SSR) technologies. For specific gene regions, the genomic DNA was amplified using primer sets that targeted gene regions in either the nuclear or the chloroplast genomes. For the nuclear gene region, we amplified the internal transcribed spacer (ITS) region of the 18S–28S nuclear ribosomal DNA (nrDNA) — which includes two spacers (ITS1 and ITS2) and the intervening 5.8S gene — with the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White *et al.* 1990) and ITS Leu (5'-GTCCACTGAACCTTATCATTTAG-3', Baum *et al.* 1998). For the chloroplast gene region, we amplified the intron of trnL (UAA) with the primer pair C (5'-CGAAATCGGTAGACGCTACG-3') and D (5'-GTTTACTTTTGGGCATGCTTCG-3'), hereafter referred to as the trnL intron region (Taberlet *et al.* 1991).

The PCRs were performed with the conditions listed in Table 1; the amplifications were carried out on a Vapo Protect Eppendorf Thermal Cycler (Eppendorf) with three replications. For each specific gene regions, we loaded 4 μL of each amplification products on 1% (w/v) agarose gels for electrophoresis in 1x TBE buffer. The gels were stained with 0.02% ethidium bromide (w/v) and were run at constant voltage (100V) for approximately 40 min. Further the images were recorded under UV fluorescence at an L-Pix Image system (Loccus Biotecnology). Amplification of a given gene region (either ITS or trnL intron) was considered positive when a single, sharp band of the expected size was visible in the gel image. We calculated the percentage of positive amplifications within each extraction period (D0, D7, D14, and D21) and within each store condition (DTT, AA, and SIL).

Table 1. Description of PCR conditions and their thermocycler programs for nuclear (ITS) and chloroplastic (trnL intron) region, and also for microsatellites primers sets of *A. peregrina* (Acol15 and Acol16), *C. fissilis* (Ced54 and Ced65) and *D. wilsonii* (DW21 and DW33).

Reagents (unit)	ITS	trnL intron	Acol15 and Acol16	Ced54 and Ced65	DW21 and DW33
Final volume (μL)	25	25	12	12	12
DNA (ng)	800	400	80	40	80
Primer Forward (μM)	0.74	0.5	0.5	0.5	0.5
Primer Reverse (μM)	0.74	0.5	0.5	0.5	0.5
dNTPs (mM)	0.29	0.2	0.21	0.21	0.21
$MgCl_2$ (mM)	2.94	2	2.5	2.5	2.5
IVB Buffer [A] (5X)	1X	1X			
IB Buffer [A] (10X)	-	-	1X	1X	-
I0 Buffer [A] (10X)	-	-	-	-	1X
Taq DNA polymerase [B] (unit)	1.25	1.25	-	-	-
Taq DNA polymerase [A] (unit)	-	-	0.8	0.8	0.4
BSA [C] (mg/mL)	-	0.02	0.1	0.03	0.01
DMSO [D] (μL)	0.07	-	-	-	-
Termocycler programs (Vapo Protect Eppendorf Thermal Cycler)					
	94ºC 4'	94ºC 5'	94ºC 3'	96ºC 2'	94ºC 3'
	35x	35x	35x	30x	35x
	(94ºC 1'	(94ºC 1'	(94ºC 30"	(94ºC 1'	(94ºC 30"
	58ºC 1'	59ºC 1'	58ºC 1'	55ºC 1'	56ºC 30"
	72ºC 45")	72ºC 1'30")	72ºC 1')	72ºC 1')	72ºC 1')
	72ºC 5'	72ºC 5'	72ºC 20'	72ºC 7'	72ºC 20'

Legends: [A] Phoneutria; [B] Promega; [C] Bovine Serum Albumin (New England Labs); [D] Dimethylsulfoxide (Sigma).

For SSR genotyping, we used three distinct fluorescent primer sets that had been developed previously for *A. peregrina* var. *peregrina*, *C. fissilis*, and *D. wilsonii*, respectively. For each species, we used two primer combinations (Table 2). Firstly, we checked the PCR with 4 μL of each amplification products on 2% (w/v) agarose gels for electrophoresis in 1x TBE buffer. The gels were stained with 0.02% ethidium bromide (w/v) and were run at constant voltage (100V) for approximately 40 min. Further the images were recorded under UV fluorescence at an L-Pix Image system (Loccus Biotecnology). Then, the fragments were separated on a 96 capillary sequencer ABI PRISM 3130x1 DNA Analyzer (Applied Biosystems), and PCR products were sized relative to a molecular size marker (ROX 500, Applied Biosystems). The fragments were scored using the software GeneMapper version 4.0 (Applied Biosystems).

Table 2. Primer sequences of the microsatellite *loci* used to test the DNA isolated from cambium. F: Forward primer; R: reverse primer.

Primers	Primer sequence (5'-3')	Array	Fluorescence	References
Acol15	F: CATATGTCCAATCAGGTTAGAC R: CTCATTTGGTGACTGTAAGC	$(GA)_{10}$	HEX	Feres *et al.* (2012)
Acol16	F: AAGGTCCAAGGGTTATGC R: GGACGTTCTCTTGTCATGC	$(TC)_{11}(CA)_5AA(CA)_5$	NED	Feres *et al.* (2012)
Ced54	F: GATCTCACCCACTTGAAAAA R: GCTCATATTTGAGAGGCATT	$(GA)_{15}(AG)_6G(GA)_5$	6-FAM	Hernández *et al.* (2007)
Ced65	F: GAGTGAGAAGAAGAATCGTGATAGC R: GAGGTTCGATCAGGTCTTGG	$(GA)_7(CA)_{14}$	HEX	Hernández *et al.* (2007)
DW21	F: ATTCACCGGATCTAAACAATGG R: TTGTTGAAGAATGGAACCAAAA	$(AG)_7$	6-FAM	Vinson *et al.* (2013)
DW33	F: CGCAGCACTCACAAGAAGAG R: CTCCTCGTTTGCCATTTCTC	$(GA)_{12}$	NED	Vinson *et al.* (2013)

Results

DNA yield

Samples of cambium tissue yielded genomic DNA (Figure 3), regardless of the treatment tested. The mean yields of genomic DNA were 425.0 ng/µL (*D. wilsonii*); 417.5 ng/µL (*A. peregrina* var. *peregrina*); 298.7ng/µL (*C. fissilis*); and 165.7 ng/µL (*C. speciosa*). For DIM1, we had the following missing data: time periods D0 and D21 of both DTT and AA; and time period D21 of sample kept in silica gel beads.

Results of the first factorial ANOVA (4x2) indicated that storage conditions, extraction periods and their interactions significantly influenced (p-value<0.05) the yield of genomic DNA (Table 3). For ANA1, the highest amount of genomic DNA (2318.3 ng/µL) was obtained at D7 when the sample had been kept on DTT. For CED3, there were two situations that yielded high concentrations of genomic DNA: at D14 and D21 in DTT (356.7 and 201.9 ng/µL, respectively) and at D0 in AA (384.5 ng/µL). For ANA3 and CEI2 the AA storage yielded the highest amount of DNA (351.1 ng/uL and 182.4 ng/uL, respectively). Results of the second factorial ANOVA (3x2) showed that storage condition influenced (p-value<0.05) the yield of genomic DNA from samples taken from ANA1 (Table 3). Regardless of the extraction period, the highest yield of genomic DNA of ANA1 was obtained in either AA or SIL (322.2 and 813.2 ng/µL, respectively). For CED2, storage condition and extraction period showed significant interaction (p-value<0.05); the highest concentration of genomic DNA (797.4 ng/µL) was obtained at D14 in SIL.

DNA purity

Regardless of the species tested, tissue samples that had been stored in SIL yielded genomic DNA that degraded quickly upon extraction. The degradation of the genomic DNA was inferred owing to the presence of low-molecular mass smears after electrophoresis (Figure 3). Genomic DNA extracted from tissue samples that were kept in either DTT or AA exhibited sharp bands of high-molecular mass, with little to no smears, which suggested that the genomic DNA remained with high integrity (Figure 3).

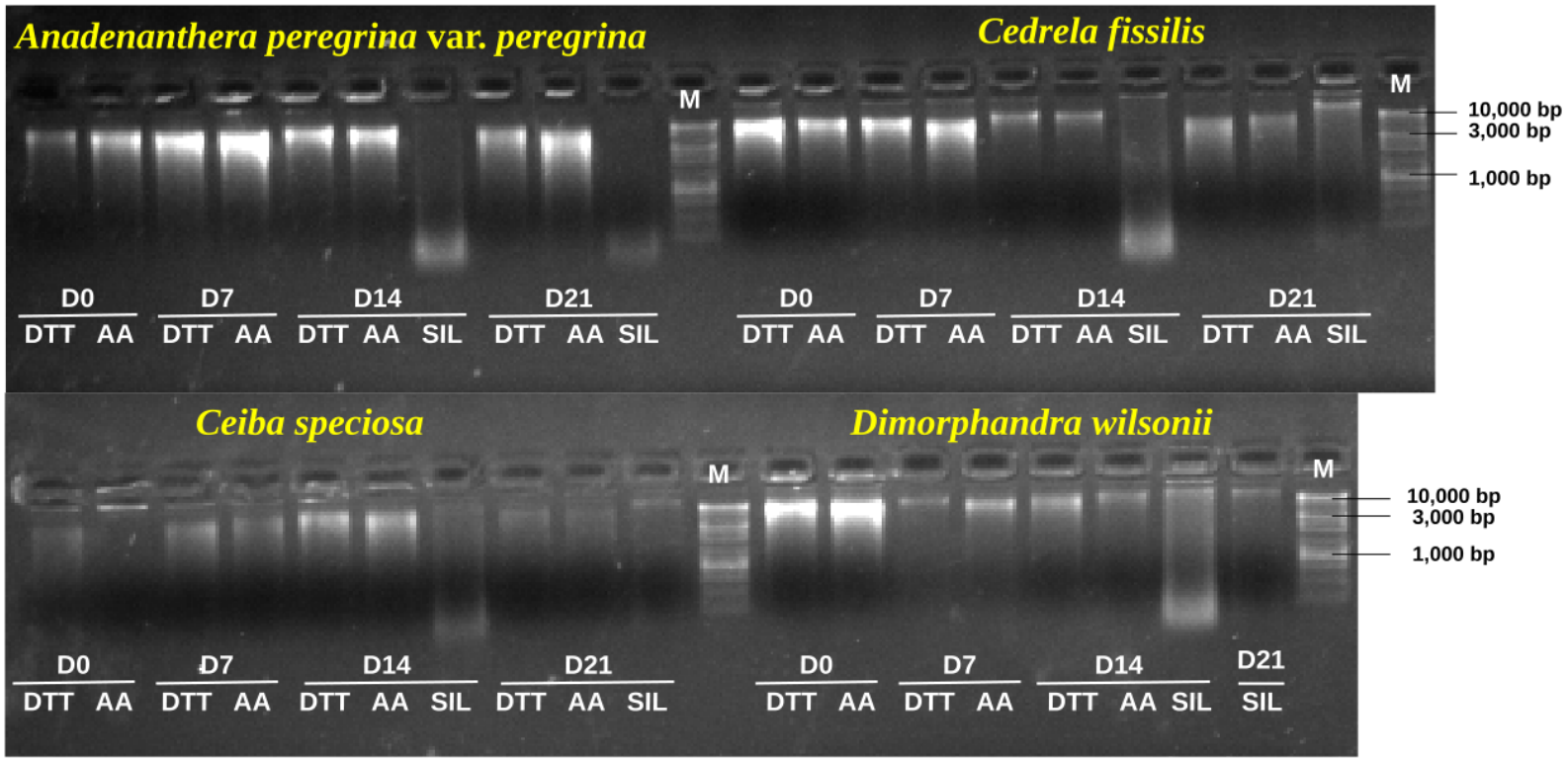

Figure 3. Electrophoresis of 1% agarose gel of the genomic DNA isolated from cambium tissue of one replicate of each treatment within the species *A. peregrina*, *C. fissilis*, *C. speciosa*, and *D. wilsonii* respectively. The treatments are each day after collection (D0, D7, D14 and D21) and store conditions (DTT, AA and SIL). M: 1kb DNA ladder (Promega).

Table 3. *P*-values of the ANOVA under two factorial analysis: the 4x2 (the DNA extraction day *vs*. the transport buffers) and the factorial 2x3 (the DNA extraction day *vs*. the storage conditions). df: degrees of freedom. See text for abbreviation of each specimen name.

	df	ANA1	ANA2	ANA3	CED1	CED2	CED3	CEI1	CEI2	CEI3
Variation Fact. 4x2										
Extraction day	3	0.00002**	ns	ns	ns	ns	0.001**	ns	ns	ns
Transport buffers	1	0.008**	ns	0.049*	ns	ns	ns	ns	0.030*	ns
Interaction	3	0.0004**	ns	ns	ns	ns	0.0001**	ns	ns	ns
Residuals	16									
Variation Fact. 2x3										
Extraction day	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Storages	2	0.019*	ns	ns	ns	ns	ns	ns	ns	ns
Interaction	2	ns	ns	ns	ns	0.029*	ns	ns	ns	ns
Residuals	12									

Legend: ns, non significant; * p-value < 0.05; and ** p-value < 0.01

The values of the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} for each variable (specimen) and treatment (extraction period and storage condition) are depicted in Figure 4. These values were taken as indicators of DNA purity. Values of the ratio A_{260}/A_{280} presented little variation among species, while values of the ratio A_{260}/A_{230} exhibited a high degree of variation (Figure 4). Regardless of the treatments, most of the samples exhibited a ratio A_{260}/A_{280} greater than 1.8 (90.9% for the samples of *D. wilsonii*; 84.4%, for *A. peregrina* var. *peregrina*; 78.9%, for *C. fissilis*; and 75.5% for *C. speciosa*). The average A_{260}/A_{280} ratios per species (± standard deviation) were 1.90 ± 0.14, for *A. peregrina* var. *peregrina*; 1.88 ± 0.18, for *C. fissilis*; 2.00 ± 0.37, for *C. speciosa*; and 1.88 ± 0.12, for *D. wilsonii*. Overall, the ratios A_{260}/A_{230} were lower than the ratios A_{260}/A_{280}: 1.49 ± 0.73, for *C. fissilis*; 1.01 ± 0.67, for *C. speciosa*; and 1.55 ± 0.46, for *D. wilsonii*. The only exception was *A. peregrina* var. *peregrina*, which showed A_{260}/A_{230} = 2.15 ± 2.31. These results suggest the genomic DNA is of high purity.

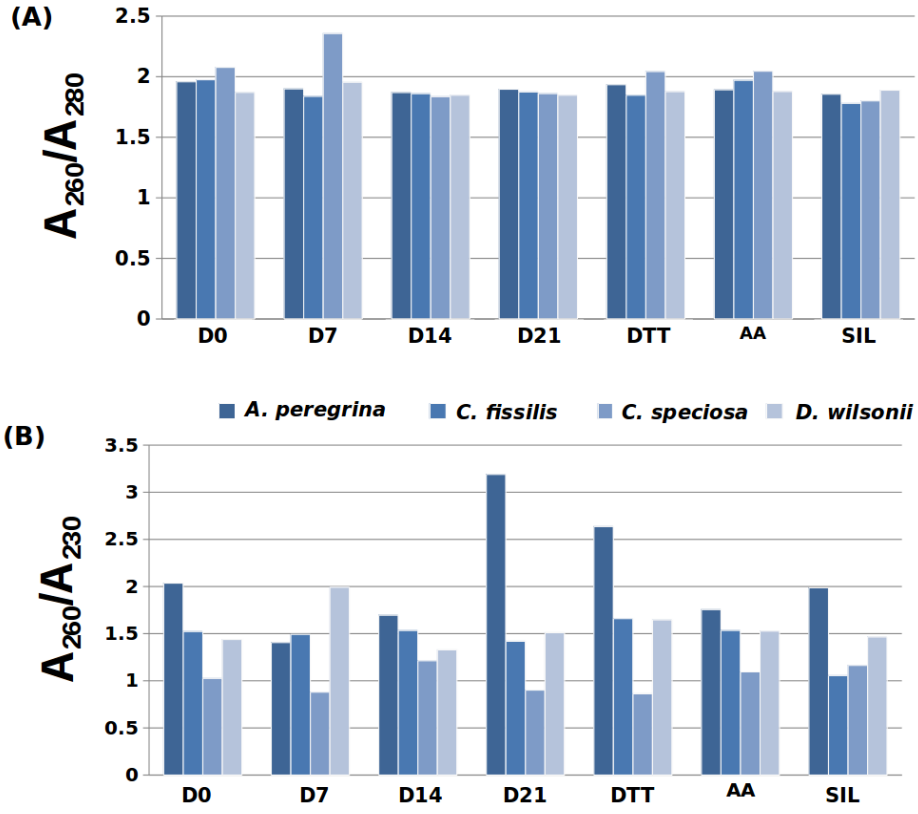

Figure 4. Chart of the NanoDrop absorbances of the average ratios (A) A_{260}/A_{280} and (B) A_{260}/A_{230} for every species (*A. peregrina*, *C. fissilis*, *C. speciosa*, and *D. wilsonii*). The results of the extraction periods (D0, D7, D14 and D21) are the averages for different store conditions, while the results of the store conditions (DTT, AA, and SIL) are the averages exhibited for every extraction periods. The averages included the three replicates of each treatment within the species.

Suitability of DNA as template for sequencing and SSR genotyping

Amplifications via PCR were successful, with the trnL intron and ITS regions reaching 89% and 76% of positive amplifications, respectively, out of 30 amplifications. *Anadenanthera peregrina* var. *peregrina* yielded the highest percentage of positive amplifications, for both trnL intron and ITS regions (96.67%, 91.11%, respectively); follow by *C. fissilis* (trnL intron, 93.3%; ITS, 83.3%), *Dimorphandra wilsonii* (trnL intron, 87.5%; ITS, 50.0%), *C. speciosa* (trnL intron, 78.9%; ITS, 61.1%). Table 4 indicates the percentage of positive amplifications considering the different treatments. Sharp bands characterised most of the amplifications, in both trnL intron region (Figure 5) and ITS region (Figure 6). Those results indicated suitability of those samples for sequencing.

Prior to submitting the SSR fragments for separation in the sequencer analyser, we visually checked the PCR products using electrophoresis in agarose gels to ensure that bands appeared as a result of the intentional amplification (Figure 7). For each of the four extraction periods (D0, D7, D14, and D21), we choose to show data from three specimens tested (ANA2, CED2, and DIM1) to illustrate the suitability of our DNA extraction protocol to SSR genotyping (Figure 8). Each chromatogram showed stutters as small peaks and the true alleles as high peaks following the stutters.

Table 4. General percentage of the positive PCR for two gene regions (trnL intron and ITS) with genomic DNA template obtained under four extraction periods (D0, D7, D14 and D21) and three storage conditions (DTT, AA and SIL). The percentages came from all PCR tried once with every sample from all three replicates of all four species.

	D0		D7		D14			D21		
	DTT	AA	DTT	AA	DTT	AA	SIL	DTT	AA	SIL
trnL intron	80.0%	83.33%	70.0%	90.0%	93.33%	100.0%	86.67%	90.0%	86.67%	96.67%
ITS	73.33%	70.0%	46.67%	73.33%	80.0%	93.33%	66.67%	86.67%	70.0%	86.67%

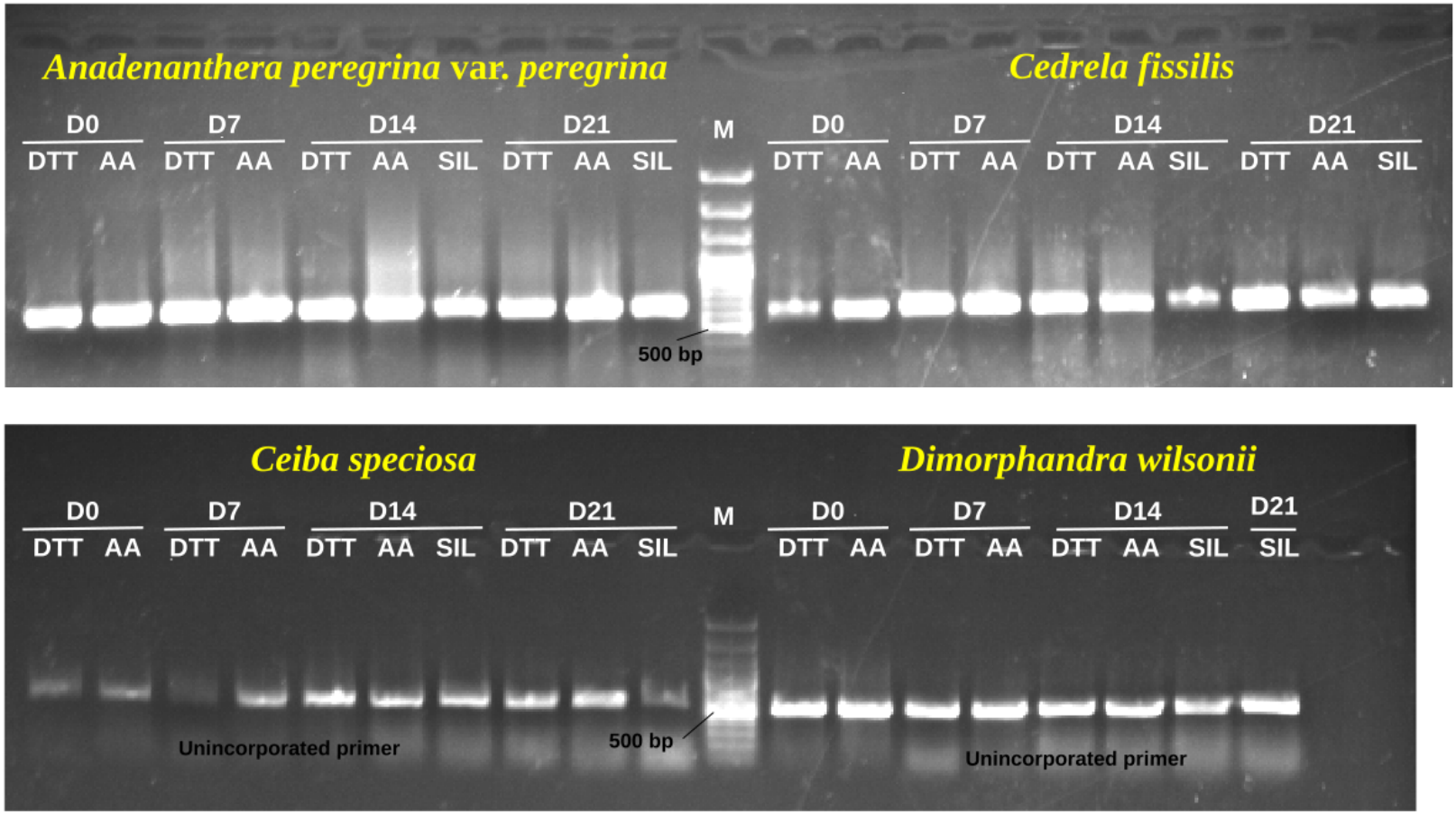

Figure 5. Electrophoresis of 1% agarose gel of the PCR that provided standard bands generated from genomic DNA from cambium tissue with the trnL intron region for one replicate of each treatment within the species *A. peregrina*, *C. fissilis*, *C. speciosa* and *D. wilsonii* respectively. For every species the bands are displayed for each extraction period (D0, D7, D14 and D21) and store conditions (DTT, AA and SIL). M: 500bp DNA ladder (Promega).

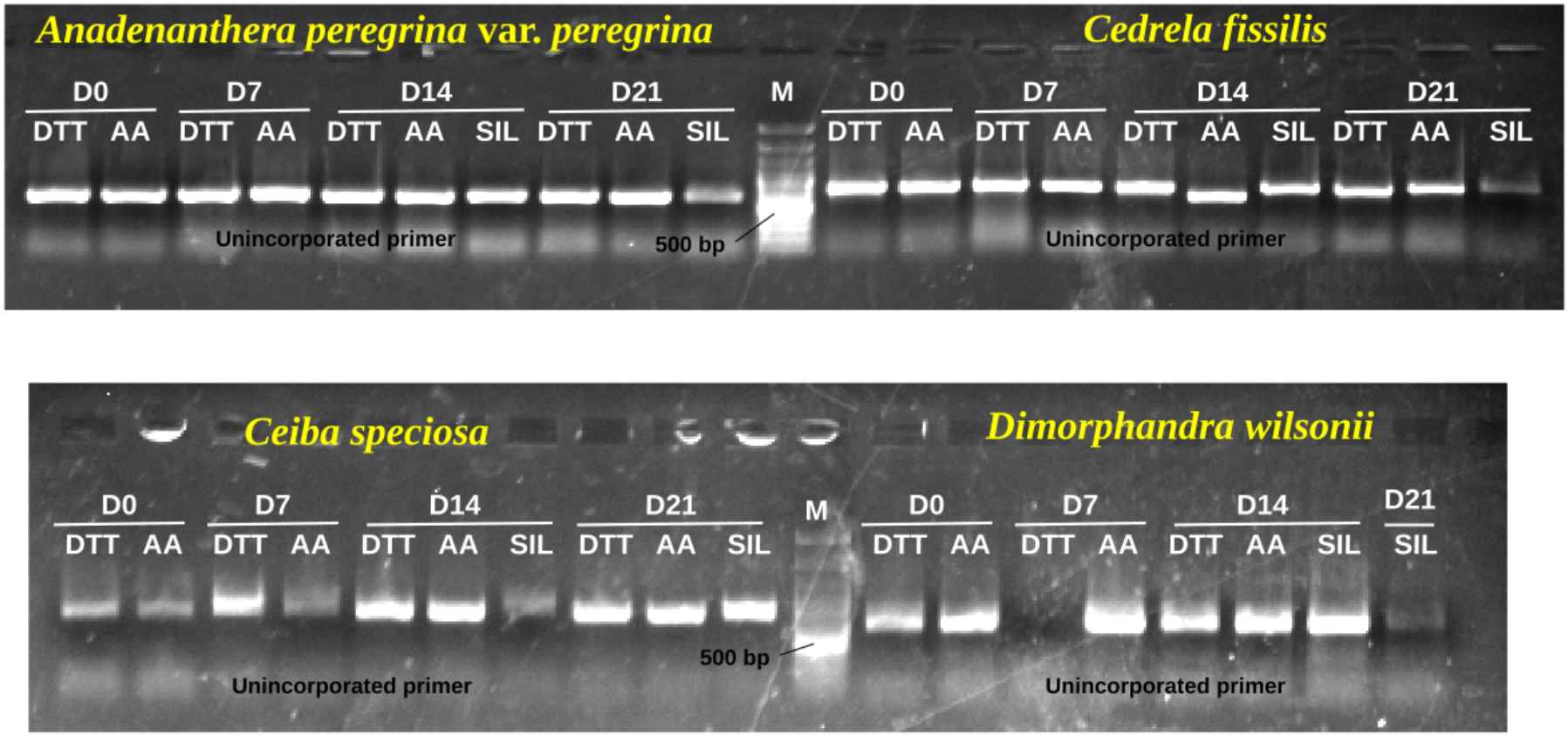

Figure 6. Electrophoresis of 1% agarose gel of the PCR that provided standard bands generated from genomic DNA from cambium tissue with the ITS region for one replicate of each treatment within the species *A. peregrina*, *C. fissilis*, *C. speciosa* and *D. wilsonii* respectively. For every species the bands are displayed for each extraction period (D0, D7, D14 and D21) and store conditions (DTT, AA, and SIL). M: 500bp DNA ladder (Promega).

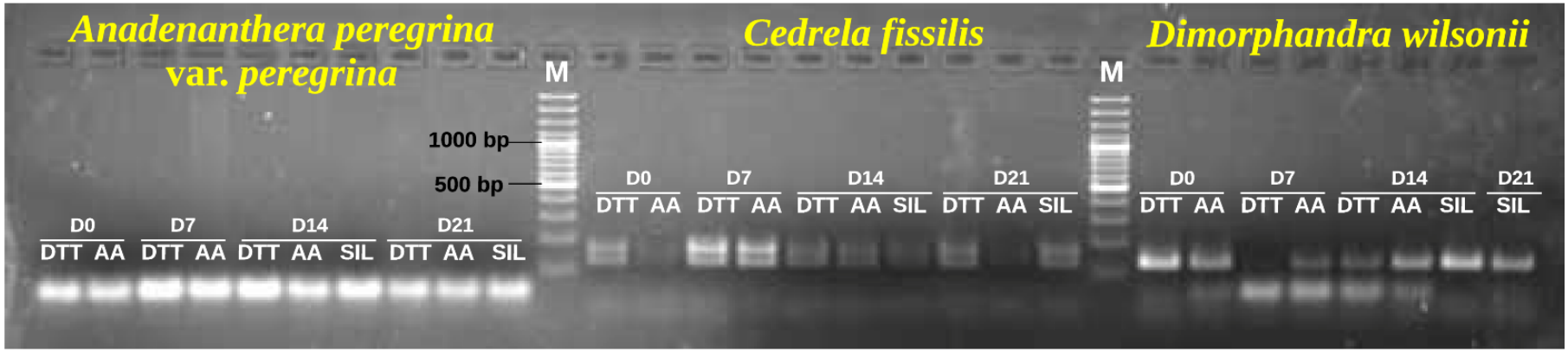

Figure 7. Electrophoresis of 2% agarose gel of the PCR that provided standard SSR bands generated with primers for one replicate of each treatment within the species *A. peregrina* (Acol15 and Acol16), *C. fissilis* (Ced54 and Ced65) and *D. wilsonii* (DW21 and DW33), respectively. For every species the SSR bands are displayed for each extraction period (D0, D7, D14 and D21) and store conditions (DTT, AA, and SIL). M: 100bp DNA ladder (Promega).

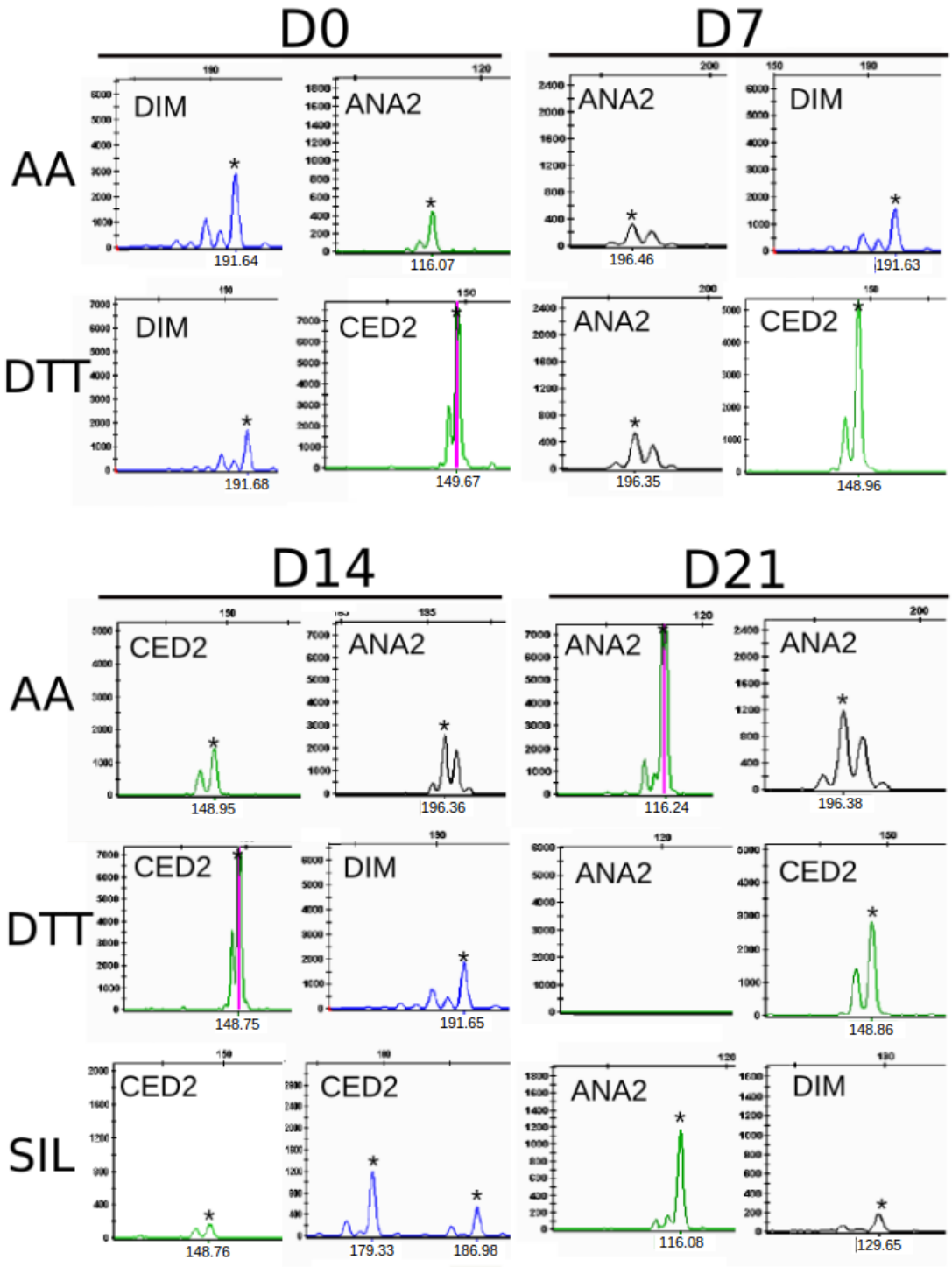

Figure 8. The peaks of the samples genotyped with SSR, viewed in the software GeneMapper. Here we example some peaks of one specimen of each species (ANA2, CED2 and DIM1) for each extraction period (D0, D7, D14 and D21) and store conditions (DTT, AA and SIL). Correct alleles are marked with asterisks. The different colours within the same specimen represent the different primers marked with different fluorescence. Only the specimen CED2 of D14 and SIL storage exhibited heterozygote genotype, with two peaks shown.

Discussion

In general, leaves are an excellent source of genomic DNA for molecular work. While working with neotropical tree species, one may come across circumstances in which leaf samples may be difficult to obtain. Many trees species - such as *C. fissilis* - can reach enormous heights, with most of the leaf-bearing branches standing high above the forest canopy. Removal of leaves from these specimens is a difficult task and may require specialised resources for tree climbing. The deciduousness of some tree species - such as that of *C. fissilis*, *D. wilsonii* and *C. speciosa* - limits the sampling to the period of the year in which the plants bear leaves. Thus, obtaining genomic DNA from cambium tissues may represent a convenient strategy during studies of genetic diversity of Neotropical tree species. Our methodology used cambium tissues as the source of genomic DNA for molecular studies of three species of Neotropical trees. The procedure was brief and consisted of simple steps, requiring neither liquid nitrogen nor additional commercial kits.

For the present study, any of the three storage conditions (DTT, AA, and SIL) were capable of preserving the quality of the genomic DNA from cambium tissues for subsequent extraction. Indeed, the SIL exhibited the DNA degradation and moderate number of positive PCR amplifications. However, the SIL storage condition exhibited higher amount of DNA when compared to the literature and also it was more convenient. For samples of *A. peregrina* var. *peregrina* stored in SIL, our methodology allowed us to recover genomic DNA (496.0 ng/mL) at a higher yield than obtained previously (364.0 ng/mL, Novaes *et al.* 2009). For recovering genomic DNA from SIL-stored sample of *D. wilsonii*, our methodology was more efficient (665.73ng/mL) compared to previously published protocols (415.5 ng/mL, Novaes *et al.* 2009). During field expeditions, silica gel is more amenable to handling than any of the buffer solutions. Once in the laboratory, the samples stored in silica can be kept at room temperature, are cost effective and require less space and maintenance for storage. We have stored leaf samples of *C. fissilis* in silica for more than ten years; those samples are routinely used as source of genomic DNA for molecular work always being viable.

Assessment among extraction periods following sampling of cambium tissues has not been reported previously. This assessment is crucial, given that samples can be submitted to DNA extraction only after the field expedition is over, which may take

days or weeks to complete. Thus, field studies would aim for a methodology that could delay the DNA extraction to the maximum extent possible without compromising the competence of the extracted DNA as template for PCR. Moreover, a methodology that would not require the samples to be maintained under sub-zero temperatures would save time, lower the overall cost of the field expedition, and avoid the requirement of keeping ice or liquid nitrogen nearby. When testing the four extraction periods (D0, D7, D14 and D21), we did not find differences on the yield of genomic DNA that would significantly impact its subsequent use as template for either sequencing (data not shown) or SSR genotyping. This finding suggested that cambium tissues can be harvested and kept under room temperature (for dried samples) and at 4ºC (for buffer-storage samples) prior to DNA extraction: a very convenient situation. In the present study, cambium tissues that have been preserved in SIL maintained their suitability for DNA extraction and yielded genomic DNA as template for PCR even after four weeks from the time they have been harvested from the tree trunk. Our data confirmed previous reports, which stated that silica provided more stable storage conditions than buffers (Colpaert *et al.* 2005).

In our experience, it was very difficult to process silica-dried samples during the first few days after taken the cambium tissues from the tree trunk. More likely, there was not enough time for full dehydration; thus pulverisation step using the Bead Beater was unproductive. We advise the use of both storage conditions: silica and buffer. The former would be very useful for long-time storage, while the latter would be useful for extracting the DNA just after harvest. We recommend the use of AA over DTT. Buffer AA is easier to prepare; ascorbic acid can be carried to the field separately and added to the remaining buffer ingredients only as needed.

One change we have made in the protocol was increase the PVP concentration of the extraction buffer, in order to improve the quality of the genomic DNA extracted. Increasing the concentration of PVP is crucial when the cambium tissues may detain a high amount of phenolic compounds (Rachmayanti *et al.* 2006). The fact that on average 82% of the DNA samples exhibited the ratio A_{260}/A_{280} above 1.8, which is the minimum rate acceptable (Sambrook and Russell 2001), indicates the good quality of our samples, by increasing the concentration of PVP. The methodology we presented herein improved previously published protocol (Novaes *et al.* 2009), because they were unable to allow PCR amplifications when frozen tree barks of *A. peregrina* and *D. mollis* were used as sources of genomic DNA, while we could amplified from cambium

tissue. The high quantity and purity of the genomic DNA was confirmed as the samples were suitable for their intended goal: PCR amplification of two gene regions (trnL intron and ITS) and SSR genotyping. Both trnL intron (from the cpDNA) and ITS (from the nuclear genome) are used widely as source of information for phylogeographic or phylogenetic studies in plants (Weiguo *et al.* 2005; Ghamkhar *et al.* 2007; Oliveira *et al.* 2010; Garcia *et al.* 2011; Cupido *et al.* 2013). Peak profiles in the chromatograms were exactly what one would anticipate for SSR genotyping. For the best of our knowledge, this is the first time a true multiplexing PCR system was carried out using genomic DNA that has been extracted from cambium tissues. Our experiments resulted in successful genotyping.

In summary, the protocol we reported herein was efficient in obtaining PCR-grade genomic DNA from samples of cambium tissue obtained from tree trunks. We tested our protocol successfully in four species of arboreal species (*Anadenanthera peregrina* var. *peregrina*, *Cedrela fissilis*, *Ceiba speciosa* and *Dimorphandra wilsonii*). We obtained amplification of PCR fragments under distinct conditions, including four extraction periods and three storage conditions. The genomic DNA was suitable for sequencing and SSR genotyping.

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CONCLUSÕES GERAIS

- Os diferentes padrões genéticos da população de altitude e o nível intermediário de estrutura genética refletem uma mistura de eventos que agiram sobre o Quaternário;

- Dispersão a longo prazo e vicariancia podem ter sido os processos mais importantes durante o LGM;

- As distribuição de habitats revelaram uma maior contração ao longo dos anos, desde LIG até o presente, sugeriando que no LGM a distribuição estivesse contraída (em relação ao LIG) e em expansão (em relação à distribuição presente);

- O uso de espécies vegetais como exmplo biológico revela um padrão complexo de diversificação, e traz novos *insights* sobre a história evolutiva da Mata Atlântica brasileira;

- O protocolo utilizado para extração de DNA de amostras de câmbio foi efetivo em obter produtos de PCR adequados para sequenciamento e para genotipagem com microssatélites;

- Não houve diferença entre os métodos de estocagem de amostra para extração, portanto sugerimos a utilização de do método mais fácil de manejar (SIL).