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DISSERTAÇÃO DE MESTRADO

Filogeografia do vinhático (*Plathymenia reticulata*, Leguminosae) e sua relação com mudanças da vegetação e do clima durante o Quaternário no Leste da América do Sul Tropical

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Renan Milagres Lage Novaes

Filogeografia do vinhático (*Plathymenia reticulata*, Leguminosae) e sua relação com mudanças da vegetação e do clima durante o Quaternário no Leste da América do Sul Tropical

Dissertação apresentada ao programa de Pós Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Mestre em Genética

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**"Filogeografia do Vinhático (*Plathymenia reticulata*, Leguminosae) e
Sua Relação Com Mudanças da Vegetação e do Clima Durante O
Quaternário No Leste da América do Sul Tropical"**

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“A mudança é a única constante na natureza”

Heráclito (535 - 475 A.C.)

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Resumo

Pouco se sabe sobre as mudanças históricas da vegetação do Leste da América do Sul Tropical (LAST) e estudos filogeográficos nesta região são escassos. Nesta dissertação foram analisados os padrões de distribuição geográfica de linhagens genealógicas da leguminosa arbórea *Plathymenia reticulata* dos biomas Mata Atlântica (MA) e Cerrado no LAST. Foram levantadas três questões relativas à evolução da espécie: 1) A atual distribuição de *P. reticulata* se manteve estável durante o Quaternário? Se não, como as populações da espécie reagiram às oscilações climáticas durante esse período? 2) A distribuição da diversidade do DNA de cloroplasto (cpDNA) corrobora a circunscrição recentemente proposta de que *P. reticulata* deve ser reconhecida como a única espécie do gênero? 3) Quais são os centros de diversidade genética e áreas prioritárias para esforços de conservação de *P. reticulata*? Procedimentos para a utilização da casca do tronco como fonte de DNA foram desenvolvidos para possibilitar ampla e eficiente amostragem de *P. reticulata*, e sua eficiência foi verificada para outras árvores da família Leguminosae da MA e do Cerrado. Quarenta e uma populações de *P. reticulata* compreendendo 220 indivíduos foram analisadas através de seqüenciamento das regiões do cpDNA *trnS-trnG* e *trnL-trnL-trnF*. Foram encontrados 18 haplótipos estruturados geograficamente. O estado de Minas Gerais, especialmente sua porção centro-norte, é um centro de diversidade genética e provavelmente a mais antiga área de ocorrência da espécie. A ele deve ser dada a maior prioridade para conservação. Por outro lado, as populações do nordeste do Brasil e do sul do Cerrado apresentaram diversidade muito baixa, exibindo quase que apenas haplótipos comuns, também encontrados na região central. O padrão geral sugere que as populações destas duas regiões se estabeleceram recentemente, a partir da região central. Recente origem da espécie ou extinção de algumas populações devido ao clima mais seco e frio durante o último máximo glacial podem ter sido responsáveis por isso. Associações a hipóteses de mudanças no passado da vegetação da América do Sul são discutidas. Aliados a outras evidências, nossos dados indicam uma divergência em andamento no gênero *Plathymenia*, mas não foram suficientes para subsidiar sua subdivisão em duas ou mais espécies.

Abstract

Little is known about the past vegetation dynamics in Tropical Eastern South America (TESA) and phylogeographical studies are still lacking. This dissertation focuses on the patterns of geographic distribution of genealogical lineages of the Leguminosae tree *Plathymenia reticulata* across the Atlantic Forest (AF) and Cerrado biomes in TESA. We raised three questions concerning the evolution of the species: 1) Was the current widespread distribution of *Plathymenia* stable during the Quaternary? If not, how did the species' populations reacted to the climatic oscillations during this period? 2) Does the chloroplast DNA diversity distribution support the recent classification proposed, that *P. reticulata* should be recognized as the single species of the genus? 3) Where are located *Plathymenia* centers of genetic diversity and priority areas for conservation of the species? A sequence of procedures for using stem bark as a source of DNA was developed to allow an extensive and efficient sampling of populations of *P. reticulata*. Their efficiency were checked for other Leguminosae trees of the AF and the Cerrado and for different storage conditions. Forty one populations comprising 220 individuals of *P. reticulata* were analyzed through sequencing the cpDNA regions *trnS-trnG* and *trnL-trnL-trnF*. Combined, the three markers resulted in 18 geographically structured haplotypes. The Brazilian state of Minas Gerais, especially its central-northern portion, is a center of genetic diversity. It is probably the most longstanding area of the distribution range of the species and should be given the highest priority for conservation. In contrast, populations from northeastern Brazil and the southern Cerrados showed very low diversity levels, almost exclusively with common haplotypes which are also found in the central region. The overall pattern suggests that the populations of those regions have been established recently, from central region sources. The recent origin of the species or the extinction of some populations due to drier and cooler climate during the Last Glacial Maximum could have been responsible for that. Associations with hypotheses of past vegetation changes in South America are discussed. Combined with other evidence, our data point to an ongoing divergence within the *Plathymenia* genus, but were not enough to support its subdivision into two or more species.

Introdução

A atual distribuição das espécies e de sua diversidade genética é produto da interação de fatores ecológicos, contingências históricas e da própria biologia da espécie (Hamrick & Godt 1996; Hewitt 1996; Schaal *et al.* 1998). O estudo da distribuição da diversidade genética pode permitir, portanto, a inferência sobre a ação desses fatores no presente e no passado e a abordagem filogeográfica tem sido muito utilizado para esse fim. O conceito de filogeografia foi introduzido por Avise *et al.* (1987) para designar o estudo da distribuição da diversidade genética num contexto geográfico e temporal. Ela busca relacionar linhagens genealógicas à sua distribuição geográfica, entre e dentro de populações, e, a partir daí, fazer inferências sobre a história evolutiva das espécies (Avise 2000). A análise filogeográfica permite inferir a respeito de diferentes aspectos desta história, como a identificação de padrões de fluxo gênico e dispersão, rotas migratórias, centros de origem, eventos de expansão e fragmentação de populações e a extensão deles, ocorrências de eventos de “gargalo de garrafa”, eventos de divergência, hibridização e introgressão entre linhagens divergentes (Comes & Kadereit 1998; Schaal *et al.* 1998; Hewitt 2001; Petit *et al.* 2005; Beheregaray 2008). Baseados nesses aspectos, associações a mudanças históricas climáticas, geológicas e ecológicas podem ser feitas, permitindo, por exemplo, a identificação de refúgios durante eras glaciais do Pleistoceno, de zonas de contato secundário entre linhagens divergentes e a datação de eventos geológicos (Comes & Kadereit 1998; Petit *et al.* 2003; Beheregaray 2008). Finalmente, a identificação de centros de diversidade genética e de linhagens raras tem implicações diretas para a conservação das espécies, inclusive daquelas de interesse econômico (Schoen & Brown 1993; Frankham 1995; Newton *et al.* 1999).

As flutuações climáticas que ocorreram durante o período Quaternário influenciaram significativamente a distribuição dos táxons e sua estruturação genética ao longo do tempo. O período Quaternário (1.8 milhões de anos atrás até o presente) é o período geológico mais recente da história do planeta, e é dividido em duas épocas, o Pleistoceno, que teve seu fim há 10.000 anos antes do presente (A.P.), e o Holoceno, que se iniciou logo em seguida (Souza *et al.* 2005). Registros geológicos indicam que

pelos menos 16 glaciações, intercaladas por épocas mais quentes (interglaciais) ocorreram ao longo desse período (Salgado-Labouriau 1994). Acompanhando as mudanças climáticas, as espécies alteraram sua distribuição, de maneira a satisfazer seus requerimentos ecológicos, levando a consideráveis migrações, fragmentações, expansões e extinções de populações (Hewitt 1996; Dynesius & Jansson 2000; Davis & Shaw 2001). As diferentes espécies e tipos vegetacionais reagiram diferentemente de acordo com suas características. Os períodos glaciais proporcionaram, em geral, condições mais frias e secas ao longo do globo terrestre e provavelmente diminuíram a distribuição de vegetações associadas a climas úmidos e quentes, como as florestas tropicais úmidas. O contrário deve ter ocorrido para vegetações associadas a climas secos e frios, como campos subtropicais, que devem ter se expandido durante estes períodos (de Oliveira *et al.* 2005). Inferências a respeito das dinâmicas vegetacional e climática do passado são feitas a partir de evidências provenientes de diferentes tipos de estudos. Estudos paleopalinológicos e paleontológicos com datações radiocarbônicas, estudos com isótopos de matéria orgânica, registros geológicos de variadas origens e modelagem computacional estão entre os tipos de fontes de informações para a inferência sobre climas passados (Webb & Bartlein 1992; Broecker & Hemming 2001; Souza *et al.* 2005). Nas últimas duas décadas, os estudos filogeográficos têm sido usados como uma fonte independente de dados sobre eventos passados e têm provado poder colaborar de forma importante para a sua reconstrução.

Estudos filogeográficos têm sido amplamente realizados, em especial com espécies de regiões do Hemisfério Norte. Hoje, a história da dinâmica dos organismos tem sido cada vez melhor conhecida em várias regiões, como por exemplo, na Europa temperada (Hewitt 1996), nos Alpes europeus (Tribsch & Schonswetter 2003), no Ártico (Abbott & Brochmann 2003) e na América do Norte (Brunsfeld *et al.* 2001). Apesar de seu alto nível de ameaça e riqueza de biodiversidade, o Leste da América do Sul Tropical (LAST; ou *Eastern Tropical South America - ETSA*) tem sido relativamente negligenciado com relação a estudos sobre a dinâmica da vegetação no passado. Mesmo com o aumento do número de estudos paleoclimáticos nos últimos anos, estudos filogeográficos são ainda muito escassos na região e o número de estudos conduzidos com espécies vegetais é notavelmente baixo (veja Beheregaray 2008). No

LAST, apenas três espécies de plantas do Cerrado foram estudadas (Olsen & Schaal 1999; Collevatti *et al.* 2003; Ramos *et al.* 2007), uma da Caatinga (Caetano *et al.* 2008) e não mais que dez espécies da Mata Atlântica (e.g., Lira *et al.* 2003; Salgueiro *et al.* 2004; Andrade *et al.* 2007; Ramos *et al.* 2008; Ribeiro *et al.* submetido)

Os estudos de filogeografia têm se baseado principalmente na análise da variabilidade dos genomas de organelas citoplasmáticas, os quais apresentam características favoráveis, como herança tipicamente uniparental, ausência de recombinação e haploidia (Birky 2001). Herança uniparental e ausência de recombinação facilitam a estimativa das relações genealógicas entre seqüências e, consequentemente, o estudo da evolução de linhagens (Schaal *et al.* 1998). Pelo fato de o genoma organelar ser haplóide e transmitido por somente um dos pais, seu tamanho populacional efetivo é menor que o do genoma nuclear, acentuando os efeitos de deriva gênica (Schaal *et al.* 1998). Enquanto estudos filogeográficos em animais baseiam-se em sua maioria no genoma mitocondrial (mtDNA), seqüências de mtDNA de plantas geralmente mostram baixas taxas de substituição de nucleotídeos e apresentam grande recombinação intramolecular. O genoma do cloroplasto (cpDNA) apresenta uma variabilidade um pouco maior e razoável conservação estrutural (Schaal *et al.* 1998) e é o genoma mais utilizado em estudos filogeográficos com plantas (Beheregaray 2008). A variação genética do cpDNA tem sido estudada principalmente através de microssatélites e seqüenciamento direto. Esta última abordagem permite a construção mais confiável das relações genealógicas entre os haplótipos, o que é altamente desejado em estudos filogeográficos (Schaal & Olsen 2000). As regiões do cpDNA utilizadas são principalmente as não-codificantes que, na grande maioria não são sujeitas a seleção e, portanto, evoluem principalmente pelos efeitos de deriva e mutação.

O gênero *Plathymenia* Benth. (Leguminosae, Mimosoideae) é neotropical e nativo da América do Sul (Warwick & Lewis 2003). Desde a descrição do gênero, duas espécies de *Plathymenia* têm sido comumente reconhecidas: *P. reticulata*, ocorrendo no Cerrado e *P. foliolosa*, na Mata Atlântica (Bentham 1842; Heringer & Ferreira 1972). As principais características utilizadas para a diferenciação entre as espécies eram o hábitat onde se encontravam, a altura da copa, o indumento da inflorescência, o

número de folíolos por folha e o número de foliolulos por folíolo (Heringer 1956; Warwick & Lewis 2003). Recentemente, Warwick e Lewis (2003) propuseram a sinonimização das duas espécies. Analisando exsicatas de ampla abrangência geográfica, eles não encontraram correlação entre morfologia e distribuição geográfica e tampouco encontraram características que diferenciassem as espécies. Todos os principais caracteres diagnósticos usados para diferenciá-las variavam de forma gradativa entre indivíduos de ambas as espécies. Eles também utilizaram a população "híbrida" relatada por Lacerda (2002), com marcadores RAPD, como uma indicação de fluxo genético entre as duas "espécies". Nesta dissertação, esta circunscrição de Warwick e Lewis (2003) é adotada.

Plathymenia reticulata Benth., conhecida popularmente como vinhático, tem uma ampla ocorrência no LAST. Entre os três domínios vegetacionais do LAST, *P. reticulata* é comumente encontrada na MA e no Cerrado, mas não o é na Caatinga xeromórfica (Fig. 1). Na primeira ocorre principalmente na floresta semi-decídua e, um pouco menos comumente, na floresta pluvial. No cerrado, cresce principalmente no cerradão e no cerrado *stricto sensu*. Os indivíduos podem atingir 30 m de altura na MA, mas não ultrapassam 12 m do Cerrado. *P. reticulata* ocorre em uma variedade de altitudes, mas raramente atingindo mais de 1.000 m (Lorenzi 1992; Warwick & Lewis 2003). É decídua, hermafrodita, polinizada por abelhas, e suas sementes dispersas pelo vento (Warwick & Lewis 2003; Goulart *et al.* 2005). Ela foi considerada pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) como uma das mais importantes e úteis espécies vegetais do Cerrado (Almeida *et al.* 1998), devido à alta qualidade de sua madeira e ao seu potencial uso para a recuperação de áreas degradadas (Heringer & Ferreira 1972). Sua madeira tem sido utilizada para diversos fins, incluindo confecção de mobiliário de luxo, acabamentos internos na construção civil e postes de cerca duradouros, tendo, portanto, significativa importância econômica (Heringer & Ferreira 1972; Lorenzi 1992). *P. reticulata* apresenta as principais características desejadas para um estudo filogeográfico focado em biogeografia histórica (Cruzan & Templeton 2000; Naciri-Graven *et al.* 2006). É uma árvore, com dispersão limitada de sementes, facilmente reconhecível no campo, mesmo sem estruturas reprodutivas, de dispersão improvável por humanos e tem distribuição ampla ao longo dos biomas em que ocorre.

Ela também não tem parentes próximos evidentes (Luckow *et al.* 2003), minimizando a probabilidade de hibridizar com espécies de outros gêneros.

Plathymenia reticulata é, portanto, um ótimo modelo para o estudo da história da vegetação do LAST. Nós utilizamos seqüências não-codificantes variáveis do DNA de cloroplasto de *P. reticulata* para verificar padrões de distribuição genética ao longo de sua distribuição geográfica e, a partir daí, compreender melhor sua história evolutiva e correlacioná-la com a dinâmica da vegetação passada no LAST. Além disso, amostrando populações de ambos os biomas, avaliamos a recente proposta de circunscrição do gênero por Warwick and Lewis (2003) com relação à variação do cpDNA. Foram levantadas três questões relativas à evolução de *P. reticulata* que guiaram nossos estudos: 1) A presente distribuição de *P. reticulata* foi estável durante Quaternário? Se não, como as populações da espécie reagiram às oscilações climáticas desse período? 2) Onde são os centros de diversidade genética e as áreas prioritárias para esforços de conservação de *P. reticulata*? 3) A distribuição da diversidade do cpDNA de *P. reticulata* suporta a classificação proposta por Warwick e Lewis (2003), que *P. reticulata* deve ser reconhecida como a única espécie do gênero?

A amostragem de um número significativo de indivíduos de espécies arbóreas para obtenção de DNA pode representar um empecilho significativo para estudos de diversidade genética. Tradicionalmente, folhas têm sido usadas como a fonte principal de DNA para esse tipo de estudo. Árvores de *P. reticulata* podem alcançar até 30 m na Mata Atlântica e seus troncos eretos, ramificando apenas a grandes alturas do solo, podem tornar o acesso a suas folhas muito difícil. Entre os procedimentos normalmente utilizados para alcançar as folhas de árvores altas estão a utilização de longos podões ou armas de fogo e até mesmo o emprego de escaladores de árvores especializados. Estes procedimentos têm vários inconvenientes: principalmente, podem ser muito demorados, ter custos elevados e estar indisponíveis em alguns casos. Durante dias chuvosos, podem ser ainda mais proibitivos, tornando o trabalho de campo algumas vezes inviável. O outro problema comum diz respeito à disponibilidade e qualidade das folhas. Espécies decíduas podem ter todas as suas folhas ausentes ou senescentes durante a estação seca, fazendo com que a amostragem de folhas nesse período seja impossível. Além disso, caso as folhas estejam severamente atacadas por

microrganismos, fungos ou pequenos invertebrados, produzindo metabólitos secundários como alcalóides (Coley & Barone 1996), a extração de DNA e sua qualidade podem ser comprometidos. Finalmente, o DNA destes organismos poderiam ser co-extraídos com o DNA das plantas e afetar análises subseqüentes. Todos estes problemas comuns podem ser facilmente superados com a amostragem da casca do tronco das árvores. Ao contrário das folhas, a casca geralmente pode ser facilmente amostrada no nível do solo, com ferramentas simples e em um tempo relativamente curto, e está disponível durante todo o ano. Por essas razões foi necessário o desenvolvimento de um protocolo que fosse eficiente em extrair o DNA de tecidos do tronco de *P. reticulata*. Em testes iniciais com três espécies, o protocolo já utilizado para extração do DNA das folhas de *P. reticulata*, assim como algumas outras variações, foi testado para sua casca sem sucesso. Dessa forma, foi necessário o desenvolvimento de um protocolo específico que aliasse praticidade, custo baixo e eficiência. O protocolo foi desenvolvido e, em seguida, sua eficiência foi testada para outras 17 espécies da família Leguminosae, nativas da Mata Atlântica e do Cerrado. Sua eficiência foi comparada entre três formas de amostragem e armazenamento de tecidos: folhas congeladas, cascas congeladas e cascas desidratadas em sílica de forma a proporcionar, ao final, diferentes alternativas para os pesquisadores interessados em estudos utilizando DNA de árvores desses dois biomas.

A dissertação está dividida em dois capítulos, redigidos na forma de artigo científico, em inglês. O primeiro artigo, metodológico, foi desenvolvido como uma ferramenta necessária para o desenvolvimento do segundo artigo. No primeiro artigo são descritos os procedimentos elaborados para a obtenção do DNA da casca do tronco de espécies arbóreas e sua eficiência é comparada entre 18 espécies arbóreas da família Leguminosae da Mata Atlântica e do Cerrado e entre diferentes formas de armazenamento. No segundo artigo, é apresentada e discutida a filogeografia de *Plathymenia reticulata* e suas relações com a história recente do Leste da América do Sul Tropical.

(a)



(b)



Figura 1 – Indivíduos de *Plathymenia reticulata* (a) na Mata Atlântica em Acaiaca/MG e (b) no Cerrado no Parque Estadual do Rio Preto/MG.

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Capítulo 1

An efficient protocol for tissue sampling and DNA isolation from the stem bark of Leguminosae trees

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ABSTRACT

Traditionally, molecular studies of plant species have used leaves as the source of DNA. However, sampling leaves from tall tree species can be quite difficult and expensive. We developed a sequence of procedures for using stem bark as a source of DNA from Leguminosae trees of the Atlantic Forest and the Cerrado. Leguminosae is an important species-rich family in these two highly diverse and endangered biomes. A modified CTAB protocol for DNA isolation is described, and details of the procedures for sampling and storage of the bark are given. The procedures were initially developed for three species, and then their applicability for 15 other species was evaluated. DNA of satisfactory quality was obtained from the bark of all species. The amounts of DNA obtained from leaves were slightly higher than from bark samples, while its purity was the same. Storing the bark frozen or by drying in silica gel yielded similar results. Polymerase chain reaction amplification worked for both plastid and nuclear genomes. This alternative for isolating DNA from bark samples of trees facilitates field work with these tree species.

KEY WORDS

DNA isolation, bark, trunk, cambium, trees, Leguminosae

INTRODUCTION

The number of biodiversity studies using molecular approaches, such as population genetics and molecular systematics, has increased considerably (e.g., Avise, 2000; Crawford, 2000). Typically, studies involving plant species have used leaves as the main source of DNA. However, sampling leaves from tall tree species can be a very laborious task and can pose a major obstacle to efficient and fast field work desirable for these studies.

Different problems can arise when depending on leaves as the only source of DNA. Among the procedures commonly used to reach the leaves of tall trees are the use of very long tree pruners or firearms and even the employment of specialized tree climbers. These procedures have several drawbacks: mainly, they can be too time-consuming, can have substantial costs and can be unavailable in some instances. During rainy days, they can be even more prohibitive, making field work sometimes impossible. The other common problem concerns the availability and quality of the leaves. Deciduous and semi-deciduous tree species can have all their leaves lost or senescent during the dry season, making the sampling of leaves in this period impossible. Moreover, if leaves are severely attacked by microbes, fungi or small invertebrates, producing secondary metabolites such as alkaloids (Coley and Barone, 1996), DNA isolation and its quality may be compromised. Finally, DNA from these organisms could be co-extracted with the plant DNA and affect further analysis. All these common problems could be easily overcome with the sampling of the stem bark from trees for DNA-based studies. In contrast to leaves, bark can usually be easily sampled from the ground level, with simple tools and in a relatively short time, and is available during all the year.

Stem bark comprises all tissues outside the vascular cambium, and in secondary states, as in tree trunks, includes the phloem, the periderm and the dead tissues outside the periderm. Among these tissues, the phloem is the best candidate for DNA isolation, as it has the greatest amount of live cells in the bark (Esau, 1977). The bark tissues can have different compounds in relation to the leaves and, due to phloem presence, can be especially rich in sugars, which are known to be a problem in plant DNA isolation and to

downstream applications (Fang *et al.*, 1992; Pandey *et al.*, 1996). Several protocols have been developed for plant DNA isolation (e.g., Rogers and Bendich, 1985; Doyle and Doyle, 1987; Csaikl *et al.*, 1998; Ribeiro and Lovato, 2007; Ivanova *et al.*, 2008), but few of them have addressed the problem of extracting DNA from trunk tissues, such as bark (e.g., Colpaert *et al.*, 2005; Rachmayanti *et al.*, 2006; Tibbits *et al.*, 2006). Most of the commercial kits for plant DNA isolation have been designed for leaves, and usually for model species (e.g., DNeasy® Plant Kit from Qiagen and the Wizard® Genomic DNA Purification Kit from Promega). Many of these protocols can be time-consuming or too expensive. Yet, none of these studies have tested the applicability of their protocols for Atlantic Forest or Cerrado species. To date, we are unaware of any population genetics study carried out on these biomes that used bark as a source of DNA.

The Atlantic Forest and the Cerrado biomes are among the most biodiverse and endangered areas of the world. They have been considered two of the 34 hotspots for biodiversity conservation in a global context (Mittermeier *et al.*, 2004). Besides, these two biomes are poorly studied and DNA-based studies are particularly scarce, especially those involving tree species. Adult trees from the Atlantic Forest can easily reach 20 to 30 meters tall (Por, 1992) and can have erect trunks that start branching just at great distances from the ground. Furthermore, many tree species from the Atlantic Forest and Cerrado are semi-deciduous or deciduous, being deprived of leaves during some months of the year.

The Leguminosae family is the third plant family in species number, with more than 18,000 species in more than 700 genera. It has great economic importance worldwide and is an important component of the main tropical vegetation communities (Lewis, 2005). Both in the Atlantic Forest and in the Cerrado, the family is one of those with the greatest number of species all over the great geographical range of these biomes (Heringer *et al.*, 1977; Oliveira and Fontes, 2000). Several tree species from the Atlantic Forest are known for their great economic value, especially due to their timber, for example Brazilian rosewood (*Dalbergia nigra*), braúna (*Melanoxylon brauna*), vinhático (*Plathymenia reticulata*) and Brazilwood (*Caesalpinia echinata*), as well as some of the Cerrado, for example, sucupira-preta (*Bowdichia virgilioides*), jacarandá-do-Cerrado (*Dalbergia miscolobium*) and again vinhático (Souza and Lorenzi, 2008).

In this study, we describe a modified CTAB method for DNA isolation of the stem bark of Leguminosae trees from the Atlantic Forest and Cerrado biomes of South America. Details of tissue sampling, storage and the DNA isolation protocol itself are given. The applicability of the methods for 18 species was checked. The performance of the protocol was compared between leaves and bark and between dried and frozen bark. This way, we hope to provide an alternative for obtaining DNA, apart from leaves, for investigators interested in molecular studies of trees from these biomes.

MATERIAL AND METHODS

Tissue sampling and storage

Leaves and bark from the plants were collected, and the first ones were frozen at -20°C while the second ones were both frozen and silica gel dried. Once dried, the bark samples were kept in refrigerators. The samples were kept under these conditions for at least one week before initial tests. Sampling was carried out during the end of the rainy season, with the exception of the bark samples from *Erythrina speciosa*, which were sampled during the middle of the dry season, when the species had no leaves at all.

Bark sampling was performed using a hammer and a chisel, common tools easily found in any hardware store. The chisel was hammered into the bark until reaching the wood of the plant, which generally offered more resistance to the chisel penetration. By doing this, we aimed to collect the inner portion of the bark, which contains the phloem (Esau, 1977). The bark thickness ranged from 0.5 to 3 cm, and a 1x1 cm square was enough to perform one isolation procedure. After collecting the inner portion, when it was possible, the remaining tissues were put back in place to assure fast bark regeneration. In order to avoid exposure of the trees to fungal diseases, we applied a Bordeaux mixture (which consists basically of a solution of copper sulfate and calcium oxide, also known in Brazil as "Calda Bordalesa") over the wound caused by the sampling procedure.

The modified CTAB method

Initial tests for DNA isolation were carried out with the classical Doyle and Doyle (1987) method with some slight modifications, using the bark of three species, *Dalbergia nigra*, *D. miscolobium* and *Plathymenia reticulata*. As these tests were not successful for one of the species initially tested, we looked for improvements that could be efficient but simpler than other procedures specially designed for bark DNA isolation. In the end, we obtained a protocol that was very efficient for the three species, and this was selected for testing with the other species. The protocol was based on that of Doyle and Doyle (1987) with main modifications based on Ferreira and Grattapaglia (1995) and Colpaert *et al.* (2005). The final protocol is described below.

Preparation of the tissue

Before the isolation procedure, thin slices from the inner portion of the bark were cut with a razor blade and were weighed. We standardized a quantity for each kind of sample: 100 mg for leaves, 125 mg for frozen bark and 75 mg for dried bark. These differences were based mainly on the amount of debris associated with each of them in the initial steps of the isolation procedure.

The DNA isolation procedure

1. Grind both tissues with mortar and pestle and liquid nitrogen until they form a fine powder. In general, grinding the bark is not difficult, being even easier than grinding leaves from some Cerrado species.
2. Immediately after thawing the samples, add 1 mL of a CTAB extraction buffer [2% CTAB; 1.4 M NaCl; 100 mM Tris-HCl (pH 8); 20 mM EDTA (pH 8); PVP 2%], 2% (20 µL) of 2-mercaptoethanol and 35 µL of proteinase K (1 mg/mL) to the powder.
3. After some more grinding, add 35 µL of 20% SDS (w/v) and mix until getting a homogeneous mixture.
4. Transfer the mixture to 2-mL tubes. For some species, especially the dried bark, some fibers may not be disrupted. In such case, these fibers must be removed from the powder, if not, they can hinder further steps.
5. Incubate tubes for 60 min at 60°C with occasional swirling.

6. After the samples cool to the room temperature, add 600 μ L of CIA [chloroform/isoamyl-alcohol (24:1)] to the tubes and homogenize them by gentle inversion for 5 min.
7. Centrifuge samples for 15 min at maximum speed and transfer the supernatant carefully to new 1.5-mL tubes.
8. Add 140 μ L of 10% CTAB (w/v) and 280 μ L of 5 M NaCl and, by gentle inversion, mix until homogenization.
9. Repeat steps 6 and 7.
10. Precipitate DNA by the addition of 1 volume of cold isopropanol and incubate tubes overnight at -20°C.
11. Centrifuge for 10 min at maximum speed and discard the supernatant.
12. Wash the pellet 2-3 times with cold 70% ethanol (v/v).
13. After the last discard, dry the pellet (usually 15 min at 37°C is enough), and then dissolve it in 30 μ L TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] with 10 μ g/mL of RNase A for 2 h at 37°C.

DNA quantification

DNA quantification and quality assessment were performed by visualization of products on agarose gels and by spectrophotometry. An aliquot of 1 μ L of total genomic DNA was used in the spectrophotometer NanoDrop™ (NanoDrop Technologies) according to the manufacturer's instructions. Two measurements were taken: the absorbance at 260 nm which reflects the DNA concentration and the ratio of the absorbances at 260 nm and 280 nm (A_{260}/A_{280} ratio), which reflects the ratio of nucleic acids to proteins in the sample (Sambrook and Russell, 2001).

DNA amplification

The suitability of the DNA obtained for polymerase chain reactions (PCR) was tested by the amplification of two widely used regions from the chloroplast and nuclear genomes, the *psbA-trnH* and the internal transcribed spacer (ITS) region of the rRNA DNA, respectively. Primers used for *psbA-trnH* and ITS regions were respectively trn H (GUG) and psb A described by Hamilton (1999) and CY1 and CY3 described by Wright

et al. (2006). PCR conditions and cycles were the same as those used by Ribeiro *et al.* (2007). Only dried bark and leaves were used for PCR tests.

Species tested

Eighteen tree species of the Leguminosae family were selected for the tests with the DNA isolation protocol, fourteen of them occurring in the Atlantic Forest biome, three in the Cerrado biome, and one in both biomes (Table 1). Selected species comprise all three subfamilies (and also the informal Cercidae) and are widespread across a recent Leguminosae phylogeny (Wojciechowski *et al.*, 2004), with representatives of the majority of the main clades. In this manner, we aimed to obtain results that could probably be extended to other species of the family. Two individuals were tested for the majority of the species, with the exception for *Bowdichia virgilioides*, *Dimorphandra mollis* and *Holocalyx balansae*, allowing us to test the reproducibility of the results.

RESULTS AND DISCUSSION

Together, the three steps for DNA isolation from bark, sampling the tissue, its storage in two different ways and the DNA isolation protocol described were very successful. It was possible to obtain enough DNA of satisfactory quality from the bark of all eighteen species tested. The amount of DNA obtained in relation to the amount of tissue used was very high, ranging from 70 ng/ μ L to more than 3,000 ng/ μ L, which corresponds to 20 ng to 850 ng of DNA for each milligram of dry tissue used, and an average of 310 ng/mg (Table 2). The DNA obtained for *Dalbergia miscolobium* was a bit brownish and could not be analyzed by spectrophotometry, but it could be done by electrophoresis. The quality of the DNA was high for most of the species, with 81% of the samples with a A_{260}/A_{280} ratio above the optimal limit of 1.8 (Sambrook and Russell, 2001). In general, leaves yielded higher quantities of DNA than bark, but there were some exceptions (Table 2). The DNA quality, in contrast, was in general comparable between leaves and bark. This enables the use of bark as an alternative as good as leaves for obtaining DNA.

For two species, *Dimorphandra mollis* and *Senna multijuga*, the protocol only was efficient with the bark samples. The mixtures obtained from leaves after the third step of the protocol were too viscous, indicating high amounts of polysaccharides. Indeed, DNA isolation from leaves of *D. mollis* is known to be difficult (Souza, H.A.V., personal communication). This result shows that for some species, using the bark could be preferable even when the leaves are easily accessible. *D. mollis*, for example, is a Cerrado species, and although its leaves are generally reachable by hand, sampling the bark could be a better choice to obtain higher quality DNA.

Differences were not observed between the dried and the frozen barks, making both kinds of storage equally satisfactory for bark DNA isolation. We did not test the storage of the tissues in CTAB buffer as this storage makes the field procedures a little more laborious, since pre-preparation of the buffer is needed. In addition, Colpaert *et al.* (2005) reported that, for their protocol, tissues stored in CTAB buffer yielded less DNA than those silica dried. Our results provide two simple and efficient ways to store the bark: frozen and dried in silica-gel. The choice between these two procedures can therefore be made according to the available resources of each field work.

For one species, *Erythrina speciosa*, the bark was sampled during the dry season, when the plants did not have any leaves at all. However, large amounts of DNA of good quality could be obtained from it, and these were comparable to those obtained from the leaves sampled at the end of the rainy season (Table 2). This result shows that the bark of this species can offer high quality DNA even in the dry season with the absence of leaves. Therefore, at least for this species, sampling would be possible all year long, even when the leaves are absent or in poor condition, making field trip scheduling more flexible. This could also be true for the other species tested, but tests are recommended before extensive sampling is made.

PCR performed for both genomic regions was successful in producing strong bands for all the samples tested (Figure 2), with the only exception for leaves from *Dimorphandra mollis*. This demonstrates that the DNA obtained was pure enough to be suitable for PCR amplifications of plastid and nuclear DNA. These regions are commonly used in plant population genetics and phylogenetics studies and the plastid

region was among the four regions pre-selected as a potential barcode for land plants (Chase *et al.*, 2007).

Some considerations are important when dealing with bark tissues. First, the freezing of the bark did not need to be done immediately in the field. For *Plathymenia reticulata* and *Dalbergia nigra*, the tissues could be maintained for four days in sealed coolers with ice or in refrigerators. Once in the laboratory, they were kept at -20°C and the DNA isolation performed well. Second, with regard to silica gel storage, special attention had to be given to the saturation of the silica. Bark tissues can be rich in sap and therefore in carbohydrates, soluble sugars and water content. This makes these tissues ideal environments for fungal development, and a few days without proper silica-gel replacement were enough to lose the entire sample. Therefore, either a large quantity of silica or more than two replacements are sometimes needed to dry the tissue completely. Finally, slight differences in the portion of the bark sampled can result in significant differences in the DNA obtained. In initial tests, we compared the inner, the middle and the outer portions of the bark, and significantly more DNA was obtained from the first one (data not shown). Therefore, correct sampling could be crucial to the success of the DNA isolation, and attention must be given to this step.

Our protocol proved to be a simple, fast, relatively inexpensive and effective method for DNA isolation from leaves and from dried and frozen bark tissues from Leguminosae trees from the Atlantic Forest and Cerrado. The Leguminosae family is a very diverse group both in terms of species and phytochemicals (Wink and Mohamed, 2003). Our protocol could successfully extract DNA from a wide range of Leguminosae species, which make us believe it would be suitable for other legume species, species of other families, and species from other biomes as well. Nevertheless, even species closely related to each other can have a great variability in their biochemical composition (Rachmayanti *et al.*, 2006). Thus, the result of the protocol could be, to some degree, particular for each species, and previous tests for other species are recommended before adopting it as a routine laboratory procedure.

In this paper, we provided different alternatives of sampling for investigators interested in conducting molecular studies in populations of Leguminosae trees from the Atlantic Forest and Cerrado. In the field, researchers can easily sample a larger number

of individuals with simple materials in a shorter time. These results can also make field trip planning easier since they provide some independence from specialized crew such as climbers, or equipment such as tree pruners or firearms, or costs associated with them. Finally, sampling can be carried out during periods when leaves are in poor condition, as in the dry season for deciduous species, and also when the integrity of leaves is affected by predators or diseases.

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Table 1. Species tested and their biomes of occurrence.

Species	Occurrence in Atlantic Forest and Cerrado
Caesalpinioideae	
<i>Bauhinia longifolia</i> D. Dietr.	Atlantic Forest
<i>Caesalpinia pluviosa</i> DC.	Atlantic Forest
<i>Hymenaea courbaril</i> L	Atlantic Forest
<i>Melanoxyylon brauna</i> Schott	Atlantic Forest
<i>Senna multijuga</i> (Rich.) H.S. Irwin & Barneby	Atlantic Forest
Mimosoideae	
<i>Anadenanthera peregrina</i> (L.) Speg.	Atlantic Forest
<i>Dimorphandra mollis</i> Benth.	Cerrado
<i>Piptadenia gonoacantha</i> (Mart.) J.F. Macbr.	Atlantic Forest
<i>Plathymenia reticulata</i> Benth.	Atlantic Forest and Cerrado
Papilionoideae	
<i>Bowdichia virgilioides</i> Kunth	Cerrado
<i>Centrolobium tomentosum</i> Guillemin ex Benth.	Atlantic Forest
<i>Dalbergia miscolobium</i> Benth.	Cerrado
<i>Dalbergia nigra</i> (Vell.) Allemao ex Benth.	Atlantic Forest
<i>Erythrina speciosa</i> Andrews	Atlantic Forest
<i>Holocalyx balansae</i> Micheli	Atlantic Forest
<i>Machaerium aculeatum</i> Raddi	Atlantic Forest
<i>Myroxylon peruiferum</i> L. f.	Atlantic Forest
<i>Platycyamus regnelli</i> Benth.	Atlantic Forest

Table 2. Summary of results for the DNA obtained for each species.

Species	Tissue	DNA conc. (ng/uL)	Yield (ng/mg)	DNA quality (A260/A280 ratio)	PCR amplification
<i>Anadenanthera peregrina</i>	L	643.2	214.4	1.96	C / N
	fB	611.0	166.6	2.01	n/a
	dB	364.0	72.8	1.96	C / N
<i>Bauhinia longifolia</i>	L	1035.2	345.1	2.02	C / N
	fB	660.9	180.2	1.83	n/a
	dB	758.3	151.7	1.94	C / N
<i>Bowdichia virgilioides</i>	L	1726.8	575.6	1.87	C / N
	fB	1751.4	477.7	1.34	n/a
	dB	1802.9	360.6	1.32	C / N
<i>Caesalpinia pluviosa</i>	L	1287.0	429.0	1.83	C / N
	fB	1436.3	391.7	1.64	n/a
	dB	1209.1	241.8	1.90	C / N
<i>Centrolobium tomentosum</i>	L	2564.0	854.7	1.74	C / N
	fB	992.8	270.8	1.49	n/a
	dB	1605.5	321.1	1.22	C / N
<i>Dimorphandra mollis</i>	L	-	-	-	- / -
	fB	704.6	192.2	1.91	n/a
	dB	415.5	83.1	1.89	C / N
<i>Dalbergia miscolobium</i>	L	1187.9	396.0	1.87	C / N
	fB	n/a	n/a	n/a	n/a
	dB	n/a	n/a	n/a	C / N
<i>Dalbergia nigra</i>	L	2413.5	804.5	1.95	C / N
	fB	750.6	204.7	1.96	n/a
	dB	707.8	141.6	1.90	C / N
<i>Erythrina speciosa</i>	L	1582.6	527.5	1.98	C / N
	fB	1041.0	283.9	1.90	n/a
	dB	1332.5	266.5	1.96	C / N
<i>Holocalyx balansae</i>	L	1934.8	644.9	1.95	C / N
	fB	763.4	208.2	1.90	n/a
	dB	2194.3	438.9	1.96	C / N
<i>Hymenaea courbaril</i>	L	889.7	296.6	1.99	C / N
	fB	724.6	197.6	1.95	n/a
	dB	642.0	128.4	1.98	C / N
<i>Machaerium aculeatum</i>	L	1763.2	587.7	1.97	C / N
	fB	420.8	114.8	1.92	n/a
	dB	843.9	168.8	1.90	C / N
<i>Melanoxylon brauna</i>	L	862.1	287.4	1.89	C / N
	fB	555.3	151.4	2.04	n/a
	dB	742.0	148.4	1.92	C / N
<i>Myroxylon perufiferum</i>	L	3177.1	1059.0	1.95	C / N
	fB	1636.9	446.4	1.92	n/a
	dB	1666.6	333.3	1.96	C / N
<i>Piptadenia gonoacantha</i>	L	973.4	324.5	1.99	C / N
	fB	379.1	103.4	1.86	n/a
	dB	284.9	57.0	1.78	C / N
<i>Platycyamus regnelli</i>	L	1426.9	475.6	2.01	C / N
	fB	794.5	216.7	2.01	n/a
	dB	1231.2	246.2	2.06	C / N
<i>Plathymenia reticulata</i>	L	662.5	220.8	1.92	C / N
	fB	69.9	19.1	1.16	n/a
	dB	99.6	19.9	1.57	C / N
<i>Senna multijuga</i>	L	-	-	-	C / N
	fB	1662.8	453.5	2.06	n/a
	dB	1037.3	207.5	2.00	C / N

Tissues are: **L**- leaf, **fB**- frozen bark, **dB**- dried bark. For PCR amplification, letters indicate successful amplification: **C** for the plastid region and **N** for the nuclear. **n/a**- not applicable. Yield was calculated based on the dry mass of the tissues. When two individuals were tested for one species, result is the mean value obtained.

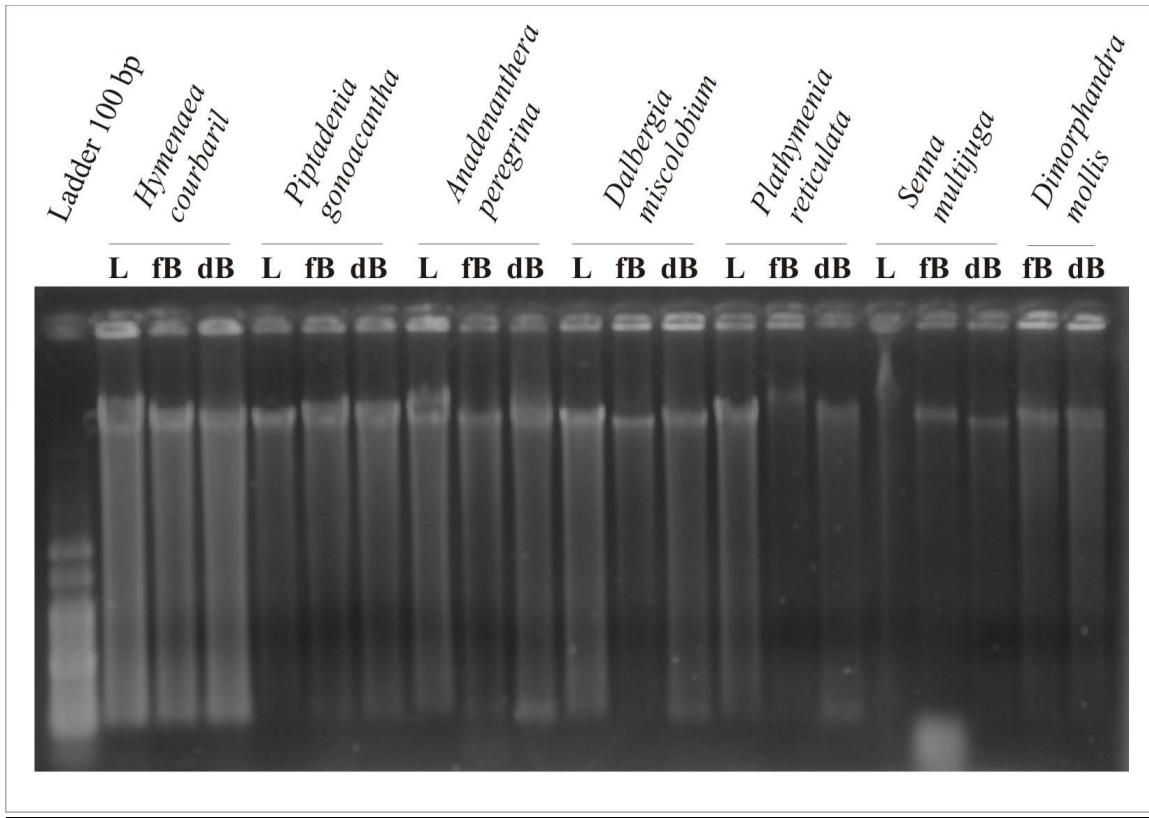


Figure 1. Total genomic DNA. **L**- leaf , **fB**- frozen bark, **dB**- dried bark.

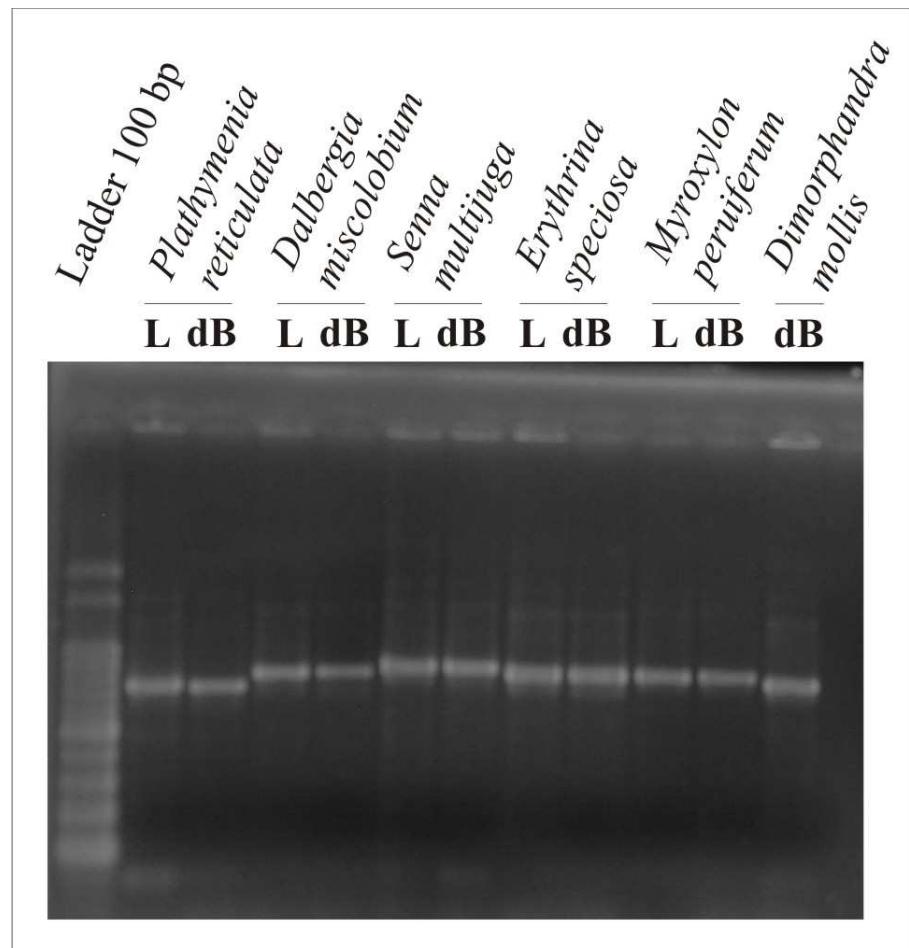


Figure 2. Amplification of the ITS region. **L**- leaf , **dB**- dried bark.

Capítulo 2

Phylogeography of *Plathymenia reticulata* (Leguminosae) reveals patterns of recent expansion towards northeastern Brazil and southern Cerrados in Tropical Eastern South America

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Abstract

Little is known about past vegetation dynamics in Eastern Tropical South America (ETSA). Here we describe patterns of chloroplast (cp) DNA variation in *Plathymenia reticulata*, a widespread tree in the ETSA Atlantic Forest (AF) and Cerrado biomes, but rarely found in the Caatinga biome. Forty one populations, comprising 220 individuals, were analyzed by sequencing the *trnS-trnG* and *trnL-trnL-trnF* cpDNA regions. Combined, they resulted in 18 geographically structured haplotypes. The Brazilian state of Minas Gerais, especially its central-northern portion, is a center of genetic diversity. It is probably the most longstanding area of the distribution range of the species and should be given the highest priority for conservation. In contrast, populations from northeastern Brazil and the southern Cerrados showed very low diversity levels, almost exclusively with common haplotypes which are also found in the central region. The overall pattern suggests that the populations of those regions have been established recently, from central region sources. The populations from northeastern Brazil originated from two colonization routes, one eastern (Atlantic) and one western (inland). The recent origin of the species or the extinction of some populations due to drier and cooler climate during the Last Glacial Maximum could have been responsible for that. Associations with hypotheses of past vegetation changes in South America are discussed. Combined with other evidence, our data point to an ongoing divergence within the *Plathymenia* genus, but were not enough to support its subdivision into two or more species.

Introduction

Past climatic oscillations have strongly influenced the current distribution of species and their genetic diversity. In pace with such changes, species shifted their ranges by tracking suitable environments that provided their ecological requirements (Hewitt 1996; Schaal *et al.* 1998; Davis & Shaw 2001). Phylogeography has made important contributions towards an understanding of past and present species distributions, and has been revealed as a valuable source of independent information about past events, enabling the identification of Pleistocene refugia, post-glacial routes and secondary contact zones, especially in the Northern Hemisphere (Hewitt 1996; Comes & Kadereit 1998; Cruzan & Templeton 2000). Moreover, the impacts of the imminent climatic change on the global biota would be better understood with an increasing knowledge of past events (Petit *et al.* 2008). Despite an increasing number of paleoclimate and paleovegetation studies on Eastern Tropical South America (ETSA) in recent years, phylogeographic studies are still scarce, especially those involving plant species. Most of them have attributed the observed genetic structure to the climatic oscillations of the Quaternary and among the few congruencies among them are a disjuncture between the southern and northern Atlantic Forest (e.g. Cardoso *et al.* 2000; Lira *et al.* 2003; Salgueiro *et al.* 2004; Andrade *et al.* 2009) and the high levels of genetic differentiation among populations (e.g. Cardoso *et al.* 2000; Collevatti *et al.* 2003; Salgueiro *et al.* 2004; Ramos *et al.* 2008; Andrade *et al.* 2009). To our knowledge, the present study on *Plathymenia reticulata* is the only one about a plant species that is widespread in both savanna and forest ETSA environments, with one of the broadest sampling efforts ever performed in these areas. With this effort, we expect to help reconstruct the past changes that occurred in ETSA during the Quaternary.

There are controversies surrounding the past climates of ETSA and the patterns of change were apparently complex, with regional variations. Climatic and vegetational changes have been recurrently reported and both cooler and drier conditions have been registered for both southeastern and northeastern ETSA during and before the Last Glacial Maximum (LGM; Ledru *et al.* 1998; Behling *et al.* 2000; Behling 2002; Behling *et al.* 2002; Anhuf *et al.* 2006; Ledru *et al.* 2006). ETSA is currently comprised of three main traditionally recognized biomes: the Atlantic Forest (AF), the Cerrado, a savanna

vegetation, and the Caatinga, a thorny woodland, sometimes classified as a seasonally dry tropical forest (SDTF; Fig. 1). The AF and Cerrado are among the 34 global hotspots for biodiversity conservation (Mittermeier *et al.* 2004). The factors that determine the occurrence of each of these biomes in ETSA are still controversial, but there is some consensus that rainfall, dry season duration and soil fertility are among the main ones (Eiten 1972; Por 1992; Rizzini 1997; Oliveira-Filho & Ratter 2002). The dry season is long and severe in the Caatinga, less pronounced in the Cerrado, and even milder or absent in the AF. The Caatinga has more fertile soils than the AF and Cerrado, where the soils are usually more dystrophic. Additionally, fire seems to play an important role in the Cerrado and is more frequent there than in the other two biomes (Eiten 1972; Oliveira-Filho & Ratter 2002). Past climate oscillations have changed the distribution of most of those factors across the landscape and, consequently, the range of the three biomes. A frequently recalled and discussed proposal about how the vegetation types responded to these climatic changes is the Pleistocene Refugia Hypothesis (Haffer 1969; Haffer & Prance 2001). This hypothesis proposes that during glaciation times, when climate was drier, forest formations persisted only in the moister areas - the Refugia - whereas in the drier areas they were replaced by more drought-tolerant vegetation types, like the Cerrado and Dry Forests. Refugia Hypothesis advocates have devoted little attention, however, to the role of some dry vegetation types during those past periods. Prado and Gibbs (1993) and Pennington *et al.* (2000) proposed the seasonally dry tropical forests (SDTF) as a general vegetation formation that might have had a wider and contiguous distribution in the past, probably invading current moist forest and Cerrado areas during the drier periods. The disjunctive SDTF pattern observed today was then formed after the increase of moisture and re-expansion of moist forest and Cerrado. On the other hand, Behling (2002) proposed that in cooler times, as in the LGM, subtropical grasslands from southern Brazil spread hundreds of kilometers northward, replacing both AF and Cerrado up to latitudes of 20°S and retreating again to the south only upon deglaciation.

Plathymenia Benth. (Leguminosae, Mimosoideae) is a Neotropical genus native to South America. It was traditionally recognized as comprised of two vicariant species: *P. foliolosa* in the AF and *P. reticulata* in the Cerrado, both known in Brazil as “vinhático”

(Bentham 1842; Heringer & Ferreira 1972). Warwick and Lewis (2003) recently revised this circumscription and synonymized *P. foliolosa* onto *P. reticulata*, thereby making the genus monospecific. They argued that there is a continuum in the characters used to distinguish them and that the differences were unrelated to geography. They also used the “hybrid” population reported by Lacerda *et al.* (2002) as an indication of gene flow between the two “species”. We adopted this latter circumscription herein. *P. reticulata* is widespread in both the AF and Cerrado, but not in the typical xeromorphic Caatinga (Fig. 1). The species occurs at a range of altitudes, but is rarely found at more than 1000 m above sea level (a.s.l.; Warwick & Lewis 2003). Two main factors seem to limit the range of *P. reticulata*: severe droughts and low temperatures. Taking only these two factors into account, it is possible to explain why *P. reticulata* is not found in the Caatinga core area (severe droughts), subtropical AF (low temperatures), and above 1000 m a.s.l. (low temperatures). If this is true, we would expect that any change in these climatic variables across ETSA would lead to range shifts of *P. reticulata* populations according to their needs, as well as of other species with similar climatic requirements.

Plathymenia reticulata exhibits some desirable features for a phylogeographic study focusing on historical biogeography (Cruzan & Templeton 2000; Naciri-Graven *et al.* 2006), mainly that it is a tree, with limited seed dispersal, not likely to be dispersed by humans, and without recognized close relatives, thus minimizing the probability of hybridization with species from other genera. It is deciduous, hermaphroditic, pollinated by small generalist bees and wasps, and wind-dispersed (Warwick & Lewis 2003; Goulart *et al.* 2005). *P. reticulata* is thus a good model to assist in understanding the past vegetation history of ETSA. We used chloroplast DNA (cpDNA) diversity to help elucidate the evolutionary history of the species and correlate it with the past vegetation dynamics of ETSA. To guide our study, we raised the following three questions on the evolution of *P. reticulata*: 1) Was the current widespread distribution of *P. reticulata* stable during the Quaternary? If not, how did the populations of this species react to the climate changes of that period? 2) Does the distribution of cpDNA diversity support the *Plathymenia* circumscription proposed by Warwick and Lewis (2003), in which *P. reticulata* should be recognized as the only species of the genus? 3) Where are the

centers of *P. reticulata* genetic diversity and the priority areas for conservation of the species?

Materials and Methods

Sampling strategy

Forty-one populations were sampled across the main vegetation types in which *P. reticulata* occurs (Table 1). Our sampling covered most of the distribution of the genus (Fig. 1), encompassing almost its entire range in the AF - corresponding to the distribution of the former *P. foliolosa* - and also most of its range in the Cerrado. A total of 220 individuals were analyzed. In the field, fertile individuals or those thicker than five cm in diameter at breast height were sampled at random. Three to ten individuals located at least 20 meters and at most two kilometers apart were sampled in each population. We classified the sampling locations into three types, according to vegetation: Atlantic Forest, Cerrado and Ecotonal Zones between the two biomes, in a total of 15, 22 and 4 populations, respectively. Samples from the source populations of the types of the two most traditionally recognized *Plathymenia* species were collected – Crato in Ceará state, for *P. foliolosa*; and Minas Gerais state, for *P. reticulata* (Warwick & Lewis 2003).

DNA isolation, amplification and sequencing

Individual trees were sampled by the collection of either leaves or bark tissue, which were dried on silica gel or frozen at -20°C. Total DNA was isolated as in Novaes *et al.* (2009). Fourteen non-coding regions of the chloroplast genome were assessed for amplification, sequencing and variation (the tested primers are available from R.M.L.N. upon request). Three of them were chosen for population analysis based on sequence quality and degree of variation: the intergenic spacers *trnS-trnG* (SG) and *trnL-trnF* (LF) and the intron *trnL* (L). The first region was amplified and sequenced with the primers from Hamilton (1999); the two latter were amplified with primers c and f and sequenced with primers c-f from Taberlet *et al.* (1991). The latter two regions (LF+L) will be addressed in combination from this point forward.

As no intra-population variation was observed in a prior screening, we decided to examine five individuals per population (when available). In doing so, we also favored analyzing more populations in detriment of a higher number of individuals per population. Exceptions were made for relatively isolated populations, for which we enlarged the number of individuals to compensate for the low number of local populations sampled (Table 1). While the SG region of all individuals was sequenced, only part of them (49%) underwent sequencing of the other two regions (LF+L). Previous results showed that these two latter regions were less variable and had a subset of the diversity found in the SG region. Therefore, if different individuals of a same population had the same SG haplotype, we assumed that this was also true for the other two markers (LF+L). When the SG haplotypes differed, all regions were sequenced. All analyzed individuals with the same SG haplotype did, in fact, present the same LF+L haplotype. Ultimately, we considered the complete haplotype for all 220 individuals.

Each polymerase chain reaction (PCR) mix contained 10 ng of genomic DNA, 1X Taq reaction buffer 1C [Phoneutria; 2.0 mM of MgCl₂, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100], 10 µg of bovine serum albumin (BSA), 200 µM dNTPs, 0.5 µM of each primer, and 1 U of Taq polymerase (Phoneutria) in a final volume of 25 µL. The amplifications were performed in Eppendorf thermocyclers using an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 1 min, and extension at 72 °C for 2 min, plus a final extension step of 72 °C for 10 min. The PCR products were visualized on 1% TAE agarose gels and bidirectionally sequenced after purification with 20% polyethylene glycol (PEG). Automated sequencing was carried out using the DYEnamic ET dye terminator sequencing Kit (GE Healthcare), followed by analysis in a MegaBACE 1000 automated sequencer (GE Healthcare) according to the manufacturer's instructions.

Data Analysis

The individual consensus sequences were aligned through ClustalW (Thompson *et al.* 1994) implemented in MEGA4 (Tamura *et al.* 2007), then carefully improved manually. The sequences were deposited in GenBank under accession number GQ141980-GQ142000. Manual alignment of the sequences was performed considering the

mechanisms of non-coding cpDNA evolution described by Kelchner (2000). To help in this task, we estimated the secondary structures using mFold (Zuker 2003), in order to visualize potential stem-loops, which can act as mutational triggers (Kelchner 2000). All detected polymorphisms were checked with the original electropherograms and, if doubt persisted, independent PCR reactions were carried out in order to confirm them. Among the variable mononucleotide repeats observed, only one, in which the number of repeats could be assigned with high confidence, was used in our analysis. Although we could infer some mutational mechanisms in our sequences, no clear method on how to treat them differentially has been proposed so far and any differential weighting that we applied would be highly arbitrary. We therefore opted to handle these variations conservatively, assigning equal weights to all of them. Each indel was considered as a single mutational event and codified as a 5th state character, except in the maximum parsimony (MP) analysis (see below). In order to assure that only high-confidence sequences would be analyzed, both extremities of both regions were excluded. All analyses were performed with the three regions in concatenation.

The genealogical relationships among the haplotypes were estimated using two network approaches, namely median-joining as implemented in Network (fluxus-engineering.com) and statistical parsimony as implemented in TCS (Clement *et al.* 2000). MP trees were also built to check for congruence between them and the networks results. The gaps were coded using the Modified Complex Indel Coding (MCIC) method (Muller 2006) implemented in Seqstate (Müller 2005). A heuristic search with 1000 replicates, random stepwise addition, tree-bisection-reconnection (TBR) branch swapping, and “Multrees” in effect was carried out in PAUP* (Swofford 1998). A strict consensus tree was built based on the most parsimonious trees obtained. Branch supports were estimated by 1000 bootstrap replicates.

Molecular diversity indexes, Tajima’s D (Tajima 1989), and Fu’s F_S (Fu 1997) neutrality tests were performed in Arlequin 3.1 (Excoffier *et al.* 2005). The molecular distances among the sequences were estimated through pairwise differences. Allelic richness after rarefaction to 20 individuals [A₍₂₀₎], corresponding to the size of the smallest of eight population groups compared, was computed in Contrib 1.02 (Petit *et al.* 1998). Between-population genetic differentiation was measured through G_{ST} and N_{ST}

using Permut 2.0 (Pons & Petit 1996). The hierarchical partitioning of genetic diversity was calculated by molecular variance analyses (AMOVA; Excoffier *et al.* 1992) in Arlequin 3.1 (Excoffier *et al.* 2005). In one of these analyses, the populations were grouped according to the traditional taxonomic subdivision of the genus (Bentham 1842; Heringer & Ferreira 1972). A spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002) was carried out to identify potential geographically correlated lineages. SAMOVA clusters populations into a user-defined number of groups (k) that are geographically homogeneous and exhibit maximal genetic differentiation, as measured by F_{CT} (Dupanloup *et al.* 2002). The SAMOVA was run with $k = 2$ to 14, with 1000 permutations.

To estimate the diversification time of the *P. reticulata* lineages, we used a Bayesian approach implemented in BEAST v. 1.4 (Drummond & Rambaut 2007). The time of the most recent common ancestor (TMRCA) of all haplotypes was estimated under a GTR+I substitution model, given by ModelTest 3.7 (Posada & Crandall 1998), and an uncorrelated lognormal relaxed clock. Three independent runs were carried out, of 10^8 generations with sampling at every 1000 generations. We used the estimated substitution rate for the *trnL-trnF* region of *Inga* species, namely 1.3×10^{-9} substitutions per site per year (Richardson *et al.* 2001). The *Inga* genus shares many features with *Plathymenia*, especially in that it belongs to the same subfamily, is composed of trees and shrubs and is of Neotropical origin.

Results

Patterns of variability

A total of 894 and 875 base pairs (bp) of aligned positions were obtained for the LF+L and SG regions, respectively (Table 2). Five sites were variable in the LF+L, four of which were substitutions and one was a mononucleotide repeat variation. A greater length variation was observed in the SG region, with sequences ranging from 733 bp to 774 bp. A large insertion/deletion (indel) of 604 bp was observed in four individuals of the jfc population, leading to only 292 bp of aligned positions, what reinforces the assumption that the SG region might be selectively neutral. This haplotype (S) was used only for some qualitative analyses since its relationship with the other haplotypes could

not be estimated. A total of 23 variation features were found in SG, specifically 11 substitutions, 11 indels (ranging from 5 to 604 bp), and one 60-bp inversion (Table 2).

After careful evaluation of these SG polymorphisms, three were excluded for the population analysis. First, the inversion was found to be involved in a hairpin of a stem-loop structure and was very homoplasious in our data. Minute inversions can have a high mutation rate and were removed from our analysis as suggested by Kelchner (2000). Second, two close-together substitutions in haplotype J seemed to be non-independent from each other, since they were complementary to each other in a stem-loop structure. One of the substitutions could have resulted from a compensatory mutation which would maintain the free energy of the stem-loop structure (Kelchner & Clark 1997), and therefore was also excluded. Finally, one indel in haplotype E could not be unambiguously aligned and was excluded from our analysis, as also suggested by Kelchner (2000). Although these latter two character exclusions reduced the variation and divergence among the haplotypes, they did not significantly affect the number of haplotypes or the network configuration. Altogether, 25 variable characters were analyzed in the 1,769 bp of the three aligned regions, resulting in a total of 18 cpDNA haplotypes (Table 2), a haplotype diversity (H_d) of 0.899, a nucleotide diversity (π) of 0.0025, and a mean number of pairwise differences among haplotypes of 4.06.

Relationship among haplotypes

Quite similar topologies were obtained from the two different network approaches used to infer the relationships among the haplotypes and only the statistical parsimony network is shown (Fig. 2a). The consensus tree showed similar results to the networks (Fig. 2b), with the main clades and structure persisting. In both approaches, we observed seven divergent lineages plus haplotypes A, B, and C, which formed a lineage in the networks but were not grouped in the MP tree. We defined each lineage as a group of haplotypes connected by no more than one mutational step and set apart from other lineages by two or more mutational steps (Fig. 2a). The only exception was lineage III, which haplotypes were connected by two mutations. Most lineages corresponded to the 1-step clades of Templeton *et al.* (1995), while the remaining ones to the 2-step clades. Two lineages (V and VIII) had only one haplotype each, and these

were the most divergent observed haplotypes. A central common haplotype was not found in the networks, but rather many divergent lineages connected by median vectors. Two of the eight lineages (I and VI) were comprised almost exclusively of Atlantic Forest (AF) populations, while most of the Cerrado populations were scattered across five lineages (II, IV, V, VII, VIII; Table 1, Fig. 2 and 3b). The remaining lineage (III) was comprised of one AF, two Cerrado, and one ecotonal population. Each ecotonal population had a distinct haplotype and lineage, thus they were scattered over the network. Five lineages have a restricted geographical distribution, while the other three (I, II and III) are widespread. These latter three lineages cover a very extensive area and each of them ranges over more than seven degrees of both latitude and longitude.

Population structure and time estimates

The populations were highly differentiated from each other, as shown by a mean G_{ST} of 0.932. This was not significantly different from the mean N_{ST} for all populations (0.938, $p = 0.32$). Only six populations (15%) showed intra-population variation, four of which were located in central Minas Gerais state (MG; Fig. 3a). By removing three of these populations (dic, ibc and vzc) which harbored highly divergent haplotypes, the new G_{ST} of 0.967 was significantly different from the N_{ST} (0.991, $p < 0.0001$). This indicates a phylogeographic structure that was being obscured by those three populations, which were probably formed by secondary contact. In one of our AMOVA analyses, the AF populations were placed in one group and those from Cerrado in another group, respectively corresponding to the traditional taxonomic subdivision of the genus into *P. foliolosa* and *P. reticulata*. The ecotonal populations were excluded from this analysis. We thereby expected to see the maximum divergence between AF and Cerrado populations, given that ecotonal sites could be intermediate to them (Lacerda *et al.* 2002; Goulart *et al.* 2005, 2006). We found that 27.6% of the variation was due to differences between these groups (AF and Cerrado), while 66.7% was due to differences among the populations within each group (Table 3).

The SAMOVAs carried out with k values ranging from 2 to 14 showed an always increasing F_{CT} . Explicit criteria are still lacking to define the best arrangement in cases like this (Dupanloup *et al.* 2002). To select the best grouping for our data, we observed

the rate of F_{CT} increase with the increase of k . The F_{CT} increased more than five percentage points (p.p.) until $k = 7$ ($F_{CT} = 0.800$), and from $k = 7$ to $k = 8$ ($F_{CT} = 0.824$) this increase was 2.4 p.p. From $k = 9$ to $k = 14$, the rise in F_{CT} was always lower than 2 p.p. The population clusters formed by $k = 7$ and $k = 8$ are shown in Table 4. With $k = 8$, the populations were grouped in almost the same way as the eight lineages observed in the network and in the MP tree (Fig. 2 and 3b), with the differences related to populations with intra-population variation composed of different lineages. For these reasons, we believe that eight groups are the best representation of the patterns of genetic differentiation among the *P. reticulata* populations.

Genetic diversity was not uniformly distributed over the *P. reticulata* range (Fig. 3 and 4). There was more genetic diversity in the Cerrado than in the AF. In the former, we detected 13 haplotypes, six lineages, an Hd of 0.881 and an $A_{(20)}$ of 7.47, as compared to six, three, 0.696 and 4.97 respectively for the latter. Taking the entire sampling area into account, the central region exhibited the highest levels of genetic diversity (Fig. 3 and 4). In MG we found 13 (72%) of the 18 haplotypes and representatives of seven (88%) of the eight lineages. Six haplotypes (33%) and three lineages (37.5%) are endemic to MG and, with the exception of haplotype F, all of the haplotypes of the interior of the network, as well as the most common ones (B, L and D), are found in this state. Highly divergent lineages are found very close to each other and some of them are found only in MG (lineages V and VIII). In most of the other states, we observed haplotypes that are either common in MG or derived from those found in that state. One region in central-north MG (between 15°-18° S and 42°-46° W) was especially diverse, containing nine haplotypes from five lineages (Fig. 3a) and showing the highest Hd (Fig. 4). The state of Goiás (GO) also exhibited high genetic diversity, with each of the three populations harboring a distinct haplotype, one of which was endemic (Q) and one (F) that was basal to lineage III.

Contrasting with this central region, two peripheral regions of the *P. reticulata* range, namely northeastern Brazil and the southern Cerrados, showed very low genetic diversity (Fig. 3 and 4). By northeastern Brazil we mean the AF to the north of the Rio Doce Valley, usually designated as the northern or northeastern AF (Oliveira-Filho & Fontes 2000; Thomas 2008), plus the four populations scattered across the caatinga

(ade, crf, jde and lee). As the southern Cerrados, we considered the Cerrado populations located at latitudes higher than 20° S (asc, fuc, mgc, smc and tlc) - comprising São Paulo state (SP), southern MG, and eastern Mato Grosso do Sul state - which roughly matches the definition of Southern Cerrados given by Ratter *et al.* (2003) and Durigan (2006). Despite their extensive areas, each of these two regions presented only three haplotypes of the 18 observed, resulting in a very low level of haplotype and nucleotide diversity within them (Fig. 4). Moreover, these are all common haplotypes that are also found in the central region (in MG and GO), with the exception of the derived haplotype R, which is endemic to the tlc population. Despite ranging over more than 1,300 km north-to-south, the 11 populations (66 individuals) from northeastern Brazil exhibited only the common haplotypes B, F and L, resulting in an Hd of 0.416 and an $A_{(20)}$ of 1.84, and 74% of them harbored haplotype B. In the southern Cerrados, five populations (30 individuals) presented only the haplotypes D, L, and R - which are all of the same lineage (II) -, an Hd of 0.577 and an $A_{(20)}$ of 1.90.

None of the statistical approaches for testing population expansions yielded significant results. The TMRCA of all haplotypes was estimated at 2.27 million years before the present (YBP), with lower and upper 95% confidence interval limits of 0.38 and 5.36 million YBP. The *Plathymenia* lineages thus probably began to diversify in the Pliocene, and most of them may have arisen during the Pleistocene.

Discussion

Distribution of genetic diversity and inferences about the past demographic history of Plathymenia reticulata

The genetic diversity of the *Plathymenia reticulata* populations was not randomly distributed in space, and contrasting patterns were observed across the sampling area. While there was extremely high genetic diversity in the central region of our sampling area, comprising the states of Minas Gerais (MG) and Goiás (GO), very low levels were detected in two peripheral areas, northeastern Brazil and the southern Cerrados. Many divergent haplotypes and lineages were found in the central region, both basal and derived and both endemic and widespread. Two highly divergent haplotypes - E and H - seem to be ancient, given their divergence from the others, and are endemic to MG.

Most of the basal haplotypes in the network were found in MG, while many of those at the tips of the network, i.e., derived haplotypes, were found only in other states. Using RAPD markers, Lacerda *et al.* (2001) also reported high levels of genetic diversity for *P. reticulata* populations in MG. The state of Goiás (GO) also exhibits some of these features, i.e., high diversity and one basal haplotype, but not comparable to what was observed in MG. These facts point to MG state, especially its central-northern portion, as the center of genetic diversity for *P. reticulata* and probably the most longstanding part of its geographic range. On the other hand, the northeastern Brazil and southern Cerrados regions, albeit their extensive area and large number of sampled populations, showed very low genetic diversity, number of haplotypes and number of lineages. The few haplotypes found therein are common to other places in the central region, with only one exception in haplotype R, which is endemic to the southern Cerrados. These patterns of variation are highly consistent with a recent expansion and colonization of both areas by migrants from the central region. A leading edge expansion by dispersants from the central region would result in a series of successive bottlenecks and founder effects, leading to allele loss and genomic homogenization of the new settlers (Hewitt 1996; Cruzan & Templeton 2000). The non-significance of our population expansion test results may be due to the low number of segregating sites, leading to low statistical power; and we were unable to use the Fay and Wu's *H* test, which can be better in such cases, because we did not have an outgroup (Fay & Wu 2000).

One reason for this recent arrival could be simply the fact that the species itself appeared and diversified only recently (the estimated TMRCA dates back to 2.27 million YBP). Assuming that *P. reticulata* first appeared in the central region of its present distribution, the species may have been unable to reach northeastern Brazil and the southern Cerrados earlier due to some constraint, such as restricted seed dispersal constraints and/or unfavorable past climate conditions. Alternatively, the species may have been able to reach those regions earlier, but became extinct due to some kind of change. In this scenario, *P. reticulata* would have been able to re-expand to those regions only upon the recent return to favorable conditions. A commonly reported cause of species range shifts are the climatic oscillations of the Pleistocene (Comes & Kadereit

1998; Davis & Shaw 2001). In ETSA, the changes caused by these fluctuations are still controversial, but any drastic changes bringing increased aridity or cold may have restricted *P. reticulata* distribution. These two kinds of changes have been commonly reported for the LGM for both northeastern Brazil and the southern Cerrados, and the ensuing history is consistent with the patterns we observed. In northeastern Brazil, increased aridity during the LGM (Behling *et al.* 2000; Anhuf *et al.* 2006; Ledru *et al.* 2006) may have been so extreme that *P. reticulata* could not have survived, and more drought-tolerant species, such as those found in the Caatinga, may have been favored. This agrees with the scenario proposed by Prado & Gibbs (1993) and Pennington *et al.* (2000), in which the Caatinga is a kind of SDTF that would have expanded its range during drier periods. In the southern Cerrados, increased cold and aridity may have favored subtropical grasslands (Behling 2002; Behling *et al.* 2002) and/or the SDTF of northern Argentina (Prado & Gibbs 1993; Pennington *et al.* 2000), which are well-adapted to those conditions, with detriment to Cerrado species that are sensitive to frost and/or severe droughts, as *P. reticulata* seems to be. A recent phylogeographic study supports a past expansion of the SDTF (Caetano *et al.* 2008). In northeastern Brazil, the ensuing wetter phases allowed forest expansion (Behling *et al.* 2000; Auler & Smart 2001; Wang *et al.* 2004) and, consequently, the expansion of *P. reticulata* from central to northeastern Brazil. In the southern Cerrados, the return of suitable, i.e. moister and warmer, conditions would have enabled the southward re-expansion of *P. reticulata*. Both recent expansions would lead to the patterns observed.

According to this scenario, the populations would have reached northeastern Brazil via at least two independent south-to-north routes. While the populations from Pernambuco (PE; populations bof and sbf), which are closer to the coast, have the same haplotype as those from eastern BA and MG, the populations from Ceará (CE; populations crf and jde), in the northeastern Brazil inland, are more closely related to the populations from northern GO and Tocantins. This suggests an eastern (Atlantic) and a western (inland) route. These results agree with the significant floristic (Santos *et al.* 2007) and phylogeographic (Carnaval & Bates 2007; Andrade *et al.* 2009) differences between Caatinga forest enclaves and the PE Centre (AF along the PE coast; Santos *et al.* 2007). However, they are in slight disagreement with the commonly reported higher

floristic relatedness of northeastern AF patches among each other than with southerly AF (Oliveira-Filho *et al.* 2006; Santos *et al.* 2007). However, Ferraz *et al.* (2004) reported that some forests from southern BA are floristically closely related to PE forests, and Andrade *et al.* (2009) reported a significantly close genetic relationship between PE and northern ES populations of *Anthurium pentaphyllum*. The persistence of a forest refuge in coastal PE as predicted by climate modeling, and the designation of southern BA as a high biodiversity area (Carnaval & Moritz 2008; Carnaval *et al.* 2009), were not supported for *P. reticulata*. In this species, all individuals from the three southern BA populations, plus the populations from northern Espírito Santo state (ES; population som) and northeastern MG (rum), presented the same haplotype, i.e., an extremely low genetic diversity. We are aware of only one phylogeographic study reporting a pattern similar to that of *P. reticulata* in northeastern Brazil. Recent expansion of the rattlesnake *Crotalus durissus* to northeastern Brazil has been reported (Quijada-Mascarenas *et al.* 2007). However, in contrast to *P. reticulata*, this species is widely found in the Caatinga and not in the rain forest zone, and the causes for the recent range expansions of *C. durissus* and *P. reticulata* were probably different. Most studies report northeastern Brazil *P. reticulata* as a distinct clade from those located to the south (Cardoso *et al.* 2000; Lira *et al.* 2003; Pellegrino *et al.* 2005; Andrade *et al.* 2007; Tchaicka *et al.* 2007; Andrade *et al.* 2009; Carnaval *et al.* 2009) and, indeed, recent expansion has been reported towards the southern AF (Cabanne *et al.* 2007; Tchaicka *et al.* 2007; Carnaval *et al.* 2009). This reinforces the hypothesis that different species may have responded differently to the climate oscillations of the Quaternary. The southern Cerrados have also been frequently reported as a distinct phytogeographical unit from the remaining Cerrado areas (Ratter *et al.* 2003; Durigan 2006). Nevertheless, the phylogeography of two Cerrado trees (Collevatti *et al.* 2003; Ramos *et al.* 2007) revealed evidence of recent colonization of the southern Cerrados from northerly sources, agreeing with the proposed scenario for *P. reticulata*.

Today, there is a mosaic of vegetation in central-north MG, including semi-deciduous forests, Cerrado and Caatinga, in a complex landscape with an altitudinal variation that includes mountain ridges more than 1000 m high. Due to this landscape complexity, aridity and cold may have impacted the vegetation heterogeneously during

glacial times, resulting in high genetic diversity. Increased aridity in some places may have led to the retraction of drought-sensitive species and expansion of drought-adapted vegetation, as for example the Caatinga. Increased cold may have caused altitudinal shifts in *P. reticulata*, promoting its descent to lower altitudes. These two changes may have enhanced fragmentation and isolation among the populations, creating perfect environments for rises in genetic drift effects and among-population differentiation (Young *et al.* 1996). Altitude and arid vegetation would therefore have acted as barriers to seed flow. Populations jfc, ric and vzc, for example, are separated by two mountain ridges and, despite the short distance that separates them (less than 150 km; Fig. 3), they harbor completely different haplotypes. Finally, the well-documented disjunction between the southern BA and Rio de Janeiro AF has also been detected in the *P. reticulata* populations. The Rio Doce Valley, in central ES, is commonly reported as the boundary of the disjunction between northern and southern AF (Thomas *et al.* 1998; Costa 2003; Lira *et al.* 2003; Pellegrino *et al.* 2005; Cabanne *et al.* 2007). Population sof, which is north of the Rio Doce, is very divergent from population dmf, located south of the river. Therefore, central ES seems to be a secondary contact zone between divergent lineages and the causes for that remain unresolved.

Our results also have implications for conservation efforts. For the long-term conservation of the genetic diversity of *P. reticulata*, it would be important to design strategies that aim to preserve most of its lineages. For such, MG is a key piece, as it houses many divergent, endemic and ancient haplotypes and lineages. These trees are sources of valuable timber (Heringer & Ferreira 1972) and these areas could be important for seed collection for silvicultural purposes. Attention should also be given to the forest fragments of northeastern Brazil. Global warming is expected to reduce the volume of precipitation in that area (Bates *et al.* 2008) and could lead to similar consequences as in glacial times. Species that are sensitive to severe drought, such as *P. reticulata*, may suffer a range reduction what could, in combination with anthropic pressure, lead to considerable biodiversity reduction in the northeastern AF. We must also highlight the importance of implementing conservation efforts in the southeastern portion of the *P. reticulata* distribution range. The two characteristic lineages of the

species in the AF were found there, and it houses the most *P. reticulata* diversity within the AF biome.

Taxonomic subdivision of the genus Plathymenia

The distribution of the *Plathymenia reticulata* cpDNA variation enabled us to identify eight lineages, but the detailed relationships among them could not be inferred. We could not discard the possibility of a monophyletic AF clade formed by lineages I and VI; thus, of one *Plathymenia* species in the AF and another in the Cerrado. However, even though most of the AF populations belong to those two lineages, our data did not allow their grouping. Furthermore, the two sampled populations from the *P. foliolosa* type specimen source locality (crf and jde, near Crato, Ceará state; Fig. 3) belonged to the same lineage of populations typical of the Cerrado (nqc and pnc), and if an AF species were to be considered, the name *P. foliolosa* would be inappropriate. The lineage to which the *P. reticulata* lectotype belonged could not be determined because details of its sampling location were not recorded. The AMOVA among groups of populations from the AF and Cerrado (Table 3), with exclusion of the populations from ecotonal sites, showed that 27.6% of the observed variation was due to differences between the groups, while most of the variation (66.7%) was due to differences among populations within the same biome. This means that, on the average, the AF populations diverge more among each other than they do from the Cerrado populations, and vice-versa. Upon analyzing 10 populations from MG using RAPD markers, Lacerda *et al.* (2002) found that 68% of the differences were among the two “species”, after excluding the ecotonal population nee. These percentages support that some genetic differentiation exists between the two former “species”, which is also corroborated by studies on the variation of fruit and seed morphology (Goulart *et al.* 2006) and vegetative phenology (Goulart *et al.* 2005). A recent ecophysiological study identified genetic differences in response to light between AF and Cerrado *P. reticulata* populations, leading the authors to suggest the existence of two ecotypes (Lemos Filho *et al.* 2008).

Our results corroborate the proposal of Lacerda *et al.* (2002), wherein population nee could be a “hybrid” between the AF and Cerrado gene pools. This population exhibited RAPD patterns that were intermediate to the AF and Cerrado populations, and

although nee is quite different from scc as concerns RAPD markers (NE and SC in Lacerda *et al.* 2002), they share the same cpDNA haplotype, along with the other typical Cerrado populations (scc, dic and vzc). Given the fact that the trees of nee present AF architectures, it is likely that this population resulted from pollen flow from AF to Cerrado populations, leading to maternal heritage from Cerrado populations and paternal heritage from the AF. The considerable synchrony of reproductive phenology between the AF and Cerrado populations (Goulart *et al.* 2005) would allow such an event.

We conclude that *Plathymenia* might be diverging into two or more species, and although some genetic differentiation has occurred, there is no morphological and ecophysiological discontinuity or complete reproductive isolation between them. In this case, a subdivision of the genus could be more misleading than helpful. Although we could not conclusively test the recent circumscription (Warwick & Lewis 2003) of the genus *Plathymenia*, we do not have evidence to refute it. Therefore, we agree that the genus *Plathymenia* should be considered monospecific at this point. Nuclear markers applied across the full distribution range of the genus could be a better choice to assess the extent of gene flow between AF and Cerrado *Plathymenia* populations, and thus the degree of reproductive isolation and genetic differentiation among them.

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Table 1. Sampling localities and cpDNA haplotypes and lineages of populations of *Plathymenia reticulata*

Population Code*	Locality, State	n	Altitude (m)	Latitude	Longitude	Biome of Origin†		
						Origin†	Haplotype(s)‡	Lineage(s)
acf	Acaiaça, MG	5	667	-20.46	-43.19	AF	A	I
ade	Andaraí, BA	5	750-950	-12.94	-41.29	E	B	I
ajf	Alto Jequitibá, MG	5	666	-20.45	-41.96	AF	C	I
apf	Apuarema, BA	5	192	-13.51	-39.45	AF	B	I
asc	Águas de Santa Bárbara, SP	5	661	-22.83	-49.23	C	D	II
bof	Bonito, PE	10	550-750	-8.48	-35.69	AF	B	I
bpf	Itabuna, BA	5	540	-14.77	-39.54	AF	B	I
cgc	Chapada Gaúcha, MG	3	700-860	-15.31	-45.62	C	E	VIII
crf	Crato, CE	7	750-950	-7.27	-39.45	AF	F	III
dic	Dores do Indaiá, MG	5	600-720	-19.45	-45.59	C	G, H	IV, V
dmf	Domingos Martins, ES	5	481	-20.38	-40.61	AF	J	VI
fnc	Faina, GO	5	432	-15.76	-50.16	C	K	VII
fuc	Furnas, MG	5	650-750	-20.67	-46.33	C	L	II
gmc	Grão Mogol, MG	5	940	-16.53	-42.88	C	D	II
goc	Gouveia, MG	4	950-1050	-18.58	-43.83	C	G	IV
ibc	Itacambira, MG	5	920	-17.05	-43.33	C	L, M	II, IV
ipf	Itapebi, BA	5	285	-15.87	-39.52	AF	B	I
jac	Japonvar, MG	5	750-850	-15.99	-44.30	C	N	VII
jde	Jardim, CE	5	750-950	-7.44	-39.34	E	F	III
jfc	Joaquim Felício, MG	5	660	-17.75	-44.17	C	M, S	IV, ?
lee	Lençóis, BA	5	400-500	-12.48	-41.35	E	L	II
mbf	Matias Barbosa, MG	5	524	-21.86	-43.34	AF	O	VI
mgc	Mogi-Guaçu, SP	5	591	-22.28	-47.15	C	L	II
nee	Ribeirão das Neves, MG	5	775	-19.67	-44.33	E	H	V
nqc	Niquelândia, GO	5	554	-14.45	-48.41	C	F	III
pnc	Porto Nacional, TO	9	230	-10.74	-48.45	C	P	III
prc	Pirenópolis, GO	5	950-1050	-15.81	-48.89	C	Q	VII
ptc	Paracatu, MG	5	726	-17.09	-46.85	C	L	II
rdf	Pq. Estadual Rio Doce, MG	5	265	-19.58	-42.50	AF	A	I
ric	Pq. Estadual Rio Preto, MG	5	940	-18.00	-43.33	C	G	IV
ruf	Rubim, MG	5	650-750	-16.70	-40.45	AF	B	I
sac	Serra das Araras, MG	3	640	-15.50	-45.35	C	K	VII
sbf	São Benedito do Sul, PE	10	507	-8.78	-35.91	AF	B	I
scc	Serra do Cipó, MG	5	776	-19.33	-43.67	C	H	V
sjf	Silva Jardim, RJ	5	45	-22.62	-42.46	AF	O	VI
smc	São Manuel, SP	5	546	-22.74	-48.46	C	D, L	II
smf	Santa Maria Madalena, RJ	5	442	-22.05	-41.99	AF	J	VI
sof	Sooretama, ES	4	53	-19.06	-40.15	AF	B	I
tlc	Três Lagoas, MS	10	330	-20.74	-51.69	C	D, L, R	II
tpc	Tupaciguara, MG	5	768	-18.53	-48.93	C	L	II
vzc	Várzea da Palma, MG	5	529	-17.71	-44.69	C	D, H	II, V

* The third letter of each population code represents the biome of origin of the population.

† AF, Atlantic Forest; C, Cerrado; E, Ecotone.

‡ The haplotypes and lineages codes were taken from the haplotype network in Fig. 2 and Table 2.

Table 2. Frequency and description of the 18 *Plathymenia reticulata* haplotypes from the two cpDNA regions combined.

Haplotype	Lineage	trnS-trnG (875 bp)																		trnL-trnF (894 bp)							
		S	Id	Id	Id	S	S	Id	Id	Id	S	S	S	S	Id	S	S	Id	Id	S	S	S	Id	S			
		1	1	1	1	2	2	2	3	3	3	3	4	4	4	6	6	1	2	5	8	8	3	4	4	3	4
	Nº individuals (percentage)	1	3	5	0	1	5	9	1	6	7	2	3	7	8	6	7	8	2	6	3	4	4	3	4		
A	I	10 (4.5%)	T	0	0	1	A	T	0	0	0	A	0	T	G	T	0	A	A	1	1	A	T	G	0	C	G
B	I	49 (22.3%)	T
C	I	5 (2.3%)	T	
D	II	21 (9.5%)	.	.	.	0	.	.	.	1	.	.	T	.	.	.	0	.	.	.	T	
E	VIII	3 (1.4%)	.	.	.	0	G	T	.	C	T	.	T	.	.	.		
F	III	17 (7.7%)	.	.	.	0	.	G	.	.	.	T	T	
G	IV	13 (5.9%)	C	.	T	G	T	
H	V	15 (6.8%)	G	T	.	.	.	0	.	G	T	1	.	C	
J	VI	10 (4.5%)	.	.	.	C	.	.	.	T	.	.	C	.	.	.	T	
K	VII	8 (3.6%)	G	.	0	.	1	.	.	T	.	1	T	
L	II	31 (14.1%)	.	1	0	.	.	1	.	T	.	.	0	.	.	.	T	.	.	.	T	
M	IV	3 (1.4%)	C	.	T	T	
N	VII	5 (2.3%)	G	.	0	.	.	.	T	.	1	T	.	.	.	T	
O	VI	10 (4.5%)	.	.	C	.	.	.	T	T	.	.	.	T	
P	III	9 (4.1%)	.	.	0	.	G	.	.	1	.	T	
Q	VII	5 (2.3%)	G	.	0	.	1	.	.	T	.	1	.	.	T	.	T	.	T	
R	II	2 (0.9%)	.	.	0	.	1	1	.	T	.	.	0	.	.	T	.	T	
S	?	4 (1.8%)	.	1	?	?	?	?	?	?	?	?	?	?	?	?	T	

The variable sites are numbered from the end of the *trnS* and *trnL-c* primers, after removal of the sequence extremities. S, substitution; Id, insertion/deletion, which lengths in bp are, in the order they are listed: 604, 21, 19, 21, 20, 19, 19, 23, 21, 11, 5, 1. In haplotype S, the “?” was due to the 604 bp indel, for which character states could not be determined.

Table 3. Partition of genetic diversity among groups of *Plathymenia reticulata* populations using AMOVA

Partition of Variation	d.f.	% of variation*
Among all populations	40	92.13
Within populations	175	7.87
Among Atlantic Forest and Cerrado without ecotonal populations	1	27.56
Among populations within groups	35	66.67
Within populations	159	5.77
Among SAMOVA groups with $k = 7^{\dagger}$	6	80.02
Among populations within groups	34	14.83
Within populations	175	5.15
Among SAMOVA groups with $k = 8^{\dagger}$	7	82.44
Among populations within groups	33	12.42
Within populations	175	5.14

* all P values ≤ 0.00000

† For group composition details, refer to Table 4

Table 4. Structure of groups of *Plathymenia reticulata* populations, as given by SAMOVA with k = 7 and k = 8.

Group	Populations	Number of populations	Biomes†
SAMOVA with k = 7 ($F_{CT} = 0.800$)			
1	acf, ade, ajf, apf, bof, bpf, ipf, rdf, ruf, sbf, sof	11	AF, E
2	asc, fuc, gmc, ibc, lee, mgc, ptc, smc, tlc, tpc	10	C, E
3	cgc, crf, jde, nqc, pnc	5	AF, C, E
4	dic, goc, jfc, ric	4	C
5	nee, scc, vzc	3	C, E
6	dmf, mbf, sjf, smf	4	AF
7	fnc, jac, prc, sac	4	C
SAMOVA with k = 8* ($F_{CT} = 0.824$)			
3	crf, jde, nqc, pnc	4	AF, C, E
8	cgc	1	C

*For k = 8, only the two groups that were different from the groups of k = 7 are shown.

† AF, Atlantic Forest; C, Cerrado; E, Ecotone.

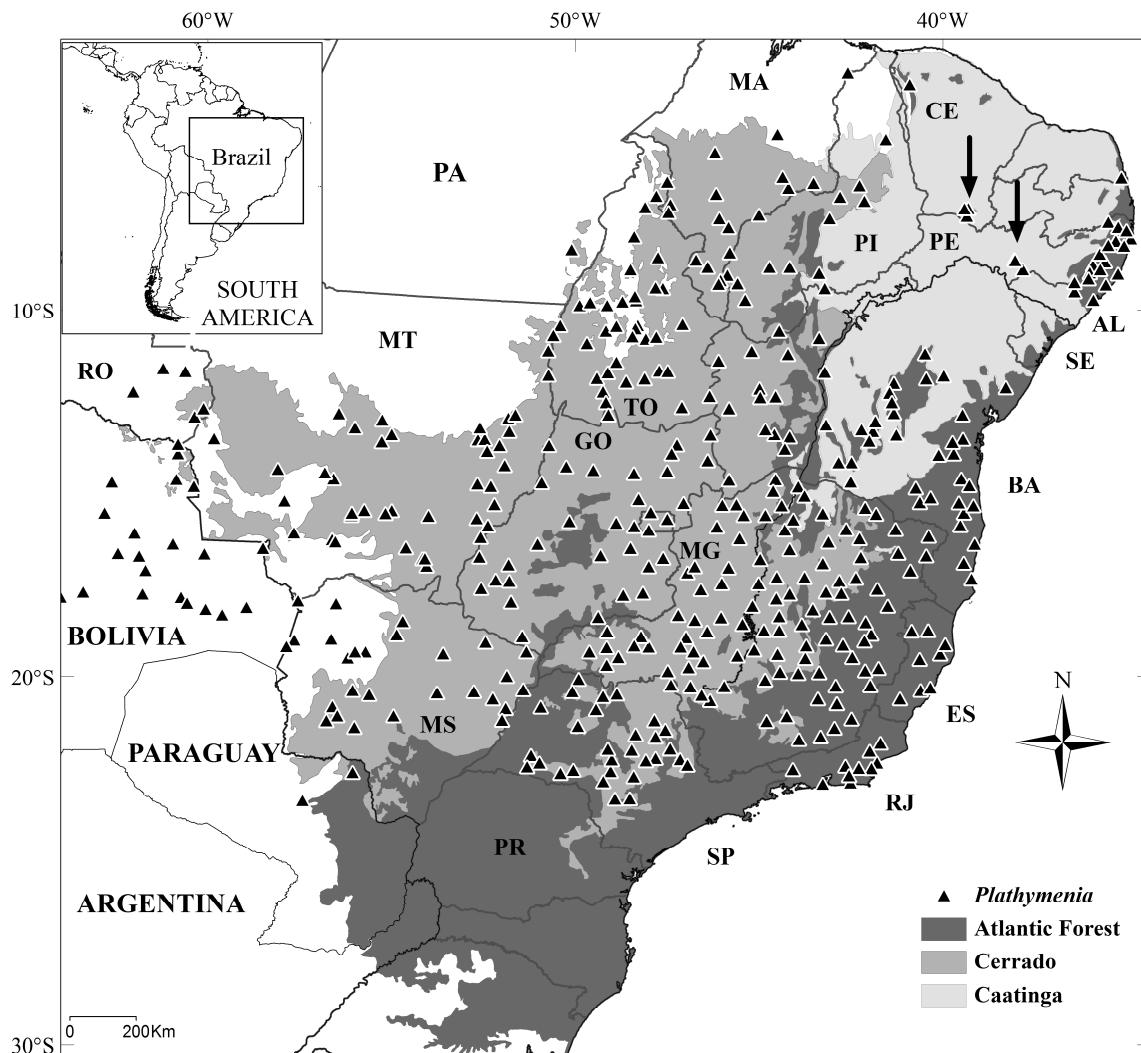


Figure 1. Geographical range of *Plathymenia reticulata* and approximate distribution of the three main biomes of Eastern Tropical South America. Each triangle corresponds to a recorded sighting of *Plathymenia*. The arrows point to forest enclaves in the northeastern caatinga where *P. reticulata* occurs. The abbreviations are for Brazilian states: AL, Alagoas; BA, Bahia; CE, Ceará; ES, Espírito Santo; GO, Goiás; MA, Maranhão; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; PA, Pará; PE, Pernambuco; PI, Piauí; PR, Paraná; RJ, Rio de Janeiro; RO, Rondônia; SE, Sergipe; SP, São Paulo; TO, Tocantins.

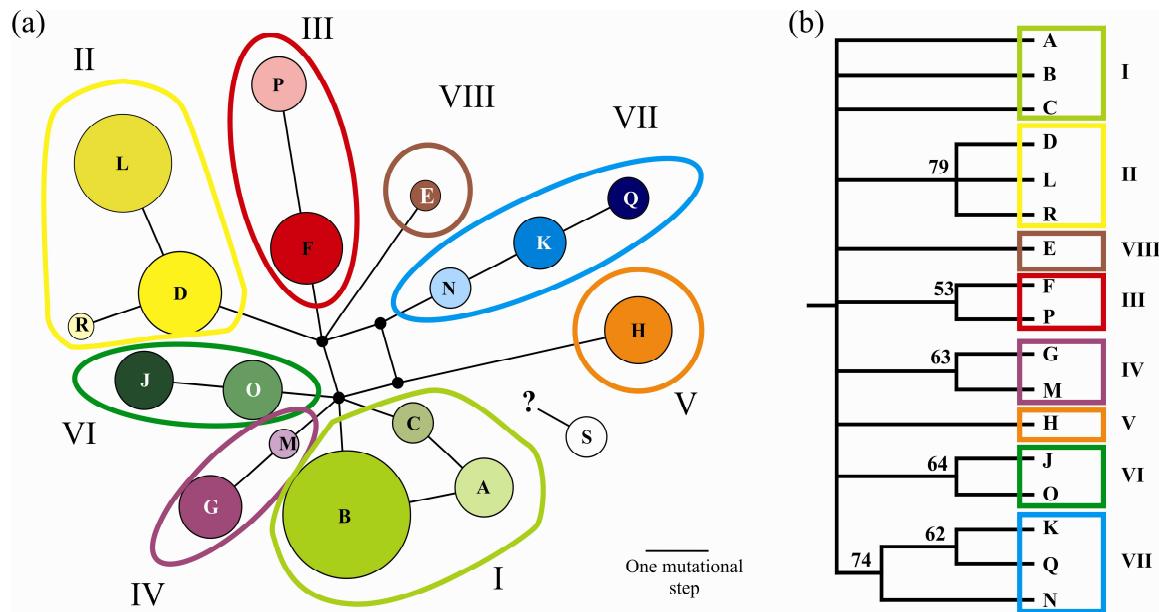


Figure 2. Estimated relationships among the 18 cpDNA haplotypes of *Plathymenia reticulata*. (a) Statistical parsimony network and (b) maximum parsimony consensus tree with bootstrap values >50. The haplotype and lineage codes correspond to those in Table 2 and Fig. 3. The empty circles (a) and rectangles (b) delimit the eight lineages; their colors are equivalent among them and to those in Fig. 3b. The full circles (a) are proportional to the number of individuals.

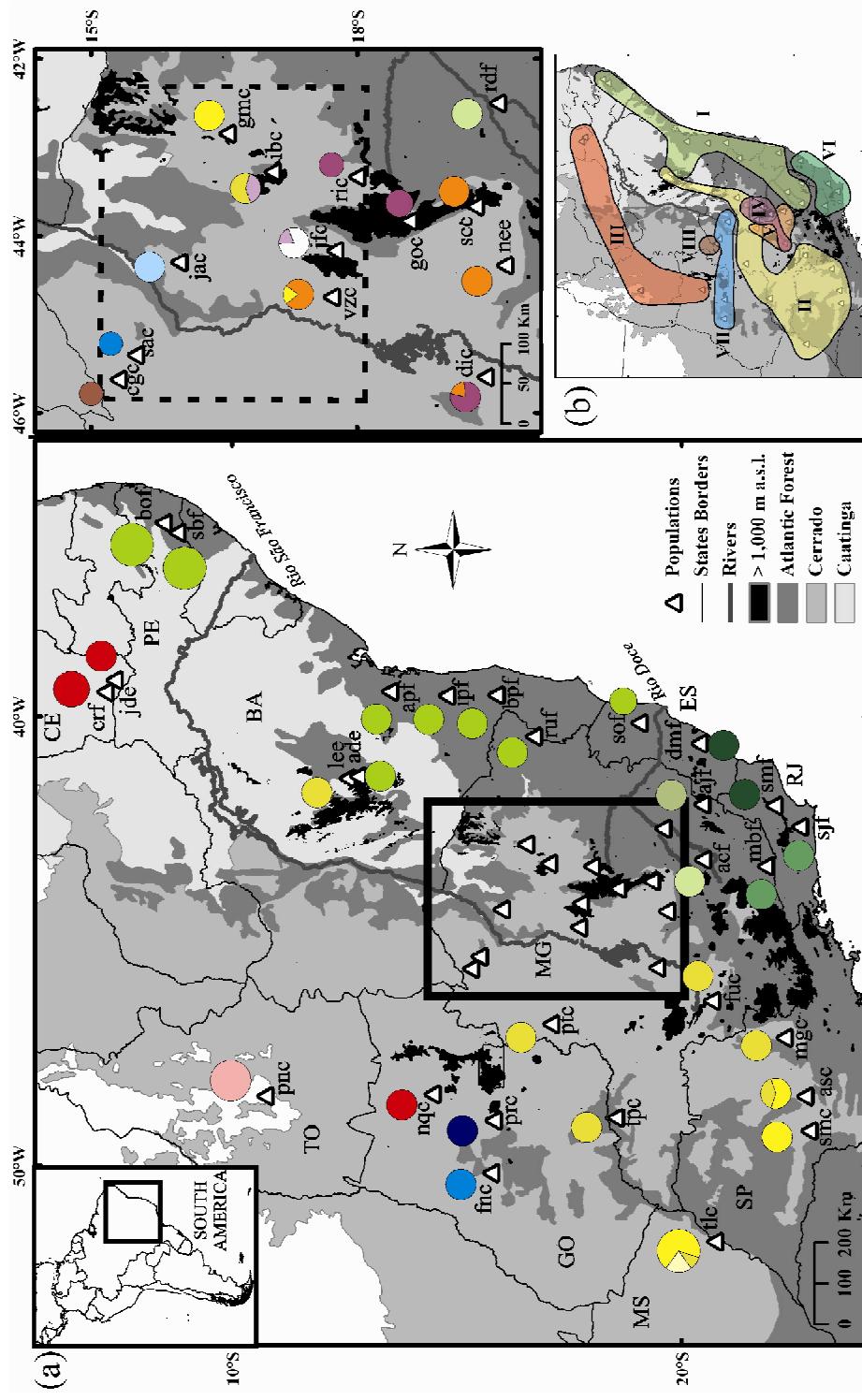


Figure 3. Distribution and frequency of (a) the 18 cpDNA haplotypes and (b) the eight lineages of the network across 41 *Plathymenia reticulata* populations. The haplotype and lineage colors match those in Fig. 2. The circles are proportional to the frequency of the haplotype. The hashed rectangle delimits the region in central-northern MG with high genetic diversity (see text). The two-letter abbreviations correspond to Brazilian states as in Figure 1; the three-letter abbreviations correspond to the population codes in Table 1 and their third letters represent the biome of origin of the population; c, Cerrado; f, Atlantic Forest; e, Ecotone. a.s.l., above sea level.

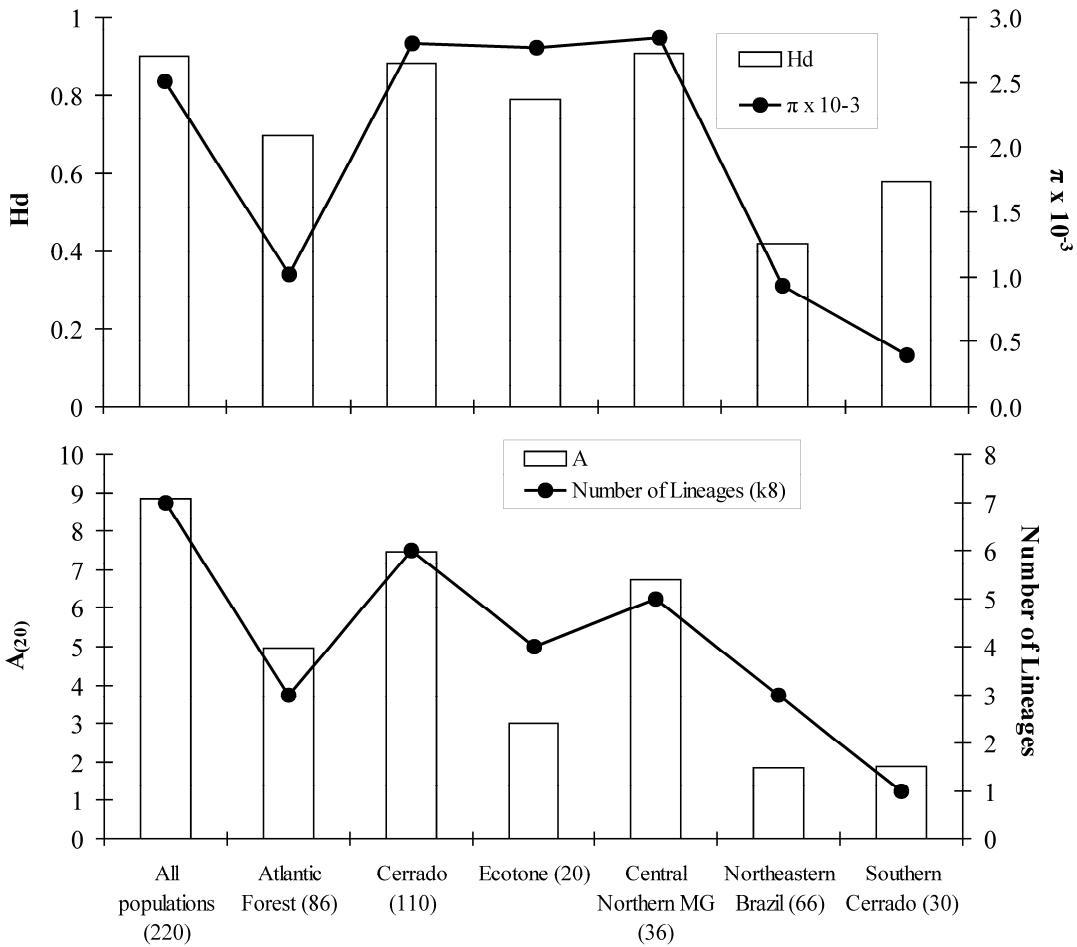


Figure 4. Comparison of summary statistics among different groupings of *Plathymenia reticulata* populations. *Upper chart*, Haplotype diversity (Hd) and nucleotide diversity (π); *Lower chart*, Allelic richness after rarefaction to 20 [$A_{(20)}$] and number of lineages. On the X axis, the numbers in parenthesis are the number of individuals in each group. For their composition details, refer to the text.

Conclusões Gerais

- Foi desenvolvido um protocolo para a obtenção do DNA de cascas do tronco de *Plathymenia reticulata*, que se mostrou eficiente também para outras 17 espécies da família Leguminosae da Mata Atlântica e do Cerrado.
- Esse protocolo representa uma alternativa para a obtenção do DNA de espécies arbóreas, permitindo o uso da casca do tronco como uma fonte de DNA e facilitando, dessa forma, a amostragem eficiente de espécies muito altas ou decíduas para trabalhos focados na análise de seu DNA.
- A diversidade genética do DNA de cloroplasto de *P. reticulata* está estruturada geograficamente ao longo da área amostrada.
- Oito linhagens divergentes foram identificadas, sete das quais podem ser encontradas no estado de Minas Gerais, que concentra alto nível de diversidade genética e é o mais provável centro de origem e diversificação de *P. reticulata*.
- As populações de *P. reticulata* do nordeste brasileiro e do sul do Cerrado exibiram diversidade genética muito baixa, apresentando quase somente haplótipos comuns encontrados também na região central.
- O padrão global sugere que as populações do nordeste brasileiro e do sul do Cerrado chegaram nessas regiões recentemente, oriundas da região central da distribuição da espécie. Origem recente da espécie e/ou extinção de populações de *P. reticulata* devido a condições mais frias e secas durante o LGM podem ter sido responsáveis por esse padrão.
- Populações de *P. reticulata* do interior do nordeste do Brasil são distintas daquelas próximas à costa e, assim, teriam chegado ao nordeste do Brasil por duas rotas independentes, uma ao leste e uma a oeste.
- A região central da amostragem da espécie, especialmente a porção centro-norte de Minas Gerais, foi identificada como uma região prioritária para a conservação de *P. reticulata*.
- Os resultados corroboraram algumas hipóteses de mudanças da vegetação e do clima durante o período Quaternário no Leste da América do Sul Tropical, contribuindo para o entendimento da dinâmica desses fatores no passado.

Também proporcionaram novas hipóteses a serem testadas por trabalhos futuros.

- Combinados com outras evidências, nossos dados sugerem uma divergência em andamento no gênero *Plathymenia*, mas não são suficientes para suportar sua subdivisão taxonômica em duas ou mais espécies.

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