RENATA ACÁCIO RIBEIRO

FILOGEOGRAFIA DE *DALBERGIA NIGRA* (JACARANDÁ-DA-BAHIA) E FILOGENIA DOS GÊNEROS *DALBERGIA*, *MACHAERIUM* E *AESCHYNOMENE* (PAPILIONOIDEAE)

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Orientadora: Profª Drª Maria Bernadete
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“Quem quer crescer não pode continuar

fazendo as mesmas coisas, da mesma maneira”

“... Viver, e não ter a vergonha de ser feliz
Cantar e cantar e cantar
A beleza de ser um eterno aprendiz
Ah meu Deus eu sei, eu sei
Que a vida devia ser bem melhor e será
Mas isso não impede que eu repita
É bonita, é bonita e é bonita
E a vida
E a vida o que é diga lá, meu irmão
Ela é a batida de um coração
Ela é uma doce ilusão, ê ô
Mas e a vida
Ela é maravida ou é sofrimento
Ela é alegria ou lamento
O que é, o que é, meu irmão
Há quem fale que a vida da gente é um nada no mundo
É uma gota é um tempo que nem dá um segundo
Há quem fale que é um divino mistério profundo
É o sopro do criador
Numa atitude repleta de amor
Você diz que é luta e prazer
Ele diz que a vida e viver
Somos nós que fazemos a vida
Como der ou puder ou quiser
Sempre desejada
Por mais que esteja errada
Ninguém quer a morte
Só saúde e sorte....”

Gonzaguinha (Viver e não ter a vergonha de ser feliz)

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SUMÁRIO

RESUMO

Estudos filogeográficos e filogenéticos são importantes para a compreensão da história evolutiva das espécies e para o delineamento de estratégias de conservação. O gênero *Dalbergia*, pertencente à subfamília Papilionoideae (Leguminosae) compreende cerca de 100 espécies de distribuição pantropical e está intimamente relacionado aos gêneros *Machaerium* e *Aeschynomene*. Muitas espécies de *Dalbergia* são economicamente importantes devido a sua valiosa madeira. Entretanto, a superexploração e a fragmentação de habitats têm tornado algumas espécies ameaçadas de extinção nos trópicos, como *D. nigra* (Jacarandá-da-Bahia), uma espécie endêmica da Mata Atlântica, considerada um dos biomas mais ameaçados do mundo. Os objetivos principais desse estudo foram: 1) analisar a diversidade genética e a estrutura filogeográfica de *D. nigra*, e 2) analisar as relações filogenéticas entre os gêneros *Dalbergia*, *Machaerium* e *Aeschynomene*, bem como analisar a concordância entre dados moleculares e a classificação infragenérica tradicional. O primeiro objetivo foi alcançado analisando a distribuição geográfica da diversidade de regiões não codificantes de DNA de cloroplasto (cpDNA) de populações de *D. nigra*. Foram amostradas 17 populações abrangendo a maioria da área de ocorrência natural de *D. nigra*, totalizando 168 indivíduos. As análises baseadas no algoritmo de Monmonier, e suportada pela AMOVA, identificaram uma diferenciação genética latitudinal que dividiu as populações de *D. nigra* em grupos norte e sul. Esta divisão foi interpretada como resultado de eventos de vicariância relacionados às mudanças climáticas do Quaternário que formaram refúgios florestais, isolando populações e conduzindo a diferenciação na região nordeste de Minas Gerais e sul da Bahia. Os resultados também mostraram que as populações localizadas em pequenos fragmentos exibem valores menores de diversidade genética que aquelas em grandes reservas, provavelmente devido a eventos recentes de fragmentação promovidos pelo homem. O segundo objetivo foi alcançado através da análise de seqüências do intron *trnL* do cpDNA e do espaçador ITS do DNA nuclear ribossomal (nrDNA) em diferentes espécies de *Dalbergia*, *Machaerium* e *Aeschynomene*. As análises de parcimônia e Bayesiana revelaram a monofilia dos gêneros *Dalbergia* e *Machaerium*. O gênero *Aeschynomene* apresentou-se parafilético, uma vez que a seção *Ochopodium* foi intimamente relacionada a *Machaeriu*m, enquanto que a seção *Aeschynomene* foi associada a outros gêneros, como *Bryaspsis* e *Soemmeringia*. Considerando o nível infragenérico de

Dalbergia, as seções *Triptolemea* e *Ecastaphyllum* foram monofiléticas, enquanto que a seção *Dalbergia* foi parafilética, concordando com a classificação baseada em morfologia. Em contraste, a classificação infragenérica tradicional do gênero *Machaerium* não mostrou nenhuma concordância com grupos moleculares, sendo a maioria dos arranjos internos fracamente ou não resolvido. Amostragens envolvendo um maior número de espécies de *Aeschynomene* e *Machaerium* juntamente com a análise de outras regiões do DNA e de dados morfológicos são necessários para determinar a exata relação filogenética entre a seção *Ochopodium* com *Machaerium*, bem como para validar a classificação infragenérica de *Machaerium*.

ABSTRACT

Phylogeographic and phylogenetic studies are important to the knowledge of evolutionary history of species and to define priority actions for their conservation. The genus *Dalbergia*, of the subfamily Papilionoideae (Leguminosae), comprises more than one hundred species, distributed pantropically and is closely related to the genera *Machaerium* and *Aeschynomene*. Many *Dalbergia* species are economically important due to their valuable timber. However, the over-exploitation and habitat fragmentation have rendered some of these species prone to extinction, as *D. nigra* (Brazilian Rosewood), an endemic species of the Brazilian Atlantic Forest, recognized as one of the most endangered biomes of the world. The main objectives of the present study were: 1) to investigate the genetic diversity and phylogeographic structure of *D. nigra*, and 2) to investigate the phylogenetic relationship among the genera *Dalbergia*, *Machaerium* and *Aeschynomene*, as well as analyse the concordance between molecular data and the traditional infrageneric classification. The first objective was obtained by the analysis of the geographic distribution of the genetic diversity of non-coding chloroplast DNA (cpDNA) regions in seventeen sampled populations of *D. nigra* throughout most of its natural range, totalling 168 individuals. The analyses based in the Monmonier's algorithm, and supported by AMOVA, identified one latitudinal genetic differentiation that divided the populations of *D. nigra* into north and south groups. This split was interpreted as a result of vicariant events related to Quaternary climatic changes that formed putative forest refuges, isolating populations and leading to the differentiation in the region of northeast of Minas Gerais and south of Bahia States. The data also showed that populations from small forest fragments present lower genetic diversity in contrast to those from great reserves, probably due to recent fragmentation events promoted by anthropogenic disturbances. The second objective was achieved by the comparative analysis of sequences from both chloroplast DNA *trnL* intron and nuclear ribosomal DNA ITS region between genera *Dalbergia*, *Machaerium* and *Aeschynomene*. The parsimony and Bayesian analyses resolved the genus *Dalbergia* and *Machaerium* as monophyletic. The genus *Aeschynomene* presented as paraphyletic, once that the section *Ochopodium* was closely related to *Machaerium*, whereas sections *Aeschynomene* was associated to other genera, such as *Bryaspsis* and *Soemmeringia*. At the infrageneric level of *Dalbergia*, the sections *Triptolemea* and *Ecastaphyllum* were monophyletic, whereas section *Dalbergia* was paraphyletic, in agreement with the

classification based in the morphology. In contrast, the traditional infrageneric classification of *Machaerium* does not show much agreement with molecular groups and most of the internal branches are associated with weak to non-existent bootstrap supports. Sampling involving a higher number of species of *Aeschynomene* and *Machaerium* species, other DNA sequences, and morphological data are needed to resolve the exact phylogenetic relationship between section *Ochopodium* and *Machaerium*, as well as to validate the infrageneric classification of *Machaerium*.

I) INTRODUÇÃO

O estudo desenvolvido durante meu mestrado em 2000-2002 enfocou a estrutura genética de *Dalbergia nigra* (Papilionoideae – tribo Dalbergieae), ameaçada de extinção e restrita a Mata Atlântica. Esse estudo teve como objetivo principal analisar o efeito da fragmentação da Mata Atlântica sobre a diversidade genética dessa espécie, através da comparação entre a população de uma grande reserva no estado de Minas Gerais, o Parque Estadual do Rio Doce, e fragmentos do seu entorno com diferentes graus de distúrbio antrópico. Os resultados mostraram que fragmentos de similar tamanho podem mostrar diferentes níveis de diversidade genética, a depender do grau de distúrbio antrópico. Os dados apontaram para a importância da adequada conservação dos pequenos fragmentos para a preservação da diversidade genética de *D. nigra* (Ribeiro *et al.* 2005). A experiência adquirida com essa espécie, a sua importância ecológica e econômica, a escassez de conhecimentos genéticos sobre ela e a necessidade desses conhecimentos para a sua conservação estimularam a realização de um trabalho mais abrangente, envolvendo o estudo da diversidade e estrutura genética em toda a área de distribuição da espécie. A importância de efeitos climáticos do Pleistoceno na modulação da diversidade e estrutura genética atual das espécies e a necessidade de estudos filogeográficos de espécies da Mata Atlântica, para melhor compreender a evolução desse bioma, também apontaram para a importância de estudos filogeográficos nessa espécie. Atualmente existem poucas populações remanescentes de *D. nigra* e, portanto, foi necessária providenciar inicialmente uma intensa pesquisa na procura de populações, através da análise de trabalhos de fitossociologia e registros em herbários, bem como comunicações pessoais. A partir desses levantamentos preliminares, diversas coletas foram realizadas para a amostragem das populações nos estados de Minas Gerais, Bahia e Espírito Santo.

O gênero *Dalbergia* apresenta 41 espécies de ocorrência no Brasil (Carvalho 1997). Várias dessas espécies exibem importância econômica devido à qualidade de sua madeira. Desse modo, além do estudo filogenético intraespecífico de *D. nigra*, considerou-se importante também realizar um estudo filogenético do gênero *Dalbergia*, baseado em marcadores moleculares, com o objetivo de caracterizar como ocorreu a diversificação do gênero no Brasil e, adicionalmente, correlacionar os dados moleculares com a classificação infragenérica tradicional baseada em dados

morfológicos. A maioria das espécies de *Dalbergia* do Brasil ocorre na região Amazônica ou exibe distribuição geográfica restrita com registros de populações naturais localizadas em um ou poucos estados brasileiros (Carvalho 1989). Considerando essas restrições, foram utilizados na análise filogenética espécimes de *Dalbergia* depositadas em herbários. Sendo assim, foi efetuada uma busca por herbários de diversas instituições de pesquisa e universidades que possuíssem diferentes espécimes de *Dalbergia* depositadas e que consentissem na retirada de uma pequena quantidade de folhas dessa espécie. Quatro herbários gentilmente permitiram essa retirada: BHCB (Depto de Botânica, UFMG, Belo Horizonte, MG), INPA (Instituto Nacional de Pesquisas da Amazônia, Manaus, AM), CEPEC (Centro de Pesquisas do Cacau, CEPLAC, Itabuna, BA) e SP (Instituto de Botânica de São Paulo, São Paulo, SP). Entretanto, foi obtido DNA de boa qualidade para seqüenciamento apenas de um pequeno número de amostras, devido, provavelmente, à alta concentração de compostos secundários nas folhas dessas espécies e degradação do DNA, principalmente do gênero *Dalbergia*. Desta maneira, antes das reações de sequenciamento foi necessário um estudo prévio para identificar um método adequado de extração de DNA do material herborizado. Considerando a proximidade filogenética do gênero *Dalbergia* com o gênero *Machaerium* e, também, a contraditória e íntima relação desses dois gêneros com *Aeschynomene* (Lavin *et al.* 2001), considerou-se importante incluir espécies desses dois gêneros nas análises filogenéticas. No total, foram obtidas amostras de 33 espécies de *Dalbergia*, 20 de *Machaerium* e 14 de *Aeschynomene*, entretanto, devido a condições do DNA, foram utilizadas na análise filogenética apenas 13, 12 e cinco espécies de cada gênero respectivamente.

Os objetivos principais dessa tese de doutorado foram:

1) analisar a diversidade genética e a estrutura filogeográfica de *D. nigra*;

2) analisar as relações filogenéticas entre os gêneros *Dalbergia*, *Machaerium* e *Aeschynomene*, bem como identificar a congruência entre dados moleculares e a classificação infragenérica tradicional desses gêneros.

Os objetivos específicos foram:

a) Selecionar regiões do DNA de cloroplasto (cpDNA) com polimorfismo intra-especifico suficiente para análises filogeográficas em *D. nigra*;

b) Determinar a diversidade e a estrutura genética de populações de *D. nigra*, associando os resultados encontrados com os efeitos da fragmentação antrópica recente;

c) Determinar a estrutura filogeográfica de *D. nigra* e associá-la com eventos históricos, como as mudanças climáticas do Quaternário, objetivando compreender a evolução da espécie e do seu bioma de ocorrência;

d) Sugerir estratégias de manejo e conservação para *D. nigra*;

e) Selecionar e adequar métodos de extração de DNA de material herborizado de espécimes do gênero *Dalbergia*;

f) Analisar a relação filogenética dentro e entre os gêneros *Dalbergia*, *Machaerium* e *Aeschynomene*.

A apresentação dessa tese de doutorado está estruturada da seguinte maneira: 1) uma revisão bibliográfica enfocando os principais temas abordados na tese, 2) três artigos resultantes dos trabalhos realizados a partir dos objetivos propostos, e por fim, 3) uma conclusão geral. Os artigos estão organizados na seguinte ordem:

Artigo 1: "Diversity and phylogeography of the endangered rosewood *Dalbergia nigra* (Papilionoidae) from the Brazilian Atlantic Forest."

Artigo 2: "Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of genus *Dalbergia*."

ARTIGO 3: "The genus *Machaerium* (Leguminosae) is more closely related to *Aeschynomene* than to *Dalbergia*: relationships inferred from combined sequence data."

II) REVISÃO DA LITERATURA

II.1) Estudos filogeográficos e mudanças climáticas do Quaternário

As plantas demonstram uma surpreendente diversidade de morfologia, adaptação e ecologia, produto de milhões de anos de divergência e diversificação de linhagens (Schaal *et al.* 1998). Caracterizar essa diversidade e conhecer os mecanismos através do qual ela surgiu é o objetivo da genética de populações e da sistemática. O conhecimento dos níveis de variação genética e da distribuição desta variação dentro e entre as populações naturais é importante para a compreensão da história evolutiva das espécies. Além disso, esse conhecimento também é de fundamental importância para o delineamento de estratégias de conservação e manejo de populações naturais (O'Brien 1994, Avise *et al.* 1995), visando à manutenção da diversidade genética das espécies.

O conceito de filogeografia foi introduzido há cerca de 20 anos (Avise *et al.* 1987) para designar o estudo da distribuição geográfica de linhagens genealógicas dentro das espécies. Os estudos de filogeografia permitem estabelecer padrões geográficos e temporais para a variação genética encontrada, permitindo inferir a evolução das espécies em seus biomas de ocorrência (Avise 1998). A análise filogeográfica em plantas baseia-se principalmente na variabilidade do cpDNA que apresenta herança uniparental, taxa relativamente baixa de mutação e seqüências não sujeitas à recombinação (Avise 1994), características estas apropriadas para estudos da filogenia intraespecífica. A estrutura genética do cpDNA é influenciada pelo parentesco histórico e fluxo gênico ancestral entre populações, bem como por eventos históricos como glaciações e mudanças climáticas ao longo do tempo geológico (Avise 1994, Schaal *et al.* 1998). Sua variação genética é revelada através de métodos de digestão por enzimas de restrição de regiões do cpDNA amplificadas por PCR (e.g., Caron *et al.* 2000, Palme *et al.* 2003, Rendell e Ennos 2003, Dorken e Barrett 2004, Magni *et al.* 2005), utilizando pares de iniciadores universais como os descritos por Taberlet *et al.* (1991), Demesure *et al.* (1995) e Dumolin-Lapègue *et al.* (1997), Grivet *et al.* (2001), entre outros. Além disso, estudos filogeográficos em plantas também se baseiam em microssatélites de cpDNA (Collevatti *et al.* 2003, Heuertz *et al.* 2004, Lira *et al.* 2003) e no seqüenciamento de pequenas regiões do cpDNA, como regiões intergênicas não

codificantes (exemplos, Wang *et al.* 1999, Su *et al.* 2005, Takayama *et al.* 2005, Zhang *et al.* 2005, Ikeda *et al.* 2006, Wu *et al.* 2006).

Recentemente, observa-se o crescente avanço de estudos filogeográficos em plantas que utilizam marcadores nucleares, como o espaçador ITS do DNA nuclear ribossomal (nrDNA), associado a marcadores de cpDNA (Franzke *et al.* 2004, Wu *et al.* 2005, Koch *et al.* 2006). Estudos comparativos do cpDNA e nrDNA podem providenciar informações complementares sobre a estrutura genética de populações naturais (McCauley 1995, Ennos et al. 1999). Em particular, o cpDNA é mais sensível a efeitos de fragmentação que o nrDNA devido ao seu menor tamanho populacional efetivo. Além disso, o fluxo gênico é menor para os genes do cpDNA, dispersos somente através de sementes na maioria das angiospermas, que para genes nucleares, dispersos tanto por sementes como por pólen (Rendell e Ennos 2003).

As extremas mudanças climáticas que ocorreram nos períodos glaciais e interglaciais do Quaternário modificaram a composição florística e a distribuição geográfica dos diferentes tipos de vegetação em várias regiões do mundo. Dados palinológicos obtidos de sedimentos de lagos e lagoas têm sido usados para reconstruir a composição da vegetação durante essa época e inferir os padrões de migração para várias espécies de plantas na Europa e América do Norte (Davis 1983, Huntley e Webb 1988). Essas mudanças climáticas periodicamente fragmentavam as áreas de florestas dentro de refúgios separados, influenciando também a distribuição geográfica da diversidade genética intraespecífica de populações de plantas. Os estudos filogeográficos, baseados em marcadores moleculares, associados aos dados palinológicos têm contribuído para o melhor entendimento da história evolutiva dos biomas na Europa e Ásia (Palmé *et al.* 2003, Petit *et al.* 2003, Franzke *et al.* 2004, Zhang *et al.* 2005, Fjellheim *et al.* 2006, Ikeda *et al.* 2006), América do Norte e Central (Dorken e Barrett 2004, Trapnell e Hamrick 2004, Magni *et al.* 2005, Schierenbeck *et al.* 2005). A maioria desses estudos associa a subdivisão genética observada nas populações de plantas às mudanças climáticas durante o Quaternário.

Na região Neotropical, as mudanças climáticas durante o Pleistoceno são consideradas fatores importantes para explicar a alta biodiversidade e endemismo nas florestas tropicais. De acordo com a teoria de refúgios (Haffer 1969, Vanzolini e Williams 1981), as florestas tropicais ficaram restritas a áreas de refúgios de clima mais

ameno durante os períodos glaciais circundadas por áreas de savanas. Os ciclos alternados de contração (fragmentação) e expansão (reconecção) das florestas resultaram em repetidos eventos de vicariância, conduzindo a diferenciação de espécies e populações (Whitmore e Prance 1987). Dados de pólen fóssil também indicam que durante o último período glacial, as condições foram mais frias e secas que nos dias atuais nos trópicos, estendendo as áreas de savanas ao norte e sul do equador e reduzindo as áreas de florestas úmidas (Hewitt 2000, Behling 2002). No Brasil, os estudos palinológicos realizados em várias localidades permitiram analisar algumas mudanças ocorridas na vegetação ao longo do tempo (Behling 1995, 1998, 2002a, 2002b, 2003, Ledru *et al.* 1996, Salgado-Labouriau *et al.* 1997, 1998). No entanto, existem poucos estudos genéticos, e principalmente filogeográficos, que permitam inferir a história evolutiva de espécies de plantas que ocorrem em importantes biomas brasileiros, como o Cerrado (Olsen e Schaal 1999, Collevatti *et al.* 2003) e a Mata Atlântica (Cardoso *et al.* 1998, Cardoso *et al.* 2000, Lira *et al.* 2003, Salgueiro *et al.* 2004, Lorenz-Lemke *et al.* 2005).

II.2) Gêneros *Dalbergia*, *Machaerium* e *Aeschynomene* (Leguminosae – Papilionoideae)

Leguminosae (Fabaceae) é a terceira maior família das Angiospermas depois das famílias Asteraceae (Compositae) e Orchidaceae, contendo cerca de 650 gêneros e mais que 18.000 espécies em todo o mundo (Polhill e Raven 1981). Está dividida em três grandes subfamílias: Caesalpinioideae, Mimosoideae e Papilionoideae. A grande diversidade da família Leguminosae não é determinada apenas por caracteres morfológicos, anatômicos e químicos, mas também pela sua capacidade de nodulação, seus modos de reprodução e sua inacreditável capacidade ecológica em ocupar quase todos ecossistemas da Terra (Sprent 2001, Doyle e Luckow 2003, Wojciechowski 2003). Os legumes são encontrados em todos continentes, ocorrendo em zonas temperadas e áridas, florestas úmidas, savanas, montanhas, planícies ou em solos salinos da região costeira (Doyle e Luckow 2003). Baseado na importância econômica e na agricultura, Leguminosae é considerada a segunda maior família depois da Poaceae (gramíneas), por produzir recursos para alimentação, óleos, fibras, madeiras, substâncias químicas, variedades horticulture e enriquecimento do solo (Wojciechowski 2003).

Considerando a grande importância econômica e ecológica das leguminosas, a evolução e a sistemática da família são temas de crescente e permanente interesse para os pesquisadores.

O recente advento de técnicas de análises moleculares por PCR e seqüenciamento permitiu a utilização de seqüências de DNA para estabelecer as relações filogenéticas entre espécies, gêneros e/ou famílias de plantas, complementando assim a grande quantidade de dados morfológicos disponíveis, principalmente para a família Leguminosae (exemplos Lavin *et al.* 2001, Gervais e Bruneau 2002, Wojciechowski 2003). As primeiras tentativas para resolver as relações entre níveis superiores dentro da família com dados moleculares foram feitos utilizando seqüências de *rbcL* (Käss e Wink 1996, Doyle *et al.* 1997). A partir dessa data, numerosos estudos filogenéticos foram realizados em vários gêneros da família. Muitos estudos têm explorado um grande número de regiões do DNA de cloroplasto (cpDNA) e, mais recentemente, algumas regiões nucleares para a reconstrução filogenética dentro da família. As regiões do cpDNA mais utilizadas nesses estudos incluem o gene *matK* e o intron flanqueador *trnK* (Hu *et al.* 2000, Miller e Bayer 2001), o intron *trnL* e espaçadores adjacentes (Bruneau *et al.* 2000, Brouat *et al.* 2001), os espaçadores intergênicos *atpB-rbcL* (Hurr *et al.* 1999) e *psbA-trnH* (Chandler *et al.* 2001), entre outras diferentes regiões (Davis *et al.* 2002). Estudos filogenéticos que tem utilizado regiões nucleares analisam majoritariamente os espaçadores internos (ITS) do DNA ribossomal (nrDNA) que incluem o gene 5.8S, altamente conservado (revisado por Baldwin *et al.* 1995). Os espaçadores de ITS providenciam sítios informativos para análises filogenéticas em nível intragenérico (Delgado-Salinas *et al.* 1999, Mayer e Bagga 2002, Stappen *et al.* 2002, Dong *et al.* 2003) e também em níveis taxonômicos superiores (Lavin *et al.* 2001, Wieringa e Gervais 2003, Varela *et al.* 2004). No entanto, para obter uma maior resolução das relações filogenéticas entre gêneros e dentro da família Leguminosae, um crescente número de estudos analisa ambos os tipos de marcadores, cpDNA e nrDNA (exemplos, Lavin *et al.* 2001, Gervais e Bruneau 2002, Wojciechowski 2003).

Papilionoideae é a maior subfamília dentro da Leguminosae com 476 gêneros e 14.000 espécies (Lewis *et al.* 2003). Análises moleculares das leguminosas apontam esta subfamília como um grupo monofilético, embora com fraco suporte na maioria das análises (Kajita *et al.* 2001), e sugerem sua divergência das outras legujminosas entre 45

e 50 milhões de anos atrás (Wojciechowski 2003). Lavin *et al.* (2001) propuseram um maior rearranjo para certas leguminosas papilionóides referidos como grupo "dalbergioides", baseado em dados moleculares (seqüências da região *matK/trnK*, intron *trnL* e espaçador ITS) e morfológicos. Este grupo inclui todos os gêneros das tribos Adesmieae, Aeschynomeneae e Dalbergieae, e Desmodieae subtribo Brynae (exceto *Andira*, *Hymenolobium*, *Vatairea* e *Vataireopsis*). Na análise filogenética, os "dalbergioides" foram divididos em três subclados bem suportados: Adesmia, Pterocarpus e Dalbergia (Lavin *et al.* 2001). Particularmente, o subclado Dalbergia inclui os gêneros *Dalbergia*, *Machaerium* e outros 14 gêneros da tribo Aeschynomeneae (sensu Rudd 1981): *Aeschynomene*, *Soemmeringia*, *Cyclocarpa*, *Kotschya*, *Smithia*, *Humularia*, *Bryaspis*, *Geissaspis*, *Weberbauerella*, *Diphysa*, *Pictetia*, *Ormocarpum*, *Ormocarpopsis* e *Peltiera* (Lavin *et al.* 2001).

O gênero *Dalbergia* (tribo Dalbergieae) compreende cerca de 100 espécies de árvores, arbustos ou lianas com distribuição pantropical, apresentando centros de diversidade na Amazônia e Indo-Asia (Polhill 1981). Muitas espécies de *Dalbergia* são economicamente importantes devido a sua valiosa madeira, entretanto, a superexploração e a fragmentação de habitats têm tornado algumas dessas espécies ameaçadas de extinção nos trópicos, como *D. nigra* (Jacarandá-da-Bahia; Carvalho 1997). No Brasil, Carvalho (1997) realizou um estudo sistemático desse gênero combinando dados de fitogeografia, de morfologia vegetativa e reprodutiva, de anatomia comparativa de folhas e frutos, bem como de flavonoídes foliares e de morfologia do pólen. O autor identificou 41 espécies de *Dalbergia* de ocorrência nos diferentes biomas do Brasil. O gênero *Dalbergia* se divide em cinco seções definidas por caracteres de inflorescência e frutos: *Dalbergia* L.f., *Triptolemea* (Mart. ex Benth.) Benth., *Selenolobium* Benth., *Pseudecastaphyllum* A.M. de Carvalho, e *Ecastaphyllum* (P. Browne) Ducke (Carvalho 1997).

O gênero *Machaerium* (tribo Dalbergieae) é predominantemente neotropical, com cerca de 130 espécies ocorrendo do México até Brasil e Argentina (Rudd 1977, Klitgaard e Lavin 2005). Estas espécies têm sido classificadas dentro de cinco séries, baseando-se na morfologia de folhas e nervuras e textura da estípula (Bentham 1860). Essas séries foram posteriormente elevadas a nível seccional por Taubert (1891): *Lineata*, *Oblonga*, *Acutifolia*, *Reticulata* e *Penninervia*. A maioria das espécies de *Machaerium* é amplamente distribuída no Brasil (Hoehne 1941), com centros de

diversidade na Bacia Amazônica (Hoehne 1941, Ducke 1949, Bastos 1987, Mackinder 1990) e no Sudeste do Brasil (Lima *et al.* 1994, Mendonça Filho 1996, Sartori e Tozzi 1998). Esse gênero é intimamente relacionado ao gênero *Dalbergia* (Polhill 1981), entretanto para esses dois gêneros, dados de seqüências de DNA são necessários para estabelecer se há concordância entre os dados morfológicos e filogenéticos.

O gênero *Aeschynomene* (tribo Aeschynomeneae) é tradicionalmente dividido em duas seções, *Aeschynomene* L. (com estípulas mediofixas) e *Ochopodium* Vogel (com estípulas basifixas), as quais incluem, respectivamente, 101 e 50 espécies com distribuição pantropical (Leonard 1954, Rudd 1955, Verdcourt 1971, Fernandes 1996, Klitgaard e Lavin 2005). Lavin *et al.* (2001) sugeriram que o gênero *Aeschynomene* seção *Ochopodium* é mais intimamente relacionado a *Machaerium*. A seção *Aeschynomene* é diagnosticada por estípulas mediofixas, característica também das espécies intimamente relacionadas, como *Bryaspsis* e *Soemmeringia*. Entretanto, os autores apontaram para a necessidade de analisar um maior número de espécies de *Aeschynomene* para obter resultados mais conclusivos da íntima relação filogenética entre o gênero *Machaerium* e a seção *Ochopodium* (Lavin *et al.* 2001).

II.3) A espécie *Dalbergia nigra* (Jacarandá-da-Bahia)

A espécie mais ameaçada do gênero *Dalbergia* é *D. nigra* (Vell.) Allemão ex Benth., conhecida no Brasil como jacarandá-da-Bahia e no exterior como *Brazilian rosewood*. Espécie característica e exclusiva da Mata Atlântica, ela ocorre desde o sul da Bahia até o litoral norte de São Paulo, abrangendo os estados de MG, RJ e ES (Carvalho 1997). É encontrada em altitudes que variam de 30 a 300 m no sul da Bahia e norte do Espírito Santo, atingindo 700 m em Minas Gerais e até 1.700 m no Rio de Janeiro (Carvalho 1994). Geralmente, *D. nigra* aparece em agrupamentos de densidade baixa, numa freqüência de 0,8 indivíduos por hectare no sul da Bahia, sua zona de maior ocorrência (Rizzini e Mattos-Filho 1974). A fenologia dessa espécie varia entre os estados brasileiros de sua ocorrência, geralmente florescendo entre os meses de setembro a maio e frutificando entre maio e janeiro (Carvalho 1994). Seu crescimento é moderado a rápido, medindo comumente a árvore adulta 15 a 25 m de altura e 40 a 80 cm de diâmetro à altura do peito (DAP), podendo atingir até 35 m de altura e 155 cm de DAP (Leão e Vinha 1975, Lorenzi 1992). Trata-se de uma planta semi-heliófita com

tolerância ao sombreamento leve a moderado na fase juvenil. A árvore adulta é usada em arborização de parques e ruas, sendo também recomendada para a recuperação do solo, por depositar razoável camada de folhas (Carvalho 1994).

Dalbergia nigra apresenta tronco tortuoso e irregular, com casca pardo-acinzentada e com descamação longitudinal. As folhas desta espécie são compostas, alternas e paripinadas, com 10 a 20 folíolos pequenos por pina, distribuídos em uma copa larga e irregular. Sua inflorescência é constituída por flores hermafroditas branco-amareladas, pequenas e perfumadas, reunidas em cachos axilares de até seis cm de comprimento, dando origem a panículas de até 20 cm (Carvalho 1994). Os frutos são do tipo legume indeiscente com 3 a 8 cm de comprimento por 18 a 22 mm de largura, apresentando de uma a duas sementes pequenas e membranáceas dispersas pelo vento (anemocórica) (Carvalho 1994). O polen é provavelmente disperso por abelhas (observação pessoal) como também observado em espécie congenérica na Índia (Mohana *et al.* 2001). Não foram encontrados na literatura dados sobre o sistema de cruzamentos de *D. nigra*; entretanto, estudo de uma espécie congenérica no Brasil (*D. miscolobium*) sugere a ocorrência de fecundação cruzada com um aparente sistema de auto-incompatibilidade (Gibbs e Sassaki 1998).

Dalbergia nigra é considerada uma das mais valiosas espécies madeireiras que ocorrem no Brasil. Sendo muito apreciada no comércio mundial desde os tempos coloniais, esta espécie foi altamente explorada, o que resultou em sua escassez nas regiões florestais mais acessíveis. Sua madeira exibe consistência pesada, coloração pardo-escura, alta resistência e longa durabilidade, sendo utilizada na fabricação de móveis de luxo, objetos decorativos e instrumentos musicais, como guitarras e caixas de piano (Lorenzi 1992, Carvalho 1994). Devido à alta fragmentação da Mata Atlântica, atualmente populações naturais de *D. nigra* são encontradas principalmente em áreas de conservação (parques e reservas) e pequenos remanescentes florestais. Estes últimos estão, na maioria das vezes, localizados em topos de morros e vertentes íngremes, onde a extração de madeira é extremamente difícil (Costa *et al.* 1998). Devido ao intenso extrativismo desta espécie, a alta fragmentação de seu habitat e à ausência de plantios de reposição, *D. nigra* está incluída, desde a década de 1990, na Lista Oficial de Espécies da Flora Brasileira Ameaçadas de Extinção (Carvalho 1997). Segundo o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA 1992) e o Apêndice I da Convenção sobre o Comércio Internacional das Espécies da

Flora e da Fauna (CITES 1992), a inclusão dessa espécie causa a proibição de sua comercialização. Além disso, *D. nigra* é classificada como vulnerável de acordo com critérios da *The World Conservation Union* (IUCN 1994), o que significa que ela apresenta alto risco de extinção na natureza em médio prazo. Entretanto, apesar da importância ecológica e econômica da *D. nigra*, o único estudo genético com esta espécie foi realizado em uma região restrita do Estado de Minas Gerais, usando marcadores aloenzimáticos (Ribeiro *et al.* 2005). Nesse estudo foram analisados os efeitos da fragmentação de habitat sobre a estrutura genética de três populações de D. nigra (plantas adultas e jovens). Os resultados mostraram que as plantas jovens da população com maior nível de distúrbio antrópico exibiu menor diversidade genética e maior diferenciação que as outras populações. Além disso, evidencia-se a conservação de pequenos fragmentos para a preservação da diversidade genética desta arbórea ameaçada de extinção (Ribeiro *et al.* 2005).

III) ARTIGOS

ARTIGO 1:

Diversity and phylogeography of the endangered rosewood *Dalbergia nigra* (Papilionoideae) from the Brazilian Atlantic Forest

Submetido para publicação.

Diversity and phylogeography of the endangered rosewood *Dalbergia nigra* (Papilionoideae) from the Brazilian Atlantic Forest

Running title: Diversity and phylogeography of *Dalbergia nigra*

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Abstract

Brazilian rosewood (*Dalbergia nigra*) is an endangered tree endemic to the Brazilian Atlantic Forest, one of the most threatened biomes of the world. There are few phylogeographic studies with plant species that could contribute to elucidate the evolutionary history of this biome. In the present study, cpDNA sequences were used to investigate the distribution of genetic diversity of *D. nigra*. Seventeen populations were sampled throughout most of its geographical range, totaling 168 individuals. In the analysis of 1297 bp from two non-coding sequences, *trnV-trnM* and *trnL*, 13 haplotypes were detected. The Monmonier's algorithm identified a latitudinal genetic differentiation that divided the populations into north and south groups. This split was supported by AMOVA and network analysis. It was interpreted as a result of vicariant events related to Quaternary climatic changes that resulted in putative forest refuges, isolating populations and leading to the differentiation between north and south groups. This geographic differentiation, corroborated by previous animal phylogeographic studies, pointed out to the occurrence of historical vicariance events in the region of northeast Minas Gerais and southern Bahia. This is probably consequence of recent fragmentation events caused by anthropogenic disturbances. Our phylogeographic analysis using a large-scale survey from an endemic species contributed to reinforce the evidence that biogeographic events had remarkable importance in the evolutionary history of the Atlantic Forest, and also contributed with new relevant data to establish priority areas for conservation.

Key words: Brazilian Atlantic Forest, conservation genetics, *Dalbergia nigra*, genetic structure, phylogeography, Quaternary climatic changes

Introduction

The extreme climatic changes that occurred between glacial and interglacial cycles modified several regions of the world interfering in the floristic composition and geographical distribution of vegetation types. Fossil pollen data indicate that during the last glacial period, the conditions were colder and drier than in present-day in the tropics, extending savannah areas from both north and south of the equator while reducing rain forest areas (Hewitt 2000; Behling 2002). In the Neotropical region the climatic changes during the Pleistocene have been considered an important factor to explain the high biodiversity and endemism in tropical forests. According to the Pleistocene Refuge Theory (Haffer 1969; Vanzolini & Williams 1981), during the glacial periods the tropical forests were restricted to warmer and moister refuges and the alternate cycles of contraction (fragmentation) and expansion (reconnection) of forests and resulted in repeated vicariant events, leading to differentiation of species or populations (Whitmore & Prance 1987). The influence of these climatic changes on the geographic distribution of intraspecific genetic diversity of plant species has contributed to test biogeography hypotheses mainly in Holoartic floristic realm, using molecular markers (e.g. Palmé *et al.* 2003; Petit *et al.* 2003; Franzke *et al.* 2004; Magni *et al.* 2005; Schierenbeck *et al.* 2005; Zhang *et al.* 2005; Grivet *et al.* 2006; Ikeda *et al.* 2006).

The influence of recent anthropogenic disturbances, such as habitat fragmentation, on the distribution of the genetic diversity of plant populations has also been stressed (reviewed by Lowe *et al.* 2005). Habitat fragmentation has transformed large regions of continuous forests into small and geographically isolated remnant fragments, often surrounded by human-modified areas (Turner 1996; Young *et al.* 1996), representing one of the major threats to biodiversity. The fragmentation could result in reduction of population size (bottleneck effect), increased genetic drift and

reduced gene flow among plant population, leading to an erosion of genetic diversity and an increase of inter-population genetic differentiation (reviewed by Young *et al.* 1996).

The Brazilian Atlantic Forest is one of the most important tropical forests in the world and it originally covered almost the entire Brazilian coastland, with area of 1.3 million km^2 (3,200 km long). After five centuries of human occupation and economic exploitation, the Brazilian Atlantic Forest has been reduced to only 7.5 % of its original area (Myers *et al.* 2000). It was cleared mainly for timber, firewood, charcoal, agriculture, cattle breeding, and urban development (Morellato & Haddad 2000). The severe reduction and the large number of endemic species promoted the inclusion of the Brazilian Atlantic Forest into the list of the 25 world conservation hotspots by Conservation International (Myers *et al.* 2000). In spite of importance of the Brazilian Atlantic Forest and the great species diversity with most of trees species (51%) being endemic (Mittermeier 1997), few studies have been carried out to analyze the genetic diversity of plant species from this biome and even fewer focusing on their phylogeographic pattern (Cardoso *et al.* 1998; Cardoso *et al.* 2000; Lira *et al.* 2003; Salgueiro *et al.* 2004; Lorenz-Lemke *et al.* 2005).

The species analyzed in this study, *Dalbergia nigra* (Vell.) Allemao ex Benth (Fabaceae – Papilionoideae), is an endangered tree species restricted to the Brazilian Atlantic Forest, popularly known as Brazilian Rosewood or "jacarandá-da-Bahia". It occurs from southern Bahia to northern São Paulo states (Carvalho 1997). From the beginning of the European colonization, *D. nigra* has been heavily logged as a source of wood for the manufacture of fine furniture and musical instruments (Lorenzi 1992; Carvalho 1994). The over-exploitation and deforestation of the Brazilian Atlantic Forest made *D. nigra* extremely rare in nature (Carvalho 1997), and extant stands are restricted

to conservation areas (parks and reserves), remote areas of difficult access and small forest remnants (Costa *et al.* 1998). According to the Official List of Threatened Brazilian Plants (IBAMA 1992) and to the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 1992), *D. nigra* is an endangered species due to the decline of its natural habitat and absence of reposition plantations. This inclusion causes the prohibition of its commercialization and international trade. Furthermore, *D. nigra* is listed as vulnerable according to the criteria of The World Conservation Union (IUCN) Red List of Threatened Species, which means that it is facing a high risk of extinction in the wild in the medium-term future (IUCN 1994). Adult trees of *D. nigra* reach 15-25 m in height and 40-80 cm in diameter at breast height (dbh) (Carvalho 1994). Data on its mating system is not available; however, studies of a congeneric species in Brazil (*D. miscolobium*) suggested it is outcrossing with an apparent self-incompatibility system (Gibbs & Sassaki 1998). Pollen of *D. nigra* is likely to be dispersed by bees (personal observation) and seeds by wind (Carvalho 1994), as it was observed in a congeneric species in India (Mohana *et al.* 2001).

To date, there has been no large-scale molecular study of *D. nigra* to determine the extent of its genetic diversity. The only available genetic study in this species was performed in a restrict region from Minas Gerais state using allozyme markers (Ribeiro *et al.* 2005). The results of that study suggested that the analyzed remnants exhibited different levels of genetic diversity, depending on the degree of anthropogenic disturbance. Moreover, the considerable genetic diversity observed in the study pointed to the importance of adequate conservation of small remnants for the preservation of the genetic diversity of *D. nigra* populations (Ribeiro *et al.* 2005).

The goal of the present study was to investigate through chloroplast DNA (cpDNA) sequences the degree and patterns of genetic diversity and phylogeographic structure in populations of *D. nigra* sampled throughout most of its geographical range. The results were also interpreted considering the different degrees of recent anthropogenic disturbance of those populations. Several questions were formulated: 1) What is the geographic distribution of the cpDNA diversity in *D. nigra*? 2) What is the relation between the geographic distribution of genetic diversity of this species and the palynological and biogeographic evidences? 3) What are the implications of the results for conservation of *D. nigra*?

Materials and Methods

Population sampling

Seventeen populations of *Dalbergia nigra* were sampled covering most of its natural distribution in the Brazilian Atlantic Forest (Fig. 1 and Table 1). Leaves were collected from about 10 individuals per population, at a minimum of 10 m apart from one another, totaling 168 individuals. Populations were located in three Brazilian States: Minas Gerais (MG), Espírito Santo (ES) and Bahia (BA). The sampling extended from 13º-20º N and 39º-45º W with elevation ranging between 58 and 870 m (Table 1). The distance between *D. nigra* populations sampled in this study varied approximately from 10 to 750 km. Two populations, RDO and VRD, were located in large biological reserves (36,000 and 22,000 ha, respectively) and one, SCR, in a small private reserve (51 ha). The other populations were located in small forest remnants. Following

collection, leaves were shipped in ice from the field and, once in the laboratory, they were stored at –70 ºC until DNA extraction was performed.

DNA extraction, amplification and sequencing

Total DNA was extracted from ground leaf using a procedure modified from CTAB (cetyltrimethyl ammonium bromide) method (Doyle & Doyle 1987), and stored at –20 ºC for further analyses. Screening for cpDNA amplification and polymorphism in *D. nigra* was conducted using 15 universal primer pairs for non-coding cpDNA regions on a subset of individuals of this study (information about primer pairs used is available on request). Of these primer pairs only seven exhibited specific amplification, but most of them showed no or very low levels of polymorphism. Then, two non-coding cpDNA regions were selected for analysis in *D. nigra* populations: *trnV-trnM* intergenic spacer and intron *trnL*. These regions were amplified and sequenced using primer pairs described by Demesure *et al.* (1995) and Taberlet *et al.* (1991). Polymerase chain reactions (PCR) were conducted in a total volume of 25 µl containing 10 ng of genomic DNA, 1X Taq buffer with 2.0 mM MgCl2 (Phoneutria), 0.2 ng of BSA (bovine serum albumin), 200 µM of each dNTP, 0.5 µM of each primer, 1U Taq DNA polymerase (Phoneutria) and, finally autoclaved deionized water to complete the volume. Amplifications were performed on Eppendorf thermocycler using an initial denaturation at 94 ºC for 4 min (*trnV-trnM*) or 2 min (*trnL*), followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing at 59 ºC (*trnV-trnM*) or 56 ºC (*trnL*) for 1 min, and extension at 72 ºC for 2 min (*trnV-trnM*) or 1 min (*trnL*), plus a final extension of 72 ºC for 7 min. Following PCR, the products were visualized on 1% TAE agarose gels stained with ethidium bromide, and purified with 20% PEG (polyethylene glycol) precipitation. Each PCR product was then double-strand sequenced using the DYEnamic ET dye terminator

sequencing Kit (GE Healthcare) following the protocol of the manufacturer. Sequencing reactions were analyzed on a MegaBACE 1000 automated sequencer (GE Healthcare). In order to determine any PCR errors or sequencing artifacts, two or more independent PCR amplifications were sequenced for samples that exhibited an unexpected polymorphism.

Data analysis

The quality of the cpDNA sequences was checked, and overlapping fragments were assembled using PHRED v. 0.20425 (Ewing & Green 1998; Ewing *et al.* 1998), PHRAP v. 0.990319 (http://www.phrap.org/) and CONSED 12.0 (Gordon *et al.* 1998) to produce high quality consensus sequences. These sequences were aligned using CLUSTAL-W (Thompson *et al.* 1994) implemented in MEGA 3.1 program (Kumar *et al.* 2004) with default gap penalties and manual verification. Sequences were deposited in GenBank under the accession numbers XXX for the *trnV-trnM* intergenic spacer and YYY for the *trnL* intron. All analyses were performed using the combined data of these two cpDNA sequences.

The distribution of the haplotypes of the *D. nigra* populations, and the indices of haplotype (h) and nucleotide (π) diversities were obtained using DNASP 4.01 (Rozas *et al.* 2003) and ARLEQUIN 3.01 (Excoffier *et al.* 2005) programs. Additionally, Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) neutrality tests and pairwise mismatch distributions were also performed with the ARLEQUIN program. Phylogenetic relationships between cpDNA haplotypes were estimated using the median-joining algorithm (Bandelt *et al.* 1999) implemented by NETWORK 4.1 program (available at www.fluxus-engineering.com). A hierarchical analysis of molecular variance (AMOVA)

was performed to assess the genetic differentiation within and among populations with the ARLEQUIN program.

The barrier was identified taking into account the relationship between the genetic distances and the geographic coordinates of the *D. nigra* populations using the Monmonier's (1973) maximum difference algorithm implemented in BARRIER 2.2 software (Manni & Guérard 2004). This method identifies the most differentiated groups of populations that are as spatially clustered as possible. It creates a Delaunay triangulation network (Delaunay 1934) to connect adjacent populations and, consequently, a set of Voronoï tessellation (polygons). Once a Voronoï tessellation is obtained, each edge is associated with the estimated value of genetic distance between the two populations that it separates. Monmonier's algorithm was then applied to identify boundaries starting from the edge for which the distance value was the highest (Manni *et al.* 2004). Moreover, a hierarchical level was added in the AMOVA analysis, as well as neutrality tests and pairwise mismatch distributions, by divising of the populations into geographic groups according to the boundary identified by Monmonier's analysis.

Results

Seven non-coding cpDNA regions were sequenced in an initial analysis, but informative variation was observed only in two of them. A total of 13 haplotypes were detected in the analyses of both cpDNA sequences, with total haplotype diversity equal to 0.7072 (Table 1). The alignment of *trnV-trnM* and *trnL* sequences had a total length of 1297 characters. Nine polymorphic sites were found among all samples of *D. nigra* comprising eight point mutations and one indel (Table 2). The highest indices of haplotype (h) and nucleotide (π) diversities were found in the population PPA from

northeastern of Minas Gerais State (due the presence of haplotypes A and G) and in two populations localized in great biological reserves, VRD and RDO (Table 1 and 2). Three of the 17 populations analyzed were monomorphic (ITA, EUN and DIO), whereas the other remaining exhibited from two to five haplotypes (Table 1 and Fig. 2A). Haplotypes B, F and N were unique to populations AMA, MED and VNI, respectively (Table 2).

The Monmonier's maximum difference algorithm suggested one main putative genetic barrier dividing the range of *D. nigra* populations into a southern and a northern group (Fig. 2B). The northern group included all *D. nigra* populations from Bahia (BA) and two populations (MED and PPA) from northeast of Minas Gerais (MG), whereas the southern group included all populations from Espírito Santo (ES) and other remaining populations from Minas Gerais (Fig. 2A). The haplotype A and G occur in high frequencies in north and south groups, respectively. However, the PPA population exhibited both haplotypes (Fig. 2A), but it was part of the northern group according Monmonier's algorithm (Fig. 2B). AMOVA analysis showed that 55% and 45% of the genetic diversity is found among and between populations considering all populations, respectively (Table 3). The clear geographical structure in *D. nigra* populations was further supported by AMOVA analysis, which revealed that approximately 62% of the genetic diversity is found between north and south groups ($P < 0.0001$, Table 3).

The relationships among haplotypes were resolved by network analysis using the median-joining algorithm (Fig. 3). Each haplotype was distinguishable from the others for one mutation, as well as the haplotypes A and G, which separated the north and south groups. The haplotypes A-F are somehow restricted to the north group, while G-N to the south group (Fig. 2A). Independent mutation events converging to a shared haplotype could explain some connections observed in the network analysis. Tajima's D

tests were not significant ($P > 0.05$) to all *D. nigra* populations (–0.753) and, to north (–1.216) and south (–1.091) groups separately. However, Fu's Fs tests were significantly negative to all samples (all populations = –6.309; north group = –3.483; south group = –3.698; $P < 0.05$) showing a significant departure from neutral model with constant population size. The mismatch distribution was unimodal to all populations (but not for each group taken separately; data not shown) what is in accordance with the expectation of a recent demographic expansion (Fig. 4).

Discussion

The phylogeographic analyses of cpDNA sequences showed that populations of *D. nigra* in the Brazilian Atlantic Forest are geographically structured. Considering all populations, *D. nigra* exhibited 55% of genetic diversity among populations (Table 3). This value was lower than the mean values observed for different species of angiosperms analyzed with cpDNA markers (G_{ST} = 64,6%), however, it was close to the value of the first quartile of the normal distribution of G_{ST} to the same species (G_{ST} = 41,6 to 87,1%, Petit *et al.* (2005). The Monmonier's algorithm (barrier analysis), the AMOVA and the network analysis identified one latitudinal genetic differentiation that divided the populations into northern and southern groups. The chloroplast haplotype network (Fig. 3) showed that these groups, corresponding to haplotypes A and G, were separated only by one mutation step.

Palynological studies of Quaternary sediment cores from several sites in South America permitted the analysis of the vegetation changes through time (Behling 1995, 1998, 2002b, 2003, Salgado-Labouriau et al. 1998). As other tropical regions, the Brazilian Atlantic Forest suffered climate changes during the Pleistocene and Holocene periods altering the vegetation distribution and promoting some genetic subdivision

across the geographical range of the species (e.g., Costa 2003, Moraes Barros *et al.* 2006). Pollen records from Southeastern Brazilian sites evidencied that during the last glacial period the subtropical grasslands and gallery forest replaced the modern tropical forest and cerrado vegetation (Behling 1998). At that period, the Atlantic rain forest was much smaller than the original distribution today due to drier conditions and was fragmented for several times (Behling 2002). The molecular data here exhibited for *D. nigra* populations, showing a latitudinal split in haplotypes distribution could be related with the last glacial events. Palynological data from Lago do Pires (17°57`S, 42°13`W) (Behling 1995) and Lagoa Nova (17°58`S, 42°12`W) (Behling 2003), located near the studied populations PPA and TOT (Table 1 and Fig. 2), indicated that during the early Holocene the landscape was dominated by savanna and small areas of gallery forest. The change to wetter climatic conditions started around 6,000-5,000 ^{14}C yr B.P. and only after about 900-600 B.P dense forest expanded throughout the studied region. The climatic changes that occurred throughout the Quaternary in the Brazilian Atlantic Forest, that may have formed the putative forest refuges, and the occurrence of subsequent migration from the refuges, may explain the division observed of *D. nigra* populations into south and north groups. Moreover, the PPA population, that exhibited haplotypes typical for each group can represent a (re) colonization from refuges in north and south groups after amelioration of the climate that followed the glaciation periods. Biogeographic evidences presented by Whitmore and Prance (1987) also support such interpretation. They found endemic centers for Neotropical plants, butterflies, and birds superimposed with the paleoecological forest refuges. Two of the refuges pointed by those authors in southeast Brazil coincide with the probable sites of remaining populations of *D. nigra* that by vicariance can resulted in the north and south groups found in the present study.

The division of species genetic variation in the Brazilian Atlantic Forest in northeast of Minas Gerais and south of Bahia States in north and south groups as revealed in *D. nigra* was also observed in similar phylogeographic studies with small mammals (Costa 2003) and sloths (Moraes Barros *et al.* 2006). In *Micoreus demerare* as also in *Metachirus nudicaudatus*, two geographically structured clades were found separating Atlantic forest into northern and southern groups (Costa 2003). Two latitudinal groups in Atlantic Forest were also identified in two sloth species, *Bradypus torquatus* (sampled in Bahia and Espírito Santo) and *B. variegates* (sampled in Bahia/Teófilo Otoni in Minas Gerais and São Paulo) (Moraes-Barros *et al.* 2006). The authors suggest that such divergences could be associated with the late Pleistocene, once the savannah areas in central Brazil separated the north and south region of the current Atlantic Forest. The similar pattern observed in these animal species and in *D. nigra* represents a strong evidence for the occurrence of vicariant events in this region (northeast of Minas Gerais and south of Bahia States) that resulted in the isolation of populations with consequent genetic differentiation.

Other phylogeographic studies in a few plant species from the Brazilian Atlantic Forest have also revealed geographic structure of the genetic diversity. Lorenz-Lemke *et al.* (2005) observed an ITS variation geographically structured in *Passiflora actinia* populations in the Atlantic Forest from south Brazil. The authors suggested that such structuration resulted probably of the Atlantic Forest expansion from northern locations into Rio Grande do Sul state, in a fragmentary way, in periods of significant climatic improvement in Holocene. Moreover, the possible formation of different glacial refuges in the Atlantic Forest were proposed to explain the phylogeographic structure observed in the tree species *Eugenia uniflora* sampled in Rio Grande do Sul, Rio de Janeiro and Pernambuco states (Salgueiro *et al.* 2004). Additionally, the same explanation was

suggested for the genetic differentiation of tree species *Caesalpinia echinata* in three geographic groups: Rio Grande do Norte, Rio de Janeiro, and Espírito Santo/Bahia states (Lira *et al.* 2003). All those examples, plus the data obtained for *D. nigra* pointed for the importance of historical climatic changes affecting the current genetic structure of the populations.

Besides historical events, the genetic structure of natural populations is also influenced by recent fragmentation of habitats promoted by anthropogenic disturbances (reviewed by Young *et al.* 1996; Lowe *et al.* 2005). Considering that the deforestation and consequent fragmentation of the Brazilian Atlantic Forest have been occurring since the beginning of the colonial period, in 1500s, it is expected different levels of genetic diversity among populations analyzed of *D. nigra* according to the degree of anthropogenic disturbance. The RDO and VRD populations that exhibited the highest values of cpDNA diversity measures are located in large biological reserves. The other populations (except PPA), located in small forest fragments, exhibited from intermediate to low levels of genetic diversity. In a previous study, Ribeiro *et al.* (2005) surveyed the allozyme diversity of three *D. nigra* populations from Minas Gerais State, corresponding to populations RDO, SCR and DIO analyzed in the present study. The first population, located in Rio Doce State Park, and other two, in small surrounding fragments, where SCR was relatively well protected while DIO exhibited a high degree of anthropogenic disturbance. Allozyme data agreed with cpDNA sequences, revealing high diversity in RDO, intermediate values in SCR and low diversity in DIO, reflecting the fragmentation effect. In the results presented here, the reduced genetic diversity observed in most of the sampled populations in small forest fragments probably reflects events that occurred after the recent fragmentation, as genetic drift and restriction on gene flow due to isolation of populations.

Studies of intraspecific genetic diversity, as phylogeographic data, may contribute to the development of conservation strategies, identifying appropriate units for conservation (Newton *et al.* 1999). *D. nigra* is an endangered species with distribution in the Brazilian Atlantic Forest, that is one of most threatened Brazilian biomes and therefore, the conservation of this species and its environment is critical. The observation, in the present study, that most of the analyzed populations collected in small fragments exhibited low genetic diversity as compared to those collected in large reserves is an evidence that this species has suffered great anthropogenic pressure. Considering that the Brazilian Atlantic Forest is currently reduced to 7.5% of the original coverage and the genetic divergence between north and south groups, all extant area must be taken into account for conservation of the genetic diversity in this species. Particularly, the northeast of Minas Gerais State constitutes a priority for conservation. In this region the evidences arisen from animal studies (Costa 2003; Moraes-Barros *et al.* 2006) plus the phylogeographic data presented here for *D. nigra* point out for this high priority. The high genetic diversity found in *D. nigra* in the northeast of Minas Gerais, due to the presence of populations exhibiting haplotypes from northern and southern groups, indicates the need of implementation of conservation units in this region of the Atlantic Forest. Moreover, the populations that exhibited haplotypes unique, as AMA, MED and VNI, also present necessity for conservation. The phylogeographic analysis presented here, using a large-scale survey from an endemic and endangered species, contributed to reinforce evidences of biogeographic events that have had great importance in the evolutionary history of the Atlantic Forest, as also presented data for priority actions for conservation.

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Figure Legends

Fig. 1 (A) Distribution area of the Brazilian Atlantic Forest: original area in 1500s (gray) and remaining area in 1990s (black) (adapted from Morellato & Haddad 2000). **(B)** Map of the 17 populations sampled for the phylogeography study of *Dalbergia nigra* in three Brazilian States: Bahia (BA), Minas Gerais (MG) and Espírito Santo (ES). See Table 1 for names of the populations.

Fig.2 (A) Geographic distribution of cpDNA haplotypes identified in the sampled populations of *Dalbergia nigra*. The haplotypes are represented in the legend. The sizes of the pie charts are proportional to the number of sampled individuals per populations. See Table 1 and Fig. 1 for description of the populations. **(B)** The first genetic boundaries (bold line) among 17 populations of *Dalbergia nigra* obtained with Monmonier's maximum difference algorithm (see text for details of the analysis). External and internal lines represent the Voronoï tessellation and Delaunay triangulation, respectively.

Fig. 3 Network analysis of genealogical relationships among 13 haplotypes of *Dalbergia nigra* based on cpDNA sequences. The haplotypes were represented for letter and color similar the figure 2. Each line between haplotypes represents a mutational step, with numbers on lines indicating the variable base pair position. The area of each circle in the network is proportional to the frequency of the haplotype across populations.

Fig. 4 Mismatch distribution histogram for cpDNA haplotypes, indicating observed and expected numbers of pairwise differences between *Dalbergia nigra* individuals.

Table 1 Sampled populations of *Dalbergia nigra* in the Brazilian Atlantic Forest and molecular diversity indexes: location, geographical coordinates (Latitude and Longitude), elevation (meters), number of individual (*n*), haplotype (*h*) and nucleotide diversities (π).

Population (abbreviation)	State	Latitude (N)	Longitude (W)	Elevation (m)	*n*	*h*	π
01. Amargosa (AMA)	BA	13º02'00"	39º36'00"	390	10	0.3556	0.00027
02. Apuarema (APU)	BA	13º51'00"	39º44'00"	296	8	0.4643	0.00039
03. Barro Preto (BPR)	BA	14º46'10"	39º32'37"	544	10	0.3556	0.00027
04. Vitória da Conquista (VCO)	BA	15º03'11"	40º44'52"	644	11	0.3273	0.00025
05. Itapebi (ITA)	BA	16º00'08"	39º35'10"	144	10	0.0000	0.00000
06. Eunapólis (EUN)	BA	16º19'11"	39º35'50"	186	6	0.0000	0.00000
07. Porto Seguro (PSE)	BA	16º53'49"	39º27'16"	81	10	0.3556	0.00027
08. Medina (MED)	MG	16º13'00"	41º29'00"	587	10	0.5111	0.00051
09. Padre Paraíso (PPA)	MG	17º06'00"	41º29'00"	780	14	0.7363	0.00072
10. Teofilo Otoni (TOT)	MG	17º51'00"	41º30'00"	334	10	0.3778	0.00043
11. Rio Doce State Park (RDO)	MG	19º42'00"	42º30'36"	262	10	0.7333	0.00084
12. Santa Cruz Particular Reserve (SCR)	MG	19º48'00"	42º43'00"	410	10	0.2000	0.00031
13. Dionísio (DIO)	MG	19º52'00"	42º46'00"	330	10	0.0000	0.00000
14. Abre Campo (ACA)	MG	20º20'04"	42º27'06"	680	10	0.4667	0.00036
15. Pará de Minas (PMI)	MG	19º53'51"	44º33'43"	870	10	0.2000	0.00015
16. Vale do Rio Doce Reserve (VRD)	ES	19º09'03"	40º00"12"	58	9	0.7222	0.00081
17. Venda Nova do Imigrante (VNI)	ES	20º18'59"	41º07'02"	790	10	0.3778	0.00031
Total					168	0.7072	0.00078

Table 2 Variable sites of the aligned sequences of cpDNA sequences in thirteen haplotypes observed in *Dalbergia nigra* populations and their distribution for each population.

	trnV-trnM						*intron trnL*																						
	0	1	3	4	5	6	0	0	5																				
	5	5	5	8	1	7	3	5	5	Populations																			
Haplotype	7	6	8	5	4	6	0	3	6	AMA	APU	BPR	VCO	ITA	EUN	PSE	MED	PPA	TOT	RDO	SCR	DIO	ACA	PMI	VRD	VNI	Total
A	T	T	T	T	A	A	A	A	A	**8**	**6**	**8**	**9**	**10**	**6**	**8**	**2**	**6**									**63**
B	.	.	.	.	.	.	.	.	T	2																	2
C	G	.	.	.	.	.	.	.	.		1		2														3
D	.	.	.	.	.	T	.	.	.		1					2	1	3									7
E	.	.	.	.	.	.	T	.	.			2						1									3
F	.	.	.	.	T	.	.	.	.								7										7
G	.	C	.	.	.	.	.	.	.									**4**	**8**	**5**	**9**	**10**	**7**	**9**	**5**	**8**	**65**
H	.	C	G	.	.	T	.	.	.										1	2							3
I	.	C	.	.	.	T	.	.	.										1	2				1	1		5
J	.	C	.	.	.	.	.	-	.											1			3		1		5
L	.	C	.	G	.	.	.	-	.												1				1		2
M	.	C	.	.	.	.	.	.	T																1	1	2
N	.	C	.	G	.	.	.	.	.																	1	1

Table 3 Analyses of molecular variance (AMOVA) for populations of *Dalbergia nigra* based on cpDNA sequences.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)	*P*-value
(1) Total populations					
Among populations	16	49.21	0.28781	55.00	<0.0001
Within populations	151	35.551	0.23544	45.00	<0.0001
(2) North vs. South groups					
Among groups	1	38.999	0.45767	61.99	<0.0001
Among populations within groups	15	10.212	0.04519	6.12	<0.0001
Within populations	151	35.551	0.23544	31.89	<0.0001

Fig. 1

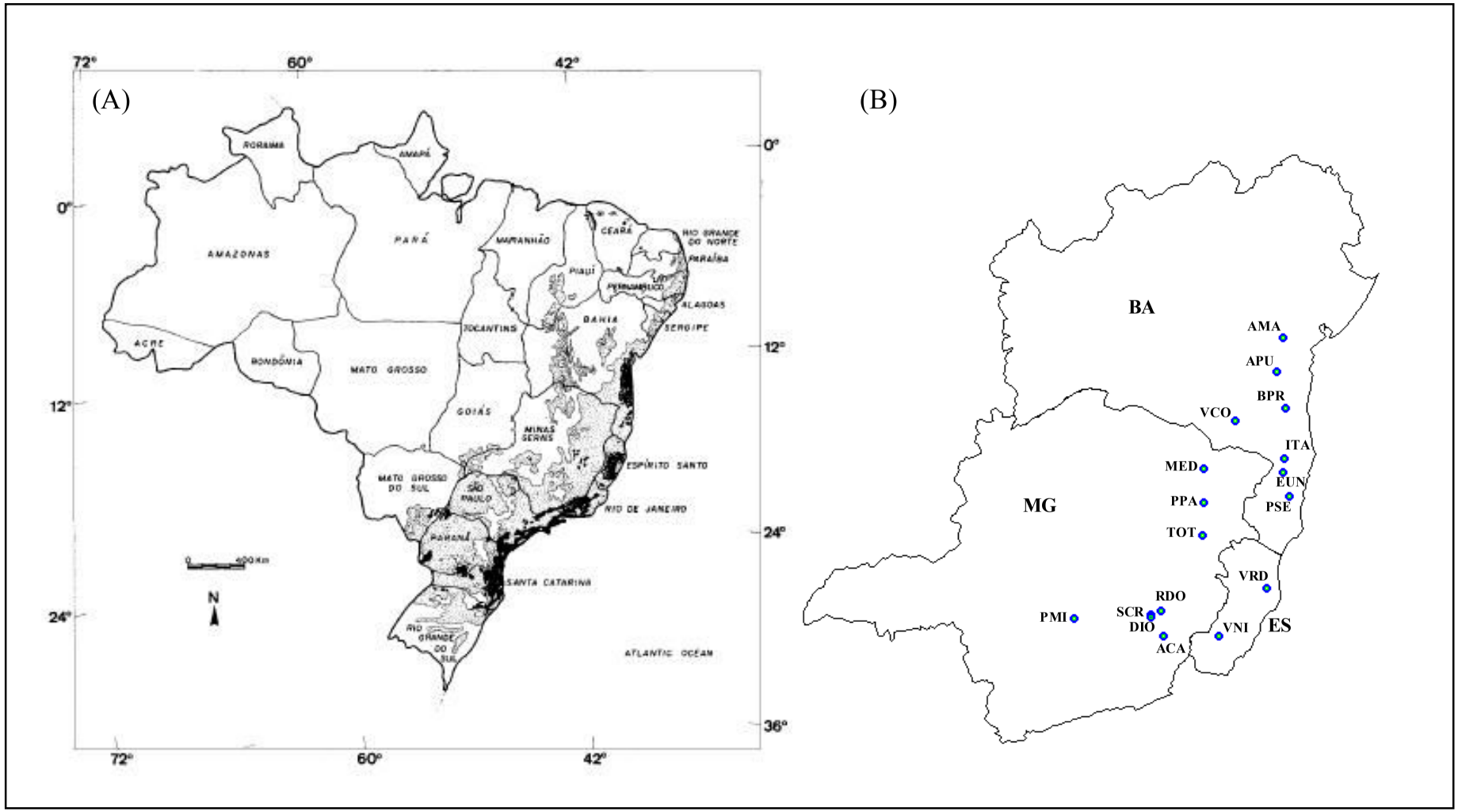
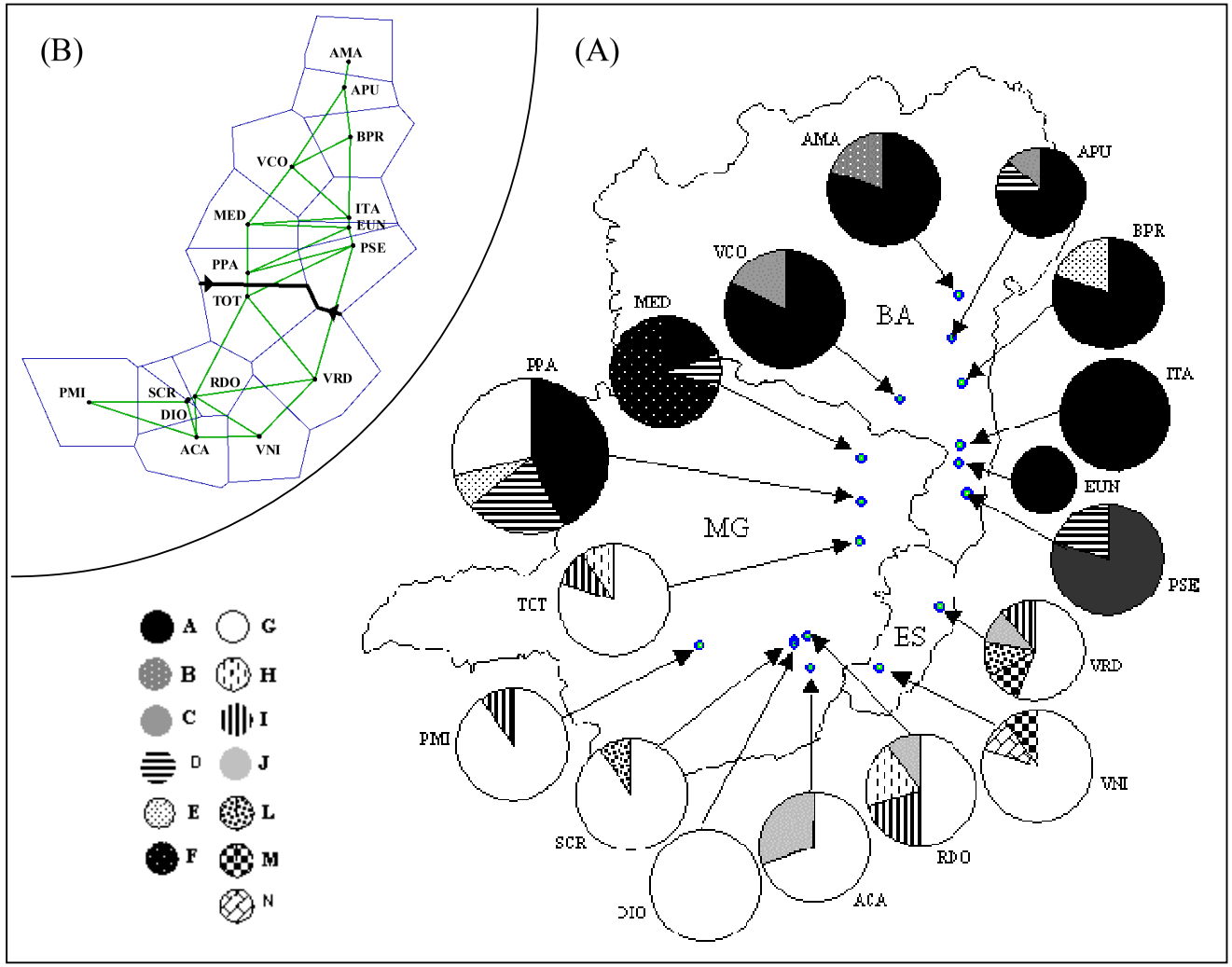

(A)
72°
60°
42°
0°
12°
24°
36°
RORAIMA
AMAPÁ
AMAZONAS
PARÁ
MARANHÃO
CEARÁ
RIO GRANDE DO NORTE
PARAÍBA
PIAUÍ
PERNAMBUCO
ALAGOAS
SERGIPE
BAHIA
TOCANTINS
ACRE
RONDÔNIA
MATO GROSSO
GOIÁS
MINAS GERAIS
ESPÍRITO SANTO
MATO GROSSO DO SUL
SÃO PAULO
RIO DE JANEIRO
PARANÁ
SANTA CATARINA
RIO GRANDE DO SUL
ATLANTIC OCEAN
N
(B)
BA
MG
ES
AMA
APU
BPR
VCO
ITA
MED
EUN
PPA
PSE
TOT
VRD
RDO
PMI
SCR
DIO
VNI
ACA

Fig. 2


(B)
AMA
APU
BPR
VCO
MED
ITA
EUN
PSE
PPA
TOT
PMI
SCR
RDO
VRD
DIO
ACA
VNI
(A)
AMA
APU
BPR
VCO
MED
BA
PPA
ITA
EUN
MG
PSE
TOT
ES
VRD
PMI
VNI
SCR
RDO
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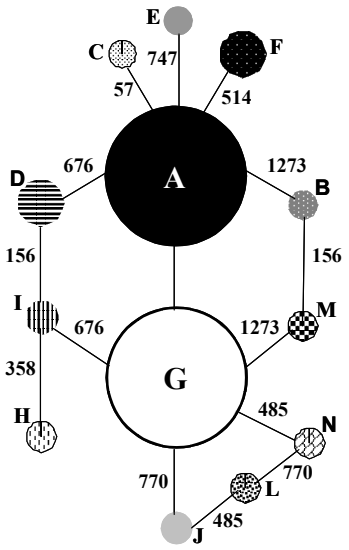
ACA
A
B
C
D
E
F
G
H
I
J
L
M
N

Fig. 3


E
C
747
F
57
514
676
A
1273
D
B
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I
676
1273
M
358
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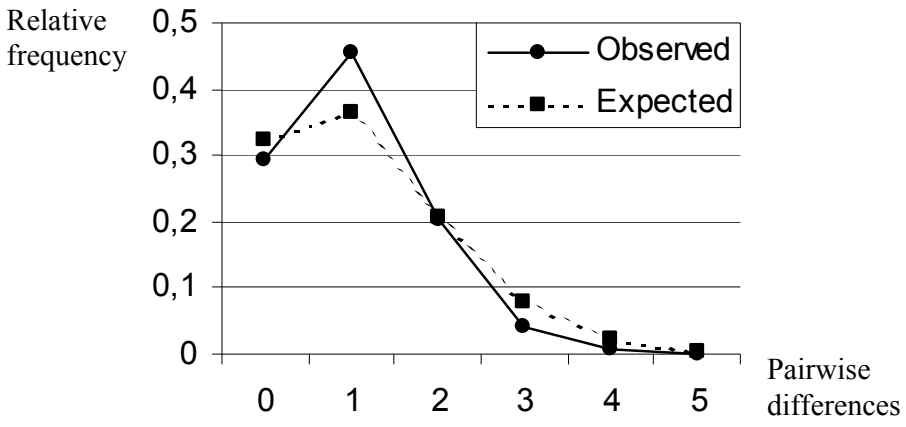
H
485
N
770
770
L
485
J

Fig. 4


Relative frequency
0,5
0,4
0,3
0,2
0,1
0
Observed
Expected
0
1
2
3
4
5
Pairwise differences

ARTIGO 2:

Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus *Dalbergia*

***Genetics and Molecular Research*, 6(1):173-187 (2007)**

Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus *Dalbergia*

Running title: DNA extraction in fresh and herbarium specimens of *Dalbergia*

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Abstract

Five published DNA extraction protocols were compared for their ability to produce good quality DNA from fresh and herbarium leaves of several species of the genus *Dalbergia*. The leaves of these species contain high amounts of secondary metabolites, which make it difficult to perform a clean DNA extraction and thereby interfering with subsequent PCR amplification. The protocol that produced the best DNA quality in most of the *Dalbergia* species analyzed utilizes polyvinylpyrrolidone to bind the phenolic compounds, a high molar concentration of NaCl to inhibit co-precipitation of polysaccharides and DNA, and LiCl for removing RNA by selective precipitation. The DNA quality of herbarium specimens was worse than that for fresh leaves, due to collecting conditions and preservation of samples. We analyzed 54 herbarium specimens, but the recovered DNA allowed successful PCR amplification in only eight. For the genus *Dalbergia*, the herbarium is an important source of material for phylogenetic and evolutionary studies; due to the occurrence of the different species in various geographical regions in Brazil, it is difficult to obtain fresh material in nature. Our results demonstrated that for *Dalbergia* species the methods used for the collection and preservation of herbarium specimens have a mayor influence on DNA quality and in the success of phylogenetic studies of the species.

Key words: *Dalbergia* species, DNA extraction, herbarium specimens, PCR amplification

Introduction

The genus *Dalbergia* (Fabaceae – Papilionoideae) comprises more than 100 species of trees, shrubs and lianas distributed pantropically (Polhill, 1981), of which 41 species occur in Brazil (Carvalho, 1997). Many of these species are economically important due to their attractive and valuable timber; however, over-exploitation and habitat fragmentation have caused them to be in danger of extinction in the tropics, such as *D. nigra* (Brazilian rosewood, Ribeiro *et al.*, 2005). Carvalho (1989) performed a systematic study of the genus *Dalbergia* in Brazil, combining reviews of taxonomic history and biogeography, and data on vegetative and reproductive morphology, comparative anatomy of leaf and fruits, as well as of leaf flavonoids and pollen morphology. The genus *Dalbergia* is recognized as having five sections defined by inflorescence and fruit characters: sects. *Dalbergia* L.f., *Triptolemea* (Mart. ex Benth.) Benth., *Selenolobium* Benth, *Pseudecastaphyllum* A.M. de Carvalho, and *Ecastaphyllum* (P.Browne) Ducke (Carvalho 1997). Such a classification reflects the phylogenetic relationships among *Dalbergia* species (Carvalho, 1989), but DNA sequence data are necessary to corroborate the relationships.

The recent advent of techniques of DNA analysis by polymerase chain reaction (PCR) and DNA sequencing has increased interest in plant systematics by using DNA sequence data to study the relationships among species, thereby complementing the enormous amount of morphological data available mainly for legume taxa (e.g., Lavin *et al.*, 2001; Gervais and Bruneau, 2002; Wojciechowski, 2003). However, to obtain accurate DNA sequence information, it is necessary to isolate good quality DNA that is relatively free from the many contaminants found in plant cells (Jobes *et al.*, 1995). Many plant species contain characteristically high amounts of polysaccharides, polyphenols and other secondary metabolites, substances known for binding firmly to

nucleic acids during DNA extraction and interfering with subsequent reactions (Pirttilä *et al.*, 2001).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Numerous protocols for DNA extraction from plants have been published (e.g., Doyle and Doyle, 1990; Scott and Playford, 1996; Csaikl *et al.*, 1998; Sharma *et al.*, 2000; Li *et al.*, 2001; Pirttila *et al.*, 2001; Drabkova *et al.*, 2002; Shepherd *et al.*, 2002; Mogg and Bond, 2003; Haymes *et al.*, 2004). Because plants contain high amounts of many different substances, it is unlikely that just one DNA extraction protocol is suitable for all plants (Loomis, 1974). To obtain good quality DNA, the utilization of fresh and young leaf tissue is ideal (Sytsma *et al.*, 1993). However, DNA has been widely isolated from desiccated leaves and material stored in silica gel or dehydrated and stored in a herbarium. In these cases, the extraction protocols frequently produce low DNA yield and quality. Satisfactory DNA quality from herbarium specimens is essential for the success of further studies using DNA sequences. For Brazilian *Dalbergia* species, herbaria are an important source of material for phylogenetic and evolutionary studies, since the species occur in different geographical regions comprising all Brazilian biomes, making it difficult to obtain fresh material from all species in nature.

This study is the first part of a phylogenetic analysis of *Dalbergia* and related genera from the Fabaceae for which it was necessary to select satisfactory methods of DNA extraction for amplification and sequencing of both chloroplast DNA (cpDNA – *trnL* intron) and nuclear ribosomal DNA (nrDNA – ITS region). Five published DNA extraction protocols were compared for their ability to produce good quality DNA from fresh and herbarium leaves of several *Dalbergia* species from four herbaria in Brazil.

Materials and Methods

Plant material

Seventy-three samples of fresh leaves and herbarium specimens from 33 species pertaining to five sections of the genus *Dalbergia* were analyzed (Table 1, Appendix A). Fresh leaves were collected from species of different geographical regions in Brazil and stored frozen at –20 ºC until DNA extraction. The dried leaves used came from herbarium specimens catalogued in four herbaria in Brazil: BHCB (Departamento de Botânica, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais), INPA (Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas), CEPEC (Centro de Pesquisas do Cacau, CEPLAC, Itabuna, Bahia) and SP (Instituto de Botânica de São Paulo, São Paulo). Herbarium codes are according to Holmgren *et al.* (1981).

Testing DNA extraction protocols

The DNA extraction protocols were analyzed in three groups of data: 1) fresh leaves from five species, 2) fresh leaves and herbarium specimens from four species, and 3) several herbarium specimens of 24 species (Table 1, Appendix A). To facilitate the distinction between fresh and herbarium specimens the letter "H" was added to its abbreviation. Total genomic DNA was extracted from fresh leaves by the protocols A to D, whereas herbarium specimens by protocols A to E (including the fresh leaves from four species in group 2). In all DNA extraction protocols 0.1 g of leaf tissue was utilized from each sample, ground in liquid nitrogen immediately prior to the procedure. Equipment and materials used in all protocols were: 1) Mortar and pestle; 2) 1.5- and 2.0-ml microcentrifuge tubes; 3) Liquid nitrogen; 4) Water bath (65 ºC); 5) Centrifuge and rotor capable of 14,000 rpm (= 17,746 *g*) and 2-ml holding tubes (e.g., centrifuge

A14 Thermo Electron Corporation, United States). All centrifugation steps were performed at 14,000 rpm.

DNA Extraction protocol A (modified - Doyle and Doyle, 1990)

As a "classical" method, we used a modified cetyl trimethyl ammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1990), which has been utilized with success in many plant species. This protocol is based on lysis and purification with CTAB that selectively precipitates DNA while maintaining the solubility of many polysaccharides.

Chemicals

- Extraction buffer: 2% CTAB; 100 mM Tris/HCl, pH 7.5; 1.4 M NaCl; 2% polyvinylpyrrolidone (PVP)-40; 20 mM ethylene diamine tetracetic acid (EDTA), pH 8.0. Add 20 µl/ml β-mercaptoethanol immediately prior to use.
- Chroloform: isoamyl alcohol 24:1 (CIA).
- Isopropanol; 70% ethanol.
- TE-RNase solution: 10 mM Tris-HCl; 1 mM EDTA, pH 8.0; 10 mg/ml RNase.

Protocol

1. To each ground leaf sample, add 1 ml of extraction buffer and incubate samples for 1 hour at 60 ºC with occasional swirling.

2. Cool samples at room temperature, add 600 µl of CIA and mix gently for 5 min.

3. Centrifuge for 15 min. Transfer the supernatant to a new tube and add equal volume of isopropanol. Mix gently and incubate at –20 ºC overnight.

4. Centrifuge for 15 min. Wash the pellet with 70% ethanol and centrifuge for 10 min. Repeat this wash one or two times.

5. Dry the DNA and dissolve the pellet in 20-50 µl (according to your quantity) of TE-RNase solution. Incubate for 1 hour at 37 ºC.

DNA Extraction protocol B (Jobes et al., 1995)

This protocol utilizes PVP to bind the phenolic compounds, a high molar concentration of sodium chloride to inhibit co-precipitation of the polysaccharides and DNA, and an improved method for removing RNA by selective precipitation with lithium chloride.

Chemicals

- Extraction buffer: 100 mM sodium acetate, pH 4.8; 100 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM dithiothreitol (DTT); 2% PVP; pH 5.5. Add 100 µg/ml proteinase K immediately prior to use.
- 20% sodium dodecyl sulfate (SDS) solution.
- 5 M potassium acetate; 8 M LiCl; 5 M NaCl.
- Isopropanol; absolute and 70% ethanol.
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Protocol

1. To each ground leaf sample, add 1 ml extraction buffer and incubate sample for 1 hour at 55 ºC with occasional swirling.

2. Add SDS to each tube (final concentration 1.5%). Mix gently and incubate for 1 hour at 55 ºC with occasional swirling.

3. Centrifuge for 10 min. Transfer the supernatant to a new tube and add 1/3 volume of 5 M potassium acetate. Mix gently and incubate for 30 min at –20 ºC.

4. Centrifuge for 10 min. Transfer the supernatant to new tube and add 0.6 volume of isopropanol. Mix gently and incubate at –20 ºC overnight.

5. Centrifuge for 10 min and carefully pour off the supernatant.

6. Dissolve the pellet in autoclaved deionized water. Add 0.5 volume of 5 M NaCl and mix well. Add two volumes of cold absolute ethanol and incubate for 30 min at –20 ºC.

7. Centrifuge for 10 min. Dissolve the pellet in autoclaved deionized water. Precipitate RNA with 1/3 volume of cold 8 M LiCl (final concentration 2 M) and incubate at –20 ºC for at least 1 hour.

8. Recover RNA by centrifugation for 15 min and carefully transfer supernatant to a new tube. Precipitate DNA by adding 0.6 volume of isopropanol and incubate for 1 hour at –20 ºC.

9. Centrifuge for 10 min. Wash the pellet with 70% ethanol and centrifuge for 5 min. Pour off ethanol and briefly dry the pellet. Dissolve the pellet in 20-50 µl of TE buffer.

DNA Extraction protocol C (Dellaporta et al., 1983)

This protocol utilizes SDS as detergent and the addition of potassium acetate resulted in the removal of some proteins and polysaccharides as a complex with the potassium-SDS precipitate.

Chemicals

- Extraction buffer: 10% SDS; 50 mM Tris/HCl; 100 mM NaCl; 10 mM EDTA, pH 8.0. Add 20 µl/ml β-mercaptoethanol and approximately 0.01 g of PVP-40 immediately prior to use.
- 5 M potassium acetate; 3 M sodium acetate.
- Isopropanol; absolute and 70% ethanol.
- TE-RNase solution; TE buffer.

Protocol

1. To each ground leaf sample, add 1 ml of extraction buffer and incubate sample for 45 min at 65 ºC with occasional swirling.

2. Add 300 µl of 5 M potassium acetate and mix gently. Incubate sample for 20 min on ice.

3. Centrifuge for 10 min. Transfer the supernatant to a new tube and add equal volume of isopropanol. Mix gently and incubate for 1 hour at –20 ºC.

4. Centrifuge for 15 min. Wash the pellet with 70% ethanol and centrifuge for 5 min.

5. Dry the DNA and dissolve the pellet in 20-50 µl of TE-RNase solution. Incubate for 1 hour at 37 ºC.

6. Precipitate the DNA by adding 10% volume of 3 M sodium acetate and two volumes of absolute ethanol. Incubate at –20 ºC overnight. Repeat steps 5 and 6 twice.

7. Dry the DNA and dissolve the pellet in 20-50 µl of TE buffer.

DNA Extraction protocol D (modified - Scott and Playford, 1996)

This protocol modifies the CTAB method by isolating membrane-bound organelles in order to remove polysaccharides and secondary metabolites. It should be suitable for a wide range of species, with particular applicability to rainforest plants with high polysaccharide or secondary metabolite content.

Chemicals

- Extraction buffer: 50 mM Tris/HCl, pH 8.0; 5 mM EDTA; 0.35 M sorbitol; 0.1% BSA; 10% PVP-6000.
- Wash buffer: 50 mM Tris/HCl, pH 8.0; 25 mM EDTA; 0.35 M sorbitol.
- CTAB buffer: 0.05M CTAB; 1 M Tris-HCl, pH 8.0; 0.5 M EDTA; 5 M NaCl.
- 3 M sodium acetate; 7.5 M ammonium acetate; 5% sarkosyl.
- CIA; absolute ethanol.
- TE-RNase solution; TE buffer.

Protocol

1. To each ground leaf sample, add 1 ml of extraction buffer at room temperature.

2. Centrifuge for 5 min. Discard the supernatant and dissolve the pellet in 400 µl of wash buffer followed by 100 µl of 5% sarkosyl and leave incubating for 15 min at room temperature.

3. Add 1 ml of CTAB buffer, mix gently and incubate at 55 ºC for 30 min.

4. Centrifuge for 5 min. Transfer the supernatant to a new tube and add equal volume of CIA. Mix gently.

5. Centrifuge for 10 min. Transfer the supernatant to a new tube and add TE-RNase solution. Incubate for 15 min at 37 ºC.

6. Precipitate the DNA by adding 10% volume of 7.5 M ammonium acetate and equal volume of absolute ethanol. Incubate at –20 ºC for 30 min.

7. Centrifuge for 15 min. Dry the DNA and dissolve the pellet in 20-50 µl of TE buffer.

DNA Extraction protocol E (Qiagen DNeasy Mini Plant Kit)

This method uses silica-gel-membrane technology and simple spin procedures to isolate high-quality DNA. Total DNA was extracted strictly following the instructions of the manufacturer.

Comparison of the extraction protocols for efficacy

The presence and quality of DNA obtained by these protocols were determined by electrophoresis on a 1% TBE agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA extract was classified according to visual inspection of coloration, which indicated oxidation of the samples (colorless, yellowish or dark). The quality of DNA obtained was estimated by measuring the A260/280

absorbance ratio using a spectrophotometer (Hitachi U-200). DNA samples were assessed for successful PCR amplification of *trnL* intron of cpDNA (~470 bp) and of ITS region of nrDNA (< 700 bp). The *trnL* intron and ITS region were amplified using primers and PCR conditions described by Taberlet *et al.* (1991), and Beyra-M. and Lavin (1999) and Delgado-Salinas *et al.* (1999), respectively.

Results

In group 1, the fresh leaves of *Dalbergia* species showed good DNA quality, with low degradation, by means of the protocols A to D. The DNA solutions from some of the samples were colored (yellowish or dark) when extracted by means of protocol A, C and D, mainly due to fast oxidation of the extract. For species bearing a high content of secondary metabolites and/or polysaccharides, protocol B was the best choice. The results for species *D. nigra*, *D. miscolobium* and *D. frutescens* were compared by electrophoresis on agarose gels (Figure 1). In group 2, fresh leaves of *D. brasiliensis*, *D. ecastaphyllum*, *D. monetaria* and *D. villosa* resulted in better DNA quality than herbarium specimens for all tested DNA extraction protocols. Figure 2 shows some of these comparisons obtained mainly through the use of protocol B (samples 1-10). Protocol E produced DNA of very good quality and no degradation from fresh leaves (Figure 2; sample 1); however, it did not produce DNA from herbarium specimens (Figure 2; sample 3). In group 3, most of the herbarium specimens yielded no DNA or DNA with a high level of degradation. DNA extraction from herbarium specimens was only achievable using protocol B (Figure 2, sample 11-17).

Of the 73 samples analyzed, only 38 yielded DNA in at least one tested protocol. The samples obtained by means of protocol B were evaluated according to DNA quality, color, spectral absorbance ratio, final concentration (ng/µl) and PCR

amplification (Table 2). Most of the herbarium specimens exhibited moderate to high degradation of DNA, yellowish or dark extract, low A260/A280 ratios and low final concentrations. In group 1, specific cpDNA amplification of the *trnL* intron was successful for all samples of fresh leaves obtained by means of protocols A to D (Figure 3, samples 1-8). However, only eight herbarium specimens exhibited successful amplification of this region (e.g., Figure 3, samples 10-14). All these samples also exhibited successful amplification of ITS region (Table 1). PCR amplifications were not successful with any of the herbarium samples that exhibited highly degraded DNA and dark extracts. Only part of herbarium samples that showed moderate DNA degradation and colorless or yellowish extract were suitable for PCR amplification.

Discussion

DNA extraction from leaves of *Dalbergia* species was complicated probably by the abundance of secondary metabolites. In a comparison of the four protocols analyzed with fresh and dried leaves, protocol B, described by Jobes *et al.* (1995), was labor intensive because several solutions needed to be prepared, demanded a great deal of time for extraction of the samples, and required a great quantity of microcentrifuge tubes. However, this protocol produced the best DNA quality in most of the *Dalbergia* species examined, both in fresh leaves and herbarium specimens. The DNA quality for herbarium specimens was worse than for fresh leaves, due to their preservation status. According to Jobes *et al.* (1995), in the presence of PVP, phenolics adhere to DNA in solution forming a colored extract around the DNA that becomes cleaner after the addition of the detergent SDS. The addition of high molar concentration of NaCl increases the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of the polysaccharides and DNA (Fang *et al.*, 1992). Finally, the addition

of LiCl selectively precipitates large RNA molecules reducing the amount of RNA present in the final DNA solution (Sambrook *et al.*, 1989). Selective precipitation has an advantage over RNAse treatment in that the RNA is removed and not simply degraded into smaller units (Storts, 1993). Protocol B also exhibited satisfactory results with *Acer rubrum*, *Magnolia grandiflora*, *Pinus* sp (Jobes *et al.*, 1995), and *Dimorphandra mollis* (Viana HA, unpublished results).

Protocols A and C demonstrated reasonable results for *Dalbergia* species, as the DNA obtained was yellowish in most of the samples. Protocol D successfully extracted DNA from many genera including *Schefflera* (Araliaceae), *Macadamia* (Proteaceae), *Dysoxylum* (Meliaceae), *Flindersia* (Rutaceae), *Sarcopteryx* (Sapindaceae), *Acacia* (Mimosaceae) and *Melicope* (Rutaceae) (Scott and Playford, 1996). However, this protocol did not exhibit satisfactory results for *Dalbergia* species, maybe due to the modification introduced in the protocol, as the samples were ground with liquid nitrogen instead of with sand. Protocol E, Qiagen DNeasy Mini Plant Kit, produced high quality DNA and no degradation in fresh leaves; however, for herbarium specimens it did not yield DNA, and consequently there was no PCR amplification, probably due to high degradation of the samples. However, considering the disadvantage of the high per-sample cost of any commercial kit, protocol E cannot be adequate for studies involving many samples of fresh leaves.

Herbarium specimens were supplied by four different herbaria for DNA analysis and complementary phylogenetic study. Each herbarium had a way of preserving the samples. The INPA herbarium informed us that the plant samples collected in the field are often immersed in alcohol due to high humidity in the Amazon rainforest. The CEPEC and SP herbarium use several chemicals in regular disinfections, whereas the BHCB herbarium stocked the samples in an enclosed space with air-conditioning (±

20ºC). We analyzed 54 herbarium specimens, but only eight yielded DNA which also suitable for successful PCR amplification, five of them pertaining to the BHCB herbarium. In fact, success in the extraction of DNA depends on methods of sampling in the field and preservation of the samples in the laboratory (see Drabkova *et al.*, 2002; Feres *et al.*, 2005). If samples are air-dried at up to 42ºC, they probably will yield high-quality DNA. Air-drying is considered to be better than the preservation of tissues in silica gel or anhydrous CaSO4 (Taylor and Swann, 1994). In general, leaves that are immersed in chemical solutions show seriously degraded DNA (Drabkova *et al.*, 2002). *Dalbergia* leaves exhibited oxidation when left under humid conditions for some time after collection, which means that it is necessary to dry or freeze them as fast as possible to better preserve DNA. Another important factor is the disinfestation methods of the herbarium collections. The material stocked at medium to low temperature and free of chemical preservatives has the best chance of yielding good quality DNA (Taylor and Swann, 1994), as in the BHCB preservation method. The satisfactory quality of DNA from herbarium specimens is essential for the success of further phylogenetic studies using DNA sequences in *Dalbergia.* Our results proved that, at least for the *Dalbergia* species tested, the methods for the collection and long-term preservation of herbarium specimens have a major influence on DNA quality and in the success of molecular phylogenetic studies.

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– Centro de Pesquisas do Cacau, CEPLAC, Itabuna, Bahia) and Inês Cordeiro (SP – Instituto de Botânica de São Paulo, São Paulo) who kindly provided the samples to analyze. This work, the research fellowship of M.B. Lovato and the doctoral fellowship of R.A. Ribeiro were supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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Appendix A. Species and Sections from herbarium specimens. Each entry includes species, locality and voucher specimen.

Section Dalbergia

Dalbergia acuta Benth.; Brazil, Minas Gerais, Januária; A. Salino & J.A. Lombardi 1692 (BHCB). *Dalbergia acuta* Benth.; Brazil, Bahia, Caetité; A.M. de Carvalho 3688 (CEPEC). *Dalbergia cuiabensis* Benth.; Brazil, Mato Grosso; H.S. Irwin 15942 (SP). *Dalbergia foliolosa* Benth.; Brazil, Minas Gerais; F.R. Couto 244 (BHCB). *Dalbergia foliolosa* Benth.; Brazil, Rio de Janeiro, Petrópolis; A.M. de Carvalho & Martini (SP). *Dalbergia glandulosa* Benth.; Brazil, Mato Grosso; A.M. Carvalho & Lewis (SP). *Dalbergia gracilis* Benth.; Brazil, Minas Gerais, Caratinga; H.C. Lima 7601 (BHCB). *Dalbergia gracilis* Benth.; Brazil, Rondônia; W.R. Anderson et al. 12220 (INPA). *Dalbergia gracilis* Benth.; Brazil, Acre, Serra Madureira; D.C. Daly et al. 8146 (CEPEC). *Dalbergia hiemalis* Malme; Brazil, Mato Grosso; A.M. de Carvalho & G.P. Lewis (SP). *Dalbergia lateriflora* Benth.; Brazil, Paraná, Guaratuba; J.M. Silva 1057 (CEPEC). *Dalbergia spruceana* (Benth.) Benth.; Brazil, Acre; C.A. Ferreira 10132 (INPA). *Dalbergia spruceana* (Benth.) Benth.; Brazil, Pará; A.P. Duarte 7264 (CEPEC). *Dalbergia spruceana* (Benth.) Benth.; Brazil, Pará, Santarém; A.M. de Carvalho (SP). *Dalbergia villosa* (Benth.) Benth.; Brazil. Minas Gerais, Itabirito; A.M. de Carvalho (BHCB). *Dalbergia villosa* (Benth.) Benth.; Brazil, São Paulo; H.F. Leitão 19878 & D.B. Azevedo (INPA). *Dalbergia villosa* (Benth.) Benth.; Brazil, Minas Gerais, Lavras; G.P.Heringer 01153 (CEPEC).

Section Triptolemea

Dalbergia brasiliensis Vogel; Brazil, Paraná; A.O.S. Vieira (BHCB). *Dalbergia brasiliensis* Vogel; Brazil, Paraná, Ponta Grossa; A.M. de Carvalho et al. 2339 (CEPEC). *Dalbergia catingicola* Harms.; Brazil, Bahia, Jequié; G.P. Lewis & A.M. de

Carvalho 980 (CEPEC). *Dalbergia cearensis* Vogel (*D. variabilis*); Brazil, Brasília (BHCB). *Dalbergia cearensis* Vogel (*D. variabilis*); Brazil, Rio de Janeiro, Petrópolis; G. Martinelli et al. 124 (INPA). *Dalbergia cearensis* Ducke (*D. variabilis*); Brazil, Bahia; B.B. Klitgaard et al. 73 (CEPEC). *Dalbergia cearensis* Ducke (*D. variabilis*); Brazil, A. Mattos Filho (SP). *Dalbergia glaziovii* Harms.; Brazil, Rio de Janeiro, Nova Friburgo; A.M. de Carvalho et al. 2334 (CEPEC). *Dalbergia glaziovii* Harms.; Brazil, Rio de Janeiro, Nova Friburgo; A.M. de Carvalho & H.C. Lima (SP). *Dalbergia hortensis* Heringer, Rizzini & Mattos; Brazil, Minas Gerais, Juiz de Fora; P.L. Krieger 11981 (CEPEC). *Dalbergia hortensis* Heringer, Rizz & Mattos; Brazil, Minas Gerais; A.M. de Carvalho (SP). *Dalbergia iquitosensis* Harms.; Brazil, Acre, Porto Velho; J.U. Santos et al. 106 (CEPEC). *Dalbergia riparia* (Marth.) Benth.; Brazil, Amazonas, Manacapuru; F.M.M. Magalhães 54 (INPA). *Dalbergia riparia* (Marth.) Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho et al. 2125 (CEPEC). *Dalbergia riparia* (Marth.) Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho (SP). *Dalbergia sampaioana* Kuhlmann & Hoehne; Brazil, Rio Janeiro; R.B. Antenor 136212 (CEPEC).

Section Selenolobium

Dalbergia foliosa Benth.; Brazil, São Paulo; J.Y.Tamasleiro et al. 88 (CEPEC). *Dalbergia inundata* Spruce ex Benth.; Brazil, Amazonas; L. Augusto 661 (INPA). *Dalbergia inundata* Spruce ex Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho et al. 2121 (CEPEC). *Dalbergia inundata* Spruce ex Benth.; Brazil, Amazonas; A.M. de Carvalho (SP). *Dalbergia revoluta* Ducke; Brazil, Acre, Cruzeiro do Sul; O.P. Monteiro 300 & C.D.A. Mota (INPA).

Section Pseudecastaphyllum

Dalbergia intermedia Vogel (D*albergia tomentosa* Benth.); Venezuela, Atures; O. Huber 1501 & J. Cerda (INPA). *Dalbergia intermedia* Vogel (D*albergia tomentosa*

Benth.); Brazil, Pará, Santarém; A.M. de Carvalho 2131 (CEPEC). *Dalbergia intermedia* Vogel (D*albergia tomentosa* Benth.); Brazil, Pará, Santarém; A.M. de Carvalho (SP).

Section Ecastaphyllum

Dalbergia amazonica (Radlk) Ducke; Ex. Herb Museu Paraensis 16971 (A. Ducke) (SP). *Dalbergia ecastaphyllum* (L.) Taub.; Brazil, Santa Catarina, Itapema; A.C. Cervi 2569 (BHCB). *Dalbergia ecastaphyllum* (L.) Taub.; Brazil, Bahia, Belmonte; A.M. de Carvalho et al. 477 (CEPEC). *Dalbergia ecastaphyllum* (L.) Taub.; Brazil, São Paulo, Peruíbe; Bianchini 352 (SP). *Dalbergia guttembergii* A.M. de Carvalho; Brazil, Amazonas; A. Ducke 35513 (CEPEC). *Dalbergia hydrophila* (Mart. Ex Benth) Hoehne; A. Ducke (SP). *Dalbergia monetaria* L. f.; Brazil, Amazonas, Humaitá; L.O.A. Teixeira et al. 1250 (INPA). *Dalbergia monetaria* L.f.; Brazil, Pará, Belém; A.M. de Carvalho et al. 2132 (CEPEC). *Dalbergia monetaria* L.f.; Brazil, Pará, Abaetatuba; A.S.L. Silva (SP). *Dalbergia riedelii* (Radlk.) Sandw.; Brazil, Amazonas; F. Magalhães 331 (INPA). *Dalbergia riedelii* (Radlk.) Sandw.; Brazil, Amazonas, Rio Negro; S. Mori et al. 21849 (CEPEC). *Dalbergia riedelii* (Radlk.) Sandw.; Brazil, Pará, Conceição do Araguaia; A.M. Carvalho (SP). *Dalbergia subcymosa* Ducke; Brazil, Amazonas, Autaz-Mirim; A. Loureiro, O.P. Monteiro & A. Miranda (INPA).

Table 1. Description of samples from fresh leaves and herbarium specimens analyzed for *Dalbergia* species.

			Herbarium specimens			
Nº	**Species**	**Fresh**	**BHCB**	**INPA**	**CEPEC**	**SP**
Group 1						
1	*D. decipularis*	DD	-	-	-	-
2	*D. elegans*	DEL	-	-	-	-
3	*D. frutescens*	DF	-	-	-	-
4	*D. miscolobium*	DM	-	-	-	-
5	*D. nigra*	DN	-	-	-	-
Group 2						
6	*D. brasiliensis*	DB	DBH	-	DBH2	-
7	*D. ecastaphyllum*	DE	DEH	-	DEH2	DEH3
8	*D. monetaria*	DMO	-	DMOH	DMOH2	DMOH3
9	*D. villosa*	DVI	DVIH	DVIH2	DVIH3	-
Group 3						
10	*D. acuta*	-	DAH	-	DAH2	-
11	*D. amazonica*	-	-	-	-	DAMH
12	*D. catingicola*	-	-	DCAH	-	-
13	*D. cearensis*	-	DCH	DCH2	DCH3	DCH4
14	*D. cuiabensis*	-	-	-	-	DCUH
15	*D. foliolosa*	-	DFOH	-	-	DFOH2
16	*D. foliosa*	-	-	-	DFLH	-
17	*D. glandulosa*	-	-	-	-	DGAH
18	*D. glaziovii*	-	-	-	DGIH	DGIH2
19	*D. gracilis*	-	DGRH	DGRH2	DGRH3	-
20	*D. guttembergii*	-	-	-	DGUH	-
21	*D. hiemalis*	-	-	-	-	DHIH
22	*D. hortensis*	-	-	-	DHH	DHH2
23	*D. hygrophila*	-	-	-	-	DHYH
24	*D. intermedia*	-	-	DTH	DTH2	DTH3
25	*D. inundata*	-	-	DIH	DIH2	DIH3
26	*D. iquitosensis*	-	-	-	DIQH	-
27	*D. lateriflora*	-	-	-	DLAH	-
28	*D. revoluta*	-	-	DREH	-	-
29	*D. riedelii*	-	-	DRIH	DRIH2	DRIH3
30	*D. riparia*	-	-	DRH	DRH2	DRH3
31	*D. sampaioana*	-	-	-	DSAH	-
32	*D. spruceana*	-	-	DSPH	DSPH2	DSPH3
33	*D. subcymosa*	-	-	DSUH	-	-

Table 2. Evaluation of samples extracted using protocol B (Jobes *et al.,* 1995) according to DNA quality, color, spectral absorbance ratio (A260/280), final concentration (ng/µl) and PCR amplification.

Species	Sample	DNA quality [1]	Color [2]	$A_{260/280}$ [3]	Cc(ng/ul) [4]	PCR [5]
Group 1						
D. decipularis	DD	1	1	1	1	1
D. elegans	DEL	1	1	1	1	1
D. frutescens	DF	1	1	1	1	1
D. nigra	DN	1	1	1	1	1
D. miscolobium	DM	1	1	1	1	1
Group 2						
D. brasiliensis	DB	1	1	1	1	1
	DBH	2	2	2	2	1
	DBH2	2	3	2	2	2
D. ecastaphyllum	DE	1	1	1	1	1
	DEH	2	1	2	2	1
D. monetaria	DMO	1	1	1	1	1
	DMOH	2	1	2	2	1
	DMOH2	3	3	2	2	2
	DMOH3	2	2	2	2	1
D. villosa	DVI	1	1	1	1	1
	DVIH	2	1	2	2	1
Group 3						
D. acuta	DAH	2	2	2	2	1
	DAH2	3	3	2	2	2
D. cuiabensis	DCUH	2	1	2	2	1
D. foliosa	DFLH	2	2	2	2	2
D. foliolosa	DFOH	2	2	2	2	1
	DFOH2	3	3	2	2	2
D. glaziovii	DGIH2	3	3	2	2	2
D. gracilis	DGRH	2	1	2	2	2
	DGRH3	3	3	2	2	2
D. hortensis	DHH2	3	3	2	2	2
D. inundata	DIH2	3	3	2	2	2
D. iquitosensis	DIQH	2	3	2	2	2
D. riparia	DRH	2	3	2	2	2
	DRH2	2	3	2	2	2
	DRH3	3	3	2	2	2
D. riedelii	DRIH	3	3	2	2	2
D. spruceana	DSPH	3	3	2	2	2
	DSPH2	3	3	2	2	2
D. tomentosa	DTH	2	3	2	2	2
	DTH2	3	3	2	2	2
D. variabilis	DVH2	3	3	2	2	2
	DVH3	3	3	2	2	2

[1] (1) High molecular weight DNA, low degradation - good quality DNA; (2) medium degradation - medium quality DNA; (3) highly degraded DNA - poor quality DNA.

[2] (1) transparent; (2) colored (yellowish); (3) brown (dark).

[3] (1) $1.8 \leq A_{260/280} \leq 2.0$; (2) $A_{260/280} \leq 1.5$

[4] (1) 500 ng/µl ≤ Cc ≤ 100 ng/µl; (2) Cc ≤ 50 ng/µl.

[5] PCR amplification of *trnL* intron and ITS region: (1) exhibited specific amplification; (2) no amplification.

Legends of figures

Fig. 1 Electrophoretic analysis of total DNA from fresh leaves of *D. nigra* (DN), *D. miscolobium* (DM) and *D. frutescens* (DF) extracted by the following four different protocols: **A**. Doyle and Doyle, 1990; **B**. Jobes *et al.*, 1995; **C**. Dellaporta *et al.*, 1983; **D**. Scott and Playford, 1996. M = 100 bp ladder.

Fig. 2 Electrophoretic analysis of total DNA from fresh and herbarium samples of *Dalbergia* species extracted by the following protocols: **1, 3.** Protocol E (Qiagen); **2, 4-17.** Protocol B (Jobes *et al.*, 1995). Samples: **1** and **2**. DVI; **3** and **4**. DVIH; **5**. DMO; **6**. DMOH; **7**. DMOH2; **8**. DMOH3; **9**. DE; **10**. DEH; **11**. DRH; **12**. DRH2; **13**. DRH3; **14**. DGRH2; **15**. DGRH3; **16**. DFOH; **17**. DFOH2. M = 100 bp ladder.

Fig. 3 Amplification of *trnL* region of cpDNA from fresh and herbarium samples of *Dalbergia* species extracted by the following protocols: **1, 5, 9-14, 16, 18-20.** Protocol A (Doyle and Doyle, 1990); **2, 6.** B (Jobes *et al.*, 1995); **3, 7.** C (Dellaporta *et al.*, 1983); **4, 8.** D (Scott and Playford, 1996); **15, 17.** E (Qiagen). Samples: **1-4.** DN; **5-8.** DEL; **9.** DE; **10.** DEH; **11.** DFOH; **12.** DCUH; **13.** DBH; **14.** DMOH; **15-16.** DVI; **17.** DVIH; **18.** DRH3; **19.** DGRH; **20.** DVH3. M = 100 bp ladder.

Fig. 1

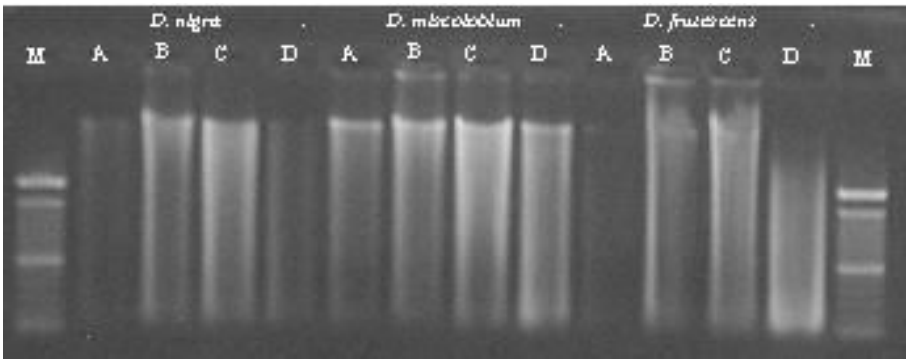


Fig. 2

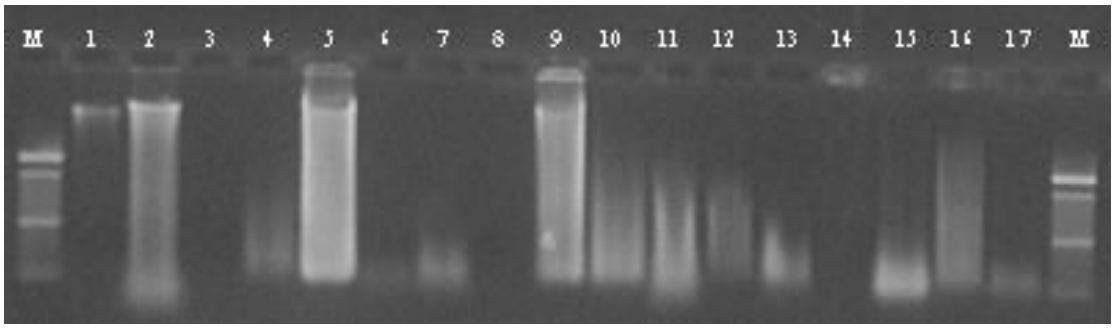
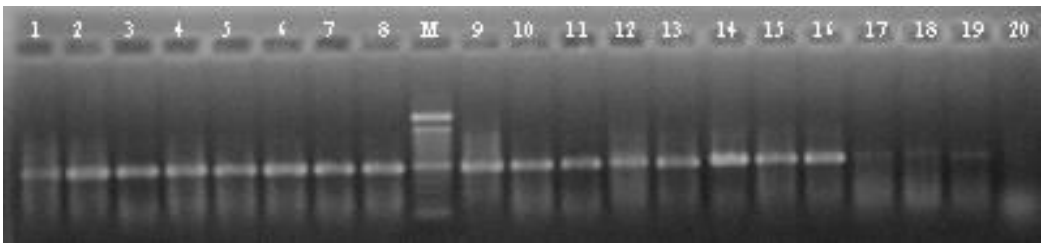


Fig. 3



ARTIGO 3:

The genus *Machaerium* (Leguminosae) is more closely related to *Aeschynomene* sect. *Ochopodium* than to *Dalbergia*: inferences from combined sequence data

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THE GENUS *MACHAERIUM* (LEGUMINOSAE) IS MORE CLOSELY RELATED TO *AESCHYNOMENE* SECT. *OCHOPODIUM* THAN TO *DALBERGIA*: INFERENCES FROM COMBINED SEQUENCE DATA

Running Title: *Dalbergia, Machaerium, Aeschynomene*

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ABSTRACT

Phylogenetic relationships among the genera *Dalbergia*, *Machaerium* and *Aeschynomene* were investigated with sequences from both the chloroplast DNA *trnL* intron and the nuclear ribosomal DNA ITS/5.8S region. A parsimony and Bayesian analysis of individual and combined data resolved a monophyletic *Dalbergia* that is sister to a clade comprising *Aeschynomene* sect. *Ochopodium* and *Machaerium*. *Aeschynomene* sects. *Aeschynomene* and *Ochopodium* are not resolved as monophyletic. Indeed, uncertainty remains as to whether sect. *Ochopodium* is sister to or nested within *Machaerium*. Sect. *Aeschynomene*, in contrast, is paraphyletic with respect to genera such as *Bryaspsis* and *Soemmeringia*, which collectively could be sister to the *Dalbergia-Macherium-Ochopodium* clade. This study thus identifies the disparate lineages of the genus *Aeschynomene* and reveals that species of this genus with basifixed stipules (i.e., sect. *Ochopodium*) should be ranked either as a distinct genus or perhaps as a subgroup within *Machaerium*. The findings of this analysis also have some bearing at the infrageneric level. Limited sampling of *Dalbergia* sects. *Triptolemea* and *Ecastaphyllum* resolves them as monophyletic, whereas sect. *Dalbergia* is paraphyletic. In contrast, the traditional infrageneric classification of *Machaerium* does not show much agreement with molecular groups and most of the internal branches of this clade are associated with weak to non-existent bootstrap supports. Additional sampling of *Aeschynomene* and *Machaerium* species, other DNA sequences, and morphological data are needed to resolve the exact relationship of sect. *Ochopodium* with *Machaerium*, as well as validate the infrageneric classification of *Machaerium*.

KEYWORDS: Dalbergia subclade, ITS/5.8S region, phylogeny, *trnL* intron

INTRODUCTION

The Leguminosae (Fabaceae) is the third largest family of flowering plants, with around 650 genera and more than 18,000 species worldwide (Polhill and Raven 1981). Traditionally, the legume family has been divided into three subfamilies: Caesalpinoideae, Mimosoideae and Papilionoideae, the latter being the largest and most diverse of the three subfamilies. Lavin et al. (2001) proposed a rearrangement for certain papilionoid legumes referred to as the "dalbergioid" group. This included all genera previously referred to the tribes Aeschynomene and Adesmieae, the subtribe Bryinae of the Desmodieae, and tribe Dalbergieae excluding *Andira* Juss., *Hymenolobium* Benth., *Vatairea* Aubl., and *Vataireopsis* Ducke. The dalbergioids were divided in three well-supported groups: the Adesmia, Pterocarpus, and Dalbergia subclades.

The Dalbergia subclade includes *Dalbergia* L. f., *Machaerium* Pers., and 14 genera from the tribe Aeschynomeneae (sensu Rudd 1981): *Aeschynomene* L., *Soemmeringia* Mart., *Cyclocarpa* Afz. ex Bak., *Kotschya* Endl., *Smithia* Ait., *Humularia* Duvign., *Bryaspis* Duvign., *Geissaspis* Wight & Arn., *Weberbauerella* Ulbrich, *Diphysa* Jacq., *Pictetia* DC., *Ormocarpum* P. Beauv., *Ormocarpopsis* R. Viguier, and *Peltiera* Labat and Du Puy (Lavin et al. 2001). The genera *Dalbergia* and *Machaerium* were traditionally assigned with 12 other genera in the tribe Dalbergieae (Polhill 1994). In the *trnL* analysis of Lavin et al. (2001), two samples of *Machaerium* were resolved as paraphyletic with respect to two samples of *Aeschynomene* sect. *Ochopodium* Vogel. The ITS analysis resolved with moderate support one *Machaerium* and three *Dalbergia* samples as monophyletic, with one sample of sect. *Ochopodium* as unresolved within the "Dalbergia" subclade. Their *matK* analysis resolved one sample of *Machaerium* as sister to two samples of sect. *Ochopodium*. The study by Lavin et al.

(2001) clearly suggested that additional sampling was needed of *Dalbergia*, *Macherium*, and *Aeschynomene* in order to investigate the conventional hypothesis that *Dalbergia* was indeed sister to just *Macherium* (Polhill 1981).

This expanded sampling effort would also bear on the infrageneric classification of not just *Aeschynomene*, but also that of *Dalbergia* and *Machaerium*. The genus *Dalbergia*, for example, comprises possibly 100 species of trees, shrubs, and lianas distributed pantropically with centers of diversity in the Amazon, Indo-Asia, and Madagascar (Prain 1904; Polhill 1981; Du Puy et al. 2002; Klitgaard and Lavin 2005). In Brazil, 41 taxa are recognized and distributed into five sections defined by inflorescence and fruit characters: *Dalbergia* L.f., *Triptolemea* (Mart. ex Benth.) Benth, *Selenolobium* Benth, *Pseudecastaphyllum* A.M. de Carvalho, and *Ecastaphyllum* (P. Browne) Ducke (Carvalho 1997). Many *Dalbergia* species produce valuable timber and over-exploitation and habitat fragmentation have rendered some of these species prone to extinction, as in the case of *D. nigra* (Brazilian rosewood; Carvalho 1997). As yet, no attempt has been made to validate the infrageneric classification of *Dalbergia*.

The genus *Machaerium* is predominantly neotropical with about 130 species occurring from Mexico to Brazil and Argentina (Rudd 1977; Klitgaard and Lavin 2005). *Machaerium isadelphum* (E. Mey.) Amshoff reaches Trinidad and Tobago and *M. lunatum* (L. f.) Ducke is disjunct in west coastal Africa (Klitgaard and Lavin 2005). These species have been classified into five series using leaflet shape and venation, and stipule texture (Bentham 1860). These series were later given sectional status by Taubert (1891): *Lineata* Benth., *Oblonga* (Benth.) Taub., *Acutifolia* Taub., *Reticulata* (Benth.) Taub., and *Penninervia* (Benth.) Taub. Most *Machaerium* species are widespread in Brazil (Hoehne 1941) with centers of diversity in the Amazon Basin (Hoehne 1941; Ducke 1949; Bastos 1987; Mackinder 1990) and in the southeastern

Brazil (Lima et al. 1994; Mendonça Filho 1996; Sartori and Tozzi 1998). As with *Dalbergia*, no study has been attempted to confirm this sectional classification of *Machaerium*.

The genus *Aeschynomene* is traditionally divided into two sections: *Aeschynomene* L. (with medifixed stipules) and *Ochopodium* Vogel (with basifixed stipules), which include 101 and 50 species, respectively (Leonard 1954; Rudd 1955; Verdcourt 1971; Fernandes 1996; Klitgaard and Lavin 2005). These sections are distributed pantropically and limited sampling of DNA sequences (Lavin et al. 2001) renders suspect the presumed close relationship of sect. *Ochopodium* with sect. *Aeschynomene*.

The current study includes more samples from the genera *Aeschynomene*, *Dalbergia,* and *Machaerium*, and mainly from the neotropical region. The intent is to use phylogenetic analysis to test the hypothesis that *Aeschynomene* sect. *Ochopodium* is more closely related to *Machaerium* than to sect. *Aeschynomene* and, likewise, that *Machaerium* in the traditional sense is not most closely related to *Dalbergia*. Congruence of phylogenetic groups in these genera with traditional infrageneric groups also can be assessed preliminarily given that sampling was designed to included multiple species from as many infrageneric taxa (sections) as possible from within *Dalbergia* and *Machaerium*.

MATERIALS AND METHODS

Plant material and DNA extraction. Species representing different sections of the genera *Dalbergia*, *Machaerium* and *Aeschynomene* were sampled. *Bryaspis lupulina* (Planch. ex Baker) J. Duvign., *Diphysa ormocarpoides* (Rudd) M. Sousa & R. Antonio, *Ormocarpum keniense* J.B. Gillett, *Ormocarpopsis itremoensis* Du Puy & Labat,

Pictetia marginata C. Wright, *Soemmeringia semperflorens* Mart., and *Weberbauerella brongniartioides* Ulbr. from the Dalbergia subclade, and *Pterocarpus rohrii* Vahl from the Pterocarpus subclade of the dalbergioid legumes, (Lavin et al. 2005) were designated as outgroups (Appendix 1). Plant samples were obtained from either field or herbarium specimens. Most the ingroup species grow in different geographical regions of Brazil, except *D. sissoo* Roxb. ex DC. and *D. congestiflora* Pittier, which are native to India and Mexico, respectively. Additionally, *A. indica* L. and *A. pfundii* Taub. are native to India and Africa (Old World – OW), whereas *A. fascicularis* Schltdl. & Cham. and *A. purpusii* Brandegee to Venezuela and México (New World – NW). Total genomic DNA was isolated from samples using a modified CTAB extraction method (Doyle and Doyle 1987). The DNA from herbarium specimens of *Dalbergia* was extracted by an alternative protocol, which removes most of the polysaccharides and secondary metabolites (Jobes et al. 1995) and yields high quality DNA from such samples (Ribeiro and Lovato 2006).

PCR and DNA sequencing. The chloroplast DNA *trnL* intron and nuclear ribosomal DNA ITS/5.8S (ITS) region were subjected to phylogenetic analysis. The *trnL* intron comprises approximately 470 base pairs (bp), is flanked by two short tRNA-encoding exons, and displays great variation and fast rate of evolution being used in studies of phylogenetic (Bakker et al. 2000). The ITS region is located between the 18S and 26S ribosomal RNA (rRNA) genes, and comprises ITS-1, 5.8S rRNA, and ITS-2 (Baldwin et al. 1995). Both the *trnL* intron and ITS region have been shown to be phylogenetic informative within genera and among closely related genera of legumes (e.g., Pennington et al. 2000; Lavin et al. 2001; Gervais and Bruneau 2002; Mayer and Bagga 2002; Kenicer et al. 2005). In contrast to the *trnK*/*matK* locus, the ITS region and the

trnL locus can be readily amplified especially from herbarium specimens. Indeed, the current study was not able to utilize *trnK*/*matK* sequences because most of the DNA isolation was performed on herbarium specimens.

In order to determine the extent of PCR errors or sequencing artifacts, two or more independent PCR amplifications were performed for each taxon. Furthermore, each PCR product was sequenced in both directions. The *trnL* intron was amplified and sequenced using primers "*c*" and "*d*" described by Taberlet et al. (1991). The ITS region was amplified and sequenced with primers described in Beyra-M. and Lavin (1999) and Delgado-Salinas et al. (1999).

PCR amplifications were typically prepared in 25 µl reactions using 1x Taq buffer containing 2.0 mM $MgCl_2$ (Phoneutria), 0.2 ng of bovine serum albumin (BSA), 200 µM of each dNTP, 0.5 µM of each primer, 1U of Taq DNA polymerase (Phoneutria), and approximately 10 ng of genomic DNA. The ITS region reaction mixture included also 2% dimethyl sulfoxide (DMSO) and 1M Betaine (N_3-trimethylglycine). These PCR enhancing agents improve yield and specificity in the amplification of GC-rich sequences (Henke et al. 1997; Frackman et al. 1998). PCR reactions were performed on Eppendorf thermocycler following standard protocols (e.g., Lavin et al. 2001). Products were cleaned using 20% polyethylene glycol (PEG) precipitation. DNA sequences were performed with the DYEnamic ET dye terminator sequencing Kit (GE Healthcare), following the protocol supplied by the manufacturer. Sequencing reactions were then analyzed on a MegaBACE 1,000 automated sequencer (GE Healthcare).

Sequence alignment and phylogenetic analyses. The initial *trnL* and ITS sequence contigs were assembled with Phred v. 0.20425 (Ewing and Green 1998; Ewing et al.

1998), Phrap v. 0.990319 (http://www.phrap.org/), and Consed 12.0 (Gordon et al. 1998) to produce high quality consensus sequences. Individual sequences were imported into CLUSTAL-W for multiple sequence alignments (Higgins et al. 1994) implemented in MEGA 3.1 software (Kumar et al. 2004) using default gap penalties. Final alignments were edited manually. All sequences have been deposited in GenBank (Appendix 1) and TreeBase (http://www.treebase.org/).

Phylogenetic analyses were performed using parsimony implemented in PAUP* (Swofford 2000). Gaps were treated as missing data because simple coding of gaps (Simmons and Ochoterena 2000) could be applied to only small clades (e.g., two accessions of the same species) that otherwise were very well supported by substitution variation. Heuristic parsimony searches were conducted with 100 random addition replicates, tree-bisection-reconnection (TBR) branch-swapping, steepest descent, and setting the maximum trees at 10,000. Bootstrap support values were calculated from 1,000 replicates each analyzed with one random addition of taxa, TBR branch swapping, and no steepest descent. The consistency index (CI; Kluge and Farris 1969) and the retention index (RI; Farris 1989) were also calculated. The *trnL* and ITS sequences were analyzed separately and in combination. The combined data set and an AIC selected substitution model using ModelTest (Posada and Crandall 1998) were subjected to Bayesian analysis using MrBayes ver. 3.1 (Huelsenbeck and Ronquist 2001). A total of 2×10^6 generations in two separate runs of four chains each was sampled every 1×10^4 generations. Thus, burnin was culled and autocorrelation avoided.

RESULTS

Sequence Characteristics. The ITS region varied in length from 607 to 641 bp among the 52 terminal taxa. The aligned sequences included 664 sites, of which 60 were variable but uninformative and 344 that were parsimony informative. The mean GC content of ITS region was 62.2%, while the mean genetic distances was 0.203 (standard deviation = 0.011). The diversity of the ITS sequences among species of *Dalbergia*, *Machaerium* and *Aeschynomene* was due more to nucleotide substitution variation than to insertions and deletions (indels). The *trnL* intron varied in length from 452 to 528 bp among the 54 terminal taxa. The aligned sequences included 577 sites, of which 53 were variable but uninformative and 102 that were parsimony informative. The mean GC content was 33.8%, while the mean genetic distances was 0.045 (0.005). The *trnL* intron data was characterized by numerous indels (e.g., gaps from one to 55 nucleotides) and a region of $(TA)_{3\text{-}6}$ dinucleotide repeats with small insertions in some species. The combined intron *trnL* and ITS sequences included 1,241 aligned sites among 54 terminal taxa. Of these, 113 were variable but uninformative and 446 were parsimony informative. The mean GC content was 49.3%, while the mean genetic distances was 0.123 (0.006). The only missing sequence data were the ITS sequences for *Aeschynomene fascicularis* and *Machaerium brasiliense* (for a total of 2.3% missing nucleotide sites).

Phylogenetic analyses. Parsimony analysis of ITS region produced 452 minimal length trees each with 1,746 steps, a CI of 0.418 (uninformative characters excluded), and an RI of 0.683. The strict consensus from the ITS analysis resolved a monophyletic *Dalbergia* sister to a clade comprising sect. *Ochopodium* nested within a paraphyletic *Machaerium* (not shown). Bootstrap support for these particular relationships averaged

higher than for the *trnL* analysis (88-93%), with the monophyly of sect. *Ochopodium* supported at 100%. Parsimony analysis of the *trnL* intron sequences produced 272 minimal length trees each with 240 steps, a CI of 0.712 (uninformative characters excluded), and an RI of 0.903. The strict consensus resolved a monophyletic *Dalbergia* sister to a clade comprising *Aeschynomene* sect. *Ochopodium* as sister to *Machaerium* (not shown). Bootstrap support for these relationships was moderate (62-87%), but the monophyly of sect. *Ochopodium* was resolved at 100%, and that of the distantly related sect. *Aeschynomene* at 93%. No conflict was noted between the two analyses with respect to clades resolved with more than 70% bootstrap support. Indeed, a partition homogeneity test (with uninformative characters excluded) suggested no clade conflict between the two data sets (p=0.193).

Parsimony analysis of the combined ITS region and *trnL* intron data produced 711 minimal length trees each with 2,005 steps, a CI of 0.442 (uninformative characters excluded), and an RI of 0.711. A strict consensus resolved a monophyletic *Dalbergia* sister to a clade comprising *Aeschynomene* sect. *Ochopodium* as sister to *Machaerium*. Bootstrap support for these relationships ranged 96-100% (Fig. 1). The distantly related sect. *Aeschynomene* was resolved with 97% bootstrap support, but as paraphyletic with respect to *Bryaspis* and *Soemmeringia*. By enforcing a monophyletic clade comprising all *Aeschynomene* species, 1182 most parsimonious trees each with a length of 2,051 were determined to be significantly longer than the unconstrained analysis via the Kishino-Hasegawa test (t=4.6858, p<0.0001) and the Templeton (Wilcoxon signed-ranks) tests (z=-4.6402, p<0.0001).

The Bayesian analysis of the combined data set using an AIC-selected GTR+G+I substitution model for both sequence regions (see Table 1 for parameter estimates of this model) generated trees with a topology highly similar to that produced

with parsimony (Fig. 2). Relationships are generally well resolved and supported, except for within *Machaerium* where several internal branches are poorly supported, as in the parsimony analysis. The Bayesian consensus most notably resolves sect. *Ochopodium* as sister to the *Machaerium* clade, in contrast to the strict consensus of the parsimony analysis (Fig. 1).

Regarding infrageneric relationships, limited sampling within *Dalbergia* suggests that the sects. *Triptolemea* (*D. brasiliensis*, *D. decipularis* and *D. frutescens*) and *Ecastaphyllum* (*D. ecastaphyllum* and *D. monetaria*) are potentially monophyletic, whereas sect. *Dalbergia* (*D. acuta*, *D. cuiabensis*, *D. elegans*, *D. foliolosa, D. miscolobium*, *D. nigra*, and *D. villosa*) is paraphyletic (Fig. 1). It was not possible obtain the sectional classification of *D. sissoo* and *D. congestiflora*. Limited sampling with *Machaerium* suggests that only sect. *Lineata* (*M. aculeatum* and *M. hirtum*) is potentially monophyletic. Sections *Acutifolia* (*M. acutifolium*, *M. stipitatum* and *M. villosum*), *Reticulata* (*M. brasiliense*, *M. glabrum*, *M. oblongifolium* and *M. opacum*), and *Oblonga* (*M. nyctitans*, *M. scleroxylon*, and *M. gracile*) were not resolved as monophyletic. In the genus *Aeschynomene*, sect. *Aeschynomene* (*A. filosa, A. indica* and *A. pfundii*) is probably not monophyletic, whereas *Ochopodium* (*A. martii*, *A. vogelii*, *A. paniculata*, *A. brasiliense*, *A. purpusii*, *A. fascicularis*) potentially is.

DISCUSSION

Our results suggest that *Dalbergia*, *Machaerium*, *Aeschynomene* sect. *Ochopodium* are each potentially monophyletic, but that *Machaerium* could be paraphyletic with respect to sect. *Ochopodium*. With very limited sampling, Lavin et al. (2001) resolved two representatives of *Ochopodium* as sister to or nested within two samples of *Machaerium*. Our analysis includes more samples of each of these taxa and

suggests that *Ochopodium* could be sister to *Machaerium*. This is equivocal, however, because it is not well supported by parsimony analysis (Fig. 1), although it is by the Bayesian analysis (Fig. 2). The genus *Aeschynomene* is with increasing certainty not monophyletic, and sect. *Ochopodium* (with basifixed stipules) should be taxonomically distinguished as a member of the clade including *Dalbergia* and *Machaerium*. Whether this separation is ranked at the genus level, or as some infrageneric taxon within *Machaerium*, will have to await additional sampling and phylogenetic analysis of *Machaerium*. Sect. *Aeschynomene* is likely paraphyletic with respect to certain genera such as *Bryaspsis* and *Soemmeringia*, as was suggested by Lavin et al. (2001). This section has been traditionally distinguished by its medifixed (peltate or appendiculate) stipules, but this trait is also found in other genera potentially of this *Aeschynomene* clade (Lavin et al. 2001), such as *Geissaspis* and *Smithia* (Rudd 1981).

Dalbergia and *Machaerium* have traditionally been considered sister lineages largely because they stood out among other woody papilionoid legumes assigned to Dalbergieae (e.g., Polhill 1981). For example, their inflorescences of helicoid cymes, small flowers mostly less than 1 cm long, and fruits winged by a flattening of the valves were individually and collectively distinctive among the Dalbergieae. In the context of *Aeschynomene* and close relatives, however, *Dalbergia* and *Machaerium* may be less outstanding. For example, small flowers arranged in helicoid cymes are characteristic of various species of *Aeschynomene* sect. Ochopodium (Lavin 1987). Not only are *Dalbergia* and *Machaerium* more closely related to genera once placed in the tribe Aeschynomeneae than they are to other Dalbergieae, they are also paraphyletic with respect to *Aeschynomene* sect. *Ochopodium*. This intimate phylogenetic relationship suggests that additional morphological similarities are expected to be found among sect. *Ochopodium*, and the genera *Dalbergia* and *Machaerium*.

***Infrageneric classification of* Dalbergia**. The most extensive systematic study of the Brazilian *Dalbergia* (Carvalho 1989) morphologically diagnosed species groups using mainly inflorescence and fruiting characteristics. Other characters such as habit, leaf, and floral morphology were not used because they were considered too variable at higher taxonomic levels. Section *Triptolemea* is characterized by generally flat-topped cymose inflorescences. Section *Ecastaphyllum* is characterized by short fasciculate inflorescences (racemose or paniculate) and semidrupaceous, orbicular or reniform fruits that bear a reticulate to rugulate venation. These morphologically distinctive groups were also resolved as monophyletic in the individual and combined ITS and *trnL* sequence analysis. According Carvalho (1989), sect. *Dalbergia* is quite heterogeneous comprising all the species that have a pyramidal paniculate inflorescence that are sometimes arranged in large leafy (or bracteate) compound panicles, and samaroid fruits with a diffuse or prominently reticulate venation over the entire surface. Carvalho (1989) suggested that *D. nigra* is very distinct because of its calyx with a glabrous tube and pilose teeth, obovate standard petals, and dark brown glossy fruits lacking a prominent venation. Notably, our molecular resolves *D. nigra* as distinct from other sect. *Dalbergia* samples (Figs. 1-2). Although the molecular phylogeny produced in this study is congruent with some of the traditionally recognized sections of *Dalbergia*, sampling is yet too limited to conclude much of significance. Further, the pantropical distribution of *Dalbergia* with centers of species diversity in Amazonia, Indo-Asia, and Madagascar, render a long term project out of reconciling the infrageneric classifications proposed by Bentham (1860), Prain (1904), and Carvalho (1989).

***Infrageneric classification of* Machaerium**. The two samples of *Machaerium* sect. *Lineata* were resolved as monophyletic, in contrast to the rest of the infrageneric groups

that showed little correspondence with phylogenetic groups (Figs. 1-2). This section is diagnosed by a unique combination of flowers with purple petals and obovate bracteoles, and leaves each with more than 25 oblong to linear leaflets having craspedromus venation (Mendonça Filho 1996). Some of the molecular phylogenetic groups of *Machaerium* resolved in this analysis are congruent with certain morphological and phytochemical characteristics. Oliveira and Gottlieb (1971) proposed the division of *Machaerium* into species groups using phytochemical data. For example, their Machaeria scleroxyla group, including *M. nyctitans* and *M. scleroxylon,* is rich in neoflavonoids, whereas their Machaeria villosa group, with *M. acutifolium*, *M. villosum*, and *M. opacum*, exhibits isoflavonoids and pterocarpans. This classification partly corroborates the infrageneric classification of Bentham (1860), as the first two species fit the diagnosis of sect *Oblonga*. Additionally, a close relationship of *M. scleroxylon* and *M. nyctitans* has been suggested from morphological data (Sartori and Tozzi 1998). The species from the Machaeria villosa group are distributed into sections *Acutifolia* and *Reticulata*. Seedling morphology (Mendonça Filho *et al.*, submitted) corroborates Rudd's (1987) suggestion that species from these two sections should be treated as one. Sampling is too limited, regardless, to compare the *Machaerium* groups that were weakly resolved in this study to groups recognized by previous classification studies of this genus. Given the geographic distribution of *Machaerium*, which is essentially confined to the neotropics, this genus should be much more amenable to thorough sampling and hence an expanded taxonomic and phylogenetic analysis. Of course, *Aeschynomene* sect. *Ochopodium* will have to be part of this future study in order to determine whether it is sister to or derived from within the *Machaerium* diversification.

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TABLE 1. Parameter estimates for the GTR + G + I model for the ITS region {1} and *trnL* intron {2}. Parameters for the ITS region and *trnL* intron were estimated separately using the unlink option in MrBayes. r = the six substitution rate estimates, pi = the base frequency estimates, alpha = the gama shape parameter estimate, pinvar = the proportion of invariant sites estimate, m = the site specific rate estimates, CI = credibility interval, and PSRF = potential scale reduction factor, uncorrected, a convergence diagnostic that should approach unity as separate Bayesian runs converge.

			95% CI			
Parameter	Mean	Variance	Lower	Upper	Median	PSRF
r(A<->C){1}	0.116126	0.000134	0.094356	0.140221	0.115234	1.003
r(A<->G){1}	0.223543	0.000329	0.189984	0.263075	0.222536	1.004
r(A<->T){1}	0.077447	0.000152	0.054724	0.100253	0.076793	0.997
r(C<->G){1}	0.058104	0.000040	0.046616	0.071381	0.057925	1.015
r(C<->T){1}	0.439832	0.000503	0.395907	0.486800	0.440419	1.015
r(G<->T){1}	0.084948	0.000093	0.066867	0.103513	0.085230	0.997
r(A<->C){2}	0.156460	0.000538	0.115980	0.207609	0.155128	0.999
r(A<->G){2}	0.129612	0.000433	0.091298	0.173656	0.128676	0.997
r(A<->T){2}	0.062150	0.000172	0.037797	0.091269	0.061203	1.000
r(C<->G){2}	0.165053	0.001019	0.110970	0.230087	0.164076	0.997
r(C<->T){2}	0.255024	0.001237	0.191910	0.325698	0.253627	1.001
r(G<->T){2}	0.231701	0.000905	0.176188	0.293511	0.230323	1.006
pi(A){1}	0.204073	0.000162	0.178790	0.228969	0.204499	0.997
pi(C){1}	0.295517	0.000156	0.270364	0.318980	0.296352	0.997
pi(G){1}	0.310665	0.000217	0.282461	0.341469	0.310234	1.002

Parameter	Mean	Variance	95% CI Lower	95% CI Upper	Median	PSRF
pi(T){1}	0.189746	0.000101	0.170031	0.209332	0.189220	1.010
pi(A){2}	0.379579	0.000344	0.342143	0.415790	0.380245	0.997
pi(C){2}	0.158323	0.000180	0.133328	0.185798	0.158023	1.023
pi(G){2}	0.173297	0.000166	0.144877	0.198543	0.172954	0.999
pi(T){2}	0.288802	0.000258	0.258975	0.321010	0.288439	1.035
alpha{1}	1.288812	0.038303	0.933951	1.685928	1.278528	0.997
alpha{2}	0.088834	0.000024	0.080717	0.099262	0.088128	1.018
pinvar{1}	0.295296	0.000767	0.232464	0.345755	0.295807	1.000
pinvar{2}	0.335447	0.001962	0.254712	0.414632	0.334573	0.998
m{1}	0.381127	0.001976	0.303762	0.472940	0.378712	1.058
m{2}	1.712187	0.002617	1.606530	1.801216	1.715084	1.058

APPENDIX. Genera, sections, and species sampled. Locality, voucher specimen, and GenBank accession numbers for [1]*trnL* intron and [2]ITS sequences are listed for each entry.

***Aeschynomene* sect. *Aeschynomene*.** *Aeschynomene filosa* Mart. Ex Benth. (1): Brazil, Minas Gerais, Januária, *A. Salino & J.A. Lombardi 1762* (BHCB), [1]EF451102, [2]EF451062. *Aeschynomene filosa* Mart. Ex Benth. (2): Brazil, Minas Gerais, Diamantina, *P.O. Moraes & J.A. Lombardi 2586* (BHCB), [1]EF451103, [2]EF451063. *Aeschynomene indica* L.: U.S.A, Lousiana, *Allen Tomas & Allen NLU3* (LSU), [1]AF208927, [2]U59892. *Aeschynomene pfundii* Taub.: Zimbabwe, National Botanic Gardens, *Lavin s.n.* (MONT), [1]AF208930, [2]AF189026. ***Aeschynomene* sect. *Ochopodium*.** *Aeschynomene brasiliana var. brasiliana* (Por) DC.: Brazil, São Paulo, São José do Rio Preto, *V. Stranghetti 765* (SP), [1]EF451126, [2]EF451087. *Aeschynomene fascicularis* Cham. & Schlect.: Venezuela, Mérida, *Lavin 5730* (MONT), [1]AF208929, [2]AF189025. *Aeschynomene martii* Benth.: Brazil, Minas Gerais, Mato Verde, *V.C. Souza 5455* (BHCB), [1]EF451127, [2]EF451088. *Aeschynomene paniculata* Willd.: Brazil, Minas Gerais, Belo Horizonte, *P.O. Morais & J.A. Lombardi 2689* (BHCB), [1]EF451125, [2]EF451086. *Aeschynomene purpusii* Brandegee: México, Oaxaca, Santiago Astata, *Lavin 5325* (MONT), [1]AF208928. *Aeschynomene vogelii* Rudd: Brazil, Minas Gerais, São Gonçalo do Rio Preto, *J.A. Lombardi 3725* (BHCB), [1]EF451128, [2]EF451089. ***Dalbergia* sect. *Dalbergia*.** *Dalbergia acuta* Benth.: Brazil, Minas Gerais, Januária, *A. Salino & J.A. Lombardi 1692* (BHCB), [1]DQ336608, [2]EF451064. *Dalbergia cuiabensis* Benth.: Brazil, Mato Grosso, *H.S. Irwin 15942* (SP), [1]EF451104, [2]EF451065. *Dalbergia elegans* AM Carvalho: Brazil, Espirito Santo, Linhares, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451105, [2]EF451066. *Dalbergia foliolosa* Benth.: Brazil,

Minas Gerais, *F.R. Couto 244* (BHCB), [1]EF451106, [2]EF451067. *Dalbergia miscolobium* Benth. (1): Brazil, Minas Gerais, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451108, [2]EF451069. *Dalbergia miscolobium* Benth. (2): Brasil, Bahia, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451109, [2]EF451070. *Dalbergia nigra* (Vell.) All. ex Benth. (1): Brazil, Minas Gerais, *J.P. Lemos Filho*, [1]EF451113, [2]EF451074. *Dalbergia nigra* (Vell.) All. ex Benth. (2): Brazil, Bahia, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451114, [2]EF451075. *Dalbergia villosa* (Benth.) Benth.: Brazil, Minas Gerais, Belo Horizonte, *J.P. Lemos Filho*, [1]EF451107, [2]EF451068. ***Dalbergia* sect. *Triptolemea*.** *Dalbergia brasiliensis* Vogel: Brazil, São Paulo, *Inês Cordeiro*, [1]EF451115, [2]EF451076. *Dalbergia decipularis* Matt. & Rizz.: Brazil, Bahia, Andaraí, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451116, [2]EF451077. *Dalbergia frutescens* (Vell.) Britton: Brazil, Espirito Santo, Linhares, *J.P. Lemos Filho s.n.* (BHCB), [1]EF451117, [2]EF451078. ***Dalbergia* sect. *Ecastaphyllum*.** *Dalbergia ecastaphyllum* (L.) Taub. (1): Brazil, Santa Catarina, Itapema, *A.C. Cervi 2569* (BHCB), [1]EF451110, [2]EF451071. *Dalbergia ecastaphyllum* (L.) Taub. (2): Brazil, Bahia, *J.P. Lemos Filho*, [1]EF451111, [2]EF451072. *Dalbergia monetaria* L.f.: Brazil, Pará, Belém, *Rafael Salomão*, [1]EF451112, [2]EF451073. ***Dalbergia* sect. undefined.** *Dalbergia congestiflora* Pittier: El Salvador, Santa Ana, Metapan, *Hughes 1253* (FHO), [1]AF208924, [2]AF068140. *Dalbergia sissoo* Roxb.: Brazil, Bahia, Ilhéus (cultivated), *J.P. Lemos Filho*, [1]EF451118, [2]EF451079. ***Machaerium* sect. *Acutifolia*.** *Machaerium acutifolium* Vogel: Brazil, Minas Gerais, Nova Ponte, *E. Tameirão Neto 2190* (BHCB), [1]EF451129, [2]EF451090. *Machaerium stipitatum* Vogel (1): Brazil, Minas Gerais, Belo Horizonte, *C.V. Mendonça 452* (BHCB), [1]EF451130, [2]EF451091. *Machaerium stipitatum* Vogel (2): Brazil, Minas Gerais, Belo Horizonte, *C.V. Mendonça 463* (BHCB), [1]EF451131, [2]EF451092. *Machaerium villosum* Vogel (1): Brazil, São Paulo, *Inês Cordeiro*, [1]EF451132,

[2]EF451093. *Machaerium villosum* Vogel (2): Brazil, Minas Gerais, Três Pontas, *C.V. Mendonça 531*, [1]EF451133, [2]EF451094. ***Machaerium* sect. *Lineata*.** *Machaerium aculeatum* Raddi: Brazil, Minas Gerais, Teixeira, *G.E. Valente 842* (BHCB), [1]EF451119, [2]EF451080. *Machaerium hirtum* (Vell.) Stelffeld: Brazil, Pernambuco, Itambé, *R.L.C. Ferreira s.n.* (BHCB), [1]EF451120, [2]EF451081. ***Machaerium* sect. *Reticulata*.** *Machaerium brasiliense* Vogel: Brazil, Minas Gerais, Belo Horizonte, *C.V. Mendonça 458* (BHCB), [1]EF451134. *Machaerium glabrum* Vogel: Brazil, Minas Gerais, Marlieria, *T.C. Sposito s.n.* (BHCB), [1]EF451135, [2]EF451095. *Machaerium oblongifolium* Vogel: Brazil, Espirito Santo, Santa Tereza, *C.V. Mendonça 570*, [1]EF451136, [2]EF451096. *Machaerium opacum* Vogel (1): Brazil, Minas Gerais, São Gonçalo do Rio Preto, *J.A. Lombardi 4068* (BHCB), [1]EF451137, [2]EF451097. *Machaerium opacum* Vogel (2): Brazil, Minas Gerais, Belo Horizonte, *C.V. Mendonça 533*, [1]EF451138, [2]EF451098. ***Machaerium* sect. *Oblonga*.** *Machaerium gracile* Benth (1): Brazil, Minas Gerais, Viçosa, *C.V. Mendonça 537*, [1]EF451139, [2]EF451099. *Machaerium gracile* Benth (2): Brazil, Espirito Santo, Santa Tereza, *C.V. Mendonça 574*, [1]EF451140, [2]EF451100. *Machaerium nyctitans* (Vell.) Benth. (1): Brazil, Minas Gerais, Igarapé, *C.V. Mendonça 455* (BHCB), [1]EF451121, [2]EF451082. *Machaerium nyctitans* (Vell.) Benth. (2): Brazil, Minas Gerais, Viçosa, *C.V. Mendonça 539*, [1]EF451122, [2]EF451083. *Machaerium scleroxylon* Tul. (1): Brazil, Minas Gerais, Januária, *A. Gotschalg & A. Salino 3979* (BHCB), [1]EF451123, [2]EF451084. *Machaerium scleroxylon* Tul. (2): Brazil, Minas Gerais, Virgem da Lapa, *E. Taimerão Neto 2531* (BHCB), [1]EF451124, [2]EF451085. **Outgroup taxa.** *Bryaspis lupulina* (Benth.) Duvign.: Sierra Leone, Waterloo, *Dawe 424* (K), [1]AF208932, [2]AF204234. *Diphysa ormocarpoides* (Rudd) M.Sousa & R. Antonio: México, Oaxaca, San Pedro Totalapan, *Saynes V. 1286* (MEXU), [1]AF208912, [2]AF068168. *Ormocarpum keniense*

Gillet: Kenya, Meru, *Faden 74/958* (MO), [1]AF208917, [2]AF068155. *Ormocarpopsis itremoensis* Du Puy & Labat: Madagascar, Fianarantsoa, Ambatofinandrahana, *Du Puy 2363* (K), [1]AF208918, [2]AF068149. *Pterocarpus rohrii* Vahl: Brazil, Manaus, Itacoatiara, *C.A. Sothers 1025* (SP), [1]EF451101, [2]EF451061. *Pictetia marginata* Sauv.: Cuba, Holguín, Sierra Nipe, *Lavin 7108* (MONT), [1]AF208910, [2]AF068176. *Soemmeringia semperflorens* Mart.: Brazil, Roraima, Ilha da Maracá, *Lewis 1600* (E), [1]AF208937, [2]AF189027. *Zygocarpum coeruleum* (Balf. f.) Thulin & Lavin: Yemen, Socotra, *Thulin & Grifi 8781* (UPS), [1]AF208914, [2]AF189037; *Weberbauerella brongniartioides* Ulbr.: Perú, Arequipa, Lomas de Mollendo, *Dillon 3909* (F), [1]AF208909, [2]AF189028.

Legends of figures

FIG. 1. One of the most parsimonious trees derived from the analysis of the combined ITS and *trnL* sequence data. OW = Old World. NW = New World.

FIG. 2. The Bayesian consensus phylogeny derived from analysis of the combined ITS and *trnL* sequence data. OW = Old World. NW = New World.

FIG. 1.

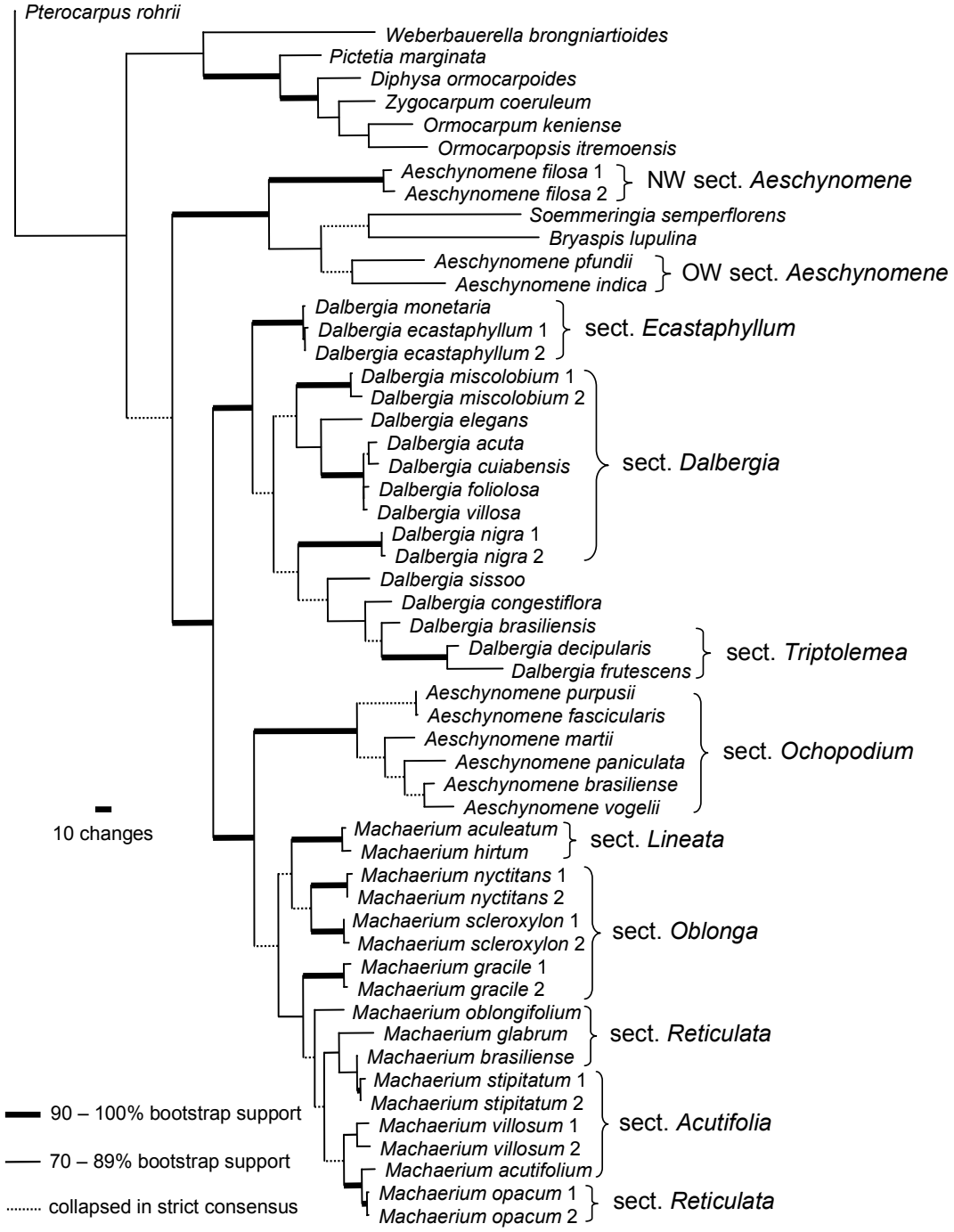
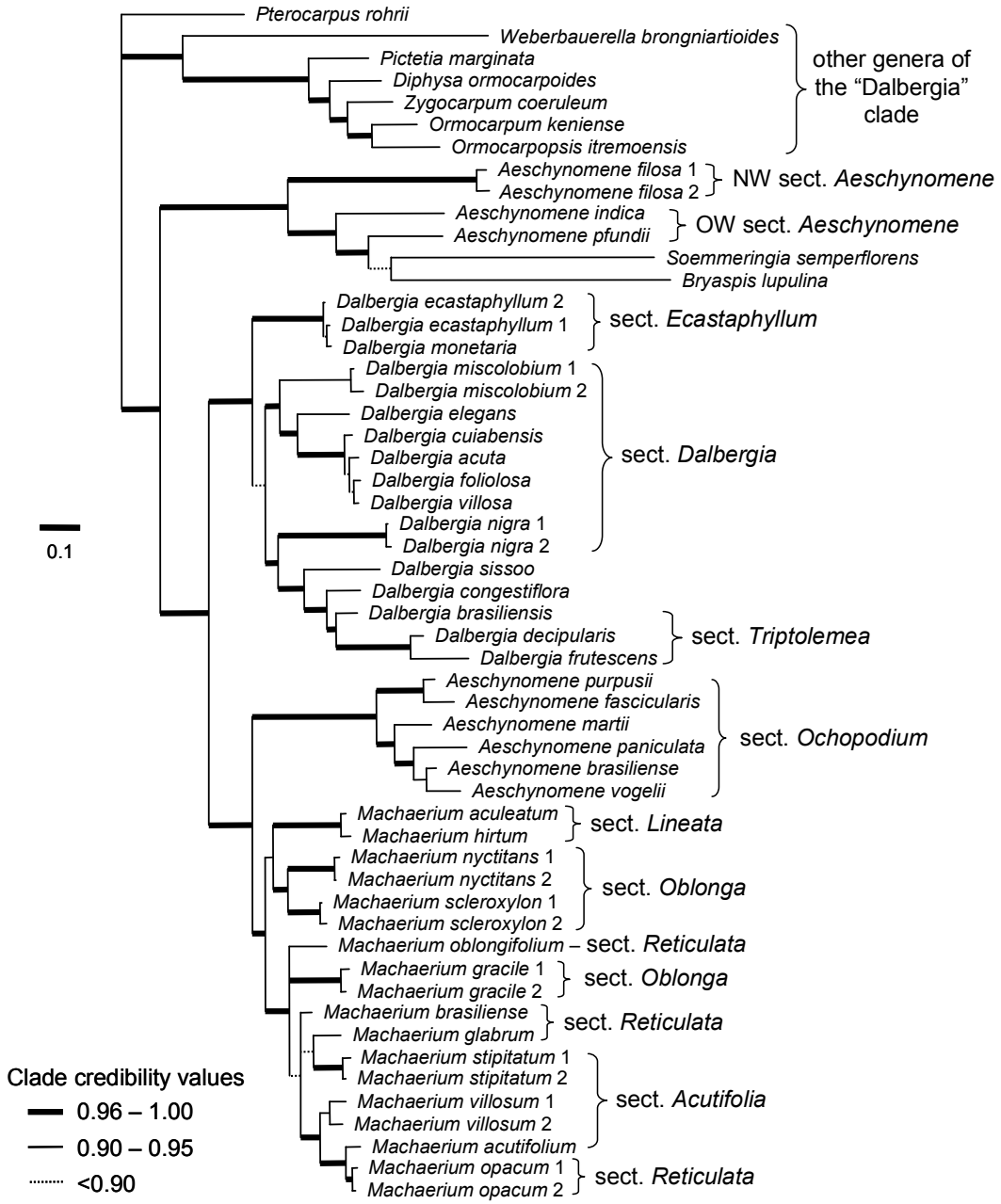

Pterocarpus rohrii
Weberbauerella brongniartioides
Pictetia marginata
Diphysa ormocarpoides
Zygocarpum coeruleum
Ormocarpum keniense
Ormocarpopsis itremoensis
Aeschynomene filosa 1
Aeschynomene filosa 2
NW sect. Aeschynomene
Soemmeringia semperflorens
Bryaspis lupulina
Aeschynomene pfundii
Aeschynomene indica
OW sect. Aeschynomene
Dalbergia monetaria
Dalbergia ecastaphyllum 1
Dalbergia ecastaphyllum 2
sect. Ecastaphyllum
Dalbergia miscolobium 1
Dalbergia miscolobium 2
Dalbergia elegans
Dalbergia acuta
Dalbergia cuiabensis
Dalbergia foliolosa
Dalbergia villosa
Dalbergia nigra 1
Dalbergia nigra 2
sect. Dalbergia
Dalbergia sissoo
Dalbergia congestiflora
Dalbergia brasiliensis
Dalbergia decipularis
Dalbergia frutescens
sect. Triptolemea
Aeschynomene purpusii
Aeschynomene fascicularis
Aeschynomene martii
Aeschynomene paniculata
Aeschynomene brasiliense
Aeschynomene vogelii
sect. Ochopodium
10 changes
Machaerium aculeatum
Machaerium hirtum
sect. Lineata
Machaerium nyctitans 1
Machaerium nyctitans 2
Machaerium scleroxylon 1
Machaerium scleroxylon 2
Machaerium gracile 1
Machaerium gracile 2
sect. Oblonga
Machaerium oblongifolium
Machaerium glabrum
Machaerium brasiliense
sect. Reticulata
Machaerium stipitatum 1
Machaerium stipitatum 2
Machaerium villosum 1
Machaerium villosum 2
Machaerium acutifolium
sect. Acutifolia
Machaerium opacum 1
Machaerium opacum 2
sect. Reticulata
90 – 100% bootstrap support
70 – 89% bootstrap support
collapsed in strict consensus

FIG. 2.


Pterocarpus rohrii
Weberbauerella brongniartioides
Pictetia marginata
Diphysa ormocarpoides
Zygocarpum coeruleum
Ormocarpum keniense
Ormocarpopsis itremoensis
other genera of the "Dalbergia" clade
Aeschynomene filosa 1
Aeschynomene filosa 2
NW sect. Aeschynomene
Aeschynomene indica
Aeschynomene pfundii
OW sect. Aeschynomene
Soemmeringia semperflorens
Bryaspis lupulina
Dalbergia ecastaphyllum 2
Dalbergia ecastaphyllum 1
Dalbergia monetaria
sect. Ecastaphyllum
Dalbergia miscolobium 1
Dalbergia miscolobium 2
Dalbergia elegans
Dalbergia cuiabensis
Dalbergia acuta
Dalbergia foliolosa
Dalbergia villosa
Dalbergia nigra 1
Dalbergia nigra 2
sect. Dalbergia
0.1
Dalbergia sissoo
Dalbergia congestiflora
Dalbergia brasiliensis
Dalbergia decipularis
Dalbergia frutescens
sect. Triptolemea
Aeschynomene purpusii
Aeschynomene fascicularis
Aeschynomene martii
Aeschynomene paniculata
Aeschynomene brasiliense
Aeschynomene vogelii
sect. Ochopodium
Machaerium aculeatum
Machaerium hirtum
sect. Lineata
Machaerium nyctitans 1
Machaerium nyctitans 2
Machaerium scleroxylon 1
Machaerium scleroxylon 2
sect. Oblonga
Machaerium oblongifolium – sect. Reticulata
Machaerium gracile 1
Machaerium gracile 2
sect. Oblonga
Machaerium brasiliense
Machaerium glabrum
sect. Reticulata
Machaerium stipitatum 1
Machaerium stipitatum 2
Machaerium villosum 1
Machaerium villosum 2
Machaerium acutifolium
sect. Acutifolia
Machaerium opacum 1
Machaerium opacum 2
sect. Reticulata
Clade credibility values
0.96 – 1.00
0.90 – 0.95
<0.90

IV) CONCLUSÕES

1. A análise filogeográfica das seqüências de cpDNA mostrou que as populações de *D. nigra* estão geograficamente estruturadas, exibindo uma diferenciação genética latitudinal que dividiu as populações em grupos norte e sul, na região do nordeste de Minas Gerais e sul da Bahia. As mudanças climáticas que ocorreram durante o Quaternário na Mata Atlântica podem ter formado refúgios, e a subseqüente migração a partir desses refúgios poderia explicar a formação desses dois grupos de populações. Evidências biogeográficas e palinológicas, bem como estudos filogeográficos em animais corroboram com esses resultados.

2. As populações de *D. nigra* localizadas em áreas de reserva florestal exibiram maiores níveis de diversidade genética do que as de pequenos fragmentos florestais, refletindo eventos que ocorreram após fragmentação antrópica recente, como deriva genética e restrição do fluxo gênico devido ao isolamento de populações.

3. Os resultados desse estudo apresentam dados relevantes para a conservação de *D. nigra*. Particularmente, a alta diversidade genética encontrada em populações de *D. nigra* no nordeste de Minas Gerais, devido à presença de haplótipos de ocorrência nos grupos norte e sul, aponta para a necessidade de implantação de unidades de conservação nesta região da Mata Atlântica.

4. A extração de DNA de boa qualidade a partir de folhas de espécies de *Dalbergia* foi problemática, provavelmente, devido à abundância de metabólitos secundários que provoca uma rápida oxidação. No entanto, foi selecionado e adaptado um protocolo que produziu DNA de boa qualidade, tanto de folhas frescas como herborizadas.

5. A qualidade do DNA extraído de amostras herborizadas de *Dalbergia* foi sempre inferior ao do obtido de folhas frescas. O método de preservação no campo (uso ou não de álcool para secagem das amostras) e no herbário (uso ou não de produtos químicos na desinfestação) influenciam na satisfatória qualidade do DNA extraído de espécimes herborizados de *Dalbergia* para análise de seqüências.

6. As análises filogenéticas utilizando seqüências de cpDNA e ITS sugeriram que os gêneros *Dalbergia* e *Machaerium* são potencialmente monofiléticos. No entanto, o gênero *Aeschynomene* não é monofilético, sendo a sect. *Ochopodium* intimamente

relacionada ao gênero *Machaerium* e a sect. *Aeschynomene* provavelmente parafilética a certos gêneros como *Bryaspsis* e *Soemmeringia*.

7. A classificação infragenérica de espécies do gênero *Dalbergia* revelou que as seções *Triptolemea* e *Ecastaphyllum* são monofiléticas, concordando com dados morfológicos (caracteres de inflorescência e fruto). No entanto, a seção *Dalbergia* foi parafilética, correspondendo a um padrão morfologicamente heterogêneo das espécies pertencentes a esta seção.

8. A análise filogenética das espécies amostradas de *Machaerium* revelou que todas seções são parafiléticas, exceto a seção *Lineata*, o que mostrou pouca correspondência entre os grupos filogenéticos e a classificação infragenérica tradicional. Alguns arranjos são congruentes com certos caracteres morfológicos e fitoquímicos. Os resultados apontam para a necessidade de um estudo filogenético mais amplo nesse gênero, envolvendo um maior número de espécies.

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