Universidade Federal do Rio Grande do Sul

Programa de Pós-Graduação em Genética e Biologia Molecular

Filogeografia e Sistemática Molecular de *Schizolobium parahyba* (Vell.) Blake (Guapuruvu) através do sequenciamento de regiões cloroplásticas e nucleares

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Orientador: Dr. Rogério Margis

Co-Orientadora: Dra. Márcia Margis-Pinheiro

Tese de doutorado

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Sumário

Resumo

A Floresta Atlântica e a Floresta Amazônica estão entre as maiores e mais diversas florestas tropicais do mundo, com muitas de suas espécies apresentando distribuição disjunta. O estudo genético molecular dessas espécies é interessante, pois podem fornecer informações sobre o relacionamento histórico entre essas diferentes regiões geográficas. Entretanto, ainda poucos são os estudos sobre a distribuição da estrutura genética nestas áreas, principalmente para espécies vegetais. O estudo da diversidade genética em espécies arbóreas é de grande importância para a manutenção das fontes de germoplasma a serem usados em práticas de reflorestamento e para espécies com uma ampla distribuição geográfica que ocupam diferentes habitats que são componentes chaves na composição de diversos ecossistemas. O gênero *Schizolobium* (Caesapinioideae) apresenta ampla distribuição nos Neotrópicos e devido ao seu rápido crescimento é amplamente utilizado em programas de reflorestamento, além de apresentar importância econômica pela utilização da madeira. O presente estudo apresenta a primeira análise genética molecular do gênero *Schizolobium*, incluindo uma ampla amostragem de populações ao longo de sua distribuição geográfica. Um conjunto de 11 marcadores moleculares (cpDNA e ITS) foram usados para investigar a evolução, posição sistemática, estimar o tempo de divergência entre as duas variedades, verificar um possível evento de especiação, estudar os padrões biogeográficos entre as florestas Atlântica e Amazônica, além de investigar a estrutura filogeográfica em *Schizolobium*. Marcadores não-neutros também foram estudados na tentativa de serem usados para investigar a variação adaptativa relacionada ao estresse hídrico. Sequências parciais dos genes P5CS de quatro espécies arbóreas (*Schizolobium parahyba*, *Ceiba pentandra*, *Bombacopsis quinata* e *Cedrela Odorata*) foram clonadas, seqüenciadas e comparadas com sequências de outras espécies. A análise filogenética indicou que eventos de duplicação ocorreram várias vezes e em diferentes frequências ao longo da evolução das monocotiledôneas e dicotiledôneas. Apesar de ter sido detectada seleção positiva em diferentes regiões do genes P5CS, uma pequena quantidade de

polimorfismo foi encontrado entre indivíduos de *Schizolobium* e não foram correlacionados com estresse hídrico. A monofilia do gênero *Schizolobium* foi bem suportada pelas análises de maxima parsimônia e Baysiana das regiões de cpDNA e de DNA nuclear. A idade do clado *Schizolobium* foi estimado em aproximadamente 15,6 milhões de anos (Mya) e as duas variedades divergiram a aproximadamente 3,1 Mya. Um elevado nível de divergência genética foi observado entre as populações de *Schizolobium* e os resultados indicam uma forte estruturação filogeográfica e um reduzido fluxo gênico entre elas. Além disso, nenhum haplótipo nuclear e de cpDNA foi compartilhado entre as duas variedades, evidenciando um isolamento entre elas. Foi observada similaridade nas sequências de cpDNA entre indivíduos de algumas populações da var. *parahyb*a na Mata Atlântica (RJ3, ES, BA1, BA2 e BA3) com indivíduos das populações da var. *amzonicum*, indicando a possibilidade da existência de retenção de polimorfismo ancestral com pouco tempo para o acúmulo de divergência nestas regiões. Todos os dados moleculares produzidos sugerem a separação das duas variedades dentro do gênero *Schizolobium*, e que a sua atual divisão taxonômica necessita de revisão. Esses dados também fornecem importantes informações genéticas que podem ser aplicadas no campo da conservação e florestamento exploratório.

Abstract

The Atlantic and the Amazon rain forests encompass the most diverse tropical forests in the world, with many species showing disjunct distribution. The molecular studies of widespread and disjunct species present particular interest, as they can provide information on the historical relationship between different geographical regions. However, there are few records about genetic structure in these areas mainly in plants species. Studies of genetic diversity of the tree species are very important to provide best practice policies for sourcing germplasm for reforestation within a range of degraded landscapes and for trees with a range of lifestyles that are key components of a diverse ecosystem composition. *Schizolobium* (Caesalpinioideae) is a widespread genus found in Neotropical forest, with a fast growing rate that make it extensively used in economically important reforestation programs that employ native trees. This study presents the first extensive molecular analysis within the genus *Schizolobium*, including a widespread sampling of populations from throughout their geographic distribution. A set of 11 molecular markers (cpDNA and nuclear) were used to address the evolution, systematic position, estimate the age of divergence between the two varieties, to study the biogeographic patterns between Atlantic and Amazonian rain forests and to investigate the phylogeographic structure of *Schizolobium*. Furthermore, non neutral markers were studied to attempt of access the adaptive variation in neotropical tree species. Partial sequences of P5CS genes from four Neotropical trees (*Schizolobium parahyba*, *Ceiba pentandra*, *Bombacopsis quinata* e *Cedrela Odorata*) were cloned and compared to those of other plant taxa. The molecular phylogenetic analysis indicated that *P5CS* duplication events have occurred several times following the emergence of flowering plants and at different frequencies throughout the evolution of monocots and dicots. Besides, positive selection was observed at different regions of *P5CS* paralogous genes, but a low polymorphism was found among individual of different areas and did not associate with water stress. The monophyletic nature of *Schizolobium* was well supported by both the Maximum Parsimony and Bayesian analyses

of the cpDNA and nuclear regions. The *Schizolobium* crown node was estimated to have arisen 15.6 million years ago (Mya) and the two varieties has been diverged approximately 3.1 Mya. High levels of genetic divergence were found among the populations of *Schizolobium* and the results indicate a strong phylogeographic structure and a reduced gene flow between them. Besides, the cpDNA and nuclear haplotypes is not sharing between the two varieties, indicated a genetic isolation between them. The cpDNA sequence similarity of some populations from Atlantic forest (RJ3, ES, BA1, BA2 e BA3) with the var. *amazonicum* was observed and this may be due retention of ancestral polymorphisms with insufficient time for the accumulation of differences in these regions. The molecular data suggest the separation of the two varieties of genus *Schizolobium*, and current taxonomic status needs revision. These data also provides important genetic information for conservation.

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Capítulo I: Introdução Geral

INTRODUÇÃO

Família Fabaceae

A família Fabaceae - Leguminosae é uma das maiores famílias de plantas dentre as angiospermas, com cerca de 730 gêneros e mais de 19.400 espécies. Os membros desta família são representados em quase todos os biomas terrestres, em regiões tropicais e temperadas, sendo que o principal elemento unificador da família é o fruto, a vagem. A família inclui muitas espécies com importância agronômica, além da sua extrema importância ecológica. A família Fabaceae é tradicionalmente dividida em três subfamílias: Caesalpinioideae, Mimosoideae e Papilonoideae (Lewis *et al.*, 2005). Considerada uma família tropical, com possível origem no último Cretáceo (65-70 Mya), as Leguminosas têm um abundante e contínuo registro fóssil desde o Terciário (Crepet, Taylor, 1985; Crepet, Taylor, 1986). Registros de ocorrência sugerem que a maioria das principais linhagens de leguminosas arbóreas tiveram maior diversificação a partir de meados do Eoceno (HERENDEEN *et al.*, 1992).

A subfamília Caesalpinioideae é parafilética e inclui aproximadamente 2250 espécies em 17 gêneros, os quais são principalmente árvores e arbustos distribuídos em regiões tropicais e subtropicais. Essa subfamília encontra-se atualmente dividida em quatro tribos: Cercideae, Deterieae, Cassiae e Caesalpineae (Lewis *et al.*, 2005). A tribo Caesalpineae encontra-se dividida em oito grupos, dentre eles o grupo *Peltophorum*, que inclui 16 gêneros, sendo 8 deles restritos da América do Sul. Análises moleculares com o gene *rbcL* sugerem que este grupo é parafilético (Kajita *et al.*, 2001), o que foi suportado

com dados do intron *trnL* (Bruneau *et al.*, 2001) e do espaçador plastidial *trnL-trnF* (Haston *et al.*, 2003; Haston *et al.*, 2005).

Gênero *Schizolobium*

O gênero *Schizolobium* está incluso no grupo *Peltophorum* e apresenta distribuição geográfica bastante ampla e disjunta, sendo encontrado desde a América Central até o Sul do Brasil (Figura 1). Desde a criação do gênero *Schizolobium,* várias espécies foram descritas e incorporadas: *S. amazonicum, S. parahyba, S. glutinosum, S. kellermaniy* e *S. excelsum.* Entretanto, em 1996, Barneby assumiu que estas cinco espécies são sinonímias da única espécie *S. parahyba* e, baseado em diferenças morfológicas, dividiu a espécie em duas variedades: *S. parahyba* var. *parahyba* e *S. parahyba* var. *amazonicum. S. parahyba* é popularmente conhecido como guapuruvu, pau-de-canoa, pinho-branco, ficheira bacuru, bageiro ou faveiro, dentre outros nomes regionais (Carvalho, 1994).

As características morfológicas que diferenciam uma variedade da outra são: *S. parahyba* var. *parahyba* apresenta pedicelos não unidos e anteras com 2,3-3,2mm, enquanto na var. *amazonicum,* os pedicelos são unidos a 2,0-6,5mm acima da base e as anteras apresentam entre 1,3-2,3mm (Barneby, 1996). Além disso, as duas variedades apresentam distribuição geográfica disjunta (Figura 1). *S.* p*arahyba* var. *amazonicum* ocorre na floresta amazônica brasileira, em áreas de floresta primária e secundária de terra firme e em várzea alta dos estados do Pará, Rondônia, Amazonas e Mato Grosso e em florestas subtropicais da America Central, Colômbia, Peru, Equador, Venezuela, e também no México. Tem como sinônimo *S. amazonicum* Huber & Ducke. *S.* p*arahyba,* var.

parahyba ocorre ao longo da Mata Atlântica, desde o Sul da Bahia até o Sul de Santa Catarina (Barneby, 1996).

Figura 1: Distribuição geográfica de *Schizolobium* mostrando a distribuição disjunta das duas variedades. Circulos represtam a distribuição da var. *parahyba* e triângulos da var. *amazonicum.* A área pontilhada mostra os locais de ocorrência.

O *Schizolobium* é uma árvore semicaducifolia, de rápido crescimento, podendo alcançar até 40m de altura e 300cm de DAP (diâmetro à altura do peito). Quando adulta apresenta copa muito ampla, de formato umbeliforme (Figura 2).

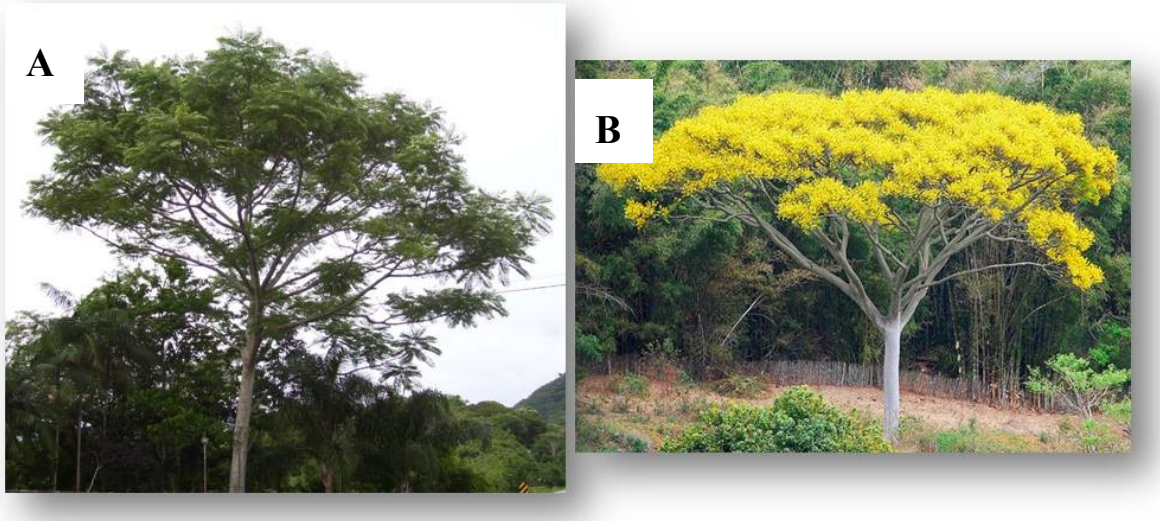

Figura 2: Árvore adulta de Schizolobium. (A) Período vegetativo. (B) Período de floração

As plantas de guapuruvu possuem raízes tabulares (Figura 3A) e o longo tronco cilíndrico apresenta fuste com até 15m de comprimento, possuindo lenticelas (Figura 3B). Apresenta folhas alternas, compostas, de até 1m de comprimento, bipinadas (Figura 3C). Suas flores formam inflorescências agrupadas em racemos terminais de até 30 cm de comprimento (Figura 3D). Os frutos são obovado-oblongo achatados, coriáceos, de coloração bege a marrom, com, em média, 16cm de comprimento e 6 cm de largura (Figura 3E). Quando os frutos estão maduros suas valvas se abrem, liberando a semente envolta pelo meso-endocarpo alado. As sementes são lisas, brilhantes, oblonga-achatadas, com tegumento duro, geralmente solitárias, medindo de 2 a 3cm de comprimento e 1,5 a 2,0cm de largura (Figura 3F) (Barneby, 1996).

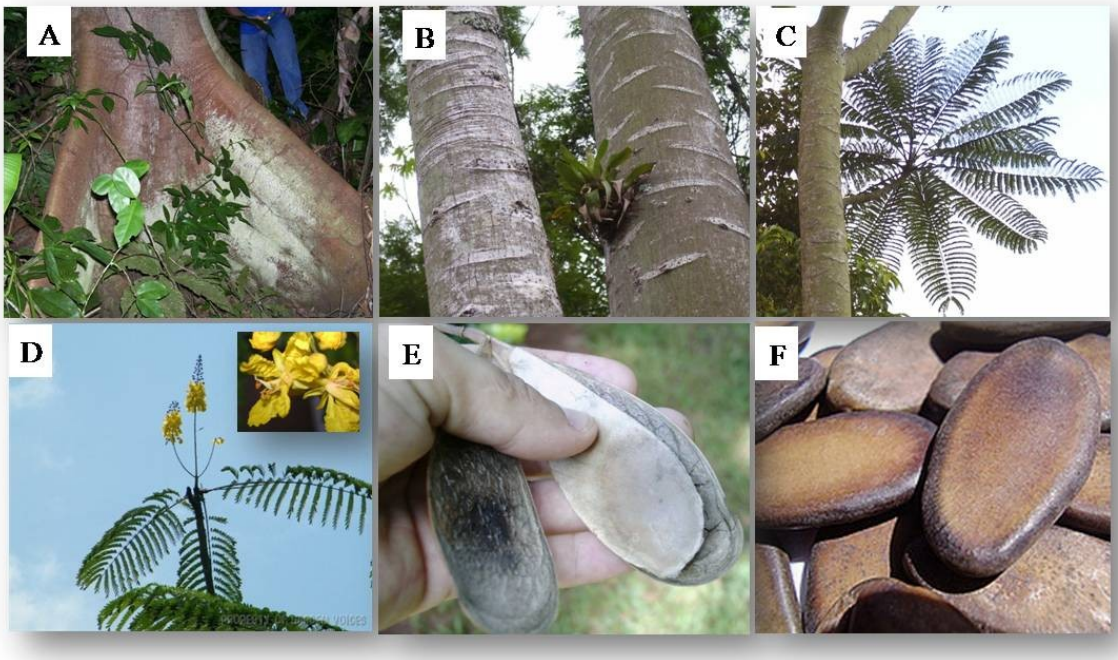

Figura 3: (A) Raízes tabulares. (B) Tronco com lenticelas. (C) Folhas compostas. (D) Inflorescências. (E) Fruto tipo sâmara. (F) Sementes.

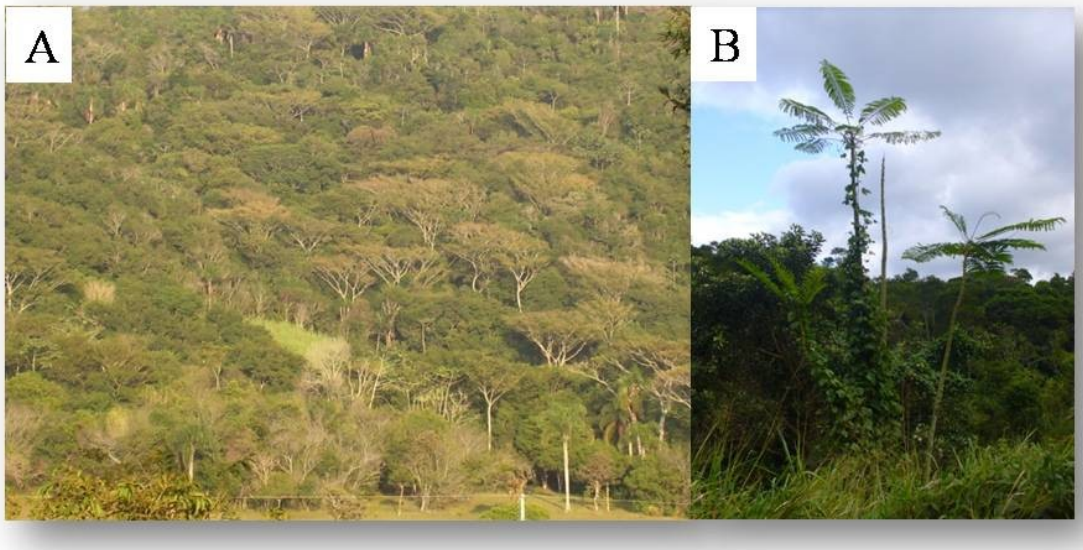

Figura 4: (A) Árvores em um fragmento de floresta Atlântica em Garopaba SC. (B) Planta jovem mostrando sua característica pioneira.

É considerada espécie pioneira, ocorrendo preferencialmente em matas em estágio inicial de sucessão, sendo essencialmente heliófita. Sua distribuição natural é irregular e descontínua, ocorrendo em grupos de várias árvores na floresta (Figura 4A) e individualmente nos estágios sucessionais (Figura 4B). Ocorre em áreas com precipitação pluvial média anual variando de 1.100mm a 2.400mm e temperatura média anual de 18,8°C a 24,3°C, em tipos climáticos tropical, subtropical de altitude e subtropical úmido (Carvalho, 1994).

Os indivíduos de guapuruvu são hermafroditas, polinizados principalmente por espécies de abelhas pequenas (*Apis mellifera, Friesella schrottkyi, Plebeia remota, Paratrigona subnuda*) (Morelatto, 1991). A floração ocorre de julho a dezembro e os frutos amadurecem entre março e agosto. O início da floração e a frutificação acontecem quando as plantas atingem entre 6 e 8 anos de idade. A dispersão dos frutos é barocórica e anemocórica e as sementes são geralmente dispersas pelo vento.

A produtividade volumétrica máxima registrada em plantas aos 10 anos de idade é de 45m^3/ha/ano e sua madeira é considerada leve (0,32 a 0,40g/cm^3), com superfície lisa, textura uniforme, alburno branco, com manchas amareladas e rosadas. Apresenta baixa resistência a organismos xilófagos, porém, sua permeabilidade às soluções preservantes é alta e é fácil de ser cortada e beneficiada. A madeira é bastante utilizada na confecção de canoas e objetos para aeromodelismo, em função da baixa densidade e na confecção de painéis, portas, brinquedos e caixas e também pode ser utilizada como chapas de compensado e laminados, é empregada na construção civil e em obras internas, além de ser considerada excelente para a produção de polpa e papel de fibra curta, levando a vantagem de ter a madeira quase branca e mole (Pietrobom, Oliveira, 2004).

A espécie apresenta, ainda, papel de destaque em programas para o reflorestamento misto de áreas degradadas visando a preservação permanente, devido principalmente ao seu rápido crescimento no campo, podendo atingir dez metros de altura no período de dois anos (Pietrobom, Oliveira, 2004).

A variedade *amazonicum* (paricá) é altamente cultivada pelas empresas madeireiras da região norte e nordeste do Brasil, principalmente nos Estados do Pará e Maranhão. Segundo o Centro de Pesquisa do Paricá (CPP) localizado no município de Dom Eliseu, no sul do Pará, que representa a grande maioria dos plantadores de paricá dos Estados do Pará e Maranhão, estima-se que, nestes Estados, existam em torno de 40.000 hectares da espécie plantados (Figura 5). No Equador, também existem muitas plantações de *Schizolobium*, sendo que a origem dos germoplasmas cultivados tem origem da Costa Rica (Canchignia-Martínes *et al.*, 2007).

Figura 5: Utilização de *Schizolobium* em programas de reflorestamento na Amazônia. Fonte: http://www.gruporosa.com.br/reflorestamento.html

Figura 6: Plantas de *Schizolobium* usadas para paisagismo

O *Schizolobium* também é utilizado em paisagismo, principalmente na região sul do país, tendo sido eleita a árvore símbolo de Florianópolis. É considerada uma espécie ornamental devido a sua intensa floração amarela e ao seu porte majestoso. Seu uso é recomendado na arborização de parques e ruas, pois suas raízes causam poucos danos a muros e calçamento, sendo também indicado para a restauração de mata ciliar em locais não sujeitos a inundações.

Muitos trabalhos vêm sendo realizados com plantas de *Schizolobium* a respeito de aspectos fisiológicos (Adami, Hebling, 2005; Coelho *et al.*, 2006; Costa *et al.*,

2006; Cruz *et al.*, 2007; de Carvalho, 2005; Filho *et al.*, 2007; Locatelli *et al.*, 2007; Melo Marques *et al.*, 2004), anatômicos (Marcati *et al.*, 2008; Pietrobom, Oliveira, 2004), bioquímicos e medicinais (de Carvalho *et al.*, 2008; Mendes *et al.*, 2008; Vale *et al.*, 2008) entre outros aspectos (Naumann *et al.*, 2008; Soares *et al.*, 2008; Zanuncio *et al.*, 2004). Entretanto, poucos trabalhos abordaram aspectos moleculares, entre eles (Canchignia-Martinez *et al.*, 2007; Freire *et al.*, 2007; Kamau *et al.*, 2003; Turchetto-Zolet *et al.*, 2009), mas nenhum tratou de aspectos filogeográficos.

Florestas Neotropicais

A região Neotropical está entre as regiões de maior biodiversidade do mundo e, por isso desperta preferência em estudos de origem da diversidade biológica (Rull, 2008). A flora Neotropical compreende aproximadamente 37% das espécies de plantas do mundo e muitas destas espécies são encontradas em florestas úmidas, as quais têm maior diversidade de plantas do que qualquer outro habitat do planeta, com mais de 90.000 espécies de plantas (Thomas, 1999). A origem de toda essa diversidade ainda permanece pouco explicada e por outro lado, algumas áreas neotropicais vêm sofrendo grande perda da biodiversidade devido à grande exploração e, foram identificadas como *hotspots* de biodiversidade e necessitam de atenção especial e programas de conservação (Myers, 2003).

O tempo e o modo de origem das espécies e a sua biodiversidade nos dias atuais ainda permanecem pouco explicadas, podendo ter sido efeitos de mudanças climáticas ocorridas no Quaternário ou ciclos glaciais do Pleistoceno (Bennett, 2004; Lovette, 2005). Muitos estudos tentam explicar o papel das mudanças climáticas

ambientais do Quaternário no mecanismo de especiação e dos modernos padrões de biodiversidade encontrados, principalmente na região Neotropical (Lister *et al.*, 2004). Os eventos de glaciações sugerem hipóteses de especiação recente, sendo que uma delas é a teoria de refúgios, onde teriam ocorrido diferenciações alopátricas entre as populações de espécies de florestas úmidas, que se tornaram isoladas umas das outras por se adaptarem a diferentes condições ambientais (Haffer, Prance, 2001; Prance, 1973). Algumas evidências de especiação no Pleistoceno em espécies arbóreas de florestas úmidas são mostradas em (Richardson *et al.*, 2001).

As florestas tropicais são conhecidas pela sua ampla biodiversidade. Exemplos de tais ecossistemas são a floresta Atlântica e Amazônica, que são as florestas tropicais mais diversas do mundo. Entre estas duas florestas existe um corredor de vegetação aberta, composto pela Caatinga ou Cerrado, que tem sido considerado uma importante barreira para a migração de muitas espécies entre elas (Prado, Gibbs, 1993). Por outro lado, estudos mostram que muitas espécies presentes nas florestas úmidas também se distribuem através do cerrado, indicando que no passado, a floresta Atlântica e Amazônica provavelmente foram contínuas e tornaram-se separadas após períodos de seca em que se formaram as savanas (Ledru, 1993). Com relação à conectividade das duas maiores florestas Neotropicais (Atlântica e Amazônica), estudos mostram que, do ponto de vista biogeográfico, a região nordeste do Brasil é particularmente interessante no que diz respeito à origem e as conexões passadas entre essas florestas (Behling, 2002; Behling *et al.*, 2000; Behling, Lichte, 1997).

A Mata Atlântica estendia-se de forma contínua ao longo da costa brasileira, desde o nordeste até o Rio Grande do Sul, penetrando até o leste do Paraguai e nordeste da Argentina. A Mata Atlântica engloba 2.428 municípios de 16 estados brasileiros. No

passado, chegou a cobrir mais de 1,5 milhões de Km^2, com 92% desta área no Brasil (Fundação SOS Mata Atlântica & INPE 2001). A Mata Atlântica é um dos ecossistemas mais ameaçados no mundo e o que resta da sua cobertura florestal é estimado em 5% da área original. Atualmente, encontra-se extremamente fragmentada, ficando reduzida a manchas disjuntas, concentradas nas regiões Sudeste e Sul, principalmente em locais de topografia acidentada. Estes remanescentes são o testemunho da formação florestal mais antiga do Brasil, estabelecida há cerca de pelo menos 70 milhões de anos (Leitão-Filho, 1987).

Os fragmentos florestais remanescentes são expostos a mudanças dramáticas nas condições microclimáticas, sendo o ambiente biótico e abiótico intensamente alterado nas áreas limítrofes (efeito de borda). Para algumas espécies de plantas, pequenas mudanças nas condições do solo e na disponibilidade de luz e água podem afetar a estrutura demográfica das populações, particularmente no estabelecimento de plântulas. Plantas de fecundação cruzada obrigatória, que dependem de polinizadores especializados para a formação de frutos e sementes, podem ser mais suscetíveis à fragmentação do habitat. Nestas espécies, a diminuição populacional e o aumento da distância entre os indivíduos podem dificultar o encontro de parceiros compatíveis.

Estudo de genes relacionados ao estresse hídrico em plantas

O estresse hídrico em plantas é um fator limitante do desenvolvimento e pode ocorrer tanto pela falta quanto pelo excesso de água, ocasionando várias respostas das plantas, que podem desenvolver mecanismos de adaptação e tolerância às novas condições. Alguns desses mecanismos estão relacionados (i) à síntese de metabólitos e

osmorreguladores (prolina e trealose); (ii) à regulação do transporte de água (aquaporinas); (iii) ao transporte de lipídios e (iv) à alterações hormonais. Todas essas mudanças fisiológicas, morfológicas e de desenvolvimento em plantas têm uma base molecular e genética, pois a adaptação e a aclimatação ao estresse ambiental resultam da integração desses eventos. Portanto, genótipos que diferem em tolerância ao estresse hídrico, sejam por falta ou por excesso de água, apresentarão diferenças qualitativas e quantitativas na expressão gênica. Uma resposta fisiológica específica ao estresse hídrico representa, na realidade, a combinação de eventos moleculares prévios, em resposta a percepção do sinal de estresse. A compreensão de como esses eventos são ativados ou desativados, como interagem entre si e quais genes estão envolvidos, é um fator de extrema importância.

A prolina é um dos osmorreguladores que se acumulam em plantas submetidas a condições de estresse hídrico (Delauney, Verma, 1993; Jimenez-Bremont *et al.*, 2006) e alguns trabalhos relatam o aumento de expressão dos genes que codificam a enzima bifuncional da rota biosintética da prolina em condições de estresse (Liu *et al.*, 1996; Silva-Ortega *et al.*, 2008; Strizhov *et al.*, 1997; Yamchi *et al.*, 2007). O gene *p5cs* já foi identificado em várias plantas, e em algumas delas foram descritas duas cópias, conhecidas como *p5cs1* e *p5cs2*, ambas codificando uma enzima bifuncional de extrema importância na rota biossintética da prolina. Entretanto, em bactérias e algas, foi descrita apenas uma cópia do gene p5cs. Estas informações ressaltam a importância do estudo da evolução destes genes, pelos mesmos poderem ser usados em análises de biodiversidade adaptativa.

Estudos filogeográficos

A filogeografia é o campo de estudos envolvido com os princípios e os processos que governam a distribuição geográfica de linhagens genéticas, especialmente aquelas dentro e entre as espécies (Avise, 2000). Inferências filogeográficas podem contribuir para um amplo estudo de ecologia e evolução em vários aspectos, tal como a identificação de regiões evolutivamente independentes. Tais abordagens ainda podem fornecer um contexto evolutivo e geográfico para as espécies e populações, compreendendo as comunidades ecológicas, permitindo a determinação das influências históricas e espaciais nas amostras de riquezas de espécies (Bermingham, Moritz, 1998; Brown, 1994; Young, 1996). Além disso, estudos filogeográficos permitem o entendimento e a identificação da história de áreas isoladas evolutivamente, podendo orientar estratégias e as prioridades para a conservação das mesmas (Bermingham, Moritz, 1998; Moritz, 2002).

Vários são os exemplos de estudos filogeográficos em plantas que discutem um panorama evolutivo de grandes áreas, sendo a maioria deles relacionados a florestas temperadas (Aoki *et al.*, 2006; Bauert *et al.*, 2007; Bettin *et al.*, 2007; Chen *et al.*, 2008a; Chen *et al.*, 2008b; Chen *et al.*, 2008c; Fujii, 2007; Ikeda, Setoguchi, 2007; Li *et al.*, 2008; Naciri, Gaudeul, 2007; Ortiz *et al.*, 2008; Ronikier *et al.*, 2008; Weising, Freitag, 2007). Por outro lado, um número limitado de estudos abordam padrões filogeográficos de espécies presentes em florestas Neotropicais (Andrade *et al.*, 2007; Lira *et al.*, 2003; Lorenz-Lemke *et al.*, 2005; Miller *et al.*, 2008; Ramos *et al.*, 2009). Poucos destes trabalhos têm disponibilizado informações a respeito dos efeitos das expansões e

contrações históricas da Mata Atlântica nos padrões de variação intra-específica e demonstram a complexidade da história deste bioma.

Estudos sobre a análise da diversidade genética de espécies vegetais nativas da Mata Atlântica (Alcantara *et al.*, 2006; Barbara *et al.*, 2007; Cardoso *et al.*, 2005; Ledru *et al.*, 2007; Margis *et al.*, 2002; Medri *et al.*, 2003; Moraes *et al.*, 1999; Salgueiro *et al.*, 2004) mostram que os níveis de estruturação genética entre populações apresentam valores de F_{ST}=0.04–0.211 (em espécies arbóreas) e de F_{ST}=0.029–0.43 (em espécies herbáceas), o que aponta para diferentes padrões da distribuição da diversidade genética e do fluxo gênico.

Sistemática Molecular

O desenvolvimento de técnicas moleculares, como PCR e sequenciamento de DNA, causou um grande impacto no campo da sistemática, desde o início da década de 1990. A sistemática molecular engloba uma serie de abordagens de relações filogenéticas, que são inferidas utilizando informações de macromoléculas dos organismos em estudo (Soltis, Soltis, 2000). Em plantas, os primeiros estudos focavam em níveis taxonômicos, no entanto, há uma crescente necessidade de ferramentas que possibilitem o estudo e o relacionamento entre gêneros e espécies recentes. Para tanto, os estudos filogenéticos utilizando variação de DNA permitem o alcance de tais informações. Com isso, o emprego da sistemática molecular vem crescendo cada vez mais, pois também permite a identificação de novas linhagens e produz dados que podem ser especialmente pertinentes para o campo da biologia da conservação (Andreasen, 2005; Moritz, 1995; Soltis, Gitzendanner, 1999). Muitos estudos recentes mostram a importância da sistemática

molecular na elucidação das relações taxonômicas de diversas espécies (Dick *et al.*, 2003; Gardner *et al.*, 2008; Karehed, Bremer, 2008; Martin-Bravo *et al.*, 2007; McNeill, Turland, 2009; Nagpal *et al.*, 2008; Nunes *et al.*, 2008; Peterson *et al.*, 2008; Redondo *et al.*, 2008; Soltis, Soltis, 2000; Torres-Perez *et al.*, 2009). Outros estudos relatam a importância da sistemática na área da conservação (Aleixo *et al.*, 2006; Boon *et al.*, 2000; Gompert *et al.*, 2006; Veron *et al.*, 2004).

Marcadores Moleculares

Os estudos de variabilidade adaptativa, sistemática molecular, diversidade genética e filogeografia podem ser feitos através de marcadores moleculares, os quais podem revelar interessantes padrões evolucionários, tais como o relacionamento entre espécies congenéricas ou populações de espécies, além da interação entre as informações genéticas com o clima e as diferentes condições ambientais nas quais as espécies estão sujeitas. Estes caracteres podem fornecer ferramentas adicionais para a compreensão de muitos fenômenos biológicos e auxiliar na conservação e manejo de muitas espécies, além de serem importantes para a compreensão da taxonomia, fenômeno de espécies criticas, isolamento de populações e a adaptação das espécies e indivíduos a estresses ambientais.

Atualmente existem diferentes técnicas da biologia molecular que permitem a detecção da variabilidade existente em nível do DNA. O sequenciamento de regiões plastidiais e nucleares são exemplos de marcadores bastante utilizados para análises filogenéticas, filogeografia, diversidade genética e variação adaptativa. Para estudos filogeográficos e filogenéticos em plantas, marcadores plastidiais e nucleares são utilizados. Entre os marcadores nucleares destacam-se as regiões ribossomais ITS (internal

transcribed spacer) (Dick *et al.*, 2007; Lorenz-Lemke *et al.*, 2005; Stahlberg, Hedren, 2008; Yamaji *et al.*, 2007) e entre os marcadores plastidiais destacam-se os espaçadores intergênicos (Dobes *et al.*, 2004; Fehlberg, Ranker, 2009; Fujii, 2007; Gong *et al.*, 2008; Lorenz-Lemke *et al.*, 2006; Tan *et al.*, 2008; Yuan *et al.*, 2008). Além dos marcadores neutros, destacam-se também a utilização de marcadores não neutros, através da análise de SNPs (Single Polimorphism Nucleotide) que possibilitam abordagens de associação adaptativa em muitas espécies de plantas. Nessa abordagem são utilizados regiões codificantes do genoma, como por exemplo, genes que estão relacionadas a algum tipo de estresse ambiental.

Os marcadores plastidiais são bastante utilizados e caracterizam-se pela frequente herança uniparental, que é uma vantagem destes marcadores para a avaliação diferencial do fluxo de pólen e sementes (Birky, 1995; Korpelainen, 2004). A análise do DNA plastidial permite a detecção de híbridos, que podem não ser identificados somente através da análise morfológica (Chen *et al.*, 2004; Fant *et al.*, 2005; Fant *et al.*, 2003; Modliszewski *et al.*, 2006; Tovar-Sanchez *et al.*, 2008).

Os internal transcribed spacer ribossomal (ITS) estão entre os marcadores nucleares mais utilizados em estudos de sistemática molecular de plantas em baixos níveis taxonômicos, devido a sua rápida taxa evolucionária e resolução do relacionamento filogenético em vários níveis taxonômicos aliados a facilidade de amplificação por PCR (Karehed *et al.*, 2008).

Objetivo Geral

A presente tese está inserida em um projeto amplo que visa contribuir para os estudos genéticos e moleculares de espécies arbóreas Neotropicais, produzindo e fornecendo fontes de informações para espécies de árvores sócio-economicamente importantes, oriundas de regiões tropicais das Américas Central e do Sul. Devido à falta de informações sobre a diversidade genética de espécies arbóreas Neotropicais, incluindo *S. parahyba*, esta tese tem como objetivo geral investigar aspectos sobre variação adaptativa, diversidade genética, fluxo gênico, e padrões filogeográficos desta espécie. O conhecimento destes itens será de grande importância para o desenvolvimento de estratégias de conservação para essa espécie e espécies relacionadas, além de possibilitar o fornecimento de informações sobre as melhores práticas em fontes de germoplasma para reflorestamento dentro de uma escala de degradação ambiental.

CAPÍTULO II: The evolution of pyrroline-5-carboxylate synthase in plants: a key enzyme in proline synthesis

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ORIGINAL PAPER

The evolution of pyrroline-5-carboxylate synthase in plants: a key enzyme in proline synthesis

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Abstract Many plants synthesize and accumulate proline in response to osmotic stress conditions. A central enzyme in the proline biosynthesis is the bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthase (P5CS) that includes two functional catalytic domains: the γ-glutamyl kinase and the glutamic-γ-semialdehyde dehydrogenase. This enzyme catalyzes the first two steps of the proline biosynthetic pathway and plays a central role in the regulation of this process in plants. To determine the evolutionary events that occurred in *P5CS* genes, partial sequences from four Neotropical trees were cloned and compared to those of other plant taxa. Molecular phylogenetic analysis indicated that *P5CS* duplication events have occurred several times following the emergence of flowering plants and at different frequencies throughout the evolution of monocots and dicots. Despite the high number of conserved residues in plant *P5CS* sequences, positive selection was observed at different regions of *P5CS* paralogous genes and also when dicots and monocots were contrasted.

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Introduction

The response of plants to osmotic stress is complex and involves several physiological and biochemical changes. Under drought stress, plants can increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes, such as polyols, sugars, betaine, glycine and proline among others (Delauney and Verma 1993). Proline participates not only in osmotic adjustment but also as a response to environmental stresses like low temperature, nutrient deficiency, exposure to heavy metals and high acidity (Delauney and Verma 1993; Parvanova et al. 2004; Goring and Thien 1979).

The pathway of proline biosynthesis was first elucidated in bacteria and begins with the ATP-dependent phosphorylation of glutamic acid by the γ-glutamyl kinase (γ-GK), encoded by the *proB* gene. The product of γ-GK is reduced to glutamic-semialdehyde (GSA) by the γ-glutamyl phosphate reductase (γ-GPR) encoded by the *proA* gene (Mahan and Csonka 1983; Orser et al. 1988). GSA cyclizes spontaneously to form Δ^1-pyrroline-5-carboxylate (P5C), which is finally reduced to proline by P5C reductase (P5CR, encoded by the *proC* gene) (Hu et al. 1992; Igarashi et al. 1997).

The proline biosynthetic route in plants resembles the bacterial pathway and uses either glutamic acid or ornithine as substrates. Under stressful conditions, proline is synthesized preferentially from glutamic acid (Delauney and Verma 1993) via two intermediates: GSA and P5C (Verdoy et al. 2006). The first two steps of proline biosynthesis are catalyzed by the bifunctional enzyme Δ1-pyrroline-5-carboxylate synthetase (P5CS) that encompasses both the

γ-glutamyl kinase and the glutamic-γ-semialdehyde dehydrogenase activities. The P5C is further reduced to proline by the Δ1-pyrroline-5-carboxylate reductase enzyme (P5CR) (Hu et al. 1992; Fujita et al. 1998; Ginzberg et al. 1998). The P5CS activity represents a rate-limiting step in proline biosynthesis, which is controlled at the level of *P5CS* transcription and through feedback inhibition of P5CS by proline (Zhang et al. 1995; Hong et al. 2000). The over-expression of the *P5CS* encoding gene in transgenic tobacco plants resulted in increased proline production and conferred tolerance of these plants to osmotic stress, confirming that P5CS is of key importance for the biosynthesis of proline in plants (Kishor et al. 1995).

The *P5CS* gene has been isolated from several plants and in some species two copies of the gene have been described. In *Arabidopsis thaliana*, the *AthP5CS1* and *AthP5CS2* genes show different temporal and spatial expression patterns. While *P5CS1* gene seems to be ubiquitously expressed in almost all organs and rapidly induced by stress, *P5CS2* is expressed in dividing cells and in response to incompatible interactions (Strizhov et al. 1997). Two *P5CS* genes were also isolated from medicago (*Medicago truncatula)* and rice. In *M. truncatula P5CS1* encodes a developmental 'housekeeping' enzyme, while *P5CS2* acts as a shoot-specific osmo-regulated isoform (Verdoy et al. 2006). In rice, *P5CS1* gene is inducible by salt, dehydration, cold, and abscisic acid (ABA), and is ubiquitously expressed in both vegetative and reproductive organs, whereas *P5CS2* is also inducible by NaCl and mannitol and is preferentially expressed in mature plants, especially in stamens (Hur et al. 2004).

A significant number of studies on physiological aspects of P5CS have been conducted, for animals, plants and bacteria (Csonka 1981; Mahan and Csonka 1983; Orser et al. 1988; Kishor et al. 1995; Savoure et al. 1995; Zhang et al. 1995; Abraham et al. 2003; Fabro et al. 2004; Su and Wu 2004; Verdoy et al. 2006; Gruszka Vendruscolo et al. 2007) and its biochemistry and function are now much better understood. However, an evolutionary analysis of these genes is still lacking. To investigate this issue, in the present work, we have cloned partial sequences of *P5CS* genes from four species of Neotropical trees, reconstructed a molecular phylogeny of *P5CS1* and *P5CS2* genes in different plant species. Our results represent the first contribution to the understanding of the evolutionary events of this gene family, which plays a key role in stress resistance and tolerance.

Materials and methods

Plant material and DNA isolation

The four species of Neotropical trees used for cloning *P5CS* genes were *Schizolobium parahyba, Bombacopsis quinata, Ceiba pentandra* and *Cedrela odorata*. Cambium from *S. parahyba* and leaves from the other three species were used to isolate genomic DNA using a CTAB method (Doyle and Doyle 1990). Briefly, 100–200 mg of plant material was powdered in liquid nitrogen and incubated with 0.7 ml of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCI pH 8.0, 0.2% β-mercaptoethanol) at 60°C for 60 min, 0.6 ml chloroform/isoamyl alcohol (24:1) was added, samples were vigorously shaken and phases were separated by centrifugation for 10 min at 12,000*g*. The upper aqueous phase was removed and mixed with two volumes of isopropanol for 10–24 h at −20°C and centrifuged for 20 min at 14,000*g*. The DNA was washed with 70% ethanol, air-dried, and dissolved in an appropriate volume of TE buffer (Tris-EDTA) and kept at −20°C before use.

Design of degenerate oligonucleotides and DNA amplification

To isolate *P5CS* partial sequence, we designed a pair of degenerate primers: the forward primer P5CSiF2 5′GGIATHTTYTGGGAYAAYGA and the reverse primer P5CSiR2 5′GCIGCIACIGCCATYTC, located in conserved sequences identified by multiple sequence alignment of *P5CS* sequences (Fig. 1a). Sequences from target species were then amplified by PCR using 10 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1 × PCR buffer, 0.05 U of Platinum Taq DNA polymerase (Invitrogen) and 5 μM of each primer in a final volume of 20 μl. The PCR cycle had an initial hot-start step at 94°C for 5 min, followed by a touchdown program with annealing temperature decreasing from 60 to 50°C in 20 cycles, followed by 30 cycles at an annealing temperature of 54°C for 15 s, with 2 min elongation at 72°C and 30 s of denaturation at 94°C. All PCR products were visualized by electrophoresis in 1.5% agarose gels stained with SYBR Gold (Invitrogen).

P5CS cloning and sequencing

Amplified PCR products of *S. parahyba* and *B. quinata* were cloned into pCR 2.1 TOPO TA vector (Invitrogen), while PCR products of the *C. pentandra* and *C. odorata* were cloned into pGEM-TEasy plasmid (Promega). The ligation products were transformed into electrocompetent *E. coli* XLI. Plasmids were purified with PureLink quick plasmid miniprep kit (Invitrogen) and sequenced by the dideoxy chain-termination method using BigDye (Applied Biosystems), using an ABI-3100 automatic sequencer (Applied Biosystems). Both DNA strands of each clone were fully sequenced using T7 and M13 universal primers. The identity of sequences was certified using blastn and tblastx algorithms against DNA plant sequences deposited

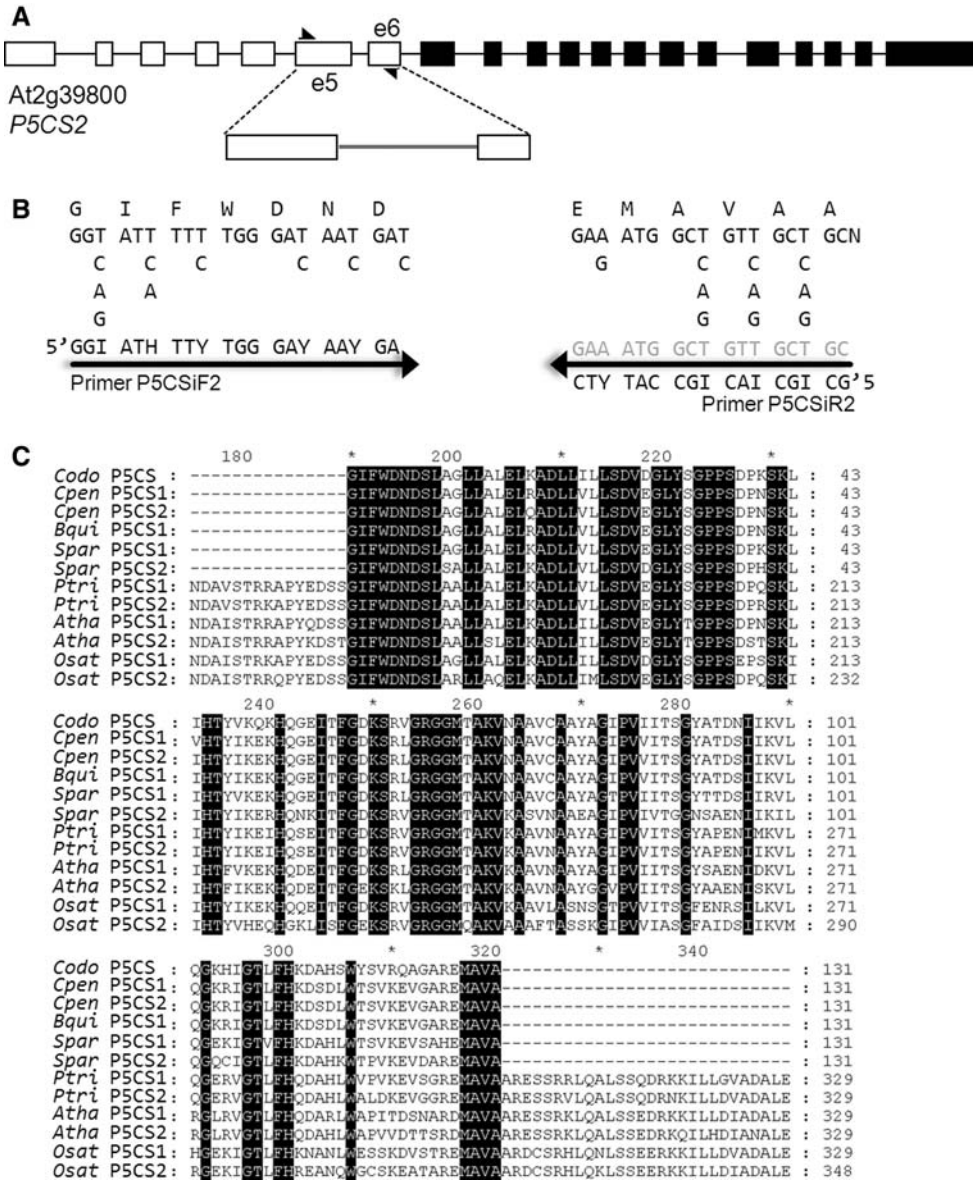

Fig. 1 **a** Structural organization of arabidopsis *P5CS2* gene with emphasis in the region amplified by P5CS degenerate primers. **b** Nucleotide and amino acid sequences of the P5CSiF2 and P5CSiR2 degenerate primers. **c** Alignment of partial P5CS amino acid sequences of *C. odorata* (*Codo*P5CS), *C. pentandra* (*Cpen*P5CS1 and 2), *B. quinata* (B*qui*P5CS), *S. parahyba* (*Spar*P5CS1 and 2), *A. thaliana* (*Atha*P5CS1 and 2), *Populus trichocarpa* (*Ptri*P5CS1 and 2) and *O. sativa* (*Osat*P5CS1 and 2). Conserved residues are in *white* with a *black* background

at NCBI (http://www.ncbi.nlm.nih.gov). Computer analyses of nucleotide and amino acid sequences were carried out using Vector-NTI software (Invitrogen).

RNA isolation, cDNA synthesis and amplification

The total RNA was extracted from young leaves and cambium samples from *S. parahyba* using the Trizol reagent (Invitrogen). Approximately 1 µg of total RNA was denaturated at 70°C and hybridized with 100 nmol of primer T23 V (5′ TTT TTT TTT TTT TTT TTT TTT TTV) or 20 nmol of specific P5CS1R2 (5′ ATT GGG CAG AGG TGG TAT GA) or P5CS2R2 (5′ CCC GCA CTC AAA GCT TAT TC) reverse primers. RNA-primer mix was added to each cDNA synthesis buffer and incubated at 40°C for 1 h in the presence of 200 U of MMLV-reverse transcriptase (Promega). The cDNAs were diluted 1:50 with deionized water and fractions of 10 µl were used in PCR reactions. Two primer-pairs were designed to amplify specifically the internal regions of P5CS1 (P5CS1F2 and P5CS1R2) or P5CS2 (P5CS2F2 and P5CS2R2). The expected size of P5CS1 and P5CS2 PCR amplified products was 167 and 219 bp, respectively.

Phylogenetic analyses of P5CS gene

The phylogenetic analysis of *P5CS* genes, sequences of cDNA and expressed sequence tags (ESTs) of several taxa were obtained from the NCBI, TIGR (http://www.tigr.org/tdb/e2kl/osal/) and DOE-JGI (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) database using systematic BLAST. The list of identified taxa with deposited *P5CS*

sequences and their accession number are displayed in Table 1. A sequence from the human *P5CS* gene was used as an outgroup for phylogenetic analysis with full sequence and a sequence from the algae *Ostreococcus lucimarinus* was used in trees constructed with ESTs and partial sequences.

Nucleotide sequences were aligned using the Alignment Explorer/CLUSTALW Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura et al. 2007) and optimized by hand, according to the respective amino acid alignments. Phylogenetic analyses were performed using the neighbor-joining (NJ) method by MEGA. The NJ method was performed for *P5CS* full-length nucleotide sequence and *P5CS* EST nucleotide sequence corresponding to 393 bp of the γGK domain cloned in each of the four Neotropical species. The molecular distances of the aligned sequences were calculated according to the *p*-distance parameter. All gap and missing data in the alignments were accounted for by pairwise deletion. Branch points were tested for significance by bootstrapping with 1,000 replications.

Both maximum-parsimony and maximum-likelihood analysis were carried out with *P5CS* EST nucleotide sequence including the partial sequence cloned in this study. The analyses were implemented in the PAUP* 4.0 software package (Swofford 2002). Maximum-parsimony trees were obtained by 100 random addition heuristic search replicates and the tree bisection–reconnection (TBR) branch-swapping option. Maximum-likelihood analysis was evaluated using bootstrap analysis with 100 repeats of bootstrap samplings. The model parameter was determined by Modeltest 3.7 (Posada and Crandall 1998).

Synonymous and non-synonymous substitutions

Pairwise synonymous (K_s) and non-synonymous (K_a) numbers of substitutions corrected for multiple hits were calculated using the DnaSP (DNA polymorphism analysis) software (Rozas et al. 2003). The K_a/K_s rate was calculated using sequences from plant species where full-length P5CS sequences were available, comparing both P5CS genes present in dicots with the duplicated genes from monocots. More refined analyses were made using all sequences listed in Table 1. A sliding window of 393 sites was used to compute the rate along three sites with a step size of the one site.

Results

Genetic structure and cloning of P5CS

The sequences of P5CS encoding genes for model species such as *Arabidopsis* (Strizhov et al. 1997), rice (Hien et al. 2003; Choudhary et al. 2005) and poplar (Dluzniewska et al. 2007) were compared to partial sequences from other plants listed in Table 1. At present, data concerning the structural organization of *Arabidopsis*, poplar and rice P5CS genes were not assembled and compared to *P5CS* from other plants. The identification and analyses of the genomic structure of *P5CS* genes in Arabidopsis, rice and poplar were performed using gene structure prediction programs and by comparison with available ESTs clones. The structural organization of exons and introns of one of two closely-related *Arabidopsis P5CS* genes are shown in Fig. 1a. The *P5CS* gene in plants is composed of 20 exons and 19 introns. The main difference observed in the duplicated copies of *P5CS* genes present in the genomes of *Arabidopsis* and rice concerns the sizes of their introns (Fig. 2a).

The amino acid alignment of arabidopsis, rice and poplar P5CS sequences allowed the identification of a conserved region and the design of degenerate primers for PCR amplification (Fig. 1b). These primers were used to amplify by PCR the *P5CS* genes from genomic DNA of four Neotropical tree species: *S. parahyba, C. odorata, C. pentandra* and *B. quinata*. The amplified region overlaps majority of the fifth and sixth *P5CS* exons and the totality of the sixth intron (Fig. 1a). Two distinct fragments were amplified from DNA samples of *S. parahyba* (Fig. 2b) and *C. pentandra* (data not shown), suggesting the presence of two copies of *P5CS* gene in their genomes. All PCR products were cloned into plasmid vectors and submitted to restriction and sequence analyses. PCR amplification products of each species encode the expected partial regions of the P5CS enzyme (Figs. 1a, c, 2a). *S. parahyba* insert presented sequences with 1,116 and 971 bp, while *C. pentandra* clones harbor fragments with 1,056 and 890 bp. The single-PCR amplified product from *C. odorata* and *B. quinata* DNA has sequence with 899 and 894 bp, respectively (Figs. 1c, 2a). These partial *P5CS* sequences were named *Bquin*P5CS (*B. quinata*), *Codo*P5CS (*C. odorata*), *Cpen*P5CS1 and *Cpen*P5CS2 (*C. pentandra*), *Spar*P5CS1, *Spar*P5CS2 (*S. parahyba*), and deposited in GenBank with accession numbers from EU292676 to EU292681, respectively (Table 1).

The cloned region of *P5CS* covers 269 and 124 bp of exons 5 and 6. This number of nucleotides is conserved in all six cloned sequences (Fig. 2a) and also in other plant sequences found in data banks. As observed in the genomic organization of arabidopsis *P5CS* genes, all differences in the size correspond to variations in the number of nucleotides in the introns (Fig. 2a). The alignment of amino acid sequences of the cloned fragment of P5CS from Neotropical trees species showed a high degree of identity with P5CS sequences from other plants (Fig. 1c). The amino acid sequences showed high identity with P5CS from

Table 1 List of species used in the study and their P5CS gene accession numbers

Organism name	Order	Family	Acronym	Accession number	nt (bp)
Asparagus officinalis	Asparagales	Asparagaceae	*Aoff* P5CS	CV289339	393
Centaurea maculosa	Asterales	Asteraceae	*Cmac* P5CS1	EH714052	393
			Cmac P5CS2	EH716682	321
Chicorium intybus			*Cint* P5CS1	EH678565	321
Helianthus exilis			*Hexi* P5CS1	EE647005	393
			Hexi P5CS2	EE632762	393
Helianthus annus			*Hann* P5CS1	DY912669	321
			Hann P5CS2	CX946653	
Helianthus tuberosus			*Htub* P5CS1	EL442123	306
			Htub P5CS2	EL454563	321
Helianthus petiolares			*Hpet* P5CS1	DY932969	333
Lactuca sativa			*Lsat* P5CS2	DW134129	393
Lactuca serriola			*Lser* P5CS1	DW109915	293
Arabidopsis thaliana	Brassicales	Brassicaceae	*Atha* P5CS1	At2g39800	2,154
			Atha P5CS2	At3g55610	2,181
Brassica napus			*Bnap* P5CS1	AF314811	2,154
			Bnap P5CS2	AF314812	2,181
Brassica rapa			*Brap* P5CS1	DN961318	369
Chorispora bungeana			*Cbun* P5CS	AY804246	2,181
Mesembryanthemum crystallinum	Caryophylales	Aizoaceae	*Mcry* P5CS	AF067967	393
Ostreococcus lucimarinus			Oluc ProB	XM001419476	447
Glycine Max	Fabales	Fabaceae	*Gmax* P5CS	AY492005	2,148
Medicago sativa			*Msat* P5CS1	X98421	2,148
Medicago truncatula			*Mtru* P5CS1	AJ278818	2,148
			Mtru P5CS2	BG455048	393
Schizolobium parahyba			***Spar* P5CS1**	EU292680	393
			***Spar* P5CS2**	EU292681	393
Vignia unguiculata			*Vung* P5CS2	AB056452	2,154
Populus trichocarpa	Malpighiales	Salicaceae	*Ptri* P5CS1	LG_X0783	2,160
			Ptri P5CS2	gw1.VIII.2487.1	2,142
Ceiba pentandra	Malvales	Malvaceae	***Cpen* P5CS1**	EU292678	393
			***Cpen* P5CS2**	EU292679	393
Bombacopsis quinata			***Bqui* P5CS2**	EU292676	393
Pinus taeda	Pinales	Pinaceae	*Ptae* P5CS	DT632653.1	393
Hordeum vulgare	Poales	Poaceae	*Hvul* P5CS2	BG368096	393
Oryza sativa			*Osat* P5CS1	Os01g62900	2,151
			Osat P5CS2	Os05g38150	2,151
			Soff P5CS2	CA209500 (4)	393
Sorghum bicolor			*Sbic* P5CS	TA26070_4558	387
Triticum aestivum			*Taes* P5CS1	TA52986_4565	2,151
			Taes P5CS2	TA56146_4565	393
Zea mays			*Zmay* P5CS	EC884238 (5)	393
Homo sapiens	Primata	Hominoidea	*Hsap* P5CS	NM_002860.3	2,220
Cedrela odorata	Sapindales	Meliaceae	***Codo* P5CS**	EU292677	393
Citrus clementina	Sapindales	Rutaceae	*Ccle* P5CS	DY292163	387
Solanum tuberosum	Solanales	Solanaceae	*Stub* P5CS	CV499774	393
Solanum lycopersicum			*Slyc* P5CS	SLU60267	2,154
Vitis vinífera	Vitales	Vitaceae	*Vvin* P5CS	VVI5686	2,178

The acronyms in bold correspond to those species for which partial *P5CS* sequences were cloned in this study

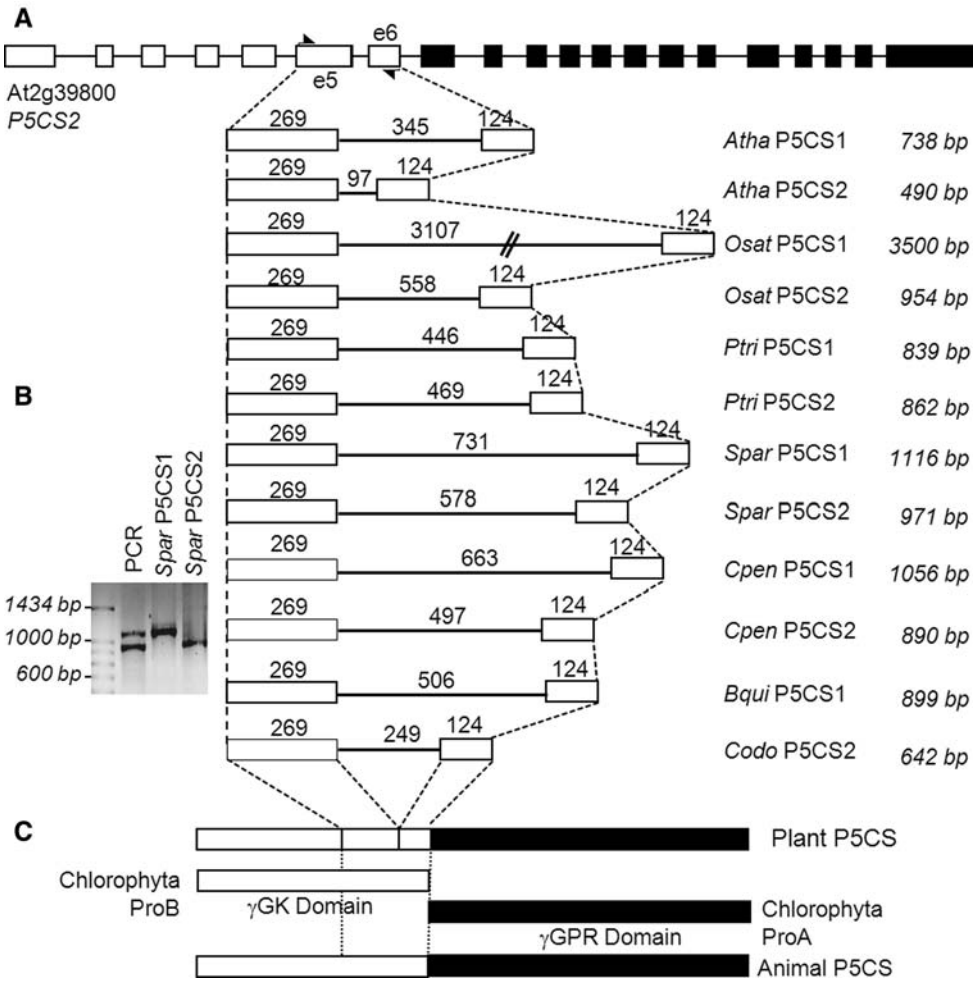

Fig. 2 **a** Comparison of the partial structure of *P5CS1* and *P5CS2* genes of *Arabidopsis thaliana, Oryza sativa, Populus trichocarpa, Schizolobium parahyba, Ceiba pentandra, Bombacopsis quinata* and *Cedrela odorata* (exons 5 and 6, intron 6). *Boxes* indicate exons and the *solid bold line* indicate introns, with *numbers* corresponding to nucleotides. The *dotted line* indicates the region cloned and used for phylogenetic analysis. **b** Agarose gel showing the two PCR amplification products of *S. parahyba P5CS* and their respective clones. **c** Distribution of γ-glutamyl kinase (γGK) and γ-glutamyl phosphate reductase (γGPR) domains present in plants, animals and the two orthologous enzymes present in algae

arabidopsis (75–77%) and rice (82–88%). In bacteria, fungi and algae, as compared to plant and animal *P5CS* genes, γGK and γGPR domains are separated and correspond to *proB* and *proA* genes respectively (Fig. 2c). A reduced but significant identity, in the range of 37 to 40%, was found among the cloned regions of plant *P5CS* and the *proB* gene from the green algae *Ostreococcus licimarinus*.

A series of two new primer-pairs were designed to hybridize inside the sequenced *P5CS* genomic fragments and bordering the deduced splicing sites. The detection of specific P5CS1 and P5CS2 PCR amplification products with 167 and 219 bp, from cDNA samples obtained from leaves and cambium, indicate that both P5CS genes from *S. parahyba* are actively transcribed in these tissues (Fig. 3). The identity of each PCR was also confirmed by direct sequencing of the PCR product.

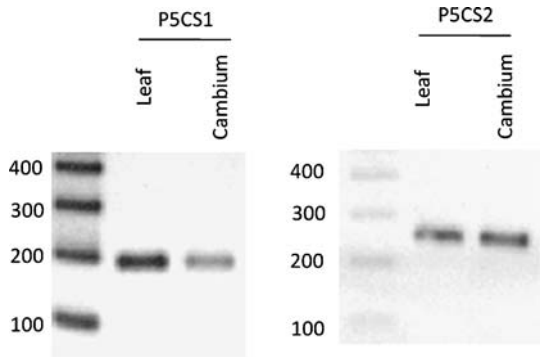

Fig. 3 Expression of *P5CS1* and *P5CS2* mRNAs in *Schizolobium parahyba*. Amplification products obtained after PCR amplification using specific primer-pairs to P5CS1 and P5CS2 on cDNAs from young leaves and cambium. DNA marker sizes (in base pairs) are indicated at the *left side* of both 2% agarose gels

Phylogeny of plant P5CS genes

Two distinct phylogenetic analyses were undertaken to reconstruct the evolutionary history of *P5CS* genes in plants. In the first analysis, a phylogenetic tree was constructed using the NJ method with a set of 16 plant sequences harboring the full-length coding sequences of *P5CS* present in public data banks (Fig. 4). Phylogenetic trees were rooted using human *P5CS* as an outgroup. Vertebrate and invertebrate *P5CS* share the same organiza-

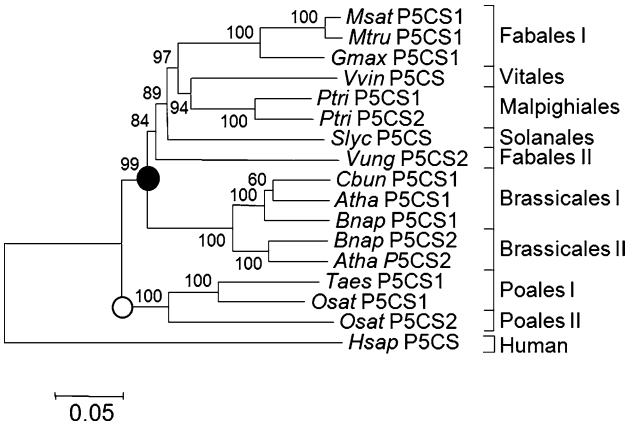

Fig. 4 Phylogenetic tree obtained from full-length cDNA sequences of *P5CS*. The tree was produced using the neighbor-joining method, p-distance and pairwise deletion analysis on a Clustal-W multialignment. Percentages refer to significant bootstrap values of 1,000 calculated trees. The *black and white circles* indicate dicot and monocot clusters, respectively

tion as plant *P5CS*, where a single-gene/polypeptide harbors two independent but sequential catalytic domains (Fig. 2c).

A second analysis, using sequences encompassing exons 5 and 6 of P5CS, allowed the comparison of a greater number of plant sequences: two families from monocots (six species), 10 families from dicots (38 species) and one species from gymnosperms (Fig. 5a, b). Three different analytical methods, namely, NJ, parsimony and maximum-likelihood analysis were compared (Fig. 5a, b). Phylogenetic trees were rooted by using *proB* (γ-glutamyl kinase) sequence of the green algae *O. lucimarinus* (Chorophyta).

The phylogenetic trees constructed with the full-length cDNA sequences were based on 2,418 unambiguously aligned positions while phylogenetic trees of partial sequences were based on 459 sites, 273 of which were informative under the parsimony criterion. The number of variable and conserved sites were 1,646 and 553 for full-length sequence phylogenetic tree, and 303 and 90 for the partial sequence tree, with an average content of T (27.6), C (18.8), A (28.3) and G (25.3) with a transition to transversion rate equal to 0.9.

Both trees, constructed with complete cDNA or partial sequences, showed the same phylogenetic profile. The P5CS sequences of monocots were clustered separately

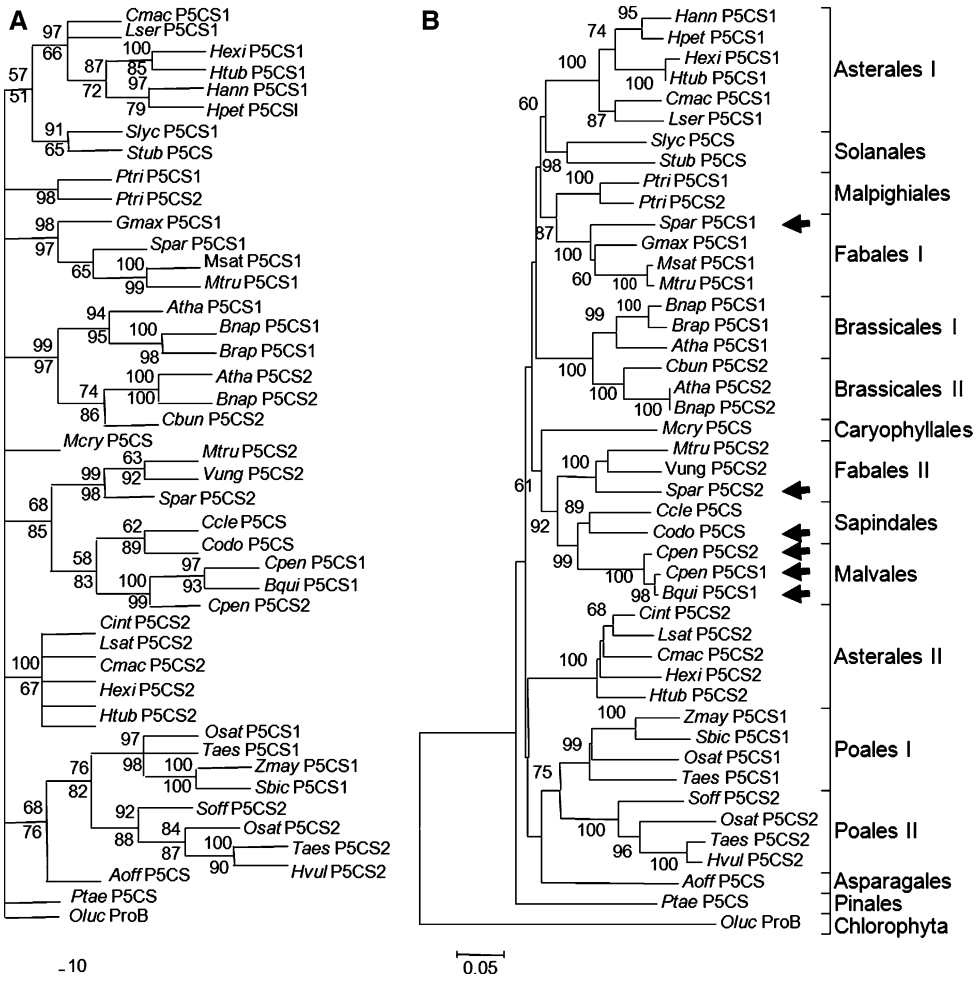

Fig. 5 Phylogenetic tree obtained from P5CS EST sequences from different plant species. Phylogenetic analysis was performed on the alignment of 44 sequences. Percentages refer to significant bootstrap values of 1,000 calculated trees. **a** Tree was produced using the neighbor-joining method, p-distance and pairwise deletion analysis on a Clustal-W multialignment. **b** Phylogenetic trees produced after a maximum-parsimony (MP) or maximum-likelihood (ML) analysis using PAUP*4.0 software. The tree constructed with MP and ML showed the same topology. Bootstrap values for MP are given above and for ML below branch lines. Organisms indicated by *arrows* correspond to sequences cloned in this study. The trees were rooted with the sequence of the green algae *Ostreococcus lucimarinus*

from the dicots, regardless of the phylogenetic method used (Figs. 4, 5a, b). Two separate clusters were obtained for Poales species in Monocots. One cluster contained all the *P5CS1* sequences while the second cluster contained all the *P5CS2* sequences of Poales. This pattern was not observed for the P5CS duplication in taxonomic orders of dicots. In fact, *P5CS1* and *P5CS2* formed independent clusters in all orders, but in some cases they are grouped, as in Brassicales, and in others, such as Fabales, they are genetically distant.

Evolution in P5CS genes

Measures of selective pressure on *P5CS*1 and *P5CS*2 were made after comparisons of nonsynonymous to synonymous substitution ratios (K_a/K_s) between monocots and dicots using the full-length cDNA sequence or the 393 bp of the γGK domain. The pairwise comparisons of *P5CS*1 and *P5CS2* genes of monocots and dicots produced an average K_a/K_s ratio of 0.07 within the full-length cDNA sequence and of 0.106 within the last 393 bp of the γGK domain (Fig. 5a, b). We also calculated K_a/K_s ratios for *P5CS1* and *P5CS2* genes between monocots (M1 and M2) and dicots (D1 and D2) groups and within each group. The average K_a/K_s was 0.109 for D1×M1, 0.114 for D2×M2, 0.237 for M1×M2 and 0.098 for D1×D2 comparisons. Nevertheless, high peaks of K_a/K_s rates were observed along the sequences, with maximum values of 34.02, 4.93, 21.5 and 7.45 in the D1×M1, D2×M2, M1×M2 and D1×D2 comparisons, respectively (Fig. 6b, c).

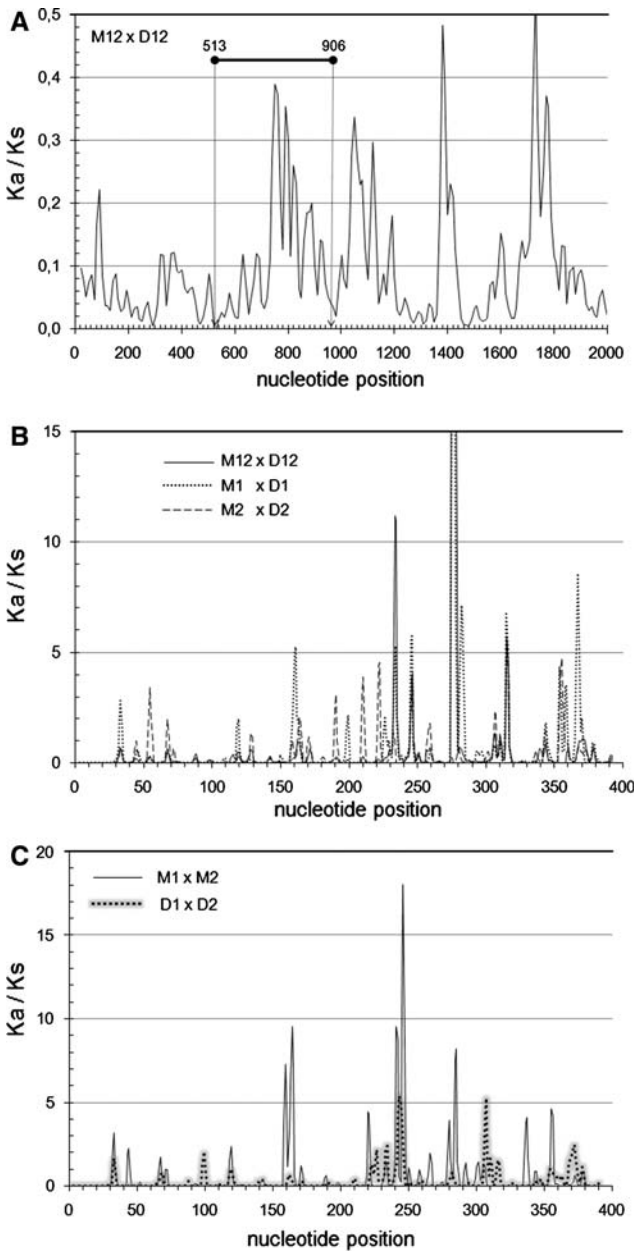

Fig. 6 Estimation of K_a/K_s rates on *P5CS* sequences. **a** Substitution rates between full-length sequences of monocots (M12) and dicots (D12). The region corresponding to the partial sequences of cloned *P5CS* from Neotropical trees is indicated. **b** Simultaneous comparison of both copies of *P5CS* from mono and dicots (M12×D12) and independent comparison of *P5CS1* (M1×D1) and *P5CS2* (M2×D2) sequences from the partial sequences of exons 5 and 6. **c** Independent comparison among monocot duplicated genes (M1×M2) and among dicots (D1×D2)

Discussion

In plants, key enzymes of metabolic pathways are generally encoded by redundant genes, which may be generated by gene duplication events during the evolutionary history of the organisms. In principle, gene duplication produces two functionally identical copies that act in a totally redundant way immediately following the duplication event. Afterward, gene duplications are often followed by sequence alterations causing changes in transcriptional regulation and contributing to evolution of functional divergence (Briggs et al. 2006). The rate-limiting step in proline synthesis is controlled by a bifunctional P5CS enzyme, which is encoded by two highly homologous genes in Arabidopsis and many other plants (Strizhov et al. 1997; Yoshiba et al. 1997; Fujita et al. 1998; Ginzberg et al. 1998).

To trace back plant *P5CS* encoding gene history, we have analyzed the P5CS nucleotide and amino acids sequences from different plant species and constructed phylogenetic trees. Our results suggest that independent duplication events have occurred throughout evolutionary history. The *A. thaliana* P5CS1 presents 86% amino acid identity with the protein sequence codified by *A. thaliana* P5CS2 gene, 96% with *Brassica napus* and 93% with P5CS from *Corispora bungeana.* The topologies observed in interspecific phylogenetic trees clearly show the existence of two groups, separating *P5CS* genes of monocots from

dicots. In addition, *P5CS* sequences corresponding to members of the order Brassicales formed a single-cluster inside the dicot group, with two internal branches independently harboring representatives of *P5CS1* or *P5CS2*. This result shows that this duplication event occurred after divergence of monocots and dicots lineages, but has also occurred later in the Brassicales.

The two *P5CS* genes found in *S. parahyba*, grouped with their correlated genes from other species of the Fabales. However, in Fabales *P5CS1* and *P5CS2* did not form a single-cluster, as observed for Brassicales. In Asterales it is found in the same clustering profile as in Fabales, indicating that at least for the Asterales, Fabales and evolutionarily related orders, the duplication of *P5CS* gene occurred early and was already present in the common ancestor that originated these orders (Fig. 7).

Among monocots, it is clear that all P5CS1 clustered together and form a separate group from P5CS2. *Osa*P5CS1, for example, has higher identity with *Triticum aestivum* and *Zea mays* (86 and 84%, respectively) than with its rice paralog, *Osat*P5CS2 (77%). The phylogenetic trees undoubtedly show the separation of the two copies of the *P5CS* gene in different clusters inside the Poales order. Therefore, duplication events occurred after monocot and dicot divergence, and seem to have occurred several times throughout evolution. These events have occurred at different frequencies in flowering plants since the divergence of monocots and dicots.

To understand enzyme evolution, it is crucial to take into account two major mechanisms namely, duplication and fusion, occurring at the gene level. In the evolutionary history of *P5CS* genes both mechanisms seem to have played an important role in the establishment of the current protein architecture. Comparing the domains γGK and γGPR in different species with *E. coli,* the *pro*A and *pro*B genes showed higher variation in γGK than in the γGPR domain. The amino-terminal domain of the P5CS protein of *V. aconitifolia* for example, showed 33.3% identity and 55.3% overall similarity to the *E. coli* proB protein while a domain with 35.7% identity and 57.9% similarity to the proA protein is located at the carboxyl end. An unexpectedly high level (42.4%) of sequence similarity was found between the *E. coli pro*B and *pro*A proteins, suggesting that they may have arisen by duplication of single common ancestral gene. The encoded proteins may have later acquired structural features conferring the respective kinase and reductase activities of the present-day enzymes. It has been proposed that the corresponding plant genes may have fused and originated the bifunctional enzyme present in plant genomes (Hu et al. 1992). A similar event of domain fusion must have occurred in animal systems since P5CS activity has been detected in mammalian cells and a single-gene encodes both functional enzymatic activities. Existence of two catalytic domains in the plant P5CS enzyme may facilitate sequential reactions in formation of γ-glutamyl phosphate, which is unstable, and its rapid conversion into GSA. In prokaryotes, as in *E. coli*, both γGK and γGPR are organized as hexameric enzymes (Hu et al. 1992).

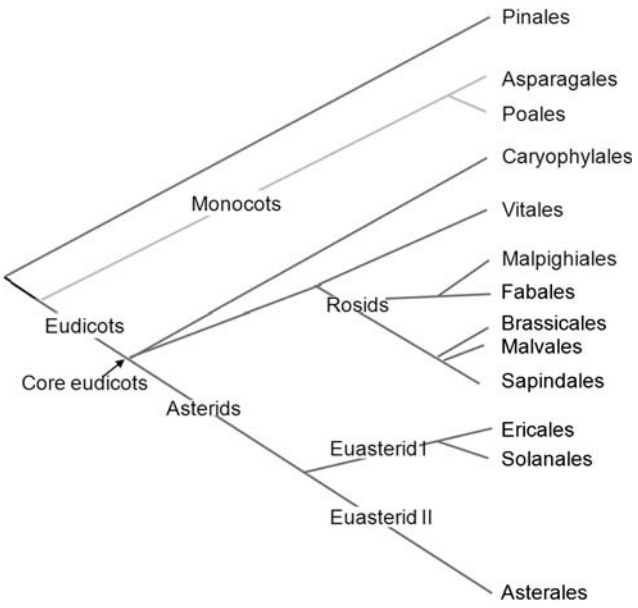

Fig. 7 Partial representation of the angiosperm phylogenetic tree with all taxonomic orders of plant used in this study (adapted from the Angiosperm Phylogenetic Group, AGP)

It has been shown that transcriptional control of the *P5CS* gene is important for the regulation of accumulation of proline during osmotic stress in plants. Previous studies demonstrated significant differences in transcriptional control of *P5CS* genes in arabidopsis (Szekely et al. 2008). Transcription of the *P5CS1* gene is inducible by salt, drought, glucose and sucrose treatments in an abscisic acid (ABA) and light-dependent fashion (Strizhov et al. 1997; Abraham et al. 2003). Steady-state *P5CS1* transcript levels are higher in most plant organs when compared to those of *P5CS2*. The latter appears to be preferentially expressed in dividing cells and in response to incompatible pathogenic interactions (Strizhov et al. 1997; Fabro et al. 2004). The analysis of arabidopsis *P5CS1* and *P5CS2* T-DNA insertion mutants allowed the characterization of functional diversification of duplicated *P5CS* genes in the developmental and stress-dependent control of proline biosynthesis. Knockout mutations of *P5CS1* resulted in the reduction of stress-induced proline synthesis, hypersensitivity to salt stress, and accumulation of reactive oxygen species. On the other hand, *P5CS2* knockout produced embryo abortion. The diversification of P5CS functions in arabidopsis reflects differences not only at the level of transcriptional regulation but also in cell-type-specific and subcellular localization of P5CS enzymes. Cellular localization studies with P5CS-GFP gene fusions indicate that P5CS1 is sequestered into

subcellular bodies in embryonic cells, while P5CS2 remains in the cytosol. In addition, although proline feeding rescues the viability of mutant embryos, *P5CS2* mutant seedlings grown in the presence of proline were not able to develop normally and fail to produce fertile plants. Promoter analysis in seedlings demonstrated that a specific expression of P5CS2-GFP occurs in leaf primordia where P5CS1-GFP levels are very low. P5CS2-GFP shows a distinct cell-type-specific and subcellular localization pattern when compared to P5CS1-GFP in root tips, leaves and flower organs. These data demonstrate that at least in *Arabidopsis* P5CS enzymes perform non-redundant functions (Szekely et al. 2008). Duplicate *P5CS* genes with differences in transcriptional regulation are also present in other plant species such as alfalfa, tomato and cactus (Fujita et al. 1998; Ginzberg et al. 1998; Farzaneh et al. 2005; Silva-Ortega et al. 2008).

It was demonstrated that juvenile plants of *S. parahyba*, subjected to two cycles of water stress in greenhouse conditions, have an increase in total soluble sugars, K^+, amino acids and proline in the leaf tissues (Carvalho 2005). *S. parahyba* had a constitutive expression of both P5CS genes in the absence of stress (Fig. 3), suggesting that this gene may be related to the adaptation and plasticity to the different environmental conditions this tree is confronted with across its wide distribution range from Mexico to Southern regions of the Atlantic Forest.

The nucleotide substitutions that occur during molecular evolution may be synonymous, which do not change the amino acid residue or non-synonymous, which result in amino acid replacement. Most non-synonymous substitutions are typically eliminated by purifying selection, which leads to a predominance of synonymous substitutions. When positive Darwinian selection occurs, non-synonymous substitutions become more frequent. Thus, the relative rates of synonymous and non-synonymous substitutions are good indicators of the amount and type of selection affecting a gene (Sharp 1997). Given K_s, the number of synonymous nucleotide substitutions per synonymous site, and K_a, the number of non-synonymous nucleotide substitutions per non-synonymous site, then the ratio K_a/K_s can be used to quantify the degree of divergence between species and to estimate the type of selection exerted on a given gene or DNA fragment. All comparisons of duplicated P5CS genes among the different species of monocots and dicots showed that positive selection has taken place at multiple locations. Duplications and positive selection could be associated with exposure of ancestral plants to more stressful habitat conditions, with the requirement of an extra copy of *P5CS* to fulfill the physiological roles of the enzyme and better regulate the proline metabolism. A more detailed approach using site directed mutagenesis on P5CS associated with phenotype analysis of mutants will be required to evaluate the extent of positive selection and to understand how selective pressure affects different copies of duplicated P5CS genes within species and among taxonomically divergent groups, and to try to determine why these duplication events occurred.

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CAPÍTULO III: Molecular systematics and evolutionary history of Neotropical tree *Schizolobium* (Fabaceae-Caesalpinioideae) inferred from nuclear and chloroplast DNA sequences.

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ABSTRACT

The Amazon and Atlantic forests are the major and more biodiverse rain forests of South America. *Schizolobium* (Caesalpinioideae) is a widespread genus found in these forests and is a fast growing tree, extensively used in reforestation that employs native trees. Based on morphological data is comprised of a single species: *Schizolobium parahyba* (Vell.) Blake, with two varieties of disjunct distribution. This study represents the first attempt to reconstruct the phylogenetic relationships within the *Schizolobium* and provides information about the evolutionary process between these forests. Our study is based on DNA sequence data from nuclear and plastid regions that were generated for 165 individuals representing the two varieties. The Maximum Parsimony and Bayesian analyses revealed the monophyletic nature of *Schizolobium* and the *Schizolobium* crown node was estimated to have arisen 15.6 million years ago. The two varieties showed high genetic differentiation, however a high genetic divergence was observed in the central portion of the Atlantic Forest biome, relative to southern areas, indicating an important area of endemism influenced by the Amazonian region. The results described here provide additional genetic information concerning the Neotropical forest species and will aid in setting conservation priorities in these regions.

Keywords: *Schizolobium,* molecular dating, cpDNA and nuclear marker, Neotropical forest, conservation genetic

1. Introduction

The Neotropics are one of the world's most species-rich regions, containing an estimated 90,000 plant species, more than any other continental area. However, these regions are notoriously endangered and understudied (Thomas, 1999). Because of their large biodiversity, these ecosystems are ideal targets for research into the origin of biological diversity (Pennington *et al.*, 2004). Some Neotropical areas are under manifest danger of biodiversity loss, and have thus been identified as biodiversity hotspots that are the focus of special conservation programs (Myers, 2003; Myers et al., 2000).

The Amazon and Atlantic forests are the major rain forests of South America, and together they encompass the most diverse tropical forests in the world. Between these two forests lies a corridor containing seasonal and open vegetation. This region, which is considered to be an important restraint to species migration between the two rain forest regions, includes the Cerrado in central Brazil, the Caatinga in north eastern Brazil, and the Chaco in Argentina and Paraguay (Prado and Gibbs, 1993). Despite their abundance, there are little data about genetic diversity, gene flow and evolution of tropical tree species in these regions of the world. In South America, the area occupied by tropical rainforest is believed to have shrunk between 100,000 and 20,000 years ago, during the Pleistocene, as a consequence of climatic changes. This has led to hypotheses of recent speciation, the most popular of which is the 'refuge' model that involves allopatric differentiation in populations of rainforest species that became isolated from each another by vegetation adapted to more xeric conditions during cool–dry periods (Prance, 1982). In general, pollen data suggest that the last glacial period was cooler and drier than present-day conditions, resulting in an

extension of savannah vegetation and a reduction in rain forest size. In addition, studies indicate past floristic connections between the Amazonian and Atlantic rain forest areas (Behling, 1998, 2002; Behling et al., 2000; Behling and Lichte, 1997).

Speciation and the differentiation of genealogical lineages are important considerations in biogeography, as they define the primary units for conservation and influence the spatial resolution of an evolutionary analysis that can provide a deep understanding of evolutionary processes. In addition, biodiversity assessments require that investigators employ stringent and rigorous methods to delimit natural species (Marshall *et al.*, 2006). The definition of species has been a major impediment for botanical studies related to speciation. Botanists have often expressed doubt that plant species even exist, based on the frequent reports of inter-specific hybrids (Arnold, 1997). These concerns were amplified by claims that gene flow within many plant species is so low that populations, rather than species, are the most inclusive reproductive units for genetic analysis (Ehrlich and Raven, 1969).

The empirical issue of species delimitation is receiving increased attention, and several novel methods have been proposed for delimiting species with a statistically precise framework (Puorto et al., 2001; Templeton, 2001; Wiens and Servedio, 2000). Non-tree-based methods delimit species on the basis of gene flow assessments (Sites and Marshall, 2003), while tree-based methods delimit species as historical lineages (Goldstein and DeSalle, 2000). However, differences in datasets and methods for delimiting species may produce ambiguous or conflicting results, due to the action of multiple evolutionary processes operating within and between populations across varying spatial-temporal scales (Sites and Marshall, 2004).

Resolution of correct systematic relationships is essential to evolutionary, biogeographic and ecological studies. Identification of these relationships enables the identification of taxonomic units, the evaluation of their divergence patterns, and allows for comparison of diversity across their ranges. However, the presence of cryptic taxa has confused many traditional studies, since they may appear morphologically similar yet be phylogenetically distinct. The use of systematic molecular techniques in studies of angiosperm evolution has resulted in the production of numerous phylogenies describing relationships across a range of evolutionary history (Soltis and Soltis, 2000). These studies are particularly important for filling in the tips of the angiosperm tree of life (Palmer et al., 2004). Phylogenetic hypotheses of the evolutionary relationships among members of the same genus provide a framework for comparative research on mechanisms of diversification and speciation (Barraclough and Nee, 2001). Molecular systematics is also a valuable technique for improving conservation, as it provides an objective means of quantifying evolutionary distinctiveness and resolving taxonomic ambiguities. There is great interest the application of molecular systematics for identification of lineages that are sufficiently distinct to warrant taxonomic status, and thus eligible for legal protection (i.e., species, subspecies, and varieties).

Species are often assumed to have evolved from a common ancestor by a process of branching followed by complete genetic isolation. Inter-specific hybridization is one of the major factors that leads to phylogenetic incongruence among loci. This is especially true for plastid and nuclear genomes with a maternal or bi-parental mode of transmission (Rieseberg et al., 1991; Rieseberg and Soltis, 1991; Soltis et al., 1991a; Soltis et al., 1991b). Species may hybridize long after speciation, resulting in problems for phylogenetic reconstruction, especially when molecular data are used

for the analysis (Spence, 1990). The discovery of cytoplasmic introgression and the lack of concordance between rDNA and cpDNA phylogenies for several plant groups reflect past hybridization and subsequent introgression (Rieseberg et al., 1996). Therefore, to avoid erroneous phylogenetic conclusions based on cpDNA data, comparisons with phylogenetic hypotheses based on nuclear gene sequences, as well as comprehensive sampling, is required (Rieseberg and Soltis, 1991).

The traditionally circumscribed Caesalpinioideae subfamily is paraphyletic and is comprised of approximately 2,250 species in 171 genera that are subdivided into four tribes: Caesalpinieae, Cassieae, Cercideae and Detarieae (Bruneau and Graham, 2008; Lewis et al., 2005). *Schizolobium* Vogel (Caesalpinieae) is a member of the *Peltophorum* group (Haston et al., 2003) and is an ecologically and economically important forest tree genera. It is largely distributed through the Americas (Atlantic Forest, Amazonian rainforest and other tropical forests of Central America) and shows good adaptation to variable climate conditions. Thus, it has great potential for wood production and other uses. It is one of the fastest growing tree species, which explains its extensive use in reforestation projects based on native trees. Furthermore, it has also been widely introduced in the tropics as an ornamental tree.

Until 1996, two species of *Schizolobium* were recognized. Collections from the Brazilian Atlantic rain forest were referred to as *S. parahyba* (Vell.) Blake, while most collections from equatorial South America and Central America were classified as *S. amazonicum* Huber ex. Ducke (Ducke, 1949). Based on morphological characteristics, Barneby (1996) postulated that the characteristics that classified *Schizolobium* into two species had become increasingly homogeneous and could not justify the existence of two species of *Schizolobium*. Therefore, Barneby (1996)

proposed that the genus *Schizolobium* should consist of only one species with two varieties: *S. parahyba* var. *parahyba* (pedicels not jointed; anthers 2.3-3.2 mm) and var. *amazonicum* (Ducke) Barneby (pedicels jointed 2-6.5 mm above base; anthers (1.2)1.3-2.3 mm) (Barneby, 1996). Differentiation of this species into two varieties is also supported by a disjoint distribution. The varietie *parahyba* is found in Brazil, from Bahia to Santa Catarina, while the varietie *amazonicum* is distributed from the Amazon Basin in Brazil and Bolivia to south-eastern Mexico (Fig. 1A). Some plant species show a geographically disjointed distribution, with the plants present in two or more remote geographical areas (Bessega et al., 2006; Ickert-Bond and Wen, 2006; Rossi et al., 2009; Singliarova et al., 2008). Processes such as ancient tectonic events (continental drift resulting in intercontinental splits in the species' range), smaller scale disjunctions that could be attributed to more recent climate change and long distance dispersal may lead to the formation of these disjoint distributions (Raven and Axelrod, 1974).

Presently, a restricted number of studies related to *Schizolobium* have focused on molecular markers. Microsatellite loci have been isolated (Kamau et al., 2003), an evolutionary analysis of the enzyme pyrroline-5-carboxylate synthase (P5CS) has been performed in *S. parahyba* and other Neotropical trees (Turchetto-Zolet et al., 2008), an analysis of the genetic structure of *Schizolobium parahyba* from the state of Rio de Janeiro (Brazil) using RAPD markers has been conduced (Freire *et al.*, 2007), and the genetic relationships among ecotypes of *Schizolobium parahyba* from Ecuador and others countries were investigated using RAPD, AFLP and SSRs (Canchignia-Martínes *et al.*, 2007).

The taxonomy of the genus *Schizolobium* has traditionally been based on morphological data. A molecular phylogenetic analysis of individuals of the two

varieties has not yet been performed. A study of the *Peltophorum* group, based on the chloroplast *trnL*, *trnL-F*, *rbcL* and *rps16* loci, was performed with individual samples of *Schizolobium* from both geographical areas (Ecuador, Mexico and Brazil) and revealed that these populations form a single clade (Haston et al., 2003, 2005).

This study presents the first extensive molecular phylogenetic analysis within the genus *Schizolobium*, and includes a widespread sampling of populations of both varieties of this species from throughout their geographic distribution. The goal of this study was (i) to investigate the evolution and origin of the genus *Schizolobium*; (ii) to verify the existence of two varieties, currently defined by morphological characteristics and presenting a disjoint geographical distribution; (iii) to estimate the age of the *Schizolobium* crown node; and (iv) to provide insights useful for conservation of *Schizolobium*. ITS (Internal transcribed spacer) nuclear markers and a set of chloroplast DNA region (cpDNA) were analyzed to reach these objectives. The monophyletic nature of the *Schizolobium* genus, its position within the *Peltophorum* clade, and molecular age estimations were also investigated in relation to other genera of the subfamily Caesalpinioideae.

2. Materials and methods

2.1. Sampling strategy

Samples from individuals of the *Schizolobium* genus were collected from fourteen sites, separated by 218 to 6,642 Km (see Table.1 in Supplementary Data, available online), covering most of the geographical range of both varieties of *Schizolobium* (Fig. 1B). The samples were collected as silica gel dried cambium and

leaf from natural populations. Voucher specimens were deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (UFRGS), in the Department of Botany. In most cases, more than one individual was sampled per population (Table1).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1987). The 5.8S nrDNA and the flanking ITS1 and ITS2 regions were PCR amplified using the ITS1 forward primer 5'GGAAGTAAAAGTCGTAACAAGG-3' and the ITS2 reverse primer 5'TCCTCCTCCGCTTATTGATATGC-3' (Table1) (White et al., 1990). To screen for variation in plastid DNA, ten regions were investigated using universal primer combinations: the *psbA-trnH* and *trnL-trnF* spacers, *rpoC1* and *rpoB* RNA Polymerase beta subunit, *accD* acetyl-CoA carboxylase beta subunit, *ndhj* NADH dehydrogenase j, *matK* gene, *ycf5* cytocrome c biogenesis protein, *rbcL-a* ribulose biphosphate carboxylase and *trnL* intron. These regions were chosen from studies describing putative markers used for barcoding in plants (Kress and Erickson, 2007; Newmaster et al., 2006; Shaw et al., 2007; Taberlet et al., 2007). Sequences of *matK* primers are based on (Wojciechowski et al., 2004)

All primer sequences used for PCR amplification and sequencing, as well as the expected sizes of the resulting fragments are described in Table1. All amplifications were conducted using 10 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1x PCR buffer, 0.05 U of Platinum Taq DNA polymerase (Invitrogen) and 5 µM of each primer, in a final volume of 20 µl. The PCR cycle used an initial hot-start step at 94 °C for 5 min, followed by 40 cycles with denaturation at 94 ^{o}C for 50 s, an annealing temperature of 48ºC (ITS1F-ITS2R, *psbA-trnH, trnL-F*, *trnL intron*) or 51ºC (*rpoC1, rpoB, accD, ndhj, ycf5, rbcL-a, matK*) for 50 s, and 50 s of elongation at 72 ºC.

All PCR products were visualized by electrophoresis on 1.5 % agarose gels stained with SYBR Gold (Invitrogen) and precipitated using 3 M Sodium Acetate and 95% ethanol.

Nuclear and plastid amplified PCR products were sequenced with the dideoxy chain-termination method using Big-Dye (Applied Biosystems) on an ABI-3100 automatic sequencer (Applied Biosystems). Sequencing was conducted in 10µL reactions with 2 µL of purified PCR product, 1 µL of primer, 2 µL of buffer 5x, 0.25 µL of Big-Dye®Terminator and 4.75 µL of milliQ water. The thermocycling program was as follows: 25 cycles of 10 s at 95 ºC, 5 s at 54 ºC and 4 min at 60 ºC. Both DNA strands were fully sequenced. Sequencing of approximately 1500 bp of the *matK* gene was performed using four additional internal primers: matK4La (forward) CCTTCGATACTGGGTGAAAGAT (500 bp); matK1100L (forward) TTCAGTGGTACGGAGTCAAATG (1100 bp); matK832R (reverse) TTGCATAGAAATGGATTCGCTCAAA (700 bp) matK1932Ra (reverse) CCAGACCGGCTTACTAATGGG (1200 bp) (Wojciechowski et al., 2004).

2.3. Editing, alignment and phylogenetic analyses

Sequences were individually checked by eye and identities were certified using the BLASTn algorithm against plant DNA sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were aligned using the Alignment Explorer/CLUSTALW Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al.*, 2007). Sequences of *Schizolobium* generated in this study were deposited in GenBank under the accession numbers FJ668603-FJ668631 and GQ167768-GQ167774. Individuals from the same population with identical sequences were represented in the subsequent analyses by only one sample.

Maximum Parsimony (MP) and Bayesian analyses of the ITS and cpDNA datasets were performed in order to access the relationships within *Schizolobium*. Parsimony analyses were conducted using Nona (Goloboff, 1993), spawned from Winclada (Nixon, 2001). Tree searches employed 1000 replicates, holding 10 trees in each search, using the tree bisection and reconnection (TBR) strategy and branch swapping. Strict consensus bootstrap percentages were computed with 1000 replicates and 10 tree bisection reconnection, holding 10 trees per replicate. For the Bayesian analysis, the datasets were analyzed using the BEAST version 1.4.8 (Drummond and Rambaut, 2007) software. Although BEAST was developed primarily to provide age estimates for a phylogeny, in this case the program also was used only to provide an estimate of the topology independent from the parsimony analysis, and no time calibration was used. Therefore, a strict molecular clock was used, with a substitution rate set to one, in combination with the GTR+I+Γ model of nucleotide substitution.

Sequences of three closely related taxa belonging to the *Peltophorum* group were used as outgroups. For the ITS analysis, unpublished sequences of *Colvillea racemosa* LWDS2147, *Conzattia multiflora* DURJ600 and *Heteroflorum* sp. CEH1845 were provided by Elspeth Haston. For the combined cpDNA analysis, *matK* and *trnL-trnF* sequences of outgroups were downloaded from GenBank: *C. racemosa* (EU361916, AY899739), *C. multiflora* (AY386918, AY899729), and *Peltophorum pterocarpum* (EU362023, AY899713). The three other partitions (*psbA-trnH*, *rpoC1*, *rpoB*) were coded as missing values for the outgroups in the cpDNA analysis, as no sequences were available in GenBank.

2.4. Dating analysis

Divergence time estimation for *Schizolobium* was based on a higher level analysis of a recently published large dataset of the Caesalpinoid legumes (Bruneau and Graham, 2008). Our dataset was constructed using the alignment of 258 *matK* sequences from the Bruneau et al. (2008) work and incorporating new sequences for 21 accessions of *Schizolobium*, comprising a concatenated plastid dataset that included the *matK* gene, and also other five plastid regions (*psbA-trnH*, *trnL-trnF*, *rpoB*, *rpoC1*) that were added to improve resolution The final aligned matrix contained 279 terminals with 3513 sites.

A molecular dating analysis was performed with the program BEAST, using the uncorrelated lognormal relaxed clock and a general time reversible model, with invariable sites and among-site rate heterogeneity (GTR+I+Γ) as the model for nucleotide substitution (as selected in Bruneau et al. (2008). Calibration points were provided by a set of legume fossils, as described in Bruneau et al. (2008), with the alteration that we excluded a few fossils in our analysis. Specifically, fossil P, which was considered redundant as another calibration point of a deeper node is constrained with the same minimum and fossils B and S, for which placement was considered problematic. Therefore, 15 fossils were used to calibrate nodes in our analysis. These calibration points were provided as minimum constraints (log-normal statistic distribution prior with mean = 0 and std =1, in BEAST). In addition, a prior with uniform distribution to the legume stem node was imposed, allowing it to vary between 60 to 70 million years ago (Mya), as employed in previous studies (Bruneau and Graham, 2008; Lewis et al., 2005). Four independent runs of 10^7 generations each were performed, sampling every 1000 generations. After the exclusion of 10^6 generations (burn-in), the results of all runs were combined. Results were determined to have

reached stationary state and convergence using Tracer version 1.4 (Drummond and Rambaut, 2007) and data from multiple runs were combined after exclusion of burn-in trees, as appropriate. The estimated parameters had sufficient effective sample sizes (ESS), in most cases well above the minimum of 200 recommended in the BEAST manual.

3. Results

3.1. Characteristics of cpDNA and ITS sequences

Nuclear and chloroplast DNA regions were successfully amplified from individuals of the *Schizolobium* genus (Table 1). Among the ten loci analyzed, six were polymorphic: ITS, *psbA-trnH*, *trnL-trnF*, *rpoB*, *rpoC1* and *matK*. The chloroplast regions corresponding to *accD*, *ndhJ*, *ycf5*, *rbcL-a* and the *trnL intron* did not show any variation among the samples analyzed, and were not used in further analysis.

The *psbA–trnH* intergenic spacer presented considerable nucleotide polymorphism between individuals of *Schizolobium.* A micro-inversion, characterized by a reverse-repeated region of 35 bp flanked by a 14 bp palindromic sequence, was one conserved polymorphism detected between the two varieties in the *psbA–trnH* region. However, individuals from Bahia (BA1 and BA2) populations, which are morphologically and geographically related to var. *parahyba*, showed the same micro-inversion pattern present in the samples of var. *amazonicum*. The *psbA-trnH* spacer also contained duplication and indel events (Fig. 2). The *trnL-trnF* intergenic spacer, *rpoB* and *rpoC1* chloroplast genes presented uniform lengths of 448, 473 and 500 bp in all samples analyzed, respectively. One polymorphic site, which also discriminated the two

varieties of *Schizolobium* (except the samples from Bahia, which grouped with individuals of var. *amazonicum*, as found in the *psbA-trnH* dataset), was found in each of these regions (Table 1). Similar polymorphisms to those observed for the *psbA–trnH* intergenic spacer were identified along the 1500 bp analyzed of the *matK* gene. However, they were much more pronounced, with 12 single nucleotide polymorphism sites (SNPs) among the sampled individuals. In contrast, no indels were found in *matK* (Table1). However, the pattern of sequence variation observed in *matK* did not distinguish between the two varieties of *Schizolobium*, as was observed for the other loci.

The amplification of the ITS region from all samples resulted in a clear single band when checked on 1.5% agarose gels (data not shown). The complete ITS region (ITS1+5.8S+ITS2) was 625 bp in length, including 227 bp for ITS1 and 181 bp for ITS2. There were no insertions or deletions, and within the 625 bp alignment, 12 positions were variable. Ten of these variable positions were related to the differentiation between the two varieties.

3.2. Phylogenetic and molecular dating analyses

The aligned ITS matrix contains 30 sequences, 27 from *Schizolobium* and 3 outgroups (species from Peltophorum group), with 561 sites analyzed and 122 excluded from the analysis because of problems in alignment. Multiple heuristic searches of 42 parsimony informative nucleotide characters of the ITS region discovered a single most parsimonious tree of 62 steps (CI = 0.87, RI = 0.94) (Fig. 3). The tree topology generated from Bayesian inference (not shown) is congruent with the tree generated by the parsimony analysis. Thus, only the posterior probabilities (PP) are included on the most parsimonious tree, together with the bootstrap percentages (BP)

(Fig. 3). The concatenated plastid dataset (*matK, psbA-trnH, trnL-trnF, rpoC1, rpoB*) alignment contains 28 sequences, 25 from *Schizolobium* and 3 outgroups (species from Peltophorum group), with 54 parsimony informative characters. Maximum parsimony analysis of the cpDNA from *Schizolobium* discovered three most parsimonious trees of 65 steps each (CI = 0.84, RI = 0.96) (Fig. 4). As shown in the ITS analysis, the tree topology generated by Bayesian inference (not shown) was congruent with the tree generated by parsimony analysis. Thus, only the posterior probabilities (PP) are included on the most parsimonious tree, together with the bootstrap percentages (BP) (Fig. 4).

The Maximum parsimony and Bayesian trees for the ITS and cpDNA regions revealed a monophyletic clade of *Schizolobium*, well supported by bootstrap and posterior probability values of 100% and 1.0, respectively (Fig. 3 and 4). The phylogenetic analysis based on the ITS sequences supports the monophyletic nature of both varieties within *Schizolobium*, with high support values in both parsimony and Bayesian analyses (Fig. 3).

The monophyly of *Schizolobium* was also well supported by the Bayesian analysis of the cpDNA sequences, although the sister group relationship remains unclear (PP=0.58). The age of the *Schizolobium* crown node was estimated to be 12.2 million years (My), with a 95% confidence interval of 5.8-19.5 My (see Fig. 1 in Supplementary Data, available online). Because of the pattern of polymorphism found in the *matK* gene, it was not possible to estimate the age of the divergence of the two varieties of *Schizolobium*. In an attempt to estimate this divergence time, we used the alignment of 258 *matK* legume sequences produced by Bruneau et al. (2008), in combination with a full chloroplast dataset of *Schizolobium* containing five plastid

regions (*psbA-trnH*, *trnL-trnF*, *rpoB*, *rpoC1* and *matK*) that were added to improve resolution at the intra-specific level. In this analysis, the estimated age for the *parahyba* clade was 3.1 My, with a 95% confidence interval of 1.2-5.4 My (see Fig. 1 in Supplementary Data, available online). This clade includes all populations from the Atlantic Forest, except those populations from Bahia (BA01 and BA2). The downside of this analysis is that most taxa (all except *Schizolobium*) were missing a substantial amount of data, which could affect divergence time estimation.

4. Discussion

4.1. Genetic differentiation of Schizolobium

This study represents the first effort to establish the phylogenetic relationships within the *Schizolobium* genus. The current taxonomy classifies *Schizolobium* as a single species containing two varieties. Until now, however, this classification had not been tested using molecular data. *Schizolobium* is a genus with a large distribution in the Neotropics. *S. parahyba* is an important Neotropical tree and the study of its evolution and biogeographical patterns is critical for aiding in genetic conservation in these rainforests. We have analyzed ten plastid regions and the internal transcribed spacer (ITS) of *Schizolobium*, covering approximately 5,300 bp.

The ITS sequences showed a high level of polymorphism, and the topology identified by the phylogenetic analysis suggests two segregated varieties, based on geographic distribution and morphological characters (Barneby, 1996). The individuals of var. *parahyba* (from Atlantic Forest) are separated from var. *amazonicum* (from Amazonian rain Forest and Central America) by 10 SNPs along the ITS sequence (Table 1).

Five of the analyzed chloroplast regions (*trnL-F, psbA-trnH, rpoC1, rpoB* and *matK*) contained polymorphisms. The 12 single nucleotide polymorphism sites in the *matK* gene did not correlate with different geographical distributions or morphological characteristics between the two varieties. In contrast, the other cpDNA loci (*trnL-F, rpoC1, rpoB*) showed variations that separate the two varieties. Curiously, the populations from Bahia (BA1 and BA2), which are morphologically classified as var. *parahyba*, have similar sequence patterns to *var. amazonicum*. The micro-inversion found in the *psbA-trnH* intergenic spacer also separated the two varieties, but the individuals from the BA1 and BA2 populations showed similar sequence to *var. amazonicum*. In addition to the micro-inversion, other variations were found in the *psbA-trnH* sequences. However, these differences did not correspond with the classification of the varieties. The populations from BA showed a higher divergence of cpDNA sequences, compared to samples from the other populations (Fig. 2).

Some physiological aspects also contribute to discrimination of the varieties of *Schizolobium*. Indeed, *S. parahyba var. parahyba* and var. *amazonicum* plants respond equally to water deficient conditions in juvenile plants by increasing total soluble amino acids, proline, total soluble sugars and K^+ content of the leaf tissues and by lowering osmotic potential values. However, the analysis of the pressure-volume curve results suggests that the varieties respond differently to water stress, and that individuals of var. *amazonicum* present a greater capacity for osmotic adaptation than individuals of var. *parahyba* (de Carvalho, 2005).

Phylogenetic and Molecular data analysis

The results of the phylogenetic analysis of the cpDNA and ITS sequences confirmed the monophyly of *Schizolobium* and the genus position within the

Peltophorum clade (Fig. 3, 4 and also Fig. 1 in Supplementary Data available online). These results are in agreement with previous work that observed the formation of one clade in *Schizolobium*, and classified it within the *Peltophorum* clade (Haston et al., 2003, 2005).

Interestingly, we observed incongruence between cpDNA and nrITS phylogenetic analyses with respect to classification of the two varieties (Fig. 3 and Fig. 4). The phylogeny based on nuclear marker (ITS) sequence showed the formation of two clades: one included the populations of varietie *parahyba* from the Atlantic Forest (Santa Catarina to Bahia) and the other included populations from the Amazon and Central American rain forests (Fig. 3). The results from this marker suggest that the two varieties are genetically distinct and are possibly in the process of speciation. However, this pattern was not observed with the cpDNA markers. In this case, we observed individuals of var. *parahyba* grouped with those of var. *amazonicum* (Fig. 4). In many instances, this lack of congruence between cpDNA and nuclear phylogenies, and this type of cyto-nuclear disequilibrium, can be explained by reticulate evolution. However, a very complex and species-level phylogeny will be needed to detail the evolutionary patterns within the *Schizolobium* taxa. This incongruence is especially evident in plastid and nuclear genomes with maternal or bi-parental modes of transmission. Some cases of incongruence may have resulted from differential lineage sorting of ancestral polymorphisms in chloroplast and nuclear genes (Comes and Abbott, 2001), as well as from evolutionary convergence (Davis *et al.*, 1998). The most extreme case is chloroplast capture, where the cytoplasm of one species is replaced by that of another species through hybridization/introgression, a process that can occur at a variety of taxonomic levels (Rieseberg and Soltis, 1991). As a result of these phenomena,

clustering taxa on the basis of chloroplast DNA often does not correspond to taxonomic units or to groups supported by analysis of morphological characteristics or groups indicated by nuclear markers (Soltis et al., 1995). In spite of the considerable amount of genetic information available concerning the evolutionary history of *Schizolobium* that are now available, we are still unable to fully understand the processes of speciation in this taxon. Therefore, a phylogeographic analysis, including a wide sampling, could help to understand the diversification patterns and the origin place of this important Neotropical tree, especially with regard to cpDNA patterns found in the northeast of the Atlantic Forest.

The cpDNA and nrITS sequences used in this phylogenetic analysis were based on previous DNA barcode studies (Edwards et al., 2008; Erickson et al., 2008; Kress and Erickson, 2007; Kress et al., 2005; Lahaye et al., 2008; Little and Stevenson, 2007; Little et al., 2008; Newmaster et al., 2006; Newmaster et al., 2008; Nielsen and Matz, 2006; Taberlet et al., 2007). DNA barcoding has been proposed as a means for species identification and has contributed to a wide range of ecological and conservation studies in which traditional taxonomic identification is not practical. Based on our results, it is important to stress that differentiation between var. *parahyba* and var. *amazonicum* was possible after nuclear ITS sequence analysis of a large number of individuals from a population. However, the cpDNA analysis showed a more complex phylogenetic relationship that did not permit differentiation between the two varieties.

Using the *matK* gene, we estimated the age of the *Schizolobium* crown node to be 12.2 My old (see Fig. 1 in Supplementary Data, available online), indicating that this species originated in the late Miocene. Through the use of the concatenate chloroplast data set, we were able to estimate the age of the *parahyba* clade

(populations from south and southeast of the Atlantic Forest) to be 3.1 My (see Fig. 1 in Supplementary Data, available online), suggesting that this clade arose in the late Pliocene. The effects of Miocene and Pliocene palaeogeographical changes on speciation are relatively well-known and are related to the building and vanishing of bridges and barriers that changed migration and isolation patterns and favoured vicariance (Coyne and Orr, 2004). Geographic gene flow barriers, such as mountain chains, rivers and others geographic characteristics, surround the main tracts of Neotropical forests and can affect the dispersal patterns of widespread species from these rain forests.

The Atlantic Forest is currently isolated from the other two largest South American forest blocks, Amazonia and the Andean Forest. However, the Atlantic Forest biota was not always isolated (Santos *et al.*, 2007). Phylogenetic studies focusing on endemic species have indicated that the evolution of the biota in the Atlantic Forest has been marked by cycles, consisting of periods in which the forest was connected to other South American forest regions, followed by periods of isolation (Prance, 1987; Prum, 1988; Rizzini, 1997; Willis, 1992). Some species have the capacity for dispersion, while others probably become isolated and, consequently, originate new species (Morrone and Crisci, 1995; Raven and Axelrod, 1972; Sanmartin et al., 2001). The mechanisms of speciation for these biotas are largely based on vicariance caused by geographical or genetic barriers.

This study sheds some light on the evolutionary history of the biota of two of the largest rainforest domains in the Neotropics: the Amazon and the Atlantic Forest. These two biomes, each harbouring a distinct flora, are currently isolated from each other by a zone of drier climates where dry forests and savannas predominate.

However, it is likely that the degree of connectivity between these two areas of rainforest may have varied throughout geological time. According to evidence from the fossil record, there are vestiges of rainforest in areas of the Northeast of Brazil, where semi-arid vegetation flourishes today. This implies that the Amazon and Atlantic forests were more strongly linked sometime in the past (Behling and Negrelle, 2001). This linkage would facilitate the interchange of species between these two biomes. In this study, it is still unclear whether the geographic distribution identified was caused by long distance dispersal or by contact between both forests followed by isolation due to climatic fluctuation. The second option seems plausible, given that the estimated time of the split between the two varieties of *Schizolobium* occurred roughly in the last 3 My. Other examples of species with disjoint distributions between the Amazon and the Atlantic forest may have been identified (Bessega et al., 2006; Colloff, 2009; Conte and Cristofolini, 2000; Gaudeul, 2006; Gomez-Zurita, 2004; Gonzales and Hamrick, 2005; Lihova et al., 2009; Orellana et al., 2009; Qian and Ricklefs, 2004; Rossi et al., 2009).

In addition, systematic molecular analysis provides information on the genetics of wide-ranging species or species groups in a continuous habitat. Our results corroborate with studies from the Atlantic forest (northeast Brazil), including the forests located north of the São Francisco River, which has been identified as an important area of endemism in South America and is influenced by the Amazonian region, making it very distinctive from other sectors of the Atlantic forest (Baker et al., 1985). In northeast Brazil, most of the Atlantic forest has been converted into agricultural land, with only 2% of the original forest remaining. In addition, the forest remnants are dispersed as small patches, surrounded by open fields. Protected areas in this region are significantly smaller than the more extensive forests in São Paulo and Southern Brazil

(da Silva and Tabarelli, 2000; Rodrigues, 2005). The results described here provide additional genetic information concerning the Neotropical forest species and will aid in setting conservation priorities in these regions.

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LEGEND OF FIGURES

Fig. 1. Native range of distribution (A) and sampled populations of *Schizolobium parahyba* (B). Circles represent var. *parahyba* and triangles var. *amazonicum*. The dotted line on the map represents the estimated limit zone of distribution between *parahyba* and *amazonicum* varieties. The populations sampled in this study showed in B are as follows: Santa Catarina (SC), Paraná (PR), São Paulo (SP1 and SP2), Rio de Janeiro (RJ1 and RJ2), Bahia (BA1 and BA2), Mato Grosso (MT), Amazonas (AM), Ecuador (EC), Colombia (CO), Costa Rica (CR) and Honduras (HO).

Fig. 2. Alignment of *psbA-trnH* spacer sequences of *S. parahyba* and *S. amazonicum* plants from different populations. The number at the left represents the number of individuals analyzed per population. The black line below the alignment represents a region corresponding to a micro-inversion.

Fig. 3. Maximum parsimony analysis of *Schizolobium* showing the single most parsimonious tree (Length = 62, steps, CI = 0.87, RI = 0.94) obtained from the analysis of ITS dataset. Bootstrap percentages after 1000 replicates are shown below and posteriori probability indicated above branches.

Fig. 4. Maximum parsimony analysis of *Schizolobium* showing the consensus of three most parsimonious tree found (Length = 65 steps, CI = 0.84, RI = 0.96) obtained from the analysis of a concatenated plastid dataset (*matK, psbA-trnH, trnL-trnF, rpoC1, rpoB*). Bootstrap percentages after 1000 replicates are shown below and posterior probability values are indicated above branches.

Table 1. Sequences of primers used for ITS and cpDNA loci amplification and sequencing, PCR expected size, sample size in each population, and number of mutations discriminating between the two varieties of *Schizolobium.*

Supplementary data

Supplementary data are available online with the following files:

Table 1. Geographical distances (in kilometers) among *Schizolobium* collected areas; Fig. 1: Phylogenetic tree comprising a concatenated plastid dataset that included the *matK* gene and other five plastid regions (*psbA-trnH*, *trnL-trnF*, *rpoB*, *rpoC1*) for *Schizolobium*. The divergence times was estimated using an uncorrelated relaxed molecular clock approach implemented in BEAST. The posterior probability values and the age estimated for *Schizolobium* are indicated by arrows.

Table 1
Sequences of primers used for ITS and cpDNA loci amplification and sequencing, PCR expected size, sample size in each population, and number of mutations discriminating between the two varieties of *Schizolobium*.

Locus	Primer sequence 5' - 3'	PCR (bp)	Polymorphic	n (sample per population)*	SNPs	Mutation [Sp:Sa]	Indels
psbA_trnH	**ACTGCCTTGATCCACTTGGC** **CGAAGCTCCATCTACAAATGG**	**363-416**	**yes**	**165/26:12:20:5:18:15:3:5:30:14:5:5:5:5**	**7**	**1****	**7**
rpoB	**ATGCAACGTCAAGCAGTTCC** **GATCCCAGCATCACAATTCC**	**473**	**yes**	**50/5:2:6:3:4:3:2:4:5:5:2:3:3:3**	**1**	**G:A**	**No**
rpoC1	**GTGGATACACTTCTTGATAATGG** **TGAGAAAACATAAGTAAACGGGC**	**500**	**yes**	**50/5:2:6:3:4:3:2:4:5:5:2::3:3:3**	**1**	**C:A**	**No**
trnL-trnF	**GGTTCAAGTCCCTCTATCCC** **ATTTGAACTGGTGACACGAG**	**448**	**yes**	**79/13:6:8:5:10:3:3:4:4:4:4:5:5:5**	**1**	**A:C**	**No**
matK	**CCTATCCATCTGGAAATCTTAG** **GTTCTAGCACAAGAAAGTCG**	**1500**	**yes**	**50/5:2:6:3:4:3:2:4:5:5:2:3:3:3**	**12**	**No**	**No**
ITS1-2	**GGAAGTAAAAGTCGTAACAAGG** **TCCTCCTCCGCTTATTGATATGC**	**663**	**yes**	**50/5:2:6:3:4:3:2:4:5:5:2:3:3:3**	**12**	**10**	**No**
accD	AGTATGGGATCCGTAGTAGG TCTTTTACCCGCAAATGCAAT	280	No	22/2:2:2:2:2:2:2:2:2:2:2:0:0:0	No	No	No
íntron trnL	CGAAATCGGTAGACGCTACG GGGGATAGAGGGACTTGAAC	587	No	33/14:5:0:2:1:2:2:2:1:2:2:0:0:0	No	No	No
ndhJ	TTGGGCTTCGATTACCAAGG TCAATGAGCATCTTGTATTTC	375	No	22/2:2:2:2:2:2:2:2:2:2:2:0:0:0	No	No	No
ycf5	GGATTATTAGTCACTCGTTGG CCCAATACCATCATACTTAC	260	No	22/2:2:2:2:2:2:2:2:2:2:2:0:0:0	No	No	No
rbcL-a	ATGTCACCACAAACAGAGACTAAAGC CTTCTGCTACAAATAAGAATCGATCTC	610	No	22/2:2:2:2:2:2:2:2:2:2:2:0:0:0	No	No	No

*Pops:
SC:PR:SP1:SP2:RJ1:RJ2:BA1:BA2:MT:AM:EC:CO:CR:HO

**The event of microinversion is represented in the figure 2.

Table 1. Sup. data

Table 1. Geographical distances (in kilometers) between *Schizolobium* collected areas.

POPs/Km	SC	PR	SP	RJ1	RJ2	BA1	BA2	MT	AM	EQ	CO	CR	HO
SC	**	218	422	724	914	1816	1709	1985	3007	4276	4231	5916	6466
PR		**	258	570	811	1638	1533	1805	2923	4155	4188	5789	6376
SP			**	379	609	1444	1324	1755	2978	4192	4176	5775	6361
RJ1				**	248	1144	1057	1814	3172	4388	4343	5958	6526
RJ2					**	976	879	1950	3328	4561	4394	6072	6642
BA1						**	140	1777	3361	4467	4218	5877	6520
BA2							**	1796	3326	4459	4202	5944	6506
MT								**	1564	2699	2521	4204	4742
AM									**	1230	1550	2965	3515
EQ										**	744	1723	2259
CO											**	1668	2271
CR												**	579
HO													**

Fig. 1.

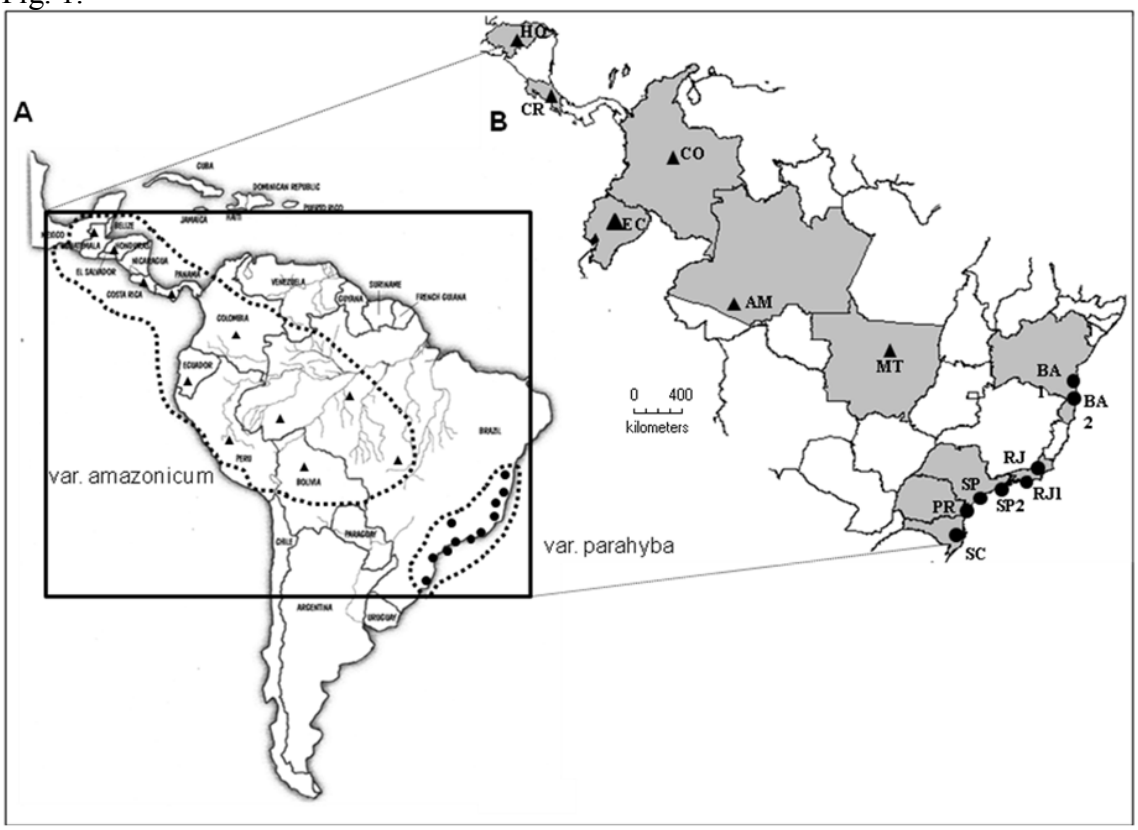

A
B
HC
CR
CO
EC
AM
MT
BA 1
BA 2
RJ
SP
RJ1
SP2
PR
SC
0 400
kilometers
var. amazonicum
var. parahyba

Fig. 2.

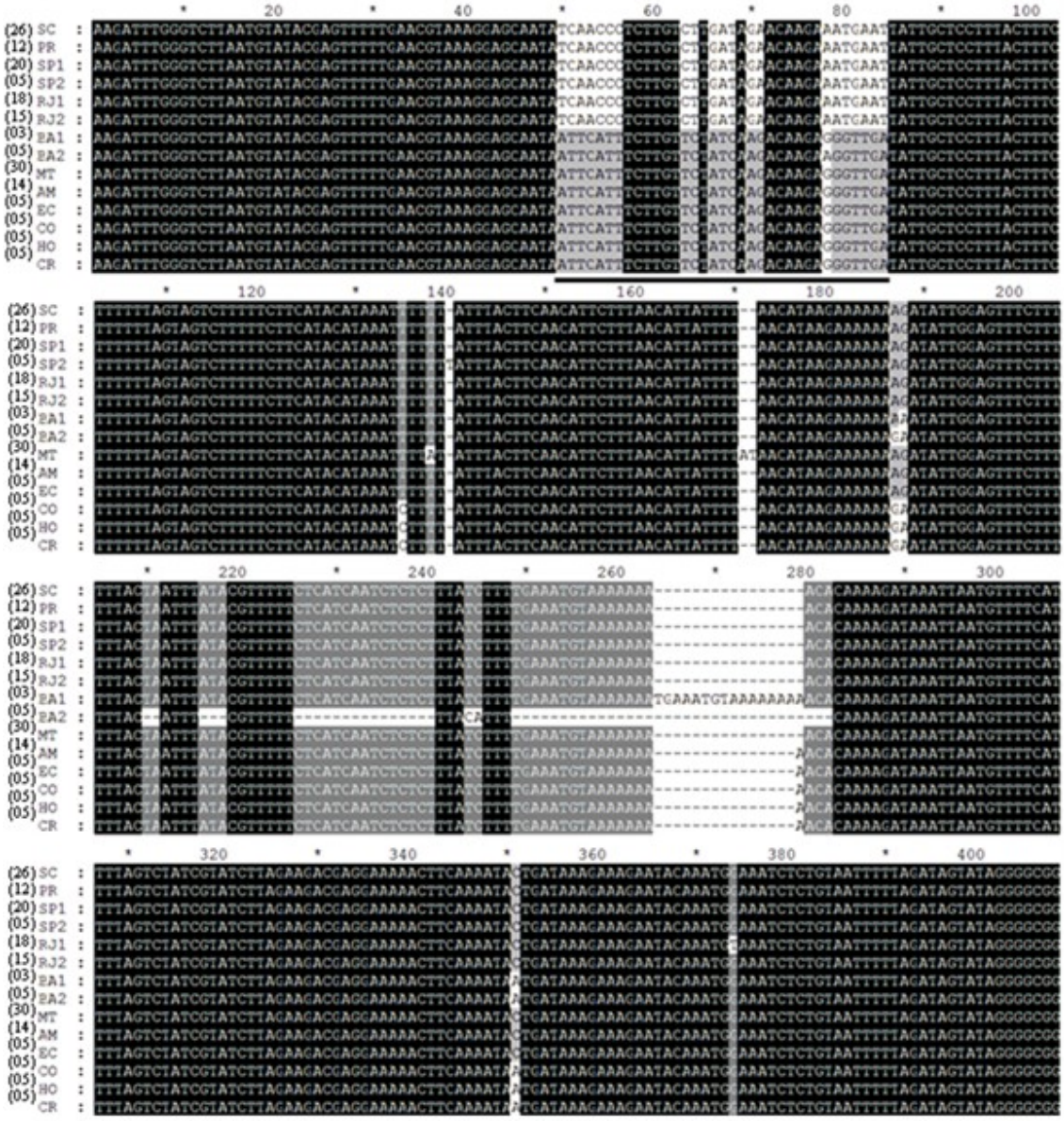


Fig. 3.

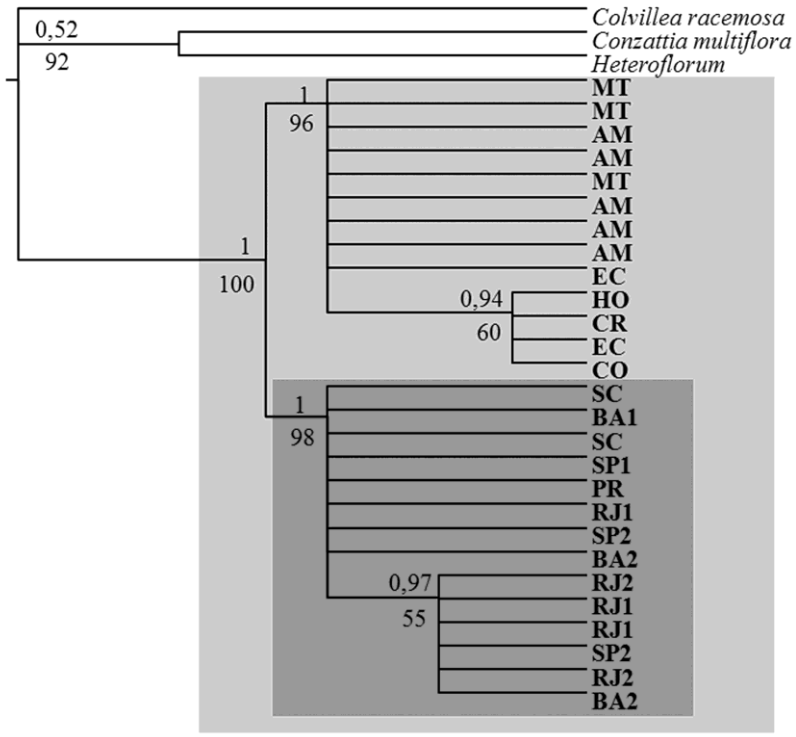
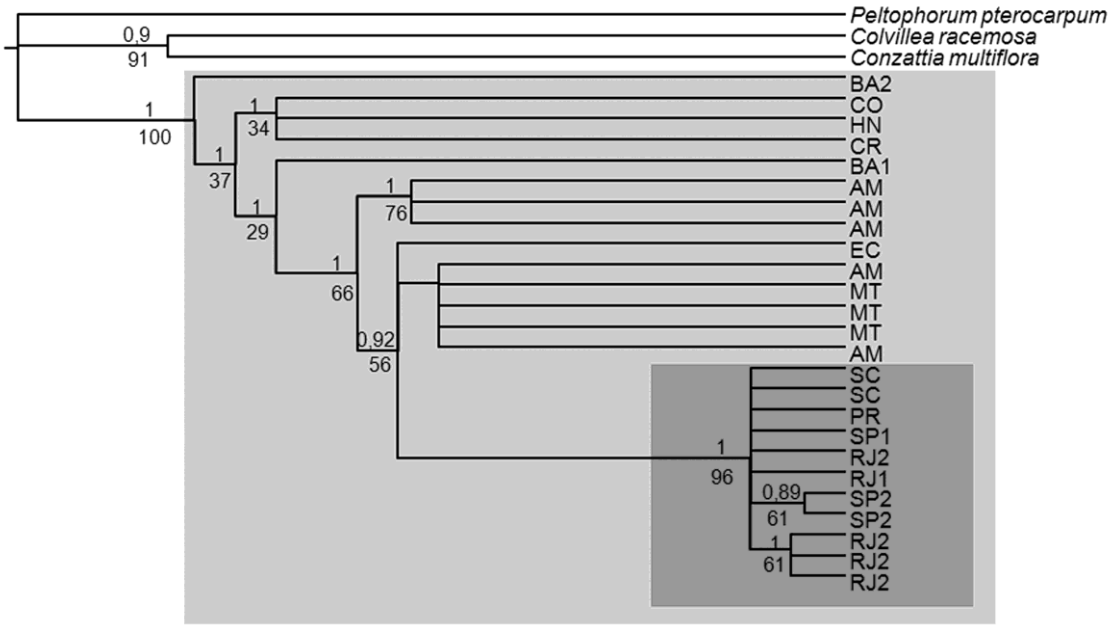

0,52
92
1
96
1
100
0,94
60
1
98
0,97
55
Colvillea racemosa
Conzattia multiflora
Heteroflorum
MT
MT
AM
AM
MT
AM
AM
AM
EC
HO
CR
EC
CO
SC
BA1
SC
SP1
PR
RJ1
SP2
BA2
RJ2
RJ1
RJ1
SP2
RJ2
BA2

Fig. 4.


Peltophorum pterocarpum
Colvillea racemosa
Conzattia multiflora
0,9
91
1
100
1
34
1
37
1
29
1
76
1
66
0,92
56
BA2
CO
HN
CR
BA1
AM
AM
AM
EC
AM
MT
MT
MT
AM
1
96
SC
SC
PR
SP1
RJ2
RJ1
0,89
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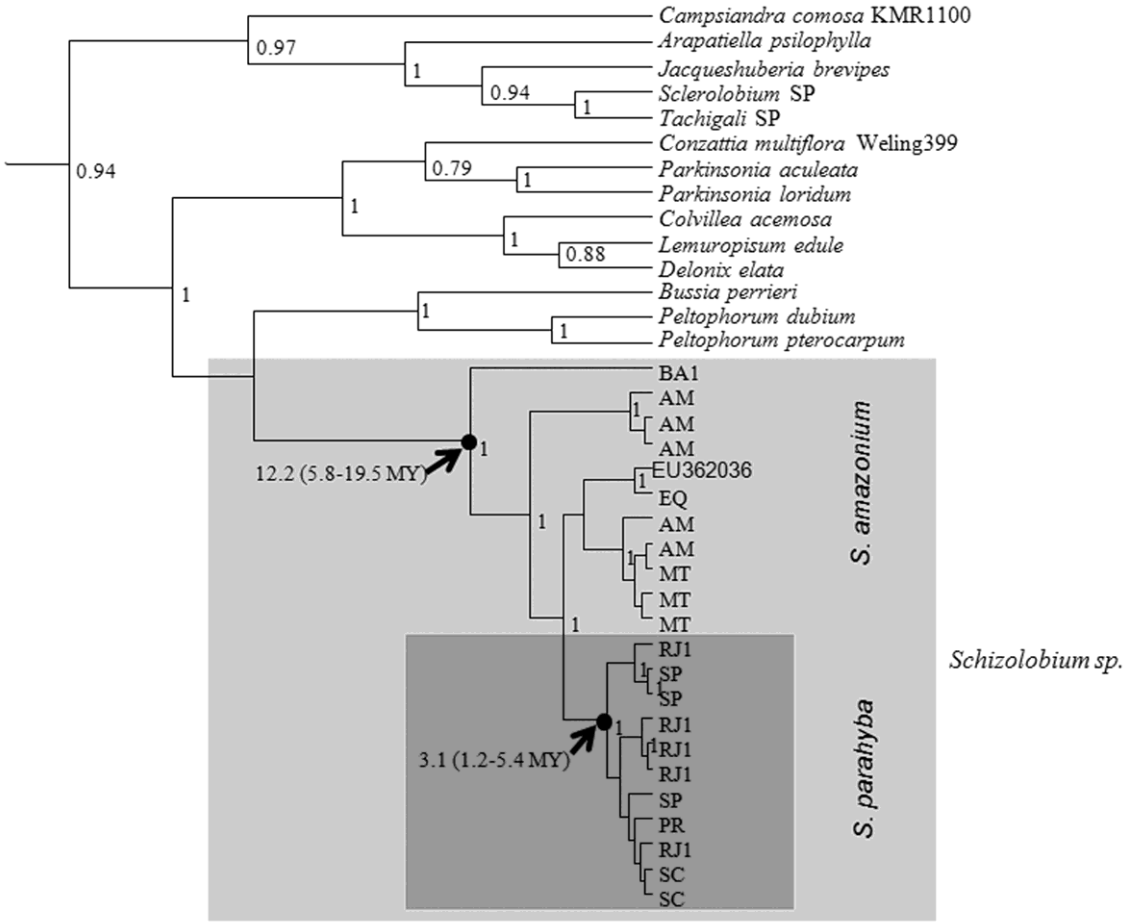
SP2
SP2
1
61
RJ2
RJ2
RJ2

Fig. 1 Sup. data



CAPÍTULO IV: Phylogeography of the disjunct Neotropical trees *Schizolobium* (Caesalpinioideae) suggests past interconnections between Amazonian and Atlantic forests

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Manuscrito a ser submetido a Molecular Ecology

Abstract

The Amazon and the Atlantic forests encompass the most diverse tropical forests in the world, with many species showing disjunct distribution between them. However yet there are few records about genetic structure in these areas. The phylogeographic studies of the widespread and disjunct species are particulary interesting because they can provide information on the historical relationship between different geographical regions. This study investigates the phylogeographic structure of genetic variation in a neotropical tree genus, *Schizolobium* Vogel (Fabaceae-Caesalpinieae), based on nuclear (ITS) and chloroplast (*psbA-trnH and trnL-trnF* spacer and *matK* gene) markers. This genus is largely distributed through of the Neotropics (Atlantic Forest, Amazonian rain Forest and tropical forest of Central America) and morphologically consists in only one species, with two varieties. High levels of genetic divergence found in *Schizolobium* and no sharing of haplotypes between the two varieties could be the result of genetic isolation between them, supporting the divergence between the two lineages. The cpDNA sequence similarity of some populations from Atlantic forest with the var. **amazonicum** was observed and this may be due historical gene flow with retention of ancestral polymorphisms. The high level of genetic diversity was found in São Paulo (SP2) and Espirito Santo (ES) populations from Atlantic forest may be associated with refuge areas and support the conservation strategies in this biome. The disjunct distribution of *Schizolobium* varieties my be result of the past climatic changes that resulted in an extension of savannah vegetation and reduction in rain forest size.

Introduction

Phylogeographic analyses can provide valuable insights into the demographic history and the past distribution of individual species, as well information about the ecological stability of the communities. Phylogeography has immediate practical importance for taxonomy and conservation management as it clarifies the genetic relationships of operational taxonomic units and identifies major demographic trends relevant to their long-term viability (Avise, 2000). In recent years, the phylogeography of Neotropical species has received more attention because of its complex biogeographical pattern, and because of the species richness and the notorious devastation of the Neotropical forests. Important phylogeographic studies have been carried out along the last decade (Castoe *et al.*, 2009; Cavers *et al.*, 2003; Couvreur *et al.*, 2007; Dutech *et al.*, 2000; Gonzalez *et al.*, 2008; Latouche-Halle *et al.*, 2003; Ramos *et al.*, 2009). However, most of these forests remain understudied with relatively few data about genetic diversity, gene flow, evolution and phylogeographic patterns.

The Neotropical flora comprises about 37% of the world's plant species and most of these species are found in rain forests, which have higher plant species diversity than any other habitat on the planet. However, much cryptic diversity awaits discovery there or will disappear before documentation (Thomas, 1999). The tempo and mode of origin and diversification of the lineage in the Neotropics is an interesting topic in evolutionary biology but is also one of the least understood and is still controversial (Daza *et al.*, 2009; Rull, 2008). The abiotic and biotic complexity of the region precludes generalizations that can be drawn regarding the historical evolutionary processes responsible for the diversity observed (Daza *et al.*, 2009). Some Neotropical areas are under manifest danger of biodiversity loss and have been identified as

biodiversity hotspots submitted to special conservation programs (Myers *et al.*, 2000) and because of the its large biodiversity, these ecosystems are preferred target for research about the origin of biological diversity (Pennington *et al.*, 2004). Knowing speciation timing, genetic mechanisms and forcing agents involved would provide fundamental clues for biodiversity conservation (Moritz 2002).

It is generally recognized that factors as changes in temperature, precipitation and sea level, the interaction of these with each other, and the topography complex have also contributed to both speciation and genetic structuring within species, but there is an ongoing debate regarding the timing of their influence in the tropics (Rull, 2006). While Quaternary climatic changes are considered as a major factor leading to speciation (Noonan, Gaucher, 2005; Rull, 2006), tropical species are typically regarded as lineages originating before the Pleistocene (Hewitt, 2000). Several evidences also have suggested that speciation has occurred at different rates and times in the Neotropics (Zarza *et al.*, 2008). Within Central America, molecular phylogenetic data suggests that forest plant species encompass both pre-Pleistocene and Pleistocene speciation events, while South American species appear to be mostly pre-Pleistocene in origin (Pennington *et al.*, 2004). In regard to genetic structuring within species and its causes, one might hypothesize that if Pleistocene events are leaving genetic signatures above the species level within the Neotropics. Widespread Neotropical species may encompass substantial genetic differentiation and structure, with the possibility of cryptic species. Hence the phylogeographic studies of the widespread species are particularly interesting because they can provide information on the historical relationship between different geographical regions and to provide information about speciation and genetic structuring within species.

The Amazon and the Atlantic Forests morpho-climatic domains of South America (Ab'Saber, 1977) which encompass the most diverse tropical forests in the world. Between these two forests lies a belt of more open vegetation, including the Argentinean and Paraguayan Chaco, the Caatinga in Northeastern Brazil, and the central Brazilian Cerrado, the latter being the second largest domain in Brazil extending over 2 million km^2 (Prado, Gibbs, 1993). This dry corridor of open vegetation has been considered as an important restraint to species migration between the two rain forest regions. The biomes composed by Amazonic and the Atlantic forests were probably a continuous in the past; becoming separated as increasing aridity in the Tertiary formed the belt of xeromorphic formations between them. The palynological record of the Quaternary showed that between 33,000 and 25,000 BP, the central Brazilian region was moister than today and was covered by Rain Forests (Ledru, 1993), and during the last glacial maximum (18,000–12,000 yr BP), the present-day corridor of xeric vegetation was covered by extensive woodland (Prado, Gibbs, 1993). These findings indicate the predominance of seasonal arboreal vegetation during most of the Pleistocene. Molecular analyses that reveal patterns of genetic diversity in these forests components are scarce. Some examples reported in literature in general come from tree species (Dick *et al.*, 2003; Kremer *et al.*, 2005; Ramos *et al.*, 2009; Ramos *et al.*, 2007), but few studies have reported the differentiation of disjoint Atlantic and Amazonian plant populations (Rossi *et al.*, 2009).

About species with disjunct distribution, it is origin has been a major concern in biogeography and many studies tried to disentangle the mechanisms involved in the origin of such distribution (Azuma *et al.*, 2001; Collevatti *et al.*, 2009; Gaudeul, 2006; Karanth, 2003; Rossi *et al.*, 2009). Disjunct distributions may be caused

by range contraction in an ancient more widely distributed species due to changes in climatic conditions that affected suitable habitat distribution (Cox, Moore, 2005). On the other hand long-distance dispersal to new suitable habitats may also be responsible for disjunct distributions.

The genus *Schizolobium* Vogel (Fabaceae-Caesalpinieae) morphologically consists in only one species, *Schizolobium parahyba,* comprising two varieties with disjunct distribution: *S. parahyba* variety *parahyba* (Atlantic Forest) and *S. parahyba* variety *amazonicum* (Amazonian rain Forest and tropical forest of Central America) (Barneby, 1996). *Schizolobium* is an ecological and economically important forest tree genera with a great potential for wood production and other uses. It is one of the fastest growing tree species, which can explain its extensive use in reforestation projects based on native trees (Carvalho, 1994). The study of these genera is very interesting because multiple distinct phylogeographic hypotheses can be proposed, especially for understanding the past floristic connections between the two largest Neotropical Forests (Amazonian and Atlantic Rain forest) and the speciation patterns in these biomes. Within this wide distribution, populations tend to be disjoint with presumably low rates of pollen and seed exchange. It therefore seems likely a priori that migration history in a complex landscape would be an important determinant of genetic structure relative to a simple isolation-by-distance model.

In this study, both nuclear and chloroplast markers were used to describe the phylogeographic structure of *S. parahyba*. Phylogeographic studies have focused primarily on organellar rather than nuclear loci because recombination and a fourfold greater effective population size greatly reduce the informativeness of individual nuclear loci. As noted by several authors (e.g. Hare 2001; Zhang & Hewitt 2003),

however the collective utility of nuclear loci is great because they represent numerous, approximately independent estimates of genealogy across the genome. Their interpretation is therefore less affected by the vagaries of lineage sorting at individual loci. Furthermore, differences in the frequencies of pre-existing nuclear polymorphisms among subdivided populations should accrue more quickly than differences among slowly evolving chloroplast haplotypes, and they can be readily translated into a genetic distance matrix regardless of recombination. Nuclear genetic structure should also have greater evolutionary consequences than that of organellar genomes and the two may be largely uncorrelated (Petit *et al*. 2005). Thus, regardless of the hypotheses to be tested, it is desirable to use both types of markers to gain an accurate view of species-level genetic structure. In this case, we have interest in understand the evolution patterns of *Schizolobium*, clarify if speciation occurred in this genus and provide genetic information to support the presence of refuge centers in the Atlantic Forests suggested by climatic modeling using other animal species as indicators (Carnaval *et al.*, 2009; Carnaval, Moritz, 2008) and understand the historical relationship between Amazonian and Atlantic forest.

Materials and Methods

Sampling strategy

A total of 286 individuals of *Schizolobium* were collected from various regions and localities, in distinct geographical areas of occurrence of both *Schizolobium* varieties and grouped into 20 populations (Table 1 and Fig. 1A). The samples were collected as silica gel dried cambium and leaf materials from natural populations located in Brazil (15 populations), Ecuador, Colombia, Costa Rica and Honduras (1 population

each). A list of collecting sites, geographical coordinates and sample size of each population is reported in Table 1.

DNA isolation, amplification and sequencing

Total genomic DNA was isolated using the CTAB method (Doyle, Doyle, 1990). The 5.8S nrDNA and flanking ITS1 and ITS2 regions were PCR amplified using ITS1 forward primer 5'GGAAGTAAAAGTCGTAACAAGG-3' and ITS2 reverse primer 5'TCCTCCTCCGCTTATTGATATGC-3 (White *et al.*, 1990). The plastid DNA was amplified using two pairs of universal chloroplast primers corresponding to fragments of *psbA-trnH* intergenic spacer (forward primer 5'ACTGCCTTGATCCACTTGGC-3' and reverse primer 5'CGAAGCTCCATCTACAAATGG-3') and *trnL-trnF* intergenic spacer (forward primer 5'GGTTCAAGTCCCTCTATCCC-3' and reverse primer 5'ATTTGAACTGGTGACACGAG-3') (Kress, Erickson, 2007; Taberlet *et al.*, 2007) and *matK* gene (forward primer 5'CCTATCCATCTGGAAATCTTAG-3' and reverse primer 5'GTTCTAGCACAAGAAAGTCG-3') (Wojciechowski *et al.*, 2004).

All amplifications were performed using 10 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1x PCR buffer, 0.05 U of Platinum Taq DNA polymerase (Invitrogen) and 5 µM of each primer in a final volume of 20 µl. The PCR cycle had an initial hot-start step at 94 °C for 5 min, followed by 40 cycles at a denaturation at 94 ^{o}C for 50 s, annealing temperature of 48ºC (ITS1F-ITS2R, *psbA-trnH,*and *trnL-F*) or 51ºC for *matK* gene for 50 s, and 50 s of elongation at 72 ºC. All PCR products were visualized by electrophoresis in 1.5 % agarose gels stained with SYBR Gold (Invitrogen) and precipitated using 3 M Sodium Acetate and 95% ethanol.

Nuclear and plastid amplified PCR products were sequenced by the dideoxy chain-termination method using Big-Dye (Applied Biosystems), using an ABI-3100 automatic sequencer (Applied Biosystems). Sequencing was conducted in 10µL reactions with 2 µL of purified PCR product, 1 µL of primer, 2 µL of buffer 5x, 0.25 Big-Dye®Terminator and 4,75 of milliQ water. The thermocycling profile was as follow: 25 cycles of 10 s at 95 ºC, 5 s at 54 ºC and 4 min at 60 ºC. Both DNA strands were fully sequenced. Sequencing of approximately 1500 bp of *matK* gene was done using four additional internal primers: matK4La (forward) CCTTCGATACTGGGTGAAAGAT (500 bp); matK1100L (forward) TTCAGTGGTACGGAGTCAAATG (1100 bp); matK832R (reverse) TTGCATAGAAATGGATTCGCTCAAA (700 bp) matK1932Ra (reverse) CCAGACCGGCTTACTAATGGG (1200 bp) (Wojciechowski *et al.*, 2004).

Editing, alignment and phylogenetic analyses

Sequences were checked by eye and the identity was certified using blastn algorithms against DNA plant sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were aligned using the Alignment Explorer/CLUSTALW Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al.*, 2007).

Haplotype Network and genetic diversity analyses

For this and all subsequent analyses, indels were coded as single binary character. Genetic relationships among nuclear and cpDNA haplotypes were inferred with Network 4.2.0.1 (Fluxus Technology Ltd. At www.Fluxus-engineering.com) using the median-joining method (Bandelt *et al.*, 1999). Molecular diversity indices (π, nucleotide diversity; h, haplotype diversity; k, mean number of nucleotide substitutions) were calculated using MEGA 3.0 and DNAsp 5.0 (Librado, Rozas, 2009).

To determine hierarchical population structure, analyses of molecular variance (AMOVA) based on pairwise differences were performed between the two varieties to determine the amount of variation attributable to differences among varieties using ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

Spatial analysis of molecular variance or SAMOVA (Dupanloup *et al.*, 2002); (ver. 1.0, http://web.unife.it/progetti/genetica/ Isabelle/samova/html) also was employed to identify spatial boundaries among the twenty sample localities. A total of 100 simulated annealing processes, for both concatenate cpDNA and ITS regions, were used to determine optimal allocation of the twenty geographic samples into two, three, four, five, six, seven, or eight groups.

Neutrality tests (Fu, Li, 1993; Tajima, 1989) for both concatenate cpDNA and ITS sequence data sets were performed in order to detect any possible deviation from neutrality which could be either an effect of natural selection or the result of a past demographic expansion. In addition, mismatch distributions were simulated under the sudden-demographic expansion and the spatial-demographic expansion models. All tests were performed using Arlequin version 3.0 (Excoffier *et al.*, 2005).

Results

DNA was extracted from 286 individuals of *Schizolobium*, sampled from 20 populations. 187 individuals are classified as var. *parahyba*, collected from Atlantic Forest and 99 to var. *amazonicum*, collected in Ecuador, Colombia, Central America and Amazonian Rain Forest, in Brazil. Sampling localities are indicated in Table 1 and

Fig.1. A total of 263 individuals were amplified and sequenced with *psb*A-*trn*H and *trn*L-*trn*F cpDNA intergenic spacer, 198 with *mat*K cpDNA gene and 129 with ITS. The differences according to the different markers it is due to difficulties encountered with the amplification and sequencing in some samples.

Genetic diversity at ITS region

The ITS aligned matrix, including ITS1+5.8S+ITS2 was 625 bp in length, of which 227 bp for ITS1 and 181 bp for ITS2. Twelve polymorphic sites were detected, of which 10 originated by comparing the two varieties, with no insertions or deletions. Nucleotide diversity was 0,094. Four ITS haplotypes were found and a significant differentiation between the two varieties was observed. All individuals collected in Atlantic Forest displayed H1 and H2 haplotypes, while all other individuals showed H3 and H4 haplotypes. The median-joining network and the relationships among the 4 ITS haplotypes are showed in Fig. 1B. Tajima's D and Fu and Li's F neutrality tests performed for each population showed no significant values (Table 2), but the global analysis of neutrality tests showed significant values (Tajima's D=3,42 and Fu and Li's F*=2,59). The F_{ST} values among the populations are showed in Table 1S (Supplementary data).

Genetic diversity from cpDNA

The *psb*A-*trn*H and *trn*L-*trn*F intergenic spacer ranged from 363 to 416 bp and 448 to 449 bp, respectively. For *psb*A–*trn*H intergenic spacer, which shows considerable nucleotide polymorphisms, including a micro-inversion event, is characterized by a reverse-repeated region of 35 bp flanked by a 14 bp palindromic sequence and insertions and deletions events. The micro-inversion was coded as a single event. A 16-bp duplication region (TGAAATGTAAAAAAAA) was found in the

populations from Bahia states (Atlantic Forest). The *trnL-trnF* spacer was characterized by 5 events (2 indels and 3 substitutions). The amplification of *matK* gene produced a fragment of 1500 bp, whit 14 polymorphic sites detected. All analyses were performed for combined data. The diversity and neutrality parameters are showed in Table 2 and the F_{ST} values among the populations are showed in Table 2S (Supplementary data). The total combined matrix presented 2322 sites, of which 28 positions were variable and 9 show with gap.

Twenty one haplotypes were found with the combined cpDNA (Fig.2). The most diverse populations in terms of detected haplotypes were SP2 and ES from Atlantic forest (four haplotype), MT and AM2 from Amazonian forest (three and four haplotypes, respectively). Populations SP1, MG, RJ1 and RJ2 had two haplotypes and all the others were monomorphic (Table 2 and Fig. 2).

Phylogeographic structure

The relationships among the detected nuclear haplotypes are shown in network reported in fig. 2 and the chloroplastic haplotypes are shown in the network reported in Fig. 3. For both matrix, the most frequent haplotype was H1, occurring in 7 out of 20 populations in concatenate cpDNA and 9 in nuclear ITS. Most haplotypes in combined cpDNA were found in only one population and no haplotype was shared between the two varieties (Fig. 2, Table 2).

The populations SP2, and ES from Atlantic Forest and AM2 and MT from Amazonian Rain Forest were the most diverse, as indicated by haplotype and nucleotide diversity indices (Table 2). The populations from Atlantic Forest (SC, PR1, PR2, RJ3, BA1, BA2 and BA3) and from Colombia (CO), Amazonian rain forest (AM1) and Central America (CR and HO) are very homogeneous. High population

pairwise F_{ST} values were estimated, especially among populations between the two varieties, based on ITS and cpDNA data (Table 1S and Table 2S, respectively). Analysis of molecular variance (AMOVA) between the two varieties based on the nuclear ITS and cpDNA showed in Table 3. AMOVA revealed that most of the variation in ITS was explained by differences among groups and low variation explained by differences between groups, while for cpDNA the highest variation is explained by differences among the populations within groups.

The SAMOVA analyses of ITS clearly indicated that there were two distinct groups of genetically defined sampling areas. In analyses where K=2, partitions of the sampling areas were identified that suggested two groups and according of the distribution of two varieties (groups: SC, PR1, PR2, SP1, SP2, RJ1, RJ2, RJ3, ES, BA1, BA2, BA3 vs. AM1, AM2, MT, EC, CO, CR, HO; F_{CT}=0.954). In analyses where K=3, an additional partition was identified that subdivided the first group into two areas, with an F_{CT} value of 0.644. With K=4 the F_{CT} increased to 0.978 and remained stable after k=5 to K=8 with F_{CT} values ranging from 0.977 to 0.981. Therefore, we suggested the division of two varieties and the presence of four geographical groups: two groups in Atlantic forest comprising SC, PR1, PR2, SP1, SP2, RJ1, BA1, BA2 vs. RJ2, RJ3, ES and BA3, one in Amazonian rain forest comprising AM1, AM2 and MT and other group comprising EC, CO, CR and HO populations. The SAMOVA analyses using cpDNA showed the presence of two main geographical groups: a comprising BA2 population and other comprising all the other populations: SC, PR1, PR2, SP1, SP2, MG, RJ1, RJ2, RJ3, ES, BA1, BA3, AM1, AM2, MT, EC; CO, CR and HO, with FCT=0,557 and no observed the separation of the two varieties in different groups.

The observed mismatch distributions for cpDNA and nuclear haplotypes (Fig. 4A and B), were not unimodal, and differed strongly from those predicted under a model of sudden range expansion. Similarly, all species-wide tests of neutrality (Tajima's D, Fu and Li's F*) showed non-significant values (Table 2). Taken together, these data do not provide evidences for recent demographic expansions in *Schizolobium*.

Discussion

Genetic diversity and genetic structure of Schizolobium

The amount of genetic variation observed in the nuclear ITS region and chloroplast DNA *psbA–trnH*, *trnL-trnF* intergenic spacer and *matK* gene in *Schizolobium* permitted inferences of genealogy, genetic structure, and historical biogeography. In the present study, using sequences of *psbA-trnH+trnL-trnF* and *matK* region, it was possible to identify that *Schizolobium* populations exhibit a high levels of genetic divergence (Table 3) as well as the values observed for other species of angiosperms where plastid DNA loci were also used (Petit *et al.*, 2005; Ramos *et al.*, 2009; Ramos *et al.*, 2007). Polimorphism patterns for the three chloroplast region were very similar but nuclear ITS presented different patterns of haplotype and nucleotide diversity. Neutrality tests and mismatch distribution showed that the populations are note in expansion. Populations of *S. parahyba* usually present a high variation in size and in proportion of adults and juveniles. Most populations had no juveniles and some were very small, such as BA1, BA3, PR1, AM1, and the lack of variation in these populations is probably related to the small size (only three individuals could be collected).

The level of divergence found with nuclear ITS was high (Table 2), but the most genetic variation in the ITS region in *S. parahyba* is distributed according to the varietal designations var. *parahyba* and var. *amazonicum*. Only four haplotypes were found with this marker and no share haplotypes between the varieties, witch shows high and significant genetic differentiation (Fig 1 and Table 3). In the plastid regions, despite the sequence similarity some Atlantic Forest populations (RJ3, ES, BA1 and BA2) with the Amazonian populations, no common haplotypes were detected between the two varieties. We observed that the haplotype H8, found in ES population (Fig. 2 and Fig. 3), connect the Atlantic forest populations with the Amazonian forest populations. This similarity may be explained by ancient gene flow and retention of ancestral polymorphisms since studies indicate past floristic connections between the Amazonian and Atlantic rain forest areas in Brazil Northeast (Behling, 1998; Behling, 2002; Behling *et al.*, 2000; Behling, Lichte, 1997). In previous studies (non published data) we showed that the estimate of split between the two varieties of *Schizolobium* occurred roughly in the last 3 million years and, according to evidences from the fossil records; there are vestiges of rainforest in areas of the Northeast of Brazil where today flourish a semi-arid vegetation. This would imply that the Amazon and Atlantic Forest were more strongly linked sometime in the past as we see today (Behling, Negrelle, 2001). Others studies also revealed connections between these two important biomes (Behling, 2002; Costa, 2003). The significant differentiation between the two varieties could be the result of genetic isolation between the varieties and these results are in accordance with the hypothesis of the occurrence of a long-standing barrier preventing gene flow between the Atlantic and Amazonian Forest populations. We postulate that open vegetations such as the Cerrado serve as a strong natural barrier for *Schizolobium*, since this species does not occur in Cerrado areas.

The haplotype pattern found and Spatial analysis of molecular variance (SAMOVA) for ITS DNA sequences markers allows to subdivide the *Schizolobium* populations into main genetically differentiated groups according to four geographic areas: two included in the Atlantic Forest biome; one in Amazonian basin and another including the populations from Ecuador, Colombia and Central America, besides indicated a clear separation of the two varieties. However, the lack of spatial correlation found with cpDNA and the incongruence between the cpDNA and nuclear are very complex and may be due the different evolutionary patterns these two genomes. The AMOVA analyses of ITS between the two varieties showed a high level of variation between them, but the AMOVA analyses of cpDNA showed that the most variation was among populations within groups. The BA1, BA2 populations from Atlantic forest and CO (Colombia), CR and HO from Central America were the most divergent populations (Table 2 and Fig. 3), also presenting low genetic diversity. These low genetic diversity may be explained by smaller size these populations.

Maternally inherited markers are frequently shared among holoarctic tree species (Heuertz *et al.*, 2006; Lexer *et al.*, 2005; Palme *et al.*, 2004; Petit *et al.*, 2002b; Rajora, Dancik, 1992), but this was not observed between the two varieties of *Schizolobium*, showing the absence of gene flow between them, strengthening its differentiation. Different results were observed for species of *Hymenaeae,* between Atlantic Forest and Cerrado (Ramos *et al.*, 2009). The 2 species (*H. coubaril* and *H. stignocarpa*) are very similar according to the genetic variation considering the pairwise distance between haplotypes and only 10.5% of the detected genetic variation is due to differences between the species, while 51.7% of it was due to differences among

populations belonging to the same. These species also shared haplotypes (Ramos *et al.*, 2009).

In Atlantic Forest, a high level of genetic diversity was found in São Paulo (SP1) and Espírito Santo (ES) that may be associated with refugia areas previously identified by climatic modeling studies (Carnaval *et al.*, 2009; Carnaval, Moritz, 2008). The Bahia Geographic areas exhibiting increased levels of genetic diversity are first candidates in the search of past refugia. These regions should be characterized by comparably stable ecological conditions during environmental fluctuations fostering the accumulation of genetic diversity as has been demonstrated in many cases (Petit *et al.*, 2003; Petit *et al.*, 2002a). Moreover, as a consequence of prolonged isolation, extant tree populations situated close to refuges should be highly divergent, especially if they were not the source of the expansion.

While sites of putative glacial refuge are expected to have higher levels of genetic variation, regions colonized after glacial periods are expected to have reduced levels of genetic variation and large geographic areas fixed for a single haplotype (Hewitt, 2000). In this case, Low levels of genetic variation and haplotype diversity in *Schizolobium* was found in South Atlantic forest (SC, PR1, and PR2) (Table 2 and Fig. 2). The populations from Santa Catarina and Parana States are genetically homogeneous and share nuclear and plastid haplotype (Fig. 1 and 3). A similar pattern was also found for other species from the Atlantic Forest (Cabanne *et al.*, 2008; Carnaval, Moritz, 2008).

In conclusion, our results support that the disjunct distribution of *Schizolobium* varieties my be result of the past climatic changes that resulted in an extension of savannah vegetation and reduction in rain forest size. However, the split

between the two varieties has been occurred before this event. In addition, the biogeographic patterns found for the different populations of *Schizolobium,* the high genetic diversity in the central portion of the Atlantic Forest biome (São Paulo e Espírito Santo States) and Amazonian forest (Mato Grosso and Amazonas State) and the high divergence of the some populations, provide important information for conservation efforts. Prior to any decision concerning conservation strategy, it is necessary to clarify whether the classification of *Schizolobium* defines Evolutionarily Significant Units (ESUs) or merely represents a geographical subdivision with no further biological or taxonomic implications. According to our results, the different groups of *Schizolobium* can be considered as independent ESUs, as they are reproductively isolated from each other and represent an important component of the evolutionary legacy of the species. Since ESUs are widely equated to conservation units, this result suggests that any conservation strategy should consider the islands of distribution separately (Moritz, 1994). In addition, these biogeographical patterns supported the connection between the Atlantic and Amazonian rainforests.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table. S1 Pairwise comparisons of F_{ST} between populations of *Schizolobium* based on the ITS region.

Table. S2 Pairwise comparisons of F_{ST} between populations of *Schizolobium* based on the concatenate *psbA-trnH/trnL-trnF intergenic* spacer and *matK* gene plastid DNA.

FIGURES OF LEGENDS

Figure 1. (A) Distribution areas populations collected of *Schizolobium parahyba* in neotropics. The black circles represents the var. parahyba and the black triangle represents the var. amazonicum distribution areas. The populations were collected from Santa Catarina (SC), Paraná (PR1 and PR2), São Paulo (SP1 and SP2), Minas Gerais (MG), Rio de Janeiro (RJ1, RJ2 and RJ3), Espírito Santo (ES), Bahia (BA1, BA2 and BA3), Mato Grosso (MT), Amazonas (AM and MA2), Ecuador (EC), Colombia (CO), Costa Rica (CR) and Honduras (HO). (B) The MJ network analysis of the relationships between haplotypes of the ncITS region ploted on the map showing the molecular divergence between the two varieties. The dotted line on the map represents the estimated limit zone of distribution between *parahyba* and *amazonicum* varieties.

Figure 2: Approximate geographic location and plastid DNA haplotype frequencies of the populations of Schizolobium studied. Circle size is proportional to sample size and colours represent the different haplotypes as shown in the key.

Figure 3: Median-Joinig network analysis of the relationships between haplotypes of the concatenate psbA-trnH and trnL-trnF plastid DNA region from 265 Schizolobium

individuals. Circle area is proportional to haplotype frequency and colours are as in map from figure 2. Lines drawn between haplotypes represent mutation events identified by the numbers corresponding to the positions at which the mutations were observed. Black points represent hypothetical haplotypes (median vector).

Figure 4. Mismatch distributions of nuclear sequences (ITS) (A) and concatenate cpDNA sequences (B).

Table 1 Geographical characteristics of the 20 *Schizolobium* populations analysed

Population	**Label**	**Latitude**	**Longitude**	**Collected variety**
Santa Catarina, Barzil	**SC**	27º2' N	48º35' W	*parahyba*
Estrada da Graciosa, Paraná, Barzil	**PR1**	25º34' N	54º29' W	*parahyba*
Fóz do Iguaçu, Paraná, Barzil	**PR2**	25º22' N	48º51' W	*parahyba*
São Paulo, Barzil	**SP1**	24º44' N	48º9' W	*parahyba*
São Paulo, Barzil	**SP2**	23º29' N	45º4' W	*parahyba*
Minas Gerais, Barzil	**MG**	20º8' N	44º6' W	*parahyba*
Rio de Janeiro, Barzil	**RJ1**	23º4' N	44º42' W	*parahyba*
Rio de Janeiro, Barzil	**RJ2**	22º29' N	43º27' W	*parahyba*
Rio de Janeiro, Barzil	**RJ3**	22º52' N	42º29' W	*parahyba*
Espírito Santo, Barzil	**ES**	19º53' N	40º5' W	*parahyba*
Bahia, Barzil	**BA1**	14º45' N	39º14' W	*parahyba*
Bahia, Barzil	**BA2**	15º5' N	39º17' W	*parahyba*
Bahia, Barzil	**BA3**	18º4' N	39º32' W	*parahyba*
Mato grosso, Barzil	**MT**	10º41' N	55º29' W	*amazonicum*
Manaus, Amazonas, Barzil	**AM1**	3º8' N	59º57' W	*amazonicum*
Boca do Acre, Barzil	**AM2**	8º43' N	69º0' W	*amazonicum*
Ecuador	**EC**	1º2' N	44º6' W	*amazonicum*
Colombia	**CO**	3º20' N	72º42' W	*amazonicum*
Costa Rica	**CR**	9º51' N	83º33' W	*amazonicum*
Honduras	**HO**	14º36' N	86º16' W	*amazonicum*

Table 2 Genetic diversity measures and neutrality tests in cpDNA regions. Sample size (n), number of variable sites (s) number of haplotypes (H), nucleotide diversity (π), Fu's FS and Tajimas D .

	concatenate cpDNA						*ITS*					
Populations	n	s	H	π	Tajima's D	Fu's F_s	n	s	H	π	Tajima's D	Fu's F_s
SC	13	0,00	1 (H1)	0,00	0,000	0,000	13	0,00	1 (H1)	0,00	0,00	0,00
PR1	2	0,00	1 (H1)	0,00	0,000	0,000	4	0,00	1 (H1)	0,00	0,00	0,00
PR2	5	0,00	1 (H1)	0,00	0,000	0,000	7	0,00	1 (H1)	0,00	0,00	0,00
SP1	7	1,00	2 (H1, H5)	0,57	1,341	0,856	7	1,00	1 (H1)	0,00	0,00	0,00
SP2	20	3,00	4 (H1, H2, H3,H4)	1,47	0,753	0,944	11	1,00	2 (H1, H2)	0,32	-0,10	0,35
MG	12	1,00	2 (H1, H5)	0,16	-1,140	-0,475	*	*	*	*	*	*
RJ1	10	0,00	2 (H4, H7)	0,46	0,000	0,818	5	1,00	2 (H1 e H2)	0,60	1,22	0,62
RJ2	18	1,00	2 (H1, H6)	0,36	0,488	0,790	13	1,00	2 (H1 e H2)	0,54	1,47	1,23
RJ3	5	0,00	1 (H9)	0,00	0,000	0,000	4	0,00	1 (H2)	0,00	0,00	0,00
ES	11	5,00	4 (H5, H8, H10, H11)	1,52	-0,811	0,163	6	0,00	1 (H2)	0,00	0,00	0,00
BA1	3	0,00	1 (H19)	0,00	0,000	0,000	3	0,00	1 (H1)	0,00	0,00	0,00
BA2	3	0,00	1 (H20)	0,00	0,000	0,000	7	0,00	1 (H1)	0,00	0,00	0,00
BA3	9	0,00	1 (H10)	0,00	0,000	0,000	3	0,00	1 (H2)	0,00	0,00	0,00
MT	26	1,00	3 (H12, H13, H14)	0,77	0,668	0,885	17	0,00	1 (H3)	0,00	0,00	0,00
AM1	4	1,00	1 (H14)	0,00	0,000	0,000	4	0,00	1 (H3)	0,00	0,00	0,00
AM2	26	5,00	3 (H14, H15, H16, H17)	2,85	1,578	3,595	10	0,00	1 (H3)	0,00	0,00	0,00
EC	5	0,00	1 (H18)	0,00	0,000	0,000	6	1,00	2 (H3 e H4)	0,33	-0,93	0,00
CO	5	0,00	1 (H21)	0,00	0,000	0,000	3	0,00	1 (H4)	0,00	0,00	0,00
CR	9	0,00	1 (H21)	0,00	0,000	0,000	6	0,00	1 (H4)	0,00	0,00	0,00
HO	5	0,00	1 (H21)	0,00	0,000	0,000	4	0,00	1 (H4)	0,00	0,00	0,00

Statistical significance of Tajima's D and Fu's Fs: Not significant, P > 0,05; *Population do not analysed

Table 2 Results of the analyses molecular variance (AMOVA) based on the nuclear ITS (a) and concatenate cpDNA (*psbA-trnH+trnL-trnF+matK*) (b) of *Schizolobium* locations

Source of variation	d.f	Sum. of squares	Variance components	Variation Percentage	Fixation indices
(a)					
Among Groups	1	302,486	5,082	95,48	FCT: 0,954
Among populations within Groups	17	20,877	0,177	3,34	FSC: 0,739
Within Populations	110	6,900	0,062	1,18	FST: 0,988
Total	128	330,264	5,323		
(b)					
Among Groups	1	148,457	1,073	25,04	FCT: 0,250
Among populations within Groups	18	472,816	2,792	65,14	FSC: 0,868
Within Populations	178	74,990	0,421	9,83	FST: 0,901
Total	197	696,263	4,287		

Fig 1.

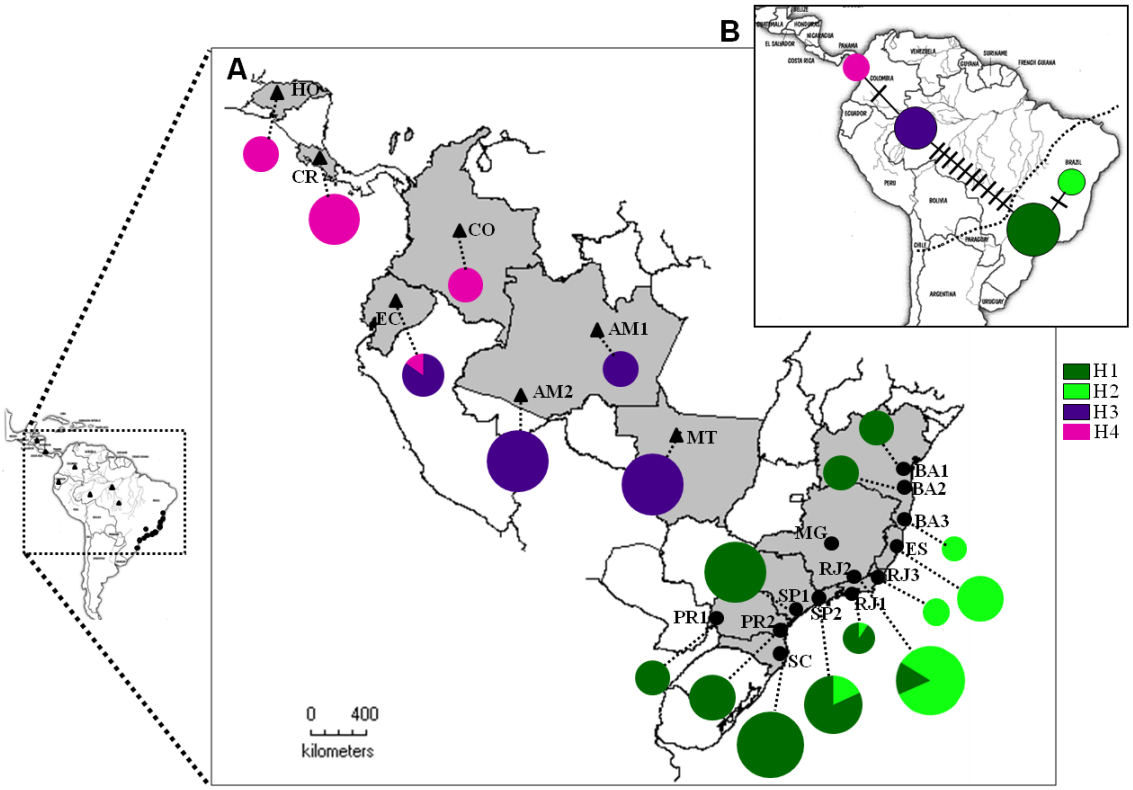

A
B
HO
CR
CO
EC
AM1
AM2
MT
BA1
BA2
BA3
ES
MG
RJ2
RJ3
RJ1
SP1
SP2
PR1
PR2
SC
0 400
kilometers
H1
H2
H3
H4

Fig. 2.

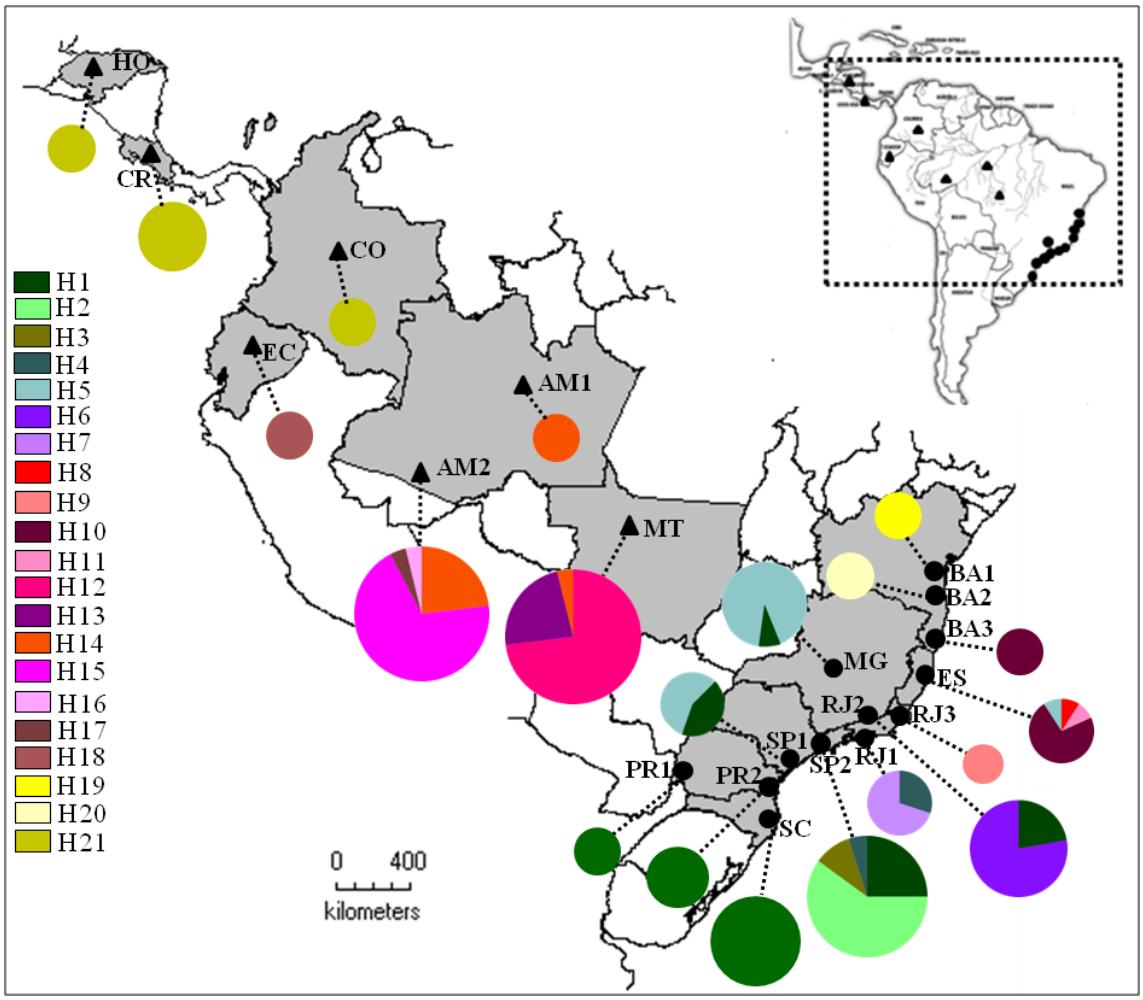
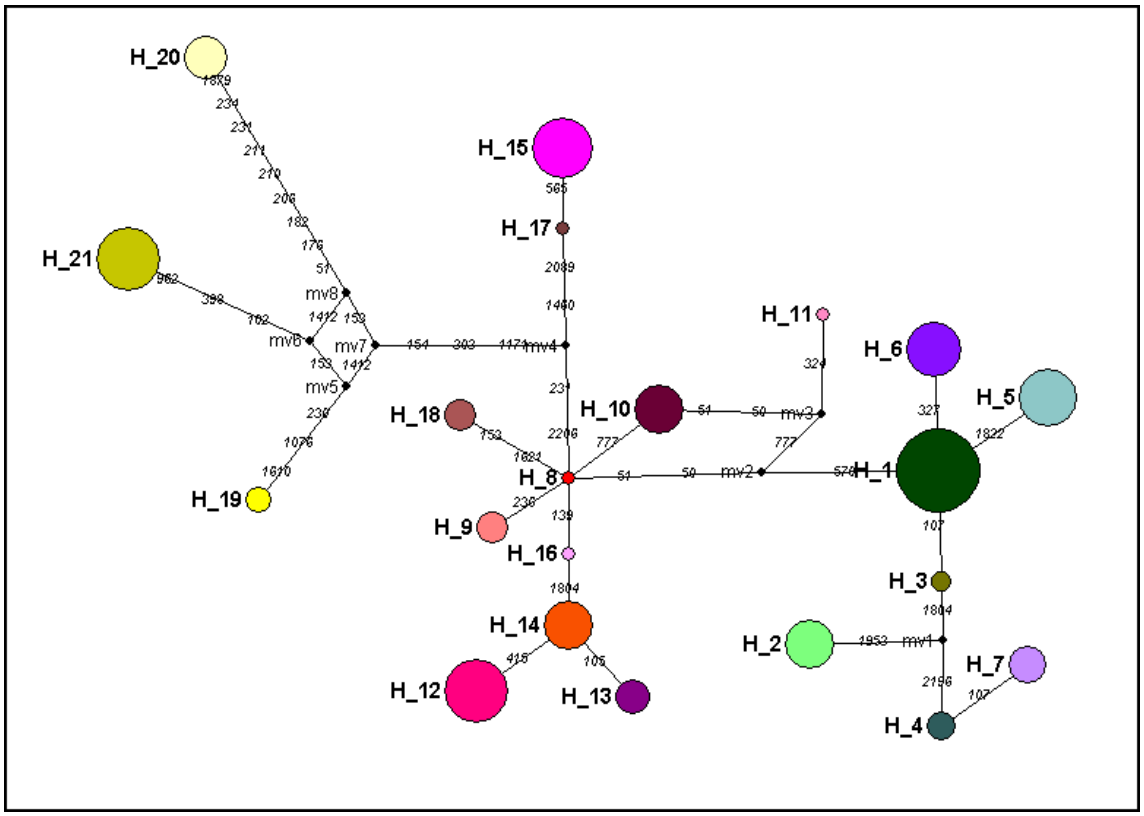

HO
CR
CO
EC
AM1
AM2
MT
BA1
BA2
BA3
MG
ES
RJ2
RJ3
SP1
SP2
RJ1
PR1
PR2
SC
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
H13
H14
H15
H16
H17
H18
H19
H20
H21
0 400
kilometers

Fig. 3.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H_15
H_17
H_21
mv8
mv6
mv7
mv4
mv5
H_11
H_6
H_5
H_18
H_10
mv3
H_8
mv2
H_1
H_19
H_9
H_16
H_3
H_14
H_2
mv1
H_7
H_12
H_13
H_4

Fig 4

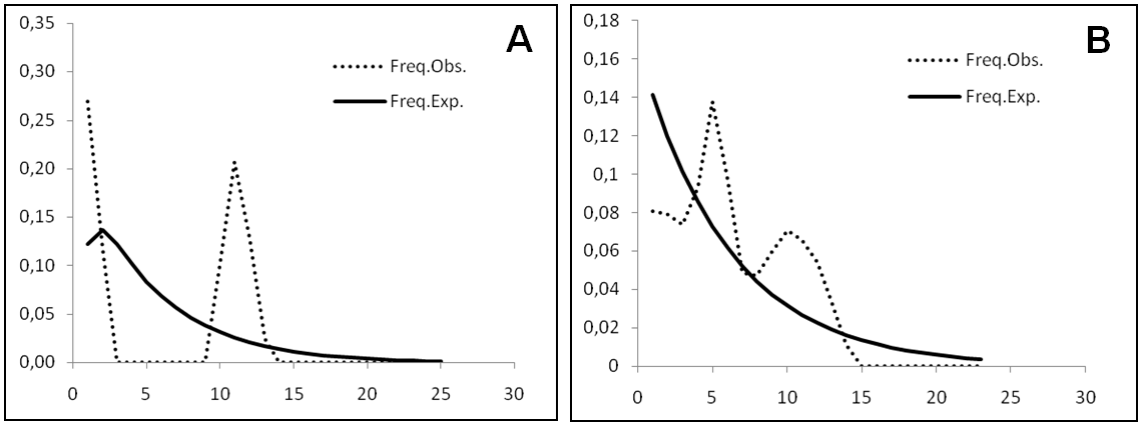
A
Freq.Obs.
Freq.Exp.
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0
5
10
15
20
25
30
B
Freq.Obs.
Freq.Exp.
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
0
5
10
15
20
25
30

Table S1

	SC	PR1	PR2	SP1	SP2	RJ1	RJ2	RJ3	ES	BA1	BA2	BA3	MT	AM1	AM2	EC	CO	CR	HO
SC																			
PR1	**0,000**																		
PR2	**0,000**	**0,000**																	
SP1	**0,000**	**0,000**	**0,000**																
SP2	**0,120**	**-0,040**	**0,040**	**0,040**															
RJ1	**0,480**	**0,190**	**0,330**	**0,330**	**-0,030**														
RJ2	0,500	**0,340**	0,410	**0,410**	**0,170**	**-0,120**													
RJ3	1,000	**1,000**	1,000	1,000	0,710	**0,450**	**0,250**												
ES	1,000	1,000	1,000	1,000	0,750	0,540	**0,300**	**0,000**											
BA1	**0,000**	**0,000**	**0,000**	**0,000**	**-0,100**	**0,120**	**0,300**	1,000	1,000										
BA2	**0,000**	**0,000**	**0,000**	**0,000**	**0,040**	**0,330**	0,410	1,000	1,000	**0,000**									
BA3	1,000	1,000	1,000	1,000	0,690	**0,390**	**0,200**	**0,000**	**0,000**	**1,000**	1,000								
MT	1,000	1,000	1,000	1,000	0,990	0,990	0,980	1,000	1,000	1,000	1,000	1,000							
AM1	1,000	1,000	1,000	1,000	0,980	0,970	0,960	1,000	1,000	1,000	1,000	1,000	**0,000**						
AM2	1,000	1,000	1,000	1,000	0,980	0,980	0,970	1,000	1,000	1,000	1,000	1,000	**0,000**	**0,000**					
EC	0,990	0,980	0,990	0,990	0,970	0,960	0,960	0,980	0,990	0,980	0,990	0,980	0,890	**0,760**	0,840				
CO	1,000	1,000	1,000	1,000	0,980	0,970	0,960	1,000	1,000	**1,000**	1,000	**1,000**	1,000	1,000	1,000	**-0,150**			
CR	1,000	1,000	1,000	1,000	0,980	0,970	0,970	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	**-0,030**	**0,000**		
HO	1,000	1,000	1,000	1,000	0,980	0,970	0,960	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	**-0,080**	**0,000**	**0,000**	

Values given in bold are not significant at P>0,05

Table S2

	SC	PR1	PR2	SP1	SP2	MG	RJ1	RJ2	RJ3	ES	BA1	BA2	BA3	MT	AM1	AM2	EC	CO	CR	HO
SC																				
PR1	**0,000**																			
PR2	**0,000**	**0,000**																		
SP1	0,616	**0,257**	**0,439**																	
SP2	0,587	**0,406**	0,494	0,552																
MG	0,913	0,839	0,871	**0,214**	0,680															
RJ1	0,912	0,824	0,863	0,822	0,565	0,906														
RJ2	0,734	**0,596**	0,657	0,680	0,666	0,832	0,869													
RJ3	1,000	1,000	1,000	0,926	0,810	0,975	0,949	0,939												
ES	0,801	**0,637**	0,712	0,715	0,732	0,809	0,825	0,810	0,595											
BA1	1,000	**1,000**	1,000	0,966	0,907	0,989	0,973	0,975	1,000	0,882										
BA2	1,000	1,000	1,000	0,983	0,936	0,994	0,985	0,983	1,000	0,940	1,000									
BA3	1,000	**1,000**	1,000	0,908	0,792	0,972	0,940	0,933	1,000	**-0,130**	**1,000**	1,000								
MT	0,914	0,878	0,891	0,888	0,838	0,915	0,890	0,910	0,839	0,768	0,941	0,963	0,827							
AM1	1,000	**1,000**	1,000	0,932	0,792	0,978	0,935	0,947	1,000	0,685	1,000	1,000	1,000	**0,412**						
AM2	0,745	0,645	0,689	0,708	0,747	0,760	0,764	0,772	0,583	0,574	0,759	0,867	0,551	0,701	0,569					
EC	1,000	1,000	1,000	0,939	0,835	0,979	0,956	0,949	1,000	0,705	1,000	1,000	1,000	0,870	1,000	0,643				
CO	1,000	1,000	1,000	0,975	0,921	0,991	0,979	0,979	1,000	0,907	1,000	1,000	1,000	0,949	1,000	0,794	1,000			
CR	1,000	1,000	1,000	0,982	0,932	0,993	0,984	0,982	1,000	0,926	1,000	1,000	1,000	0,955	1,000	0,817	1,000	**0,000**		
HO	1,000	**1,000**	1,000	0,975	0,921	0,991	0,979	0,979	1,000	0,907	1,000	1,000	1,000	0,949	1,000	0,794	1,000	**0,000**	**0,000**	

Values given in bold are not significant at P>0,05

Capítulo V: Considerações Finais

CONSIDERAÇÕES FINAIS

No ano de 2006 foi iniciado o projeto SEEDSOURCE, que visa produzir e fornecer fontes de informações para aproximadamente 50 espécies de árvores sócio-economicamente importantes, oriundas de regiões tropicais das Américas Central e do Sul. Essas informações baseiam-se em estudos sobre variação adaptativa, diversidade genética, fluxo gênico e capacidade regenerativa de 12 espécies, combinado com as informações já disponíveis, sendo interpretadas usando análise de meta-dados e procedimentos de modelagem. Com essa abordagem integrada, o projeto fornece as melhores práticas sobre as fontes de germoplasma para reflorestamento dentro de uma escala de degradação ambiental, e para árvores de diferentes nichos, que são componentes chaves da composição de diversos ecossistemas. Estas informações são individualmente adaptadas e focadas para as diferentes partes interessadas.

O projeto conta com a participação de diversos grupos de pesquisadores de Instituições de vários países: Alemanha, Brasil, Itália, Estados Unidos, Equador, Costa Rica, Guiana Francesa, França, Inglaterra, Austrália e Escócia e abrange três aspectos principais: i) estudo da biodiversidade usando marcadores não neutros, através da análise da variabilidade em genes candidatos relacionada ao estresse hídrico; ii) estudo de filogeografia, através de marcadores neutros e iii) genética de populações (diversidade intra e inter populações). *Schizolobium parahyba* (Fabaceae-Caesalpinioideae) é uma das 12 espécies estudadas no projeto SEEDSOURCCE.

Este trabalho representa o primeiro estudo genético molecular dentro do gênero *Schizolobium*. Um amplo número de marcadores cloroplásticos e nucleares foi utilizado, permitindo o entendimento da evolução, biogeografia, sistemática e filogeografia de *Schizolobium*, além de contribuírem para a compreensão de questões relacionadas à evolução e diversidade genética de outras espécies arbóreas Neotropicais. Por outro lado, o conhecimento da diversidade genética de *Schizolobium*, bem como de outras espécies arbóreas também contribui para fornecer informações necessárias sobre a origem e utilização de germoplasma, para assegurar que os sistemas de produção utilizem materiais mais adaptados, maximizando a produção, sem afetar a diversidade genética, num contexto de reflorestamento e produção de madeira. Alguns trabalhos desenvolvidos por membros do projeto SEEDSOURCE relacionados a este trabalho podem ser citados (Andre *et al.*, 2008; Cavers *et al.*, 2005a; Cavers *et al.*, 2005b; Colpaert *et al.*, 2005; Dick *et al.*, 2003; Dick *et al.*, 2007; Dick, Heuertz, 2008; Dick, Wright, 2005; Kremer *et al.*, 2005; Kremer, Reviron, 2004; Lemes *et al.*, 2007; Lemes *et al.*, 2003; Petit *et al.*, 2008).

Estudo de genes relacionados ao estresse hídrico para espécies arbóreas

Uma das abordagens do projeto SEEDSOURCE é o estudo de genes relacionados ao estresse hídrico em espécies arbóreas. O objetivo é a identificação de polimorfismos (single nucleotide polimorphisms, SNPs) em uma série de genes candidatos envolvida em estresse hídrico em plantas, tais como genes da família das aquaporinas, genes envolvidos na síntese de osmorreguladores, tais como prolina e trealose, entre outros. Um conjunto destes genes foi selecionado e as sequências gênicas presentes em diferentes tipos de plantas alinhadas para o desenho de iniciadores degenerados e posterior

amplificação em todas as espécies arbóreas de interesse. Os produtos de amplificação foram clonados e seqüenciados para o desenho de iniciadores específicos para cada espécie, para, posteriormente realizar amplificação em um grande número de indivíduos de diferentes áreas de ocorrência. Cada grupo de pesquisa do projeto SEEDSOURCE ficou responsável pelo estudo e clonagem de um gene, sendo o gene P5CS (Δ1-pirrolina 5–carboxilato sintase) envolvido na síntese de prolina um dentre os genes escolhidos.

A sequência parcial (exons 5 e 6 e o íntron 6) do gene p5cs foi clonada e sequenciada para quatro espécies arbóreas Neotropicais: *Schizolobium parahyba*, *Cedrela odorata*, *Bombacopsis quinata* e *Ceiba pentandra*. A partir disso, duas abordagens foram feitas: na primeira, realizou-se um estudo sobre a evolução desses genes em plantas, através da reconstrução filogenética dos mesmos, para tentar entender o evento de duplicação em plantas, bem como a bi-funcionalidade da enzima P5CS (Turchetto-Zolet *et al.*, 2009); na segunda, iniciadores específicos para *S. parahyba* foram desenhados com o objetivo de realizar uma análise de polimorfismo em indivíduos em toda a sua distribuição geográfica.

Para o estudo sobre a evolução dos genes P5CS, buscaram-se todas as possíveis sequências depositadas nos bancos de dados e foram construídas duas diferentes filogenias: uma utilizando somente a sequência completa de diversas plantas e a outra utilizando somente as sequências parciais, incluindo assim as sequências clonadas das 4 espécies arbóreas, bem como um maior número de plantas. Os resultados dessas análises sugerem que eventos independentes de duplicação ocorreram ao longo da história evolutiva desses genes e que a duplicação ocorreu após a divergência entre mono e dicotiledôneas. Eventos de duplicação durante a história evolutiva dos organismos, principalmente plantas, podem ocorrer nas principais enzimas de vias metabólicas. Tais

eventos de duplicação produzem cópias idênticas que atuam de maneira redundante logo após a duplicação, mas que podem sofrer alterações causando mudanças na regulação transicional e assim contribuindo para a evolução da divergência funcional. Em muitos casos, uma das cópias pode ter perda de função e não mais ser expressa. Neste trabalho, foi mostrado através de análise de expressão por RT-PCR que na espécie *S. parahyba*, as duas cópias do gene *p5cs* são expressas em condições normais. Além disso, foi estimado as taxas de *Ka/Ks* e todas as comparações dos genes P5CS duplicados entre as diferentes espécies mostrou a existência de seleção positiva, o que pode estar associado com a exposição de plantas ancestrais aos diferentes condições ambientais ao longo da evolução. A existência de duas cópias em plantas pode ser requerida para um aumento das funções fisiológicas da enzima e para melhor regular o metabolismo da prolina nas condições adversas.

Com o desenho dos iniciadores específicos, foi possível amplificar e seqüenciar essa região do gene P5CS em uma ampla amostragem de indivíduos de *Schizolobium*. Entretanto, apenas um pequeno nível de polimorfismo foi identificado e não foi possível identificar uma correlação com estresse hídrico (Veja alinhamento Anexo I). A maioria dos SNPs encontrados está localizada na região do íntron e somente dois grupos de indivíduos foram encontrados: um contendo indivíduos da variedade *parahyba* (Mata Atlântica) e o outro contendo indivíduos da variedade *amazonicum* (Floresta Amazônica). Tendo em vista a falta de polimorfismo encontrado no gene P5CS, partiu-se para o estudo da sistemática, biogeografia e filogeografia através de marcadores neutros.

Estudo de Sistemática Molecular, Biogeografia e filogeografia

Através de uma ampla amostragem de indivíduos de *Schizolobium*, representado as duas zonas de ocorrência de ambas as variedades, foi possível verificar a posição sistemática do gênero e dentro do grupo *Peltophorum,* e estimar a idade do surgimento de gênero, verificar a monofilia e produzir dados moleculares para sugerir um possível evento de especiação dentro do gênero, além de contribuir com informações pertinentes no campo da conservação.

Um total de 10 regiões do cloroplasto e uma nuclear foram analizadas. Destas, cinco regiões cpDNA e uma região nuclear foram polimórficas. Através delas foi possível construir filogenias que permitiram verificar que o gênero *Schizolobium* é monofilético e confirmar a sua posição dentro do grupo *Peltophorum*, como já havia sido verificado em análises filogenéticas anteriores do grupo (Haston *et al.*, 2003; Haston *et al.*, 2005).

A filogenia construída com sequências de ITS possibilitou verificar uma separação entre populações da variedade *parahyba* (Mata Atlântica) e populações da variedade *amazonicum* (Floresta Amazônica e América Central), com valores altos de *bootstrap* e probabilidade *posterior*. Doze sítios polimórficos foram encontrados e destes 10 estão relacionados com a separação entre as duas variedades. Os espaçadores ITS1 e ITS2 constituem uma valiosa fonte de caracteres moleculares usados para reconstruir filogenias em plantas (Bessega *et al.*, 2006; Hsiao *et al.*, 1995; Nanni *et al.*, 2004; Schnabel *et al.*, 2003; Whitcher, Wen, 2001).

Dentre as cinco regiões polimórficas do cloroplasto, quatro apresentaram um sítio que mostra a separação entre as duas variedades, mas o curioso é que populações

da região da Bahia (Nordeste do Brasil) apresentaram o mesmo padrão neste sítio do que aquelas populações da variedade *amazonicum*. Com isso, foi observado que as filogenias construídas com os diferentes genomas (nuclear e do cloroplasto) apresentaram incongruência e um caso de evolução reticulada pode ser observado. Esses resultados levam a hipótese da existência de resíduos de polimorfismo ancestral (Comes, Abbott, 2001) e evolução convergente (Davis *et al.*, 1998). Alguns exemplos de incongruências entre os genomas nucleares e cpDNA foram identificados em plantas (Kim, Donoghue, 2008; Lihova *et al.*, 2006; Morgan *et al.*, 2009; Nishimoto *et al.*, 2003; VanRaamsdonk *et al.*, 1997). No caso de *Schizolobium*, acredita-se na hipótese de retenção de polimorfismo ancestral no genoma do cloroplasto, pois existem muitas evidências da existência de conexões passadas entre as duas maiores florestas tropicais dos Neotrópicos: floresta Atlântica e floresta Amazônica, ocorridas antes da última glaciação no período do quaternário, no nordeste Brasileiro (Behling, 2002; Behling *et al.*, 2000; Behling, Lichte, 1997; Cabanne *et al.*, 2008). Acredita-se que nesta região as duas florestas mantinham contato, além de serem identificadas zonas de refúgios interglaciais nesta região (Carnaval *et al.*, 2009; Carnaval, Moritz, 2008).

A análise do gene *matK* possibilitou estimar a idade do gênero *Schizolobium* em aproximadamente 13 MY, indicando o surgimento no último Mioceno. Através do uso das cinco regiões do cloroplasto combinadas (*matK+ rpoB+rpoC1+trnL-F+psbA-trnH*) foi possível estimar a idade do clado que agrupa os indivíduos do sul e sudeste da Mata Atlântica em aproximadamente 3 MY, sugerindo que este clado surgiu no final do Plioceno. Os resultados indicam a presença de barreiras para o fluxo gênico (pólen e semente) entre as duas florestas, o que pode ser explicado pela presença do Cerrado entre elas. Na Mata Atlântica, pelos resultados revelados através dos dados nucleares, observa-se

que o fluxo de pólen é contínuo. Entretanto, pelos resultados dos dados de cpDNA, observa-se uma barreira de fluxo de sementes entre o Sul/Sudeste e Nordeste da Mata Atlântica. Os efeitos das mudanças paleogeográficas do Mioceno e do Plioceno na especiação são relativamente bem conhecidas e estão relacionados com o surgimento de barreiras e isolamento de muitas espécies (Coyne, Orr, 2004). Algumas espécies possuem a capacidade de dispersão a longas distâncias, enquanto outras podem torna-se isoladas originando novas espécies (Morrone, Crisci, 1995; Sanmartin *et al.*, 2001).

Esse estudo possibilitou verificar diferentes padrões genéticos entre as duas variedades, corroborando com os padrões morfológicos entre elas, assim como a sua ocorrência disjunta. Além disso, os resultados poderão contribuir para o estudo evolutivo das duas maiores biotas dos Neotrópicos: A Floresta Atlântica e Amazônica e auxiliar nos esforços para a conservação genética nessas áreas.

Esse trabalho permitiu a identificação e interpretação dos padrões de diversidade dos genomas nuclear e cloroplasto de *Schizolobium,* uma importante espécie arbórea nativa de floretas Neotropicais, ao longo de uma ampla distribuição geográfica. Indivíduos foram coletados dos mais diversos locais de ocorrência da mesma e a estrutura geográfica da diversidade genética foi realizada. Um gene e dois espaçadores intergênicos do cloroplasto e as regiões ribossomais ITS foram analisados em um grande número de indivíduos de *Schizolobium* representando as duas variedades AM uma ampla área de ocorrência. Os resultados apresentados na presente tese indicaram que a diversidade intraspecífica do genoma do cloroplasto foi bem maior do que a encontrada para o genoma nuclear e possivelmente haja um restrito fluxo gênico mediado pela semente em relação ao fluxo de pólen, o que pode ser observado na baixa diversidade genética do cpDNA das populações do Sul da Mata Atlântica (SC, PR1, PR2 e SP1), Colômbia (CO) e América

Central (CR e HO) em relação as demais populações. Esses dados corroboram com a dispersão restrita observada para sementes anemocóricas.

Foram encontrados 21 haplótipos com a combinação dos espaçadores do cloroplasto *psbA-trnH+trnL-trnF* e o gene *matK*, enquanto que com o ITS, apenas 4 haplótipos foram identificados. Desses 4 haplótipos, dois estão presentes em populações da variedade *parahyba* (Mata Atlântica) e dois em populações da variedade *amazonicum* (base da floresta Amazônica e América Central) e nenhum haplótipo foi compartilhado entre as duas variedades. Uma separação bem consistente foi observada entre as duas variedades com esse marcador, corroborando com a distribuição geográfica disjunta e as diferenças na morfologia da flor. Além disso, os valores de F_{ST} foram altos (0,859). Os haplótipos encontrados para o genoma do cloroplasto também não são compartilhados entre as duas variedades, apesar de ser observada uma maior proximidade das populações RJ3, ES (sudeste do Brasil) e BA1, BA2 e BA3 (nordeste do Brasil) com aquelas da variedade *amazonicum* (base da Amazônia e América Central). Esse resultado não foi observado com o genoma nuclear, o que indica que no passado possivelmente houve contato entre as florestas Atlântica e Amazônica e que esse contato provavelmente ocorreu próximo a Região Nordeste. Esses resultados estão de acordo com alguns trabalhos que relatam as interconexões entre essas florestas antes dos eventos de mudanças climáticas ocorridas no quaternário (Behling, 1995; Behling, 1998; Behling, 2002; Behling *et al.*, 2000; Behling, Lichte, 1997; Cabanne *et al.*, 2008).

Ao contrário da baixa diversidade encontrada nas populações do Sul da Mata Atlântica, da Colômbia e da América Central, um alto grau de diversidade foi observado nas populações localizadas na região sudeste da Mata Atlântica (SP, ES, BA) e na floresta Amazônica (AM2, MT), indicando que essas regiões possivelmente estão

relacionadas com zonas de refúgios. Na Mata Atlântica, trabalhos com espécies animais têm demonstrado que a região de São Paulo e da Bahia correspondem a refúgios e associadas com *hotspots* de conservação dentro do bioma (Carnaval *et al.*, 2009; Carnaval, Moritz, 2008).

Capítulo VI: Referências Bibliográficas dos Capítulos I e V

Referências Bibliográficas

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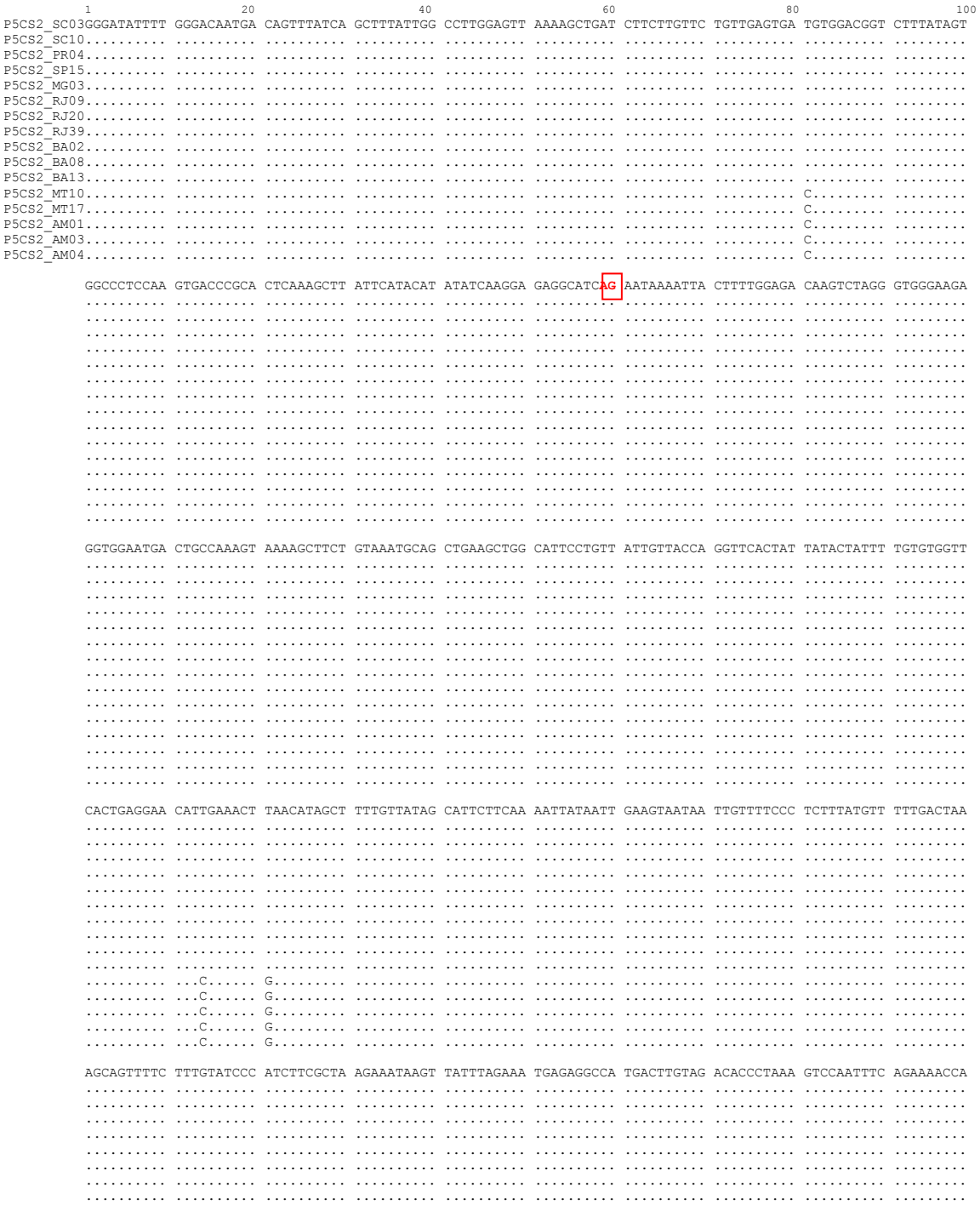
ANEXO I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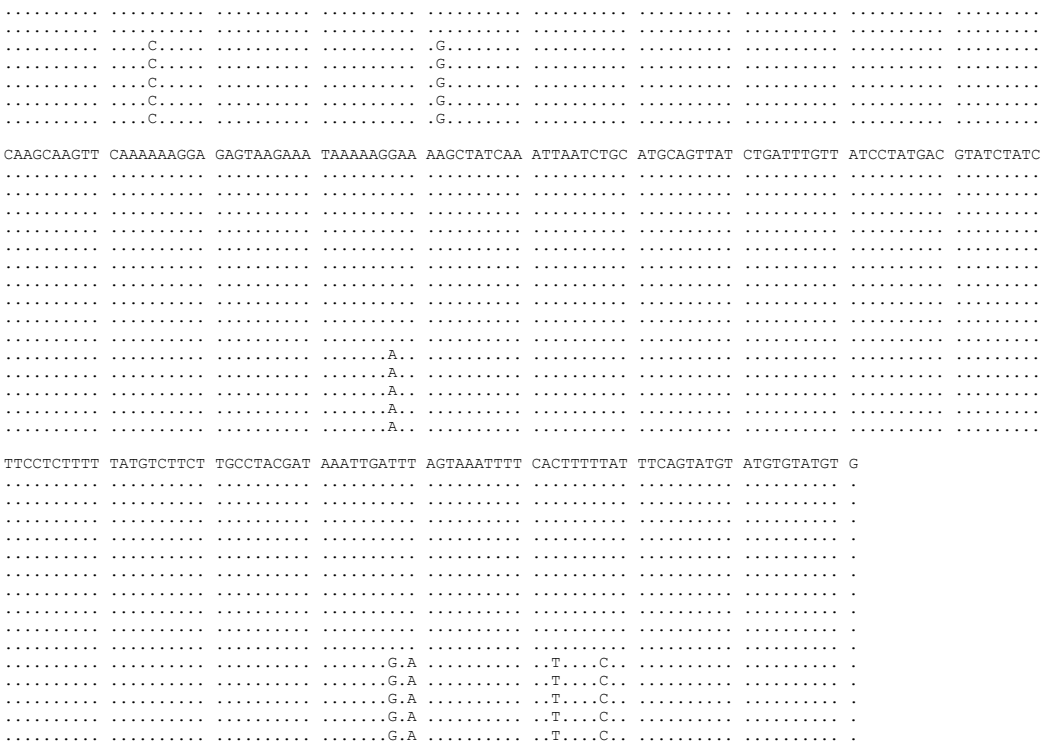

Fig. 1. Alinhamento do exon 5 e intron 6 do gene P5CS2, mostrando os polimorfismos encontrados entre indivíduos de *Schizolobium* de diferentes localidades. A caixa vermelha indica o início do íntron 6.