Mariana Vargas Cruz

Isolamento de microssatélites, taxonomia molecular e diversidade genética de *Plathymenia* (Leguminosae)

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

Orientadora: Prof. Dra. Maria Bernadete Lovato
Universidade Federal de Minas Gerais

Belo Horizonte

Instituto de Ciências Biológicas

2012

Dedico este trabalho aos meus pais.

Agradecimentos

Agradeço à FAPEMIG (APQ-01741-10) ao CNPq (472423/2010-0) e ao PRONEX (História Natural CBB-APQ-00336-09) pelo suporte financeiro, e à CAPES pela bolsa de mestrado concedida desde setembro de 2010.

À Prof. Dra. Maria Bernadete Lovato, por ter aceitado me orientar, por todos os ensinamentos, por ter acreditado em mim e me dado a chance de poder desenvolver este trabalho junto a ela. Sua dedicação e gosto pela área de pesquisa em que atua são para mim motivo de admiração e inspiração que contribuíram e que certamente ainda irão contribuir muito para as minhas decisões profissionais futuras.

Aos meus pais, por sempre terem me dado total apoio nas escolhas que fiz, por todo o incentivo e investimento, imprescindíveis para cada uma de minhas conquistas, pela educação que me deram e pelo o amor, carinho, paciência e dedicação ilimitados.

Aos amigos e professores dos grupos de pesquisa dos laboratórios GenePop, LDGH e LBEM pelos ensinamentos teóricos e práticos laboratoriais, que me foram passados de forma esclarecedora e muito paciente, indispensáveis para a realização da minha pesquisa. A todas as meninas e aos (escassos) meninos do GenePop, sou infinitamente grata pela amizade, pelo amor, pela convivência extremamente agradável, pelo companheirismo, pelos ombros e abraços nos momentos difíceis e pelas constantes risadas, que me encorajaram e facilitaram o meu processo.

Agradeço ao Juliano Leal e ao Anderson Chaves também pela amizade e pelo suporte na geração da biblioteca genômica de *Plathymenia*, no sequenciamento e nas genotipagens. Ao Prof. Dr. José Pires de Lemos Filho e ao amigo Marcos Toledo, pela ajuda no delineamento do projeto e nas coletas.

A todos os professores e aos profissionais da secretaria e da limpeza, agradeço por colaborarem para o bom funcionamento da pós-graduação em Genética da UFMG.

E, por fim, agradeço a todos os amigos de Vitória e de BH, especialmente ao meu irmão, à Marlene, à Camila, à Maria, à Gabi e à Carol, que acompanharam esse processo mais de perto, me deram forças pra conseguir chegar até aqui, me divertiram e me fizeram rir. Foi fundamental.

Índice

RESUMO GERAL 1

GENERAL ABSTRACT 2

INTRODUÇÃO GERAL 3

REFERÊNCIAS BIBLIOGRÁFICAS 8

CAPÍTULO 1 "Isolation and characterization of microsatellite markers for *Plathymenia reticulata* Benth. (Leguminosae)" 10

ABSTRACT 11

INTRODUCTION 12

METHODS AND RESULTS 13

CONCLUSIONS 16

TABLES 17

LITERATURE CITED 20

CAPÍTULO 2 "Genetic differentiation and gene flow between forest and savanna populations of a neotropical tree: revisiting the case of *Plathymenia*" 22

ABSTRACT 23

INTRODUCTION 24

MATERIAL AND METHODS 27

Characterization of the collect sites and sampling 27

DNA isolation, amplification and genotyping 28

Data analysis 29

RESULTS 30

TABLES ……..... 34

FIGURES ……..… 40

DISCUSSION ……..…. 43

LITERATURE CITED ……..…… 48

CONCLUSÕES FINAIS …… 54

Resumo

A taxonomia do gênero *Plathymenia*, leguminosa arbórea amplamente distribuída pela América do Sul, tem sido muito discutida desde sua descrição. Na mais recente revisão taxonômica do gênero, as duas espécies originalmente descritas foram consideradas uma só espécie. Alguns anos depois, estudos ecofisiológicos demonstraram a existência de diferenças adaptativas relacionadas às diferenças ambientais entre Mata Atlântica e Cerrado, o que levou os pesquisadores a considerar a existência de ecótipos relativos aos dois biomas. No presente estudo, marcadores microssatélites foram desenvolvidos especificamente para *Plathymenia* e caracterizados em 51 indivíduos presentes em duas populações amostradas em áreas de Cerrado. Os loci isolados demonstraram alto grau de polimorfismo e foram ainda utilizados a fim de investigar a ocorrência de fluxo gênico entre populações presentes na Mata Atlântica e no Cerrado. Um total de 159 indivíduos foram amostrados em duas populações localizadas em áreas core de Cerrado, em duas populações de áreas core de Mata Atlântica, e em quatro outras populações, localizadas em regiões ecotonais, onde ocorre a transição de um bioma para o outro. Foi verificado que o habitat em que as populações se encontram está mais relacionado com a diferenciação genética do que a distância geográfica que existe entre as populações. Ao comparar as populações presentes nas áreas core da Mata Alântica e do Cerrado, foram encontradas diferenças genéticas que estão de acordo com a classificação dos indivíduos em dois ecótipos adaptados a condições ambientais distintas encontradas em cada um dos biomas. Contudo, altos níveis de fluxo gênico foram observados nas populações presentes em áreas ecotonais, indicando que não existem mecanismos de isolamento reprodutivo completo entre os ecótipos de *Plathymenia*. Nossos resultados corroboram, portanto, com a última revisão taxonômica que considera o gênero *Plathymenia* monoespecífico.

Palavras chave: Cerrado, ecótipos, ecótones, hibridização, Mata Atlântica, microssatélites, *Plathymenia reticulata*.

Abstract

The taxonomy of the genus *Plathymenia*, leguminous tree widely distributed in South America, has been much discussed since its description. In the most recent taxonomic revision of the genus, the two species originally described were considered a single species. Some years later, ecophysiological studies have demonstrated the existence of adaptive differences related to environmental differences between Atlantic Forest and Cerrado, which led researchers to consider the existence of different ecotypes between the two biomes. In the present study, microsatellite markers were developed specifically for *Plathymenia* and characterized in 51 individuals present in two populations sampled in areas of Cerrado. The isolated loci showed a high degree of polymorphism and were further used to investigate the occurrence of gene flow between populations present in the Atlantic Forest and Cerrado. A total of 159 individuals were sampled in two populations located in core areas of Cerrado, in two populations of core areas of Atlantic Forest, and in four other populations, located in ecotonal regions where the transition from one biome to another occurs. It was found that the habitat in which populations were sampled more is more related to the genetic differentiation found than the geographical distance between populations. When comparing the populations present in the core areas of Cerrado and Atlantic Forest we have found genetic differences that are consistent with the classification of individuals into two ecotypes adapted to different environmental conditions found in each of the biomes. However, high levels of gene flow were observed in the populations present in the ecotone areas, indicating that there are no mechanisms for complete reproductive isolation between the two ecotypes *Plathymenia*. Our results corroborate, therefore, with the latest taxonomic revision that considers the genus *Plathymenia* monospecific.

Key words: Cerrado, ecotypes, ecotone, hybridization, Atlantic forest, microsatellite, *Plathymenia reticulata*.

Introdução Geral

A diversidade genética de uma espécie e sua estruturação espacial são determinadas tanto por eventos históricos como recentes (Schaal *et al.,* 1998; Ribeiro *et al.,* 2010). Investigar os perfis de diversidade genética e dos fatores que os influenciam é fundamental para entender os processos evolutivos, bem como para fornecer informações para a implementação de estratégias de conservação e restauração de populações (Moritz, 1994; Crandall *et al.*, 2000) e para o manejo e uso sustentado da flora nativa, de maneira a evitar erosão genética nas populações naturais.

Um dos marcadores moleculares mais informativos utilizados para estudos genético-populacionais em populações naturais são os microssatélites, que consistem em sequências simples de nucleotídeos repetidas em tandem (SSR, *Simple Sequence Repeat*) contendo no máximo 100pb, ampla e uniformemente distribuídas pelo o genoma e que apresentam geralmente alto grau de polimorfismo, resultante da presença de diferentes números de elementos simples repetidos (Ferreira e Grattapaglia, 1996). A grande adequação dos microssatélites para os estudos de estrutura genética e fluxo gênico deve-se ao fato desses marcadores apresentarem características favoráveis para este fim, como: 1) Codominância; 2) Alto grau de polimorfismo, com grande conteúdo informativo por cada *locus* analisado.

Os *loci* microssatélites são identificados por amplificação da região alvo por meio da Reação em Cadeia da Polimerase (PCR), seguida por eletroforese em gel de poliacrilamida de alta resolução ou pelo uso de sequenciador de DNA, que permite distinguir alelos que diferem em tamanho, muitas vezes por poucos pares de bases. Para se identificar regiões SSR é feita uma triagem da biblioteca genômica de um indivíduo representante da espécie de interesse com sondas biotiniladas de oligonucleotídeos repetidos i*n tandem*, que são recuperadas e clonadas em um vetor, para subsequente seqüenciamento dessas regiões (Ferreira e Grattapaglia, 1996). Uma desvantagem dos microssatélites é que identificar as regiões com esses *loci* numa biblioteca genômica envolve grande quantidade de trabalho laboratorial e

demanda um tempo considerável. Entretanto, uma vez desenhados e sintetizados os iniciadores, o protocolo consiste basicamente em PCRs, eletroforeses e, finalmente, genotipagem das amostras. Além disso, tem sido observado, tanto em plantas como em animais, que vários iniciadores de microssatélites desenvolvidos para uma espécie podem amplificar *loci* de espécies próximas (Dayanandan *et al.,* 1997; Parker *et al.,* 1998), o que justifica ainda mais o esforço para o desenvolvimento desses marcadores.

Com um conjunto de *loci* microssatélites ainda se pode eficientemente realizar identificação individual, análise de paternidade e parentesco entre indivíduos ou diferenciação entre populações, podendo fornecer informações de *loci* individuais para cálculo de frequências alélicas em estudos populacionais (Parker *et al.,* 1998) e inferir sobre a história evolutiva das populações, uma vez que ajudam a compreender a fronteira da espécie e verificar hibridizações intraespecíficas (Lexer *et al.,* 2005; Drummond e Hamilton, 2007). Deve-se citar também a capacidade de detectar os níveis de endogamia das populações, fator altamente importante para avaliar os problemas advindos da depressão endogâmica. Devido à sua alta taxa de evolução, os microssatélites são muito promissores para análise do efeito de eventos recentes, como da fragmentação de habitats por atividades antrópicas, na diversidade genética intrapopulacional, na estrutura genética e no fluxo gênico (White *et al.,* 1999; Collevatti *et al.,* 2001).

O gênero *Plathymenia* (Leguminosae, Mimosoideae) descrito por Benth. (1842) é exclusivamente neotropical, nativo da América do Sul, onde ocorre amplamente pelo Cerrado e pela a Mata Atlântica (Warwick e Lewis, 2003) (Fig.1). No cerrado, os indivíduos crescem principalmente no cerradão, mas também no cerrado *stricto sensu*. Na Mata Atlântica, ocorrem principalmente na floresta semi-decídua, mas também são encontrados na floresta pluvial. Os indivíduos são fenotipicamente distintos nos dois biomas, podendo atingir 30 m de altura na Mata Atlânica, mas não ultrapassam 12 m no Cerrado (Lorenzi, 1992; Warwick e Lewis, 2003). Algumas características anatômicas da madeira também diferenciam indivíduos presentes em biomas distintos (Toledo, 2010). As árvores são decíduas, hermafroditas, polinizadas por abelhas, e suas sementes são dispersadas pelo vento (Warwick e Lewis, 2003; Goulart *et al.*, 2005). *Plathymenia reticulata* foi considerada

pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) como uma das mais importantes e úteis espécies vegetais do Cerrado (Almeida *et al.,* 1998) devido à alta qualidade de sua madeira e ao seu potencial uso para a recuperação de áreas degradadas (Heringer e Ferreira, 1972). Sua madeira tem sido utilizada para diversos fins, incluindo confecção de mobiliário de luxo, acabamentos internos na construção civil e postes de cerca duradouros, tendo, portanto, significativa importância econômica (Heringer e Ferreira, 1972; Lorenzi, 1992).

Desde a descrição do gênero, duas espécies de *Plathymenia* eram comumente reconhecidas: *P. reticulata*, ocorrendo no Cerrado e *P. foliolosa*, na Mata Atlântica, ambas conhecidas popularmente como Vinhático. As principais características utilizadas para a diferenciação entre as espécies eram o habitat, a altura da copa, o indumento da inflorescência, o número de folíolos por folha e o número de foliólolos por folíolo (Heringer, 1956; revisado por Warwick e Lewis, 2003). Contudo, em 2003, Warwick e Lewis propuseram a sinonimização das duas espécies, adotando o epíteto *P. reticulata*. Por meio de análises de exsicatas de ampla abrangência geográfica, eles não encontraram correlação entre morfologia e distribuição geográfica, tampouco características que diferenciassem as duas espécies. Todos os principais caracteres diagnósticos usados para diferenciá-las variavam de forma gradativa entre indivíduos das duas espécies. Eles ainda utilizaram a população híbrida relatada no trabalho de genética populacional desenvolvido com marcadores RAPD por Lacerda *et al.* (2002) como uma evidência da ocorrência de fluxo gênico entre as duas espécies.

Num estudo sobre a fenologia de *P. reticulata*, Goulart *et al.* (2005) observaram maior variação na fenologia dentro das populações e dos biomas do que entre os diferentes biomas. Foi observado ainda que populações de biomas distintos florescem em sincronia, o que favorece, portanto, a ocorrência de fluxo gênico em regiões ecotonais. Num outro estudo que comparou a morfologia do fruto e da semente em populações de Cerrado e de Mata, Goulart *et al.* (2006) observaram, contudo, que algumas características adaptativas mostraram-se significativamente distintas entre populações dos dois biomas, embora valores intermediários tenham sido relatados para populações de regiões ecotonais. Os

autores sugeriram a existência de dois ecótipos da espécie, um adaptado ao Cerrado e o outro, à Mata Atlântica.

Em um trabalho sobre a filogeografia de *P. reticulata* com marcadores de cpDNA (Novaes *et al.,* 2010), não foram encontradas diferenças entre populações da Mata (antiga *P. foliolosa*) e do Cerrado. Entretanto, o DNA de cloroplasto tem herança materna e baixa taxa de mutação, não sendo um bom marcador para a discriminação de táxons filogeneticamente próximos, uma vez que espécies recentemente separadas podem compartilhar polimorfismos ancestrais. Desta forma, este estudo não foi conclusivo a respeito da circunscrição taxonômica do gênero *Plathymenia*.

Estudos sobre os fatores ambientais associados com diferenças genéticas e fenotípicas entre populações são importantes para compreender os processos evolutivos. Informações genéticas ajudam a resolver incertezas taxonômicas e a definir unidades de manejo dentro de espécies, as quais têm implicações diretas para a conservação (Frankham *et al.,* 2002). Os estudos com marcadores moleculares, notadamente os microssatélites, podem ser utilizados para auxiliar, em conjunto com outros marcadores e com informações a respeito da ecologia das espécies, na sua delimitação taxonômica.

Este trabalho analisou populações de *Plathymenia reticulata* presentes em áreas core do Cerrado e da Mata Atlântica, bem como populações de áreas ecotonais e teve os seguintes objetivos:

(1) Isolar e caracterizar marcadores microssatélites polimórficos na espécie;

(2) Estimar a diversidade genética das populações, utilizando os marcadores microssatélites desenvolvidos;

(3) Verificar o grau de divergência entre as populações dos biomas Mata Atlântica e Cerrado e testar a hipótese de fluxo gênico entre populações dos dois biomas, utilizando os marcadores microssatélites desenvolvidos.

Desta forma, pudemos testar a hipótese de ocorrência de fluxo gênico entre populações de *Plathymenia* do Cerrado e da Mata Atlântica e a hipótese de que ambas as formas devem ser consideradas uma única espécie, de acordo com a

última revisão taxonômica do gênero, proposta por Warwick e Lewis (2003). Medidas de conservação para *P. reticulata* são sugeridas na conclusão do trabalho, com base nos resultados obtidos.

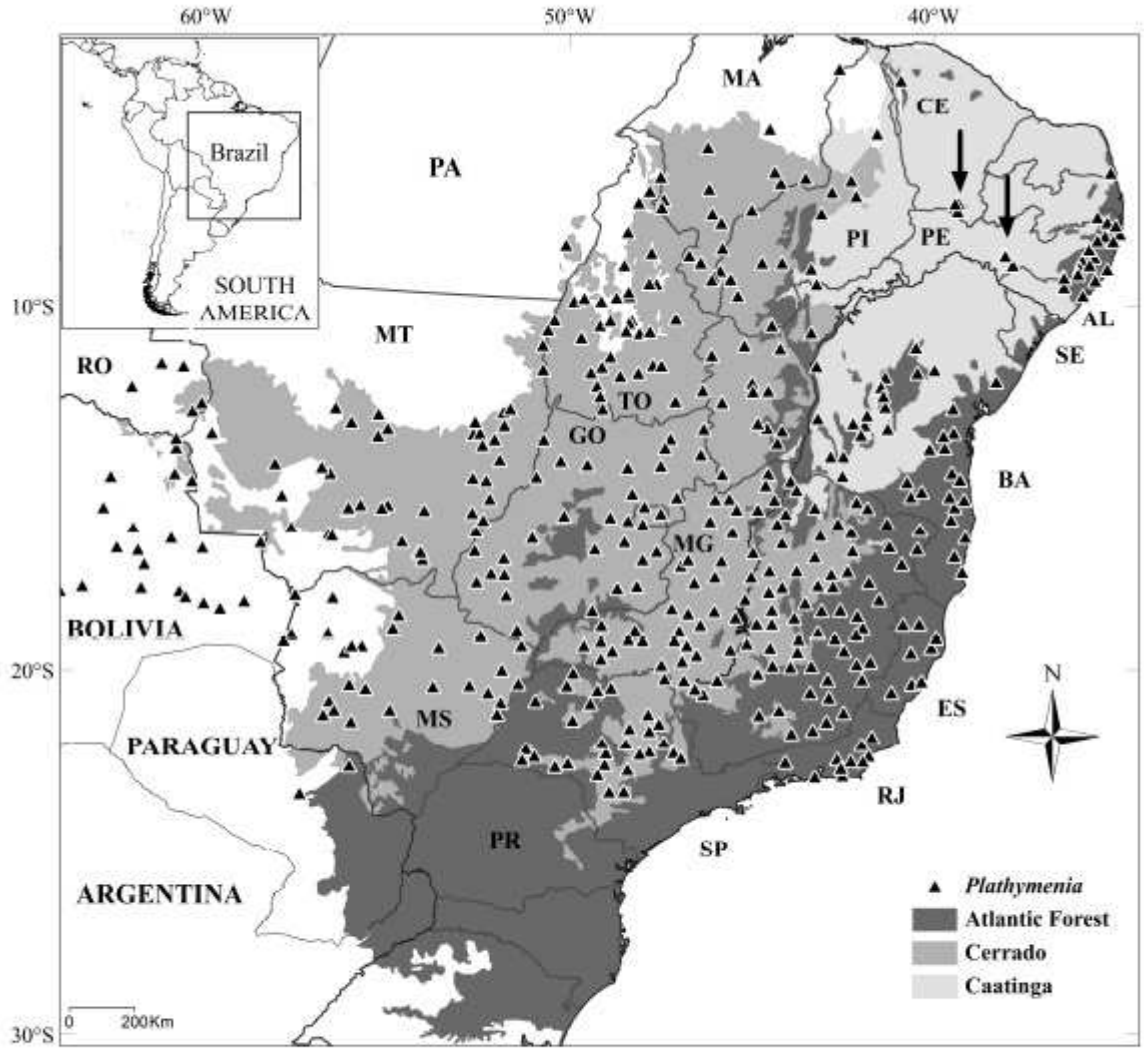

Fig. 1: Distribuição de *Plathymenia reticulata*. A espécie não ocorre na Caatinga, as setas indicam enclaves de Mata Atlântica. (Fonte: Novaes, R. M. L., 2009).

Estrutura da Dissertação

Este estudo apresenta dois capítulos, ambos redigidos na forma de artigos científicos. O Capítulo 1, “Isolation and characterization of microsatellite markers for *Plathymenia reticulata* Benth. (Leguminosae)” apresenta o isolamento e caracterização de nove marcadores microssatélites específicos para *Plathymenia*, utilizando biblioteca enriquecida. Esse artigo foi aceito para publicação no periódico *American Journal of Botany*. O Capitulo 2, intitulado “Genetic differentiation and gene flow between forest and savanna populations of a neotropical tree: revisiting the case of *Plathymenia*” analisa a diversidade genética, a divergência e o fluxo gênico entre populações de áreas core da Mata Atlântica e no Cerrado, e de áreas ecotonais entre esses biomas.

Referências Bibliográficas

Almeida SP, Proença CEB, Sano SM (1998) Cerrado: Espécies Vegetais Úteis EMBRAPA - CPAC, Planaltina, DF.

Bentham G (1842) On *Mimoseae. Hook. Journ. Bot.*, **4**, 333-334.

Collevatti RG, Grattapaglia D, Hay JD (2001) Population genetic structure of the endangered tropical tree species *Caryocar brasiliense*, based on variability at microsatellite loci. Molecular Ecology 10: 349-356.

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000). Considering evolutionary processes in conservation biology. Trends in Ecology and Evolution 15: 90-295.

Dayanandan S, Bawa KS e Kesseli R (1997) Conservation of microsatellites among tropical trees (Leguminosae). American Journal of Botany 84:1658-1663.

Drummond CS, Hamilton MB (2007) Hierarchical components of genetic variation at a species boundary: population structure in two sympatric varieties of Lupinus microcarpus (Leguminosae). Molecular Ecology 16: 753–769.

Ferreira ME, Grattapaglia D (1996) Introdução ao uso de marcadores moleculares em análise genética. 2ª. ed. Brasília, EMBRAPA/CENARGEN, 220 pp.

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to Conservation Genetics. Cambridge University Press.

Goulart MF, Lemos JP, Lovato MB (2005) Phenological variation within and among populations of *Plathymenia reticulata* in Brazilian Cerrado, the Atlantic Forest and transitional sites. Annals of Botany 96, 445-455.

Heringer EP (1956) O gênero *Plathymenia.* Anais da Sociedade Botânica do Brasil 7, 55-64. 2231–2242.

Heringer EP, Ferreira MB (1972) Árvores úteis no cerrado (I): Vinhático: o gênero P*lathymenia* Benth. *P. foliolosa* Benth. e *P. reticulata* Benth., vinhático da mata e vinhático do campo (par vicariante). Cerrado 5, 28-34.

Lacerda DR, Lemos JP, Acedo MDP, Lovato MB (2002) Molecular differentiation of two vicariant neotropical tree species, *Plathymenia foliolosa* and *P. reticulata* (Mimosoideae), inferred using RAPD markers. Plant Systematics and Evolution 235: 67-77.

Lexer C, Fay MF, Joseph JA, Nica MS, Heinze B (2005) Barrier to gene flow between two ecologically divergent Populus species, P. alba (white poplar) and P. tremula (European aspen): the role of ecology and life history in gene introgression. Molecular Ecology 14: 1045–1057.

Lorenzi H (1992) Árvores Brasileiras. Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil. Plantarum Ltda, Nova Udessa, SP.

Moritz C (1994). Defining Evolutionary Significant Units for conservation. Tree 9: 373-375.

Novaes R M L, Lemos filho JP, Ribeiro RA, Lovato MB (2010) Phylogeography of *Plathymenia reticulata* (Leguminosae) Reveals patterns of recent range expansion towards northeastern Brazil and southern Cerrados in Eastern Tropical South America. Molecular Ecology 19: 985-998.

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA (1998) What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79:361-382.

Ribeiro RA, Lemos-Filho, J.P, Ramos, ACS, lovato MB (2010) Phylogeography of the rosewood *Dalbergia nigra* (Fabaceae): insights into the evolutionary history and conservation of the Brazilian Atlantic Forest. Heredity (published on line on June 2, 2010): 1-12. DOI: 10.1038/hdy.2010.64.

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. Molecular Ecology 7:465-474.

Toledo MM (2010) Anatomia da madeira, incremento radial, fenologia e relações hídricas em ecótipos de *Plathymenia reticulata* Benth. (Leguminosae, Mimosoideae). Tese de doutorado, Universidade Federal de Minas Gerais, Belo Horizonte.

Warwick MC, Lewis GP (2003) Revision of *Plathymenia* (Leguminosae-Mimosoideae). Edinburgh Journal of Botany 60, 111-119.

CAPÍTULO 1

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *PLATHYMENIA RETICULATA* (FABACEAE)

Mariana V. Cruz, Jacqueline G. Rodrigues, Helena A. V. Souza and Maria Bernadete Lovato[1].

Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brasil.

[1]Autor para correspondência: lovatomb@icb.ufmg.br

ABSTRACT

Premise of the study: Microsatellite loci were isolated and characterized for use in population genetic studies of *Plathymenia reticulata* (Fabaceae), a tropical tree widespread in the Atlantic Forest and Cerrado biomes of South America.

Methods and results: Nine microsatellite markers were developed using a SSR-enriched library. Polymorphism was analyzed in 51 individuals from two populations. All loci were polymorphic, with the number of alleles per loci ranging from 5 to 15 (mean number of alleles was 10.22). Observed and expected heterozygosities per loci and population ranged from 0.313 to 1.000 and 0.280 to 0.869, respectively.

Conclusions: These highly informative loci are potentially useful to estimate population genetic structure and to understand evolutionary processes and taxonomy of the species.

Key-words: Genetic diversity; microsatellite; *Plathymenia reticulata*.

INTRODUCTION

Plathymenia reticulata Benth. is a neotropical tree, native to South America, widespread in Atlantic Forest (a tropical forest) and Cerrado (a savanna vegetation). It is a hermaphroditic tree that exhibits entomophily (by most generalist wasps and bees) and anemochory (Warwick and Lewis, 2003). The species can be used for restoration efforts and its high quality wood is suitable for joinery and luxurious furnishings (Lorenzi, 1992). Due to habitat and to morphological differences, the genus was traditionally comprised by two vicariant species: *P. reticulata*, in Cerrado and *P. foliolosa*, in the Atlantic Forest. However, in 2003, Warwick and Lewis proposed the synonimization of the two species. Based on the analysis of herbarium specimens covering a wide geographic range, they did not find an association between morphology and geographical distribution, nor features to differentiate species.

In a study about phylogeography of *P. reticulata*, chloroplast DNA sequences did not differentiate forest populations (formerly recognized as *P. foliolosa*) from the savanna populations (Novaes *et al.*, 2010). However, cpDNA is maternally inherited and has a low mutation rate, being therefore not a good marker for discriminating closely related taxa, as recently separated species may share ancestral polymorphisms. Thus, our objective was to isolate microsatellites for *Plathymenia reticulata*, because they are powerful markers for analyzing effects of recent events in genetic structure at several spatial scales, population differentiation, gene flow and also to study the molecular taxonomy of *Plathymenia*.

METHODS AND RESULTS

The genomic DNA of one individual of *Plathymenia reticulata* from Belo Horizonte, Brazil, was extracted using the CTAB buffer protocol to construct the microsatellite enriched library following the protocol described by Billotte *et al.* (1999). Vouchers of this individual and of the sampled populations were deposited in the herbarium of the Departamento de Botânica da Universidade Federal de Minas Gerais – BHCB (see Appendix 1 for voucher information). Approximately 5µg of DNA were digested with the restriction enzyme *RsaI* (Promega, Madison, Wisconsin, USA). Fragments resulting from digestion were ligated to adapters *Rsa21 (3' CTCTTGCTTACGCGTGGACTA 5')* and *Rsa25 (3' TAGTCCACGCGTAAGCAA 5')*. Subsequently, the digested DNA was subjected to polymerase chain reaction (PCR) using primers with sequences complementary to the sequences of the adapters. PCR products were purified with PEG 8000. To select the fragments with microsatellites, genomic DNA was enriched in repetitive biotinylated oligonucleotide probes - $(CT)_8$, $(GT)_8$, $(GATA)_4$ and $(GACA)_4$. Fragments hybridized to oligos were captured using magnetic beads coated with streptavidin (Promega). After the selection, another PCR was performed and the selected fragments were cloned in the plasmid vector pGEM-T Easy Vector (Promega) and transformed into competent XL1-blue *Escherichia coli*. Transformed cells were plated on agar with X-galactosidase. Single white colonies were submitted to PCR using the universal M13 forward and reverse primers.

Three hundred eighty-four positive clones were sequenced using M13 universal primers and the DYEnamic ET-terminator Kit (GE Healthcare, Buckinghamshire, United Kingdom) on a MegaBACE 1000 automated DNA

sequencer (GE Healthcare). Sequences were analyzed in Mega 4.0 (Tamura *et al.*, 2007) to delete the plasmid sequence. Repeats were found with the Gramene Project's SSR identification tool (http://www.gramene.org/db/markers/ssrtool). A total of 25 primer pairs were designed using the software Primer 3 Plus (Untergasser *et al.*, 2007) for amplifying fragments containing microsatellite inserts with ten or more repeat motifs. Forward primers were appended with a M13 tag (5'-TTTTCCCAGTCACGAC-3') to allow amplification with a tailed fluorescent dye M13 primer for subsequent genotyping.

Polymorphism was analysed in fifty-one individuals from two Cerrado core populations of *P. reticulata*: Pirenópolis (PRC, 15°48'44"S 48°53"28"W) and Várzea da Palma (VZC, 17°42'37"S 44°41'24"W). Genomic DNA was amplified using the following conditions: 10–20 ng of DNA, PCR buffer (50 mM KCl, 10 mM Tris-HCl pH8.4, 0.1% Triton X-100, Phoneutria, Belo Horizonte, Brazil), 2.5 mM of $MgCl_2$ for primers Pre5 and Pre8 and 0.86 mM of $MgCl_2$ for the remaining primers, 0.25 mM of each dNTP, 0.05 µM forward primer, 0.10 µM reverse primer, 0.10 µM M13 fluorescent-labeled primer, 1U Taq polymerase (Phoneutria), totalizing a volume of 13µL. A Mastercycler thermocycler (Eppendorf, Hamburg, Germany) was used in the following cycling conditions for primers Pre9, Pre11 and Pre15: initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 54–66°C depending on the primer (Table 1) for 1 min, extension at 72°C for 1 min followed by 8 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 50 min. To amplify loci Pre5, Pre8, Pre10, Pre16, Pre23 and Pre25, other PCR parameters were used: initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 58–67°C

depending on the primer (Table 1) for 1 min, extension at 72°C for 1 min followed by 25 cycles at 94°C for 30 s, 52°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 50 min.

Genotyping was performed on a MegaBACE 1000 automated sequencer, using 0.1% Tween 20 and ROX-500 Size Standard (GE Healthcare). Alleles were identified using the MegaBACE Fragment Profiler version 1.2 software (GE Healthcare). Observed and expected heterozygosties, number of alleles and deviations from Hardy– Weinberg Equilibrium were estimated for each locus and each population, using the software Arlequin version 3.1 (Excoffier *et al.*, 2005). With Cervus version 3.0 (Kalinowski *et al.*, 2007), two probabilities of paternity exclusion were estimated for each population, based on the sampled trees: Pr(Ex1), the probability of paternity exclusion when the offspring is sampled, but the mother is not and Pr(Ex2), the probability of paternity exclusion when both mother and offspring are sampled. Pairwise linkage disequilibrium was tested using a randomization based test with Bonferroni correction with software Fstat version 2.9.3.2 (Goudet, 2001). Presence of null alleles and scoring errors at each locus was tested using Micro-Checker version 2.2.3 (van Oosterhout *et al.*, 2004).

From the 25 primer pairs, nine amplified the fragment of interest clearly. All of them were polymorphic in both analyzed populations (Table 2). The number of alleles per locus ranged from 5 to 15 across both populations (Table 1) with an average number of alleles of 10.22. The observed heterozygosity in the PRC population ranged from 0.588 to 0.882 and, in the VZC population, from 0.313 to 1.000. The expected heterozygosity ranged from 0.582 to 0.854, in the PRC population and from 0.280 to 0.869, in VZC (Table 2). Only the locus Pre10 in the population PRC

showed significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$) (Table 2). This deviation is likely to be due to null alleles detected in this locus, since it was the only locus in which null alleles were detected. Probabilities of paternity exclusion for all combined loci in population PRC was 0.98446 [Pr(Ex1)] and 0.99918 [Pr(Ex2)]. In population VZC, these probabilities were 0.98640 [Pr(Ex1)] and 0.99929 [Pr(Ex2)]. Linkage disequilibrium was observed only between two loci (Pre15 and Pre25, $P = 0.05$), in population PRC.

CONCLUSIONS

The nine microsatellites isolated and characterized in this study are highly informative and may be appropriate tools to access several population genetic parameters in *Plathymenia reticulata*, such as gene flow, genetic structure, kinship and paternity. These studies will improve our understanding of the evolutionary history of *P. reticulata* and may also be applied to taxonomy, sustainable management and conservation of the species.

TABLES

TABLE 1. Characterization of 9 microsatellite loci developed in *Plathymenia reticulata* . The forward and reverse sequence, repeat array, annealing temperature (*Ta*), allele range size (bp), number of alleles per locus and GenBank accession number are shown for each primer pair.

Locus	Primer sequence (5'—3')	Repeat Array	*Ta*	Allele range size (bp)	No. of Alleles	GenBank ID
Pre5	F: *CTGCGATGGAATGCACTACA R: AGAGCAATAGAGATCCTATCGTAGA	$(AC)_{16}$	63	172-202	11	JN840010
Pre8	F: *GCACTCTGTTCCATCCCATT R: ATGTTGCAGGGGAACTATGG	$(AC)_{14}$	66	184-204	7	JN840011
Pre9	F: *GTGTGAGGGTATTGAAGCAACAGG R: ACGGCAAGCATACATTAGATCCCA	$(CA)_{14}$	54	310-344	10	JN840012
Pre10	F: *GCTCATGTCTCACTCCACGA R: CCTACCACGAAATTCCTCCA	$(GA)_{16}$	64	196-222	12	JN840013
Pre11	F: *GTTTGGTGCTTGTGAATGCC R: ACCTAACCCTCATCTCCCCAAT	$(GA)_{27}$	60	127-159	12	JN840014
Pre15	F: *GCTTCACACGCTCTTCTCCT R: TATGGGGTTTGTGCCTCTTC	$(GATA)_6\,(GA)_{12}\,GG\,(GA)_{10}$	66	218-252	12	JN840015
Pre16	F: *CAGGGAATGGTGAAAGTGAAA R: GCATCAGTGGCTAGCATGTC	$(CT)_{13}\,(CA)_8$	67	210-236	8	JN840016
Pre23	F: *CACCCTCACCGAGTCAGATT R: CGAGTTGTTCACACCCAGAA	$(TG)_{10}$	60	221-233	5	JN840017
Pre25	F: *GACCAGTTGGTGGTCCAGTT R: CGTTGCTGTGGTTTCTTCTG	$(AG)_{11}$	58	189-253	15	JN840018

*M13 tag (TTTTCCCAGTCACGAC) added to 5′ end of forward primer for amplification with fluorescently labeled M13.

TABLE 2. Results of primer screening in two populations of *Plathymenia reticulata* . Locus name, number of alleles per locus (NA), observed (HO) and expected heterozygosity (HE) and paternity exclusion probabilities [Pr(Ex1) and Pr(Ex2)] are shown for two populations: PRC (Pirenópolis, 15°48'44"S/48°53"28"W) and VZC (Várzea da Palma,17°42'37"S/44°41'24"W). Sample size (N) for each population is shown in parentheses.

	P. reticulata									
	PRC population (N = 34)					VZC population (N = 17)				
Locus	N_A	H_O	H_E	*Pr(Ex1)*	*Pr(Ex2)*	N_A	H_O	H_E	*Pr(Ex1)*	*Pr(Ex2)*
Pre5	9	0.875	0.854	0.5178	0.6856	8	0.750	0.861	0.5006	0.6711
Pre8	5	0.774	0.641	0.2204	0.3847	7	0.688	0.720	0.2939	0.4662
Pre9	9	0.882	0.839	0.481	0.654	6	0.706	0.709	0.2759	0.4428
Pre10	8	0.588	0.846*	0.500	0.6712	8	0.529	0.604	0.2063	0.3886
Pre11	9	0.706	0.737	0.3469	0.5334	8	0.846	0.837	0.4464	0.6227
Pre15	7	0.645	0.768	0.3616	0.5398	9	0.938	0.877	0.5307	0.6965
Pre16	5	0.818	0.675	0.2448	0.4118	8	0.750	0.831	0.4526	0.6297
Pre23	4	0.625	0.582	0.1749	0.3281	3	0.313	0.280	0.0369	0.1325
Pre25	11	0.700	0.779	0.3872	0.5648	9	1.000	0.869	0.5104	0.6793
MEAN	7.444	0.735	0.747	0.98446	0.99918	7.333	0.724	0.732	0.98640	0.99929

Notes: *significant departures from Hardy-Weinberg equilibrium at $P < 0.05$.

APPENDIX 1. List of vouchers of *Plathymenia reticulata* used in this paper.

Code	Country	Locality (State)	Voucher Number BHCB
BHZ	Brazil	Belo Horizonte (Minas Gerais)	150648
VZC	Brazil	Várzea da Palma (Minas Gerais)	152888
PRC	Brazil	Pirenópolis (Goiás)	97924

LITERATURE CITED

BILLOTTE, N., P. J. L. LAGODA, A. M. RISTERUCCI, AND F. C. BAURENS. 1999. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277-288.

EXCOFFIER, L., G. LAVAL, AND S. SCHNEIDER. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47-50.

GOUDET, J. 2001. FSTAT version 2.9.3.2, a program to estimate and test gene diversities and fixation indices. Institute of Ecology, Lausanne, Switzerland. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed on 10 May 2011].

KALINOWSKI, S.T., M.L. TAPER AND T.C. MARSHALL. 2007. Revising how the computer program Cervus accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.

LORENZI, H. 1992. Árvores Brasileiras. Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil. *Plantarum Ltda*, Nova Udessa, SP.

NOVAES, R.M.L., J.P. LEMOS FILHO, R.A. RIBEIRO, AND M.B. LOVATO. 2010. Phylogeography of *Plathymenia reticulata* (Leguminosae) Reveals patterns of recent range expansion to wards northeastern Brazil and southern Cerrados in Eastern Tropical South America. *Molecular Ecology* 19: 985-998.

TAMURA, K., J. DUDLEY, M. NEI, AND S. KUMAR. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.

UNTERGRASSER A., H NIJVEEN, X. RAO, T. BISSELING, R. GEURTS, AND J.A. LEUNISSEN. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35:W71-W74.

VAN OOSTERHOUT, C., W. F. HUTCHINSON, D. P. M. WILLS, AND P. SHIPLEY. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4(3): 535-538.

WARWICK, M.C., AND G.P. LEWIS. 2003. Revision of *Plathymenia* (Leguminosae - Mimosoideae). *Edinburgh Journal of Botany* 60: 111-119.

CAPÍTULO 2

GENETIC DIFFERENTIATION AND GENE FLOW BETWEEN FOREST AND SAVANNA POPULATIONS OF A NEOTROPICAL TREE: REVISITING THE CASE OF *PLATHYMENIA*

Mariana V. Cruz[1], Jacqueline G. Rodrigues[1], José P. Lemos Filho[2] and Maria Bernadete Lovato[1,3].

[1]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brasil.

[2]Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Minas Gerais, Brasil.

[3]Autor para correspondência: lovatomb@icb.ufmg.br

ABSTRACT

The species status of a vicariant pair of species belonging to the Brazilian tree genus *Plathymenia* has been discussed for many decades. Individuals encountered in contrasting biomes are phenotypically distinguishable, but evidences of gene flow between them were demonstrated in a previous work developed with dominant marker. The objectives of this study was to investigate the occurrence of gene flow between populations of different habitats through the use of microsatellite markers and to test the hypothesis that the geographical distance is the main factor determining the distribution of genetic diversity. We found that genetic traits are associated with the biome where the individuals are located rather than with the geographical distance between populations. High levels of gene flow were observed in areas where both morphological types coexist, at the border of the biomes, in ecotonal areas, and no admixed individuals were found in central areas. The results corroborates with the latest revision of the taxa that classifies *Plathymenia* as a monospecific genus and support previous hypothesis of the existence of two different ecotypes of *Plathymenia*, since, in distinct biomes, individuals maintain distinct gene pools. It characterizes the ecotypes as different evolutionary units that require, therefore, separate management.

Key words: Atlantic forest, *Plathymenia reticulata*, Cerrado, ecotone, ecotypes, microsatellite, hybridization.

INTRODUCTION

Abrupt changes in phenotypes and genotypes often occur in contact zones between habitats that differ in subtle characteristics and can be a consequence of divergent selection that leads to different adaptations, or of secondary contact after differentiation in isolation (Barton and Hewitt, 1985; Wiens, 2003). Relationship and gene flow between closely related taxa at incomplete levels of divergence can be estimated by the use of molecular methods, which allow studying the distribution of alleles in nuclear loci and represent therefore an important tool to investigate the limits of species (Moritz, 2002; Pettengill and Neel, 2011). Different environmental conditions play an important role in the origin of species, since adaptation to each environment drives to divergent natural selection (Wiens, 2003).

The genus *Plathymenia* occurs in the biomes of Cerrado and Atlantic forest, mainly in Brazil, but it is also encountered in Surinam, Bolivia and Paraguay (Warwick and Lewis, 2003). These two biomes are considered "hot spots" for conservation due to its high levels of biodiversity, endemism and degree of threat (Myers *et al.*, 2000).

Among all savanna vegetations in the world, Brazilian Cerrado has the richest flora, with over 10,000 plant species and several xeromorphic vegetation types, from herbaceous fields with no trees to forest formations with an open canopy (Klink and Machado, 2005; Myers *et al.*, 2000). The original extent of the primary vegetation was ca. 1.7 million km² but, since it has been threatened by human activities, it was rapidly reduced to less than 20% of the original area (Myers *et al.*, 2000). The Atlantic forest is a typical tropical forest, with a mesic environment and over 20,000 plant species, of which approximately 40% are endemic (Myers *et al.*, 2000).

Originally it covered an area of approximately 1.5 million km² of coastal and inland regions, but today it is estimated that this biome was reduced to less than 16% of its original coverage (Ribeiro *et al.*, 2009).

In these contrasting habitats, many taxa exhibit vicariant pairs, including the individuals of the genus *Plathymenia*, which also show some contrasting morphological differences that, since its first description, by Bentham (1842), has been generating doubts about its taxonomy. Bentham recognized two species: *P. reticulata* Benth., occurring in the Cerrado, and *P. foliolosa* Benth., encountered in the Atlantic forest. In 1925 though, Ducke reduced the genus to a single species, yet, some decades later, Heringer (1956) reconsidered the existence of two distinct species. Observing herbarium specimens, Lewis (1987) over again cast doubt on the distinctness of both species. The morphological features that Bentham (1842, 1876) used to distinguish *P. reticulata* from *P. foliolosa* were the habitat in which individuals were found, height, inflorescence indumentum and number of pinnae per leaf and leaflets per pinna.

The first work on population genetics of *Plathymenia* used RAPD markers to compare specimens from both biomes and its results launched another discussion on the taxonomy of the genus (Lacerda *et al.*, 2002). Genetic differences between the two species were found, but a single population of *P. foliolosa* sampled in a transition area between the biomes presented characteristic markers of *P. reticulata*, suggesting the occurrence of gene flow between species. This finding provided support to the taxonomic study developed by Warwick and Lewis (2003), who considered the genus monospecific, using the epiteto *P. reticulata*. Based on analysis of herbarium specimens sampled throughout the whole range of the genus,

the authors neither find features that distinguished individuals from the two biomes, nor significant correlation between geography and morphological characteristics. Instead, they found a continuum in the variation of the characters previously used to distinguish species.

A phenological study found no difference in the reproductive phenology between Cerrado and Atlantic forest populations of *Plathymenia* (Goulart *et al.*,2005). They demonstrated that individuals bloom almost synchronously in both habitats, which represents, thus, an opportunity for gene flow between individuals from forest and Cerrado. Adaptive traits to the different ecological conditions of the two biomes have been reported in natural populations of the species suggesting the existence of two distinct ecotypes (Goulart *et al.*, 2006; Lemos-Filho *et al.*, 2008). When compared to individuals from the Cerrado, forest individuals showed enhanced seed dispersion abilities (Goulart *et al.*, 2006), greater potential for hydraulic conductivity (Toledo, 2010) and greater phenotypic plasticity in several morphological and physiological traits related to light availability (Goulart *et al.*, 2011). For some of the studied traits, intermediate values were found in individuals from ecotonal areas between the two biomes, corroborating with previous evidences of gene flow previously related by Lacerda *et al.* (2002).

In the present study we use nuclear microsatellite markers in order to analyze differentiation and gene flow among populations of *Plathymenia reticulata* from Cerrado and Atlantic forest. We test the hypothesis suggested by Lacerda *et al.* (2002) of occurrence of gene flow between Cerrado and Atlantic forest individuals of *Plathymenia*, detected by the use of dominant markers (RAPD) in a single ecotonal population. Here, we use four populations from the core areas, two from the Cerrado

and two from the Atlantic forest, as well as four other ecotonal sites, where individuals with typical morphology of Cerrado occur near to those with forest morphology. According to the hypothesis of occurrence of gene flow, we predicted that in the core areas, populations from different biomes would show higher genetic differences than in ecotonal sites. In addition, private alleles to an ecotype from core area could be found in alternative ecotype from ecotonal areas. Based on the results, we discussed the pertinence of the proposition of Warwick and Lewis (2003) of considering the genus monospecífic.

MATERIAL AND METHODS

Characterization of the collect sites and sampling

The Atlantic forest is a tropical rainforest, where the climate is moist and characterized by high temperatures, with an annual average of 25° C and high levels of rainfalls well distributed throughout the year (0 to 60 days dry). In these environments predominate the latosol of low natural fertility. The vegetation in the coastal Atlantic forest is dense and evergreen. The inland Atlantic forest has a semi deciduous physiognomy and, as occurs in the Cerrado, is characterized by seasonal rainfalls (Mittermeier *et al.*, 1999). The Cerrado is characterized as a xeromorphic savanna, defined by seasonal climate, with about six months of severe drought and its soil is acid and aluminium-rich, with low nutrient availability. The vegetation is mainly scrub, with shrubs and scattered trees (IBGE, 1992). In the ecotone areas between these biomes, generally, there is a mosaic of patches of Atlantic forest within the Cerrado and Cerrado-like areas within the Atlantic forest, very similar to

their respective core habitats, instead of a gradual transition from one biome to the other or an intermediate vegetation type (Goulart *et al.*, 2011).

Eight populations of *Plathymenia* were sampled (Table 1, Fig. 1), totalizing 159 individuals that were classified into two very characteristic ecotypes, occurring in the two distinct biomes, named in this work as: (I) Atlantic forest ecotype and (II) Cerrado ecotype, as suggested by Goulart *et al.* (2006). The two ecotypes were distinguished by size (Cerrado ecotype individuals did not exceed 10 m in height of the canopy, while the forest ecotype individuals measured from 15 to 30 m), trunk characteristics (forest ecotype individuals had straight trunk and Cerrado ecotype, tortuous and twisted trunk) and characteristics of the bark (which was more suberous in Cerrado ecotype). A total of four populations were sampled for each ecotype. The sampled populations represent three different habitats: (1) Atlantic forest core, with two populations sampled; (2) Cerrado core, with two populations sampled and (3) ecotone areas between the two biomes, where both ecotypes occur close to each other, with four populations sampled (Fig. 1, Table 1). Among the four populations sampled in these ecotone areas, two presented individuals with typical characteristics of the Atlantic forest ecotype and the other two, of Cerrado ecotype.

DNA isolation, amplification and genotyping

Leaves or bark tissues were dried on silica gel before being frozen at -20°C. Genomic DNA was isolated from bark tissue as in Novaes *et al.* (2009). For leaf samples, DNA was extracted using the CTAB buffer protocol.

Nine microsatellite markers developed specific for *Plathymenia reticulata* from an individual with Cerrado ecotype traits (Cruz *et al.*, 2012) were tested. Loci Pre9 and Pre25 did not amplify in most individuals of Atlantic forest core populations and were therefore excluded from subsequent analysis. The seven remaining loci amplified in the PCR conditions described by Cruz *et al.* (2012). PCR products were genotyped on a MegaBACE 1000 automated sequencer, using 0.1% Tween 20 and ROX-500 Size Standard (GE Healthcare). Alleles were identified by size using the MegaBACE Fragment Profiler version 1.2 software (GE Healthcare).

Data analysis

The following parameters were estimated with the software Arlequin version 3.1 (Excoffier *et al.*, 2005): the observed and expected heterozygosities and the deviations from Hardy–Weinberg equilibrium for each locus and over all loci within populations and the F_{ST} between all pairs of populations was calculated and then we tested for isolation by distance by Mantel tests with 10,000 permutations. Wright's coefficient of inbreeding (F_{IS}) in each population was calculated with GENEPOP 3.4 (Raymond and Rousset, 1995). With the software Fstat version 2.9.3.2 (Goudet, 2001), we tested pairwise linkage disequilibrium using a randomization based test with Bonferroni correction and calculated the number of alleles and the number of alleles that would be expected for each population, if they all had eight individuals, which was the lowest number of individuals genotyped for each locus (allele richness). Scoring errors and presence of null alleles at each locus were tested using

Micro-Checker version 2.2.3 (van Oosterhout *et al.*, 2004). For comparing levels of diversity between groups of populations paired-T tests were used.

In Arlequin version 3.1 (Excoffier *et al.*, 2005), analysis of molecular variance (AMOVA) was calculated to infer the hierarchical distribution of genetic variation estimating the F-statistics (Excoffier *et al.,* 1992), which are analogous to Wright's hierarchical fixation indices under the island model of gene flow (Wright, 1951). Four independent AMOVAs were performed, with the genetic diversity being hierarchically partioned by: (1) all populations and individuals within populations; (2) different core biomes (Atlantic forest core and Cerrado core), populations within core biomes and individuals within populations; (3) different habitats (Atlantic forest core, Cerrado core, Atlantic forest ecotype in ecotonal area and Cerrado ecotype in ecotonal area), populations within habitats and individuals within populations; and (4) different ecotypes (Atlantic forest and Cerrado ecotypes), populations within ecotypes and individuals within populations. Permutation tests for the AMOVAs were conducted to determine if the levels of differentiation were significantly different from zero.

The software STRUCTURE (Pritchard *et al.*, 2000) was used to perform a Bayesian clustering of individuals into predefined numbers of clusters (K), based on admixture model, with correlated allelic frequencies. The K value was set from 1 to 12, with 10 independent runs for each value, 100,000 generations as burnin and 500,000 generations sampled in the Monte Carlo Markov chain (MCMC). The optimal number of clusters was determined by the *ad hoc* statistics, ΔK (Evanno *et al.* 2005), and the log of probability, LnP(D) (Pritchard *et al.*, 2000).

RESULTS

The mean observed (H_O) and expected heterozygosities (H_E), allelic richness and F_{IS} of each population in each habitat are presented in Table 2. Null alleles were not detected in the sampled loci and linkage disequilibrium was not observed between any pair of loci. All loci were polymorphic and most did not deviate from the Hardy-Weinberg equilibrium in any population, except for Pre5 in the populations SJF, SUF, NEF and COC, and for Pre10, in PRC and NEF (data not shown). The inbreeding coefficient (F_{IS}) ranged from 0.017 (PRC) to 0.128 (NEF), but only populations PRC (F_{IS}=0.017), VZC (F_{IS}=0.043), IPF (F_{IS}=0.038) and SUF (F_{IS}=0.042) showed significant heterozygote deficits. Mean observed and expected heterozygosities between all sampled populations were 0.648 and 0.686, respectively. The mean allelic richness, based on minimum population size of eight individuals, was 5.3, varying from 3.89 (SJF) to 6.67 (SUC). The mean number of alleles sampled per locus was 6.52, varying from 4.43 (IPF) to 7.86 (SUC) (Table 2).

For all the diversity indices (H_O, H_E and allelic richness) the average between populations of the same habitats was calculated. The same pattern was observed for all parameters: the highest levels of diversity were presented by populations of Cerrado in ecotonal areas, followed by Cerrado core populations; intermediate level was presented by forest populations in ecotonal area and the lowest levels, by forest core populations (Table 2). The statistical analysis using paired-T tests to compare total diversity between groups of populations showed that, for allelic richness, the Cerrado in the ecotone is significantly ($P<0.05$) more diverse than all the other groups and, for total heterozigosity, the Cerrado in ecotone was significantly ($P<0.05$) more diverse than forest ecotype, in core or ecotone areas (Table 3).

Of the 108 different alleles detected across all loci and all populations, 48 (44.4%) were present in the Cerrado core populations and absent in the Atlantic forest core. Most of these alleles (75%) were also found in Cerrado populations in the ecotones, however, a considerable proportion (39.6%) was also detected in forest populations present in the ecotonal areas. Thirty-one alleles (28.7%) that occurred in forest core populations were absent in the Cerrado core, and15 (48.4%) among these were also present in the forest individuals in ecotone areas, whereas 11 (35.5%) occurred in Cerrado individuals in the transition areas (Table 4). Thirty alleles were sampled exclusively in Cerrado ecotype populations and 20 were sampled only in the forest ecotype individuals. It means that almost half (46.29%) of the sampled alleles were unique to one or to the other ecotype, and the remainder alleles (53.71%) were shared.

According to the results of the first hierarchical AMOVA, 18.89% of the total variation was due to differences between the sampled populations (Table 5). In the three remaining AMOVAs, the sampled populations were grouped in three different ways, in order to check to which grouping would be assigned most part of the total variation. In the second hierarchical AMOVA, where populations of ecotone areas were excluded from the analysis, 22.2% of the total variation was attributed to differences between two groups of population: forest core populations and Cerrado core populations. When populations from ecotone areas were included together with core areas, the difference between ecotypes decreased to 13.4%. The third AMOVA revealed that differences between groups of populations that occur in different habitats account for 12.2% of total diversity. In all AMOVAs most part of the genetic diversity was found within populations (Table 5).

Pairwise F_{ST} values highlight the differentiation between populations (Table 6). The greatest pairwise F_{ST} were observed between populations from core areas of distinct biomes, ranging from 0.418 to 0.496. The great difference observed may be due to physical impossibility of gene flow and not by some reproductive isolation mechanism, since geographical distances between the populations are very large. However, populations of the same ecotype sampled in core areas, which are also far apart from each other, showed much lower F_{ST} values (F_{ST}=0.117 between Cerrado core populations and F_{ST}=0.139 between forest core populations). On the other hand, in the ecotonal area, where geographical distance could not be an obstacle to gene flow, the genetic differentiation between the two ecotypes measured by F_{ST} was much lower than between populations of distinct core areas, ranging from 0.075 to 0.266. In the ecotone areas, the genetic differentiation between populations of the same ecotype was 0.042 (in Cerrado ecotype) and 0.106 (in forest ecotype). Thus, in these areas there are populations of different ecotypes with lower genetic differentiation than populations of the same ecotype. The Mantel test showed no correlation (r=0.00018, p=0.4668) between pairwise F_{ST} and geographic distances. These results indicate that genetic distances between populations are not related to geographic distances between them.

Bayesian clustering carried out in STRUCTURE inferred the uppermost hierarchical level of structure at K=2 genetic clusters with the *ad hoc* statistics, ΔK, albeit more clusters were inferred by the log of probability of data, LnP(D) (Fig. 2). Clustering results are shown in Figure 3. At K=2, all individuals from the Atlantic forest core populations were very strongly assigned to one cluster and all individuals of the Cerrado core populations, to the other. Most individuals of the forest ecotype in

ecotone areas were more strongly related to the same group to which forest core individuals belonged. It was likewise for individuals of Cerrado ecotype in transition areas compared to Cerrado core individuals. However, 43% (N=29) of the individuals from the ecotone areas were highly heterogeneous, with an admixture coefficient (Q) between 0.1 and 0.9.

TABLES

Table 1 Sampled populations of *Plathymenia reticulata*.

Population	ID	Ecotype	Vegetation type	Geographical coordinates	N
Cerrado core					*51*
Pirenópolis	PRC	Cerrado	Xeromorphic savanna	15°51'07" S, 48°57'32" W	34
Várzea da Palma	VZC	Cerrado	Xeromorphic savanna	17°35'52" S, 44°43'51" W	17
Atlantic Forest core					*40*
Itapebi	IPF	Atlantic forest	Dense tropical rainforest	15°57'03" S, 39°32'02" W	14
Silva Jardim	SJF	Atlantic forest	Dense tropical rainforest	22°39'03" S, 42°23'31"W	26
Ecotone					*68*
Parque Estadual do Sumidouro	SUF	Atlantic forest	Semideciduous tropical forest	19°32'30" S, 43°55'24" W	14
Parque Estadual do Sumidouro	SUC	Cerrado	Xeromorphic savanna	19°32'42" S, 43°55'52" W	15
Ribeirão das Neves	NEF	Atlantic forest	Semideciduous tropical forest	19°46'01"S, 44°05'13"W	24
Contagem	COC	Cerrado	Xeromorphic savanna	19°54'13" S, 44°03'16" W	15
Total					159

N, number of sampled individuals.

Table 2 Diversity indices for the sampled populations of *Plathymenia*.

Population	ID	N	Ho	He	Alellic richness (Na)	Fis
Cerrado core		*51*				
Pirenópolis	PRC	34	0.719	0.729	5.12 (6.71)	0.017*
Várzea da Palma	VZC	17	0.687	0.716	5.76 (7.29)	0.043*
Mean			*0.703*	*0.723*	*5.44 (7.00)*	*0.03*
Atlantic forest core		*40*				
Itapebi	IPF	14	0.499	0.517	3.94 (4.43)	0.038*
Silva Jardim	SJF	26	0.483	0.543	3.89 (5.57)	0.114
Mean			*0.491*	*0.53*	*3.92 (5.00)*	*0.076*
Ecotone (Cerrado ecotype)		*30*				
Parque Estadual do Sumidouro	SUC	15	0.766	0.817	6.67 (7.86)	0.07
Contagem	COC	15	0.771	0.81	6.52 (7.57)	0.063
Mean			*0.769*	*0.813*	*6.60 (7.71)*	*0.067*
Ecotone (Atlantic forest ecotype)		*38*				
Parque Estadual do Sumidouro	SUF	14	0.734	0.758	6.21 (7.29)	0.042*
Ribeirão das Neves	NEF	24	0.523	0.599	4.31 (5.43)	0.128
Mean			*0.629*	*0.679*	*5.26 (6.36)*	*0.085*

N, number of individuals; Ho, observed heterozygosity; He, expected heterozygosity; Na, number of alleles (in brackets). Allelic richness is based on a minimum sample size of eight individuals.

* Statistically significant values of Fis ($p<0.05$).

Table 3 Differences between groups using paired-T tests. Groups identified by different letters are significantly different ($p<0.05$).

Groups of populations	He	Allele richness
Cerrado core	0.761 (AB)	7.92 (B)
Atlantic forest core	0.565 (B)	5.79 (B)
Cerrado ecotype in ecotonal area	0.826 (A)	9.191 (A)
Atlantic forest ecotype in ecotonal area	0.689 (B)	7.104 (B)

Table 4 Range size of alleles of each habitat and exclusive alleles among populations of Cerrado and Atlantic forest.

	Range size of alleles				Exclusive alleles	
Locus	*Cerrado core*	*Atlantic forest core*	*Cerrado Ecotone*	*Atlantic forest Ecotone*	*Cerrado core*	*Atlantic forest core*
Pre5	176-202	152-188	162-202	162-190	178[C, F], 190[C, F], 192[C], 194[C], 202[C]	152, 160, 162[C, F], 164[F], 166, 168, 170[C], 172[C, F], 174[F]
Pre8	184-204	176-182	178-204	176-198	184[C, F], 186[C, F], 192, 196[C], 198[C, F], 202, 204[C]	176[F], 178[C, F], 180[F], 182
Pre10	196-222	162-256	196-250	204-250	196[C], 198[C], 200[C], 206[C, F], 208[C], 210, 214[C], 216[C], 218[C], 222	162, 224[C, F], 244, 246[F], 248[C], 250[C, F], 252, 256
Pre11	127-159	129-153	127-149	127-149	127[C, F], 131, 133[C, F], 135[C, F], 137[C, F], 141[C], 147[C, F], 149[C, F], 159	153
Pre15	218-252	222-264	216-268	216-268	218, 224, 232[F], 234[C, F], 236[C], 242, 248[C], 252	222[C, F], 230[C, F], 240, 264
Pre16	210-236	210-218	210-236	210-236	220[C, F], 224[C], 226[C, F], 228[C, F], 230[C, F], 232[C, F], 236[C, F]	212[C, F], 214[C, F], 218
Pre23	221-233	219-227	221-231	221-227	231[C], 233	219, 227[C, F]

[C], allele also present in populations of Cerrado ecotype in ecotonal area.
[F], allele also present in populations of Atlantic forest ecotype in ecotonal area.

Table 5 Results of the hierarchical AMOVAs for 159 individuals of two distinct ecotypes of *Plathymenia reticulata* ocurring in four different habitats: Atlantic forest core, Cerrado core, Atlantic forest in ecotone and Cerrado in ecotone.

Source of variation	d.f.	Sum of squared deviation	Percentage of total variance	F-statistics
(1) Populations				
Among populations	7	137.55	18.9	
Within populations	310	603.12	81.1	F_{ST} = 0.189
Total	317	740.67		
(2) Core biomes				
Between core biomes	1	69.49	22.2	F_{CT} = 0.222
Among populations within core biomes	2	23.44	8.6	F_{SC} = 0.110
Within populations	178	344.64	69.2	F_{ST} = 0.308
Total	181	437.57		
(3) Habitats				
Among habitats	3	100.72	12.2	F_{CT} = 0.122
Among populations within habitats	4	36.84	8.1	F_{SC} = 0.092
Within populations	310	603.12	79.7	F_{ST} = 0.203
Total	317	740.67		
(4) Ecotypes				
Among ecotypes	1	67.56	13.4	F_{CT} = 0.134
Among populations within ecotypes	6	69.99	10.0	F_{SC} = 0.116
Within populations	310	603.12	76.5	F_{ST} = 0.235
Total	317	740.67		

d.f., degrees of freedom.

Table 6 Matrix of geografic distances in kilometers (above diagonal) and pairwise Fst* (below diagonal) between all sampled populations of *Plathymenia reticulata*.

Population	VZC	PRC	IPF	SJF	NEF	COC	SUC	SUF
VZC		490.503	583.229	610.485	249.523	265.03	231.477	231.441
PRC	0.11664		1009.446	1021.126	674.086	686.36	671.853	672.302
IPF	0.4255	0.41855		800.174	641.205	648.614	612.898	612.021
SJF	0.49578	0.43448	0.13934		364.576	349.67	379.227	379.215
NEF	0.32263	0.31425	0.20463	0.22703		15.531	29.487	30.259
COC	0.09829	0.11676	0.26708	0.28008	0.1595		42.379	42.379
SUC	0.05663	0.10363	0.38624	0.37147	0.266	0.04183		0.912
SUF	0.12734	0.13862	0.17706	0.17487	0.10568	0.07515	0.07464	

* All values of Fst were significant ($P<0.05$).

FIGURES

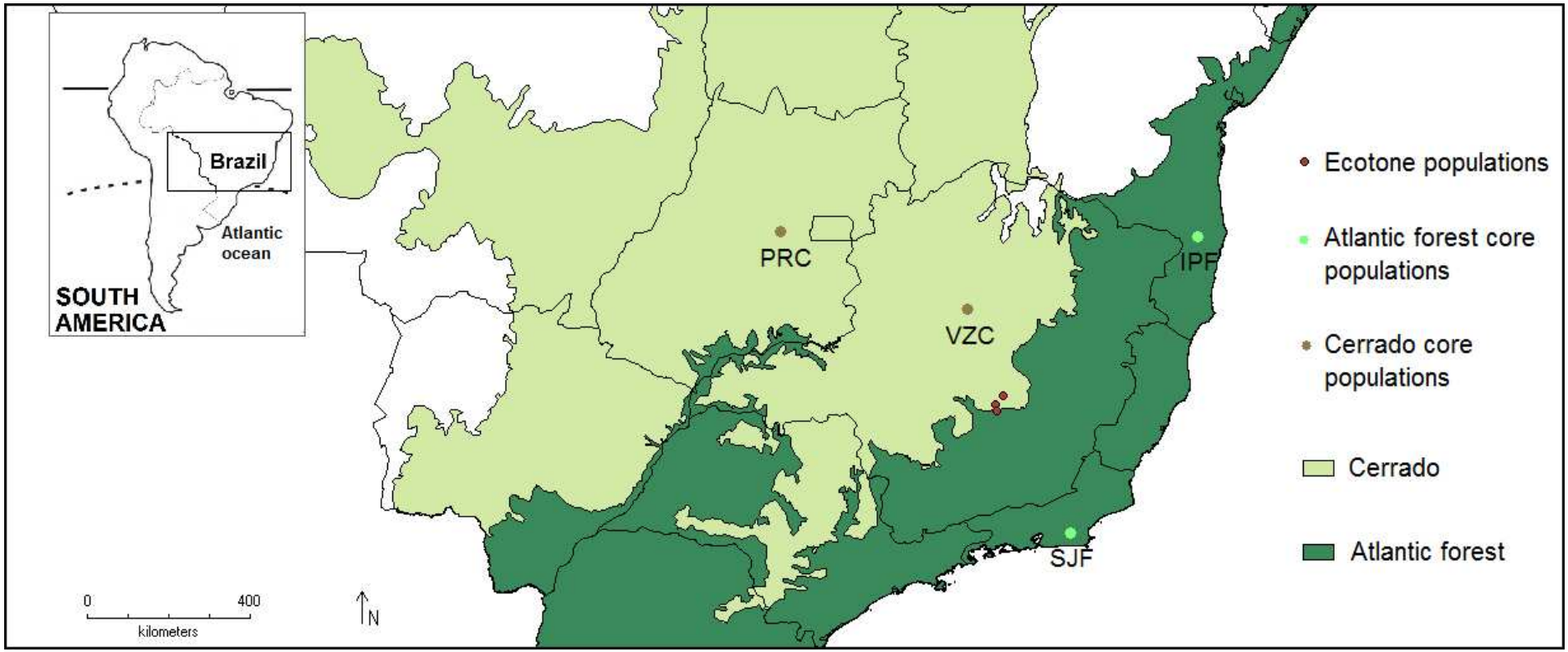

Fig 1 Sampled populations of *Plathymenia* and distribution of the biomes in which they were collected.

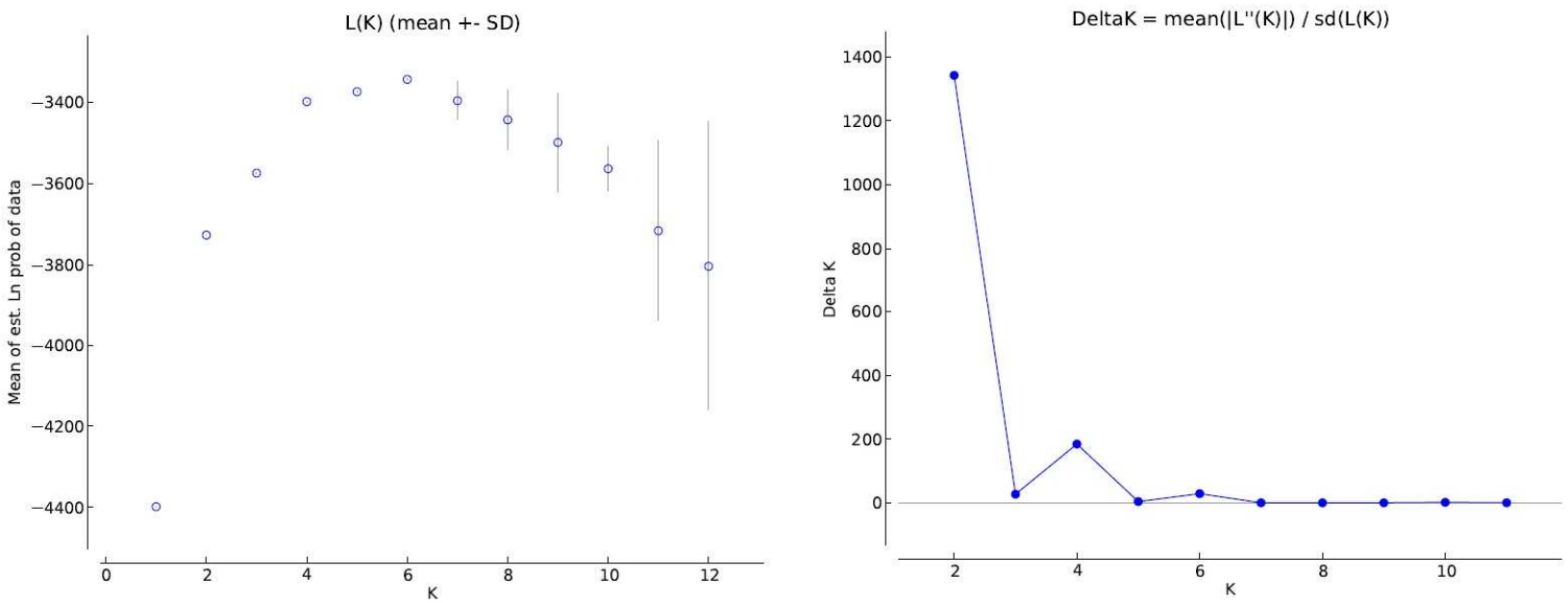

Fig. 2 Estimation of the number of clusters found by Bayesian clustering with STRUCTURE. K, number of clusters; mean of estimated Ln probability of data, probability of data for a given K, after 10 independent runs; SD, standard deviation; DeltaK, *ad hoc* statistic to estimate the uppermost value of K.

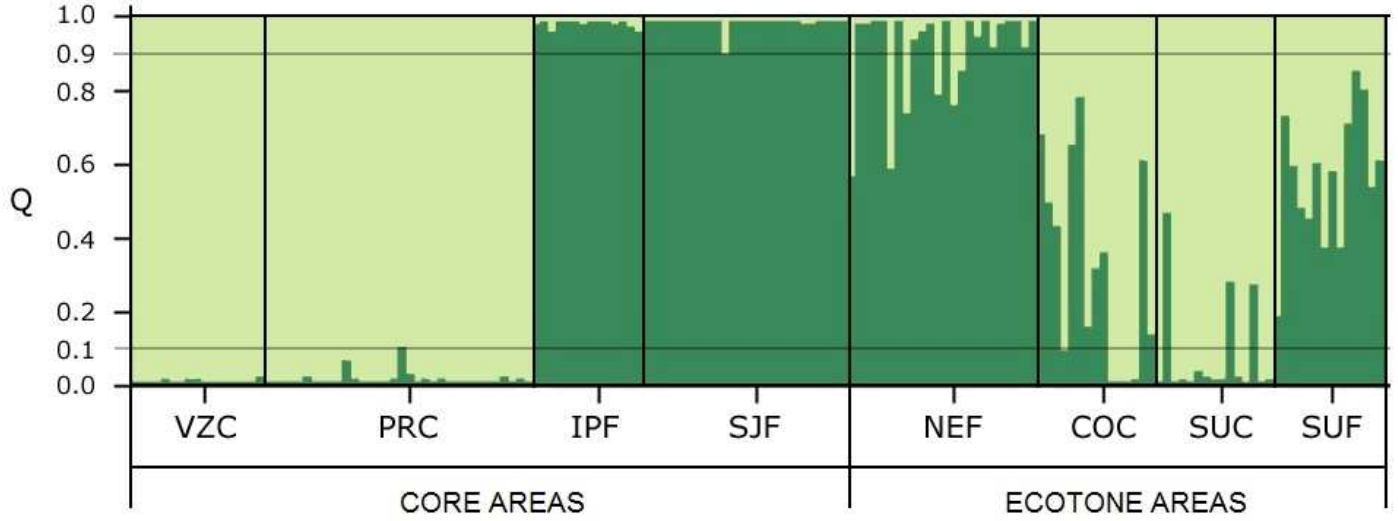

Fig. 3 Graphical representation of genetic structure in the microsatellite data for 159 individuals of *Plathymenia* obtained by two clusters. Q, admixture coefficient for each individual, represented by the vertical bars. The third letter of each Population ID represents the biome in which the population was sampled; C, Cerrado; F, Atlantic forest.

DISCUSSION

In this study we have shown the occurrence of genetic differences in neutral nuclear loci (microsatellite) between populations of *Plathymenia* located in the Brazilian Atlantic forest and Cerrado. In Bayesian analysis, individuals from core areas were grouped in a way that was concordant with the habitats in which they were sampled. Both Cerrado core populations were grouped in one cluster and the Atlantic forest core individuals were grouped in the other. The two populations sampled in the Atlantic forest core (IPF and SJF) were 800 km apart and yet they were more genetically similar than IPF and VZC, which were located closer from each other (583km), but belonged to different habitats. However, the genetic differences were not great, although individuals of different ecotypes present visually distinguishable morphology even in the ecotone areas. The AMOVA results showed that only 13.4% of the total genetic diversity was due to differences between the two ecotypes. Yet, when we removed from the analysis the populations from the ecotone areas, genetic diversity attributed to differences between the populations from the core of the biomes increased to 22.2% of the total diversity. In all analysed loci, private alleles to an ecotype in core areas are found in the alternative ecotype in ecotone areas and the Bayesian analysis also showed that in the ecotones there is a great proportion of admixed individuals. These are all strong evidences of high levels of gene flow between the two ecotypes occurring when they are located close to each other, in the ecotones. Genetic differences found between the ecotypes in this study are consistent with differences found in a previous study with the species, with RAPD markers (Lacerda *et al.*, 2002). The authors also found markers that were typical to Cerrado individuals from a population of individuals with typical forest

morphology, located in an ecotonal zone where the two ecotypes coexisted. The same ecotone population sampled by Lacerda *et al.* (2002) was one of the populations used in the present work (NEF).

Goulart *et al.* (2005, 2011) evaluated morphological and physiological traits in the wild and in experiments of common garden and demonstrated the presence of adaptive differences between populations from Cerrado and from the Atlantic forest, leading to consider the existence of different ecotypes in the two distinct biomes. Compared to the results present in the present word, these studies showed a similar pattern of differentiation between the groups of populations: for most of the morphological and physiological traits analyzed, populations from ecotones exhibited intermediate values between core populations from Cerrado and forest, which is also consistent with the hypothesis of occurrence of gene flow between ecotypes in these areas.

This pattern of differentiation between populations of core areas and occurrence gene flow in areas of borders of two ecosystems, detected with microsatellite markers, was found in plant species as *Lupinus microcarpus* (Drummond and Hamilton, 2007), *Casearia sylvestris* (Cavallari *et al.*, 2010), two oak species of the genus *Quercus* (De Dios, Benito-Garzón and Sainz-Ollero, 2006), as well in animal species, as mouse lemurs of Madagascar (Gligor *et al.*, 2009) and in sympatric species of the genus *Callipepla* (Gee, 2004). The study developed by Cavallari *et al.* (2010) is noteworthy, since they studied two varieties of *Casearia sylvestris* that, just as *Plathymenia reticulata*, occur in the Cerrado, in the Atlantic forest and in transition areas in Brazil. They also found two groups genetically distinct that closely matched the morphological classification of the sampled individuals, with

admixed individuals in the ecotonal sites. According to Drummond and Hamilton (2007), this finding may suggest shared ancestral polymorphism. However, in the case of *Plathymenia*, this is unlikely, since that there are much more shared alleles between ecotypes in ecotonal areas than between ecotypes in core areas. Thus, the pattern found in the sampled populations of *Plathymenia* is better explained by secondary contact after divergence due to geographic isolation. However, as suggested by Wiens (2007), a single method that distinguishes gene flow from recent divergence, that integrates data from multiple loci, that determines species limits without a priori definition and that allows estimating the statistical support for decisions in species level woefully does not exist yet.

A phylogeographic study with cpDNA regions performed by Novaes *et al.* (2010), found that the two lineages of *Plathymenia* began to diversify in the early Pleistocene (from 2.59 to 0.01 million years ago). After the last glaciation, with the re-expansion of the Atlantic forest, the gene flow between individuals of different ecotypes present in the transition areas between the biomes might have been facilitated by the morphological similarity of flowers and by the synchrony of phenology related to reproduction among individuals of the two lineages, reported by Goulart *et al.* (2005).

Higher levels of diversity were observed within populations of *Plathymenia* sampled in Cerrado areas than in Atlantic forest areas. This could be, in part, due to the fact that the microsatellite markers used in this study were isolated from an individual with morphology of Cerrado ecotype, and it is known that heterologous primers show less diversity (Lopes *et al.*, 2010). However, this probably is not the main cause, since great diversity in Cerrado individuals was also found with RAPD

markers (Lacerda *et al.*, 2001) and with cpDNA (Novaes *et al.*, 2010). The highest levels of genetic diversity were found in the Cerrado ecotype from ecotone areas, since in these regions are found alleles that are exclusive from one biome and from the other. Higher levels of diversity in the ecotones were also observed in *Casearia sylvestris*, by Cavallari *et al.* (2010). It is likely that populations from Cerrado have maintained larger size than forest populations.

In summary, this study shows that the populations of *Plathymenia* occurring in the Cerrado and in the Atlantic forest are genetically more differentiated than populations located in the same biome. Other studies (Goulart *et al.*, 2005, 2006, 2011; Toledo, 2010) showed important adaptative differences between populations from different environments, which justified the classification of different ecotypes. Despite the great morphological differences observed in adult plants in relation to the external architecture and the considerable genetic differentiation between populations located in the core areas of the distinct biomes, the present study showed a high degree of gene flow in areas of sympatry, i.e., in ecotonal areas. There is, though, no measure of genetic diversity that determines the species boundaries (Morgan-Richards and Wolff, 1999). Regardless of whether the gene flow has been established after secondary contact of populations that had not yet reproductively isolated, or if the populations in the ecotones represent areas of parapatric speciation, we do not have evidences for considering populations of different biomes as representing different biological species. Thus, our data corroborates with the latest revision of the taxon that classifies *Plathymenia* as a monospecific genus and support previous hypothesis of the existence of two different ecotypes of *Plathymenia*, since in distinct biomes, individuals maintain distinct gene

pools. The genetic differences found in neutral loci and differences in adaptive characters encountered in previous studies point to consider populations from different biomes as representing different evolutionary significant units (ESU) requiring separate management (Moritz, 1995). The admixture of both ESUs can disturb the genetic combination that guarantees the success of individuals in the core areas of the biomes. As it was suggested by Lemos Filho *et al.* (2008), this should be considered in plans of habitat restoration or reintroduction of the species, to improve the chances of individuals to respond successfully to the distinct environmental challenges.

LITERATURE CITED

Almeida SP, Proença CEB, Sano SM (1998) *Cerrado: Espécies Vegetais Úteis* xiii: 280-283. Planaltina: EMBRAPA - CPAC.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 133-148.

Barton NH (2001) Speciation. *Trends in Ecology and Evolution*, **16**, 325.

Beierkuhnlein C, Thiel D, Jentsch A, Willner E, Kreyling J (2009). Ecotypes of european grass species respond differently to warming and extreme drought. *Journal of Ecology*, **99**, 703-713.

Bentham G (1842) On *Mimoseae. Hook. Journ. Bot.*, **4**, 333-334.

Bentham G (1876) *Flora Brasiliensis,* Leipzig, R. Oldenbourg.

Cavallari MM, Gimenes MA, Billot C, Torres RB, Zucchi MI, Cavalheiro AJ, Bouvet, JM (2010) Population genetic relationships between *Casearia sylvestris* (Salicaceae) varieties occurring sympatrically and allopatrically in different ecosystems in south-east Brazil. *Annals of Botany*, **106**, 627-636.

Cruz MV, Rodrigues JG, Souza HAV, Lovato MB (2012) Isolation and characterization of microsatellite markers for *Plathymenia reticulata* (Leguminosae). Submitted to the *American Journal of Botany* on the 24th of October, 2011.

Ducke WA (1925) As leguminosas do Estado do Pará. *Arquivos do Jardim Botânico do Rio de Janeiro,* **4**, 209-343.

De Dios RS, Benito-Garzón M, Sainz-Ollero H (2006) Hybrid zones between two European oaks: a plat community approach. *Plant Ecology*, **187**, 109-125.

Drummond CS, Hamilton MB (2007) Hierarchical components of genetic variation at a species boundary: population structure in two sympatric varieties of *Lupinus microcarpus* (Leguminosae). *Molecular Ecology*, **16**, 753-769.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters using the software Structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620.

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479-491.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online,* **1**, 47-50.

Gee JM (2004) Gene flow across a climatic barrier between hybridizing avian species, California and Gambel's quail (*Callipepla californica and C. gambelii*). *Evolution*, **58(5)**, 1108-1121.

Gligor M, Ganzhorn JU, Rakotondravony D, Ramilijaona OR, Razafimahatratra E, Zischler H, Hapke A (2009) Hybridization between mouse lemurs in an ecological transition zone in southern Madagascar. *Molecular Ecology*, **18**, 520-533.

Goulart MF, Lemos JP, Lovato MB (2005) Phenological variation within and among populations of *Plathymenia reticulata* in Brazilian Cerrado, the Atlantic Forest and transitional sites. *Annals of Botany*, **96**, 445-455.

Goulart MF, Lemos JP, Lovato MB (2006) Variability in fruit and seed morphology among and within populations of *Plathymenia* (Leguminosae-Mimosoideae) in

areas of the Cerrado, the Atlantic Forest, and transitional sites. *Plant Biology,* **8**, 112-119.

Goulart MF, Lovato MB, Barros FV, Valladares F, Lemos-Filho JP (2011) Which extent is plasticity to light involved in the ecotypic differentiation of a tree species from savanna and forest? *Biotropica*, 1-9.

Goudet J (2001) FSTAT version 2.9.3.2, a program to estimate and test gene diversities and fixation indices. Institute of Ecology, Lausanne, Switzerland. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed on 10 May 2011].

Heringer EP (1956) O gênero *Plathymenia*. *Annais da Sociedade Botânica do Brasil,* **7,** 55-64.

Heringer EP, Ferreira MB (1972) Árvores úteis no cerrado (I): Vinhático: o gênero *Plathymenia* Benth. *P. foliolosa* Benth. e *P. reticulata* Benth., vinhático da mata e vinhático do campo (par vicariante). *Cerrado,* **5**, 28-34.

IBGE (1992) Manual técnico da vegetação brasileira. Rio de Janeiro: IBGE. 92p.

Klink CA, Machado RB (2005) Conservation of the Brazilian Cerrado. *Conservation Biology*, **19**, 707-713.

Lacerda DR, Acedo MDP, Lemos JP, Lovato B (2001). Genetic diversity and structure of natural populations of *Plathymenia reticulata* (Mimosoideae), a tropical tree from the Brazilian Cerrado. *Molecular Ecology,* **10**, 1143-1152.

Lacerda DR, Lemos JP, Acedo MDP, Lovato MB (2002). Molecular differentiation of two vicariant neotropical tree species, *Plathymenia foliolosa* and *P. reticulata* (Mimosoideae), inferred using RAPD markers. *Plant Systematics and Evolution,* **235**, 67-77.

Lemos Filho JPd, Goulart MF, Lovato MB (2008). Populational approach in ecophysiological studies: the case of *Plathymenia reticulata*, a tree from Cerrado and Atlantic Forest. *Brazilian Journal of Plant Physiology,* **20**: 205-216.

Lewis GP (1987) *Legumes of Bahia,* Kew, Royal Botanic Gardens.

Lopes DM, Campos LAD, Salomao TMF, Tavares MG (2010). Comparative study on the use of specific and heterologous microsatellite primers in the stingless bees *Melipona rufiventris* and *M. mondury* (Hymenoptera, Apidae). *Genetics and Molecular Biology*, **33**, 390-393.

Mittermeier RA, Fonseca GAB, Rylands AB, Mittermeier CG (1999) Atlantic forest. *In hot spots: earth's biologically richest and most endangered terrestrial ecoregions (Mittermeier RA, Myers N, Gil PR, Mittermeier CG, eds.)*, Mexico City: CENEX, 137-147.

Morgan-Richards M, Wolff K (1999) Genetic structure and differentiation of *Plantago major* reveals a pair of sympatric sister species. *Molecular Ecology*, **8**, 1027-1036.

Moritz C (1995) Use of molecular phylogenies for conservation. *Philosophical transactions of the Royal Society of London B*. **349**, 113-118.

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematics Biology*, **51(2)**, 238-254.

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

Novaes RML, Rodrigues JG, Lovato MB (2009) An efficient protocol for tissue sampling and DNA isolation from the stem bark of Leguminosae trees. *Genetics and Molecular Research*, **8**, 89-96.

Novaes RML, Lemos Filho JP, Ribeiro RA, Lovato MB (2010) Phylogeography of *Plathymenia reticulata* (Leguminosae) reveals patterns of recent range expansion towards northeastern Brazil and southern Cerrados in Eastern Tropical South America. *Molecular Ecology*, **19**, 985-998.

Pettengill JB, Neel MC (2011) A sequential approach using genetic and morphological analyses to test species status: the case of united states federally endangered *Agalinis acuta* (Orobanchaceae). *American Journal of Botany*, **98**, 859-871.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945-959.

Raymond M, Rousset F (1995) Genepop (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248-249.

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

Rodríguez F, Derelle E, Guillou L, Le Gall F, Vaulot D, Moreau H (2005). Ecotype diversity in the marine picoeukaryote *Ostreococcus* (Chlorophyta, Prasinophyceae). *Environmental Microbiology*, **7(6)**, 853-859.

Shimono Y, Watanabe M, Hirao AS, Wada N, Kudo G (2009). Morphological and genetic variations of *Potentilla matsumurae* (Rosaceae) between fellfield and snowbed populations. *American Journal of Botany*, **96(4)**, 728-737.

Toledo MM (2010) Anatomia da madeira, incremento radial, fenologia e relações hídricas em ecótipos de *Plathymenia reticulata* Benth. (Leguminosae, Mimosoideae). Tese de doutorado, Universidade Federal de Minas Gerais, Belo Horizonte.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4(3)**, 535-538.

Warwick MC, Lewis GP (2003) Revision of *Plathymenia* (Leguminosae-Mimosoideae). *Edinburgh Journal of Botany,* **60**,111-119.

Wiens JJ (2004) Speciation and ecology revisited: Phylogenetic niche conservatism and the origin of species. *Evolution*, **58(1)**, 193-197.

Wiens JJ (2007) Species delimitation: new approaches for discovering diversity. *Systematics Biology*, **56(6)**, 875-878.

Wright S (1951) The genetical structure of populations. *Annals of Eugenics*, **15**, 323-354.

Conclusões Finais

O trabalho de isolamento de microssatélites e o estudo da diversidade e estrutura genética de populações de *Plathymenia reticulata* por meio desses marcadores nos permitiram chegar às seguintes conclusões:

(1) Os nove microssatélites isolados e caracterizados para *Plathymenia reticulata* mostraram ter alto grau de polimorfismo e são, portanto, bastante informativos para estudos genético-populacionais;

(2) Sete dos nove *loci* isolados puderam ser genotipados em todas as oito populações amostradas neste estudo e foram eficientes para diferenciá-las, bem como para diferenciar os indivíduos dentro das populações;

(3) Nos ambientes ecotonais, foi detectado fluxo gênico em níveis elevados entre as populações dos biomas de Mata Atlântica e de Cerrado, indicando que o par vicariante provavelmente não desenvolveu mecanismos de isolamento reprodutivo completo;

(4) Populações que estão sob condições ambientais muito distintas, nos cores dos dois biomas, apresentam diferenças genéticas que corroboram com os resultados de trabalhos ecológicos desenvolvidos previamente com a espécie, o que justifica a classificação dos indivíduos em dois ecótipos;

(5) Embora as diferenças genéticas encontradas não sejam suficientes para considerar os ecótipos como espécies biológicas diferentes, nossos resultados nos levam a considerar populações de diferentes ecótipos como representantes de diferentes Unidades Evolutivas Significativas (*Evolutionary Significant Units*, ESU) que requerem ser manejadas separadamente; o cruzamento entre indivíduos de ecótipos distintos pode perturbar a combinação genética que garante o sucesso dos indivíduos nas áreas core dos biomas.